Differential Processing and Sorting of Nerve growth Factor and Brain- Derived Neurotrophic Factor in Hippocampal Neurons

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ABSTRACT

We have examined the biosynthesis, post-translational processing and sorting of neurotrophins in both constitutive and regulated cells infected with a pro-NGF, pro-BDNF and/or pro-NT-3 encoding vaccinia virus. Our results show that: 1) pro-BDNF is generated as a 32 kDa precursor that is N-glycosylated and glyco-sulfated in its prodomain. The precursor undergoes N-terminal cleavage to generate mature BDNF (14 kDa) as well as a truncated form of the precursor (28 kDa). 2) Both 32 and 28 kDa BDNF are released into media and are able to stimulate TrkB auto-phosphorylation. 3) The production of 28 kDa BDNF, unlike that of mature BDNF, occurs in the ER and is generated by a novel enzyme called subtilisin/kexin isozyme-1 (SKI-1) at the $RGLT^{57}\downarrow SL$ site. 4) Results obtained from pulse-chase experiments, secretagogue-induced release, and immunocytochemistry suggest that, NGF and NT-3 are primarily secreted constitutively while BDNF is principally directed to the regulated secretory pathway. 5) In contrast to homodimeric NT-3, NT-3/BDNF heterodimer is primarily sorted to the regulated secretory pathway suggesting that for the NT-3/BDNF heterodimer, the presence of a single proBDNF chain is sufficient for packaging into large dense-core secertory vesicles and subsequent regulated release. 6) Blocking furin activity in AtT-20 cells with α1-PDX as well as increasing the level of expression of NGF and NT-3 precursors partially directed them into the regulated secretory pathway. Therefore, neurotrophins can be sorted into either the constitutive or regulated secretory pathways, and sorting may be regulated by the efficiency of furin cleavage in the TGN. This mechanism may explain how neuron-generated neurotrophins can act both as survival factors and as neuropeptides.

RESUMÉ

Nous avons étudié la biosynthèse, les modifications post-traductionnelles et le routage cellulaire des neurotrophines en infectant des cellules à voie constitutive ou régulée par les virus de la vaccine recombinants pro-NGF, pro-BDNF et/ou pro-NT-3. Des marquages métaboliques suivis d'immunoprécipitations et d'analyses sur gels dénaturants ont permis de montrer que: 1) le pro-BDNF est synthétisé sous la forme d'un précurseur de 32 kDa N-glycosylé et glyco-sulfaté et engendre par clivage N-terminal le BDNF mature ainsi qu'un intermédiaire de 28 kDa, 2) les formes de 28 et 32 kDa sont sécrétées et capables de stimuler l'auto-phosphorylation de TrkB, 3) la forme de 28 kDa, contrairement au BDNF mature, est produite dans le RE et est due à un clivage au site $RGLT^{57}\downarrow SL$ par une nouvelle enzyme, SKI-1 (subtilisin/kexin-isozyme-1). Des expériences de marquages suivis d'une chasse, de sécrétion induite et d'immunocytochimie suggèrent que: 4) le NGF et la NT-3 sont essentiellement sécrétés constitutivement alors que le BDNF transite par la voie régulée, 5) contrairement aux homodimères NT-3, les hétérodimères BDNF/NT-3 transitent par la voie régulée, ce qui suggère que la présence d'une molécule de BDNF par hétérodimère suffise à conduire à leur accumulation au sein de vésicules denses et à leur sécrétion par la voie régulée. Enfin, 6) le blocage de l'expression de la furine par l'α1-PDX dans des cellules AtT-20, ainsi que l'augmentation de l'expression des précurseurs du NGF et de la NT-3, mènent au routage d'une partie de ces précurseurs par la voie régulée. Les neurotrophines peuvent donc emprunter les voies constitutive ou régulée et leur routage être affecté par l'efficacité de clivage de leur précurseur par la furine dans le TGN. Ce mécanisme pourrait expliquer comment les neurotrophines synthétisées par les neurones peuvent agir en tant que facteur de survie ou de neuropeptide.

TABLE OF CONTENTS:

LIST OF ABBREVIATIONS:

ACTH	adrenocorticotrophic hormone
APS	ammonium persulfate
α1-AT	α1-antitrypsin
α1-PDX	α1-antitrypsin Portland
BDNF	Brain-derived neurotrophic factor
BFA	brefeldin A
CL	cell lysate
CM	conditioned media
CNS	central nervous system
CPE	carboxypeptidase E
DRG	dorsal root ganglion
E	embryonic day
ELH	egg-laying hormone
ELISA	enzyme linked immunosorbent assays
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FCS	fetal calf serum
GFP	green fluorescent protein
HCG	human choriogonadotropin
hrBDNF	human recombinant brain derived neurotrophic factor
I.P.	immunoprecipitation
kDa	kilodalton
LDCV	large dense core vesicle
LTD	long-term depression
LTP	long-term potentiation
NGF	Nerve growth factor
NT	Neurotrophin
$P75^{LNTR}$	low-affinity neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	prohormone/precursor convertase
PDGF	platelet-derived growth factor
Pfu	plaque-forming units
PMSF	phenylmethyl sulphonyl fluoride
PNS	peripheral nervous system
POMC	pro-opiomelanocortin
SCG	superior cervical ganglion
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SREBP	sterol regulatory element-binding protein
SEM	standard error of the mean

SKI-1	subtilisin/kexin-isozyme-1
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween-20
TEMED	N,N,N^{1}, N^{1}- Tetramethylethlyenediamine
TGF-_	transforming growth factor _
TGN	*trans*-Golgi network
Trk	tropomyosin kinase receptor
VV	vaccinia virus

LIST OF FIGURES

MANUSCRIPTS AND AUTHORSHIP

As stated in the "Guidance Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

"An alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following: 1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.) 2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. 3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. 4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) insufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the

thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers. 6. When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition. 7. Irrespective of the internal and external examiners reports, if the oral defence committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis."

CONTRIBUTION TO PAPERS

Chapter 2

Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Seidah NG and Murphy RA (1999). Biosynthesis and Post-translational Processing of the Precursor to Brain-Derived Neurotrophic Factor. Submitted in J. Biol. Chem.

My contribution to this paper included all the experiments shown in this paper. Jasi Atwal helped to establish the Trk binding and phosphorylation assay used in this paper. Hooman Farhadi helped in producing figures 1 and 8. Sangeeta Pareek has done the primary works on metabolic labelling and immunoprecipitation of BDNF. I also wrote the first draft of the paper with the help of Hooman Farhadi. This draft was edited with the help of Drs Nabil Seidah, and Richard Murphy.

Chapter 3

Seidah NG, Mowla SJ, Hamelin J, Mamarbachi AM, Benjannet S, Toure BB, Basak A, Munzer JS, Marcinkiewicz J, Zhong M, Barale JC, Lazure C,

Murphy RA, Chretien M, Marcinkiewicz M (1999) subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. ***Proc Natl Acad Sci U S A*** **96:1321-6.**

Most of the work in this paper performed by Dr. Nabil Seida either personally or with the help of his technicians. My contribution to this paper included the finding that SKI-1 enzyme is responsible for the producing of 28 kDa BDNF. This finding was also very important to confirm the biological activity of the enzyme. I also showed the site of cleavage of pre-proBDNF to produce 32 and 28 kDa BDNF by microsequencing. This finding was also very important since it demonstrated that in contrast to other PCs, which cleaves precursors at monobasic or dibasic residues, SKI-1 cleaves precursors at non-basic (Threonine) residues.

Chapter 4

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. ***J Neurosci*** **19:2069-80.**

My contribution to this paper included all the experiments shown in this paper. I also wrote the first draft of the paper with the help of Hooman Farhadi. He also helped in producing figures 5 and 12. Sangeeta Pareek helped to establish the BDNF immunoprecipitation protocol used in this and other papers. Dr Stephen Morris produced NGF-GFP and BDNF-GFP constructs. He also generated the figure 10A. Kevin Petrecca helped us to produce confocal figures. The final draft was edited with the help of Drs Nabil Seidah, Wayne Sossin and also mostly by Dr. Richard Murphy.

Chapter 5

Farhadi HF*, Mowla SJ*, Petrecca K, Morris SJ, Seidah NG, and Murphy RA (2000) Neurotrophin-3 sorts to the constitutive secrtetory pathway of

hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with Brain-derived neurotrophic factor. ***J Neurosci*** **20:4059-68.**

This paper is a joint first authorship between Hooman Farhadi and myself. The role of hetero-dimerization in the sorting of NTs was originally performed in NGF/BDNF heterodimer that was more related to the story of differential sorting of NGF and BDNF. But since those results were not convincing enough I tested the idea for NT-3/BDNF heterodimer, which is more stable than NGF/BDNF heterodimer. This paper is a tight collaboration between us and both of us were involved in designing and producing all of the results. Kevin Petrecca has helped us in producing confocal figures. In addition, we wrote the first draft of the paper. This draft was edited with the help of Drs NG Seidah and RA Murphy.

* Denotes co-author

RATIONALE AND OBJECTIVES:

The main purpose of this study was to determine how NTs are produced, sorted and released in cells containing both constitutive and regulated secretory pathway, including hippocampal neurons. NTs have been traditionally considered as trophic factors released constitutively by neuronal target tissues and are necessary for survival and differentiation of innervating neurons. In the CNS, NTs are produced exclusively by neurons and there are increasing evidence considering NTs as a potential modulator of synaptic plasticity, a role which reqires the regulated release of neurotrophins. A precise interpretation of these new data requires a deep understanding of how neurons produce and release NTs. Our main objectives in this thesis are:

1) To determine the biosynthesis and post-translational modification of proBDNF and compare it with previous published data obtained in our lab for proNGF and proNT-3.
2) To determine how NTs are sorted and released by hippocampal neurons.
3) To determine the mechanism underlying sorting of NTs.
4) To determine the sorting fate of NGF/BDNF and NT-3/BDNF heterodimers.

CHAPTER 1

LITERATURE REVIEW

PREFACE

The aim of this chapter is to provide background to the work presented in the following chapters. The first part provides a general introduction to Neurotrophin (NT) family and their receptors, with an emphasis on their differential roles in neuronal survival and synaptic plasticity. I will also review relevant literature on the processing and sorting of NTs. The second part provides a literature review on proprotein convertases (PCs), which we now know process pro-NTs. The third part summarizes relevant literature on protein sorting within constitutive and regulated secretory pathway, with an emphasis on the mechanism of sorting. Taken together, this information will help establish the rational and objectives of the thesis.

NEUROTROPHINS

Neurotrophins (NTs) are an important family of neurotrophic factors that regulate neuronal survival, differentiation and plasticity in both the peripheral (PNS) and central nervous systems (CNS; reviewed by Levi-Montalcini, 1987; Thoenen 1995; Snider and Lichman 1996). This family of proteins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). NTs are small (~13 kDa) and highly basic (PI 9-10.5) proteins that naturally exist as homodimers (and possibly heterodimers; Bothwell and Shooter 1977). NTs exert their biological activity by inducing dimerization and autophosphorylation of their appropriate Trk receptors (Jing et al., 1992). Activated Trk receptors in turn initiate a cascade of intracellular events, which ultimately affect the survival, differentiation, and morphology of the neurons expressing them. NTs have common structural features and share ~50% sequence identity. Six cysteine residues in the mature domain of the NTs are conserved, giving rise to three disulfide bonds that stabilize the three dimensional structure of the protein (McDonald et al., 1991).

Nerve Growth Factor

Nerve growth factor (NGF) was the first member of NT family to be discovered almost half a century ago. Levi-Montalcini and Hamburger (1951) discovered that mouse sarcoma fragments implanted into the body wall of chick embryos induce enlargement of neurons in dorsal root and sympathetic ganglia. This finding suggested the presence of a soluble growth factor from sarcoma cells that could influence neuronal growth. The putative factor was named nerve growth factor (NGF). Subsequent work showed that co-culturing superior cervical ganglia (SCG) or dorsal root ganglia (DRG) next to the mouse sarcoma induced a hallo of outgrowing neurites from the ganglia (Levi-Montalcini and Hamburger 1953). This in-vitro bioassay was used as a bioassay for purifying NGF from mouse

submandibular glands, which are a rich source of the protein (Cohen and Levi-Montalcini 1956; Cohen 1960).

In the PNS, NGF is made by tissues that are innervated by NGF-dependent neurons (sympathetic neurons and a sub-population of sensory neurons; Levi-Montalcini, 1987). The level of NGF synthesis in these tissues is extremely low (~ 1 ng/g tissue). NGF sensitive neurons express the high-affinity NGF receptor, TrkA (Kaplan et al., 1991). In the CNS, NGF acts on cholinergic neurons in forebrain, that project to the hippocampus and neocortex and that are involved in learning, memory and attention. Substantial evidence suggests that NGF is a target-derived neurotrophic factor for these neurons, which express TrkA. The expression of NGF in the brain is mostly restricted to hippocampus and neocortex (Phillips et al., 1990).

Brain-derived neurotrophic factor

Because NGF only supports a limited set of neurons, the existence of additional neurotrophic factors has long been postulated. However, the identification of such factors was hampered by their extremely low abundance. It took a long time and much effort to isolate and characterize from brain extracts the next member of the family, brain-derived neurotrophic factor (BDNF; Barde et al., 1982). BDNF's primary structure is similar to that of NGF. BDNF is absolutely conserved among mammals implying remarkably strong evolutionary constraints on its structures (Yancopoulos et al., 1990). In contrast to other NTs, BDNF expression is mostly restricted to CNS neurons. Its expression is relatively low in the embryo, but increases significantly after birth to reach its highest in the adult (Yancopoulos et al., 1990). Expression is highest in the hippocampus and cortex, two regions known to undergo synaptogenesis during development, implicating BDNF as an important molecule in this process (McAllister et al., 1999). BDNF expression in brain is much higher than that of NGF; for example, in the adult mouse hippocampus BDNF mRNA is at least 50 times more abundant than the NGF mRNA (Hofer et al., 1990).

The biological actions of BDNF are distinctly different from that of NGF. In particular, BDNF supports the survival of retinal ganglion cells and placode-derived peripheral sensory neurons, which are not affected by NGF. BDNF can also be anterogradly transported (Altar et al., 1997; Smith et al., 1997; Fawcett et al., 1998; Caleo 2000), is able to elicit an action potential in central neurons (Kafitz et al., 1999), and can modulate synaptic plasticity (reviewed by McAllister et al., 1999).

Neurotrophin-3 and other members of Neurotrophin family

The highly conserved sequences of NGF and BDNF led to the rapid cloning of the other members of mammalian NT family including NT-3 (Maisonpierre et al., 1990) and NT-4/5 (Berkemeier et al., 1991; Ip et al., 1992). The same strategy has been used to clone NT-6 (Gotz et al., 1994) and NT-7 (Lai et al., 1998) in fish. These new members of the NT family were cloned and sequenced without prior purification of the proteins.

When the sequences of NT-3 and NT-4 were analyzed, the common characteristics were revealed with NGF and BDNF. In addition to a 50% identity in primary biochemical sequence, the proforms of these neurotrophins contain a protease cleavage sequence (Arg-X-Arg/Lys-Arg) which joining the prodomain with the mature domain. Cleavage at this site gives rise to the mature NTs. The proforms of the NTs also contain a conserved N-linked glycosylation site just eight amino acids upstream of the putative cleavage site. Similar to BDNF, mature NT-3 is also absolutely conserved in mammals. This extreme degree of evolutionary conservation has not been reported for any other secretory proteins (Yancopoulos et al., 1990).

Similar to NGF, NT-3 is widely expressed in both neuronal and non-neuronal tissues. The expression pattern of NT-3 in CNS is essentially reciprocal to that of BDNF, in that levels are high in the embryonic brain and dramatically decreases as neurons mature. The highest level of NT-3 expression correlates with those crucial periods in early neural development in which neurogenesis, and neuronal

differentiation occur. Thus, NT-3 might be involved in regulating the proliferation or differentiation of neuronal precursors (Maisonpierre et al., 1990; Yancopoulos et al., 1990).

Neurotrophin homo- and hetero-dimerization

The NT family belongs to the cysteine knot super family, a large family of proteins with a conserved core of three intertwined disulfide bonds. Members of this family include transforming growth factor _ (TGF- _), platelet-derived growth factor (PDGF), and human choriogonadotropin (hCG). All cystine knot growth factors are non-globular _-sheets and exist as homo- and heterodimers (Cheifetz et al., 1987; Cleason-Welsh et al., 1989; Ogawa et al., 1992). The dimers of these factors are held together by an inter-subunit disulfide bridge. In contrast, NTs exist in solution as non-covalent but very stable dimers (Bothwell and Shooter 1977; Radziejewski et al., 1992). NT dimerization seems to be essential for the dimerization and the activation of Trk receptors (Jing et al., 1992).

X-ray crystallography of mouse NGF (McDonald et al., 1991) revealed that each NGF subunit is made up by two pairs of anti-parallel _-sheet strands that contribute to the molecule's flat and elongated shape. The amino acid residues involved in the hydrophobic dimer interface are highly conserved among all NTs, in contrast to the more variable and exposed loop regions. This high degree of similarity at the dimer interface and also similarities among members of the cystine knot family suggest that NT heterodimers might also occur. The formation of NT heterodimers was originally explored in vitro by two separate studies (Radziejewski and Robinson 1993; Jungbluth et al., 1994) in which all four NTs were found to readily form heterodimers. Homodimer subunit exchange was promoted by treatment with urea, low pH, acetonitrile or guanidine hydrochloride. Heterodimers containing NGF protomer were less stable than heterodimers of the other NTs and gradually rearranged into the parent homodimers. It was later demonstrated that NGF, BDNF and NT-3 are all capable of forming heterodimers intracellularly when mammalian

cells were co-infected (Jungbluth et al., 1994) or co-transfected (Heymach and Shooter 1995) with constructs coding for the proforms of the proteins.

The NT-3/BDNF heterodimer, which is highly stable, interacts with the extracellular domains of the TrkB and TrkC receptors (Philo et al., 1994) and induces TrkB autophosphorylation similar to that of BDNF or NT-3 homodimers (Arakawa et al., 1994). Its action was also indistinguishable from the homodimers in DRG and SCG neuronal survival assay. However, Jungbluth et al., (1993) showed that NT-3/BDNF is at least ten times less active compared with a mixture of BDNF and NT-3 homodimers in all neuronal survival assays that were used. The heterodimer was still able to interact with TrkB and TrkC receptors as efficiently as the BDNF and NT-3 homodimers. Adding to the complexity, NT-3/BDNF heterodimer behaved like NT-3 in the sympathetic neuron survival assay, whereas it was intermediate between the two homodimers in promoting dopamine uptake in cultures of rat substantia nigra (Arakawa et al., 1994).

One intriguing question is whether NT heterodimers form in vivo. The finding that different NTs can be simultaneously produced by the same cell (Kokaia et al., 1993; Miranda et al., 1993) and also the finding that certain heterodimers are biologically active suggests that heterodimers may form in vivo. However, the extremely low level of NT expression makes it almost impossible to detect NT heterodimers in vivo.

Neurotrophin receptors

NT-responsive neurons possess at least two kinds of receptors, low-affinity and high-affinity receptors with a molecular weight of approximately 75 and 140 kDa, respectively. The 75-kDa protein (P75, also called the low-affinity neurotrophin receptor $P75^{LNTR}$) is a trans-membrane glycoprotein, which shares structural homology to the tumor necrosis factor receptor (Chao 1994). The P75 receptor lacks, at its cytoplasmic domain, a kinase domain required for intracellular signal

transduction. However, it can facilitate ligand binding to and enhance signaling through high affinity Trk receptors and also initiate signaling through the ceramide pathway (Chao and Hempstead 1995). P75 receptor contains an intracellular region resembling the death domain found in apoptotic receptors (Chapman 1995). Recent in vitro and in vivo studies have shown that P75 receptor can mediate apoptosis (Barret and Barlett 1994; Frade and Barde 1999). Recently, a novel protein was identified (NARGE: Neurotrophin Receptor-interacting MAGE homolog) which is interact with the P75 receptor and is required for P75 receptor-dependent apoptosis (Salehi et al., 2000). The 140-kDa protein tyrosine kinase-containing receptor (Trk) is able to mediate the effects of NTs acting alone (Kaplan et. al., 1991). TrkA, the first receptor discovered, mediates the biological effects of NGF. Two other members of the Trk receptor family (145-kDa proteins) were discovered later and named TrkB (Klein et al., 1989; 1991) and TrkC (Lambelle et al., 1991). TrkB specifically binds BDNF or NT-4/5 and TrkC specifically binds NT-3. In addition to binding to TrkC, NT-3 also activates TrkA and Trk B to a lesser extent.

Trk receptors mediate almost all of the biological activity of NTs. Consistently: transgenic mice lacking functional Trk receptors show almost identical phenotypes to those having a null mutation for NTs (Snider 1994). Consistent with the very restricted expression of NGF, TrkA expression is also restricted to only a very few neuronal types in the CNS. In contrast, TrkB and TrkC are widely distributed throughout the brain (Klein et al., 1989; 1990; 1991). Upon binding, NTs induce dimerization and phosphorylation of their cognate Trk receptors. Activated receptors in turn initiate a cascade of intracellular signals via multiple signaling pathways (Segal and Greenberg 1996).

Each trk gene is capable of producing multiple transcripts. Also, truncated forms of TrkB and TrkC receptors exist that lack intracellular kinase domains (Chao and Hemstead 1995). Truncated receptors are present on glial cells and could potentially modulate NT activity by controlling the amount and duration of NTs availability to the NT-sensitive neurons (Biffo et al., 1995).

PHYSIOLOGY OF THE NEUROTROPHINS

Neurotrophins as survival factors

In the vertebrate nervous system, neurons are initially overproduced, and a large number are eliminated during a period of developmentally programmed cell death (Oppenheim, 1991). This critical period coincides with the formation of synapses between neurons and their targets. A limited number of survival factors, including the NTs, are released by target tissues and can suppress the cell death program in innervating neurons (reviewed by Yuen et al., 1996). These factors are released in small amounts, which forces neurons to compete with each other for a limited supply. The dependence of neurons on factors derived from their targets is the basis of the *neurotrophic hypothesis*. Neurotrophic factors are produced not only by non-neuronal target cells, but also by neurons.

In the PNS, injection of NGF antibody into pregnant rats (Levi-Montalcini et al., 1960) almost completely eliminates SCG neurons, and also reduces the size and number of the sensory neurons. Consistently, transgenic mice carrying a disrupted NGF or trkA gene show severe sensory and sympathetic neuropathies (Smeyne et al., 1994). On the other hand, overexpression of NGF in sympathetic target tissues causes hypertrophy of sympathetic neurons (Albers et al., 1994).

In the CNS, NGF expression is restricted to subpopulation of neurons in cortex, hippocampus and olfactory bulb (Large et al., 1986; Whittemore and Seiger 1987) all targets of basal forebrain cholinergic neurons. The later projection neurons express mRNA for both p75 and TrkA receptors (Hefti et al., 1986; Kordower et al., 1994; Sobreviela et al., 1994; Holtzman et al., 1995). Accordingly, it was proposed that NGF produced in the hippocampus was necessary for the survival of the basal forebrain cholinergic neurons, and that retrograde mechanisms were responsible for this survival response (Hefti et al., 1989).

Further works on NT and trk knockout mice showed depletion of specific subsets of peripheral neurons homozygous for NTs or trk genes (reviewed by Snider 1994). In contrast, the survival of CNS neurons appears to be unaffected by a deletion of a single member of NT family.

Neurotrophins and synaptic plasticity

Despite considerable evidence that neuronal activity influences the organization and function of circuits in the developing and adult brain, the existence of extracellular signaling molecules that translate activity into structural and functional changes in synapses remain unknown. In recent years, NTs have emerged as attractive candidates for such signaling molecules (reviewed by Lo 1995, Thoenen 1995, Bonhoeffer 1996, Snider & Lichtman 1996, McAllister et al., 1999). As a mediator of synaptic plasticity, NTs must fulfill at least three criteria (McAllister et al., 1999): First, the expression of NTs and their receptors must be in the relevant areas in the CNS during times of developmental or adult plasticity. Second, NTs must be able to regulate aspects of neuronal function that change activity in neuronal plasticity, including neuronal morphology and connectivity, membrane excitability and synaptic function. Finally, NT expression and secretion must be activity dependent.

While recent evidence suggests that BDNF can fulfill almost all of these requirements, convincing evidence does not exist for the synaptic modulatory role of other NTs (see below).

Distribution of NTs and their receptors in CNS

Whereas NGF expression is mostly restricted to defined areas of the CNS, BDNF and NT-3 as well as their receptors (TrkB and TrkC) are widely expressed especially in areas of the brain that undergo plasticity such as cerebellum, hippocampus, and cerebral cortex. BDNF (Ernfors et al., 1990; Maisonpierre et al., 1990) and its

receptor (TrkB; Klein et al., 1989; 1990) is the most widely distributed of the NTs in the mature brain. Highest levels of expression are in hippocampus and other projection areas of basal forebrain cholinergic neurons, where the expression of BDNF mRNA is estimated to be at least 50 times greater than NGF's mRNA (Hofer et al., 1990). The expression of NT-3 is greater than NGF but lower than BDNF (Maisonpierre et al., 1990).

In addition to their specific patterns of expression in the adult CNS, NTs and their receptors are developmentally regulated (reviewed by Davies 1994). BDNF and NGF mRNA levels significantly increase postnatally, while over the same time period, the expression of NT-3 is down regulated (Maisonpierre et al 1990, Friedman et al 1991).

Regulation of neurotrophin synthesis by activity

In the CNS, in contrast to PNS, NTs are predominantly expressed by neurons under physiological conditions (Thoenen et al., 1987, Philips et al., 1990). Regulation of NT expression by neuronal activity was first discovered in the hippocampus. NGF and BDNF (but not NT-3) mRNA levels are rapidly and significantly increased after limbic seizures in the hippocampus (Ernfors et al., 1991; Isackson et al., 1991). The transient up-regulation of BDNF mRNA is particularly dramatic, achieving more than a 6-fold increase within 30 min after seizure activity begins (Ernfors et al., 1991). Interestingly, similar manipulations decrease NT-3 mRNA expression (Castren et al 1993, Elmer et al 1996, Mudo et al 1996). In rats, expression of TrkB and TrkC transiently increase between postnatal day 1 (P1) and P14 in several different brain regions, correlating with maximal neuronal growth, differentiation, and synaptogenesis (Ernfors et al 1990, Dugich-Djordjevic et al 1992, Ringstedt et al 1993, Altar et al 1994, Knusel et al 1994). Manipulations mimicking neuronal activity are particularly effective in regulating NT expression in dissociated neuronal cultures. Depolarization of cultured hippocampal neurons and cerebellar granule cells dramatically increases the levels of BDNF and NGF mRNAs (Lu et al 1991,

Zafra et al 1991, 1992, Berzaghi et al 1993, Berninger et al 1995, Bessho et al 1993, Lindholm et al 1994). Conversely, BDNF and NGF levels are down regulated in the presence of δ-aminobutyric acid (GABA) through activation of GABAA receptors (Zafra et al 1991, 1992, Berzaghi et al 1993, Berninger et al 1995).

More physiological levels of activity have also been shown to strongly regulate BDNF mRNA levels. However, NT-3 is not directly regulated by neuronal activity (Lindholm et al., 1994). Induction of long-term potentiation (LTP) in hippocampal slices for example, rapidly and selectively increases BDNF mRNA levels, with little or no effect on the other NTs (Patterson et al 1992, Castren et al 1993, Dragunow et al 1993). Physiological stimuli, such as light, also regulate the expression of BDNF and TrkB in the visual system (Castren et al 1992, Schoups et al 1995, Bozzi et al 1995, Rocamora et al 1996).

Effects of Activity on Neurotrophin Secretion

In the PNS, NGF is synthesized in a variety of neuronal target tissues containing only constitutive secretory pathway (reviewed by Levi-Montalcini 1987), and the regulation of synthesis and release is independent of neuronal input (Shelton and Reichardt 1986, Rohrer et al., 1988). Accordingly, NGF is released constitutively and in a calcium-independent manner (Barth et al., 1984). However, recent evidence suggests that in the CNS, NTs are primarily produced by neurons and they may be released through regulated secretory pathways. Using enzyme linked immunosorbent assays (ELISA) technique; Blochl and Thoenen (1995) have shown that NGF is secreted by primary cultures of hippocampal neurons, overexpressing NGF, in both constitutive and regulated pathways. The activity-dependent secretion of NGF is induced by KCl, carbacol, or glutamate and showed some unusual features distinct from those of the regulated secretion of neurotransmitters and neuropeptides (DeCamilli and Jahn 1990, Thureson-Klein and Klein 1990, Matteoli and Decamilli 1991). The secretion is independent of extracellular calcium but dependent on extracellular sodium. Further, by immunohistochemistry, NGF is localized in ER-

like compartments in both perikaryon and neuronal processes (Blochl et al., 1996). In contrast to the previous report, Goodman and colleagues (1996) showed that in BDNF-overexpressing hippocampal cultures BDNF secretion is increased at least five fold by depolarization, in a calcium-dependent manner. Consistent with this evidence, endogenous BDNF in hippocampal neurons has been localized to large dense-core vesicles, which are potentially capable of undergoing regulated release (Fawcett et al 1997, Smith et al 1997). In other studies, overexpressed NGF, BDNF and NT-3 could undergo calcium-dependent regulated release in response to secretogogues (Heymach et al., 1996). Further immuno-histochemical techniques co-localize all of the over-expressed NTs within LDCVs (Moller et al., 1998).

There is also some evidence that NTs themselves can cause the release of NTs (Canossa et al., 1997: Kruttgen et al., 1998). Addition of NT-3 or NT-4/5, but not NGF, increased the release of BDNF in hippocampal cultures infected with an adenovirus expressing BDNF (Canossa et al., 1997). The non-NMDA channel blocker drug, blocked glutamate induced release of BDNF, but had no effect on NT induced release suggesting that NT induced release of NT is not acting indirectly through the activation of glutamate (Canossa et al., 1997).

Together, the *in vitro* data suggest that NTs have the ability to be sorted within the regulated secretory pathway. However, caution must be taken to interpret the experiments described above. Many of them have relied on overexpression of NTs in dissociated cultures and release evoked by strong, non-physiological stimuli such as chronic depolarization. Accordingly, there is little evidence to suggest whether endogenous NTs can be sorted and released by regulated secretory pathway *in vivo*. The only suggestion for the regulated release of endogenous NTs comes from localization of endogenous BDNF which is localized in large dense-core vesicles of axon terminals in lamina II of lumbar spinal cord (Michael et al., 1997) and in the LP2 fraction of rat synaptosomes isolated from rat brain (Fawcett et al., 1997). So far, nothing is known about the sorting of endogenous NGF and NT-3.

Neurotrophin regulation of synaptic plasticity

Short term effects

NTs have both short and long term effects on synaptic transmission and the intrinsic excitability of target neurons. Lohof and colleagues (1993) were the first to show a direct influence of exogenous BDNF and NT-3, but not NGF, on synaptic transmission in neuromuscular synapses. Later, several other groups demonstrated that acute application of exogenous BDNF, NT4/5, or NT3 can alter or potentiate synaptic transmission in rat hippocampal cultures and slices (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995). Kang and Schuman (1995) found that BDNF and NT-3, but not NGF, potentiate glutamatergic transmission at Schaffer collateral-CA1 synapses in adult rat hippocampal slices via what appears to be a presynaptic mechanism. The effects of BDNF and NT-3 are rapid, increasing synaptic efficacy by 3-fold within 1 hr of NT application. With regard to potentiation of synaptic strength in CA1, BDNF and NT3 seem to be equally effective. However, using two highly specific, function-blocking antibodies against BDNF and NT3, Chen et al., (1999) show that only BDNF but no other NTs are likely to be involved in potentiation of these synapses.

NTs may be important in the normal development of LTP. Work with mice carrying a null mutation in NT genes showed that the lack of endogenous BDNF (but not the other NTs) leads to impaired LTP (Korte et al., 1995; Patterson et al., 1996). Importantly, it was also shown that re-expression of the BDNF gene (Korte et al., 1996) or treatment of slices with recombinant BDNF (Patterson et al., 1996) were both able to restore LTP in slices taken from BDNF-deficient mutant mice. In contrast, a conditional knock out of the NT-3 gene failed to show any involvement of NT-3 in synaptic transmission or LTP in the adult rat hippocampus (Ma et al., 1999).

Long term effects: Morphological plasticity

NTs have also potent long-term (stays for days or more) effects on the signaling properties of target neurons especially their strong regulation of gene expression and neuronal morphology. The long-term regulatory effects of NGF on the morphology of both developing and adult PNS neurons are well known (reviewed by Purves et al., 1988). Similar, but more complex, morpho-regulatory effects of NTs have been reported in CNS (McAllister et al., 1995). NTs promote axon growth and branching. Cohen-Corey and Fraser (1995) showed that substantial increase in collateral arborization of *Xenopus* retinal ganglion cell axons take place in response to tectal application of BDNF. Interestingly, BDNF stimulates axon arborization at the time when synaptic connections are normally being established and remodeled. Adding to the complexity, robust growth promoting effects of NTs on dendrites have also been observed (Snider 1988; McAllister et al., 1995). NTs rapidly increase the length and complexity of dendrites of pyramidal neurons in the developing neocortex (McAllister et al., 1995). On the other hand, removing the effects of endogenous NTs by using Trk "receptor bodies" showed dramatic changes on cortical dendritic arborization. (McAllister et al., 1997).

NTs have also been hypothesized to regulate the number of synapses per neuron. In transgenic mice over-expressing BDNF, in noradrenergic neurons using the dopamine-β-hydroxylase (DBH) promoter, the number of synapses increased more than two-fold (Causing et al., 1997). Correspondingly, a decrease in synaptic innervation density was found in BDNF knockout mice.

PROTEIN BIOSYNTHESIS AND POST-TRANSLATIONAL PROCESSING

Entering the ER

Co-translational translocation

Secretory proteins of eukaryotic cells are initially expressed as a pre- (pro) protein with an N-terminal signal sequence, which directs co-translational entry of the protein through a 'translocon' complex into the ER lumen (Blobel and Dobberstein 1975; Walter and Johnson 1994). Once positioned in the ER, the translocated protein undergo a series of post-translational modification including the removal of signal peptide, addition of N-link core carbohydrates and transient association with molecular chaperons which help the nascent protein to fold correctly. All signal peptides contain a hydrophobic core region, but, despite this, their overall length and amino acid composition are very different. Signal sequences are usually released from the precursor protein by signal peptidase during passage of the growing polypeptide chain through the ER membrane (Dalbey and Heijine 1992; Klappa et al., 1996). Recently, it has become clear that signal peptides may even have their own functions after being cleaved from the parent protein (Martoglio and Dobberstein 1998).

N-linked glycosylation

The covalent addition of N-linked core oligosaccharides to proteins occurs co-translationally soon after translocation of the nascent polypeptide into the lumen of the endoplasmic reticulum (ER). The N-glycosylation occurs at Asn residue in the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline or aspartic acid; Kornfeld and Kornfeld 1985). However, the presence of the consensus sequence does not ensure glycosylation, as many proteins contain the sequence remain un-glycosylated (Allen et al., 1995). The process begins with the *en bloc* transfer of a high mannose precursor oligosaccharide ($Glucose_3$-$Mannose_9$-N-$Acetulglucosamine_2$) to the proteins entering the ER lumen. The precursor oligosaccharide is held in the ER membrane by a special lipid molecule called dolichol, and the transfer catalyzed by a membrane-bound 'oligosaccharyl transferase' enzyme as early as Asn residue emerges in the ER lumen during protein translocation (Kornfeld and Kornfeld 1985). The antibiotic 'tunicamycin' inhibits the function of dolichol phosphate as an acceptor of N-acetyl glucosamine and thereby

prevents N-glycosylation (Kornfeld and Kornfeld 1985). While a role for N-linked glycosylation in the folding of glycoproteins is widely accepted, the mechanisms underlying this function are still unclear. For some glycoproteins, N-linked oligosaccharides are required for their overall stability, while for others the presence of the sugars is only needed during the folding process. Some proteins, such as IgD, fold more efficiently without sugars (Helenius1994).

Quality control

The main job of endoplasmic reticulum is to deliver properly folded proteins to their site of action and contains a sophisticated quality-control system to monitor and prevent abnormal proteins from being delivered. ER-associated degradation of misfolded and incompletely folded proteins is mainly carried out by the enzyme 'proteasome', which is located in the cytosol (Kopito 1997). The degradation process can be divided into two steps: In the first step, misfolded or unassembled proteins are recognized by ER chaperones such as calnexin and BiP. In the second step, the complex then retrotranslocated through the Sec61 channel into the cytosol, to be degraded by proteasome enzyme (Plemer et al., 1997).

From ER to Golgi

Ribosome-free membranes of the rough ER facing the cis-Golgi are called transitional elements (TE) and represent the ER exit sites (Bannykh et al., 1998). Newly synthesized secretory cargo proteins are packaged into coat protein II (COPII[1])-coated vesicles and tubules (Barlowe et al., 1994). COPII-coated buds transform into coated vesicles that rapidly shed their coats and fuse with ER-Golgi intermediate compartment (Hauri et al., 2000). Until recently, secretory proteins were taught to exit the ER by a default or 'bulk-flow' pathway mechanism (Rothman

[1] There are several classes of protein coats, which provides driving energy for vesicle buddings. The three best classes are : 1) COPII, which mediate ER to Golgi traffic. 2) COPI, which mediate retrograde traffic from the Golgi to the ER and for traffic between the cisterna of the Golgi and 3) clathrin which mediate post-Golgi and endocytic vesicular trafficking.

1987). However, recent data suggest that some classes of proteins are actively recruited into the ER export carriers. Albumin and VSV G, for instance, are concentrated in ER-derived vesicles of mammalian cells (Mizuno and Singer 1993; Balch et al., 1994). Such Findings imply that the exit from ER might need some signals to be facilitated. ER exit of VSV G and other cargo molecules, for example, requires a cytoplasmic, di-acidic (Asp-X-Glu, where X represents any amino acid) anterograde targeting signal (Nishimura and Balch 1997).

The Golgi-derived COPI vesicles are involved in a retrograde pathway from Golgi to ER (Cosson et al., 1998). ER-Golgi intermediate compartment is the main station for COPI-mediated retrograde transport; however, the Golgi and TGN are also involved to a lesser extent (Griffiths et al., 1995; Shima et al., 1999). Such a pathway is very essential for the maintenance of ER membranes, and retrieval of escaped resident ER proteins (Kelly 1999).

Post-translational modification of secretory proteins within Golgi

Glycosylation

En bloc attachment of N-linked oligosaccharide chain and the initial trimming of the sugar chain occurs in the ER (Kornfeld and Kornfeld 1985). Further processing, additions and modifications, occurs in the Golgi apparatus in a very highly ordered steps (Balch et al., 1984; Kornfeld and Kornfeld 1985). Further processing of the sugar chains in the Golgi complex produces two main classes of N-linked oligosaccharide: 1) High mannose oligosaccharides which contain just two N-acetylglucosamines and many mannose residues and 2) Complex oligosaccharides which contain several N-acetylglucosamines as well as a variable number of galactose and sialic acid residues and in some cases, fucose (Kornfeld and Kornfeld 1985). The two forms could be distinguished by a highly specific endoglycosidase (Endo H) enzyme. High mannose sugars are Endo H-sensitive while complex sugars are End H-resistant.

In addition to further modifying N-linked oligosaccharides. The O-linked glycosylation of Thr and Ser occurs in the Golgi apparatus. O-linked oligosaccharide chains are generally much simpler in structure than N-linked oligosaccharides (Jentoft 1990; Van den Steen 1995). Usually, N-acetylgalactosamine is added first, followed by the addition of more sugar residues, ranging from just a few to ten or more.

Proteolytic processing

A variety of polypeptide hormones, neuropeptides, and growth factors are initially synthesized as part of inactive precursor proteins in which biologically active peptides are flanked by either mono- or di-basic residues. The recent discovery of a novel family of precursor processing endoproteases has greatly improved our understanding of the complex mechanisms underlying the maturation of precursor-derived proteins. These proteases are calcium-dependent serine endoproteases related to the bacterial subtilisin and the yeast kexin enzymes (reviewed by Seidah et al., 1994; Nacayama 1997; Zhou et al., 1999), and hence have been called subtilisin/kexin-like proprotein convertases (SPCs) or more simply PCs. So far, seven members of this family in mammals have been identified and characterized (furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, LPC/PC7/PC8/).

Mammalian family of subtilisin/kexin-like proprotein convertases (PCs)

General structure of PCs

All seven members of PCs have well conserved signal peptides, prodomains, catalytic domains, and P domains (a downstream domain of about 150 amino acids required for correct folding and catalytic activity of the protein; Seidah et al., 1994; Rouillé et al., 1995) but differ in their C-terminal domains. The prodomain, is autocatalytically removed through cleavage at an Arg-X-Lys-Arg↓ site during maturation of the convertases. This intramolecular cleavage allows PCs to exit the

ER (Vey et al., 1994). However, the prodomain remains attached non-covalently until the cleaved inactive proenzyme reaches the *trans* Golgi network (TGN) where the more acidic and calcium-enriched environment facilitates dissociation of the prodomain (Anderson et al., 1997). A second cleavage within the prodomain then precludes further inhibitory interactions, resulting in full activation of the enzyme (Anderson et al., 1997).

A catalytic domain of approximately 240 residues (Zhou et al., 1999) follows the prodomain. The catalytic domain is highly conserved; in particular, the characteristic catalytic triad of the serine protease (ordered Asp, His, and Ser along the polypeptide chain) are present at corresponding positions in all members, except for PC2, where the Asn residue is replaced by an Asp. The sequences flanking these residues are conserved as well. The sequence substrate for the PCs is Lys-Arg↓ or Arg-Arg↓ (Rouillé et al., 1995, Seidah et al., 1998). However, additional basic residues at the P4 and/or P6 position also contribute to substrate recognition. Furin preferentially recognizes the motif Arg-X-Lys/Arg-Arg↓ but also is known to cleave Arg-X-X-Arg↓ sites in some precursors (Molloy et al., 1992; Nakayama 1997).

The P domain is also well conserved among eukaryotic convertases, including yeast Kexin, but is absent in bacterial subtilisins. The variable C-terminal regions of the PCs play a role in their subcellular routing (Creemers et al., 1998; Seidah et al., 1998). Towards the C-terminus, furin, PACE4 and PC5/A and B have a Cys-rich domain. Furin and PC7 also have a transmembrane domain near the C-terminus and are localized in the TGN. Another convertase with a transmembrane domain, PC5B, is also localized in the Golgi area, although it appears not to concentrate in the TGN (De Bie et al., 1996). PACE4 differs from the above constitutive pathway convertases in lacking a transmembrane anchor.

Tissue distribution of PCs

The mammalian PCs function in either the regulated or constitutive secretory pathway. Furin, PACE4, PC5, and PC7 are expressed in a broad range of tissues and cell lines where their active forms are localized in the TGN and small secretory vesicles of the constitutive pathway. The expression of PC4 is highly restricted to testicular spermatogenic cells (Nakayama et al., 1992). In contrast, the expression of PC1 and PC2 is limited to neuroendocrine system and brain, where they act on prohormone and neuropeptide precursors in secretory vesicles of the regulated secretory pathway (Rouille et al., 1995; Seidah et al., 1998).

α1-PDX, a potent inhibitor of furin-like enzymes

In human, the 394- amino acid α1-antitrypsin (α1-AT) is the physiological inhibitor of neutrophil elastase (Kurachi et al., 1981; Perlmutter and Pierce 1989). Its inhibitory role mediate by the formation of a stable tetrahedral adduct between the active site Serine of elastase and Met^{358} (Ala-Ile-Pro-Met^{358}) in α1-AT (Matheson et al., 1991). A naturally occurring mutation, known as α1-AT Pittsburgh, at the α1-AT reactive site (Ala-Ile-Pro-$\underline{Arg}^{358}$), has changed the specificity of this molecule from an inhibitor of elastase into an inhibitor of thrombin (Owen et al., 1983). A second mutation was constructed by Anderson et al., (1993) which contains in its reactive site $\underline{Arg}^{355}$-Ile-Pro-$\underline{Arg}^{358}$, the minimal sequence required for efficient processing by furin. This new variant, called α1-AT Portland (α1-PDX), is greater than 3000-fold more effective than α1-AT Pittsburgh at inhibiting furin in vitro. The full-length α1-PDX (64 kDa) is primarily localized within the TGN and inhibits PC-mediated processing of precursors primarily within the constitutive secretory pathway (Benjannet et al., 1997).

Constitutive and regulated secretory pathway

Proteins can be secreted from eukaryotic cells by either a constitutive or a regulated secretory pathway (reviewed by Kelly 1985; Burgess and Kelly 1987; Arvan and Castle, 1998). In constitutive pathway, proteins are secreted almost as fast as they are

synthesized in a calcium-independent manner. Since their transport vesicles have such a short half-life, it is very hard to find them in light or electron microscopy. Vesicles do not accumulate in a large intracellular pool and secretion is not coupled to extracellular clues (Burgess and Kelly 1987). Alteration of secretion is achieved by altering the level of protein synthesis. Specialized secretory cells such as endocrine, exocrine and neuronal cells contain a regulated secretory pathway in addition to the constitutive one. These cells are specialized to release, for a brief period of time, large amounts of proteins at a level much higher than their synthesis rate. The regulated secretory pathway can be distinguished from the constitutive one with at least three criteria (Kelly 1985): 1) Newly synthesized proteins destined for regulated release are retained inside the cell and concentrated as high as 200-fold during their passage from TGN to the mature secretory vesicle (Salpeter and Farquhar 1981). 2) The cytoplasm of regulated cells is filled with secretory vesicles that have a half-life of days and appears as a punctuate pattern of localization in immunocytochemistry. The secretory vesicles are large (100-500nm) and due to the condensation of secretory proteins often appear as electron-opaque in electron micrographs, which refers as large-dense core vesicles (LDVC). 3) Upon stimulation by an extracellular signal such as KCl and cAMP, secretory granules fuse, in a calcium-dependent manner with the plasma membrane and release their contents. Since regulated cells contain both a constitutive and regulated pathway, sorting must exist to target proteins into the appropriate pathway (Arvan and Castle 1998).

The mechanism of sorting in the TGN

It is now well documented that regulated proteins must possess sorting signals in order to be actively targeted into the regulated pathway. Proteins lacking such sorting signals will be released through constitutive or default secretory pathway (Griffiths and Simons 1986; Thiele and Huttner 1998; Traub and Kornfeld 1997).

The molecular mechanisms that target a particular protein to the regulated pathway are poorly understood but at least two non-exclusive theories have been

proposed. According to the first model, there is little distinction of secretory proteins at the level of entry into immature secretory vesicle[2]. Subsequent vesicular exit from immature secretory vesicle via efficient or inefficient means is viewed as the critical step for differential sorting. Proteins to be sorted to the regulated pathway would selectively aggregate in the presence of high Ca^{2+} and low pH, which exist in immature secretory vesicles (Kelly 1985; Tooze 1991; Yoo 1996). Condensation of regulated secretory proteins is expected to limit the ability of these molecules to escape from maturing granules and be retained in secretory vesicles while soluble proteins are removed from maturing vesicle in the process of constitutive-like vesicle budding (Tooze and Huttner 1990; Kuliawat and Arvan 1992). The second model involves a receptor-mediated mechanism in which specific structural domains within regulated proteins or protein aggregates recognized by specific receptors localized on the membranes of TGN or immature secretory vesicles (Chung et al., 1989; Arrandale and Dannies 1994). Mannose-6-phosphate-receptor-mediated sorting of newly synthesized lysosomal hydrolases into clathrin-coated vesicles that are targeted to lysosome represents the classical example of this model (Kornfeld and Mellman 1989). Recently, carboxypeptidase E (CPE) has been proposed as a sorting receptor for the regulated proteins in neuroendocrine cells, in a manner completely independent of its enzymatic activity (Cool et al., 1997). However, such a potential role was down played with the fact that in Cpe^{fat}/ Cpe^{fat} mice (expressing a CPE point mutant that misfolds and fails to be exported from the ER) proinsulin exhibits normal entry into the regulated secretory pathway (Varlamov et al., 1997; Irminger et al., 1997).

Both models imply that regulated proteins must have structural sorting signals that permit homo- or hetero-aggregation and/or receptor binding in order to be packaged in LDCV. However, the nature of the sorting signals is not clear yet. Comparison of the sequences of proteins released from regulated pathway failed to

[2] Although it has been widely recognized that the segregation of proteins between the constitutive and regulated pathways occurs in TGN, more recent evidence suggests that both types of proteins are co-packaged into immature secretory vesicles (von Zastrow and Castle 1987; Kuliawat and Arvan 1992)

reveal any conserved common sorting sequences. Nevertheless, a disulfide bond-stabilized loop in the N-terminal domain of chromogranin B (Chanat et al., 1993; Kromer et al., 1998; Glombik et al., 1999) and pro-opiomelanocortin (POMC; Cool et al., 1995) was proposed to be essential for targeting these precursors to the regulated pathway. There is also emerging evidence considering dibasic cleavage site of regulated precursors as potential sorting signals. A single mutation of the Arg-Lys dibasic that is normally processed to yield somatostatin 14 led to the constitutive release of pro-somatostatin (Brakch et al., 1994). Similarly, both site-directed mutations of the native cleavage site of pro-renin and/or the premature removal of prodomain in TGN (by inserting an Arg at P4 position) prevented the regulated secretion of renin (Brechler et al., 1996). Using the same methodology, Feliangeli and colleagues (submitted) demonstrated that the dibasic residues in the C-terminal domain of pro-neurotensin/neuromedin N are also essential for its sorting to the regulated secretory pathway. Accordingly, while over-expression of furin diminishes the efficiency of sorting in the regulated pathway, introducing PC1 within regulated secretory cells that normally lack PC1 has been found to increase the efficiency of sorting of insulin (Arvan and Castle 1998).

SUMMARY

Neurotrophins (NTs) are traditionally thought to be secretory proteins that regulate long-term survival and differentiation of neurons. Recent studies have revealed a previously unexpected role for NT in synaptic plasticity in a variety of neuronal populations. Nerve growth factor (NGF) is a well-documented target-derived trophic factor in the peripheral nervous system. Considerable evidence suggests that NGF also function in a target derived retrograde transport mode in the brain. For example, NGF is synthesized in the target hippocampus and cortex and interacts with the TrkA receptors on terminals projecting from basal forebrain cholinergic neurons. Spatial separation of NGF and TrkA gene expression is consistent with the target mechanism of action. A series of recent studies have provided strong evidence suggesting an additional novel role for BDNF in modulating synaptic plasticity. For

example, exogenous BDNF can elicit action potential in central neurons and also potentiate synaptic transmission. Endogenous BDNF is also important to the normal development of LTP, a cellular model for learning and memory. To understand how NTs can act as a survival factor or a modulator of synaptic plasticity requires knowing how neurons, which contain both constitutive and regulated pathway, produce and release NTs.

CHAPTER 2:

BIOSYNTHESIS AND POST-TRANSLATIONAL PROCESSING OF THE PRECURSOR TO BRAIN-DERIVED NEUROTROPHIC FACTOR

PREFACE

To understand the molecular mechanisms governing the sorting, targeting and release of neurotrophins (NTs) require a good understanding on how NTs are being produced, and post-transitionally modified. The post-translational modification of pro-NGF and pro-NT3 have been already studied and published through a collaboration between our lab and Dr. Seidah's (Seidah et al 1996a, 1996b). However, nothing had been known about the post-translational processing of pro-BDNF. This has been primarily due to the lack of a good immunoprecipitating antibody against BDNF. The recent production and characterization of an affinity-purified antibody against BDNF thus provides an opportunity to overcome the problem. Our data presented in this chapter, reveals two major findings: 1) the existence of a 28 kDa form which led us to identification of a novel enzyme, Subtilasin-Kexin-Isozyme-1 (SKI-1, would be discussed in chapter 3). 2) The fact that pro-BDNF, compare to other NTs, is poorly processed by constitutive cells and that it might have a role in the differential sorting of NTs (would be discussed in chapters 4 and 5).

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Biosynthesis and Post-translational Processing of the precursor to Brain-Derived Neurotrophic Factor

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ABSTRACT

We have examined the biosynthesis and post-translational processing of the brain-derived neurotrophic factor precursor (pro-BDNF) in cells infected with a pro-BDNF-encoding vaccinia virus. Metabolic labeling, immunoprecipitation, and SDS-PAGE reveal that pro-BDNF is generated as a 32 kDa precursor that is N-glycosylated and glyco-sulfated on a site located within the pro-domain. Some pro-BDNF is released extracellularly and is biologically active as demonstrated by its ability to mediate TrkB phosphorylation. The precursor undergoes N-terminal cleavage within the trans-Golgi network and/or immature secretory vesicles to generate mature BDNF (14 kDa). Small amounts of a 28 kDa protein that is immunoprecipitated with BDNF antibodies is also evident. This protein is generated in the endoplasmic reticulum through an N-terminal cleavage of pro-BDNF at the $RGLT^{57}\downarrow SL$ site. Cleavage is abolished when Arg^{54} is changed to Ala (R54A) by in vitro mutagenesis. Blocking the generation of 28 kDa BDNF has no effect on the level of mature BDNF and blocking the generation of mature BDNF with α1-PDX, an inhibitor of furin-like enzymes, does not lead to accumulation of the 28 kDa form. These data suggest that 28 kDa pro-BDNF is not an obligatory intermediate in the formation of the 14 kDa form in the constitutive secretory pathway.

Running title: Biosynthesis of BDNF

Keywords: BDNF, precursor, protein processing, glycosylation, sulfation, TrkB

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) along with nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are members of the neurotrophin family of trophic factors (Snider, 1994). The neurotrophins play essential roles in the development, survival and function of a wide range of neurons in both the peripheral and central nervous systems.

The neurotrophins have a number of shared characteristics, including similar molecular weights (13.2 - 15.9 kDa), isoelectric points (in the range of 9-10), and approximately 50% identity in primary structure. They exist in solution as non-covalently bound dimers. Six cysteine residues conserved in the same relative positions give rise to three intra-chain disulfide bonds (Maisonpierre et al., 1990; 1991). The neurotrophins interact with two cell surface receptors, the low affinity P75 receptor (Barker, 1998), and the Trk family of high affinity tyrosine kinase receptors (Kaplan et al., 1991). NGF preferentially binds TrkA, BDNF and NT4/5 bind TrkB and NT-3 binds TrkC (and TrkA to a lesser extent).

Sequence data predict that mature neurotrophins are generated through the proteolytic processing of higher molecular weight precursors (31-35 kDa), a process that has been extensively studied with respect to the production of NGF (Edwards et al., 1988; Seidah et al., 1996). Almost nothing is known, however, about the biosynthesis and post-translational processing of the other members of the neurotrophin family. Recent data from our laboratory show that cells with a regulated secretory pathway, including CNS neurons, release mature (i.e. fully processed) NGF (Mowla et al., 1999) and NT-3 (Farhadi et al., 2000) via the constitutive secretory pathway, while mature BDNF is packaged in vesicles and released through the regulated pathway (Mowla et al., 1999). Furthermore, BDNF is contained in a microvesicular fraction of lysed brain synaptosomes consistent with its anterograde transport in large dense core vesicles (Fawcett et al., 1997). Differences in the intracellular sorting of neurotrophins may arise, at least in part,

from differences in the chemistry and processing of their precursors. Therefore defining how neurotrophins are generated within a cell will be key to understanding how neurotrophins are released and function within the nervous system.

In this study, we monitored the biosynthesis and post-translational processing of the precursor to BDNF (pro-BDNF) using a vaccinia virus (vv) expression system together with metabolic labeling, immunoprecipitation, and SDS-PAGE. Data show that pro-BDNF is produced as a 32 kDa precursor that undergoes N-glycosylation and glycosulfation on residues located within the pro-domain of the precursor. N-terminal cleavage of the precursor generates mature BDNF as well as a minor truncated form of the precursor (28 kDa) that arises by a different processing mechanism than mature BDNF. Site-directed mutagenesis data suggest that 28 kDa BDNF is not an obligatory intermediate in the formation of the mature form. Data also demonstrate that pro-BDNF could be biologically active, as determined by its ability to promote TrkB auto-phosphorylation.

MATERIALS AND METHODS

Cell culture - AtT-20, COS-7 and LoVo cells were cultured as reported previously (Seidah et al., 1996). A human glioma (U373) cell line and a variant (U373/PDX) that stably expresses α1-PDX, an inhibitor of furin-like enzymes (Anderson et al., 1993), was generously provided by Dr Gary Thomas (Vollum institute, Portland Oregon).

Vaccinia virus (vv) infections and metabolic labeling - Purified recombinant vv containing the full-length coding region of human pro-BDNF was prepared and used to infect cells as described previously (Seidah et al., 1996). U373 and U373/PDX glial cells, and AtT-20 cells were grown in 60 mm dishes and exposed to virus for 30 min or 2 h, respectively. The cells were incubated in medium without virus overnight and either pulsed or pulse-chase labeled at 37 °C for specified time intervals. For pulse-chase experiments, infected cells were incubated in cysteine/methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for 1 h, and then received 1.5 ml of the same medium containing 0.2 mCi/ml [^{35}S] Cys/Met (Dupont- New England Nuclear) for 30 min. For the chase, cells were bathed for specified intervals in DMEM medium containing 10% FCS plus excess (2x) cysteine and methionine.

In experiments assessing sulfation, AtT-20 cells were labeled for 3 h with [$Na_2{}^{35}SO_4$] (0.5 mCi) (Dupont-New England Nuclear) in methionine/cysteine/SO_4-free RPMI-1640 medium (Gibco). Sodium chlorate (1 mM) was added to the medium in some experiments to inhibit sulfation and in others, tunicamycin (5 µg/ml) was added to inhibit N-linked glycosylation. In both cases, the drugs were present in the medium during the 60-min pre-incubation period and throughout the pulse-chase period.

Immunoprecipitation and microsequencing - Radiolabeled BDNF was immunoprecipitated from cell lysates and conditioned medium as previously described (Mowla et al., 1999). We used an affinity-purified antibody to BDNF (Yan et al., 1997, kindly supplied by Amgen) at a concentration of 0.5 µg/ml. Samples were analyzed by 13-22% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed for 1 h in 40% methanol and 10% acetic acid, treated with ENHANCE (Dupont-New England Nuclear) for 1 h, washed in 10% glycerol for 1 h and dried for 4 h at 60°C. Micro-sequencing was carried out on samples from conditioned medium that were [^{3}H] Leu-labeled and eluted from SDS-containing gels following electrophoresis. Micro-sequencing was performed using an Applied Biosystem gas-phase sequenator model 470A, as previously described (Benjannet et al., 1992).

Endoglycosidase H (endo H) and N-glycanase treatment - vv:BDNF infected AtT-20 cells were metabolically labeled with [^{35}S] Cys-Met, conditioned medium was collected and treated with antibody to BDNF, and the precipitates dissolved in 100 µl of reaction buffer with or without endo H (10 units; Boehringer Mannheim) or N-glycanase (1.5 units; Oxford GlycoSystems). Samples were incubated overnight at 37 °C. Endo H digestions were carried out in 100 mM sodium citrate buffer, pH 5.5, and N-glycanase digestions in 20 mM sodium phosphate buffer (pH 7.5) containing 50 mM EDTA.

Transient Transfection of R54A BDNF mutant in COS-7 Cells - Using the LipofectAMINE reagent (Life Technologies, Inc.), we transfected 60-70% confluent COS-7 cells with pcDNA3 recombinants of either the wild-type or R54A mutant form of pro-BDNF. After a 5 hr incubation in serum- and antibiotic-free DMEM, the cells were incubated for another 48 hr in DMEM plus 10% fetal calf serum. Two days after transfection, cells were metabolically labeled for 6 hr, cell lysates and conditioned media were collected, immunoprecipitated, and resolved by 13-22% gradient SDS-PAGE.

TrkB phosphorylation assay - For these studies, we obtained relatively pure preparations of the BDNF precursor by co-infecting LoVo cells, an epithelial cell line that is deficient in endogenous furin-like enzymes (Takahashi et al., 1993), with vv:BDNF and vv:α1-PDX (Anderson et al., 1993). The cells were incubated in virus-free medium for 10 h, followed by 4 h incubation in serum-free medium, which was subsequently collected for testing. To isolate fully processed BDNF generated under similar conditions, we co-infected LoVo cells with vv:BDNF and vv:Furin, to ensure that the precursor was cleaved, and collected conditioned medium 6 h later. Media collected from uninfected and wild-type vaccinia virus-infected (vv:WT) LoVo cells were used as controls. To test for biological activity, we used NIH 3T3 cells that overexpress TrkB, prepared and generously provided by Dr. David Kaplan (Montreal Neurological Institute). The cells were bathed in conditioned medium for 5 min, following which cell lysates were immunoprecipitated with panTrk 203 antibody (Hempstead et al., 1992). The pellets were dissolved in sample buffer, fractionated by SDS-PAGE using an 8% gel, and transferred onto a 0.2 μm nitrocellulose membrane for Western blotting. The replicas were probed overnight at 4°C with a monoclonal phosphotyrosine antibody (UBI, Lake Placid, NY) diluted 1:10,000 in TBS supplemented with 0.1% Tween 20, and for an additional 1 h with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000). Immunoreactivity was observed using enhanced chemiluminescence (NEN Life Science Products).

RESULTS

Antibody specificity - To characterize the specificity of the BDNF antibody and to monitor its effectiveness in immunoprecipitations, we over-expressed BDNF in AtT-20 cells using a vaccinia virus (vv) encoding the full-length precursor to hBDNF. Samples of cell lysate and conditioned medium were divided equally and immunoprecipitated with non-immune serum, antibody to BDNF, or BDNF antibody with excess rhBDNF (5 ng/μl). As seen in Figure 1, in both cell lysate and conditioned media, the antibody to BDNF specifically immunoprecipitated three proteins migrating at approximately 32, 28, and 14 kDa. None of these proteins reacted with non-immune serum (NI), and none were immunoprecipitated in the presence of excess rhBDNF. We therefore conclude that the 32 kDa protein is unprocessed pro-BDNF, the 28kDa protein a truncated form of pro-BDNF, and the 14 kDa protein fully processed mature BDNF.

Pro-BDNF processing in vv:BDNF infected AtT-20 cells – To understand the relationship of the different forms of the BDNF precursor to mature BDNF, we carried out pulse chase studies using AtT-20 cells infected with recombinant vv:BDNF, a system we have used previously (Mowla et al., 1999, Farhadi et al., 2000). Figure 2A shows that 32 kDa BDNF precursor is apparent in cell lysates as early as 10 min after the cells were radiolabeled and increased in intensity through 30 min of pulse incubation. In cells labeled for 20 min, a slightly higher molecular weight band is apparent that resolves into a doublet in cells chased for 4 h. This material likely represents differentially glycosylated and sulfated forms of the BDNF precursor (see below). Over the 8 h chase period, the 32 kDa and, to a lesser extent, the minor higher molecular weight bands decreased in intensity while levels of the 14 kDa mature BDNF band increased, suggesting a precursor-product relationship. The 28 kDa band appeared as early as 10 min pulse and its level increased by 1 hour chase. The intensity of the band decreased significantly thereafter. Figure 2B reveals that significant amounts of the 32 kDa BDNF precursor, the 28 kDa form, and mature BDNF are released into conditioned medium during the 8 h chase period.

Pro-BDNF is N-glycosylated - N-glycanase treatment of the 32 kDa BDNF precursor and the truncated 28 kDa form of the precursor reduces their apparent size to around 27 kDa and 24 kDa, respectively, indicating that these proteins contain N-linked complex carbohydrates (Fig. 3). N-glycanase treatment has no effect on the apparent molecular size of mature BDNF (14 kDa). Treatment with endo H, which removes high mannose sugar moieties, only partially digests the 32 and 28 kDa BDNF (Fig. 3), suggesting that the precursor released into the conditioned medium contains a heterogeneous mixture of complex and high mannose sugars. Note that both pro-BDNF and 28 kDa BDNF appear as doublets with the lower band being endo H sensitive while the higher band is endo H resistant.

In order to define the importance of glycosylation in the generation of BDNF from its precursor, we infected AtT-20 cells with vv:BDNF and metabolically labeled the cells in the presence or absence of 5 μg/ml of tunicamycin, an inhibitor of N-glycosylation. Cells were metabolically labeled for 30 min followed by a 2 h chase period, in the presence or absence of tunicamycin. Figure 4 shows that tunicamycin greatly reduced the signal intensity of the BDNF precursors as well as mature BDNF (compare the level of labeling in the left and right panels of Fig. 4). In addition, the apparent molecular weight of the BDNF precursor in cell lysate and in conditioned medium was reduced from 32 kDa to approximately 27 kDa. The result suggests that glycosylation may play an important role in stabilizing the BDNF precursor during its processing and subcellular trafficking. Tunicamycin did not alter the molecular size of mature BDNF (14 kDa), as expected since this form of the protein is not N-glycosylated.

Pro-BDNF is glycosulfated - Metabolic labeling of vv:BDNF infected AtT-20 cells with [$^{35}SO_4$] Na_2 (Fig. 5A) reveals that pro-BDNF as well as the truncated 28 kDa form of the precursor are sulfated. Mature BDNF, in contrast, is not sulfated. Furthermore, treatment of the sulfated species with N-glycanase (Fig. 5B) completely removes the radioactive signal, demonstrating that sulfation occurs on carbohydrate groups.

To determine whether sulfation is essential for the processing and/or secretion of pro-BDNF, we labeled AtT-20 cells expressing pro-BDNF with [^{35}S] Cys-Met for 30 min. and then chased the cells for 2 h in the presence or absence of sodium chlorate (1 mM) (Baeuerle et al., 1986). This treatment reduced by 97% the levels of [$^{35}SO_4$] that were incorporated into protein immunoprecipitates measured in conditioned medium at the end of the chase period (data not shown). The result showed that exposure to sodium chlorate had no detectable effect on processing of pro-BDNF or on secretion of mature BDNF (data not shown).

Generation of 28 kDa BDNF occurs in the ER - In order to determine where in the cell the 28 kDa form of BDNF is generated, we metabolically labeled vv:pro-BDNF infected cells with [^{35}S] Cys-Met for 3 h in the presence or absence of brefeldin A (BFA, 5 μg/ml), a molecule that inhibits anterograde vesicular transport from the ER (Fernandez et al., 1997). The cells were analyzed immediately or after a further 2h chase period without BFA. Figure 6 shows that BFA had no effect on the generation of the 28 kDa form of pro-BDNF, but it did inhibit the generation of the 14 kDa form of mature BDNF. This effect was reversed when the cells were chased two hrs in the absence of BFA. These results suggest that the 28 kDa form of BDNF can be generated in the ER while the mature form of BDNF, as already shown (Mowla et al., 1999), is generated in the *trans*-Golgi network or a post-Golgi compartment.

N-terminal sequence of 28 kDa BDNF - The data presented above show that cell lysates and conditioned media of AtT-20 cells infected with vv:pro-BDNF generate a truncated form of BDNF with an apparent molecular weight of 28 kDa. We also detected this molecule in several other cell lines as well as in primary cultures of mouse hippocampal neurons infected with the same vv construct (data not shown). In a separate study, we showed that a novel enzyme (SKI-1, subtilisin-kexin-isozyme-1) is able to increase the level of 28 kDa BDNF when co-expressed with pro-BDNF in COS-7 cells (Seidah et al., 1999). N-terminal micro-sequencing of [^{3}H]-Leu labeled 28 kDa BDNF revealed a unique cleavage site at $R^{54}GLT^{57}\downarrow SL$ (shown in

Fig 7A). To determine whether endogenous 28 kDa BDNF is also cleaved at the same site, we mutagenized Arg^{54} (which lies at the P4 position) to Ala. This residue potentially could serve as a recognition signal for this kind of subtilase (Seidah et al., 1999). Processing of the R54A pro-BDNF mutant results in unchanged levels of mature 14 kDa BDNF with no significant generation of the 28 kDa protein (Fig 7B). This result demonstrates that the endogenous protein is indeed cleaved at the same site, and that, as seen for other PC substrates, Arg at the P4 is critical for efficient cleavage (Fig. 7B).

28 kDa BDNF is not an obligatory intermediate in the generation of mature BDNF- In this study, we introduced a vv encoding pro-BDNF into a cell line (U373 glial cells) that stably expresses the furin-inhibitor α1-PDX (Fawcett et al., 1997). Figure 8 shows that inhibiting furin–like enzymes abolishes the formation of mature BDNF but has no effect on the generation of the 28 kDa protein. Also, as shown in Fig 7B, transient expression of the Arg54Ala mutant in COS-7 cells abolishes the generation of 28 kDa BDNF without affecting the level of mature BDNF. Taken together, these results strongly suggest that the 28 kDa species does not constitute an obligatory intermediate in the normal processing of the BDNF precursor in the constitutive secretory pathway.

Pro-BDNF is biologically active - Significant amounts of unprocessed pro-BDNF are secreted into conditioned media under our experimental conditions, a result that led us to question whether the precursor, if released in vivo, could be biologically active. To test this idea, we set out to generate unprocessed pro-BDNF by co-infecting LoVo cells, which are already deficient in furin activity (Takahashi et al., 1993), with vv:BDNF along with vv:α1-PDX. By blocking the activity of all furin-like enzymes in the cell, we were able to obtain conditioned medium containing pro-BDNF and the 28 kDa BDNF without detectable amounts of mature BDNF (Fig. 10A). As a control for this study, we collected medium from LoVo cells co-infected with vv:BDNF and vv encoding furin (vv:Furin) (Seidah et al., 1996), conditions that

favor the processing of pro-BDNF to mature BDNF (Fig. 10B). Conditioned medium from these cells contained small amounts of unprocessed pro-BDNF.

Figure 10C shows that medium collected from both cell types induce robust TrkB auto-phosphorylation in NIH 3T3 cells that over-express the TrkB receptor. Medium conditioned by cells infected with wild-type vv had no effect. We conclude from these data that once released from a cell, the intact BDNF precursor containing small amounts of the 28 kDa form of BDNF has the potential to be biologically active. We do not know the precise contribution of the 28 kDa form of pro-BDNF to this activity since we were unable to obtain sufficient amounts of the protein for testing in the absence of pro-BDNF or mature BDNF.

DISCUSSION

Because biosynthesis of neurotrophins normally occurs at low levels in neurons and non-neuronal cells, it is impossible to analyze endogenous neurotrophin processing with currently available techniques. Therefore, in this study, we used a vaccinia virus expression system to overexpress pro-BDNF and to study its processing in a variety of cell lines as well as in primary cultures of mouse hippocampal neurons. We have used similar methods previously to monitor the biosynthesis and post-translational processing of pro-NGF (Seidah et al., 1996).

Using the BDNF antibody provided by Amgen, as well as a commercially available antibody from Santa Cruz (data not shown), we detected three BDNF-related products in vv:BDNF infected AtT 20 cells and hippocampal neurons, namely 32 kDa, 28 kDa, and 14 kDa forms of the protein (Fig. 1). Results indicate that pro-BDNF is synthesized as a 32 kDa precursor that is processed within 1 h to give rise to mature BDNF (14 kDa). We also observed a significant amount of unprocessed pro-BDNF being released into conditioned medium by AtT-20 cells and hippocampal neurons (Mowla et al., 1999), cells that can release proteins both by the regulated and constitutive secretory pathways. In parallel studies, we did not observe precursor release when similar methods were used to monitor processing and release of the precursors of NGF (Mowla et al., 1999) or NT-3 (Farhadi et al., 2000). Indeed, previous work by others has shown that large amounts of the precursors of proteins released by the regulated secretory pathway, such as pro-opiomelanocorticotrophin (POMC), are also constitutively released from AtT-20 cells (Fernandez et al., 1997). Although these differences could simply reflect over-expression of pro-BDNF saturating the sorting machinery in the trans-Golgi network (Mowla et al., 1999), constitutive release of the precursor could also be of biological significance. In that regard, BDNF mRNA is present in the dendrites of hippocampal neurons in culture (Crino et al., 1996; Tongiori et al., 1997), and as yet, we know nothing about the chemistry or fate of the BDNF protein synthesized within dendrites. It is possible

that pro-BDNF could be produced in dendrites and released, in part, in an unprocessed form, for as yet unknown purposes.

In this study, we have shown that medium containing pro-BDNF interacts with the TrkB receptor and activates its autophosphorylation (Fig. 10). Previous work has shown that a BDNF mutant containing an extension of 19 amino acids upstream of the cleavage site of mature BDNF (25 kDa BDNF) is biologically active (Kolbeck et al., 1994). Also, Edwards and colleagues (Edwards et al., 1988) have reported that pro-NGF is biologically active, but at a level 10-20 fold below that of mature NGF. Taken together, these findings suggest that complete processing of pro-neurotophins may not be an absolute requirement for biological activity.

During transit through the secretory pathway, the BDNF precursor is glycosylated (Fig. 3), presumably at the single putative consensus sequence for N-linked glycosylation (N-X-T/S) six residues upstream of the cleavage site that generates mature BDNF. This glycosylation site is conserved in the same position in all neurotrophins, suggesting a critical role for N-linked glycosylation in neurotrophin maturation and/or trafficking. Pro-BDNF is released into conditioned medium as a mixture of endo H-sensitive (un-trimmed) and endo H-resistant (trimmed) sugars. Both the 32 and 28 kDa forms of BDNF appear as doublets, the upper band being endo H resistant and the lower band is endo H sensitive (Fig. 3). The importance of carbohydrates in the folding of proteins has been well-documented (Kornfeld and Kornfeld 1985). In the case of NGF, blocking N-glycosylation with tunicamycin prevents the entry of pro-NGF into the Golgi apparatus and its subsequent secretion (Seidah et al., 1996). In this study, blocking N-glycosylation of pro-BDNF significantly reduced the level of radiolabelling of both pro-BDNF and mature BDNF (Fig. 4), which may be due to incorrect folding diminishing the half-life of newly synthesized protein. Our results also demonstrate that oligosaccharide chains attached to the pro-domain of the BDNF precursor are sulfated (Fig. 5), as has been previously reported for pro-NGF (Seidah et al., 1996). Blocking sulfation with sodium chlorate (Baeuerle and Huttner 1986) did not affect

processing and release of pro-BDNF. This result is consistent with the recent finding of Van Kuppereld and colleagues (Van Kupperfeld et al., 1997) that protein sulfation is not required for the transport, sorting, or proteolytic processing of proteins directed to the regulated secretory pathway.

We have also identified a 28 kDa protein that is a cleavage product of the BDNF precursor in addition to 14 kDa mature BDNF but which is generated through a distinct processing pathway. The protein is precipitated by antibodies to BDNF and is present only in cells expressing pro-BDNF. Furthermore, its processing in U373 glial cells (Fig. 8) is not affected by αl-PDX, an inhibitor of the furin-like enzymes that likely generate mature BDNF from pro-BDNF in cells that contain a constitutive but not regulated secretory pathway. These results strongly suggest that the 28 kDa molecule is not processed by the known prohormone convertases, but rather by some other processing system within the cell.

Recent studies from our laboratories revealed that the 32 kDa BDNF precursor is a substrate for a newly identified subtilisin/kexin-like enzyme, called SKI-1 (Seidah et al., 1999). Co-expression of pro-BDNF and SKI-1 produced sufficient 28 kDa BDNF for N-terminal microsequencing, which revealed that cleavage occurs at the $R^{54}GLT^{57}\downarrow SL$ site (Fig.7A). To determine whether the 28 kDa BDNF we detected is generated at the same cleavage site, we mutagenized Arg^{54}, which lies at the P4 position relative to the cleavage site and is potentially important for recognition by this kind of enzyme (Seidah et al., 1999). Processing of the R54A pro-BDNF mutant did not yield significant amounts of 28 kDa BDNF, suggesting that the 28 kDa precursor is cleaved at the same site (Fig. 7B). Thus, while the 28kDa form of pro-BDNF is clearly evident in our samples, we do not whether the protein is biologically important.

Two lines of evidence suggest that the generation of mature BDNF in the constitutive pathway does not require initial processing of pro-BDNF to the 28 kDa form. First, in the U373-PDX cell line (a constitutive secreting cell line expressing

α1-PDX), generation of 14 kDa BDNF is abolished but there is no accumulation of 28 kDa BDNF, as would be expected if the latter were an intermediate product (Fig 8). Second, Ala substitution of the P4 Arg (Arg54Ala) abolished the generation of the 28 kDa form without affecting the level of mature BDNF production (Fig 7B).

Much is yet to be learned about the BDNF precursor. For example, we do not know whether the intact precursor (32 kDa) and the 28 kDa form of the precursor, both of which can be released constitutively from cells, could have biological roles of their own distinct from mature BDNF. Furthermore, in cells with both the regulated and constitutive secretory pathways, pro-BDNF is preferentially processed and released from the regulated pathway while pro-NGF (Mowla et al., 1999) and pro-NT3 (Farhadi et al., 2000) are in the constitutive secretory pathway. Differential targeting may well arise because of structural differences in the pro-domains of the neurotrophin precursors or because of differential processing. Studies currently underway are targeted toward solving these issues.

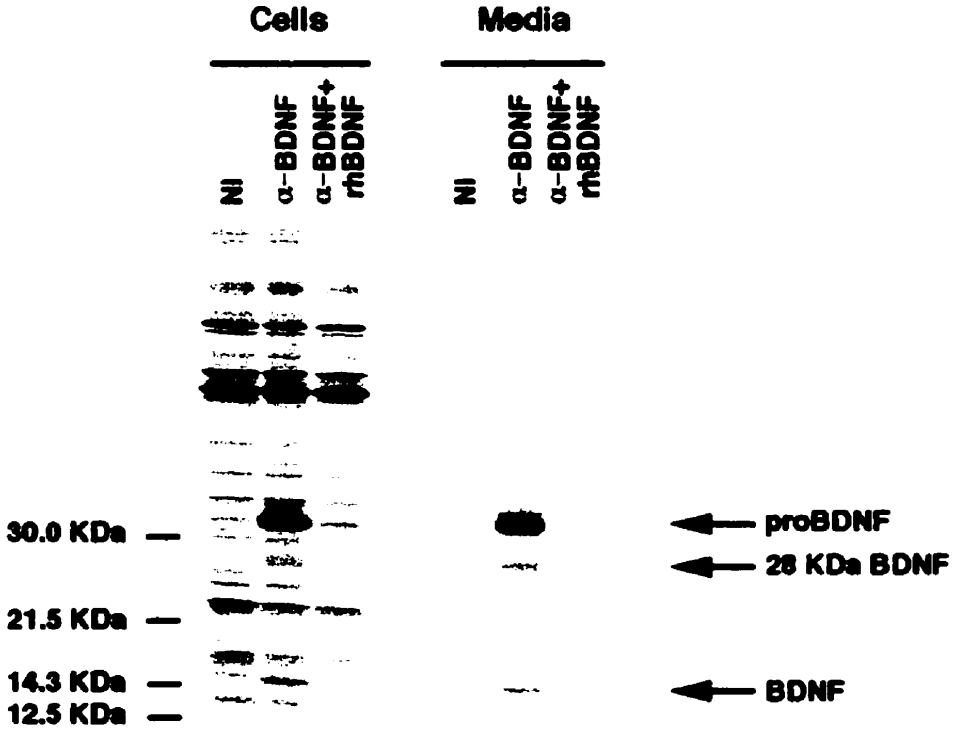
Cells
Media
NI
α-BDNF
α-BDNF+ rhBDNF
NI
α-BDNF
α-BDNF+ rhBDNF
30.0 KDa
21.5 KDa
14.3 KDa
12.5 KDa
proBDNF
28 KDa BDNF
BDNF

FIGURE 2.1 **Immunoprecipitations with the BDNF antibody**. AtT-20 cells were infected for 1 h with vv:BDNF and labeled with [^{35}S] Cys-Met for 4 h. Cell lysates and media were equally divided into three tubes and immunoprecipitated with either non-immune serum (NI), antibody to BDNF (α-BDNF), or α-BDNF with excess recombinant human BDNF (rhBDNF).

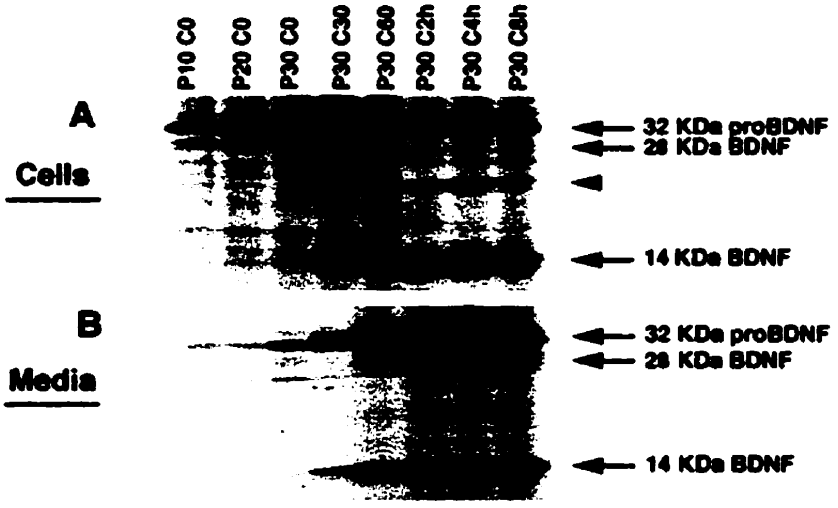
P10 C0
P20 C0
P30 C0
P30 C30
P30 C60
P30 C2h
P30 C4h
P30 C8h
A
Cells
32 KDa proBDNF
28 KDa BDNF
14 KDa BDNF
B
Media
32 KDa proBDNF
28 KDa BDNF
14 KDa BDNF

FIGURE 2.2 **Pulse-chase labeling of AtT-20 cells infected with vv:BDNF.** Infected cells were labeled with [^{35}S] Cys-Met for 10, 20, and 30 min without chase or pulsed for 30 min and then exposed to a chase medium containing excess unlabeled cysteine and methionine for 0.5, 1, 2, 4, and 8 h. Cell lysates (A) and conditioned media (B) were immunoprecipitated and analyzed separately by SDS-PAGE.

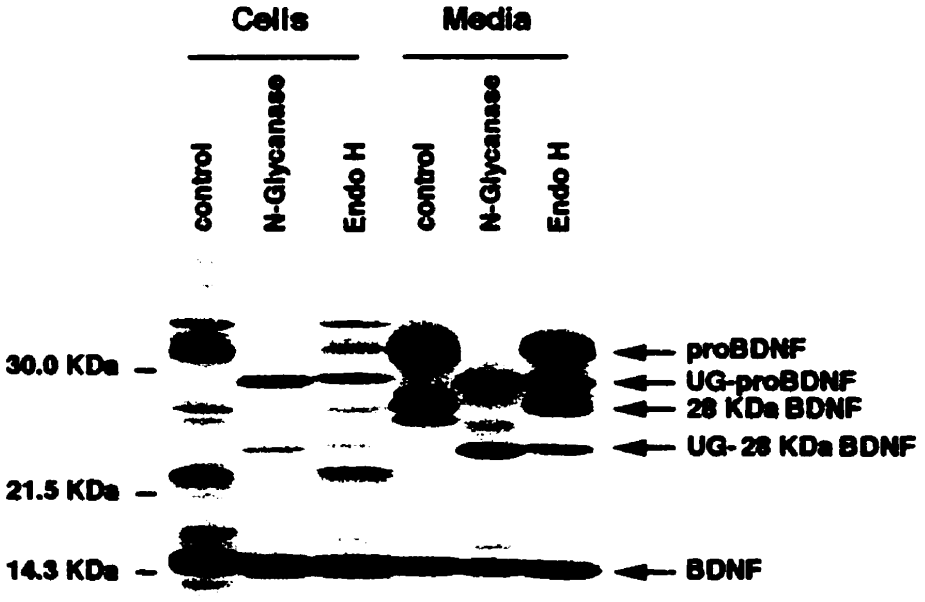
Cells
Media
control
N-Glycanase
Endo H
control
N-Glycanase
Endo H
30.0 KDa
21.5 KDa
14.3 KDa
proBDNF
UG-proBDNF
28 KDa BDNF
UG-28 KDa BDNF
BDNF

FIGURE 2.3 **Pro-BDNF but not mature BDNF is glycosylated**. AtT-20 cells were infected with vv:BDNF, incubated overnight in medium without virus, and labeled with [^{35}S] Cys-Met for 3 h. Conditioned media were collected, and either incubated with pre-immune serum (PI), or antibody to BDNF. Following immunoprecipitation, the samples were incubated in the absence (-) or presence (+) of endoglycosidase H or N-glycanase, and analyzed by SDS-PAGE.

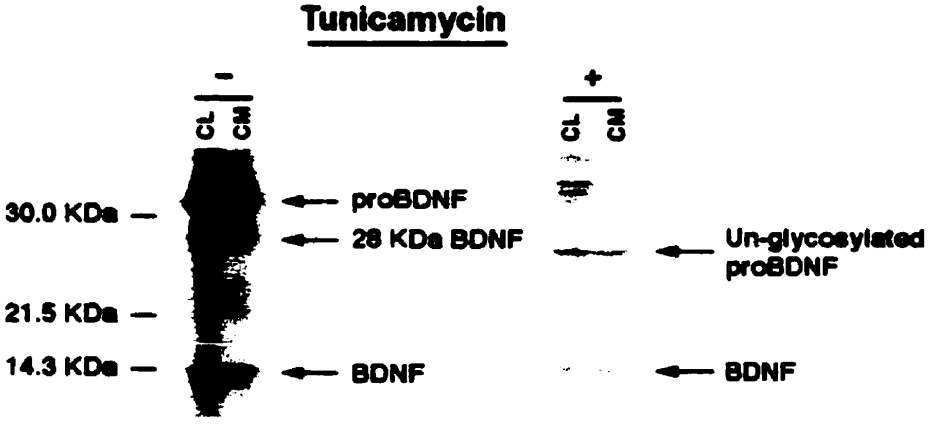
Tunicamycin
-
+
CL
CM
CL
CM
30.0 KDa
21.5 KDa
14.3 KDa
proBDNF
28 KDa BDNF
BDNF
Un-glycosylated proBDNF
BDNF

FIGURE 2.4 **N-glycosylation increases the stability of pro-BDNF**. AtT-20 cells were infected with vv:BDNF, pulse-labeled with [^{35}S] Cys-Met for 30 min and chased for 2 h in the absence (-) or presence (+) of 5 μg/ml tunicamycin. Immunoprecipitates from cell lysates (CL) and conditioned media (CM) were resolved by SDS-PAGE and exposed to film.

A
P 3 hours
CL
CM
B
N-Glycanase
–
+
32 KDa proBDNF
28 KDa BDNF
14 KDa BDNF

FIGURE 2.5 **pro-BDNF is glycosulfated**. A) AtT-20 cells were infected with vv:BDNF for 2 h, incubated overnight without virus, then labeled with [$Na_2{}^{35}SO_4$] for 3 h. Cell lysates (CL) and conditioned media (CM) were immunoprecipitated with antibodies to BDNF and the precipitate analyzed by SDS-PAGE. Pro-BDNF (32 kDa) along with a minor (28 kDa) form of the precursor (see below) are sulfated but mature BDNF is not. B) Samples of CM shown in panel A were incubated with (+) or without (-) N-glycanase, showing that sulfation occurs on carbohydrate chains within the precursor.

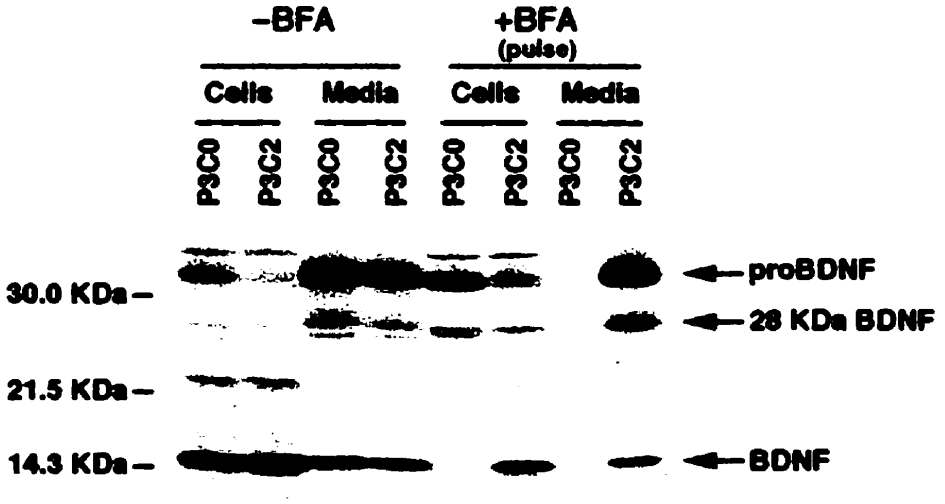
-BFA
+BFA
(pulse)
Cells
Media
Cells
Media
P3C0
P3C2
P3C0
P3C2
P3C0
P3C2
P3C0
P3C2
30.0 KDa –
21.5 KDa –
14.3 KDa –
proBDNF
28 KDa BDNF
BDNF

FIGURE 2.6 **Generation of 28 kDa BDNF occurs in the ER**. COS-7 cells were infected with vv:BDNF for 1 h, post infected for overnight and were metabolically labeled with [^{35}S] Cys-Met for 3 h in the presence (+) or absence (-) of brefeldin A (BFA, 5 μg/ml). Cells were then chased for 2 h in the absence of BFA.

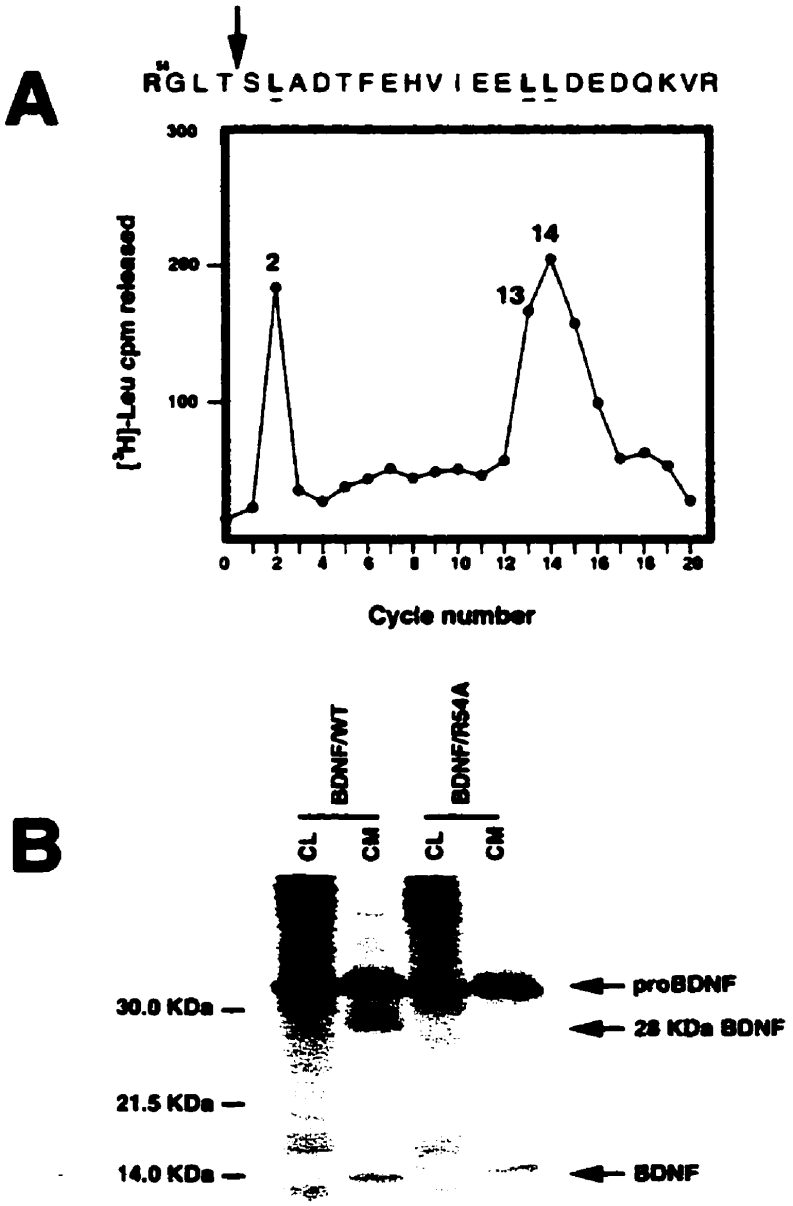
A
R G L T S L A D T F E H V I E E L L D E D Q K V R
300
200
100
[3H]-Leu cpm released
2
13
14
0 2 4 6 8 10 12 14 16 18 20
Cycle number
B
BDNF/WT
BDNF/R54A
CL
CM
CL
CM
30.0 KDa
21.5 KDa
14.0 KDa
proBDNF
28 KDa BDNF
BDNF

FIGURE 2.7 **Identification of the cleavage site within pro-BDNF that generates 28 kDa BDNF.** A) N-terminal microsequence analysis of [^{3}H] Leu-labeled 28 kDa BDNF. The N-terminal sequence of the 28 kDa product in COS-7 cells infected with vv:BDNF and vv:SKI-1 revealed a [^{3}H]Leu at positions 2, 13, and 14. This result demonstrates that 28 kDa BDNF is generated by a unique cleavage at Thr^{57} (arrow) in the sequence $R^{54}GLT^{57}\downarrow$SLADTFEHVIEELLD (top panel). B) Transient expression of the wild-type and R54A mutant form of pro-BDNF in COS-7 cells. COS-7 cells were transfected with expression constructs of the wild type (WT) or the Arg 54 to Ala mutant (R54A) form of pro-BDNF. Two days after transfection, cells were metabolically labeled with [^{35}S] Cys-Met for 6 hr, cell lysates (CL) and conditioned media (CM) were collected, immunoprecipitated with a BDNF-specific antiserum, and resolved by SDS-PAGE.

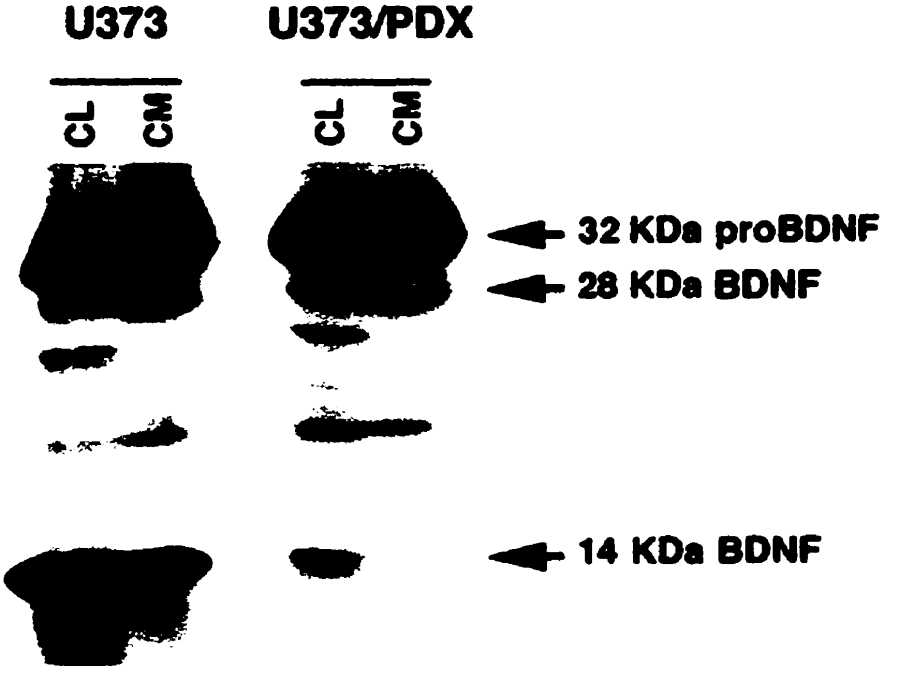
U373
U373/PDX
CL
CM
CL
CM
32 KDa proBDNF
28 KDa BDNF
14 KDa BDNF

FIGURE 2.8 **αl-PDX does not inhibit the generation of the 28 kDa form of BDNF**. U373 and U373-PDX cell lines were infected with vv: BDNF for 30 min, incubated overnight without virus, and labeled with [^{35}S] Cys-Met for 3 h. Cell lysates (CL) and conditioned media (CM) were immunoprecipitated and resolved by SDS-PAGE.

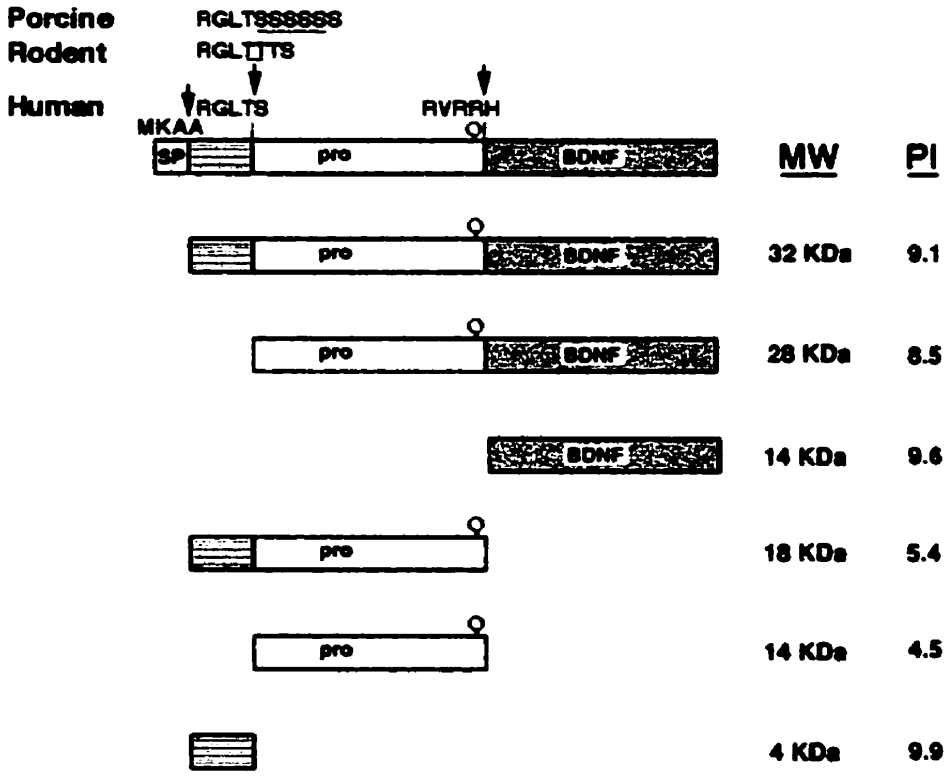
Porcine
Rodent
Human
RGLTSSSSSS
RGL□□TS
RGLTS
MKAA
RVRRH
SP
pro
BDNF
MW
PI
32 KDa
9.1
28 KDa
8.5
14 KDa
9.6
18 KDa
5.4
14 KDa
4.5
4 KDa
9.9

FIGURE 2.9 **Schematic diagram of pro-BDNF processing products.** At least six products are generated as a result of endogenous pre-pro-BDNF processing. N-terminal microsequencing reveals that the bands appearing in the 32 kDa range represent the BDNF precursor (the consensus cleavage site is indicated by the arrow in the sequence CMKA↓A) while RGLT↓S represents the consensus cleavage site generating the 28 kDa BDNF from the precursor .

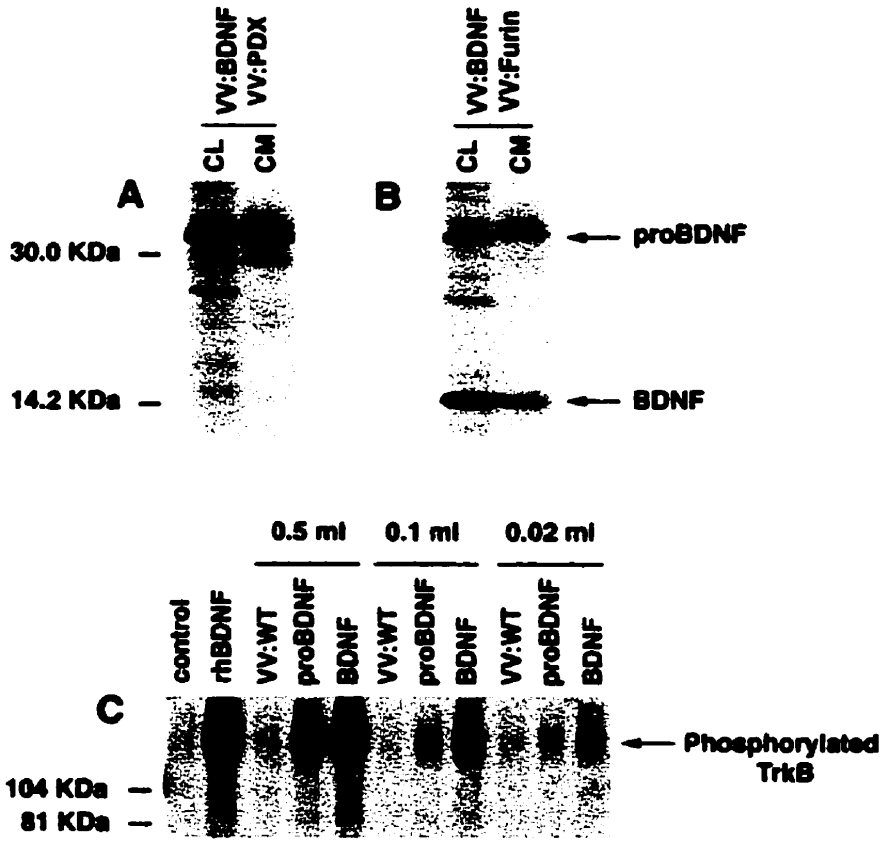
A
B
VV:BDNF
VV:PDX
VV:BDNF
VV:Furin
CL
CM
CL
CM
30.0 KDa
14.2 KDa
proBDNF
BDNF
0.5 ml
0.1 ml
0.02 ml
control
rhBDNF
VV:WT
proBDNF
BDNF
VV:WT
proBDNF
BDNF
VV:WT
proBDNF
BDNF
C
104 KDa
81 KDa
Phosphorylated
TrkB

FIGURE 2.10 **BDNF- and pro-BDNF-stimulated TrkB auto-phosphorylation.** (A and B) Metabolic labeling of LoVo cells co-infected with vv:BDNF/α1-PDX (A) or vv:pro-BDNF/Furin (B). The cells were labeled for 4 h and cell lysates (CL) and conditioned media (CM) were immunoprecipitated and analyzed by SDS-PAGE. C) Western blot analysis of TrkB phosphorylation levels in NIH 3T3-TrkB cells exposed for 5 min to either of the following media. Conditioned media from un-infected LoVo cells (control), DMEM with 100 μg/ml recombinant human BDNF (rhBDNF), conditioned medium from LoVo cells infected with wild-type vv (vv:WT), and conditioned medium from LoVo cells co-infected with vv: BDNF/vv:α1-PDX (pro-BDNF, B), or vv:BDNF and vv:Furin (BDNF, C). The cell lysates were immunoprecipitated with the pan Trk 203 antibody and analyzed by SDS-PAGE. Levels of phosphorylated TrkB were analyzed on Western Blot replicas with a phosphotyrosine antibody.

ACKNOWLEDGEMENT

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CHAPTER 3:

MAMMALIAN SUBTILISIN/KEXIN ISOZYME SKI-1: A WIDELY EXPRESSED PROPROTEIN CONVERTASE WITH A UNIQUE CLEAVAGE SPECIFICITY AND CELLULAR LOCALIZATION.

PREFACE

In the previous chapter, we reveal the post-translational modification of pro-BDNF. In doing so, we have identified a truncated form of pro-BDNF which appears to be generated via an alternative secretory pathway. The 28 kDa BDNF can be generated in ER and α1-PDX, a potent inhibitor of PC enzymes within constitutive pathway, does not affect its production. These findings suggest that a non-PC family enzyme might be involved in this process. In search of the enzyme responsible for the generation of 28 kDa BDNF, we have found, through collaboration with Dr Seidah's lab, a novel enzyme, subtilisin/kexin isozyme-1 (SKI-1). The enzyme is related to but distinct from PC family. Following works reveal that SKI-1 is capable of generating 28 kDa BDNF by cleaving pro-BDNF at $RGLT^{57}\downarrow S$; an interesting finding which identified SKI-1 as the first mammalian subtilisin-like enzyme capable of processing substrates at non-basic residues. Also the fact that generation of 28 kDa BDNF can occur in the ER, introduce SKI-1 as the earliest enzyme in the secretory pathway capable of processing its substrates.

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Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization

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ABSTRACT

Using reverse transcriptase-PCR and degenerate oligonucleotides derived from the active-site residues of subtilisin/kexin-like serine proteinases, we have identified a highly conserved and phylogenetically ancestral human, rat, and mouse type I membrane-bound proteinase called subtilisin/kexin-isozyme-1 (SKI-1). Computer databank searches reveal that human SKI-1 was cloned previously but with no identified function. In situ hybridization demonstrates that SKI-1 mRNA is present in most tissues and cells. Cleavage specificity studies show that SKI-1 generates a 28-kDa product from the 32-kDa brain-derived neurotrophic factor precursor, cleaving at an RGLTSL bond. In the endoplasmic reticulum of either LoVo or HK293 cells, proSKI-1 is processed into two membrane-bound forms of SKI-1 (120 and 106 kDa) differing by the nature of their N-glycosylation. Late along the secretory pathway some of the membrane-bound enzyme is shed into the medium as a 98-kDa form. Immunocytochemical analysis of stably transfected HK293 cells shows that SKI-1 is present in the Golgi apparatus and within small punctate structures reminiscent of endosomes. In vitro studies suggest that SKI-1 is a Ca^{2+}-dependent serine proteinase exhibiting a wide pH optimum for cleavage of pro-brain-derived neurotrophic factor.

INTRODUCTION

Limited proteolysis of inactive precursors to produce active peptides and proteins generates biologically diverse products from a finite set of genes. Most often, such processing occurs at either monobasic or dibasic residues as a result of cleavage by mammalian serine proteinases related to bacterial subtilisin and yeast kexin (Seidah et al., 1998a; Steiner 1998). These enzymes, known as proprotein convertases (PCs), cleave a variety of precursors at the consensus (R/K)-(Xaa)n-R sequence, where Xaa is any amino acid except Cys and n = 0, 2, 4, or 6 (Seidah et al., 1998a; Seidah et al., 1998b; Steiner 1998).

Less commonly than cleavage at basic residues, bioactive products also can be produced by limited proteolysis at amino acids such as L, V, M, A, T, and S (Seidah et al., 1998b). This type of cellular processing has been implicated in the generation of bioactive peptides such as - and -endorphin (Ling et al., 1976), the C-terminal glycopeptide fragment 1-19 of provasopressin (Burbach et al., 1986), platelet factor 4 (Gupta et al., 1995), the metalloprotease ADAM-10 (Rosendahl et al., 1997), site 1 cleavage of the sterol regulatory element-binding proteins (SREBPs) (Duncan et al., 1997), as well as in the production of the Alzheimer's amyloidogenic peptides A40, -42, and -43 (Checler 1995). Processing of this type occurs either in the endoplasmic reticulum (ER) (Duncan et al., 1997), late along the secretory pathway, within secretory granules (Burbach et al., 1986; Ling et al., 1976), at the cell surface, or in endosomes (Checler 1995; Gupta et al., 1995; Rosendahl et al., 1997). The proteinases responsible for these cleavages are not yet identified.

We hypothesized that an enzyme (or enzymes) distinct from, but related to, PCs may generate polypeptides by cleavage at nonbasic residues. To test that idea, we employed a reverse transcriptase-PCR (RT-PCR) strategy similar to the one used to identify the PCs (Seidah 1995), except that we used degenerate oligonucleotides closer to bacterial subtilisin than to yeast kexin. This resulted in the isolation of a

cDNA fragment encoding a putative subtilisin-like enzyme from human cell lines. This partial sequence was identical to a segment of a human myeloid cell-derived cDNA reported by Nagase et al. (Nagase et al., 1995). Preliminary results demonstrated that this putative proteinase cleaves pro-brain-derived neurotrophic factor (pro-BDNF) (Seidah et al., 1999; S.J.M. N.G.S., and R.A.M., unpublished results).

In this paper, we show that the sequences of rat, mouse, and human orthologues of this putative type I membrane-bound subtilisin-kexin-isoenzyme, which we called SKI-1, exhibit a high degree of sequence conservation. Tissue distribution analysis by both Northern blots and in situ hybridization revealed that SKI-1 mRNA is widely expressed. A vaccinia virus recombinant and a stable transfectant of human SKI-1 in HK293 cells allowed the analysis of its biosynthesis and intracellular localization. Finally, we present data demonstrating that SKI-1 cleaves at a specific T residue within the N-terminal segment of pro-BDNF. SKI-1 is thus identified as a mammalian secretory subtilisin/kexin-like enzyme capable of cleaving a proprotein at nonbasic residues.

MATERIALS AND METHODS

PCR and Sequencing - Most RT-PCRs were performed using a Titan One Tube RT-PCR system (Boehringer Mannheim) on 1 μg of total RNA isolated from a human neuronal cell line (IMR-32), mouse corticotrophic cells (AtT20), or rat adrenal glands. The active-site degenerate primers were as follows: His (sense) 5'-GGICA(C,T)GGIACI(C,T)(A,T)(C,T)(G,T)(T,G)IGCIGG-3' and Ser (antisense) 5'-CCIG(C,T)IACI(T,A)(G,C)IGGI(G,C)(T,A)IGCIACI(G,C)(A,T)IGTICC-3', based on the sequences GHGT(H,F)(V,C)AG and GTS(V,M)A(T,S)P(H,V)V(A,T)G, respectively. The amplified 525-bp products were sequenced on an automated laser fluorescence DNA sequencer (Pharmacia). To obtain the full-length sequence of rat and mouse SKI-1, we used PCR primers based on the human (Nagase et al., 1995) and mouse sequences, in addition to 5' (Edwards et al., 1991) and 3' (Lusson et al., 1993) RACE amplifications. At least three clones of the amplified cDNAs were sequenced. The GenBank accession numbers of the 3,788-bp mouse mSKI-1 cDNA and 3,895-bp rat rSKI-1 are AF094820 and AF094821, respectively.

Infection, Transfection, and Metabolic Labeling - Human SKI-1 (nucleotides 1-4338) (Nagase et al., 1995) in Bluescript (a generous gift from N. Nomura, Kazusa DNA Research Institute, Chiba, Japan; accession no. D42053) was digested with SacII (nucleotides 122-4338) and inserted into the vector PMJ602, and a vaccinia virus recombinant was isolated. The PMJ602 construct was also digested with 5' KpnI/3' NheI and cloned into the KpnI/XbaI sites of pcDNA3 (Invitrogen), and the cDNA was transfected into HK293 cells by using Lipofectin. A number of stable transfectants resistant to G418 and positive on Western blots using an SKI-1 antiserum (see below) were isolated, and one of them (clone 9), was investigated further. Either vaccinia virus-infected or -transfected cells were pulsed for 20 min with [^{35}S]cysteine and then chased for various times in the presence or absence of either tunicamycin (5 μg/ml) or brefeldin A (2.5 μg/ml). Media and cell lysates were immunoprecipitated with SKI-1 antisera directed against either amino acids 634-651 or 217-233, or a pro-SKI-1 antiserum directed against the prosegment comprising

amino acids 18-188 (Fig. 3.1). Immune complexes were resolved by SDS/PAGE on an 8% polyacrylamide/N [tris(hydroxymethyl)methyl]glycine gel (Seidah et al., 1996).

Northern Blots, in Situ Hybridizations, and Immunocytochemistry - Northern blot analyses (Marcinkiewicz et al., 1998) were done on total RNA from adult male rat tissues by using either a TRIzol reagent kit (Life Technologies, Gaithersburg, MD) or a Quick Prep RNA kit (Pharmacia) and on poly(A)+ RNA of (male + female) rat adult tissues (BIO/CAN, Montreal). The blots were hybridized overnight at 68°C in the presence of [^{32}P]UTP SKI-1 cRNA probes, which consisted of the antisense of nucleotides 655-1249 of rat SKI-1. For in situ hybridization on newborn rats, the same rat sense and antisense cRNA probes were labeled with uridine and cytosine 5'-[-[^{35}S]thio]triphosphate (Marcinkiewicz et al., 1998; Seidah et al., 1998a). For immunofluorescence staining we used a rabbit anti-SKI-1 antiserum at a 1:100 dilution and rhodamine-labeled goat anti-rabbit IgGs diluted 1:20 (Marcinkiewicz et al., 1998). Red SKI-1 immunostaining was compared with green-staining patterns of both fluorescein-labeled concanavalin A (Con A; Molecular Probes), an ER marker, or fluorescein-conjugated wheat germ agglutinin (WGA; Molecular Probes), a Golgi marker (Lippincott-Schwartz et al., 1989).

Ex Vivo and in Vitro pro-BDNF Processing - A vaccinia virus recombinant of human SKI-1 (vv:SKI-1) was isolated as described for human pro-BDNF (vv:BDNF) (Seidah et al., 1996). The vaccinia virus recombinants of α1-antitrypsin Pittsburgh (α1-PIT; vv:PIT) and α1-antitrypsin Portland (α1-PDX; vv:PDX) (Anderson et al., 1993) were generous gifts from G. Thomas (Vollum Institute, Portland, OR). COS-7 cells (4 _ 106) were coinfected with 1 plaque-forming unit (pfu) per cell of vv:BDNF and either the wild-type virus (vv:WT) alone at 2 pfu per cell or with 1 pfu per cell of each virus in the combinations [vv:SKI-1+vv:WT], [vv:SKI-1+vv:PIT], and [vv:SKI-1+vv:PDX]. At 10 h postinfection, cells were pulsed for 4 h with 0.2 mCi of [^{35}S]cysteine/[^{35}S]methionine (DuPont). Media and cell extracts were immunoprecipitated with a BDNF antiserum (ref. 19; provided by Amgen) at 0.5

μg/ml, and the proteins were resolved on SDS/PAGE 13-22% gradient gels (Seidah et al., 1996). [^{35}S]Met-labeled 32-kDa pro-BDNF and [^{3}H]Leu-labeled 28-kDa BDNF were sequenced as described (Paquet et al., 1994). For in vitro analysis, 32-kDa pro-BDNF obtained from the media of LoVo cells infected with vv:BDNF was incubated overnight with the shed form of SKI-1 obtained from cells coinfected with vv:SKI-1 and vv:PDX, either at different pH values or at pH 6.5 in the presence of selected inhibitors: pepstatin (1 μM), antipain (50 μM), cystatin (5 μM), E64 (5 μM), soybean trypsin inhibitor (SBTI, 5 μM), 0.5 M phenylmethylsulfonyl fluoride (PMSF) + 50 μM para-amino-PMSF (pAPMSF), o-phenanthroline (5 mM), and EDTA (10 mM). The products were resolved by SDS/PAGE on a 15% polyacrylamide gel, blotted, and then probed with a BDNF antiserum (Santa Cruz Biotechnology) at a dilution of 1:1,000.

RESULTS

Protein Sequence Analysis of SKI-1 - We first aligned the protein sequences within the catalytic domain of PC7 (Seidah et al., 1996b), yeast subtilases, and bacterial subtilisins, together with that of a novel Plasmodium falciparum subtilisin-like enzyme called pf-SUB2 (J.-C.B., unpublished results). This led to the choice of conserved amino acids GHGT(H/F)(V/C)AG and GTS(M/V)A(T/S)P(H/V)V(A/T)G around the active sites His and Ser, respectively. Thus, using degenerate oligonucleotides coding for the sense His and antisense Ser consensus sequences, we initiated a series of RT-PCRs on total RNA and isolated a 525-bp cDNA fragment from the human neuronal cell line IMR-32. This sequence was found to be 100% identical to that reported for a human cDNA called KIAA0091 obtained from a myeloid KG-1 cell line (Nagase et al., 1995) and 88% identical to that of a 324-bp expressed sequence tag (accession no. H31838) from rat PC12 cells. The full-length rat and mouse cDNA sequences were obtained after RT-PCR amplifications of total RNA isolated from rat adrenal glands and PC12 cells and from mouse AtT20 cells. As shown in Fig. 3.1, alignment of the protein sequence deduced from the cDNAs revealed that rat and mouse SKI-1 share 98% sequence identity and a 96% identity to human SKI-1. Interestingly, within the catalytic domain (Asp^{218} _ Ser^{414}) the sequence similarity between the three species is 100%. Analysis of the predicted amino acid sequence suggests a 17-aa signal peptide, followed by a putative prosegment beginning at Lys^{18} and extending for some 160-180 aa. The proposed catalytic domain encompasses the typical active sites Asp^{218}, His^{249}, and Ser^{414} and the oxyanion hole Asn^{338}. This domain is followed by an extended C-terminal sequence characterized by the presence of a conserved growth factor/cytokine receptor family motif $\underline{C}^{849}\underline{L}DDSHRQK\underline{DC}F\underline{W}^{861}$. This sequence is followed by a potential 24-aa hydrophobic transmembrane segment and a less-conserved 31-aa cytosolic tail that, remarkably, consists of 35% basic residues. Some of the clones isolated from rat adrenal glands suggested the existence of alternatively spliced rSKI-1 mRNAs in which the segments coding for amino acids 430-483 or 858-901 are absent. Finally, the phylogenetic tree derived from the alignment of the catalytic

domain of SKI-1 with subtilases (Siezen and Leunissen 1997) suggests that it is an ancestral protein that is closer to plant and bacterial subtilases than to either yeast or mammalian homologues (not shown).

Tissue Distribution of SKI-1 mRNA - Northern blot analyses of SKI-1 mRNA in adult male rat reveal that rSKI-1 mRNA is widely expressed and is particularly rich in anterior pituitary, thyroid, and adrenal glands (Fig. 3.2A). A Northern blot of poly (A)+ RNA obtained from mixed adult male and female rat tissues also showed a wide distribution and a particular enrichment in liver (Fig. 3.2B). Similarly, analysis of 24 different cell lines (Seidah et al., 1994) revealed a ubiquitous expression of SKI-1 mRNA (not shown).

In situ hybridization data obtained in a day 2 postnatal rat also provided evidence of a widespread, if not ubiquitous, distribution of rSKI-1 mRNA. Fig. 3.3 shows at the anatomical level the presence of SKI-1 mRNA in developing skin, striated muscles, cardiac muscles, bones, and teeth as well as brain and many internal organs. Strong hybridization signals were detectable in the retina, cerebellum, pituitary, submaxillary, thyroid, and adrenal glands, molars, thymus, kidney, and intestine. Evidence for the cellular expression of rSKI-1 mRNA was obtained from analysis of the relative labeling densities per cell in selected tissues, based on a semiquantitative analysis of emulsion autoradiographs (not shown). In the central nervous system rSKI-1 mRNA labeling was mostly confined to neurons, whereas ependymal cells and supportive glial cells, such as presumed astrocytes, oligodendrocytes, and microglia, exhibited 5- to 30-fold-less labeling per cell. In addition, within the peripheral nervous system, trigeminal ganglia revealed a 5- to 10-fold greater expression in neurons as compared with presumptive Schwann cells. Labeling was observed in most of the glandular cells in the anterior and intermediate lobes of the pituitary as well as in the pituicytes of the pars nervosa. A semiquantitative comparison in the adult and newborn rat pituitary gland, submaxillary gland, thymus, and kidney demonstrated an overall 2-fold-decreased labeling of rSKI-1 mRNA with age (not shown).

Biosynthesis of hSKI-1 - To define the molecular forms of human SKI-1 we generated both a vaccinia virus recombinant (vv:SKI-1) and a stable transfectant in HK293 cells. Three antisera were produced against amino acids 18-188 (prosegment), 217-233, and 634-651 of SKI-1. Expression of vv:SKI-1 in four different cell lines revealed that the enzyme is synthesized as a 148-kDa proSKI-1 zymogen, which is processed progressively into 120-, 106-, and 98-kDa proteins (Fig. 3.4). Only the 148-kDa form is recognized by the prodomain antiserum, whereas all four forms react with the other two antisera. Processing of the 148-kDa proSKI-1 into the 120- and 106-kDa forms occurs in the ER based on the presence of these proteins in cells preincubated with the fungal metabolite brefeldin A (Fig. 3.4; ref. 24). Furthermore, preincubation with tunicamycin revealed only two bands (Fig. 3.4), suggesting that the presumably membrane-bound 106- and 120-kDa forms differ by their N-glycosylation. At the 3-h chase time, results reveal the secretion of a 98-kDa shed form (sSKI-1) recognized by both SKI-1 antisera (Fig. 3.4) but not by the proSKI-1 antiserum (not shown). Similar SKI-1-related forms were seen in stably transfected HK293 cells after a 4-h pulse labeling with [^{35}S]methionine (not shown).

Intracellular Localization of SKI-1 - Double-staining immunofluorescence was used to compare the intracellular localization of the stably transfected human SKI-1 in HK293 cells with that of either the ER or Golgi markers Con A and WGA (Lippincott-Schwartz et al., 1989), respectively. The data show that SKI-1 exhibits (i) perinuclear staining, colocalizing with Con A fluorescence, presumably corresponding to the ER (not shown); (ii) paranuclear staining colocalizing with WGA fluorescence, suggesting the presence of SKI-1 in the Golgi (Fig. 3.5 A and B), and (iii) punctate staining observed in the cytoplasm and within extensions of a few cells (Fig. 3.5A). Some, but not all, of the punctate immunostaining matched that observed with WGA. This suggests that SKI-1 localizes in the Golgi but may sort to other organelles, including lysosomal and/or endosomal compartments. An indication of lysosomal/endosomal localization was provided by the analysis of SKI-1 immunofluorescence within cells preincubated for 4 h with 10 mM leucine methyl

ester, a specific lysosomal/endosomal protease inhibitor (Reeves et al., 1981). The results showed a net increase in the proportion of cells exhibiting punctate staining as compared with control cells (Fig. 3.5 A and B). The relative proportions of SKI-1 in cellular organelles and their dependence on culture conditions are now amenable to evaluation by subcellular fractionation and electron microscopy.

Enzymatic Activity and Cleavage Specificity of SKI-1 - To prove that SKI-1 is a proteolytic enzyme we examined its ability to cleave five different potential precursor substrates, including pro-opiomelanocortin, pro-atrial natriuretic factor, HIV gp160, pro-nerve growth factor, and pro-BDNF. Cellular coexpression of vv:SKI-1 with the vaccinia virus recombinants of each of the above precursors revealed that only pro-BDNF was cleaved intracellularly by SKI-1. Thus, upon expression of vv:BDNF alone in COS-7 cells we observed a partial processing of pro-BDNF (32 kDa) into the known, major 14-kDa BDNF product (Seidah et al., 1996a) and the minor production of a previously observed (Marcinkiewicz et al., 1998; S.J.M., N.G.S., and R.A.M., unpublished results) but still undefined 28-kDa product (Fig. 3.6A). Upon coexpression of pro-BDNF and SKI-1, a net increase in the level of the secreted 28-kDa BDNF is evident, without significant alteration in the amount of 14-kDa BDNF (Fig. 3.6A). To examine whether the 28-kDa product results from cleavage at a basic residue or at an alternative site, we first coexpressed pro-BDNF, SKI-1, and either α1-PIT or α1-PDX, which are inhibitors of thrombin and PC cleavages, respectively (Anderson et al., 1993; Benjannet et al., 1997). The results show that different from α1-PIT, the serpin α1-PDX selectively blocks the production of the 14-kDa BDNF and that neither α1-PIT nor α1-PDX affects the level of the 28-kDa product. This finding shows that α1-PDX effectively inhibits the endogenous furin-like enzyme(s) responsible for the production of the 14-kDa BDNF (Seidah et al., 1996a), but does not inhibit the ability of SKI-1 to generate the 28-kDa product. Thus, it is likely that the generation of the 28-kDa BDNF takes place via an alternate cleavage. Incubation of the cells with brefeldin A or the Ca2+ ionophore A23187 revealed that the 28-kDa pro-BDNF is formed in the ER and that this cleavage is Ca^{2+}-dependent (not shown).

In Fig. 3.6B, we present the N-terminal microsequence analysis of [^{35}S]Met-labeled 32-kDa pro-BDNF and [^{3}H]Leu-labeled 28-kDa BDNF. The sequence of the 32-kDa form revealed the presence of an [^{35}S]Met at position 3 (Fig. 3.6B), which is in agreement with the proposed sequence of human pro-BDNF (Maisonpierre et al., 1991) resulting from the removal of an 18-aa signal peptide cleaved at GCMLA^{18}↓APMK site. The N-terminal sequence of the 28-kDa product revealed a [^{3}H]Leu at positions 2, 13, and 14 (Fig. 3.6B). This result demonstrates that the 28-kDa BDNF is generated by a unique cleavage at Thr^{57} in the sequence RGLT^{57}↓SLADTFEHVIEELL (Maisonpierre et al., 1991).

To prove that SKI-1 is directly responsible for the production of the 28-kDa BDNF at the novel Thr-directed cleavage, pro-BDNF was incubated at various pH values with concentrated medium of vv:SKI-1-infected Schwann cells. A similar preparation obtained from wild-type vaccinia virus-infected cells served as control. The data show that SKI-1 exhibits a wide pH-dependence profile revealing activity at both acidic and neutral pH values from pH 5.5 to 7.3 (Fig. 3.7A). Analysis of the inhibitory profile of this reaction revealed that metal chelators such as EDTA and o-phenanthroline or a mixture of the serine proteinase inhibitors PMSF and pAPMSF effectively inhibits the processing of pro-BDNF by SKI-1. The inhibition by EDTA is expected because, like all PCs, SKI-1 is a Ca^{2+}-dependent enzyme. The unexpected inhibition by 5 mM o-phenanthroline may be a result of excess reagent because at 1 mM only 25% inhibition is observed (not shown). All other class-specific proteinase inhibitors (aspartyl-, cysteinyl-, and serine proteases- of the trypsin type) proved to be inactive.

DISCUSSION

This work provides evidence for the existence of a mammalian secretory Ca^{2+}-dependent serine proteinase of the subtilisin/kexin type that selectively cleaves at nonbasic residues. Thus, SKI-1 processes the 32-kDa human pro-BDNF at an **K**AGS**R**GL**T**↓**SL** sequence, generating a 28-kDa form, which may have its own biological activity (S.J.M., N.G.S., and R.A.M., unpublished results). Such a cleavage site is close to the consensus site deduced from a large body of work done with the PCs, whereby an (**R/K**)-(X)n-**R**↓X-(**L/I/V**), (where n = 0, 2, 4, or 6) motif is favored by most PCs (Nakayama 1997; Seidah et al., 1998a; Seidah et al., 1998b; Steiner 1998). Note that in the SKI-1 site, P1 Arg is replaced by Thr and an aliphatic Leu is present at P2', an amino acid also favored by PCs (Nakayama 1997; Seidah et al., 1998a; Seidah et al., 1998b; Steiner 1998). Several proteins are known to be cleaved after Thr. These include human antiangiogenic platelet factor 4 (Gupta et al., 1995; QCLCVKTT↓SQ), the neuroendocrine -endorphin (Ling et al., 1976; KSQTPLVT↓LF), and ADAM-10 metalloprotease (Rosendahl et al., 1997; LLRKKRTT↓SA).

Interestingly, comparison of the phylogenetically highly conserved sequence of pro-BDNF revealed an insertion of hydroxylated amino acids (threonine and serine) just after the identified SKI-1 cleavage site of human pro-BDNF. Thus, in rat and mouse pro-BDNF, two threonines are inserted (RGLTTTSL), and in porcine pro-BDNF, five serines are added (RGLTSSSSSSL) (Maisonpierre et al., 1991). These observations raise a number of questions: (i) Do these insertions affect the kinetics of pro-BDNF cleavage by SKI-1? In that context, it was published recently that rat pro-BDNF is also cleaved into a 28-kDa protein (Haubensak et al., 1998). (ii) Does SKI-1 recognize both single and pairs of Thr and Ser and combinations thereof? (iii) Is the presence of a basic residue at P4, P6, or P8 critical for cleavage?

Another question that arises is whether SKI-1 can cleave at residues other than Thr. In that context, after submission of this manuscript, Sakai et al.

Demonstrated that the sequence of hamster S1p responsible for the site 1 cleavage of SREBPs is almost identical to the presently reported human, mouse, and rat SKI-1 (Sakai et al., 1998). In this model, within the lumen of the ER, S1p cleaves SREBP-2 at an **R**SV**L**↓SF sequence, where Arg at P4 is very critical, whereas the P1 Leu could be replaced by a number of other amino acids (Duncan et al., 1997). Our in vitro data show that sSKI-1 does not cleave small fluorogenic substrates of sequence **R**GL**T**-MCA, **R**GLTT**T**-MCA, or **R**SV**L**-MCA (MCA is 4-methylcoumaryl-1-amide), suggesting that it has an extended substrate-specificity pocket. In agreement, preliminary data show that SKI-1 specifically cleaves at neutral pH a 27-mer synthetic peptide of sequence GGAHDSDQHPHSGSG**R**S V**L**↓S**F**ESGSGG, representing the luminal amino acids 504-530 of human SREBP-2 (Duncan et al., 1997) (B.B.T. and N.G.S., unpublished data). We have shown that this synthetic peptide is efficiently processed by SKI-1 in vitro, paving the way for refined kinetic analyses.

Biosynthetic analysis of the zymogen processing of proSKI-1 demonstrated an ER-associated removal of the prosegment (Fig. 3.4). Furthermore, analysis of the ^{35}SO4-labeled SKI-1 demonstrated the presence of only sulfated 106- and 98-kDa forms but not that of either the 148 proSKI-1 or the 120-kDa SKI-1a forms (not shown). Because sulfation occurs in the trans-Golgi network, this confirms that the removal of the prosegment occurs in the ER. As with furin and PC5-B (Seidah et al., 1998a; Steiner 1998; Seidah et al., 1998b; de Bie et al., 1996) the membrane-bound 106-kDa SKI-1 is transformed into a soluble 98-kDa form. The secreted 98-kDa sSKI-1 is enzymatically active because it processes pro-BDNF in vitro (Fig. 3.7). Attempts to sequence the SDS/PAGE-purified [^{3}H]Leu- and Val-labeled 148- and 98-kDa forms resulted in ambiguous results, suggesting that SKI-1 is refractory to N-terminal Edman degradation. Presently, we are unable to define the zymogen cleavage site leading to the formation of the 120-kDa SKI-1a and 106-kDa SKI-1b deduced by pulse-chase studies (Fig. 3.4). Examination of the prosegment sequence (Fig. 3.1), the species-specific pro-BDNF motif potentially recognized by SKI-1, the sequence of the luminal portion of SREBP-2 (see above), and the alignment of SKI-1

with other subtilases (Siezen and Leunissen 1997) suggests three possible conserved sites: **R**A S**L**^{167}_SL GS, **R**HS**S**^{182}_RRLL, and **R**RL**L**^{186}-RAIP. These predict cleavages at motifs containing a P4 Arg and a P1 either Leu or Ser.

Phylogenetic structural analysis of the predicted amino acid sequence of SKI-1 reveals that this serine proteinase is closer to plant and bacterial subtilases than it is to yeast and mammalian PCs. The 100% conservation of the catalytic domain sequence, although striking and suggestive of an important function, is not far from the 98% similarity between human and rat PC7 (Seidah et al., 1998b; Seidah et al., 1996b). The sequence C-terminal to the catalytic domain of SKI-1 is very different from that of any of the known PCs. In fact, although PCs have a typical P-domain critical for the folding of these enzymes (for reviews see refs. Seidah et al., 1998a; Seidah et al., 1998b; Steiner 1998), we did not find the hallmark sequences (Lipkind et al., 1998; Seidah et al., 1998b) of the P-domain within the SKI-1 structure. Instead, different from the PCs, we find a conserved growth factor/cytokine receptor motif of which functional importance will need to be addressed, especially because this motif is partly missing in alternatively spliced forms (Fig. 3.1). Finally, the highly basic nature of the cytosolic tail of SKI-1 (Fig. 3.1) may be critical for its probable cellular localization within endosomal/lysosomal compartments (Fig. 3.5), similar to the importance of basic residues for the accumulation of the -amidation enzyme PAM in endosomal compartments (S. L. Milgram, personal communication).

The wide tissue distribution of SKI-1 mRNA transcripts suggests that this enzyme processes numerous precursors in various tissues. Furthermore, the observed developmental down-regulation of the level of its transcripts also suggests a functional importance during embryonic development. That SKI-1 can cleave C-terminal to Thr, Leu, and, possibly, Ser residues suggests that, like the combination of PCs and carboxypeptidases E and D (Varlamov and Fricker 1998), a specific carboxypeptidase also may be required to trim out the newly exposed C-terminal hydroxylated or Leu residues.

SKI-1 is closest to the pyrolysin branch of the six-membered family of subtilisin-like proteinases (Siezen and Leunissen 1997) and we believe is the first known mammalian subtilase cleaving at sites other than basic amino acids. That other eukaryotic subtilases exist is supported by a recent report on the structure of a soluble subtilisin-like enzyme called PfSUB-1 found in Plasmodium falciparum (Blackman et al., 1998) and exhibiting a 29% sequence identity to SKI-1. This enzyme, which is closest to the subtilisin branch of subtilases (Siezen and Leunissen 1997), localizes to granular-like compartments and presumably cleaves at a Leu-Asn bond (Blackman et al., 1998). Therefore, because only mammalian members of the kexin (PCs) and pyrolysin (SKI-1) subfamilies have been identified, could it be that the other four subtilase subfamilies (Siezen and Leunissen 1997) have their mammalian counterparts?

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SKI-1, subtilisin/kexin-isozyme-1; PC, proprotein convertase; ER, endoplasmic reticulum; BDNF, brain-derived neurotrophic factor; SREBP, sterol regulatory element-binding protein; RT-PCR, reverse transcriptase-PCR; vv, vaccinia virus; PDX, α1-antitrypsin Portland; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride.

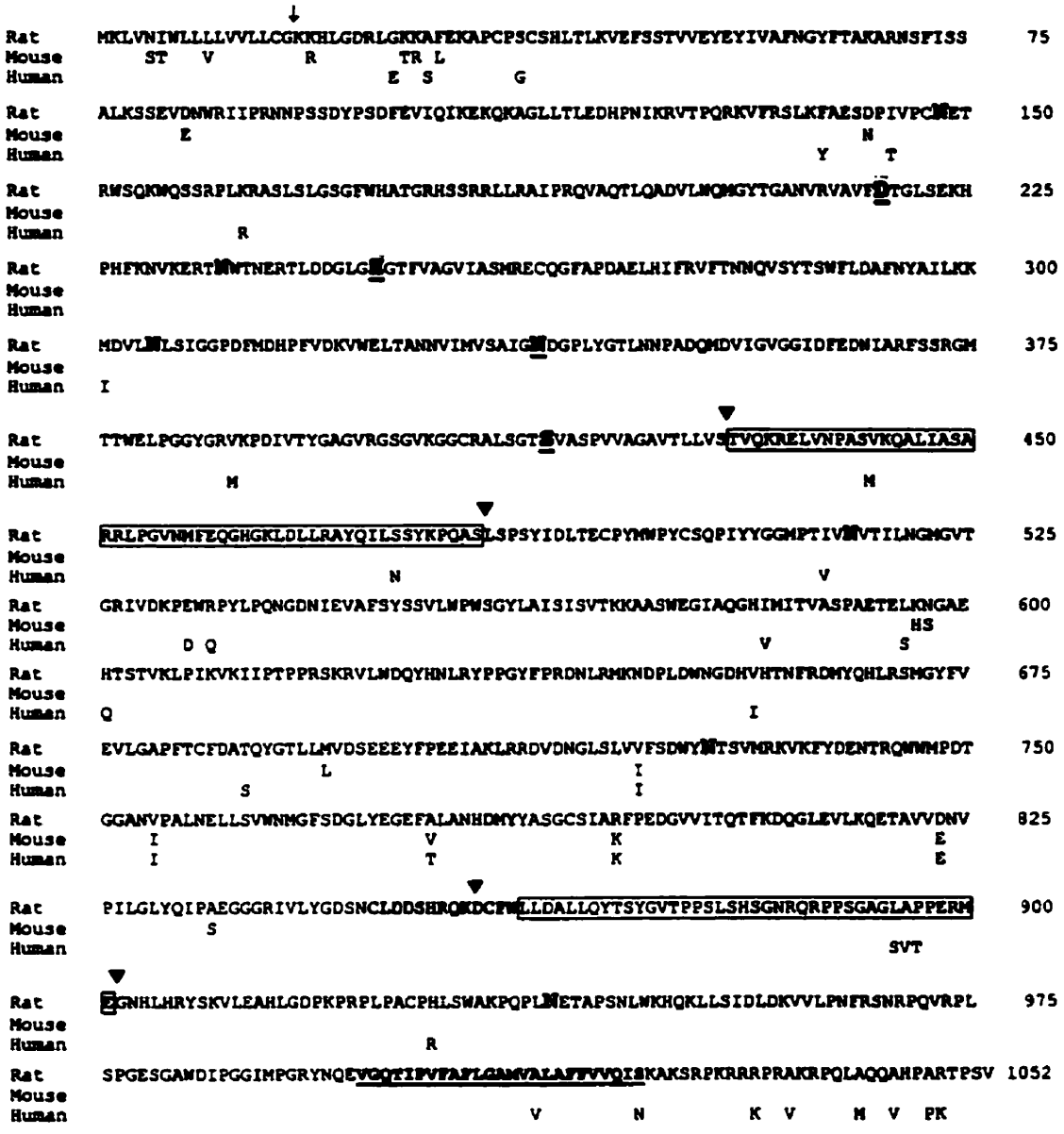
Rat MKLVNIWLLLLVVLLCGKKHLGDRLGKKAFEKAPCPSCSHLTLKVEFSSTVVEYEYIVAFNGYFTAKARNSFISS 75
Mouse ST V R TR L
Human E S G
Rat ALKSSEVDNWRIIPRNNPSSDYPSDFEVIQIKEKQKAGLLTLEDHPNIKRVTPQRKVFRSLKFAESDPIVPC ET 150
Mouse E N
Human Y T
Rat RWSQKMQSSRPLKRASLSLGSGFWHATGRHSSRRLLRAIPRQVAQTLQADVLNQMGYTGANVRVAVFD TGLSEKH 225
Mouse
Human R
Rat PHFKNVKERT WTNERTLDDGLG GTFVAGVIASMRECQGFAPDAELHIFRVFTNNQVSYTSWFLDAFNYAILKK 300
Mouse
Human
Rat MDVL LSIGGPDFMDHPFVDKVWELTANNVIMVSAIG DGPLYGTLNNPADQMDVIGVGGIDFEDNIARFSSRGM 375
Mouse
Human I
Rat TTWELPGGYGRVKPDIVTYGAGVRGSGVKGGCRALSGT VASPVVAGAVTLLVS TVQKRELVNPASVKQALIASA 450
Mouse
Human M M
Rat RRLPGVNMFEQGHGKLDLLRAYQILSSYKPQAS LSPSYIDLTECPYMWPYCSQPIYYGGMPTIV VTILNGMGVT 525
Mouse
Human N V
Rat GRIVDKPEWRPYLPQNGDNIEVAFSYSSVLWPWSGYLAISISVTKKAASWEGIAQGHIMITVASPAETELKNGAE 600
Mouse HS
Human D Q V S
Rat HTSTVKLPIKVKIIPTPPRSKRVLWDQYHNLRYPPGYFPRDNLRMKNDPLDWNGDHVHTNFRDMYQHLRSMGYFV 675
Mouse
Human Q I
Rat EVLGAPFTCFDATQYGTLLMVDSEEEYFPEEIAKLRRDVDNGLSLVVFSDNY TSVMRKVKFYDENTRQWWMPDT 750
Mouse L I
Human S I
Rat GGANVPALNELLSVWNMGFSDGLYEGEFALANHDMYYASGCSIARFPEDGVVITQTFKDQGLEVLKQETAVVDNV 825
Mouse I V K E
Human I T K E
Rat PILGLYQIPAEGGGRIVLYGDSNCLDDSHRQKDCPW LLDALLQYTSYGVTPPSLSHSGNRQRPPSGAGLAPPERM 900
Mouse S
Human SVT
Rat GNHLHRYSKVLEAHLGDPKPRPLPACPHLSWAKPQPL ETAPSNLWKHQKLLSIDLDKVVLPNFRSNRPQVRPL 975
Mouse
Human R
Rat SPGESGAMDIPGGIMPGRYNQ EVGQTIPVFAFLGAMVALAFVVQIS KAKSRPKRRRPRAKRPQLAQQAHPARTPSV 1052
Mouse
Human V N K V M V PK

FIGURE 3.1 **Comparative protein sequences of SKI-1 deduced from rat, mouse, and human cDNAs.** The position of the predicted end of the 17-aa signal peptide is shown by an arrow. The active sites Asp^{218}, His^{249}, and Ser^{414} as well as the oxyanion hole Asn^{338} are shown as bold, shaded, and underlined characters. The positions of the six potential N-glycosylation sites are emphasized in bold. The conserved shaded sequence fits the consensus signature for growth factors and cytokine receptor family. Each of the two boxed sequences was absent (_) in a number of rat clones. The predicted transmembrane segment is in bold and underlined.

Liver
Thymus
Spleen
Kidney
Heart
Brain
3
4
5
6
Kb
B

Adrenals
Thyroid
Striatum
Hippocam
Hypothal
Pineal
Pit-AP
Pit-NIL
Submax
Thyroid
2.37
4.40
7.46
Kb
Prostate
Ovary
Uterus
A

FIGURE 3.2 **Northern blot analysis of the expression of SKI-1 in adult rat tissues.** (A) Five micrograms of male rat total RNA was loaded in each lane: pituitary anterior (AP) and neurointermediate (NIL) lobes and submaxillary gland (submax). (B) Two micrograms of poly(A)+ RNA of (male + female) Sprague-Dawley rat adult tissues. The estimated size of rat SKI-1 mRNA is about 3.9 kb.

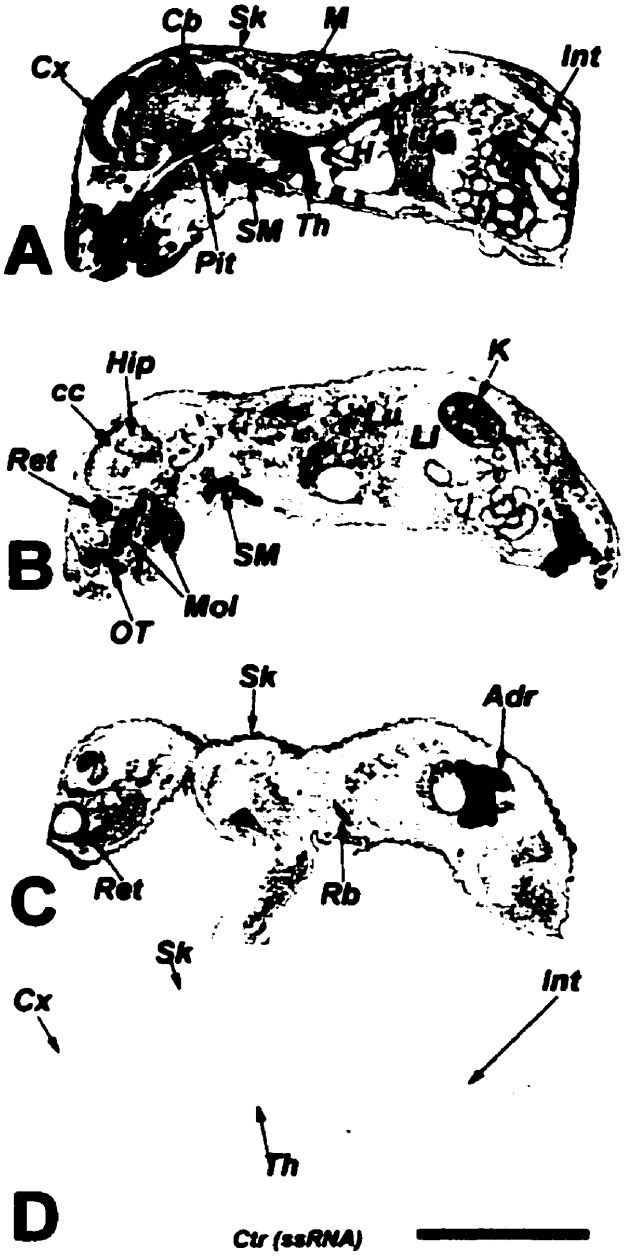
Cb
Sk
M
Cx
Int
H
SM
Th
A
Pit
Hip
K
cc
Li
Li
Ret
SM
B
Mol
OT
Sk
Adr
Ret
Rb
C
Sk
Int
Cx
Th
D
Ctr (ssRNA)

FIGURE 3.3 **In situ hybridization of rSKI-1 mRNA in a 2-day-old rat**. In situ hybridization is shown at anatomical resolution on x-ray film using an ^{35}S-labeled antisense riboprobe (A-C) and sense control riboprobe (D). Adr, adrenal gland; Cb, cerebellum; cc, corpus callosum; Cx, cerebral cortex; H, heart; Int, intestine; K, kidney; Li, liver; Lu, lungs; M, muscles; Mol, molars; OT, olfactory turbinates; Pit, pituitary gland; Rb, ribs; Ret, retina; Sk, skin, SM, submaxillary gland; Th, thymus. [_4; bar (D) = 1 cm.]

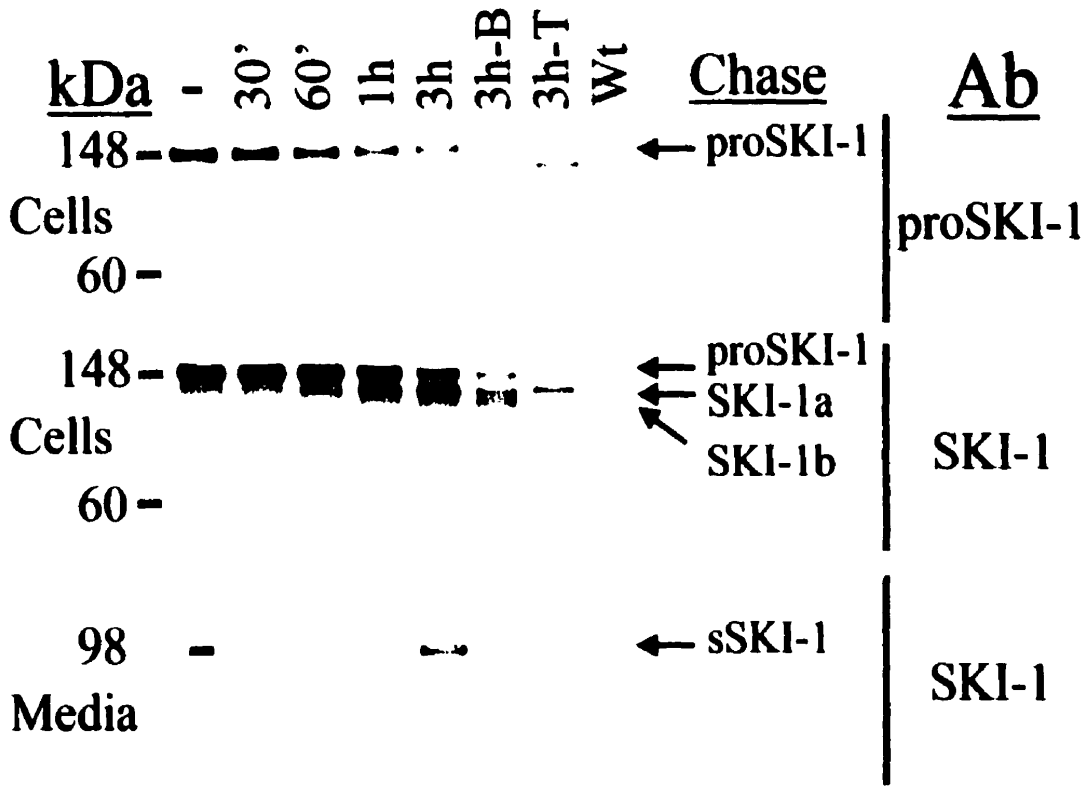
[35S]Cys, Pulse 20' in LoVo cells
kDa
-
30'
60'
1h
3h
3h-B
3h-T
Wt
Chase
Ab
148
proSKI-1
Cells
60
proSKI-1
148
proSKI-1
SKI-1a
SKI-1b
Cells
60
SKI-1
98
sSKI-1
Media
SKI-1

FIGURE 3.4 **Biosynthetic analysis of SKI-1 in LoVo-C5 cells overexpressing vv:SKI-1.** Cells were pulsed for 20 min with [^{35}S]cysteine and chased for 30 min, 1 h, and 3 h in the absence or presence of either brefeldin A (3h-B) or tunicamycin (3h-T). The control represents the 3-h chase period for cells infected with the wild-type virus (Wt). Media and cell lysates were immunoprecipitated with either a SKI-1 antiserum (Ab: SKI-1; against amino acids 634-651) or a proSKI-1 antiserum. The arrows point to the 148-, 120-, 106-, and 98-kDa forms immunoprecipitated.

FIGURE 3.5 **hSKI-1 immunoreactivity in stably transfected HK293 cells.** Black and white representation of the comparative double (red and green) fluorescence staining using an SKI-1 antiserum (directed against amino acids 634-651) (A and B) and fluorescein isothiocyanate-labeled WGA (A' and B') in control (A and A') and leucine methyl ester (LME)-treated (B and B') cells. Thin arrows emphasize the observed punctate staining, which is enhanced in the presence of LME. Large arrows point to the coincident staining of SKI-1 and WGA. [_900; bar (B') = 10 μm.]

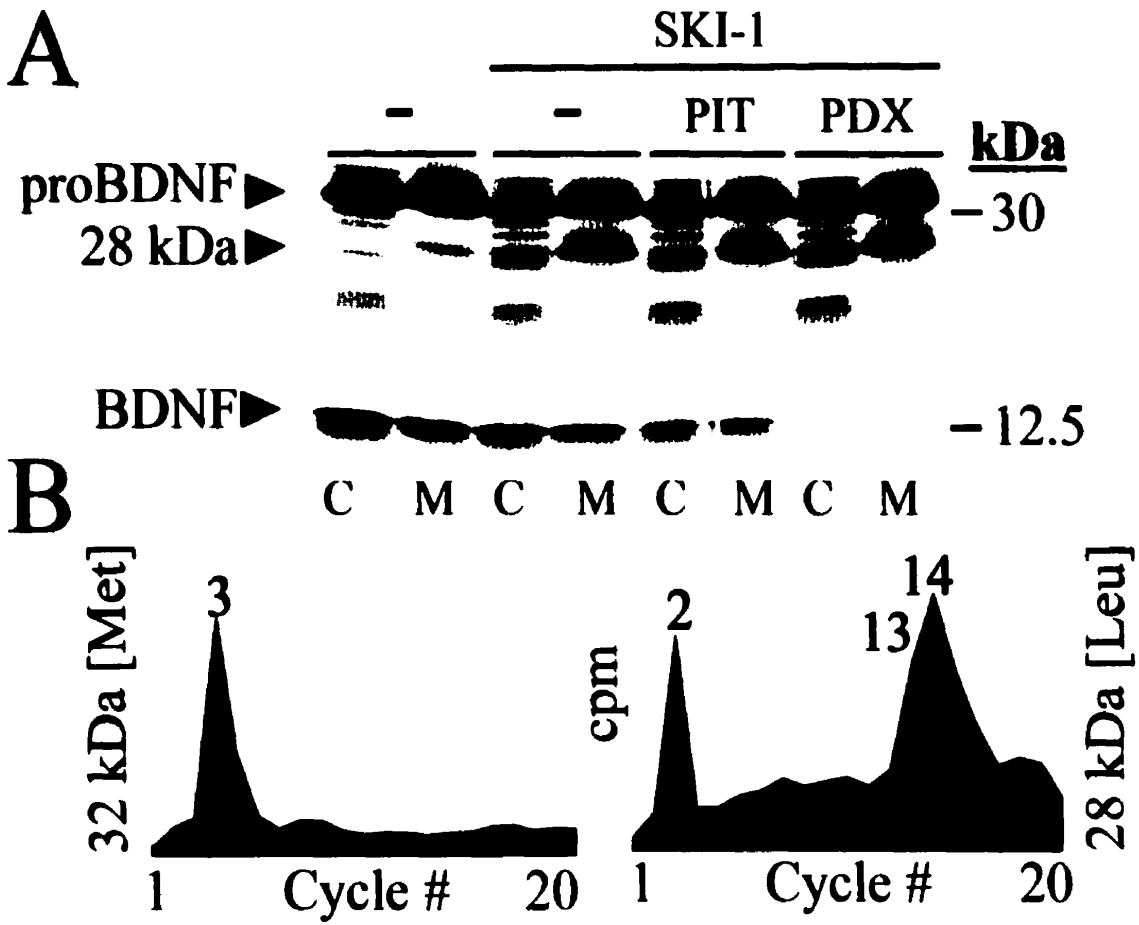
A
SKI-1
-
-
PIT
PDX
kDa
proBDNF
28 kDa
30
BDNF
12.5
B
C M C M C M C M
32 kDa [Met]
3
1
Cycle #
20
cpm
2
13
14
1
Cycle #
20
28 kDa [Leu]

FIGURE 3.6 **Processing of pro-BDNF by SKI-1.** (A) COS-7 cells were infected with vv:BDNF and either vv:WT (-) or vv:SKI-1 in the presence of either vv:PIT or vv:PDX. The cells were labeled metabolically with [^{35}S]cysteine/[^{35}S]methionine for 4 h, and the media (M) and cell lysates (C) were immunoprecipitated with a BDNF antiserum before SDS/PAGE analysis. The autoradiogram shows the migration positions of pro-BDNF (32 kDa), the 28-kDa BDNF produced by SKI-1, and the 14-kDa BDNF. (B) Microsequence analysis of the [^{35}S]Met-labeled 32-kDa pro-BDNF (maximal scale, 1,000 cpm) and [^{3}H]Leu-labeled 28-kDa BDNF (maximal scale, 250 cpm).

A
WT
SKI-1
pH
6.0
6.5
7.3
6.0
5.5
proBDNF
28 kDa
B
Pepstatin
Antipain
Cystatin
E-64
SBTI
PMSF
o-Phen
EDTA
proBDNF
28 kDa

FIGURE 3.7 **In vitro processing profile of pro-BDNF by SKI-1.** (A) pH dependence of the processing of pro-BDNF by SKI-1. (B) Inhibitor profile of the processing of pro-BDNF to the 28-kDa BDNF by the same SKI-1 preparation as in A. The reaction was performed overnight at 37°C, pH 6.0.

CHAPTER 4:

DIFFERENTIAL SORTING OF NERVE GROWTH FACTOR AND BRAIN-DERIVED NEUROTROPHIC FACTOR IN HIPPOCAMPAL NEURONS.

PREFACE

The previous chapters, reveals how pro-BDNF is being produced and post-translationally modified. These data along with the previous published data in our lab (Seidah et al., 1996) showing post-translational modification of pro-NGF and pro-NT-3 would provide some necessary background information on our attempt to understand how neurons sort and release NTs. In the present chapter, using different approaches we have tested the secretory pathway taken by NGF and BDNF in the primary cultures of mouse hippocampal neurons. Our results reveal a constitutive release of NGF by hippocampal neurons consistence with a survival role of NGF in CNS. In contrast pro-BDNF is processed and released via regulated secretory pathway of hippocampal neurons suggesting a potential modulatory role for BDNF in synaptic function and plasticity.

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Differential Sorting of Nerve Growth Factor and Brain-Derived Neurotrophic Factor in Hippocampal Neurons

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ABSTRACT

Nerve growth factor (NGF) is released through the constitutive secretory pathway from cells in peripheral tissues and nerves where it can act as a target-derived survival factor. In contrast, brain-derived neurotrophic factor (BDNF) appears to be processed in the regulated secretory pathway of brain neurons and secreted in an activity-dependent manner to play a role in synaptic plasticity. To determine whether sorting differences are intrinsic to the neurotrophins or reflect differences between cell types, we compared NGF and BDNF processing in cultured hippocampal neurons using a Vaccinia virus expression system. Three independent criteria (retention or release from cells after pulse-chase labeling, depolarization-dependent release, and immunocytochemical localization) suggest that the bulk of newly synthesized NGF is sorted into the constitutive pathway, whereas BDNF is primarily sorted into the regulated secretory pathway. Similar results occurred with AtT-20 cells, including those transfected with cDNAs encoding neurotrophin precursor-green fluorescent protein fusions. The NGF precursor, but not the BDNF precursor, is efficiently cleaved by the endoprotease furin in the trans-Golgi network (TGN). Blocking furin activity in AtT-20 cells with α1-PDX as well as increasing the expression of NGF precursor partially directed NGF into the regulated secretory pathway. Therefore, neurotrophins can be sorted into either the constitutive or regulated secretory pathways, and sorting may be regulated by the efficiency of furin cleavage in the TGN. This mechanism may explain how neuron-generated neurotrophins can act both as survival factors and as neuropeptides.

INTRODUCTION

Numerous cell types secrete neurotrophins, including CNS and PNS neurons and non-neuronal cells in peripheral tissues. Once released, neurotrophins promote neuronal survival and plasticity by interacting with specific receptors on the membranes of target neurons (for review, see Thoenen, 1995; Snider and Lichtman, 1996). We know much about sites of neurotrophin production and utilization, but we know little about the mechanisms that regulate neurotrophin release from cells.

Most secretory proteins are synthesized as high molecular weight precursors that translocate into the endoplasmic reticulum (ER) and then to the Golgi stacks. There they are post-translationally modified (Loh, 1993) and cleaved by endoproteases that separate active peptides from inactive precursors. Many precursors are cleaved within the trans-Golgi network (TGN) by furin or furin-like enzymes that act on the COOH-terminal side of multibasic sites (generally Arg-X-Lys/Arg-Arg) (Hosaka et al., 1991) after which they can be constitutively released (Dubois et al., 1995). In neurons, most neuropeptides are cleaved within the regulated secretory pathway not by furin-like enzymes but by prohormone convertases 1 and 2 (PC1 and PC2) (Rouille et al., 1995), which cleave precursors in immature secretory granules before or after granules bud from the TGN. Thus, proteolytic maturation of proteins destined for regulated secretion occurs at a later time point and in a different subcellular compartment than does proteolysis of constitutively secreted proteins.

Neurotrophin processing can occur in either the constitutive or the regulated secretory pathways. Fibroblasts and Schwann cells contain the constitutive secretory pathway only. They also produce furin, which cleaves neurotrophin precursors in vitro (Bresnahan et al., 1990; Seidah et al., 1996a,b), and bioactive NGF (Bunge, 1994; Singh et al., 1997), brain-derived neurotrophic factor (BDNF) (Acheson et al., 1991), and neurotrophin-3 (NT-3) (Cartwright et al., 1994). NGF can also be processed in the regulated pathway of cells exposed to viruses or plasmids encoding

the NGF precursor (Edwards et al., 1988; Heymach et al., 1996; Canossa et al., 1997; Kruttgen et al., 1998).

BDNF processing appears to occur within the regulated pathway in cells that have both secretory mechanisms, including neurons. Depolarization releases BDNF from virus-infected hippocampal neurons (Goodman et al., 1996). BDNF has been detected in large dense-core vesicles of sensory neurons (Michael et al., 1997) and in brain synaptosomes (Fawcett et al., 1997). These data are consistent with a growing number of studies showing that BDNF, but not NGF, is anterogradely transported in neurons [Altar et al. (1997); Fawcett et al. (1998); for review, see Altar and DiStefano (1998)].

In this study, we used a Vaccinia virus (VV) expression system to directly compare the sorting of NGF and BDNF in hippocampal neurons and AtT-20 cells. Pulse-chase labeling, immunocytochemistry, and depolarization-dependent release studies suggest that under identical experimental conditions, NGF is primarily sorted to the constitutive secretory pathway, and BDNF is sorted to the regulated secretory pathway. Inhibiting furin-like enzymes alters the processing of pro-NGF but not pro-BDNF, and cold-block methods that inhibit protein exit from the TGN prevent cleavage of pro-BDNF but not pro-NGF. In addition, blocking furin activity directs some pro-NGF to the regulated pathway, suggesting that sensitivity to furin-mediated cleavage may be an important determinant in regulating neurotrophin sorting.*

* Some of these results have been published previously in abstract form (Mowla et al., 1997).

MATERIALS AND METHODS

Cell cultures - Hippocampal neurons were prepared according to the method of Banker and Cowan (1977) as modified by Brewer et al. (1993). Briefly, the hippocampus was dissected from embryonic day 18 (E18) mice (Charles River, Montreal, Canada), exposed to trypsin, dissociated mechanically, and grown in 60 mm collagen/poly-L-lysine-coated dishes. Cells from two litters of mice were plated into six dishes. Cultures were maintained in serum-free Neurobasal medium (Life Technologies, Gaithersburg, MD) containing 0.5 mM glutamine and 1_ B27 supplement (Life Technologies). Schwann cell cultures were prepared from neonatal rat sciatic nerve as described previously (Pareek et al., 1993). AtT-20 cells and COS 7 cells were cultured as reported previously (Seidah et al., 1996a). We also used an AtT-20 cell line stably transfected with α1-PDX cDNA that has been described previously (Benjannet et al., 1997). Special care was taken to ensure that cells were distributed in equal numbers in dishes that were to be used for group comparisons.

VV recombinants and infections - Purified recombinant VVs containing the full-length coding regions of mouse pro-NGF and human pro-BDNF (generously provided by Regeneron Pharmaceuticals, Tarrytown, NY) were constructed as described previously (Seidah et al., 1996a,b). VVs coding for α1-PDX were kindly provided by Dr. Gary Thomas (Vollum Institute, Portland, OR). Separate plates of cells were infected with VV encoding pro-NGF or pro-BDNF. In one series of studies, we coinfected AtT-20 cells with VV encoding pro-NGF and α1-PDX. VV infections were performed as described previously (Seidah et al., 1996a), except that we used a multiplicity of infection (MOI) of 1 followed by an incubation of 8-10 hr in virus-free medium before metabolic labeling. Under our experimental conditions, there was no evidence of cell death in cells exposed to VVs for the times indicated in each experiment.

Green fluorescent protein-neurotrophin fusions - cDNAs coding for pro-BDNF and pro-NGF were amplified using primers that eliminated the stop codons and created

restriction sites for inserting neurotrophin cDNAs in frame with the coding sequence of green fluorescent protein (GFP) from EGFP-N1 (Clontech, Cambridge, UK). The GFP coding region was inserted near the region coding for the C terminus of the mature neurotrophin. Thus, the NGF-GFP construct coded for amino acids 1-304 of pro-NGF, and the BDNF-GFP construct coded for amino acids 1-250 of pro-BDNF. Clones were sequenced manually (Sequenase; United States Biochemical Corporation, Cleveland, OH). AtT-20 cells growing on poly-L-lysine-coated coverslips were transfected with neurotrophin-GFP constructs using lipofectamine (Life Technologies). Three days later the cells were fixed in 4% paraformaldehyde in PBS and analyzed by epifluorescence using a Zeiss Axioskop microscope with a 40_ objective.

To determine whether GFP-labeled neurotrophins were properly processed, we metabolically labeled the cells for 6 hr with [^{35}S] cysteine-methionine (Cys-Met) Translabel 48 hr after cells were transfected with the constructs, collected cell lysates and conditioned medium, exposed them to neurotrophin antibodies, and analyzed the immunoprecipitates by SDS-PAGE, as described below. We also analyzed the biological activity of secreted GFP-tagged neurotrophins by testing conditioned medium in a Trk autophosphorylation bioassay. Conditioned media obtained from nontransfected COS 7 cells or cells transfected with NGF, NGF-GFP, BDNF, or BDNF-GFP were incubated for 5 min with NIH 3T3 cells engineered to express Trk A (for NGF) or Trk B (for BDNF). The cells were lysed and immunoprecipitated with anti-pan Trk 203 antibody, fractionated by SDS-PAGE, and probed on Western blot replicas with a phosphotyrosine antibody, according to the methods of Hempstead et al. (1992).

Metabolic labeling and immunoprecipitation - For pulse-chase experiments, we incubated infected cells with 1.5 ml of Cys-Met-free DMEM containing 10% FCS and 0.5 mCi/ml [^{35}S] Translabel (ICN Biochemicals, Montréal, Québec, Canada) (70% methionine, 30% cysteine) for 30 min. Pro-BDNF contains 10 methionines as compared with four in pro-NGF, and mature BDNF contains three methionines as

compared with one in mature NGF. These differences, together with higher concentrations of methionine in the Translabel, explain why pro-BDNF and mature BDNF tend to label more heavily than pro-NGF and NGF in most figures showing metabolic labeling. Cells were washed, and the medium was replaced with an equal volume of DMEM containing 10% FCS plus twofold excess concentrations of nonradioactive cysteine and methionine for the times indicated (chase periods). In some experiments, hippocampal neurons were incubated at 20°C for 3 hr in medium containing Translabel to monitor the effects of cold conditions on precursor processing.

In all experiments, conditioned media and cell lysates were brought to final volumes of 1.5 ml, 750 μl of which was subjected to immunoprecipitation. Samples immunoprecipitated with nonimmune rabbit IgG showed no bands corresponding to standards of neurotrophin precursors or products.

Immunoprecipitations were performed as described previously (Seidah et al., 1996a). For NGF, we used an affinity-purified rabbit anti-NGF IgG described previously (Murphy et al., 1993; Seidah et al., 1996a). BDNF immunoprecipitations were performed using an antibody kindly supplied by Amgen and characterized previously (Fawcett et al., 1997; Yan et al., 1997). Cell lysates and conditioned media were analyzed by electrophoresis on a 13-22% SDS-PAGE. Gels were fixed in 40% methanol and 10% acetic acid, treated with ENHANCE (DuPont NEN, Boston, MA), and washed in 10% glycerol, all for 1 hr. Dried gels were analyzed by a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA), and radioactivity in each band was quantitated using the ImageQuant program. Levels of radioactivity were within the linear range of the device. Statistical significance was determined using the Student's t test on a minimum of triplicate experiments.

To monitor the effects of depolarization on neurotrophin release, we infected hippocampal neurons with recombinant viruses, metabolically labeled the cells for 30 min, and washed and incubated the cells in medium containing excess

nonradioactive methionine and cysteine for 4 hr. The cells were exposed to tissue culture medium supplemented with or without KCl (56 mM) and $CaCl_2$ (5.8 mM) for 15 min. Conditioned media and cell lysates were collected, immunoprecipitated, and fractionated by SDS-PAGE. Neurotrophin levels were estimated and compared by phosphorimager analysis. In a control experiment, we examined KCl-induced release of endogenous secretogranin II using immunoprecipitation methods. VV/NGF-infected cultures of hippocampal neurons were treated as above, and conditioned media and cell lysates were immunoprecipitated with an antibody to rat secretoneurin kindly provided by Dr. Reiner Fischer-Colbrie (Department of Pharmacology, Innsbruck University, Austria).

Immunocytochemistry and confocal microscopy - VV/NGF-BDNF-infected AtT-20 cells and primary cultures of hippocampal neurons as well as controls consisting of uninfected cells or cells infected with wild-type VVs were rinsed with PBS, fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and permeabilized in 0.1% Triton X-100 for 10 min. The cells were preincubated for 20 min in HEPES-buffered saline (HBS) containing 10% FCS to reduce nonspecific antibody binding and exposed to 1:2000 dilutions of primary antibodies overnight at 4°C. The cells were washed three times with HBS (5 min each) and incubated 1 hr with CY3-conjugated goat antirabbit antibody (Jackson Laboratory, Bar Harbor, ME) diluted 1:2000 in HBS containing 10% goat serum. Cells were washed three times in HBS and mounted in a Tris-buffered glycerol mounting medium (Sigma, St. Louis, MO).

Cells were analyzed by confocal laser scanning microscopy with a Zeiss LSM 410 inverted confocal microscope using a 63_, 1.4 NA objective. Cells were excited at 543 nm and imaged on a photomultiplier after passage through FT 590 and LP 590 filter sets. The confocal images represent one confocal level (a depth of ~1 μm) that contains the cell nucleus along with as many cell processes as were possible to image, to evaluate the peripheral distribution of secretory vesicles. There were no perceptible differences in the distribution of NGF and BDNF immunoreactivity

when we scanned below and above the nucleus. All images were printed on a Kodak XLS 8300 high-resolution printer.

In some studies we used epifluorescence microscopy to compare in VV-infected AtT-20 cells the distribution of NGF and BDNF immunoreactivity with TGN38, a marker of the trans-Golgi network (Luzio et al., 1990), and ACTH, which is packaged in secretory vesicles of AtT-20 cells. Antibody to TGN38 raised in guinea pig (kindly provided by Drs. G. Banting and W. Garten, University of Texas, Southwestern, Dallas, TX) was used at a 1:50 dilution and visualized using an FITC-conjugated secondary antibody raised in goat (Jackson Laboratory) diluted 1:50 in HBS containing 10% normal goat serum. ACTH localization was performed using a monoclonal antibody (Cortex Biochem) at a dilution of 1:1000, visualized with a CY2-conjugated goat anti-mouse secondary antibody (Jackson Laboratory) diluted 1:1000 in HBS containing normal goat serum.

RESULTS

Differential retention of NGF and BDNF in hippocampal neurons - Figure 4.1 compares neurotrophin processing in hippocampal neurons infected with recombinant viruses encoding either pro-NGF or pro-BDNF. Figure 4.1A shows that over an 8 hr chase period, pro-NGF processing gives rise to mature NGF. The NGF precursor (35 kDa) is evident in cell lysates at the start of the chase period, and within 30 min, glycosylated higher molecular weight forms of the precursor (39-42 kDa) (Seidah et al., 1996a) are evident. Levels of the precursor remain steady in cell lysates for up to 2 hr but decrease thereafter. Small amounts of the precursor are also evident in conditioned medium sampled at 4 and 8 hr. Mature NGF (13.2 kDa) is visible in conditioned medium after 2 hr, and at 4 hr, levels are higher than in the corresponding cell lysates. Phosphorimager analysis revealed that in samples collected at 8 hr, 3.0 times (±0.7 SEM) as much mature NGF is released into medium than is retained within cell lysates.

Figure 4.1B shows that pro-BDNF is also processed by hippocampal neurons. Pro-BDNF (32 kDa) is evident within cell lysates in all time periods tested, with levels decreasing in samples collected at 4 and 8 hr when levels of processed product increase. In contrast to pro-NGF, significant levels of the BDNF precursor are also evident in conditioned media in all samples collected after 1 hr, apparently because of constitutive release of the protein. Mature BDNF (14.2 kDa) is evident within cell lysates by 1 hr and remains detectable throughout the 8 hr period of analysis. The amount of BDNF retained in cell lysates exceeds the amount released into conditioned medium by 4.0-fold (±1.5 SEM) in samples collected at 8 hr.

Figure 4.2 presents data obtained from triplicate experiments on hippocampal neurons performed as shown in Figure 4.1. The figure compares the amount of processed NGF or BDNF in cell lysates as a function of the total amount of processed neurotrophin in cell lysates and conditioned media. Significantly higher

levels of BDNF are retained within cell lysates as compared with NGF as early as 1 hr after chase, and the differences increase over the 8 hr chase period.

To determine whether hippocampal neurons are unique in their ability to retain more BDNF than NGF, we repeated the pulse-chase experiments shown in Figure 4.1 in AtT-20 cells, a well established cell line that contains both the regulated and constitutive secretory pathways (Burgess and Kelly, 1987). Figure 4.3 shows that AtT-20 cells, like hippocampal neurons, release more NGF into conditioned medium than they retain in cell lysates; the opposite occurs with BDNF. Therefore, in both neurons and AtT-20 cells, most newly synthesized and processed NGF is released from cells, whereas most processed BDNF is retained in cell lysates.

To determine whether retention of BDNF is only a characteristic of cells with the regulated secretory pathway, we repeated the experiments with constitutively secreting rat Schwann cells. Figure 4.4 shows that pro-BDNF is processed by Schwann cells. By 4 hr chase, slightly higher levels of processed BDNF are evident in conditioned media than in cell lysates. By 8 hr, both mature BDNF and pro-BDNF are evident only in conditioned medium. Therefore, Schwann cells process pro-BDNF and release it, along with the BDNF precursor, into conditioned medium. Thus, the retention of processed BDNF by hippocampal neurons and AtT-20 cells is likely caused by differences in the secretory pathways of these cells and Schwann cells.

Immunocytochemical localization of BDNF and NGF - We used immunocytochemistry and confocal microscopy to assess the intracellular locations of NGF and BDNF immunoreactivity in recombinant virus-infected cells. Figure 4.5 shows BDNF immunoreactivity localized to punctate structures throughout the cytoplasm and in the tips of processes in both AtT-20 cells (Fig. 4.5A) and hippocampal neurons (Fig. 4.5B). In contrast, NGF immunoreactivity is distributed in the perinuclear cytoplasm of both AtT-20 cells (Fig. 4.5C) and hippocampal neurons (Fig. 4.5D) and was seldom detected as punctate in either cell type. Neither

NGF nor BDNF immunoreactivity was evident in uninfected cells or in cells infected with wild-type viruses (data not shown). Detection of BDNF immunoreactivity within vesicle-like structures is consistent with the idea that BDNF is processed within the regulated secretory pathway.

Figure 4.6 compares the distribution of BDNF and NGF immunoreactivity with that of endogenous TGN38 and ACTH in virally infected AtT-20 cells. Cells were immunostained for BDNF (A,B,G,H), NGF (C,I), ACTH (E,H), and TGN38 (D,F,G,I). Immunoreactivity for BDNF (A) and TGN38 (D) colocalize (G) as does immunoreactivity for NGF (C) and TGN38 (F) within the perinuclear region (I). BDNF immunoreactivity is also located in punctate structures that are distributed within the cytoplasm and processes of AtT-20 cells (A,B) in a manner indistinguishable from ACTH (E). In some vesicles, the two proteins colocalize (H). In contrast, NGF immunoreactivity was never seen in punctate structures under these experimental conditions. Taken together, these data suggest that BDNF is located in the TGN, as expected before sorting, and packaged within large dense-core vesicles similar to those containing endogenously produced ACTH. NGF is also located in the TGN but is not packaged or concentrated within large dense-core vesicles, which is consistent with NGF being released through the constitutive secretory pathway.

Our results suggest clear differences between the sorting of NGF and BDNF. To test whether these results arose simply as a result of the Vaccinia virus infection method, we transfected AtT-20 cells with constructs coding for GFP fused to the C-terminal region of pro-BDNF or pro-NGF and examined the distribution of the fusion proteins by epifluorescence microscopy. Figure 4.7A shows that pro-BDNF-GFP fluorescence was localized within punctate structures in the cytoplasm and tips of cell processes. In contrast, pro-NGF-GFP (Fig. 4.7B) was distributed diffusely within the cell, never within punctate structures. NGF-GFP fluorescence was also less intense than that of BDNF-GFP, perhaps because of its failure to be concentrated in vesicles and its constitutive release from the cell. The differential distribution of GFP-labeled pro-BDNF and pro-NGF was similar to that obtained

using the Vaccinia virus infection method. Haubensak et al. (1998) recently reported results similar to ours with respect to the localization of BDNF-GFP fusion proteins.

We performed metabolic labeling and SDS-PAGE analyses to determine whether pro-BDNF- and pro-NGF-GFP-labeled constructs were appropriately translated and processed in these experiments. However, these experiments were unsuccessful in AtT-20 cells because of low transfection efficiency. We repeated the experiments in COS 7 cells and found that both pro-NGF-GFP and pro-BDNF-GFP were processed appropriately, without cleavage of the GFP tag, as reported previously (Haubensak et al., 1998) (Fig. 4.7C). We also determined that medium conditioned by COS 7 cells that had been transfected with pro-NGF-GFP and pro-BDNF-GFP was fully active in inducing Trk A and Trk B autophosphorylation, respectively, in NIH 3T3 cells engineered to express the receptors (Fig. 4.7D). These data indicate that GFP-tagged pro-BDNF and pro-NGF are processed appropriately and that conditioned media containing the precursor and mature forms of the proteins can activate their cognate receptors. Data monitoring the distribution of the neurotrophin-GFP fusion proteins further confirm our VV data indicating clear differences in the sorting and intracellular distribution of NGF and BDNF.

Depolarization-induced release of BDNF from hippocampal neurons - If BDNF is in the regulated secretory pathway, depolarization should promote its release. Figure 4.8 shows that BDNF levels in conditioned medium nearly doubled when hippocampal neurons were exposed to KCl; however, depolarization had no effect on NGF release. Depolarization did not promote the release of pro-BDNF or pro-NGF under these experimental conditions (data not shown). To be certain that infecting hippocampal neurons with the NGF-coding virus had not altered the regulated secretory pathway of hippocampal neurons, we monitored the effects of KCl depolarization on the release of endogenously produced secretogranin II, which is present in the regulated pathway. Figure 4.9 shows that KCl treatment effectively promoted secretogranin II release in cells infected with pro-NGF encoding VV.

Differential processing of pro-NGF and pro-BDNF by furin - To further test whether BDNF and NGF are differentially sorted in cells containing both a regulated and constitutive secretory pathway, we performed three additional sets of experiments. In the first, we tested the effects of lowered temperature on the processing of the neurotrophin precursors. Cold temperatures (20°C) allow precursor proteins to enter the TGN but inhibit vesicle budding from the TGN, which is an essential step for processing proteins in immature secretory granules of the regulated secretory pathway (Matlin and Simons, 1983). Therefore, precursor processing at 20°C must occur within the TGN. For these studies, we infected hippocampal neurons with recombinant VVs, metabolically labeled the cells, incubated them for 3 hr at 20°C, and analyzed the intracellular content of newly synthesized NGF and BDNF in cell lysates by immunoprecipitation methods. Figure 4.10 shows that significant amounts of mature NGF were generated from the NGF precursor under cold-block conditions, whereas cold block totally inhibited the generation of mature BDNF from the BDNF precursor. Therefore pro-NGF, but not pro-BDNF, is cleaved within the TGN, probably by furin.

We also compared pro-NGF and pro-BDNF processing in the presence of α1-PDX, an α1-anti-trypsin derivative that selectively interferes with furin's ability to process precursor proteins within the TGN (Anderson et al., 1993; Watanabe et al., 1995; Vollenweider et al., 1996). In these studies, we monitored neurotrophin processing in AtT-20 cells coinfected with VVs encoding either pro-NGF or pro-BDNF with or without VVs coding for α1-PDX. Figure 4.11 shows that α1-PDX had no detectable effect on pro-BDNF processing. However, α1-PDX did increase the amount of pro-NGF released constitutively into conditioned medium, a result that was similar to those obtained when we monitored pro-BDNF processing in hippocampal neurons and AtT-20 cells (Figs. 4.1, 4.3). Identical results (data not shown) were obtained when we infected neurotrophin-encoding viruses into a stably transfected AtT-20 cell line overexpressing α1-PDX (Benjannet et al., 1997).

The finding that α1-PDX caused the constitutive release of pro-NGF into conditioned medium suggested to us that α1-PDX might be altering sorting of NGF

within the cell, an idea confirmed by immunocytochemistry. Figure 4.12B shows that in AtT-20 cells that stably overexpress α1-PDX, pro-NGF encoding viruses cause the accumulation of NGF immunoreactivity in punctate vesicles within the cell cytoplasm and tips of cell processes that does not occur in the absence of α1-PDX. Figure 4.12C shows that at least some of the NGF in these cells can be released into conditioned medium in response to extracellular cAMP, which is consistent with the protein being sorted to the regulated secretory pathway. Taken together, these data suggest that α1-PDX, which partially inhibits the processing of pro-NGF in the TGN, targets some NGF processing to the regulated secretory pathway, where its release can be promoted by extracellular cues.

Finally, Edwards et al. (1988) reported that cAMP causes the release of NGF from VV-infected AtT-20 cells, data that led them to suggest that NGF is processed and released by the regulated secretory pathway. In that study, cells were infected with an MOI of 10-20, as opposed to 1 MOI in our study. The probable explanation for differences in their results and ours is presented in Figure 4.13. Immunocytochemical data show that increasing levels of viral infection shifts the intracellular distribution of NGF from a diffuse to a punctate pattern (Fig. 4.13a). Furthermore, cells receiving 1 MOI do not release NGF in response to cAMP, but cAMP-induced NGF release is seen when cells are exposed to 5 and 10 MOI (Fig. 4.13b), data that agree with those presented by Edwards et al. (1988). (In the figure, compare CM3 in the presence and absence of cAMP). In contrast, reducing by up to 50-fold the MOI of VV coding for BDNF had no effect on the punctate localization of BDNF in infected cells (data not shown). Therefore, sorting of BDNF to the regulated pathway is likely not attributable to concentration effects arising from the level of viral infection.

DISCUSSION

Pulse-chase studies show that hippocampal neurons and AtT-20 cells retain more newly synthesized BDNF than they release. BDNF immunoreactivity is evident in punctate, vesicle-like structures distributed throughout the cell cytoplasm, including in the tips of cell processes, and cell depolarization induces BDNF release. Thus, BDNF appears to sort primarily to the regulated secretory pathway. In contrast, hippocampal neurons and AtT-20 cells release more NGF than they retain. NGF immunoreactivity is distributed diffusely within the perinuclear cytoplasm, presumably within the endoplasmic reticulum and Golgi apparatus, and depolarization fails to promote NGF's release into conditioned medium. Thus, NGF appears to be processed and released in the constitutive pathway. These results are not unique to the Vaccinia virus expression system because NGF and BDNF were differentially distributed as well within cells transfected with cDNAs coding for GFP-pro-neurotrophin fusion proteins.

Furin appears to cleave pro-NGF in the cells we tested. Pro-NGF processing is unaffected by cold-block conditions that inhibit the exit of proteins from the TGN (Matlin and Simons, 1983), suggesting that NGF processing occurs within the TGN. In contrast, cold block totally inhibited the processing of pro-BDNF. Pro-BDNF processing likely occurs in immature secretory vesicles after they bud from the TGN, which is an early step in the regulated secretory pathway. Also, α1-PDX, a competitive inhibitor of furin, increased levels of NGF precursor in conditioned medium, which is consistent with its inhibiting pro-NGF processing within the TGN. α1-PDX did not affect pro-BDNF processing. In the presence of α1-PDX, NGF immunoreactivity appeared in punctate structures similar to those in cells infected with pro-BDNF encoding viruses, and some NGF precursor was constitutively secreted. Furthermore, depolarization released small amounts of NGF into conditioned medium. These results are similar to those obtained with pro-BDNF in the absence of α1-PDX. Thus, inhibiting furin activity induces some pro-NGF to be

sorted to the regulated secretory pathway. Similar effects have been observed for pro-opiomelanocortin (Benjannet et al., 1997).

Cleavage by furin or furin-like enzymes within the TGN may be one factor determining whether neurotrophins are sorted into the constitutive or regulated secretory pathways. Studies with the precursor for egg-laying hormone (pro-ELH) in mollusks may explain how this mechanism could work. Pro-ELH contains bioactive peptides on both the C- and amino-terminal sides of a furin cleavage site (Sossin et al., 1990). The C-terminal side of the precursor is sorted into the regulated secretory pathway after furin cleavage, and the amino-terminal side is released constitutively, degraded, or sorted into a separate regulated secretory pathway (Jung and Scheller, 1991). In cells with low furin levels, the precursor avoids cleavage, and both sides of the precursor are sorted into the same regulated secretory vesicles (Klumperman et al., 1996). Thus, sorting of the amino-terminal active peptides into dense-core secretory vesicles occurs only in the absence of furin cleavage, and different cells with different amounts of furin sort the same neuropeptide differently.

A similar situation may occur with neurotrophin processing. Sorting into the regulated pathway may require signals within the pro-domain or near the consensus cleavage site. When cleaved by furin, the NGF precursor may lose these signals, and the mature cleaved protein is sorted into the constitutive pathway for release. In that regard, inserting furin-sensitive cleavage sites into pro-insulin (Yanagita et al., 1992) and pro-renin (Oda et al., 1991), which are normally processed by the regulated pathway, redirects these proteins into the constitutive pathway. In the absence of furin cleavage, the precursor remains intact, and sorting signals that direct the protein to the regulated pathway become functional. This may explain why pro-BDNF, which likely eludes furin cleavage in the TGN, is targeted to the regulated pathway where its processing appears to occur in secretory granules.

Differences between pro-BDNF and pro-NGF processing may therefore arise because of the furin sensitivity of their pro-protein processing cleavage sites. In pro-

NGF (Arg-Ser-Lys-Arg↓Ser) the site is highly suited to furin processing, whereas the analogous site in pro-BDNF (Arg-Val-Arg-Arg↓His) is less suitable because of the replacement of Ser in the +1 position (P1) of NGF with His in the P1 of BDNF (Seidah et al., 1996a). Sequences with His at P1 show reduced sensitivity to furin-mediated cleavage (Ogi et al., 1990; Matthews et al., 1994).

Our results are consistent with this model. In constitutively secreting cells with moderate levels of furin-like enzymes, such as fibroblasts and Schwann cells (M. Marcinkiewicz and N. G. Seidah, unpublished observations), pro-NGF and pro-BDNF are cleaved, with only low levels of unprocessed precursor secreted constitutively. In AtT-20 cells and hippocampal neurons with lower levels of furin (Seidah et al., 1994), pro-NGF is cleaved efficiently, and NGF is released constitutively. When furin cleavage is inhibited with α1-PDX, pro-NGF is not cleaved efficiently, some sorting occurs into the regulated secretory pathway, where some of the protein can be released by depolarization, and some unprocessed precursor is secreted constitutively. In contrast, pro-BDNF avoids furin cleavage and is sorted into the regulated secretory pathway, and some unprocessed precursor is secreted constitutively into conditioned medium. Constitutive release of other precursors normally processed in the regulated pathway has also been reported (Kelly et al., 1983; Moore et al., 1983; Brechler et al., 1996)

An increasing number of reports suggest that BDNF, but not NGF, is anterogradely transported within axons in brain neurons to carry out a number of physiological actions [Altar et al. (1997); Fawcett et al. (1998); for review, see Altar and DiStefano (1998)]. Also, BDNF is enriched in a microvesicular fraction of rat brain synaptosomes along with synaptotagmin, a protein associated with synaptic and large dense-core vesicles in nerve terminals (Fawcett et al., 1997). BDNF immunoreactivity is also present in mossy fiber terminals in the hippocampus (Conner et al., 1997; Fawcett et al., 1997) and in large dense-core vesicles of axon terminals in lamina II of lumbar spinal cord (Michael et al., 1997). BDNF can be transported anterogradely in neurons within the visual system (von Bartheld et al.,

1996) and released by depolarization in a calcium-dependent mechanism from virus-infected hippocampal neurons (Goodman et al., 1996). Taken together, these data are consistent with BDNF being packaged within secretory vesicles of the regulated pathway. Presumably these granules are targeted to axons, although transport may occur to other parts of the cell as well.

Fewer studies have monitored the processing of NGF in cells containing the regulated pathway. Edwards et al. (1988) reported that AtT-20 cells secrete VV-encoded NGF in response to cAMP, suggesting regulated release. In that study, they infected cells with an MOI of 10-20 as opposed to an MOI of 1 in our study, which explains why their results and ours differ (Fig. 4.13). Overloading the furin pathway beyond its capacity may drive NGF into the regulated pathway, as does inhibiting furin with α1-PDX (Figs. 4.11, 4.12). In a similar manner, overexpressing β2-microglobulin in pancreatic cells drives the protein from the constitutive pathway into secretory vesicles of the regulated pathway (Allison et al., 1991). In contrast, reducing infectivity from 1 to 0.02 MOI had no effect on the sorting of BDNF, although we cannot rule out the possibility that local concentration effects contributed to BDNF's sorting into the regulated pathway.

Heymach et al. (1996) reported that AtT-20 cells release NGF as well as BDNF and NT-3 in response to secretagogues. It may be that in those studies the level of NGF production was above the threshold levels in which NGF is shunted from the constitutive pathway into the regulated pathway. Heymach et al. (1996) used transfection instead of infection, expressed the neurotrophins using a different promoter, and treated cells with secretagogues for a longer time, which may further explain why their conclusions differ from ours. Blochl and Thoenen (1995) hypothesized that there is both constitutive and regulated sodium-dependent release of NGF from neurons. They suggest that release occurs independent of extracellular calcium, which is essential for protein release in the regulated pathway (DeCamilli and Jahn, 1990), including the release of BDNF (Goodman et al., 1996). Canossa et al. (1997) extended these findings by reporting that neurotrophins can also induce

neurotrophin release from neurons (also see Kruttgen et al., 1998). These conflicting data need to be investigated further.

Our results provide a mechanism whereby neurotrophins in brain neurons can act either as survival factors or as neuropeptides. When neurotrophins are cleaved by furin, the bioactive peptide may be released constitutively to promote neuronal survival. Perhaps this explains how hippocampal neurons constantly provide NGF to innervating cholinergic neurons in the basal forebrain. In contrast, BDNF may avoid furin cleavage and be sorted into the regulated secretory pathway where it is processed by PC1 (Seidah et al., 1996a) and released in an activity-dependent manner similar to other neuropeptides. This may be the mechanism that allows BDNF to alter synaptic transmission, connectivity, and synaptic plasticity in an activity-dependent manner (Ghosh, 1996). Also, perhaps neurons modulate the physiological fates of neurotrophins by regulating furin levels and thus the intracellular sorting of the neurotrophins they produce.

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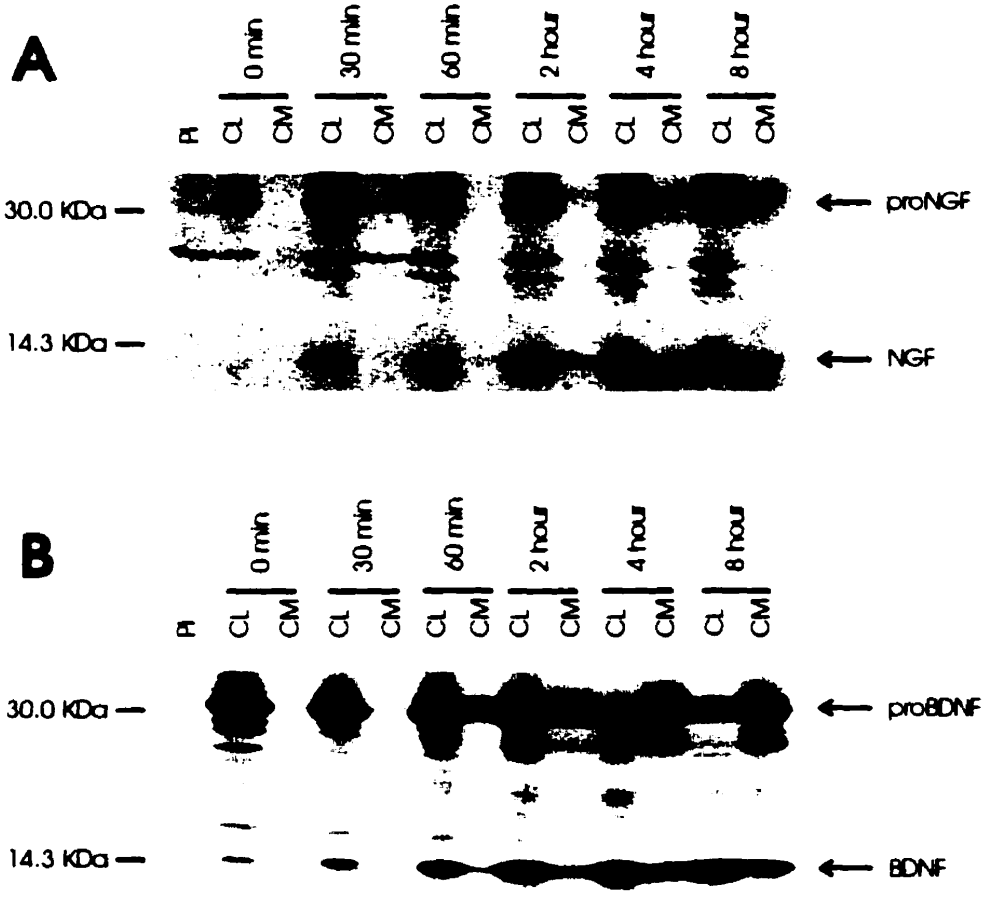

A
0 min
30 min
60 min
2 hour
4 hour
8 hour
PI
CL
CM
30.0 KDa
14.3 KDa
proNGF
NGF
B
0 min
30 min
60 min
2 hour
4 hour
8 hour
PI
CL
CM
30.0 KDa
14.3 KDa
proBDNF
BDNF

FIGURE 4.1 **Pulse-chase metabolic labeling of pro-NGF (A) and pro-BDNF (B) in cultures of hippocampal neurons.** Separate plates of cells were infected with VV encoding the NGF precursor or the BDNF precursor for 1 hr and postincubated in fresh medium without virus for 10 hr. Cells were exposed to medium containing [^{35}S] Cys-Met for 30 min and chased for 0, 0.5, 1, 2, 4, and 8 hr. Identical volumes (750 μl) of cell lysates (CL) and conditioned media (CM) were immunoprecipitated with antibodies to NGF or BDNF or with nonimmune serum (NI; a cell lysate sample) and electrophoresed on 13-22% SDS gradient gels. Dried gels were exposed to a phosphorimaging screen.

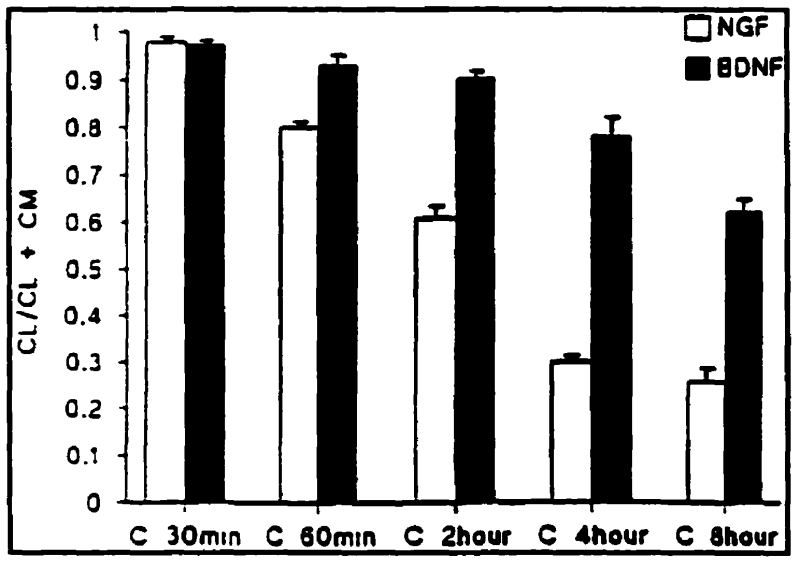

NGF
BDNF
CL/CL + CM
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C 30min
C 60min
C 2hour
C 4hour
C 8hour

FIGURE 4.2 **Kinetics of NGF and BDNF retention in hippocampal neurons.** Experiments in Figure 4.1 were repeated three times, and the combined results were analyzed by the ImageQuant program. The ratio of mature NGF and BDNF in cell lysates (CL) was compared with the total NGF and BDNF in CL + conditioned medium (CM). Data show that significantly larger amounts of BDNF are retained in CL than NGF.

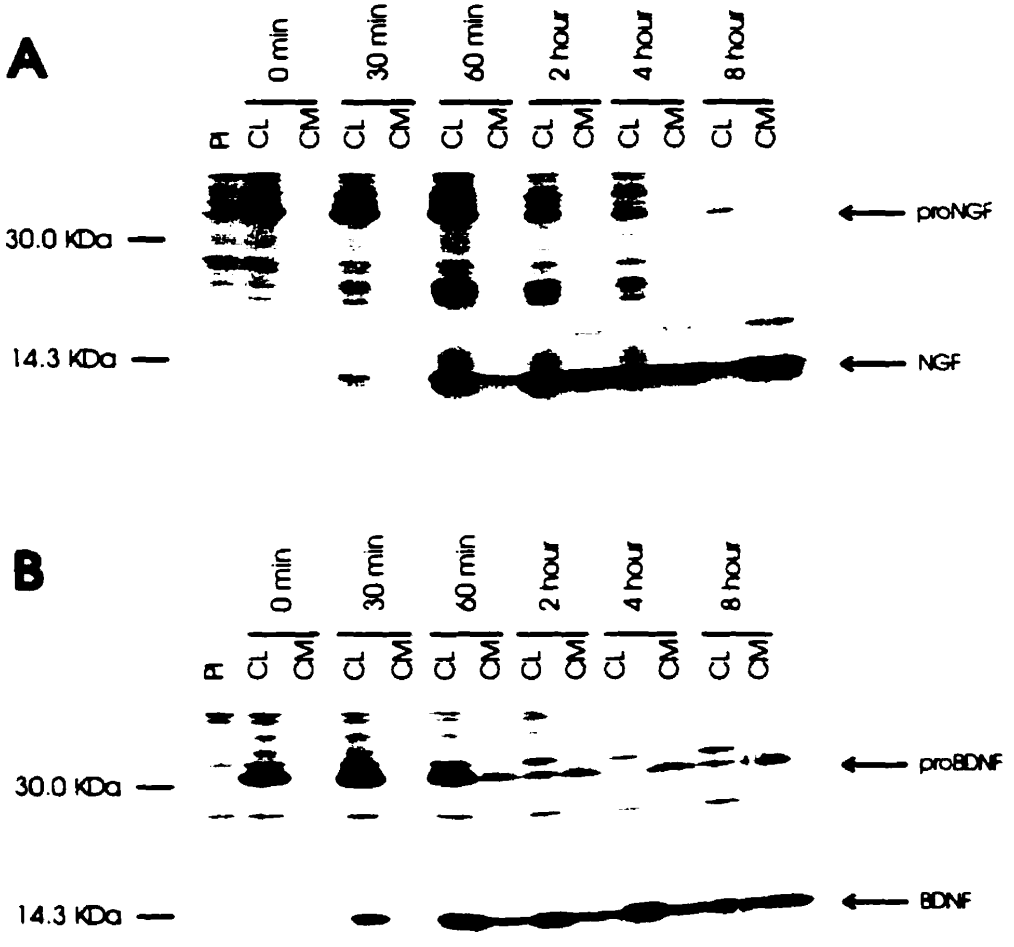

A
0 min
30 min
60 min
2 hour
4 hour
8 hour
PI
CL
CM
30.0 KDa
14.3 KDa
proNGF
NGF
B
proBDNF
BDNF

FIGURE 4.3 **Pulse-chase metabolic labeling of pro-NGF (A) and pro-BDNF (B) production and processing in VV-infected AtT-20 cell cultures.** Methods were identical to those described in the legend to Figure 4.1. NGF and BDNF and their precursors were measured in conditioned medium (CM) and in cell lysates (CL). NI is a sample of CL precipitated with nonimmune serum.

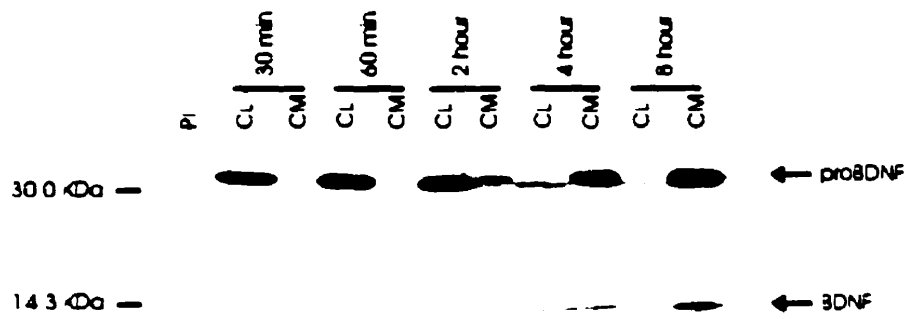

PI
CL
CM
30 min
60 min
2 hour
4 hour
8 hour
300 kDa
143 kDa
proBDNF
BDNF

FIGURE 4.4 **Pulse-chase metabolic labeling of primary rat Schwann cells infected with VV encoding pro-BDNF.** Methods were identical to those described in the legend to Figure 4.1. BDNF and its precursor were measured in cell lysates (CL) and conditioned media (CM). NI is a CL sample precipitated with nonimmune serum.

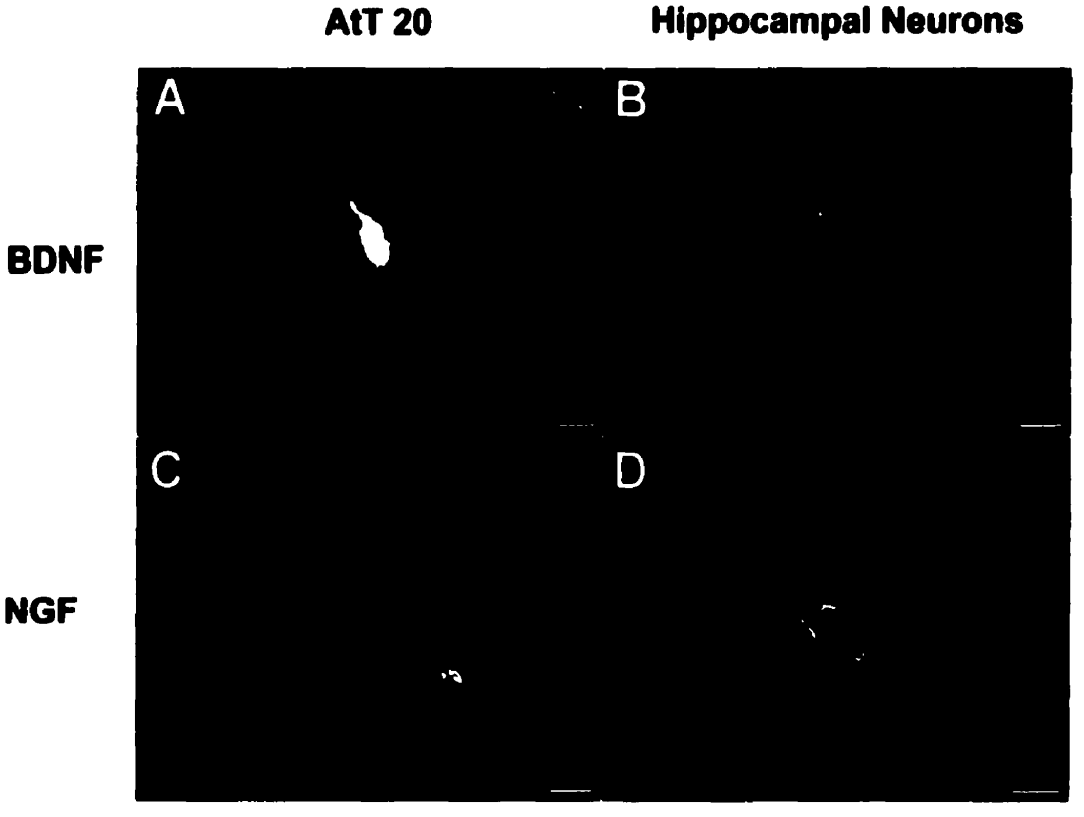
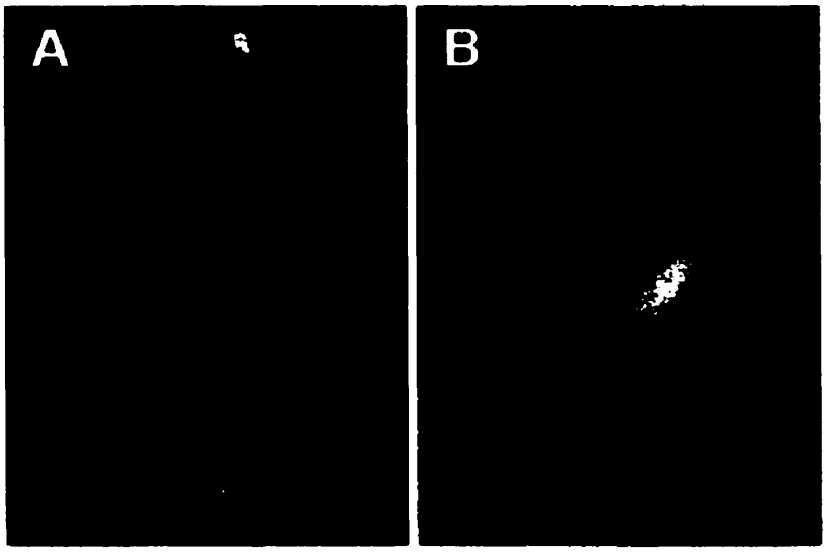

AtT 20
Hippocampal Neurons
A
B
BDNF
C
D
NGF

FIGURE 4.5 **Confocal microscopy of AtT-20 cells (A, C) and hippocampal neurons (B, D) infected with VV encoding pro-NGF (C, D) or pro-BDNF (A, B).** Cells were infected for 1 hr and postincubated in the absence of virus for another 8 hr. The cultures were fixed and treated with antibodies against NGF or BDNF, followed by CY3-conjugated goat anti-rabbit IgG. Scale bar, 10 μm.

FIGURE 4.6 **Double-label immunocytochemistry comparing the distribution in infected AtT-20 cells of BDNF and NGF immunoreactivity with immunostaining of endogenous TGN38 and ACTH.** BDNF immunoreactivity (A, B) colocalizes with TGN38 (D) in the perinuclear region and with ACTH in the cytoplasm and cell processes (E). G is a composite of A and D; H is a composite of B and E. NGF immunoreactivity (C) colocalizes with TGN38 (F) in the perinuclear region but is not detectable in cell processes. I is a composite of C and F.


A
B


C
control
NGF
NGF-GFP
control
BDNF
BDNF-GFP
CL
CM
KDa
97.7
69.0
proNGF-GFP
proBDNF-GFP
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proNGF
BDNF-GFP
proBDNF
30.0
21.5
NGF
BDNF

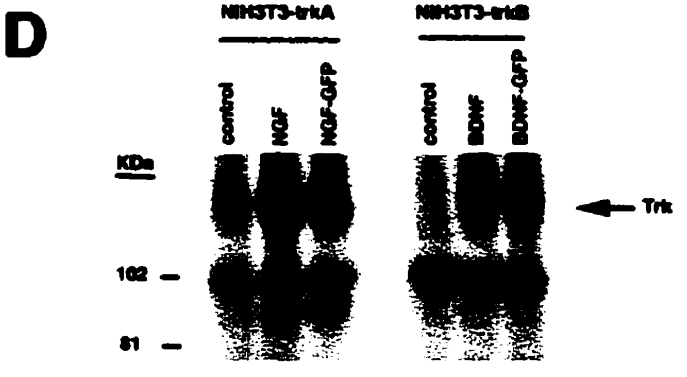

D
NIH3T3-trkA
NIH3T3-trkB
control
NGF
NGF-GFP
control
BDNF
BDNF-GFP
KDa
Trk
102
81

FIGURE 4.7 **Expression of pro-neurotrophin-GFP fusion proteins in AtT-20 cells.** Cells were plated on poly-L-lysine-coated coverslips and transfected using lipofectamine with cDNAs encoding either (A) pro-NGF-GFP or (B) pro-BDNF-GFP. Three days later, the cells were analyzed by fluorescence microscopy. C, Immunoprecipitation and SDS-PAGE of metabolically labeled GFP fusion proteins from transfected COS 7 cells. D, Conditioned medium from pro-NGF-GFP or pro-BDNF-GFP expressing COS 7 cells activate Trk A and Trk B phosphorylation, respectively, in NIH 3T3 cells engineered to overexpress either receptor.

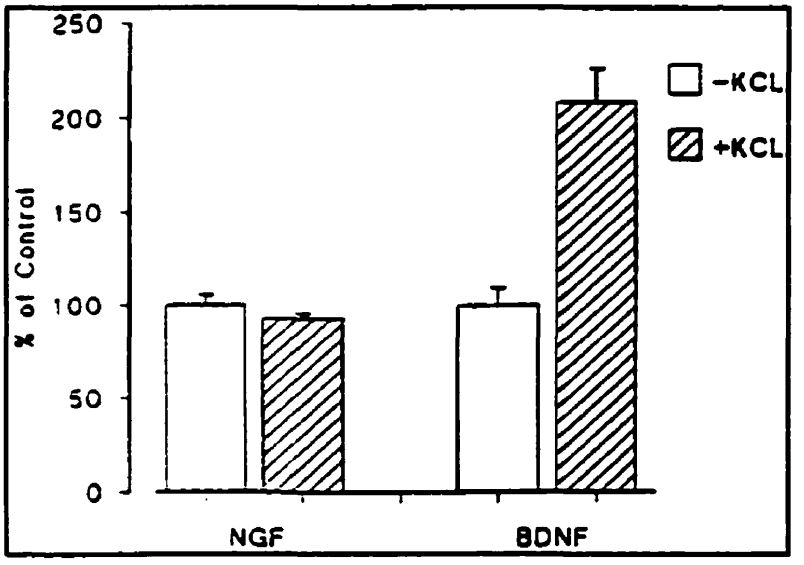

250
200
150
100
50
0
% of Control
-KCL
+KCL
NGF
BDNF

FIGURE 4.8 **KCl-induced release of BDNF but not NGF from hippocampal neurons.** Hippocampal neurons from E18 mice were cultured for 7 d and infected for 1 hr with VV encoding pro-NGF or pro-BDNF. After 10 hr in medium without virus, the cells were labeled for 30 min with [^{35}S] Cys-Met, incubated in medium without radiolabel for 4 hr, and treated with medium with or without KCl and $CaCl_2$ for 15 min. Conditioned media were immunoprecipitated with antibodies to NGF or BDNF and electrophoresed on a SDS gel. Results were analyzed on a phosphorimager and are an average (±SEM) of three independent experiments.

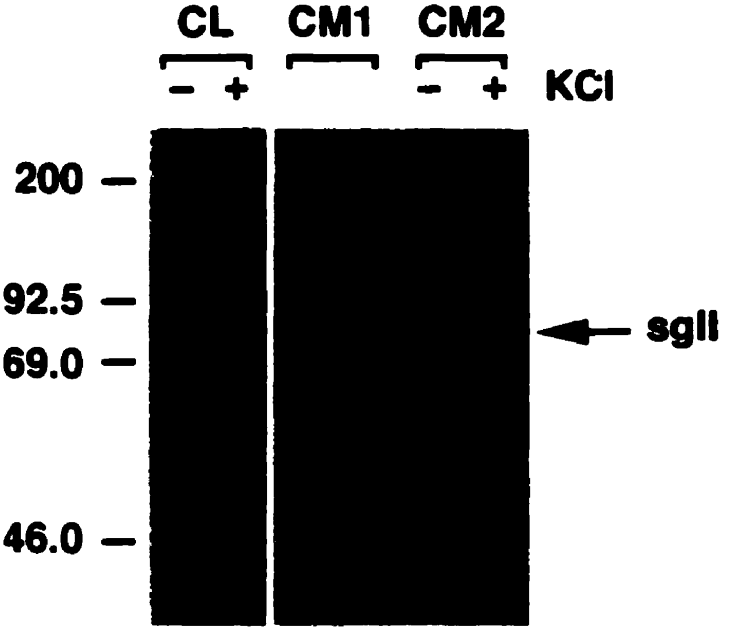

CL
CM1
CM2
- +
- +
KCl
200
92.5
69.0
46.0
sgII

FIGURE 4.9 **Release of secretogranin II (sgII) from hippocampal neurons infected with VV coding for pro-NGF**. Eight hours after neurons were exposed to VV, the cells were pulsed for 30 min with medium containing [^{35}S] Cys-Met. The cells were chased for an additional 4 hr, after which samples of conditioned medium were analyzed (CM1), and again 30 min later after the addition (+) or in the absence (-) of KCl (50 mM) added to the culture medium (CM2).

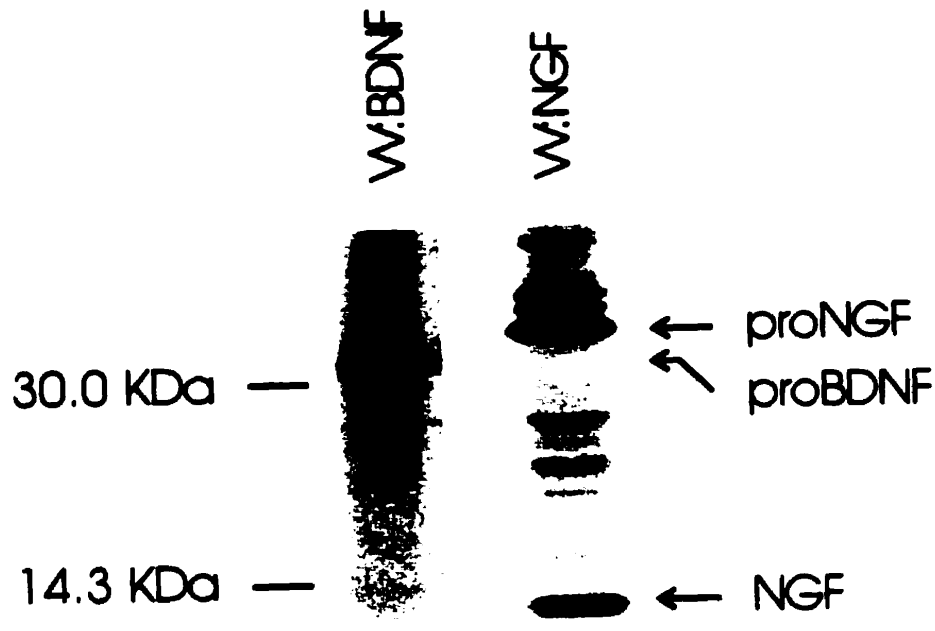

W:BDNF
W:NGF
30.0 KDa
14.3 KDa
proNGF
proBDNF
NGF

FIGURE 4.10 **Cold-block experiments**. Hippocampal neurons were infected with VV encoding pro-NGF or pro-BDNF for 1 hr, and metabolically labeled at 20°C for 3 hr. Cell lysates were prepared, immunoprecipitated with antibodies to NGF or BDNF, and electrophoresed on SDS gels.

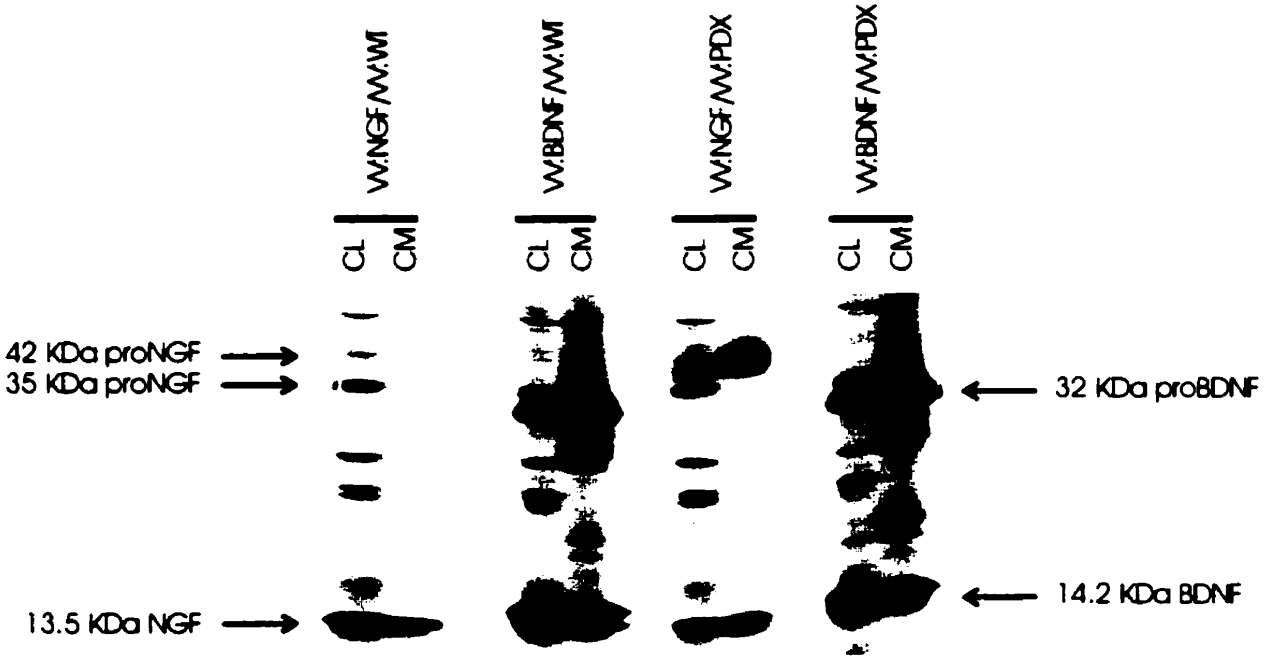
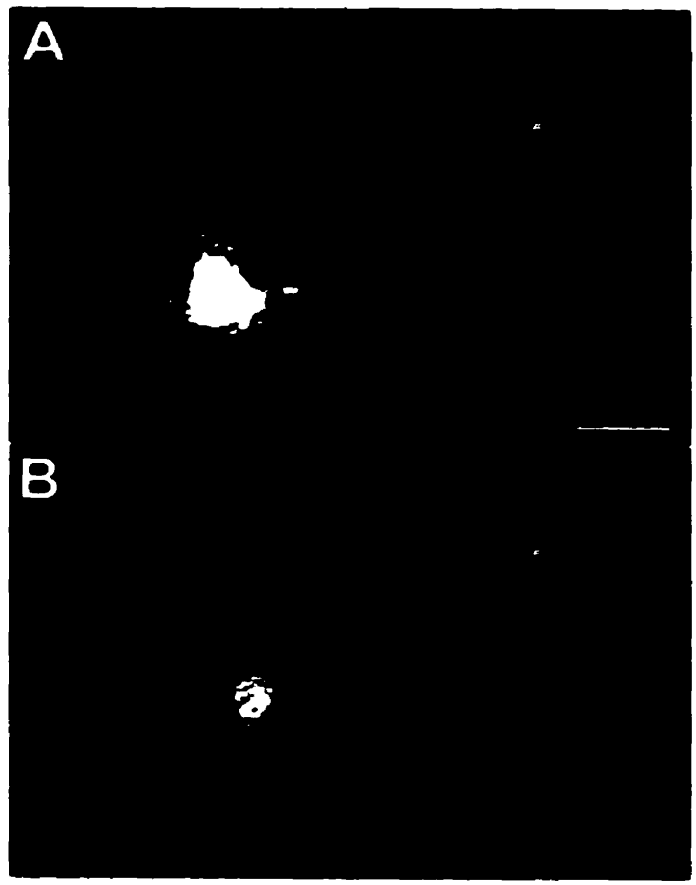

W:NGF/W:WT
W:BDNF/W:WT
W:NGF/W:PDX
W:BDNF/W:PDX
CL
CM
42 KDa proNGF
35 KDa proNGF
13.5 KDa NGF
32 KDa proBDNF
14.2 KDa BDNF

FIGURE 4.11 **The effects of α1-PDX on pro-NGF processing in AtT-20 cells.** AtT-20 cells were infected for 2 hr with VV encoding pro-NGF or pro-BDNF with or without VV encoding the furin inhibitor α1-PDX. Cells were incubated in virus-free medium for 10 hr and metabolically labeled for 3 hr. Cell lysates (CL) and conditioned media (CM) were collected, immunoprecipitated, and analyzed by SDS gel electrophoresis.


A
B

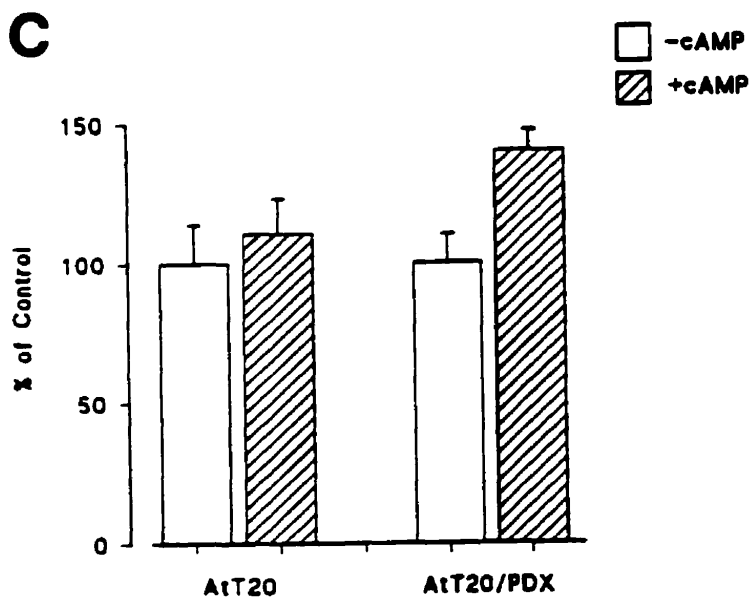

C
-cAMP
+cAMP
150
100
50
0
% of Control
AtT20
AtT20/PDX

FIGURE 4.12 **The effects of α1-PDX on NGF sorting in AtT-20 cells.** AtT-20 cells (A) or AtT-20 cells that stably express α1-PDX (B) were infected for 1 hr with VV encoding pro-NGF, postinfected for 10 hr, and prepared for immunocytochemistry. In C, AtT-20 cells with or without stably expressed α1-PDX were infected with VV encoding pro-NGF for 1 hr, postinfected for 10 hr in control medium, pulsed with medium containing [35S] Cys-Met for 2 hr, chased for 3 hr, and treated with medium with or without 5 mM cAMP for 3 hr. Cell lysates and conditioned media were immunoprecipitated and analyzed by SDS-PAGE. Results were analyzed on a phosphorimager and report an average (±SEM) of three independent experiments.

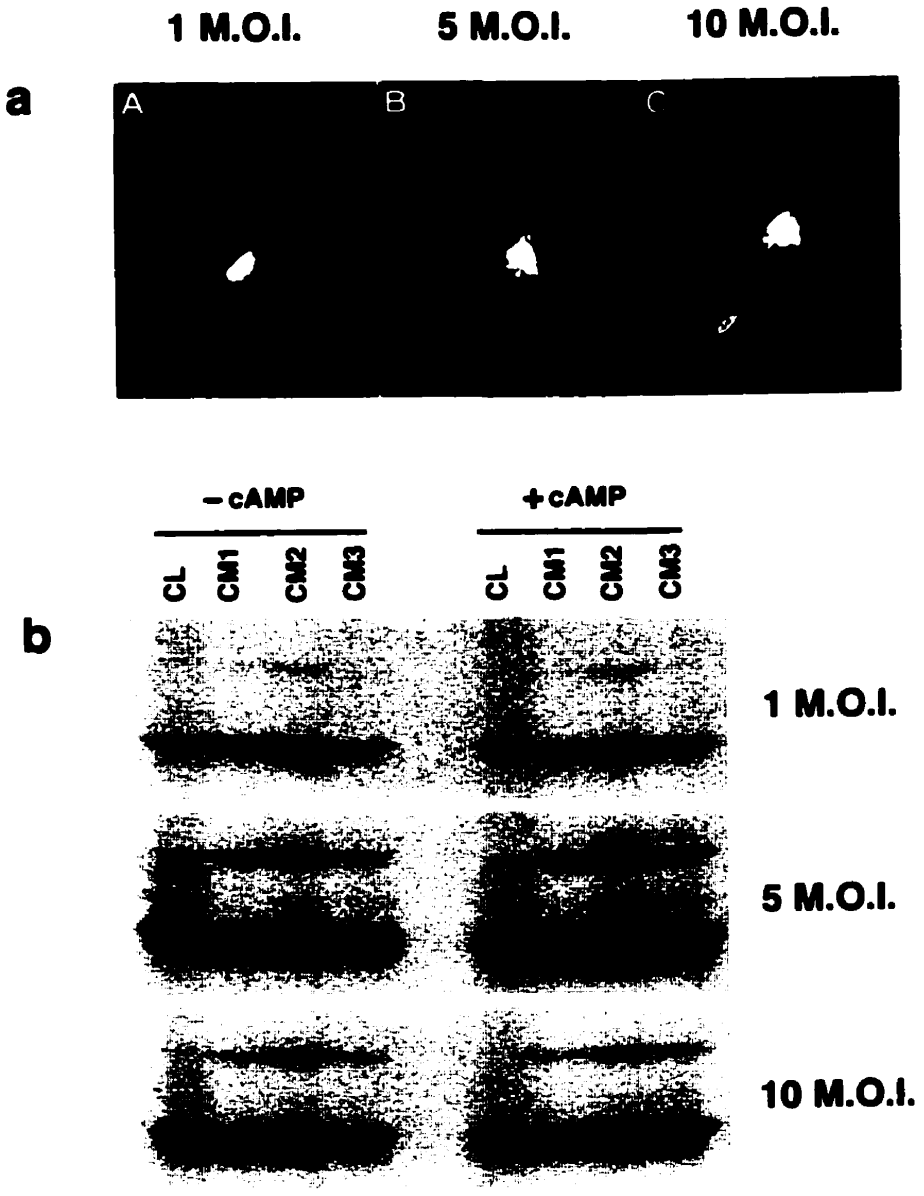

1 M.O.I.
5 M.O.I.
10 M.O.I.
a
A
B
C
−cAMP
+cAMP
CL
CM1
CM2
CM3
CL
CM1
CM2
CM3
b
1 M.O.I.
5 M.O.I.
10 M.O.I.

FIGURE 4.13 **Overexpression of NGF results in missorting of NGF from the constitutive to the regulated secretory pathway.** a. AtT-20 cells were infected for 1 hr with 1 (A), 5 (B), or 10 (C) MOI of VV coding for pro-NGF, postinfected for 8 hr, fixed, and prepared for immunocytochemistry using an antibody to NGF followed by a CY3-conjugated secondary antibody. Cells were analyzed by confocal microscopy. b. AtT-20 cells were infected with 1 (A), 5 (B), or 10 (C) MOI for 1 hr followed by a 4 hr postinfection and 3 hr incubation in medium containing [^{35}S] Translabel. Conditioned media were collected (CM1), the cells were chased for 3 hr, and media was again collected (CM2). 8-bromo-cAMP (5 mM) was then added to some cultures, and cells were incubated for an additional 3 hr, after which media were collected (CM3) and the cells were lysed (CL). NGF was immunoprecipitated from all samples, and the precipitate was analyzed by SDS-PAGE. Comparison of CM3 samples shows that NGF release can be stimulated by cAMP from cells infected with 5 or 10 MOI but not from cells receiving 1 MOI.

CHAPTER 5:

NEUROTROPHIN-3 CAN BE DIVERTED FROM THE CONSTITUTIVE TO THE REGULATED SECRETORY PATHWAY OF HIPPOCAMPAL NEURONS BY DIMERIZATION WITH BRAIN-DERIVED NEUROTROPHIC FACTOR

PREFACE

In the previous chapter We have shown that pro-NGF is primarily processed and released through constitutive secretory pathway of hippocampal neurons while pro-BDNF is sorted and released via regulated secretory pathway. Motivated by the previous reports demonstrating that NTs are able to form heterodimers; we started to evaluate whether NGF can be re-routed to the regulated secretory pathway by means of heterodimerization with BDNF. Using the same criteria as mentioned in previous chapter, we were unable to show convincing data pointing to a role for heterodimerization in the re-routing of NGF to the regulated secretory pathway. Considering the fact that heterodimer between NT-3 and BDNF (NT-3/BDNF) is unusually stable, we decided to pursue the idea for NT-3. Indeed, our results showed that pro-NT-3 is primarily processed and released constitutively similar to what we observed for pro-NGF. Our results also strongly reveals that NT-3 can be re-directed into regulated secretory pathway by means of co-expression with pro-BDNF.

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Neurotrophin-3 Sorts to the Constitutive Secretory Pathway of Hippocampal Neurons and Is Diverted to the Regulated Secretory Pathway by Coexpression with Brain-Derived Neurotrophic Factor

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¶ The first two authors contributed equally to this work.

ABSTRACT

Hippocampal neurons release nerve growth factor (NGF) through the constitutive secretory pathway, thus allowing the protein to be continuously available for promoting nerve cell survival. In contrast, hippocampal neurons use the regulated secretory pathway to process brain-derived neurotrophic factor (BDNF), which alters synaptic activity when released acutely from dense-core vesicles. Thus, understanding how neurons sort and deliver neurotrophins may provide clues to their functions in brain. In this study, we monitored the processing and delivery of neurotrophin-3 (NT-3). Pulse-chase studies, immunocytochemistry, and secretagogue-induced release experiments were performed on cultured hippocampal neurons and AtT-20 cells infected with vaccinia viruses encoding the NT-3 precursor (pro-NT-3). Results show that most newly synthesized NT-3 is released through the constitutive secretory pathway as a result of furin-mediated endoproteolytic cleavage of pro-NT-3 in the trans-Golgi network. Pro-NT-3 can also be diverted into the regulated secretory pathway when cells are treated with α1-PDX, a selective inhibitor of furin-like enzymes, or when pro-NT-3 expression is increased by transient transfection methods. In cells coinfected with viruses coding for pro-NT-3 and pro-BDNF, NT-3 is sorted into the regulated pathway, stored in secretory granules, and released in response to extracellular cues together with BDNF, apparently as a result of heterodimerization, as suggested by coimmunoprecipitation data. Taken together, these data show that sorting of the NT-3 precursor can occur in both the constitutive and regulated secretory pathways, which is consistent with NT-3 having both survival-promoting and synapse-altering functions.

Key words: neurotrophin; NT-3; BDNF; constitutive pathway; regulated secretory pathway; heterodimer

INTRODUCTION

Neurotrophins are synthesized as high molecular weight precursors containing a prodomain linked to the amino terminus of the mature protein. The two components are separated by endoproteolytic cleavage within either the constitutive or regulated secretory pathways. Understanding how neurotrophins are processed in these pathways may help explain their physiological functions.

Hippocampal neurons and AtT-20 cells in culture process nerve growth factor (NGF) within the constitutive secretory pathway (Mowla et al., 1999). The NGF precursor is cleaved within the trans-Golgi network (TGN) by the endoprotease furin, a member of the subtilisin/kexin-like family of proteases (for review see Seidah et al., 1998; Zhou et al., 1999). Constitutive release of NGF occurs soon after the molecule is synthesized. Thus, as a result of being processed in the constitutive pathway, NGF is continuously available to cells that require it, which is consistent with hippocampal neurons producing NGF as an apoptosis-inhibiting survival factor for basal forebrain cholinergic neurons (for review, see Yuen et al., 1996).

In contrast, hippocampal neurons process brain-derived neurotrophic factor (BDNF) within the regulated secretory pathway (Goodman et al., 1996; Mowla et al., 1999). Intact pro-BDNF is shunted from the TGN into immature secretory granules where it is likely cleaved by prohormone convertase 1 (PC1), another member of the subtilisin/kexin-like endoprotease family (Seidah et al., 1996b; Mowla et al., 1999). BDNF is stored within dense-core vesicles (Fawcett et al., 1997; Michael et al., 1997) (for review, see Altar and DiStefano, 1998), and once released, presumably in response to extracellular cues, it can induce changes in neuronal structure (Ventimiglia et al., 1995; Fawcett et al., 2000), membrane depolarization (Kafitz et al., 1999), and changes in synaptic function (for review, see McAllister et al., 1999). Likewise, neurotrophin-3 (NT-3) may regulate neuronal depolarization (Kafitz et al., 1999) and synaptic plasticity (Kang and Schuman, 1995), but as yet little is known about its intracellular sorting in vivo.

Neurotrophins are normally synthesized as noncovalently linked homodimers consisting of two identical chains. When different neurotrophins are coexpressed within the same cell, however, they also form heterodimers. Heterodimers of BDNF and NT-3 are stable, whereas heterodimers involving NGF are not (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Heymach and Shooter, 1995; Robinson et al., 1995). BDNF/NT-3 heterodimers can induce autophosphorylation of Trk receptors, promote the survival of sympathetic neurons in vitro, and induce dopamine uptake in cultures of substantia nigra neurons (Arakawa et al., 1994; Philo et al., 1994). However, the physiological functions of heterodimers remain unknown.

In this study, we monitored the sorting of NT-3 by infecting hippocampal neurons and AtT-20 cells with vaccinia viruses (VVs) encoding pro-NT-3. Pulse-chase studies and immunocytochemistry show that processed NT-3 is primarily released through the constitutive secretory pathway. Inhibition of furin-mediated cleavage or overexpression of pro-NT-3 shifts pro-NT-3 sorting into the regulated secretory pathway. NT-3 is also sorted to the regulated secretory pathway when it is coexpressed with BDNF. Immunoprecipitation data suggest that the transfer of NT-3 to the regulated pathway occurs as a result of NT-3 and BDNF heterodimerizing. Thus, either secretory pathway can sort NT-3. [1]

[1] Some of these results have been published previously in abstract form (Farhadi et al., 1998).

MATERIALS AND METHODS

Cell Cultures - Hippocampal neurons were prepared according to the method of Banker and Cowan (1977) as modified by Brewer et al. (1993). Briefly, the hippocampus was dissected from embryonic day 18 (E18) mice (Charles River, Montreal, Quebec, Canada), exposed to trypsin, dissociated mechanically, and grown in 60 mm collagen/poly-L-lysine-coated dishes. Cells from two litters of mice were plated into six dishes. Cultures were maintained in serum-free Neurobasal medium (Life Technologies, Gaithersburg, MD) containing 0.5 mM glutamine and 1_ B27 supplement (Life Technologies). AtT-20 cells and COS-1 cells were cultured as reported previously (Seidah et al., 1996a). AtT-20 cells are a neuroendocrine cell line that has been used extensively for studying the regulated secretory pathway (Moore et al., 1983). We also used an AtT-20 cell line stably transfected with α1-PDX cDNA that has been described previously (Benjannet et al., 1997). Special care was taken to ensure that cells were distributed in equal numbers in dishes that were to be used for group comparisons.

Vaccinia virus (VV) recombinants and infections - Purified recombinant VVs containing the full-length coding regions of mouse pro-NGF, human pro-BDNF, and human pro-NT-3 (generously provided by Regeneron Pharmaceuticals) were constructed as described previously (Seidah et al., 1996a,b). VVs coding for α1-PDX were kindly provided by Dr. Gary Thomas (Vollum Institute, Portland, OR). Separate plates of cells were infected as described previously (Seidah et al., 1996a) with VV encoding pro-NGF, pro-BDNF, or pro-NT-3, generally at a multiplicity of infection (MOI) of 1. We incubated the cells for 8-10 hr in virus-free medium before metabolic labeling. In some experiments, plates were coinfected with pro-BDNF and either pro-NT-3 or pro-NGF, using an MOI of 0.5 for each. Under our experimental conditions, there was no evidence of cell death after exposure to VVs for the times indicated.

Construction of expression vectors and transfections - In some experiments, we transfected cells with DNA using the lipofectamine reagent (Life Technologies). cDNAs corresponding to the full-length coding regions of human pro-BDNF and human pro-NT-3 were subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). AtT-20 cells growing on poly-L-lysine-coated coverslips were transfected using lipofectamine with a constant amount of DNA (2 μg DNA/2 ml medium) that was composed of expression vector alone (with no insert) together with 0.1, 0.5, 1, or 2 μg of pro-BDNF or pro-NT-3 plasmid DNA. After 5 hr of incubation, the transfection medium was diluted 1:1 with DMEM/20% fetal calf serum, and after 2 d, coverslips were processed for immunostaining (see below).

Metabolic labeling and immunoprecipitation - For pulse-chase experiments, we incubated infected cells with 1.5 ml of Cys-Met-free DMEM containing 10% FCS and 0.5 mCi/ml [35S] Translabel (ICN Biochemicals, Costa Mesa, CA) (70% methionine, 30% cysteine) for 30 min. Pro-BDNF contains eight methionines as compared with three in pro-NT-3, and mature BDNF contains three methionines as compared with none in mature NT-3. These differences, together with higher concentrations of methionine than cysteine in the Translabel, explain why, in Figures showing the results of metabolic labeling experiments, pro-BDNF and mature BDNF label more heavily than pro-NT-3 and NT-3. For the chase periods, cells were washed, and the medium was replaced with an equal volume of DMEM containing 10% FCS plus twofold excess concentrations of nonradioactive cysteine and methionine for the times indicated. In all experiments, conditioned media and cell lysates were brought to final volumes of 1.5 ml, 750 μl of which was subjected to immunoprecipitation. Samples immunoprecipitated with nonimmune rabbit IgG showed no bands corresponding to standards of neurotrophin precursors or products.

Immunoprecipitations were performed as described previously (Seidah et al., 1996a). For NT-3, we used an affinity-purified rabbit anti-NGF IgG that recognizes NT-3 (Murphy et al., 1993; Seidah et al., 1996a). BDNF immunoprecipitations were performed using an antibody kindly supplied by Amgen and characterized previously

(Fawcett et al., 1997; Yan et al., 1997). Cell lysates and conditioned media were analyzed by electrophoresis on a 13-22% SDS-PAGE. Gels were fixed in 40% methanol and 10% acetic acid, treated with ENHANCE (Dupont NEN, Boston, MA), and washed in 10% glycerol, all for 1 hr. Dried gels were analyzed by a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA), and radioactivity in each band was quantitated using the ImageQuant program. Levels of radioactivity were within the linear range of the device. Statistical significance was determined using the Student's t test on a minimum of triplicate experiments.

To monitor the effects of depolarization on neurotrophin release, we infected hippocampal neurons with recombinant viruses, metabolically labeled the cells for 30 min, and washed and incubated the cells in medium containing excess nonradioactive methionine and cysteine for 4 hr. The cells were exposed to tissue culture medium supplemented with or without KCl (56 mM) and CaCl2 (5.8 mM) for 15 min. Conditioned media and cell lysates were collected, immunoprecipitated, and fractionated by SDS-PAGE. Neurotrophin levels were estimated and compared by PhosphorImager analysis. In a previous control experiment (Mowla et al., 1999), we confirmed that KCl induces the release of endogenous secretogranin II in cultures of VV:NGF-infected hippocampal neurons to confirm that the regulated secretory pathway is fully functional in cells infected with VV constructs (Mowla et al., 1999).

Immunocytochemistry and confocal microscopy - We visualized VV-infected AtT-20 cells and primary cultures of hippocampal neurons as well as controls consisting of uninfected cells or cells infected with wild-type VVs. Cells were rinsed with PBS, fixed for 25 min in 4% paraformaldehyde/15% picric acid in 0.1 M phosphate buffer, pH 7.4, and incubated in PBS containing 20% horse serum for 30 min to reduce nonspecific binding. The cells were incubated with 1 μg/ml of affinity-purified anti-NT-3 (Chemicon, Temecula, CA) in PBS/0.2% Triton X-100 overnight at 4°C, washed three times with PBS/0.05% Tween-20 (5 min each), and incubated for 1 hr with CY3-conjugated goat anti-rabbit antibody (Jackson Laboratory, Bar Harbor, ME) diluted 1:2000 in PBS/0.05% Tween-20 containing 10% goat serum. Cells were

washed three times in PBS and mounted in a Tris-buffered glycerol mounting medium (Sigma, St. Louis, MO). In control experiments, the anti-NT-3 antibody showed no detectable cross-reactivity by immunocytochemistry with either NGF or BDNF (data not shown).

Double-label immunocytochemistry was also performed on VV-infected AtT-20 cells to compare the distribution of NT-3 and NT-3/BDNF with that of TGN38, a marker of the TGN (Luzio et al., 1990), and ACTH, which is packaged in secretory vesicles. Antibody to TGN38 raised in guinea pig (kindly provided by Drs. G. Banting and W. Garten) was used at a 1:50 dilution and visualized using an FITC-conjugated secondary antibody raised in goat (Jackson Laboratory) diluted 1:50 in PBS containing 10% goat serum. ACTH was localized with a monoclonal antibody (Cortex Biochem) at a dilution of 1:1000, visualized with a CY2-conjugated goat anti-mouse secondary antibody (Jackson Laboratory) diluted 1:1000 in PBS containing 10% goat serum.

Cells were analyzed by confocal laser scanning microscopy using a Zeiss LSM 410 inverted confocal microscope and a 63_, 1.4 NA objective. Cells were excited at 543 nm and imaged on a photomultiplier after passage through FT 590 and LP 590 filter sets. The confocal images represent one confocal level (a depth of ~1 μm) that includes the cell nucleus along with as many cell processes as were possible to capture, the goal being to evaluate the distribution of secretory vesicles. No differences were evident in the distribution of NT-3 or NT-3/BDNF immunoreactivity when we scanned at various levels below or above the nucleus.

RESULTS

NT-3 is released constitutively from hippocampal neurons and AtT-20 cells - To determine the pathway by which NT-3 is processed and released, we did the following: (1) measured the retention or release of processed NT-3 from virally infected cells after pulse-chase labeling; (2) determined whether agents that promote vesicle exocytosis promote the release of NT-3; and (3) used immunocytochemistry to visualize the intracellular localization of NT-3 in virally infected cells.

Figure 5.1 shows the results of 30 min pulse-chase studies performed over 8 hr to monitor the processing of pro-NT-3 in primary cultures of hippocampal neurons (Fig. 5.1A) and in AtT-20 cells (Fig. 5.1B). In both cell types, pro-NT-3 (33.5 kDa) is processed to mature NT-3 (14.5 kDa). The precursor is detectable in cell lysates at the start of the chase period, and levels decrease thereafter; by 8 hr of chase, the precursor is barely detectable. Intact pro-NT-3 is not detectable in conditioned medium at any time point in either cell type. Mature NT-3 is visible in cell lysates at the beginning of the chase period, but over time, levels in cell lysates decrease. Over the same time period, NT-3 levels in conditioned medium increase, and by 2 hr they exceed those in cell lysates. Therefore, most newly processed NT-3 is rapidly released from both hippocampal neurons and AtT-20 cells.

We were surprised that levels of pro-NT-3 were so low in these cells and that processed NT-3 was evident immediately after the 30 min pulse period. In our previous studies, levels of pro-NGF and pro-BDNF were much higher at the beginning of the chase period, attributable in part to increased labeling efficiency (see Materials and Methods). Also, significant processing of pro-NGF and pro-BDNF was not evident until 30 min after the chase began. To test whether these differences were caused by pro-NT-3 being processed more efficiently in the constitutive pathway than either pro-NGF or pro-BDNF, we compared precursor processing in virally infected COS-1 cells, a cell line that contains only the constitutive secretory pathway. Results show that abundant amounts of processed

NGF and BDNF are detectable in cell lysates and conditioned medium at the end of the test period (Fig. 5.2). As well, significant levels of unprocessed pro-NGF and pro-BDNF are detectable in cell lysates and in conditioned medium, which suggests that some precursor escapes proteolysis, perhaps by overwhelming the processing capacity of the cell (Mowla et al., 1999). In contrast, pro-NT-3 is detectable in cell lysates but not in conditioned medium, whereas processed NT-3 is evident in both. One explanation for these data is that pro-NT-3 is more efficiently processed within the constitutive secretory pathway than either pro-NGF or pro-BDNF.

We used pulse-chase labeling methods similar to those used in Figure 5.1 to determine whether the endoprotease furin, which is present in the constitutive pathway, is involved in pro-NT-3 processing in AtT-20 cells. For this experiment, we used AtT-20 cells that have been stably transfected with α1-PDX, an α1-anti-trypsin structural variant that selectively inhibits furin-mediated cleavage of precursor proteins in the TGN (Anderson et al., 1993; Watanabe et al., 1995; Vollenweider et al., 1996; Benjannet et al., 1997). In cells expressing α1-PDX (Fig. 3), significant levels of pro-NT-3 are released into conditioned medium over the 8 hr chase period, but levels of processed NT-3 are markedly reduced (compare Fig. 3 with Fig. 1B). Therefore, inhibition of furin-mediated cleavage prevents the processing of pro-NT3 and the efficient generation of mature product.

The finding that α1-PDX prevents the processing of pro-NT-3 and induces its release into conditioned medium is identical to that obtained previously with pro-NGF (Mowla et al., 1999). In that study, we determined that inhibiting furin cleavage with α1-PDX caused the shunting of pro-NGF from the constitutive to the regulated secretory pathway (Mowla et al., 1999). We also observed that some pro-BDNF is constitutively released in the course of its processing within the regulated secretory pathway (Mowla et al., 1999). Together, these results suggest that inhibiting furin-mediated cleavage of pro-NT-3 may also direct the precursor from the constitutive to the regulated secretory pathway.

To test this idea, we monitored the effects of cAMP on NT-3 release from AtT-20 cells in the presence or absence of α1-PDX. We also used immunocytochemistry to analyze the distribution of NT-3 under both experimental conditions. Results show that cAMP stimulates the release of NT-3 from AtT-20 cells in the presence (Fig.5.4, right panel) but not in the absence (left panel) of α1-PDX. This result is consistent with immunocytochemical data (Fig. 5.5) showing that in the absence of α1-PDX (Fig. 5.5A), NT-3 immunoreactivity is distributed in the perinuclear cytoplasm and not in cell processes, an appearance identical to that previously reported for NGF (Mowla et al., 1999). However, in the presence of α1-PDX (Fig. 5.5B), punctate NT-3 immunoreactivity is evident throughout the cytoplasm and in the tips of cell processes, which is consistent with earlier results monitoring the distribution of BDNF in wild-type cells and NGF in α1-PDX-treated cells (Mowla et al., 1999). Thus NT-3, which is normally processed and released from the constitutive pathway, can be rerouted to the regulated secretory pathway when furin-mediated cleavage within the TGN is inhibited.

In earlier studies, Heymach et al. (1996) and Moller et al. (1998) showed that NT-3 is packaged in dense-core vesicles of AtT-20 cells and PC12 cells and released in response to secretagogues or cell depolarization. To explain why their results differ from ours, we investigated whether elevating levels of pro-NT-3 expression could redirect NT-3 from the constitutive to the regulated secretory pathway, as has been shown previously for pro-NGF (Mowla et al., 1999). Initially, we determined that infecting AtT-20 cells with 5, 10, and 25 MOIs of VV encoding pro-NT-3 resulted in only a small amount of pro-NT-3 being redirected into the regulated secretory pathway, as determined by the ability of cAMP to induce NT-3 release (data not shown). Similar infection levels were extremely effective in rerouting NGF from the constitutive to regulated secretory pathway (Mowla et al., 1999). We suspect that the differences are attributable to pro-NT-3 being more efficiently processed than pro-NGF within the constitutive secretory pathway, as shown in Figure 5.2. To achieve higher intracellular concentrations of pro-NT-3, we opted for

a lipofectamine transfection method similar to that used by Heymach et al. (1996) and Moller et al. (1998).

Lipofectamine transfection of pro-NT-3 DNA (0.1 μg with 1.9 μg vector DNA/2 ml of culture medium) resulted in NT-3 immunoreactivity in AtT-20 cells that is diffusely distributed in the perinuclear cytoplasm (Fig. 5.6A), as seen earlier (compare with Fig. 5.5A). However, when we transfected 0.5 or 1 μg of pro-NT-3 DNA (with vector DNA to a total of 2 μg/2 ml of culture medium), NT-3 immunoreactivity is evident in punctate structures that extend into cell processes, which is consistent with the appearance of secretory proteins processed in the regulated secretory pathway (Fig. 5.6B). For comparison, we show in Figure 5.6C the punctate localization of BDNF immunoreactivity in cells exposed to only 0.1 μg of pro-BDNF with 1.9 μg of vector DNA/2 ml of culture medium. Pro-BDNF is processed within the regulated secretory pathway (Mowla et al., 1999). Thus, cells transfected with high concentrations of pro-NT-3 DNA process the protein in the regulated secretory pathway, which likely explains why our results with VV infection methods differ from those of Heymach et al. (1996) and Moller et al. (1998) using lipofectamine transfection.

NT-3 is sorted to the regulated secretory pathway when coexpressed with pro-BDNF - Because NT-3 is processed in the constitutive pathway and BDNF is processed in the regulated pathway (Mowla et al., 1999), we questioned what would happen if we coexpressed precursors to the two proteins within the same cell. Others have shown that neurotrophins readily heterodimerize (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Philo et al., 1994; Heymach and Shooter, 1995; Robinson et al., 1995; Treanor et al., 1995), but nothing is known about the mechanisms regulating the processing, sorting, and release of heterodimers within cells.

For these studies, we first analyzed the specificity of our antibodies, because previous studies have shown that antibodies to, one neurotrophin can cross-react on

Western blots with the other (Murphy et al., 1993). In these studies we infected AtT-20 cells for 1 hr with 1 MOI of VV coding for either pro-NT-3 or pro-BDNF, incubated the cells for 8 hr without virus, metabolically labeled the cells for 3 hr, and immunopreciptated cell lysates and conditioned media with antibodies to NGF (for NT-3) or BDNF. In some experiments, we coinfected cells with 0.5 MOI of VV coding for pro-NT-3 and pro-BDNF to ensure that the total level of viral infection (1 MOI) was held constant.

Figure 5.7 (left side) shows that antibody to NGF immunoprecipitates NT-3 but not BDNF or pro-BDNF. Similarly, antibody to BDNF (Fig. 5.7, right side) immunoprecipitates BDNF and pro-BDNF but not NT-3. In contrast, in cells coinfected with VV coding for pro-NT-3 and pro-BDNF, antibody to NGF immunoprecipitates in conditioned medium NT-3 as well as a protein migrating in a position identical to that of pro-BDNF (Fig. 5.7, left side). We interpret this result to mean that NT-3 and pro-NT3 are associating with the BDNF precursor. In cell lysates as well as conditioned medium, the NGF antibody immunoprecipitates a doublet consisting of a higher molecular weight band (probably arising from precipitation of NT-3 alone and with BDNF) as well as a lower molecular weight band (BDNF) that precipitates because of association with NT-3. The right side of Figure 5.7 shows that antibodies to BDNF precipitate in cell lysates, and to a lesser extent in conditioned medium, a doublet consisting of a lower band (probably arising from precipitation of the BDNF alone or with NT-3) as well as an upper band (NT-3). Results identical to those shown in Figure 5.7 were obtained when the same experiments were performed in cultures of hippocampal neurons (data not shown).

In short, NT-3-specific antibodies precipitate BDNF, and BDNF-specific antibodies precipitate NT-3 only when the two neurotrophins are coexpressed within cells. We strongly suspect that these two independent but mutually consistent results arise from NT-3 and BDNF forming heterodimers intracellularly. Immunoprecipitation methods similar to ours were used previously by Jungbluth et al. (1994) and Heymach and Shooter (1995) to characterize neurotrophin

heterodimers, including NT-3/BDNF. It should be noted that coinfection with wild-type and BDNF-encoding VV and coinfection with VV:pro-NGF and VV:pro-BDNF did not result in coprecipitated neurotrophins (data not shown), probably because of the relative instability of NGF/BDNF heterodimers, as reported previously (Radziejewski and Robinson, 1993; Arakawa et al., 1994).

Evidence in support of the idea that coexpression of NT-3 with BDNF results in some NT-3 being shunted from the constitutive to the regulated secretory pathway is shown in Figure 5.8. The bar graph (Fig. 5.8A) shows the amount of processed NT-3 in cell lysates as a function of the total amount of NT-3 present in cell lysates and conditioned media. Results indicate that more NT-3 is retained within cells when pro-NT-3 is coexpressed with pro-BDNF-encoding virus as compared with wild type. This result suggests that NT-3 and BDNF are noncovalently associated through heterodimerization and that association of the two leads to intracellular retention of NT-3, perhaps within dense-core vesicles. Examination of a typical SDS gel used in this analysis (Fig. 5.8B) shows that NT-3 and BDNF, which are coprecipitated by the NGF (NT-3-reactive) antibody, are evident within the cell lysate and conditioned medium, suggesting that coinfected cells synthesize and release the NT-3 and BDNF together. In companion experiments, coinfection with VV:pro-BDNF and VV:pro-NGF had no effect on NGF retention as compared with the wild-type coinfection control (data not shown).

The idea that NT-3 is diverted into the regulated secretory pathway is further confirmed by secretion data presented in Figure 5.9. When pro-NT-3 is coexpressed with pro-BDNF, processed NT-3 can be released from AtT-20 cells (Fig. 5.9A) in response to cAMP and from hippocampal neurons by KCl depolarization (Fig. 5.9B). Thus, release of NT-3 that is coexpressed (and presumably dimerized) with BDNF appears to be regulated by the same extracellular signals that regulate the release of homodimeric BDNF (Mowla et al., 1999). Cell lysates and conditioned media immunoprecipitated with NGF antibodies contained both NT-3 and BDNF, further

indicating that the two neurotrophins are synthesized and released together in our culture system.

Finally, we used immunocytochemistry and confocal microscopy to compare the intracellular distributions of NT-3 and BDNF when they are singly expressed or coexpressed in hippocampal neurons. Figure 5.10 shows that in hippocampal neurons infected with 1 MOI of VV:pro-NT-3, NT-3 immunoreactivity is distributed in the perinuclear cytoplasm (Fig. 5.10A), as was seen in AtT-20 cells (Fig. 5.5). However, in cells coinfected with 0.5 MOI of VV encoding pro-NT-3 and pro-BDNF, NT-3 immunoreactivity (Fig. 5.10C) is localized in punctate structures distributed throughout the cell cytoplasm and in the tips of cell processes, an appearance similar to that of BDNF immunoreactivity in cells infected with pro-BDNF alone (Fig. 5.10B). In control studies, coexpression of pro-NT-3 and pro-NGF did not change the distribution of NT-3, as assessed by immunocytochemistry (data not shown). Furthermore, coexpression of pro-BDNF and pro-NGF did not divert NGF from the constitutive to the regulated pathway as assessed by either immunoprecipitation or immunocytochemistry (data not shown).

Figure 5.11 compares the distribution in virally infected AtT-20 cells of NT-3 alone and NT-3 coexpressed with BDNF with that of endogenous TGN38, a Golgi marker, and endogenous ACTH, which is packaged within secretory granules of AtT-20 cells. In cells infected with VV:pro-NT-3 alone, immunoreactivity for NT-3 (Fig. 5.11A) and TGN38 (Fig. 5.11B) colocalize in the perinuclear cytoplasm (Fig. 5.11C). In contrast, in cells coinfected with VV encoding pro-NT-3 and pro-BDNF, NT-3 immunoreactivity (Fig. 5.11D) is localized in the perinuclear cytoplasm as well as in punctate structures within the cytoplasm and tips of cell processes. In the same cells, ACTH immunoreactivity is distributed in punctate structures within cell processes (Fig. 5.11E). In a subpopulation of vesicles, NT-3 and ACTH immunoreactivity colocalize (Fig. 5.11F).

DISCUSSION

Data in this study show that NT-3 is processed and released from the constitutive secretory pathway of hippocampal neurons and AtT-20 cells. Pulse-chase experiments reveal that the NT-3 precursor is rapidly cleaved in cells infected with VV encoding the NT-3 precursor (Fig. 5.1), with processed NT-3 being evident 30 min after cells are exposed to medium containing radiolabeled amino acids. Over the next 8 hr, most processed NT-3 is released into conditioned medium, a result similar to that reported previously for NGF (Mowla et al., 1999). Immunocytochemical analysis shows that NT-3 is diffusely distributed within the perinuclear cytoplasm (Figs. 5.5, 5.10, 5.11), and colocalizes with TGN38, a marker of the trans-Golgi network (Figs. 5.11A-C). Secretagogues (Figs. 5.4, 5.9A) and KCl-induced depolarization (Fig. 5.9B) do not induce the release of radiolabeled NT-3 from virus-infected AtT-20 cells or hippocampal neurons, respectively. Thus, NT-3 release is constant and not dependent on extracellular cues, which is similar to the results we obtained for NGF (Mowla et al., 1999).

Pro-NGF, pro-BDNF, and pro-NT-3 were cleaved in COS-1 cells, which is a constitutively secreting cell line that does not have a regulated pathway. Most pro-BDNF and some pro-NGF (but not pro-NT-3) were released into conditioned medium (Fig. 5.2). This result could be attributable to pro-BDNF and to a lesser degree pro-NGF not being cleaved as effectively as pro-NT-3 by furin or furin-like enzymes within the TGN. The consensus cleavage site of pro-NT-3 (Arg-Arg-Lys-Arg↓Tyr) is ideally suited for furin-mediated processing, probably even more so than that of pro-NGF (Arg-Ser-Lys-Arg↓Ser) (Decroly et al., 1994). Basic residues at positions -4, -2, -1 are conducive to processing in the constitutive secretory pathway (Watanabe et al., 1992). However, the presence of an additional Arg residue at position -3 in pro-NT-3 may enhance even more the ability of furin to cleave the protein at this site.

Although it has yet to be shown directly with neurotrophin precursors, several lines of evidence suggest that sensitivity to furin-mediated cleavage within the TGN is an important factor in determining whether a protein is sorted to the constitutive or regulated secretory pathway (Brechler et al., 1996). In this study, blocking furin activity with α1-PDX inhibited pro-NT-3 processing in the constitutive pathway and resulted in the constitutive release of unprocessed pro-NT-3 (Fig. 5.3). In addition, α1-PDX treatment caused a shift in the appearance of NT-3 immunoreactivity from diffuse and perinuclear to punctate and distributed throughout the cell cytoplasm, including in the tips of cell processes (Fig. 5.5). Also, processed NT-3 was released by cAMP treatment (Fig. 5.4), which is characteristic of proteins within the regulated secretory pathway. Insertion of furin-sensitive cleavage sites into precursors that are normally processed in the regulated secretory pathway favors release through the constitutive secretory pathway (Oda et al., 1991; Yanagita et al., 1992), which is further evidence of the importance of furin sensitivity in the sorting decision (Jung and Scheller, 1991; Brechler et al., 1996; Mowla et al., 1999).

Increasing the MOI of vaccinia virus also shifted pro-NGF from the constitutive to the regulated secretory pathway (Edwards et al., 1988; Mowla et al., 1999), probably by saturating the ability of furin to cleave pro-NGF as a substrate. However, increasing MOI had little effect on the subcellular localization of NT-3 (data not shown), probably because pro-NT-3 is especially well cleaved in the TGN by furin. In contrast, lipofectamine transfection methods (Fig. 5.6) resulted in NT-3 immunoreactivity becoming localized within punctate structures throughout the cytoplasm and in the tips of cell processes. Lipofectamine transfection may yield higher intracellular concentrations of neurotrophin precursors in the comparatively small number of cells that are transformed (<10%) when compared with vaccinia virus infection (>90% cells infected). These differences in intracellular levels of the protein can be inferred by comparing NT-3 immunoreactivity in lipofectamine-transfected cells (Fig. 5.6B) with that of vaccinia virus-infected cells (Fig. 5.11A). The differences probably explain why Heymach et al. (1996) and Moller et al. (1998)

detected NT-3 within the regulated secretory pathway of AtT-20 and PC12 cells. Similarly, increasing expression levels altered the intracellular distribution of 2-microglobulin in pancreatic cells of transgenic mice (Allison et al., 1991). Exogenously applied NT-3 is as effective as BDNF in potentiating synaptic efficacy in hippocampal CA1 neurons, which suggests that NT-3, like BDNF, may normally access the synapse through its activity-dependent release from presynaptic neurons (Kang and Schuman, 1995). However, removing endogenous NT-3 has no effect on long-term potentiation (LTP) in mouse hippocampal CA1 neurons (Chen et al., 1999; Ma et al., 1999). In contrast, blocking endogenous BDNF reduces LTP (Korte et al., 1995). Thus, exogenously applied NT-3 may mimic the effects of BDNF through mechanisms that are unrelated to the way NT-3 normally accesses the synapse (Chen et al., 1999).

Neurotrophin heterodimers have not yet been detected in vivo, even with antibodies that recognize them specifically (Kolbeck et al., 1999). However, neurotrophins can form stable heterodimers either when renatured together or coexpressed in cells (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Philo et al., 1994; Heymach and Shooter, 1995; Robinson et al., 1995; Treanor et al., 1995). Thus, cells that coexpress neurotrophins such as hippocampal neurons (Maisonpierre et al., 1990; Schecterson and Bothwell, 1992) could produce heterodimeric forms of these proteins. The NT-3/BDNF heterodimer is especially stable (Arakawa et al., 1994), which has allowed its crystal structure to be resolved (Robinson et al., 1995).

In our experiments, coexpressing pro-NT-3 and pro-BDNF resulted in the retention of NT-3 within vesicle-like structures that were distributed throughout the cytoplasm and within cellular processes of both AtT-20 cells and hippocampal neurons, a distribution that was identical to that of BDNF (Mowla et al., 1999). NT-3 was also released together with BDNF in response to secretagogues or depolarization. These data together with our coimmunoprecipitation studies strongly suggest that in our experimental system, NT-3 and BDNF heterodimerize, as shown

previously by others (Philo et al., 1994; Heymach and Shooter, 1995). However, confirming that idea will require isolating the heterodimers to purity and characterizing them chemically, which we have not done.

As yet, we do not know how NT-3/BDNF heterodimers form. Protein dimerization normally occurs between monomers of unprocessed precursors in the endoplasmic reticulum (Danielsen, 1990; Zhu et al., 1996), which would yield pro-NT-3/pro-BDNF heterodimers. However, heterodimers could also exist between NT-3, which is efficiently processed in the TGN, and pro-BDNF, which is processed in immature secretory granules. Indeed, in our coimmunoprecipitation experiments, we detect within conditioned medium pro-BDNF along with mature NT-3 and BDNF. Further processing of the NT-3/pro-BDNF heterodimer likely occurs in immature secretory granules to yield heterodimers of mature forms of NT-3 and BDNF.

Others have noted previously that heterodimerization can alter the intracellular trafficking of proteins. For example, the common -subunit of lutropin, follitropin, and chorionic gonadotropin is constitutively secreted when produced alone; however, heterodimerization with the appropriate -subunit causes the heterodimer to be sorted and released by the regulated secretory pathway (Blomquist and Baenziger, 1992; Bielinska et al., 1994). Apparently, a single chain of a protein destined for the regulated secretory pathway contains sufficient information to reroute the entire heterodimeric complex.

The finding that pro-NT-3 can be released from either the constitutive or regulated secretory pathways suggests that NT-3 could have multiple functions. The release of homodimeric NT-3 from the constitutive secretory pathway would allow NT-3 to be constantly available as a differentiation and survival-promoting factor for neurons, which appears to be its role during development (for review, see Chalazonitis, 1996). In contrast, when NT-3 heterodimerizes with BDNF, as may occur when both are highly expressed in adult hippocampus (Maisonpierre et al., 1990), NT-3 is sorted to the regulated secretory pathway, where the NT-3/BDNF

heterodimer is packaged within vesicles that are released in response to activity. Under these conditions, NT-3 acting at TrkC receptors or NT-3/BDNF heterodimers acting in concert at TrkC and TrkB receptors (Philo et al., 1994) could regulate synaptic transmission and plasticity. Thus, understanding how NT-3 is processed in specific populations of neurons at different times

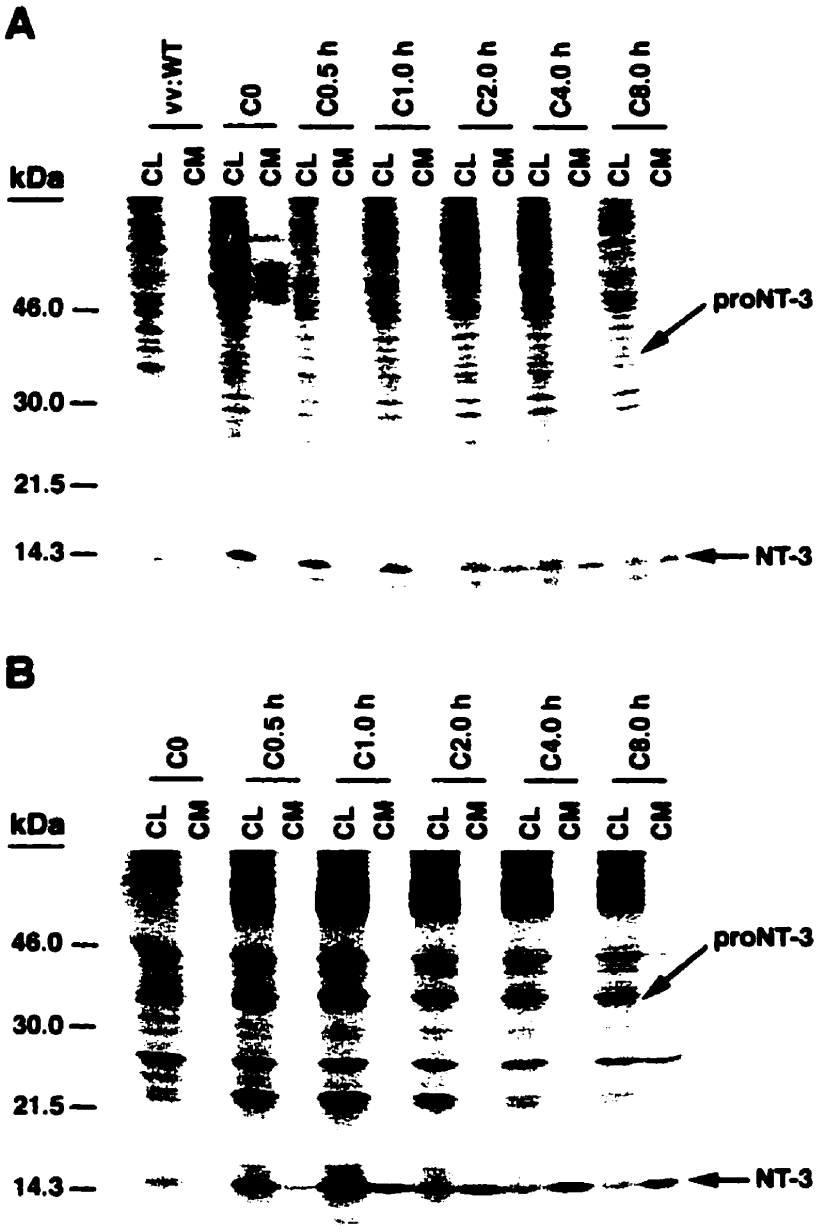

A
vv:WT
C0
C0.5 h
C1.0 h
C2.0 h
C4.0 h
C8.0 h
kDa
CL
CM
46.0
30.0
21.5
14.3
proNT-3
NT-3
B
C0
C0.5 h
C1.0 h
C2.0 h
C4.0 h
C8.0 h
kDa
CL
CM
46.0
30.0
21.5
14.3
proNT-3
NT-3

FIGURE 5.1 **Pulse-chase metabolic labeling of pro-NT-3 in primary cultures of hippocampal neurons (A) and AtT-20 cells (B).** Cells were infected with VV encoding the NT-3 precursor for 1 hr and postincubated in fresh medium without virus for 8 hr. Cells were then exposed to medium containing [^{35}S] Cys-Met for 30 min and chased for 0, 0.5, 1, 2, 4, and 8 hr. Identical volumes (750 µl) of cell lysates (CL) and conditioned media (CM) were incubated with antibodies to NGF, which immunoprecipitate NT-3, and electrophoresed on 13-22% SDS gradient gels.

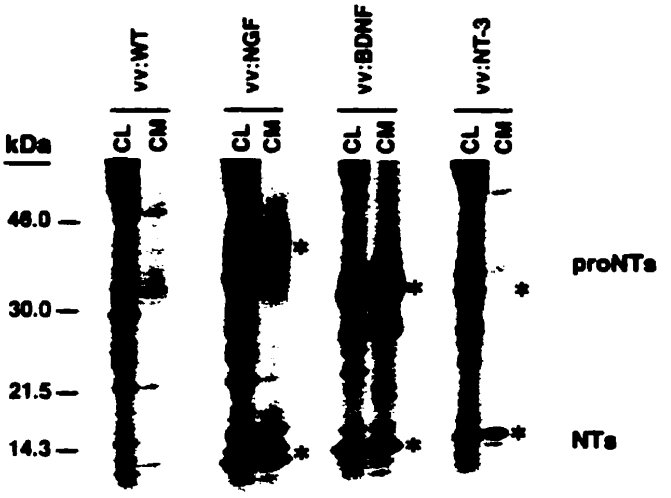
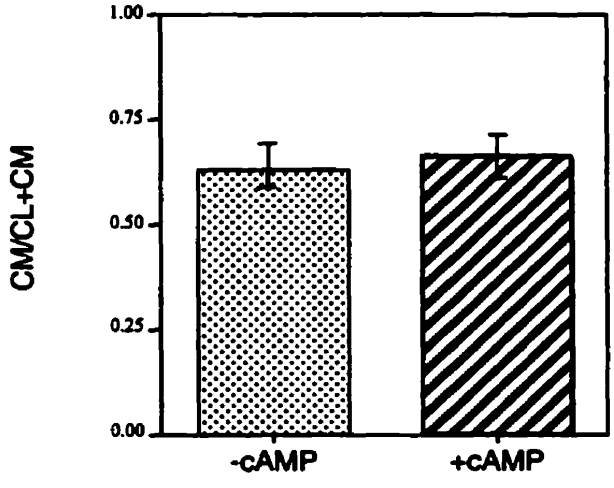

vv:WT
vv:NGF
vv:BDNF
vv:NT-3
CL
CM
kDa
46.0
30.0
21.5
14.3
proNTs
NTs

FIGURE 5.2 **Differential processing of neurotrophin precursors in COS-1 cells, which contain only the constitutive secretory pathway.** Cells were infected at an MOI of 1 with either wild-type VV (vv:WT) or VV encoding pro-NGF, pro-BDNF, or pro-NT-3. The cells were postincubated in the absence of virus for another 8 hr and metabolically labeled for 3 hr. Identical volumes of cell lysates (CL) and conditioned media (CM) from vv:WT-, vv:NGF-, and vv:NT-3-infected cells were immunoprecipitated with an NGF antibody that recognizes both NGF and NT-3. vv:BDNF-infected cells were immunoprecipitated with a BDNF-specific antibody.


C0
C0.5 h
C1.0 h
C2.0 h
C4.0 h
C8.0 h
kDa
CL
CM
30.0
21.5
14.3
proNT-3
NT-3

FIGURE 5.3 **Inhibition of pro-NT-3 processing in AtT-20 cells expressing α1-PDX.** AtT-20 cells stably expressing the furin inhibitor α1-PDX were infected for 30 min with VV encoding pro-NT-3. Cells were then incubated in virus-free medium for 8 hr, metabolically labeled for 30 min, and chased for up to 8 hr. Cell lysates (CL) and conditioned media (CM) were immunoprecipitated and analyzed by SDS-PAGE.

AtT-20



AtT-20/PDX

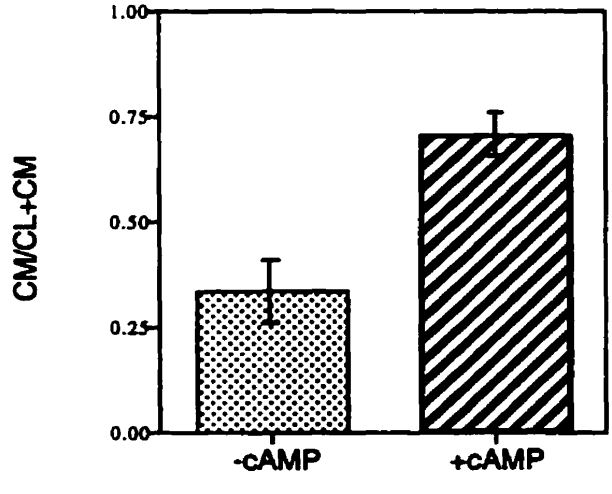


FIGURE 5.4 **cAMP-induced release of NT-3 from AtT-20 cells expressing α1-PDX.** Cells were infected with VV:pro-NT-3 for 1 hr, incubated in virus-free medium for 8 hr, metabolically labeled for 3 hr, chased for 3 hr, and treated for 3 hr with medium with or without 5 mM cAMP. CL and CM were immunoprecipitated and the amount of processed, mature NT-3 was analyzed by SDS-PAGE. Analysis was performed on a PhosphorImager, and values represent an average (±SEM) of three independent experiments.

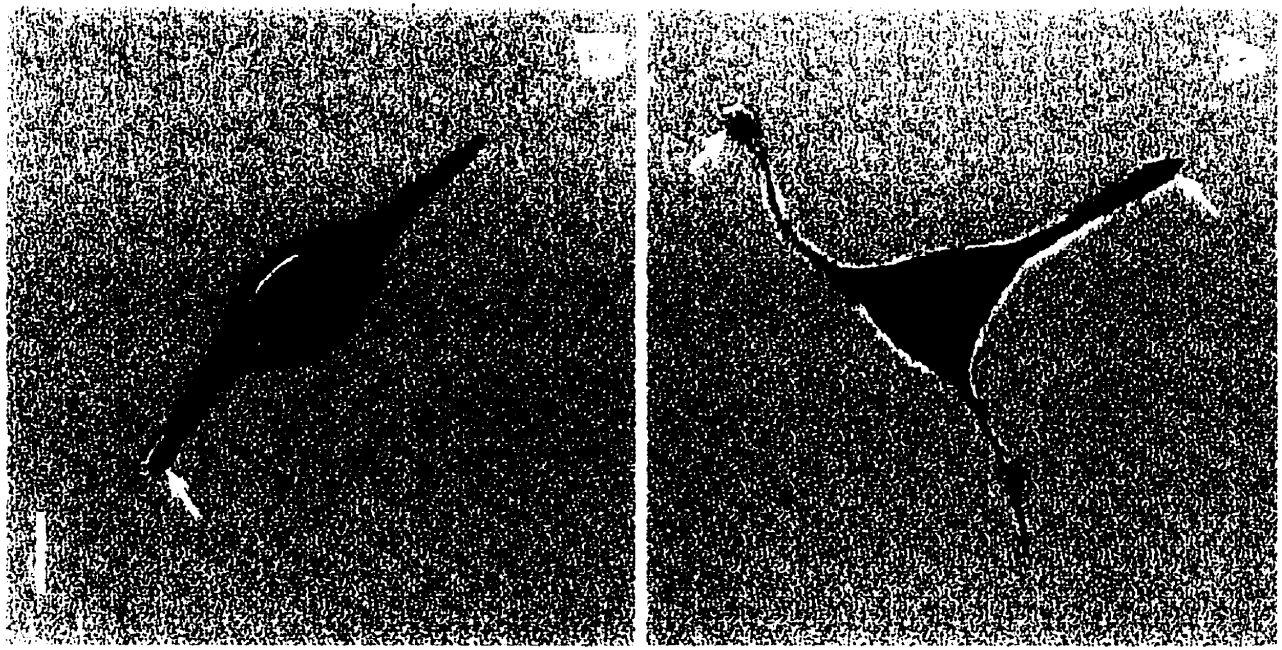


FIGURE 5.5 **Confocal microscopy of AtT-20 (A) and AtT-20/α1-PDX cells (B) infected with VV encoding pro-NT-3.** Cells were infected for 1 hr and postincubated in the absence of virus for another 8 hr. The cultures were fixed and treated with antibodies against NT-3 (Chemicon), followed by CY3-conjugated goat anti-rabbit IgG. Scale bar, 10 μm. Photomicrographs were obtained by overlaying confocal fluorescence images over transmitted light images. Scale bar, 10 μm.

FIGURE 5.6 **Overexpression of NT-3 results in rerouting from the constitutive to the regulated secretory pathway.** AtT-20 cells were transfected with a construct encoding either pro-NT-3 (A, B) or pro-BDNF (C), and immunocytochemistry using NT-3 (A, B) and BDNF (C) antibodies was performed as described in Figure 5.5. In A, cells were lipofectamine-transfected with 0.1 μg of pro-NT-3 DNA (and 1.9 μg vector DNA). In B, cells were transfected with 2 μg pro-NT-3 DNA. In C, cells were transfected with 0.1 μg of pro-BDNF (and 1.9 μg vector DNA). Scale bar, 10 μm.

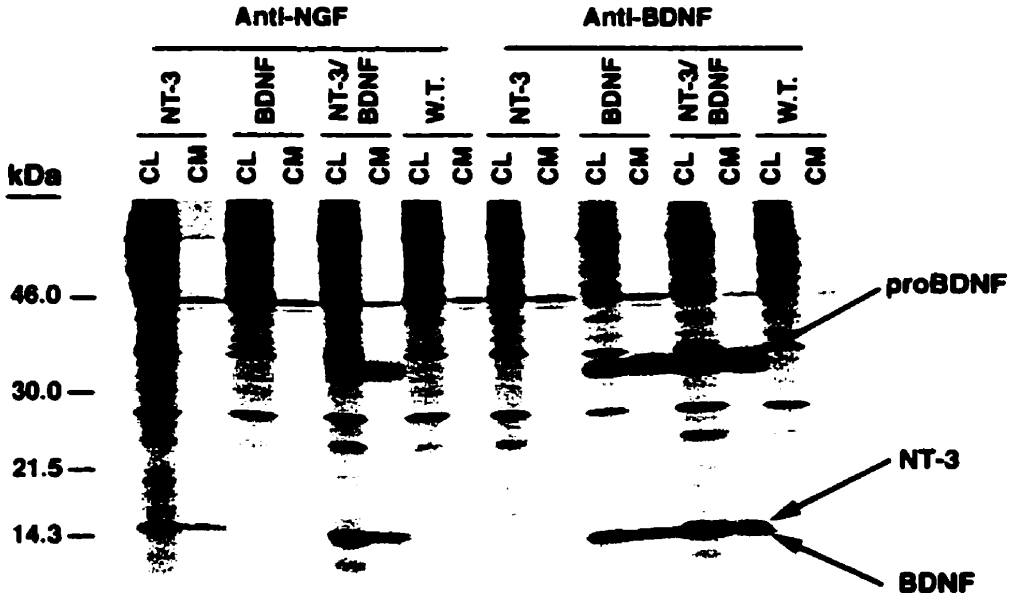

Anti-NGF
Anti-BDNF
NT-3
BDNF
NT-3/ BDNF
W.T.
CL
CM
kDa
46.0
30.0
21.5
14.3
proBDNF
NT-3
BDNF

FIGURE 5.7 **Immunprecipitation of cell extracts and conditioned medium from cells infected with 1MOI of wild type (W.T) VV, VV:pro-NT-3, VV:pro-NGF, VV:pro-BDNF, alone, or coinfected with 0.5 MOI of pro-NT-3 and pro-BDNF.** AtT-20 cells were infected for 1 hr with the viruses indicated, postincubated for 8 hr, and metabolically labeled for 3 hr. Cell lysates and conditioned media were immunoprecipitated with either an anti-NGF antibody (left side) or a BDNF antibody (right side).

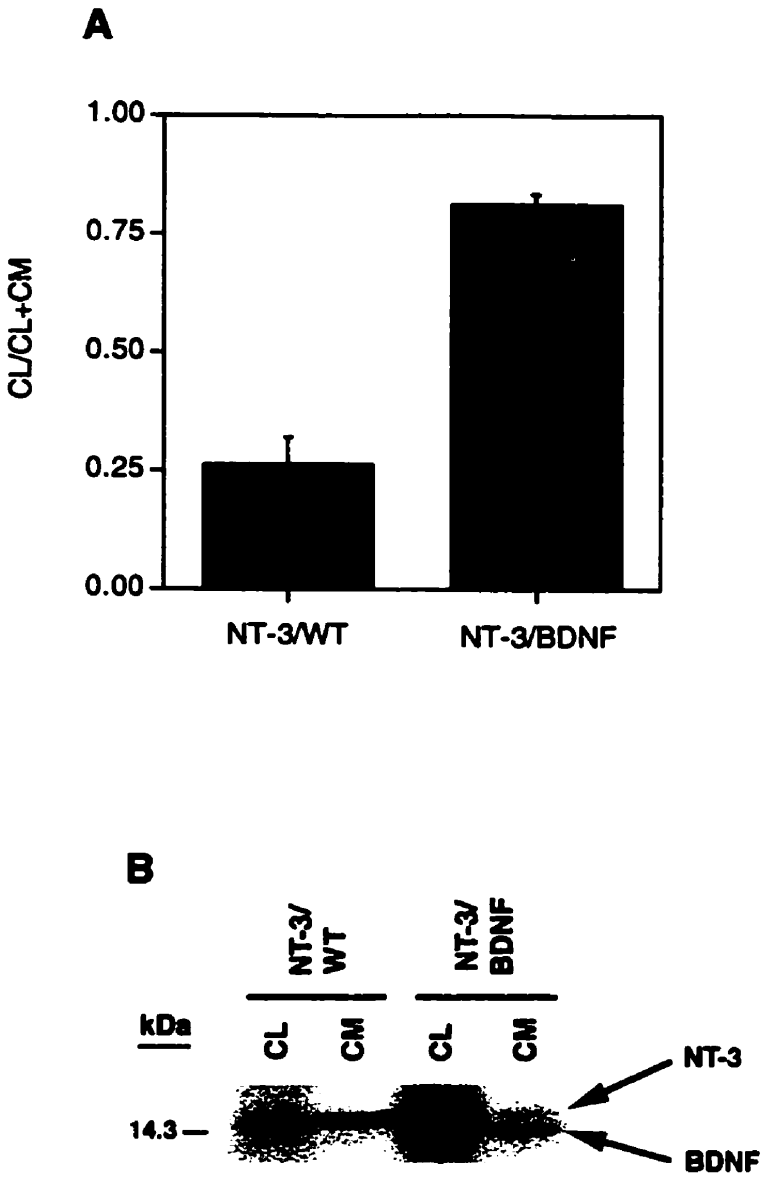
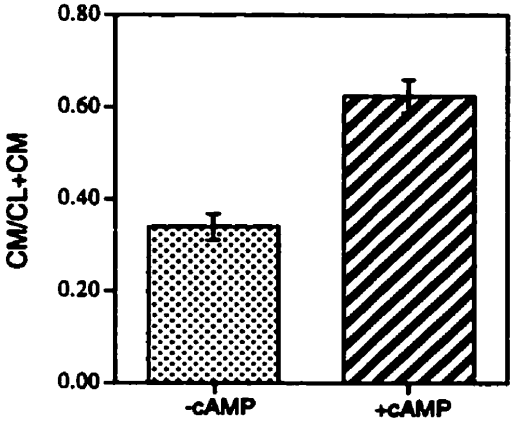

A
1.00
0.75
0.50
0.25
0.00
CL/CL+CM
NT-3/WT
NT-3/BDNF
B
NT-3/ WT
NT-3/ BDNF
kDa
CL
CM
CL
CM
14.3
NT-3
BDNF

FIGURE 5.8 **NT-3/BDNF is retained in hippocampal neurons.** A. The methodology in Figure 5.1A (involving a 4 hr chase only) was repeated three times with cells infected with either VV:pro-NT-3/VV:WT or VV:pro-NT-3/VV:pro-BDNF. The NGF antibody used for immunoprecipitations. Results were analyzed on a PhosphorImager and are an average (±SEM) of the ratio of mature NT-3 in cell lysates (CL) over the total amount of NT-3 in CL + conditioned medium (CM). B. A representative SDS gel from the experiments in A showing the NT-3/BDNF heterodimer in the cell conditioned medium.

A

AtT-20

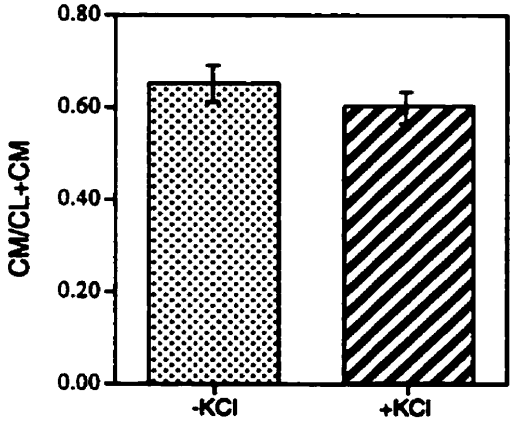
NT-3/BDNF



B

Hippocampal Neurons

NT-3



NT-3/BDNF

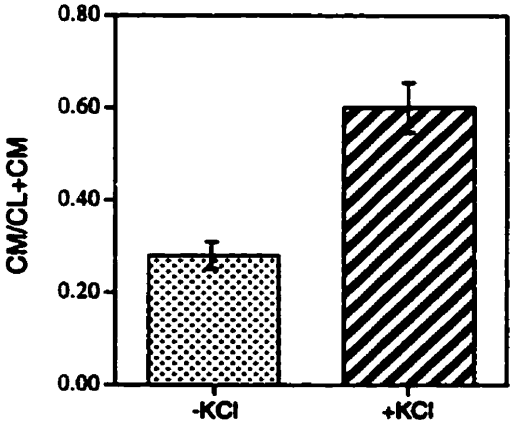


FIGURE 5.9 **Secretagogue-induced release of NT-3/BDNF but not NT-3.** A, AtT-20 cells coinfected with VV encoding pro-NT-3 and VV encoding pro-BDNF were processed using the methodology described in Figure 5.4. B. Hippocampal neurons from E18 mice were cultured for 7 d and infected for 1 hr with either (1) VV encoding pro-NT-3 or (2) VV encoding pro-NT-3 and VV encoding pro-BDNF. After 8 hr in medium without virus, the cells were labeled for 30 min with [^{35}S] Cys-Met, incubated in medium without radiolabel for 4 hr, and treated with medium with or without KCl and $CaCl_2$ for 15 min. Cell lysates and conditioned media were immunoprecipitated with the antibody to NGF and electrophoresed on an SDS gel. Results were analyzed on a PhosphorImager and are an average (±SEM) of three independent experiments.

FIGURE 5.10 **Confocal microscopy of hippocampal neurons infected with pro-NTs.** Hippocampal neurons were infected with 1 MOI VV encoding either pro-NT-3 (A) or pro-BDNF (B); in C, the cells were coinfected with 0.5 MOI each of VV:pro-NT-3 and VV:pro-BDNF. Immunocytochemistry was performed with the NT-3 antibody in A and C and the BDNF antibody in B. Scale bar, 10 μm.

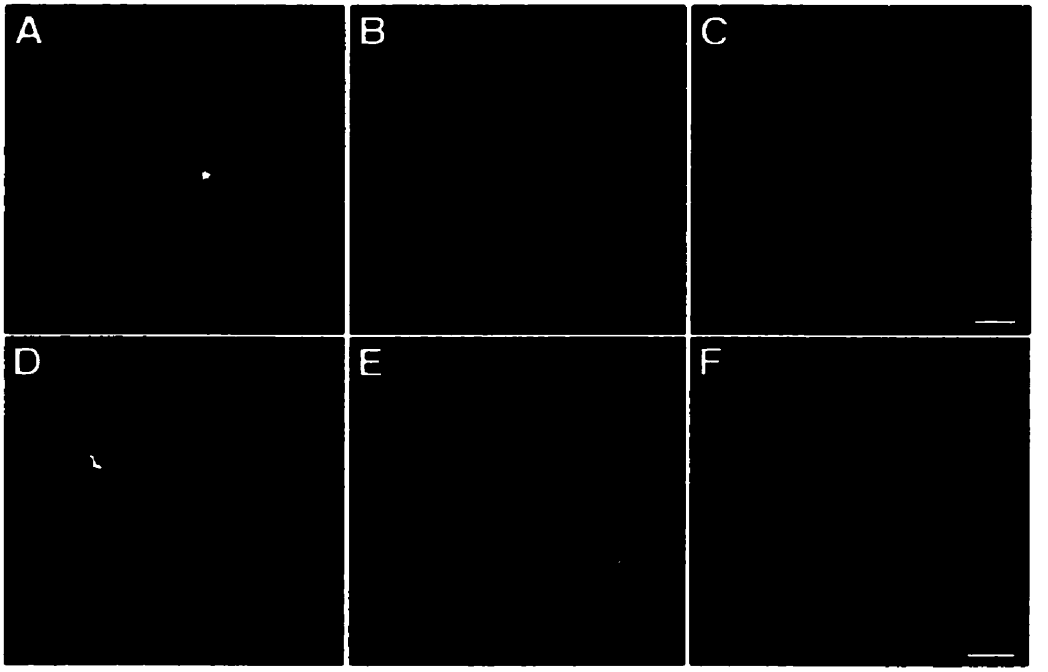

A
B
C
D
E
F

FIGURE 5.11 **Double-label immunocytochemistry comparing the distribution in infected AtT-20 cells of NT-3 and NT-3/BDNF with that of endogenous TGN38 and ACTH.** . NT-3 immunoreactivity (A) colocalizes with TGN38 (B) in the perinuclear region as seen in C (NT-3 in red and TGN38 in green). NT-3 immunoreactivity in cells coinfected with pro-NT-3 and pro-BDNF (D) colocalizes with ACTH (E) primarily in the tip of the cellular process as seen in F (NT-3 in red and ACTH in green). Scale bar, 10 μm.

CHAPTER 6

GENERAL DISCUSSION:

I. Biosynthesis and post-translational modification of pro-BDNF

The biosynthesis and post-translational processing of pro-NGF and pro-NT-3 have been already studied through collaboration between our lab and Dr Seida's (Seidah et al., 1996a, 1996b). Our previous data show that pro-NGF is N-glycosylated and glycosulfated within its pro-segment and this process is necessary for the exit of the precursor from the endoplasmic reticulum and its eventual processing and secretion (Seidah et al., 1996a). The data also reveal that in both constitutive and regulated secretory cells types furin, and to a lesser extent PACE4 and PC5/6-B, are the best candidate to process pro-NGF (Seidah et al., 1996a) and pro-NT3 (Seidah et al., 1996b) into their mature forms. Nothing was known, however, about the biosynthic pathway and post-translational modification of pro-BDNF. This has been primarily due to the lack of a good immunoprecipitating antibody against BDNF. The recent production and characterization of an affinity-purified antibody against BDNF (Yan et al., 1997) thus provides a good opportunity to study the post-translational processing of pro-BDNF and compare the results with the previous data obtained from pro-NGF and pro-NT-3.

Endogenously, NTs are produced at extremely low levels in neurons and non-neuronal cells and, thus, is almost impossible to be detected with currently available techniques. Therefore, in order to overcome the problem we used a vaccinia virus expression system to over-express pro-BDNF in a variety of constitutive and regulated cell lines as well as in primary cultures of mouse hippocampal neurons. Our data revealed that pro-BDNF is produced as a 32 kDa precursor, which in its prodomain undergoes N-glycosylation and glycosulfation. The glycosylation and sulfation presumably occurs at a single putative consensus sequence for N-linked glycosylation (Asn-X-Thr/Ser) eight residues upstream of the cleavage site of mature BDNF (Mowla et al., 1998). The glycosylation site is conserved at the same position

within the pro-domains of all NTs (Maisonpierre et al., 1990) suggesting a critical role for N-glycosylation in NT processing and/or secretion. The importance of carbohydrates in the folding of proteins has been well documented (Kornfeld and Kornfeld, 1985). Blocking N-glycosylation of pro-NGF by tunicamycin treatment prevented the entry of the precursor into the Golgi apparatus and its subsequent processing and secretion (Seidah et al., 1996a). Similarly, inhibition of N-glycosylation of pro-BDNF by tunicamycin dramatically reduced the level of radiolabeled signal in both pro-BDNF and mature BDNF in SDS-PAGE. This might be due to the effects of incorrect folding of the precursor and/or a reduction of the half-life of the un-glycosylated proteins. In the lysate of the cells treated with tunicamycin, however, we did not detect any protein fragments (bands) corresponding to the degradation of pro-BDNF within the ER. This might be due to the sensitivity of our antibody to detect such truncated forms. Using a pro-BDNF which is tagged in its both N-terminus and C-terminus would provide an approach to test that idea. On the other hand, blocking the activity of the degrading enzymes in the ER and cytosol, such as 'proteasome' enzyme may prevent the potential degradation of the precursor and led to the accumulation of the precursor in the ER. In any case, more detailed works required to confirm a direct link between glycosylation and degradation of pro-BDNF. The oligosaccharide chain is also sulfated, as we demonstrated previously for pro-NGF (Seidah et al., 1996a). While the glycosylation is demonstrated to be important for the stability of the protein, inhibition of sulfation with sodium chlorate (Baeuerle and Huttner, 1986) did not affect the processing or release of pro-BDNF, however. This result is consistent with the recent finding of Van Kuppereld et al., (1997) who reported that protein sulfation is not required for the transport, sorting, or proteolytic processing of proteins that transit through the regulated secretory pathway.

pro-BDNF undergoes N-terminal cleavage to generate the mature form (14 kDa) as well as a minor truncated form (28 kDa). Both 32 kDa precursor and 28 kDa form are significantly released into conditioned media along with mature form. In ongoing studies, we do not observe precursor release when similar methods were

used to monitor the processing and release of the precursors of NGF (Mowla et al., 1999) and NT-3 (Farhadi et al.,2000). However, previous works by others have shown that significant amounts of un-processed precursors, such as pro-opiomelanocorticotropin (POMC) are also constitutively released by the cells contain regulated secretory pathway (Fernandez et al., 1997). The constitutively released pro-BDNF might have its own biological function. In this regards, high levels of BDNF mRNA has been detected in the dendrites of hippocampal neurons in culture (Crino and Eberwine, 1996; Tongiorgi et al., 1997), suggesting that the BDNF precursor may be synthesised within dendrites (Crino and Eberwine, 1996). Thus, it is theoretically possible that pro-BDNF produced in dendrites could avoid cleavage by furin (primarily resides in *trans*-Golgi network) and/or PC1 (primarily resides in secretory vesicles) and be released in an unprocessed form, for as yet unknown purposes. Indeed, in this study we have shown that pro-BDNF is biologically active, as determined by its ability to induce TrkB autophosphorylation.

Within the constitutive pathway, α1-PDX (an α1-antitrypsin derivative that selectively interferes with furin's ability to process precursor proteins within the TGN, Anderson et al., 1993; Watanabe et al., 1995; Vollenweider et al., 1996) inhibits the cleavage of pro-BDNF into the mature form but has no effect on the production of the 28 kDa form. Further works show that 28 kDa form, but not the mature BDNF, is insensitive to BFA treatment, a molecule that inhibits anterograde vesicular transport from the ER (Fernandez et al., 1997). An interesting finding which suggest that generation of 28 kDa BDNF occurs within ER, while mature BDNF is generated within a post-ER compartment (Mowla et al., 1999). Taken together, these findings reveal that the generation of the 14 and 28 kDa products occur within two separate processing pathways. Till now, we do not know the biological importance of the 28 kDa production. For the future work, it is very important to determine the biological role of the 28 kDa BDNF. This requires purification of 28 kDa form by FPLC or directly generating 28 kDa BDNF by putting a signal peptide just N-terminal to the cleavage site of 28 kDa form. Also, a potential biological role for the peptides generated from the prodomain of the pro-

BDNF should also been considered. In that regard, it has been reported that two peptides arising from the pro-domain of pro-NGF have their own biological activity *in vitro* (Dicou et al., 1997).

II. The enzyme SKI-1 is responsible for the generation of the 28 kDa BDNF

In the search of the enzyme responsible for the processing of the 32 kDa pro-BDNF into the truncated 28 kDa form, we tested, through collaboration with Dr. Seidah's lab, the involvement of the known convertase enzymes along with a newly cloned enzyme, mammalian subtilisin/kexin isozyme 1 (SKI-1). SKI-1 is a highly conserved, calcium-dependent serine endoproteinas and has been cloned in Dr. Seidah's lab by using RT-PCR and degenerate primers derived from the active-site residues of subtilisin/kexin like enzyme (Seidah et al., 1999). While co-expression of pro-BDNF with PC enzymes such as furin, PC1 and PC2 failed to increase the generation of the 28 kDa form, co-expression of pro-BDNF with SKI-1 enzyme significantly increased the intensity of the 28 kDa band (Seidah et al., 1999). We have further shown that the generation of the 28 kDa form by SKI-1 is insensitive to α1-PIT and α1-PDX, which are inhibitors of thrombin- and convertase-mediated cleavage, respectively. Thus, it is likely that the generation of the 28 kDa pro-BDNF takes place via an alternate processing pathway. Finally, the N-terminal microsequencing of the 28 kDa product generated by SKI-1 demonstrates that this protein is generated by a unique cleavage at Thr^{57} in the sequence: R<u>GLT</u>^{57}↓S. The later result identifies SKI-1 as the first known mammalian subtilisin-like enzyme capable of cleaving their substrates at non-basic residues.

Interestingly, comparison of the phylogenetically highly conserved sequence of pro-BDNF revealed an unusual insertion of hydroxylated amino acids (threonine and serine) just after the identified SKI-1 cleavage site of human pro-BDNF. Thus, in rat and mouse pro-BDNF, two threonines (R<u>GLT</u>TTSL), in porcine pro-BDNF, five serines (R<u>GLT</u>SSSSSSL), in bovine pro-BDNF three serine (R<u>GLT</u>SSSSL) and in cavpo pro-BDNF eight serine (R<u>GLT</u>SSSSSSSSSL) are inserted. Interestingly,

while the cleavage site itself is highly conserved within the known pro-BDNF sequences. in Ximpa pro-BDNF the cleavage site is changed but nevertheless into a new cleavage site REELPSLT↓DT. The new site contains a Threonine at P1, a leucine at P2 and an Argenine at P8 positions, N-terminal to the cleavage site. Our later site-directed mutagenesis study revealed that SKI-1 strongly prefers a Leucine at P2 site (Mowla et al., 2000). These observations raise a number of questions: (1) do these insertions affect the kinetics of pro-BDNF cleavage by SKI-1? A comparison of the processing of human, rat and pig BDNF revealed that all of them are processed into 28 kDa and mature forms with a comparable ratio (Mowla et al., 1999b). (2) Is the presence of a basic residue at P4, P6, or P8 positions critical for the detection of the cleavage site by SKI-1 enzyme? To answer this question we have compared the processing of wild-type pro-BDNF along with a series of pro-BDNF mutants in which the amino acids around the SKI-1 cleavage site have been changed. Replacing Arg^{54} at P4 position by Ala (AGLT↓S), significantly diminished, but not eliminated the generation of 28 kDa BDNF (Mowla et al., 2000). Interestingly, when we co-expressed pro-BDNF with SKI-1, the generation of 28 kDa BDNF completely blocked (Mowla SJ, Murphy RA and Seidah NG, in preparation). The later finding suggests that possibly other enzymes are involved in the generation of 28 kDa BDNF in a lesser extent and that overexpression of SKI-1 compete with the activity of these potential enzymes (Mowla SJ, Murphy RA and Seidah NG, in preparation). Our data has also shown that the presence of basic amino acids at the P6 and P8 positions are not necessary, but nevertheless, their presence can improve the efficiency of the processing (Mowla et al., 2000). (3) What is the reason for having multiple Ser/Thr residues just C-terminus to the cleavage site. In that context, a comparison of the size of BDNF containing bands in human and rat BDNF reveals that the 32 kDa and 28 kDa rat BDNF appears slightly higher than human ones in 13-22% gradient SDS-PAGE. We have also observed an additional band above 32 kDa rat pro-BDNF. An observation that is consistence with the post-translational modification (O-glycosylation and/or O-phosphorylation) of the inserted Threonines (Mowla et al., 1999b). The generation of 14 kDa BDNF (in constitutive pathway) is not affected by

the insertion of Ser/Thr or site-directed Ski-1 cleavage site mutants (Mowla et al., submitted).

Another question that arises is whether SKI-1 is able to cleave substrates at residues other than Thr. In that context, Sakai et al. demonstrated that the sequence of hamster Site-1 protease (S1P) responsible for the site-1 cleavage of sterol regulatory element-binding proteins (SREBPs)[1] is almost identical to the human, mouse, and rat SKI-1 (Sakai et al., 1998). In this model, within the lumen of the ER, S1P/SKI-1 cleaves SREBP-2 at an RSVL↓SF sequence, where Arg at P4 is absolutely required, whereas the P1 Leu could be replaced by a number of other amino acids (Duncan et al., 1997). The flexibility of P1 position was later on confirmed by our results obtained from site-directed mutagenesis. We have shown that Thr at P1 position can be replaced by a number of amino acids. However, replacing Thr at P1 position by Glu, almost completely blocked the generation of 28 kDa BDNF while replacing Thr with Arg increased the efficiency of the cleavage. The later finding reveals that SKI-1 is capable of cutting both basic and non-basic residues (Mowla et al., 2000).

The wide tissue distribution of SKI-1 mRNA transcripts suggests that this enzyme processes numerous precursors in various tissues. The wide distribution of the enzyme might also be simply a reflection of wide distribution of a single substrate with a general or housekeeping function. Interestingly, SKI-1 is highly expressed in hippocampus where BDNF is also highly expressed (Seidah et al.,

[1] SREBPs are a three-membered family of transcription factors modulating the transcription of genes encoding enzymes of cholestrol biosynthesis and uptake from plasma lipoproteins (reviewed by Brown and Goldstein 1999). SREBPs are bound to membranes of the ER and nuclear envelope in a hairpin orientation where the COOH- and NH2-terminals project into the cytosol. The NH2-terminal domains of SREBPs are transcription factors of the basic-helix-loop-helix-leucine zipper family and for being functional must be proteolytically released from membranes by two sequential cleavages (Brown and Goldstein 1997). The first cleavage is catalyzed by S1P/SKI-1 in the lumen of ER, separating the SREBPs into two haves, both of which remain membrane-bound. The second cleavage within the membrane-spaning segment of NH2-terminal intermediate fragment by S2P, a hydrophobic protein that appears to be a zinc metalloprotase, releases the NH2-terminal to enter the nucleus. Build up of sterols within cells, block the proteolytic release process by selectively inhibiting cleavage by S1P/SKI-1. These regulated proteolytic cleavages are ultimately responsible for controling the level of cholestrol in membranes, cells and blood (reviewed by Brown and Goldstein 1999).

1999; Maisonpierre et al., 1991). Furthermore, the observed developmental down-regulation of the level of its transcripts also suggests a functional importance during embryonic development. SKI-1 is closest to the pyrolysin branch of the six-member family of subtilisin-like proteinases (Siezen, 1997) and we believe it is the first known mammalian subtilase capable of cleaving substrates at sites other than basic amino acids. For the future works, it is very important to determine other substrates for SKI-1 enzyme. Also, our ongoing site-directed mutagenesis studies aimed to determine the minimal sequence requirement for SKI-1 cleavage activity would help us to design specific inhibitor to the enzyme and also to find a better strategy to search for the other potential members of the SKI family.

III. Differential sorting of NGF and BDNF in hippocampal neurons

NTs are traditionally considered as trophic factors released constitutively and at extremely low levels by non-neuronal target tissues in the peripheral nervous system (Levi-Montalcini et al., 1960). According to neurotrophic hypothesis, neurons that contain appropriate receptor for theses factors (TrkA for NGF, TrkB for BDNF/NT-4 and TrkC for NT-3) compete with other neurons to have access to this limited survival factors (Yuen et al., 1996). Neurons that fail to have correct access to their targets are eliminated during a period of developmentally programmed cell death (Oppenheim, 1991). Works in central nervous system, however, have revealed some deviation to that classical view. For example, in CNS, NTs are exclusively produced by neurons (Thoenen et al., 1987, Philips et al., 1990), and more surprisingly in some areas (such as hippocampus) they are co-expressed along with their designated receptors (Klein et al., 1989; 1990; Maisonpierre et al., 1990). Further works revealed an involvement of NTs in modulating synaptic plasticity (reviewed by Lo 1995, Thoenen 1995, Bonhoeffer 1996, Snider & Lichtman 1996, McAllister et al., 1999). Being a modulator of synaptic plasticity requires that the release of NTs being regulated in an activity-dependent manner (McAllister et al., 1999). In contrast, being a survival factor requires constitutive release of NTs. To understand how NTs

can function as either a survival factor or a regulator of synaptic plasticity requires a deep understanding of how NTs are produced and released by neurons.

We used three independent criteria (retention or release from cells after pulse-chase metabolic labeling, depolarization-dependent release, and immunocytochemical localization) to determine the secretory pathway taken by NTs in hippocampal neurons. Our results provide evidence suggesting that NGF and BDNF are primarily sorted to constitutive and regulated secretory pathways, respectively (Mowla et al., 1999a). We have further demonstrated that the differential sensitivity of pro-NGF and pro-BDNF to furin-mediated cleavage plays a key role in their sorting. pro-NGF is efficiently cleaved by furin in TGN in the cells we tested. Accordingly, 20°C cold-block conditions that inhibit the exit of proteins from the TGN (Matlin and Simons, 1983) did not inhibit the production of mature NGF by primary cultures of mouse hippocampal neuron. This finding suggests that the proteolytic processing of pro-NGF occurs within the TGN. In contrast, 20° C cold block condition totally inhibited the processing of pro-BDNF into mature form, with no effects on the production of 28 kDa BDNF. An interesting finding that suggest cleavage of pro-BDNF into 28 kDa form occurs earlier than mature form and that the production of mature BDNF likely occurs in a post-TGN compartment (i.e. immature or mature secretory vesicle).

When furin activity is inhibited in AtT-20 cells with α1-PDX, a potent inhibitor of PC enzymes within constitutive secretory pathway, pro-NGF is not cleaved efficiently in TGN. A portion of intact precursor is sorted within secretory vesicles where it can further be cleaved by other enzymes residing in secretory vesicles such as PC1. The remaining unprocessed precursor that does not enter secretory vesicles is released constitutively, mimicking the constitutive release of pro-BDNF in the absence of α1-PDX. Thus, inhibiting furin-like activity in TGN, mis-sort some pro-NGF to the regulated secretory pathway. Similar effects have been observed for POMC (Benjannet et al., 1997).

The efficiency of furin-mediated cleavage may be one important factor determining the sorting of NTs within secretory pathway. This hypothesis is already well documented for egg-laying hormone precursor (pro-ELH). Pro-ELH contains bioactive peptides on both the C- and N-terminal sides of a furin cleavage site (Jung and Scheller, 1991). The peptide derived from C-terminal side of the cleavage site is sorted into the regulated secretory pathway, while the peptide derived from the amino-terminal side of the cleavage site is released constitutively, degraded, or sorted into a separate regulated secretory vesicle (Jung and Scheller, 1991). Cells with low furin levels, are not efficiently process the precursor in TGN, and both N- and C-terminal sides of the precursor are sorted into the same regulated secretory vesicles (Klumperman et al., 1996). Thus, sorting of the N-terminal active peptides into dense-core secretory vesicles occurs only in the absence of furin cleavage. Accordingly, the level of furin within different cells would dictate the fate of N-terminal derived peptide.

We hypothesized that a similar situation may also occur with NT sorting. In our model, sorting of NTs into the regulated secretory pathway may require signals within the pro-domain or around the consensus cleavage site. Pro-NGF is a very good substrate for furin and efficiently processed by the enzyme within TGN (the sorting compartment for regulated pathway). Losing its putative signal(s), the mature NGF is released via constitutive pathway. This model is consisting with some previous reports showing the importance of furin-mediated cleavage in the sorting of proteins. For example, inserting furin-sensitive cleavage sites (addition of an Arg at P4 position: RX(K/R)R into pro-insulin (Yanagita et al., 1992) and pro-renin (Oda et al., 1991), which are normally processed by enzymes within the regulated secretory pathway, lead to the constitutive release of these proteins. On the other hand, pro-BDNF is not a good substrate for furin, the intact protein containing the putative sorting signal(s) escape furin cleavage in the TGN and targeted to the regulated secretory pathway where it can be processed by PC1 in (immature) secretory granules (Seidah et al., 1996b).

Therefore, differential sensitivity of pro-NGF and pro-BDNF to furin-mediated proteolytic processing might be the key into their differential sorting. The cleavage site of pro-NGF (Arg-Ser-Lys-Arg↓Ser) is highly suited to furin processing, whereas the pro-BDNF cleavage site (Arg-Val-Arg-Arg↓His) is much less suitable for furin. The reduced efficiency of pro-BDNF cleavage site to furin is not fully understood. One possibility is the replacement of Ser in the P1' position of the cleavage site of pro-NGF with His in the P1' of pro-BDNF. Previous works has shown that sequences with His at P1' have reduced sensitivity to furin-mediated cleavage (Ogi et al., 1990; Matthews et al., 1994). Another possibility for the reduced furin-mediated cleavage sensitivity of pro-BDNF could be its acidic pro-domain. Interestingly, while all mature NTs share similar basic isoelectric points (PI; ranging between 9-10), the PI for their pro-domains are significantly different. The pro-BDNF pro-domain is quite acidic (PI 5.1), in contrast to the considerably basic pro-domain of pro-NGF (PI 11.4) and pro-NT-3 (PI 9.0). The potential importance of the acidic domain in the furin-mediated processing or sorting of pro-BDNF is presently unknown.

A large number of studies suggest that NGF and BDNF might have different functions. NGF is primarily expressed by non-neuronal target tissues in the PNS and also a very restricted area in the CNS, all targets of basal forebrain cholinergic neurons. Therefore, NGF is primarily acting as a target-derived survival factor (requires a continuous release) in both PNS and CNS neurons. On the other hand, BDNF is primarily expressed by CNS neurons in the areas that undergo extensive synaptic plasticity, such as hippocampus (reviewed by Altar and DiStefano 1998). Consistently, there are increasing evidence considering BDNF as a potential modulator of synaptic plasticity (a function which requires a regulated release). A differential targeting of NGF and BDNF is also reported. BDNF, but not NGF, is anterogradly transported within axons on neurons to carry out a number of autocrine or paracrine physiological actions (Altar and DiStefano 1998; Fawcett et al., 1998).

Our results on sorting of BDNF within regulated secretory pathway are consistent with some previous works: Goodman et al., (1996) have reported that BDNF can be released by depolarization and in a calcium-dependent manner from virus-infected hippocampal cultures. Also, Fawcett et al., (1997) have shown enrichment of BDNF in a vesicular fraction of rat brain synaptosomes (treated with kianic acid) along with synaptotagmin, a protein associated with synaptic and large dense-core vesicles in nerve terminals. Finally and more interestingly, endogenous BDNF immunoreactivity is also localized in large dense-core vesicles of axon terminals in lamina II of lumbar spinal cord (Michael et al., 1997). Taken together, these data are consistent with our findings showing BDNF being packaged within secretory vesicles and released via regulated secretory pathway.

Fewer studies have monitored the sorting of NGF in cells containing the regulated secretory pathway. Edwards et al. (1988) reported that AtT-20 cells secrete vv:encoded NGF in response to cAMP, suggesting a regulated release for NGF. Also, Heymach et al. (1996) reported that AtT-20 cells release NGF as well as BDNF and NT-3 in response to secretogogues. In the later study, they expressed the NTs using a different promoter, and treated cells with secretogogues for a longer time, which may further explain why their conclusions differ from ours. Blochl and Thoenen (1995) hypothesized that there is both constitutive and regulated sodium-dependent release of NGF from neurons. They suggest that release occurs independent of extracellular calcium, an essential requirment for the regulated release (DeCamilli and Jahn, 1990), including the release of BDNF (Goodman et al., 1996).

To find out why we got different conclusion on NGF release from the one obtained by Edwards et al., (whom have used the same methodology as ours except for the higher level of infection); we repeated the experiment with higher M.O.I. of infections. Our results show that increasing the level of expression of pro-NGF is able to mis-sort a proportion of mature NGF to the regulated secretory pathway demonstrating that over-loading of furin-mediated cleavage may drive some NGF

into the regulated pathway. This scenario is analogous to that seen we inhibit furin activity with α1-PDX. Overloading the furin activity beyond its capacity may drive some pro-NGF into the regulated pathway. This finding suggests that cautions should be exercised when over-expressing proteins that are normally expressed at very low levels. Also this finding implies that, in contrast to NGF, BDNF is not only sorted to the regulated pathway because of its lack of a suitable cleavage site for furin but also because its expression at the level of protein is at least 100 times of that for NGF (Altar and DiStefano 1998).

In conclusion, our results provide a mechanism whereby NTs in brain neurons can act either as survival factors or as a modulator of synaptic plasticity. When NTs are cleaved by furin in TGN, the bioactive peptide may be released constitutively to promote neuronal survival. Perhaps this explains how hippocampal neurons constantly provide NGF to innervating cholinergic neurons in the basal forebrain. In contrast, BDNF may avoid furin cleavage and be sorted into the regulated secretory pathway where it is processed by PC1 (Seidah et al., 1996a) and released in an activity-dependent manner similar to other neuropeptides. This may be the mechanism that allows BDNF to alter synaptic transmission, connectivity, and synaptic plasticity in an activity-dependent manner.

IV. Constitutively secreted NT-3 can be rerouted to the regulated secretory pathway by dimerization with pro-BDNF

NTs exist in solution as non-covalent but very stable dimers (Bothwell and Shooter 1977; Radziejewski et al., 1992). The amino acid residues involved in the hydrophobic dimer interface are highly conserved among all NTs, in contrast to the more variable and exposed loop regions. This high degree of similarity at the dimer interface and also the fact that some cell types such as hippocampal neurons express more than one NT, suggest that NT heterodimers might also occur (Radziejewski and Robinson 1993; Jungbluth et al., 1994). The formation of NTs heterodimers, both *in vitro* and *ex vivo*, and also their isolation and characterization is extensively studied (see the literature review). Initially, and motivated by our findings that NGF and

BDNF are differentially sorted to the constitutive and regulated secretory pathways, respectively, we started to evaluate whether NGF can be re-directed to the regulated secretory pathway by way of dimerization with pro-BDNF. We have tested the formation of NGF/BDNF heterodimers in our system by means of co-immunoprecipitation. Our NGF antibody is able to immunoprecipitate BDNF, only if it is co-expressed by NGF. Our results show that while cAMP slightly induced the release of the NGF/BDNF heterodimer, the increase fell below the statistically significant level. Also, our unbiased observer (Kevin Petrecca) was unable to distinguish between NGF/NGF and NGF/BDNF co-infection cultures in terms of detecting punctate versus diffuse immunocytochemical distribution of NGF. Taken together, we were unable to show convincing results pointing to a role for heterodimerization in the re-routing of NGF to the regulated secretory pathway.

In parallel, we conducted studies on the processing and sorting of neurotrophin-3 (NT-3). Results obtained from pulse-chase experiments, secretogogues-induced release, and immunocytochemistry all suggest that pro-NT-3 is efficiently cleaved by furin and NT-3 and released constitutively, as is the case for NGF. Since the NT-3/BDNF heterodimer is much more stable than the one formed between NGF and BDNF monomers (Arakawa et al., 1994), we were prompted to repeat the above experiments to determine the fate of NT-3 when is co-expressed with pro-BDNF. Similar to our previous results, co-infection of AtT-20 cells with vv:pro-NT-3 and vv:pro-BDNF, lead to the detection of the NT-3/BDNF heterodimer by immunoprecipitation and SDS-PAGE. We have further shown that co-expression of pro-NT-3 with pro-BDNF is able to re-route NT-3 within regulated secretory pathway. The mis-sorting of NT-3 to the regulated secretory pathway induced by dimerization with pro-BDNF is much more efficient than the effect of increasing the level of over-expression of NT-3 and also the effect of _1-PDX.

One very important issue, which has been left to be answered in future, is whether NTs heterodimers can form in vivo and also the potential biological importance of such heterodimers. It is also very important to determine where inside the cells the

formation of heterodimers occur and also to determine whether post-translational modifications of NTs (such as glycosylation and sulfation) has some roles in the event.

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APPENDICES

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Biochemistry

Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization

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ABSTRACT **Using reverse transcriptase–PCR and degenerate oligonucleotides derived from the active-site residues of subtilisin/kexin-like serine proteinases, we have identified a highly conserved and phylogenetically ancestral human, rat, and mouse type I membrane-bound proteinase called subtilisin/kexin-isozyme-1 (SKI-1). Computer databank searches reveal that human SKI-1 was cloned previously but with no identified function. *In situ* hybridization demonstrates that SKI-1 mRNA is present in most tissues and cells. Cleavage specificity studies show that SKI-1 generates a 28-kDa product from the 32-kDa brain-derived neurotrophic factor precursor, cleaving at an RGLT ↓ SL bond. In the endoplasmic reticulum of either LoVo or HK293 cells, proSKI-1 is processed into two membrane-bound forms of SKI-1 (120 and 106 kDa) differing by the nature of their N-glycosylation. Late along the secretory pathway some of the membrane-bound enzyme is shed into the medium as a 98-kDa form. Immunocytochemical analysis of stably transfected HK293 cells shows that SKI-1 is present in the Golgi apparatus and within small punctate structures reminiscent of endosomes. *In vitro* studies suggest that SKI-1 is a Ca^{2+}-dependent serine proteinase exhibiting a wide pH optimum for cleavage of pro-brain-derived neurotrophic factor.**

Limited proteolysis of inactive precursors to produce active peptides and proteins generates biologically diverse products from a finite set of genes. Most often, such processing occurs at either monobasic or dibasic residues as a result of cleavage by mammalian serine proteinases related to bacterial subtilisin and yeast kexin (1, 2). These enzymes, known as proprotein convertases (PCs), cleave a variety of precursors at the consensus (**R**/**K**)-(Xaa)$_n$-**R** ↓ sequence, where Xaa is any amino acid except Cys and n = 0, 2, 4, or 6 (1–3).

Less commonly than cleavage at basic residues, bioactive products also can be produced by limited proteolysis at amino acids such as L, V, M, A, T, and S (3). This type of cellular processing has been implicated in the generation of bioactive peptides such as α- and γ-endorphin (4), the C-terminal glycopeptide fragment 1–19 of provasopressin (5), platelet factor 4 (6), the metalloprotease ADAM-10 (7), site 1 cleavage of the sterol regulatory element-binding proteins (SREBPs) (8), as well as in the production of the Alzheimer's amyloidogenic peptides Aβ40, -42, and -43 (9). Processing of this type occurs either in the endoplasmic reticulum (ER) (8), late along the secretory pathway, within secretory granules (4, 5), at the cell surface, or in endosomes (6, 7, 9). The proteinases responsible for these cleavages are not yet identified.

We hypothesized that an enzyme (or enzymes) distinct from, but related to, PCs may generate polypeptides by cleavage at nonbasic residues. To test that idea, we employed a reverse transcriptase–PCR (RT-PCR) strategy similar to the one used to identify the PCs (10), except that we used degenerate oligonucleotides closer to bacterial subtilisin than to yeast kexin. This resulted in the isolation of a cDNA fragment encoding a putative subtilisin-like enzyme from human cell lines. This partial sequence was identical to a segment of a human myeloid cell-derived cDNA reported by Nagase *et al.* (11). Preliminary results demonstrated that this putative proteinase cleaves pro-brain-derived neurotrophic factor (proBDNF) (ref. 12; S.J.M. N.G.S., and R.A.M., unpublished results).

In this paper, we show that the sequences of rat, mouse, and human orthologues of this putative type I membrane-bound subtilisin-kexin-isoenzyme, which we called SKI-1, exhibit a high degree of sequence conservation. Tissue distribution analysis by both Northern blots and *in situ* hybridization revealed that SKI-1 mRNA is widely expressed. A vaccinia virus recombinant and a stable transfectant of human SKI-1 in HK293 cells allowed the analysis of its biosynthesis and intracellular localization. Finally, we present data demonstrating that SKI-1 cleaves at a specific T ↓ residue within the N-terminal segment of proBDNF. SKI-1 is thus identified as a mammalian secretory subtilisin/kexin-like enzyme capable of cleaving a proprotein at nonbasic residues.

MATERIALS AND METHODS

PCR and Sequencing. Most RT-PCRs were performed using a Titan One Tube RT-PCR system (Boehringer Mannheim) on 1 μg of total RNA isolated from a human neuronal cell line (IMR-32), mouse corticotrophic cells (AtT20), or rat adrenal

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Abbreviations: SKI-1, subtilisin/kexin-isozyme-1; PC, proprotein convertase; ER, endoplasmic reticulum; BDNF, brain-derived neurotrophic factor; SREBP, sterol regulatory element-binding protein; RT-PCR, reverse transcriptase–PCR; vv, vaccinia virus; PDX, α1-antitrypsin Portland; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF094820 and AF094821).

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glands. The active-site degenerate primers were as follows: His (sense) 5′-GGICA(C,T)GGIACI(C,T)(A,T)(C,T)(G,T)(T,G)IGCIGG-3′ and Ser (antisense) 5′-CCIG(C,T)IACI-(T,A)(G,C)IGGI(G,C)(T,A)IGCIACI(G,C)(A,T)IGTICC-3′, based on the sequences GHGT(H,F)(V,C)AG and GTS-(V,M)A(T,S)P(H,V)V(A,T)G, respectively. The amplified 525-bp products were sequenced on an automated laser fluorescence DNA sequencer (Pharmacia). To obtain the full-length sequence of rat and mouse SKI-1, we used PCR primers based on the human (11) and mouse sequences, in addition to 5′ (13) and 3′ (14) RACE amplifications. At least three clones of the amplified cDNAs were sequenced. The GenBank accession numbers of the 3,788-bp mouse mSKI-1 cDNA and 3,895-bp rat rSKI-1 are AF094820 and AF094821, respectively.

Infection, Transfection, and Metabolic Labeling. Human SKI-1 (nucleotides 1–4338) (11) in Bluescript (a generous gift from N. Nomura, Kazusa DNA Research Institute, Chiba, Japan; accession no. D42053) was digested with *Sac*II (nucleotides 122–4338) and inserted into the vector PMJ602, and a vaccinia virus recombinant was isolated. The PMJ602 construct was also digested with 5′ *Kpn*I/3′ *Nhe*I and cloned into the *Kpn*I/*Xba*I sites of pcDNA3 (Invitrogen), and the cDNA was transfected into HK293 cells by using Lipofectin. A number of stable transfectants resistant to G418 and positive on Western blots using an SKI-1 antiserum (see below) were isolated, and one of them (clone 9), was investigated further. Either vaccinia virus-infected or -transfected cells were pulsed for 20 min with [^{35}S]cysteine and then chased for various times in the presence or absence of either tunicamycin (5 μg/ml) or brefeldin A (2.5 μg/ml). Media and cell lysates were immunoprecipitated with SKI-1 antisera directed against either amino acids 634–651 or 217–233, or a pro-SKI-1 antiserum directed against the pro-segment comprising amino acids 18–188 (Fig. 1). Immune complexes were resolved by SDS/PAGE on an 8% polyacrylamide/*N*-[tris(hydroxymethyl)methyl]glycine gel (15).

Northern Blots, *in Situ* Hybridizations, and Immunocytochemistry. Northern blot analyses (16) were done on total RNA from adult male rat tissues by using either a TRIzol reagent kit (Life Technologies, Gaithersburg, MD) or a Quick Prep RNA kit (Pharmacia) and on poly(A)$^+$ RNA of (male + female) rat adult tissues (BIO/CAN, Montreal). The blots were hybridized overnight at 68°C in the presence of [^{32}P]UTP SKI-1 cRNA probes, which consisted of the antisense of nucleotides 655-1249 of rat SKI-1. For *in situ* hybridization on newborn rats, the same rat sense and antisense cRNA probes were labeled with uridine and cytosine 5′-[γ-[^{35}S]thio]triphosphate (1, 16). For immunofluorescence staining we used a rabbit anti-SKI-1 antiserum at a 1:100 dilution and rhodamine-labeled goat anti-rabbit IgGs diluted 1:20 (16). Red SKI-1 immunostaining was compared with green-staining patterns of both fluorescein-labeled concanavalin A (Con A; Molecular Probes), an ER marker, or fluorescein-conjugated wheat germ agglutinin (WGA; Molecular Probes), a Golgi marker (17).

***Ex Vivo* and *in Vitro* proBDNF Processing.** A vaccinia virus recombinant of human SKI-1 (vv:SKI-1) was isolated as described for human proBDNF (vv:BDNF) (15). The vaccinia virus recombinants of α1-antitrypsin Pittsburgh (α1-PIT; vv:PIT) and α1-antitrypsin Portland (α1-PDX; vv:PDX) (18) were generous gifts from G. Thomas (Vollum Institute, Portland, OR). COS-7 cells (4×10^6) were coinfected with 1 plaque-forming unit (pfu) per cell of vv:BDNF and either the wild-type virus (vv:WT) alone at 2 pfu per cell or with 1 pfu per cell of each virus in the combinations [vv:SKI-1+vv:WT], [vv:SKI-1+vv:PIT], and [vv:SKI-1+vv:PDX]. At 10 h postinfection, cells were pulsed for 4 h with 0.2 mCi of [^{35}S]cysteine/[^{35}S]methionine (DuPont). Media and cell extracts were immunoprecipitated with a BDNF antiserum (ref. 19; provided by Amgen) at 0.5 μg/ml, and the proteins were resolved on SDS/PAGE 13–22% gradient gels (15). [^{35}S]Met-labeled 32-kDa proBDNF and [^{3}H]Leu-labeled 28-kDa BDNF were sequenced as described (20). For *in vitro* analysis, 32-kDa proBDNF obtained from the media of LoVo cells infected with vv:BDNF was incubated overnight with the shed form of SKI-1 obtained from cells coinfected with vv:SKI-1 and vv:PDX, either at different pH values or at pH 6.5 in the presence of selected inhibitors: pepstatin (1 μM), antipain (50 μM), cystatin (5 μM), E64 (5 μM), soybean trypsin inhibitor (SBTI, 5 μM), 0.5 M phenylmethylsulfonyl fluoride (PMSF) + 50 μM *para*-amino-PMSF (pAPMSF), *o*-phenanthroline (5 mM), and EDTA (10 mM). The products were resolved by SDS/PAGE on a 15% polyacrylamide gel, blotted, and then probed with a BDNF antiserum (Santa Cruz Biotechnology) at a dilution of 1:1,000.

RESULTS

Protein Sequence Analysis of SKI-1. We first aligned the protein sequences within the catalytic domain of PC7 (21), yeast subtilases, and bacterial subtilisins, together with that of a novel *Plasmodium falciparum* subtilisin-like enzyme called pf-SUB2 (J.-C.B., unpublished results). This led to the choice of conserved amino acids GHGT(H/F)(V/C)AG and GTS(M/V)A(T/S)P(H/V)V(A/T)G around the active sites His and Ser, respectively. Thus, using degenerate oligonucleotides coding for the sense His and antisense Ser consensus sequences, we initiated a series of RT-PCRs on total RNA and isolated a 525-bp cDNA fragment from the human neuronal cell line IMR-32. This sequence was found to be 100% identical to that reported for a human cDNA called KIAA0091 obtained from a myeloid KG-1 cell line (11) and 88% identical to that of a 324-bp expressed sequence tag (accession no. H31838) from rat PC12 cells. The full-length rat and mouse cDNA sequences were obtained after RT-PCR amplifications of total RNA isolated from rat adrenal glands and PC12 cells and from mouse AtT20 cells. As shown in Fig. 1, alignment of the protein sequence deduced from the cDNAs revealed that rat and mouse SKI-1 share 98% sequence identity and a 96% identity to human SKI-1. Interestingly, within the catalytic domain ($Asp^{218} \rightarrow Ser^{414}$) the sequence similarity between the three species is 100%. Analysis of the predicted amino acid sequence suggests a 17-aa signal peptide, followed by a putative prosegment beginning at Lys^{18} and extending for some 160–180 aa. The proposed catalytic domain encompasses the typical active sites Asp^{218}, His^{249}, and Ser^{414} and the oxyanion hole Asn^{338}. This domain is followed by an extended C-terminal sequence characterized by the presence of a conserved growth factor/cytokine receptor family motif C849LDDSHRQKDCFW^{861}. This sequence is followed by a potential 24-aa hydrophobic transmembrane segment and a less-conserved 31-aa cytosolic tail that, remarkably, consists of 35% basic residues. Some of the clones isolated from rat adrenal glands suggested the existence of alternatively spliced rSKI-1 mRNAs in which the segments coding for amino acids 430–483 or 858–901 are absent. Finally, the phylogenetic tree derived from the alignment of the catalytic domain of SKI-1 with subtilases (22) suggests that it is an ancestral protein that is closer to plant and bacterial subtilases than to either yeast or mammalian homologues (not shown).

Tissue Distribution of SKI-1 mRNA. Northern blot analyses of SKI-1 mRNA in adult male rat reveal that rSKI-1 mRNA is widely expressed and is particularly rich in anterior pituitary, thyroid, and adrenal glands (Fig. 2*A*). A Northern blot of poly(A)$^+$ RNA obtained from mixed adult male and female rat tissues also showed a wide distribution and a particular enrichment in liver (Fig. 2*B*). Similarly, analysis of 24 different cell lines (23) revealed a ubiquitous expression of SKI-1 mRNA (not shown).

In situ hybridization data obtained in a day 2 postnatal rat also provided evidence of a widespread, if not ubiquitous, distribution of rSKI-1 mRNA. Fig. 3 shows at the anatomical level the presence of SKI-1 mRNA in developing skin, striated

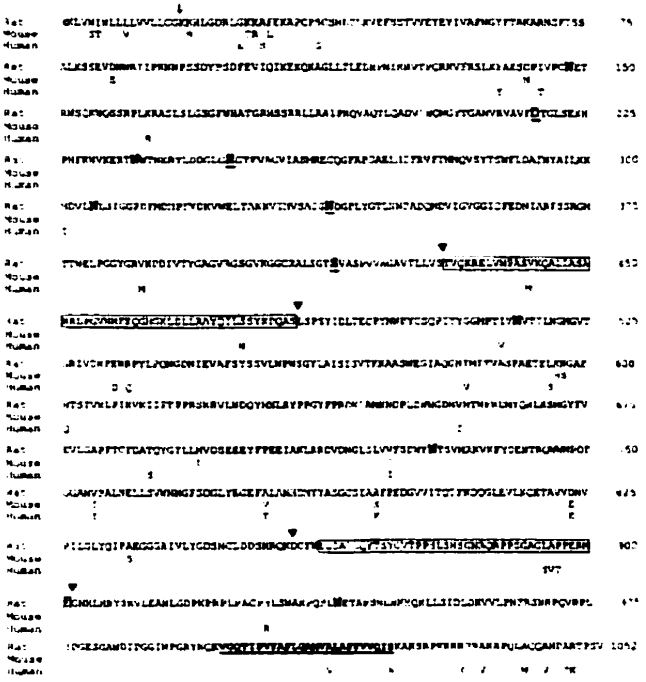

FIG. 1. Comparative protein sequences of SKI-1 deduced from rat, mouse, and human cDNAs. The position of the predicted end of the 17-aa signal peptide is shown by an arrow. The active sites Asp^{218}, His^{249}, and Ser^{414} as well as the oxyanion hole Asn^{338} are shown as bold, shaded, and underlined characters. The positions of the six potential N-glycosylation sites are emphasized in bold. The conserved shaded sequence fits the consensus signature for growth factors and cytokine receptor family. Each of the two boxed sequences was absent (▼) in a number of rat clones. The predicted transmembrane segment is in bold and underlined.

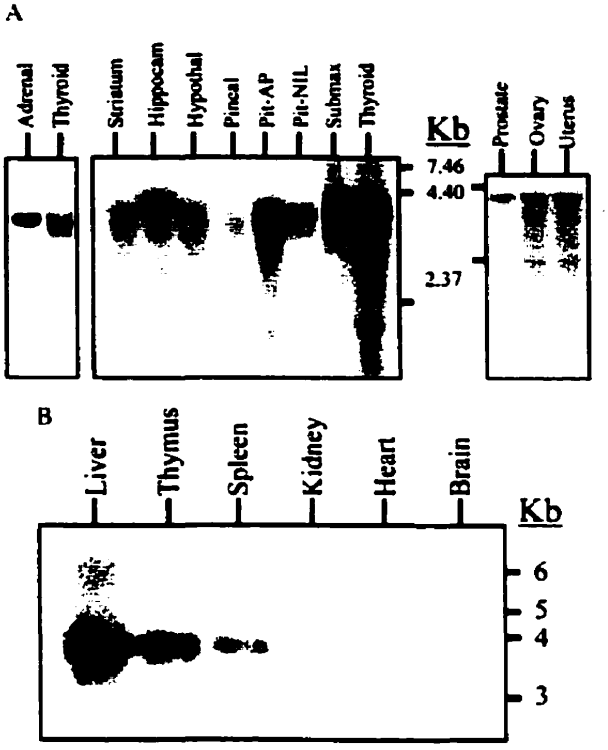

FIG. 2. Northern blot analysis of the expression of SKI-1 in adult rat tissues. (*A*) Five micrograms of male rat total RNA was loaded in each lane: pituitary anterior (AP) and neurointermediate (NIL) lobes and submaxillary gland (submax). (*B*) Two micrograms of poly(A)$^+$ RNA of (male + female) Sprague–Dawley rat adult tissues. The estimated size of rat SKI-1 mRNA is about 3.9 kb.

muscles, cardiac muscles, bones, and teeth as well as brain and many internal organs. Strong hybridization signals were detectable in the retina, cerebellum, pituitary, submaxillary, thyroid, and adrenal glands, molars, thymus, kidney, and intestine. Evidence for the cellular expression of rSKI-1 mRNA was obtained from analysis of the relative labeling densities per cell in selected tissues, based on a semiquantitative analysis of emulsion autoradiographs (not shown). In the central nervous system rSKI-1 mRNA labeling was mostly confined to neurons, whereas ependymal cells and supportive glial cells, such as presumed astrocytes, oligodendrocytes, and microglia, exhibited 5- to 30-fold-less labeling per cell. In addition, within the peripheral nervous system, trigeminal ganglia revealed a 5- to 10-fold greater expression in neurons as compared with presumptive Schwann cells. Labeling was observed in most of the glandular cells in the anterior and intermediate lobes of the pituitary as well as in the pituicytes of the pars nervosa. A semiquantitative comparison in the adult and newborn rat pituitary gland, submaxillary gland, thymus, and kidney demonstrated an overall 2-fold-decreased labeling of rSKI-1 mRNA with age (not shown).

Biosynthesis of hSKI-1. To define the molecular forms of human SKI-1 we generated both a vaccinia virus recombinant (vv:SKI-1) and a stable transfectant in HK293 cells. Three antisera were produced against amino acids 18–188 (prosegment), 217–233, and 634–651 of SKI-1. Expression of vv:SKI-1 in four different cell lines revealed that the enzyme is synthesized as a 148-kDa proSKI-1 zymogen, which is processed progressively into 120-, 106-, and 98-kDa proteins (Fig. 4). Only the 148-kDa form is recognized by the prodomain antiserum, whereas all four forms react with the other two antisera. Processing of the 148-kDa proSKI-1 into the 120- and 106-kDa forms occurs in the ER based on the presence of these proteins in cells preincubated with the fungal metabolite brefeldin A (Fig. 4; ref. 24). Furthermore, preincubation with tunicamycin revealed only two bands (Fig. 4), suggesting that the presumably membrane-bound 106- and 120-kDa forms differ by their N-glycosylation. At the 3-h chase time, results reveal the secretion of a 98-kDa shed form (sSKI-1) recognized by both SKI-1 antisera (Fig. 4) but not by the proSKI-1 antiserum (not shown). Similar SKI-1-related forms were seen in stably transfected HK293 cells after a 4-h pulse labeling with [^{35}S]methionine (not shown).

Intracellular Localization of SKI-1. Double-staining immunofluorescence was used to compare the intracellular localization of the stably transfected human SKI-1 in HK293 cells with that of either the ER or Golgi markers Con A and WGA (17), respectively. The data show that SKI-1 exhibits (*i*) perinuclear staining, colocalizing with Con A fluorescence, presumably corresponding to the ER (not shown); (*ii*) paranuclear staining colocalizing with WGA fluorescence, suggesting the presence of SKI-1 in the Golgi (Fig. 5 *A* and *B*), and (*iii*) punctate staining observed in the cytoplasm and within extensions of a few cells (Fig. 5*A*). Some, but not all, of the punctate immunostaining matched that observed with WGA. This suggests that SKI-1 localizes in the Golgi but may sort to other organelles, including lysosomal and/or endosomal compartments. An indication of lysosomal/endosomal localization was provided by the analysis of SKI-1 immunofluorescence within cells preincubated for 4 h with 10 mM leucine methyl ester, a specific lysosomal/endosomal protease inhibitor (25). The results showed a net increase in the proportion of cells exhibiting punctate staining as compared with control cells (Fig. 5 *A* and *B*). The relative proportions of SKI-1 in cellular organelles and their dependence on culture conditions are now amenable to evaluation by subcellular fractionation and electron microscopy.

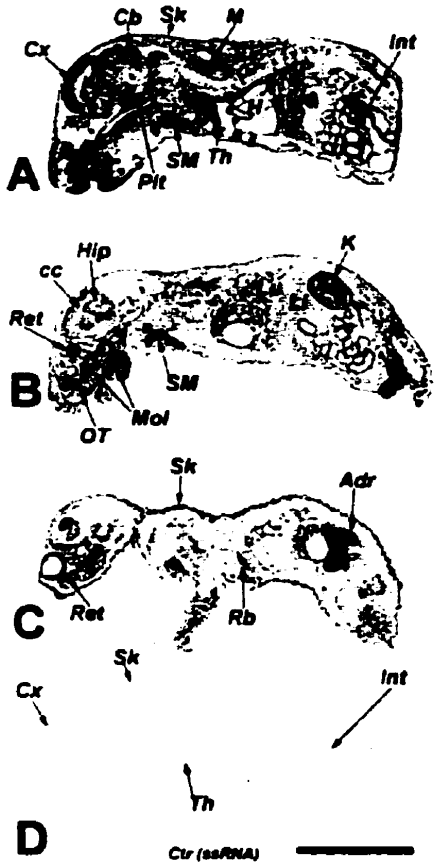

FIG. 3. *In situ* hybridization of rSKI-1 mRNA in a 2-day-old rat. *In situ* hybridization is shown at anatomical resolution on x-ray film using an ^{35}S-labeled antisense riboprobe (*A–C*) and sense control riboprobe (*D*). *Adr*, adrenal gland; *Cb*, cerebellum; *cc*, corpus callosum; *Cx*, cerebral cortex; *H*, heart; *Int*, intestine; *K*, kidney; *Li*, liver; *Lu*, lungs; *M*, muscles; *Mol*, molars; *OT*, olfactory turbinates; *Pit*, pituitary gland; *Rb*, ribs; *Ret*, retina; *Sk*, skin, *SM*, submaxillary gland; *Th*, thymus. [×4; bar (*D*) = 1 cm.]

Enzymatic Activity and Cleavage Specificity of SKI-1. To prove that SKI-1 is a proteolytic enzyme we examined its ability to cleave five different potential precursor substrates, including pro-opiomelanocortin, pro-atrial natriuretic factor, HIV gp160, pro-nerve growth factor, and proBDNF. Cellular coexpression of vv:SKI-1 with the vaccinia virus recombinants of each of the above precursors revealed that only proBDNF was cleaved intracellularly by SKI-1. Thus, upon expression of vv:BDNF alone in COS-7 cells we observed a partial processing of proBDNF (32 kDa) into the known, major 14-kDa BDNF product (15) and the minor production of a previously observed (ref. 16; S.J.M., N.G.S., and R.A.M., unpublished results) but still undefined 28-kDa product (Fig. 6*A*). Upon coexpression of proBDNF and SKI-1, a net increase in the level of the secreted 28-kDa BDNF is evident, without significant alteration in the amount of 14-kDa BDNF (Fig. 6*A*). To examine whether the 28-kDa product results from cleavage at a basic residue or at an alternative site, we first coexpressed proBDNF, SKI-1, and either α1-PIT or α1-PDX, which are inhibitors of thrombin and PC cleavages, respectively (18, 26). The results show that different from α1-PIT, the serpin α1-PDX selectively blocks the production of the 14-kDa BDNF and that neither α1-PIT nor α1-PDX affects the level of the 28-kDa product. This finding shows that α1-PDX effectively inhibits the endogenous furin-like enzyme(s) responsible for the production of the 14-kDa BDNF (15), but does not inhibit the ability of SKI-1 to generate the 28-kDa product. Thus, it is likely that the generation of the 28-kDa BDNF takes place via an alternate cleavage. Incubation of the cells with brefeldin A or the Ca^{2+} ionophore A23187 revealed that the 28-kDa proBDNF is

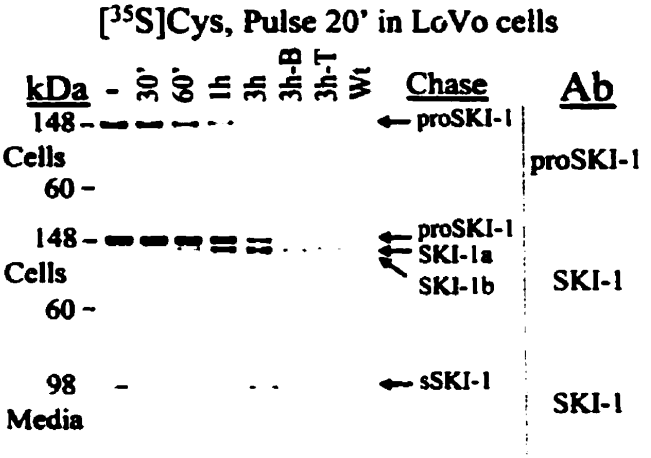

FIG. 4. Biosynthetic analysis of SKI-1 in LoVo-C5 cells overexpressing vv:SKI-1. Cells were pulsed for 20 min with [^{35}S]cysteine and chased for 30 min, 1 h, and 3 h in the absence or presence of either brefeldin A (3h-B) or tunicamycin (3h-T). The control represents the 3-h chase period for cells infected with the wild-type virus (Wt). Media and cell lysates were immunoprecipitated with either a SKI-1 antiserum (Ab: SKI-1; against amino acids 634–651) or a proSKI-1 antiserum. The arrows point to the 148-, 120-, 106-, and 98-kDa forms immunoprecipitated.

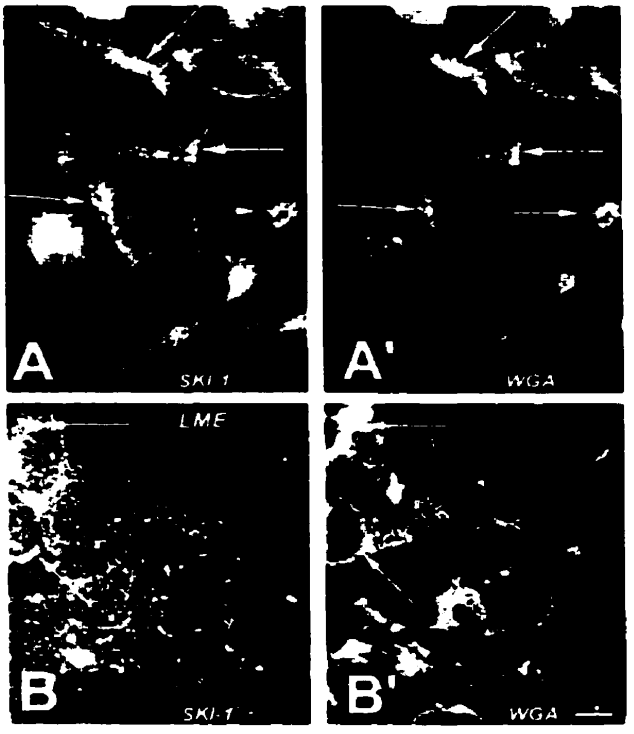

FIG. 5. hSKI-1 immunoreactivity in stably transfected HK293 cells. Black and white representation of the comparative double (red and green) fluorescence staining using an SKI-1 antiserum (directed against amino acids 634–651) (*A* and *B*) and fluorescein isothiocyanate-labeled WGA (*A'* and *B'*) in control (*A* and *A'*) and leucine methyl ester (LME)-treated (*B* and *B'*) cells. Thin arrows emphasize the observed punctate staining, which is enhanced in the presence of LME. Large arrows point to the coincident staining of SKI-1 and WGA. [×900; bar (*B'*) = 10 μm.]

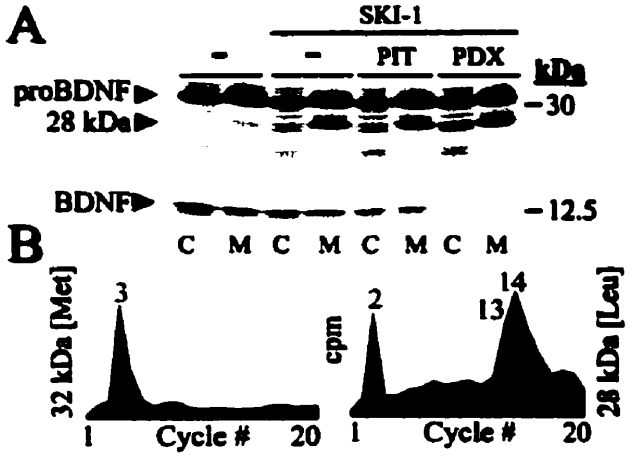

FIG. 6. Processing of proBDNF by SKI-1. (*A*) COS-7 cells were infected with vv:BDNF and either vv:WT (−) or vv:SKI-1 in the presence of either vv:PIT or vv:PDX. The cells were labeled metabolically with [^{35}S]cysteine/[^{35}S]methionine for 4 h, and the media (M) and cell lysates (C) were immunoprecipitated with a BDNF antiserum before SDS/PAGE analysis. The autoradiogram shows the migration positions of proBDNF (32 kDa), the 28-kDa BDNF produced by SKI-1, and the 14-kDa BDNF. (*B*) Microsequence analysis of the [^{35}S]Met-labeled 32-kDa proBDNF (maximal scale, 1,000 cpm) and [^{3}H]Leu-labeled 28-kDa BDNF (maximal scale, 250 cpm).

formed in the ER and that this cleavage is Ca^{2+}-dependent (not shown).

In Fig. 6*B*, we present the N-terminal microsequence analysis of [^{35}S]Met-labeled 32-kDa proBDNF and [^{3}H]Leu-labeled 28-kDa BDNF. The sequence of the 32-kDa form revealed the presence of an [^{35}S]Met at position 3 (Fig. 6*B*), which is in agreement with the proposed sequence of human proBDNF (27) resulting from the removal of an 18-aa signal peptide cleaved at GCMLA^{18} ↓ APMK site. The N-terminal sequence of the 28-kDa product revealed a [^{3}H]Leu at positions 2, 13, and 14 (Fig. 6*B*). This result demonstrates that the 28-kDa BDNF is generated by a unique cleavage at Thr^{57} in the sequence RGLT^{57} ↓ SLADTFEHVIEELL (27).

To prove that SKI-1 is directly responsible for the production of the 28-kDa BDNF at the novel Thr-directed cleavage, proBDNF was incubated at various pH values with concentrated medium of vv:SKI-1-infected Schwann cells. A similar preparation obtained from wild-type vaccinia virus-infected cells served as control. The data show that SKI-1 exhibits a wide pH-dependence profile revealing activity at both acidic and neutral pH values from pH 5.5 to 7.3 (Fig. 7*A*). Analysis

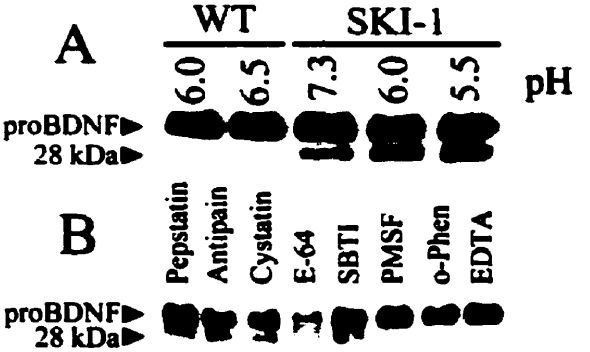

FIG. 7. *In vitro* processing profile of proBDNF by SKI-1. (*A*) pH dependence of the processing of proBDNF by SKI-1. (*B*) Inhibitor profile of the processing of proBDNF to the 28-kDa BDNF by the same SKI-1 preparation as in *A*. The reaction was performed overnight at 37°C, pH 6.0.

of the inhibitory profile of this reaction revealed that metal chelators such as EDTA and *o*-phenanthroline or a mixture of the serine proteinase inhibitors PMSF and pAPMSF effectively inhibits the processing of proBDNF by SKI-1. The inhibition by EDTA is expected because, like all PCs, SKI-1 is a Ca^{2+}-dependent enzyme. The unexpected inhibition by 5 mM *o*-phenanthroline may be a result of excess reagent because at 1 mM only 25% inhibition is observed (not shown). All other class-specific proteinase inhibitors (aspartyl-, cysteinyl-, and serine proteases- of the trypsin type) proved to be inactive.

DISCUSSION

This work provides evidence for the existence of a mammalian secretory Ca^{2+}-dependent serine proteinase of the subtilisin/kexin type that selectively cleaves at nonbasic residues. Thus, SKI-1 processes the 32-kDa human proBDNF at an KAGSRGLT ↓ SL sequence, generating a 28-kDa form, which may have its own biological activity (S.J.M., N.G.S., and R.A.M., unpublished results). Such a cleavage site is close to the consensus site deduced from a large body of work done with the PCs, whereby an (R/K)-(X)$_n$-R ↓ X-(L/I/V), (where n = 0, 2, 4, or 6) motif is favored by most PCs (1–3, 28). Note that in the SKI-1 site, P1 Arg is replaced by Thr and an aliphatic Leu is present at P2′, an amino acid also favored by PCs (1–3, 28). Several proteins are known to be cleaved after Thr. These include human antiangiogenic platelet factor 4 (ref. 6; QCLCVKTT ↓ SQ), the neuroendocrine α-endorphin (ref. 4; KSQTPLVT ↓ LF), and ADAM-10 metalloprotease (ref. 7; LLRKKRTT ↓ SA).

Interestingly, comparison of the phylogenetically highly conserved sequence of proBDNF revealed an insertion of hydroxylated amino acids (threonine and serine) just after the identified SKI-1 cleavage site of human proBDNF. Thus, in rat and mouse proBDNF, two threonines are inserted (RGLTTT—SL), and in porcine proBDNF, five serines are added (RGLTSSSSS—SL) (27). These observations raise a number of questions: (*i*) Do these insertions affect the kinetics of proBDNF cleavage by SKI-1? In that context, it was published recently that rat proBDNF is also cleaved into a 28-kDa protein (29). (*ii*) Does SKI-1 recognize both single and pairs of Thr and Ser and combinations thereof? (*iii*) Is the presence of a basic residue at P4, P6, or P8 critical for cleavage?

Another question that arises is whether SKI-1 can cleave at residues other than Thr. In that context, after submission of this manuscript, Sakai *et al.* demonstrated that the sequence of hamster S1p responsible for the site 1 cleavage of SREBPs is almost identical to the presently reported human, mouse, and rat SKI-1 (30). In this model, within the lumen of the ER, S1p cleaves SREBP-2 at an RSVL ↓ SF sequence, where Arg at P4 is very critical, whereas the P1 Leu could be replaced by a number of other amino acids (8). Our *in vitro* data show that sSKI-1 does not cleave small fluorogenic substrates of sequence RGLT-MCA, RGLTTT-MCA, or RSVL-MCA (MCA is 4-methylcoumaryl-1-amide), suggesting that it has an extended substrate-specificity pocket. In agreement, preliminary data show that SKI-1 specifically cleaves at neutral pH a 27-mer synthetic peptide of sequence GGAHDSDQHPHSGSGRSVL ↓ SFESGSGG, representing the luminal amino acids 504–530 of human SREBP-2 (8) (B.B.T. and N.G.S., unpublished data). We have shown that this synthetic peptide is efficiently processed by SKI-1 *in vitro*, paving the way for refined kinetic analyses.

Biosynthetic analysis of the zymogen processing of proSKI-1 demonstrated an ER-associated removal of the prosegment (Fig. 4). Furthermore, analysis of the $^{35}SO_4$-labeled SKI-1 demonstrated the presence of only sulfated 106- and 98-kDa forms but not that of either the 148 proSKI-1 or the 120-kDa SKI-1a forms (not shown). Because sulfation occurs in the trans-Golgi network, this confirms that the removal of the prosegment occurs in the ER. As with furin and PC5-B (1–3,

24) the membrane-bound 106-kDa SKI-1 is transformed into a soluble 98-kDa form. The secreted 98-kDa sSKI-1 is enzymatically active because it processes proBDNF *in vitro* (Fig. 7). Attempts to sequence the SDS/PAGE-purified [^{3}H]Leu- and Val-labeled 148- and 98-kDa forms resulted in ambiguous results, suggesting that SKI-1 is refractory to N-terminal Edman degradation. Presently, we are unable to define the zymogen cleavage site leading to the formation of the 120-kDa SKI-1a and 106-kDa SKI-1b deduced by pulse–chase studies (Fig. 4). Examination of the prosegment sequence (Fig. 1), the species-specific proBDNF motif potentially recognized by SKI-1, the sequence of the luminal portion of SREBP-2 (see above), and the alignment of SKI-1 with other subtilases (22) suggests three possible conserved sites: **R**ASL^{167} ↓ SLGS, **R**HSS^{182} ↓ RRLL, and **RR**LL^{186} ↓ RAIP. These predict cleavages at motifs containing a P4 Arg and a P1 either Leu or Ser.

Phylogenetic structural analysis of the predicted amino acid sequence of SKI-1 reveals that this serine proteinase is closer to plant and bacterial subtilases than it is to yeast and mammalian PCs. The 100% conservation of the catalytic domain sequence, although striking and suggestive of an important function, is not far from the 98% similarity between human and rat PC7 (3, 21). The sequence C-terminal to the catalytic domain of SKI-1 is very different from that of any of the known PCs. In fact, although PCs have a typical P-domain critical for the folding of these enzymes (for reviews see refs. 1–3), we did not find the hallmark sequences (3, 31) of the P-domain within the SKI-1 structure. Instead, different from the PCs, we find a conserved growth factor/cytokine receptor motif of which functional importance will need to be addressed, especially because this motif is partly missing in alternatively spliced forms (Fig. 1). Finally, the highly basic nature of the cytosolic tail of SKI-1 (Fig. 1) may be critical for its probable cellular localization within endosomal/lysosomal compartments (Fig. 5), similar to the importance of basic residues for the accumulation of the α-amidation enzyme PAM in endosomal compartments (S. L. Milgram, personal communication).

The wide tissue distribution of SKI-1 mRNA transcripts suggests that this enzyme processes numerous precursors in various tissues. Furthermore, the observed developmental down-regulation of the level of its transcripts also suggests a functional importance during embryonic development. That SKI-1 can cleave C-terminal to Thr, Leu, and, possibly, Ser residues suggests that, like the combination of PCs and carboxypeptidases E and D (32), a specific carboxypeptidase also may be required to trim out the newly exposed C-terminal hydroxylated or Leu residues.

SKI-1 is closest to the pyrolysin branch of the six-membered family of subtilisin-like proteinases (22) and we believe is the first known mammalian subtilase cleaving at sites other than basic amino acids. That other eukaryotic subtilases exist is supported by a recent report on the structure of a soluble subtilisin-like enzyme called PfSUB-1 found in *Plasmodium falciparum* (33) and exhibiting a 29% sequence identity to SKI-1. This enzyme, which is closest to the subtilisin branch of subtilases (22), localizes to granular-like compartments and presumably cleaves at a Leu ↓ Asn bond (33). Therefore, because only mammalian members of the kexin (PCs) and pyrolysin (SKI-1) subfamilies have been identified, could it be that the other four subtilase subfamilies (22) have their mammalian counterparts?

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Differential Sorting of Nerve Growth Factor and Brain-Derived Neurotrophic Factor in Hippocampal Neurons

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Nerve growth factor (NGF) is released through the constitutive secretory pathway from cells in peripheral tissues and nerves where it can act as a target-derived survival factor. In contrast, brain-derived neurotrophic factor (BDNF) appears to be processed in the regulated secretory pathway of brain neurons and secreted in an activity-dependent manner to play a role in synaptic plasticity. To determine whether sorting differences are intrinsic to the neurotrophins or reflect differences between cell types, we compared NGF and BDNF processing in cultured hippocampal neurons using a Vaccinia virus expression system. Three independent criteria (retention or release from cells after pulse–chase labeling, depolarization-dependent release, and immunocytochemical localization) suggest that the bulk of newly synthesized NGF is sorted into the constitutive pathway, whereas BDNF is primarily sorted into the regulated secretory pathway. Similar results occurred with AtT 20 cells, including those transfected with cDNAs encoding neurotrophin precursor–green fluorescent protein fusions. The NGF precursor, but not the BDNF precursor, is efficiently cleaved by the endoprotease furin in the *trans*-Golgi network (TGN). Blocking furin activity in AtT 20 cells with α1-PDX as well as increasing the expression of NGF precursor partially directed NGF into the regulated secretory pathway. Therefore, neurotrophins can be sorted into either the constitutive or regulated secretory pathways, and sorting may be regulated by the efficiency of furin cleavage in the TGN. This mechanism may explain how neuron-generated neurotrophins can act both as survival factors and as neuropeptides.

Key words: NGF; BDNF; precursor; furin; constitutive secretion; regulated secretion; neurotrophin

Numerous cell types secrete neurotrophins, including CNS and PNS neurons and non-neuronal cells in peripheral tissues. Once released, neurotrophins promote neuronal survival and plasticity by interacting with specific receptors on the membranes of target neurons (for review, see Thoenen, 1995; Snider and Lichtman, 1996). We know much about sites of neurotrophin production and utilization, but we know little about the mechanisms that regulate neurotrophin release from cells.

Most secretory proteins are synthesized as high molecular weight precursors that translocate into the endoplasmic reticulum (ER) and then to the Golgi stacks. There they are post-translationally modified (Loh, 1993) and cleaved by endoproteases that separate active peptides from inactive precursors. Many precursors are cleaved within the *trans*-Golgi network (TGN) by furin or furin-like enzymes that act on the COOH-terminal side of multibasic sites (generally Arg-X-Lys/Arg-Arg) (Hosaka et al., 1991) after which they can be constitutively released (Dubois et al., 1995). In neurons, most neuropeptides are cleaved within the regulated secretory pathway not by furin-like enzymes but by prohormone convertases 1 and 2 (PC1 and PC2) (Rouille et al., 1995), which cleave precursors in immature secretory granules before or after granules bud from the TGN. Thus, proteolytic maturation of proteins destined for regulated secretion occurs at a later time point and in a different subcellular compartment than does proteolysis of constitutively secreted proteins.

Neurotrophin processing can occur in either the constitutive or the regulated secretory pathways. Fibroblasts and Schwann cells contain the constitutive secretory pathway only. They also produce furin, which cleaves neurotrophin precursors *in vitro* (Bresnahan et al., 1990; Seidah et al., 1996a,b), and bioactive NGF (Bunge, 1994; Singh et al., 1997), brain-derived neurotrophic factor (BDNF) (Acheson et al., 1991), and neurotrophin-3 (NT-3) (Cartwright et al., 1994). NGF can also be processed in the regulated pathway of cells exposed to viruses or plasmids encoding the NGF precursor (Edwards et al., 1988; Heymach et al., 1996; Canossa et al., 1997; Kruttgen et al., 1998).

BDNF processing appears to occur within the regulated pathway in cells that have both secretory mechanisms, including neurons. Depolarization releases BDNF from virus-infected hippocampal neurons (Goodman et al., 1996). BDNF has been detected in large dense-core vesicles of sensory neurons (Michael et al., 1997) and in brain synaptosomes (Fawcett et al., 1997). These data are consistent with a growing number of studies showing that BDNF, but not NGF, is anterogradely transported

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in neurons [Altar et al. (1997); Fawcett et al. (1998); for review, see Altar and DiStefano (1998)].

In this study, we used a Vaccinia virus (VV) expression system to directly compare the sorting of NGF and BDNF in hippocampal neurons and AtT 20 cells. Pulse–chase labeling, immunocytochemistry, and depolarization-dependent release studies suggest that under identical experimental conditions, NGF is primarily sorted to the constitutive secretory pathway, and BDNF is sorted to the regulated secretory pathway. Inhibiting furin-like enzymes alters the processing of pro-NGF but not pro-BDNF, and cold-block methods that inhibit protein exit from the TGN prevent cleavage of pro-BDNF but not pro-NGF. In addition, blocking furin activity directs some pro-NGF to the regulated pathway, suggesting that sensitivity to furin-mediated cleavage may be an important determinant in regulating neurotrophin sorting.

Some of these results have been published previously in abstract form (Mowla et al., 1997).

MATERIALS AND METHODS

Cell cultures. Hippocampal neurons were prepared according to the method of Banker and Cowan (1977) as modified by Brewer et al. (1993). Briefly, the hippocampus was dissected from embryonic day 18 (E18) mice (Charles River, Montreal, Canada), exposed to trypsin, dissociated mechanically, and grown in 60 mm collagen/poly-L-lysine-coated dishes. Cells from two litters of mice were plated into six dishes. Cultures were maintained in serum-free Neurobasal medium (Life Technologies, Gaithersburg, MD) containing 0.5 mM glutamine and 1× B27 supplement (Life Technologies). Schwann cell cultures were prepared from neonatal rat sciatic nerve as described previously (Pareek et al., 1993). AtT 20 cells and COS 7 cells were cultured as reported previously (Seidah et al., 1996a). We also used an AtT 20 cell line stably transfected with α1-PDX cDNA that has been described previously (Benjannet et al., 1997). Special care was taken to ensure that cells were distributed in equal numbers in dishes that were to be used for group comparisons.

VV recombinants and infections. Purified recombinant VVs containing the full-length coding regions of mouse pro-NGF and human pro-BDNF (generously provided by Regeneron Pharmaceuticals, Tarrytown, NY) were constructed as described previously (Seidah et al., 1996a,b). VVs coding for α1-PDX were kindly provided by Dr. Gary Thomas (Vollum Institute, Portland, OR). Separate plates of cells were infected with VV encoding pro-NGF or pro-BDNF. In one series of studies, we coinfected AtT 20 cells with VV encoding pro-NGF and α1-PDX. VV infections were performed as described previously (Seidah et al., 1996a), except that we used a multiplicity of infection (MOI) of 1 followed by an incubation of 8–10 hr in virus-free medium before metabolic labeling. Under our experimental conditions, there was no evidence of cell death in cells exposed to VVs for the times indicated in each experiment.

Green fluorescent protein–neurotrophin fusions. cDNAs coding for pro-BDNF and pro-NGF were amplified using primers that eliminated the stop codons and created restriction sites for inserting neurotrophin cDNAs in frame with the coding sequence of green fluorescent protein (GFP) from EGFP-N1 (Clontech, Cambridge, UK). The GFP coding region was inserted near the region coding for the C terminus of the mature neurotrophin. Thus, the NGF–GFP construct coded for amino acids 1–304 of pro-NGF, and the BDNF–GFP construct coded for amino acids 1–250 of pro-BDNF. Clones were sequenced manually (Sequenase; United States Biochemical Corporation, Cleveland, OH). AtT 20 cells growing on poly-L-lysine-coated coverslips were transfected with neurotrophin–GFP constructs using lipofectamine (Life Technologies). Three days later the cells were fixed in 4% paraformaldehyde in PBS and analyzed by epifluorescence using a Zeiss Axioskop microscope with a 40× objective.

To determine whether GFP-labeled neurotrophins were properly processed, we metabolically labeled the cells for 6 hr with [^{35}S] cysteine-methionine (Cys-Met) Translabel 48 hr after cells were transfected with the constructs, collected cell lysates and conditioned medium, exposed them to neurotrophin antibodies, and analyzed the immunoprecipitates by SDS-PAGE, as described below. We also analyzed the biological activity of secreted GFP-tagged neurotrophins by testing conditioned medium in a Trk autophosphorylation bioassay. Conditioned media obtained from nontransfected COS 7 cells or cells transfected with NGF, NGF–GFP, BDNF, or BDNF–GFP were incubated for 5 min with NIH 3T3 cells engineered to express Trk A (for NGF) or Trk B (for BDNF). The cells were lysed and immunoprecipitated with anti-pan Trk 203 antibody, fractionated by SDS-PAGE, and probed on Western blot replicas with a phosphotyrosine antibody, according to the methods of Hempstead et al. (1992).

Metabolic labeling and immunoprecipitation. For pulse–chase experiments, we incubated infected cells with 1.5 ml of Cys-Met-free DMEM containing 10% FCS and 0.5 mCi/ml [^{35}S] Translabel (ICN Biochemicals, Montréal, Québec, Canada) (70% methionine, 30% cysteine) for 30 min. Pro-BDNF contains 10 methionines as compared with four in pro-NGF, and mature BDNF contains three methionines as compared with one in mature NGF. These differences, together with higher concentrations of methionine in the Translabel, explain why pro-BDNF and mature BDNF tend to label more heavily than pro-NGF and NGF in most figures showing metabolic labeling. Cells were washed, and the medium was replaced with an equal volume of DMEM containing 10% FCS plus twofold excess concentrations of nonradioactive cysteine and methionine for the times indicated (chase periods). In some experiments, hippocampal neurons were incubated at 20°C for 3 hr in medium containing Translabel to monitor the effects of cold conditions on precursor processing.

In all experiments, conditioned media and cell lysates were brought to final volumes of 1.5 ml, 750 μl of which was subjected to immunoprecipitation. Samples immunoprecipitated with nonimmune rabbit IgG showed no bands corresponding to standards of neurotrophin precursors or products.

Immunoprecipitations were performed as described previously (Seidah et al., 1996a). For NGF, we used an affinity-purified rabbit anti-NGF IgG described previously (Murphy et al., 1993; Seidah et al., 1996a). BDNF immunoprecipitations were performed using an antibody kindly supplied by Amgen and characterized previously (Fawcett et al., 1997; Yan et al., 1997). Cell lysates and conditioned media were analyzed by electrophoresis on a 13–22% SDS-PAGE. Gels were fixed in 40% methanol and 10% acetic acid, treated with ENHANCE (DuPont NEN, Boston, MA), and washed in 10% glycerol, all for 1 hr. Dried gels were analyzed by a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA), and radioactivity in each band was quantitated using the ImageQuant program. Levels of radioactivity were within the linear range of the device. Statistical significance was determined using the Student's *t* test on a minimum of triplicate experiments.

To monitor the effects of depolarization on neurotrophin release, we infected hippocampal neurons with recombinant viruses, metabolically labeled the cells for 30 min, and washed and incubated the cells in medium containing excess nonradioactive methionine and cysteine for 4 hr. The cells were exposed to tissue culture medium supplemented with or without KCl (56 mM) and $CaCl_2$ (5.8 mM) for 15 min. Conditioned media and cell lysates were collected, immunoprecipitated, and fractionated by SDS-PAGE. Neurotrophin levels were estimated and compared by phosphorimager analysis. In a control experiment, we examined KCl-induced release of endogenous secretogranin II using immunoprecipitation methods. VV/NGF-infected cultures of hippocampal neurons were treated as above, and conditioned media and cell lysates were immunoprecipitated with an antibody to rat secretoneurin kindly provided by Dr. Reiner Fischer-Colbrie (Department of Pharmacology, Innsbruck University, Austria).

Immunocytochemistry and confocal microscopy. VV/NGF–BDNF-infected AtT 20 cells and primary cultures of hippocampal neurons as well as controls consisting of uninfected cells or cells infected with wild-type VVs were rinsed with PBS, fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and permeabilized in 0.1% Triton X-100 for 10 min. The cells were preincubated for 20 min in HEPES-buffered saline (HBS) containing 10% FCS to reduce nonspecific antibody binding and exposed to 1:2000 dilutions of primary antibodies overnight at 4°C. The cells were washed three times with HBS (5 min each) and incubated 1 hr with CY3-conjugated goat anti-rabbit antibody (Jackson Laboratory, Bar Harbor, ME) diluted 1:2000 in HBS containing 10% goat serum. Cells were washed three times in HBS and mounted in a Tris-buffered glycerol mounting medium (Sigma, St. Louis, MO).

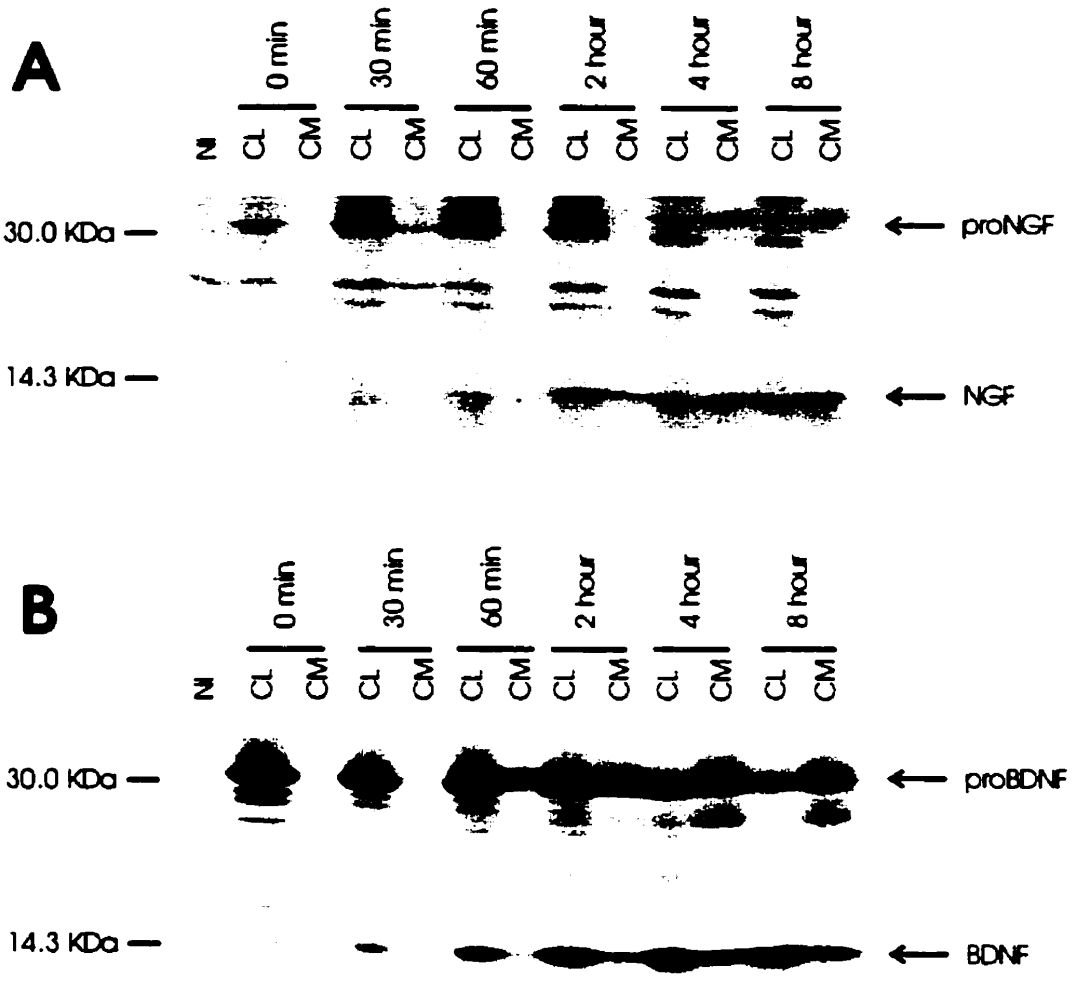

Figure 1. Pulse–chase metabolic labeling of pro-NGF (*A*) and pro-BDNF (*B*) in cultures of hippocampal neurons. Separate plates of cells were infected with VV encoding the NGF precursor or the BDNF precursor for 1 hr and postincubated in fresh medium without virus for 10 hr. Cells were exposed to medium containing [^{35}S] Cys-Met for 30 min and chased for 0, 0.5, 1, 2, 4, and 8 hr. Identical volumes (750 μl) of cell lysates (*CL*) and conditioned media (*CM*) were immunoprecipitated with antibodies to NGF or BDNF or with nonimmune serum (*NI*; a cell lysate sample) and electrophoresed on 13–22% SDS gradient gels. Dried gels were exposed to a phosphorimaging screen.

Cells were analyzed by confocal laser scanning microscopy with a Zeiss LSM 410 inverted confocal microscope using a 63×, 1.4 NA objective. Cells were excited at 543 nm and imaged on a photomultiplier after passage through FT 590 and LP 590 filter sets. The confocal images represent one confocal level (a depth of ~1 μm) that contains the cell nucleus along with as many cell processes as were possible to image, to evaluate the peripheral distribution of secretory vesicles. There were no perceptible differences in the distribution of NGF and BDNF immunoreactivity when we scanned below and above the nucleus. All images were printed on a Kodak XLS 8300 high-resolution printer.

In some studies we used epifluorescence microscopy to compare in VV-infected AtT 20 cells the distribution of NGF and BDNF immunoreactivity with TGN38, a marker of the *trans*-Golgi network (Luzio et al., 1990), and ACTH, which is packaged in secretory vesicles of AtT 20 cells. Antibody to TGN38 raised in guinea pig (kindly provided by Drs. G. Banting and W. Garten, University of Texas, Southwestern, Dallas, TX) was used at a 1:50 dilution and visualized using an FITC-conjugated secondary antibody raised in goat (Jackson Laboratory) diluted 1:50 in HBS containing 10% normal goat serum. ACTH localization was performed using a monoclonal antibody (Cortex Biochem) at a dilution of 1:1000, visualized with a CY2-conjugated goat anti-mouse secondary antibody (Jackson Laboratory) diluted 1:1000 in HBS containing normal goat serum.

RESULTS

Differential retention of NGF and BDNF in hippocampal neurons

Figure 1 compares neurotrophin processing in hippocampal neurons infected with recombinant viruses encoding either pro-NGF or pro-BDNF. Figure 1*A* shows that over an 8 hr chase period, pro-NGF processing gives rise to mature NGF. The NGF precursor (35 kDa) is evident in cell lysates at the start of the chase period, and within 30 min, glycosylated higher molecular weight forms of the precursor (39–42 kDa) (Seidah et al., 1996a) are evident. Levels of the precursor remain steady in cell lysates for up to 2 hr but decrease thereafter. Small amounts of the precursor are also evident in conditioned medium sampled at 4 and 8 hr. Mature NGF (13.2 kDa) is visible in conditioned medium after 2

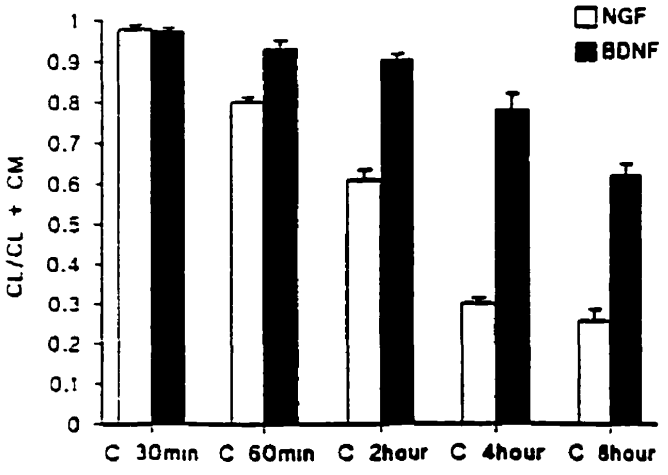

Figure 2. Kinetics of NGF and BDNF retention in hippocampal neurons. Experiments in Figure 1 were repeated three times, and the combined results were analyzed by the ImageQuant program. The ratio of mature NGF and BDNF in cell lysates (*CL*) was compared with the total NGF and BDNF in CL + conditioned medium (*CM*). Data show that significantly larger amounts of BDNF are retained in CL than NGF.

hr, and at 4 hr, levels are higher than in the corresponding cell lysates. Phosphorimager analysis revealed that in samples collected at 8 hr, 3.0 times (±0.7 SEM) as much mature NGF is released into medium than is retained within cell lysates.

Figure 1*B* shows that pro-BDNF is also processed by hippocampal neurons. Pro-BDNF (32 kDa) is evident within cell lysates in all time periods tested, with levels decreasing in samples collected at 4 and 8 hr when levels of processed product increase. In contrast to pro-NGF, significant levels of the BDNF precursor are also evident in conditioned media in all samples collected after 1 hr, apparently because of constitutive release of the protein. Mature BDNF (14.2 kDa) is evident within cell lysates by 1 hr and remains detectable throughout the 8 hr period of analysis. The amount of BDNF retained in cell lysates exceeds the amount released into conditioned medium by 4.0-fold (±1.5 SEM) in samples collected at 8 hr.

Figure 2 presents data obtained from triplicate experiments on hippocampal neurons performed as shown in Figure 1. The figure compares the amount of processed NGF or BDNF in cell lysates as a function of the total amount of processed neurotrophin in cell lysates and conditioned media. Significantly higher levels of BDNF are retained within cell lysates as compared with NGF as early as 1 hr after chase, and the differences increase over the 8 hr chase period.

To determine whether hippocampal neurons are unique in their ability to retain more BDNF than NGF, we repeated the pulse–chase experiments shown in Figure 1 in AtT 20 cells, a well established cell line that contains both the regulated and constitutive secretory pathways (Burgess and Kelly, 1987). Figure 3 shows that AtT 20 cells, like hippocampal neurons, release more NGF into conditioned medium than they retain in cell lysates; the opposite occurs with BDNF. Therefore, in both neurons and AtT 20 cells, most newly synthesized and processed NGF is released from cells, whereas most processed BDNF is retained in cell lysates.

To determine whether retention of BDNF is only a characteristic of cells with the regulated secretory pathway, we repeated the experiments with constitutively secreting rat Schwann cells. Figure 4 shows that pro-BDNF is processed by Schwann cells. By 4 hr chase, slightly higher levels of processed BDNF are evident in conditioned media than in cell lysates. By 8 hr, both mature BDNF and pro-BDNF are evident only in conditioned medium. Therefore, Schwann cells process pro-BDNF and release it, along with the BDNF precursor, into conditioned medium. Thus, the retention of processed BDNF by hippocampal neurons and AtT 20 cells is likely caused by differences in the secretory pathways of these cells and Schwann cells.

Immunocytochemical localization of BDNF and NGF

We used immunocytochemistry and confocal microscopy to assess the intracellular locations of NGF and BDNF immunoreactivity in recombinant virus-infected cells. Figure 5 shows BDNF immunoreactivity localized to punctate structures throughout the cytoplasm and in the tips of processes in both AtT 20 cells (Fig. 5*A*) and hippocampal neurons (Fig. 5*B*). In contrast, NGF immunoreactivity is distributed in the perinuclear cytoplasm of both AtT 20 cells (Fig. 5*C*) and hippocampal neurons (Fig. 5*D*) and was seldom detected as punctate in either cell type. Neither NGF nor BDNF immunoreactivity was evident in uninfected cells or in cells infected with wild-type viruses (data not shown). Detection of BDNF immunoreactivity within vesicle-like structures is consistent with the idea that BDNF is processed within the regulated secretory pathway.

Figure 6 compares the distribution of BDNF and NGF immunoreactivity with that of endogenous TGN38 and ACTH in virally infected AtT 20 cells. Cells were immunostained for BDNF (*A,B,G,H*), NGF (*C,I*), ACTH (*E,H*), and TGN38 (*D,F,G,I*). Immunoreactivity for BDNF (*A*) and TGN38 (*D*) colocalize (*G*) as does immunoreactivity for NGF (*C*) and TGN38 (*F*) within the perinuclear region (*I*). BDNF immunoreactivity is also located in punctate structures that are distributed within the cytoplasm and processes of AtT 20 cells (*A,B*) in a manner indistinguishable from ACTH (*E*). In some vesicles, the two proteins colocalize (*H*). In contrast, NGF immunoreactivity was never seen in punctate structures under these experimental conditions. Taken together, these data suggest that BDNF is located in the TGN, as expected before sorting, and packaged within large dense-core vesicles similar to those containing endogenously produced ACTH. NGF is also located in the TGN but is not packaged or concentrated within large dense-core vesicles, which is consistent with NGF being released through the constitutive secretory pathway.

Our results suggest clear differences between the sorting of NGF and BDNF. To test whether these results arose simply as a result of the Vaccinia virus infection method, we transfected AtT 20 cells with constructs coding for GFP fused to the C-terminal region of pro-BDNF or pro-NGF and examined the distribution of the fusion proteins by epifluorescence microscopy. Figure 7*A* shows that pro-BDNF–GFP fluorescence was localized within punctate structures in the cytoplasm and tips of cell processes. In contrast, pro-NGF–GFP (Fig. 7*B*) was distributed diffusely within the cell, never within punctate structures. NGF–GFP fluorescence was also less intense than that of BDNF–GFP, perhaps because of its failure to be concentrated in vesicles and its constitutive release from the cell. The differential distribution of GFP-labeled pro-BDNF and pro-NGF was similar to that obtained using the Vaccinia virus infection method. Haubensak et al. (1998) recently reported results similar to ours with respect to the localization of BDNF–GFP fusion proteins.

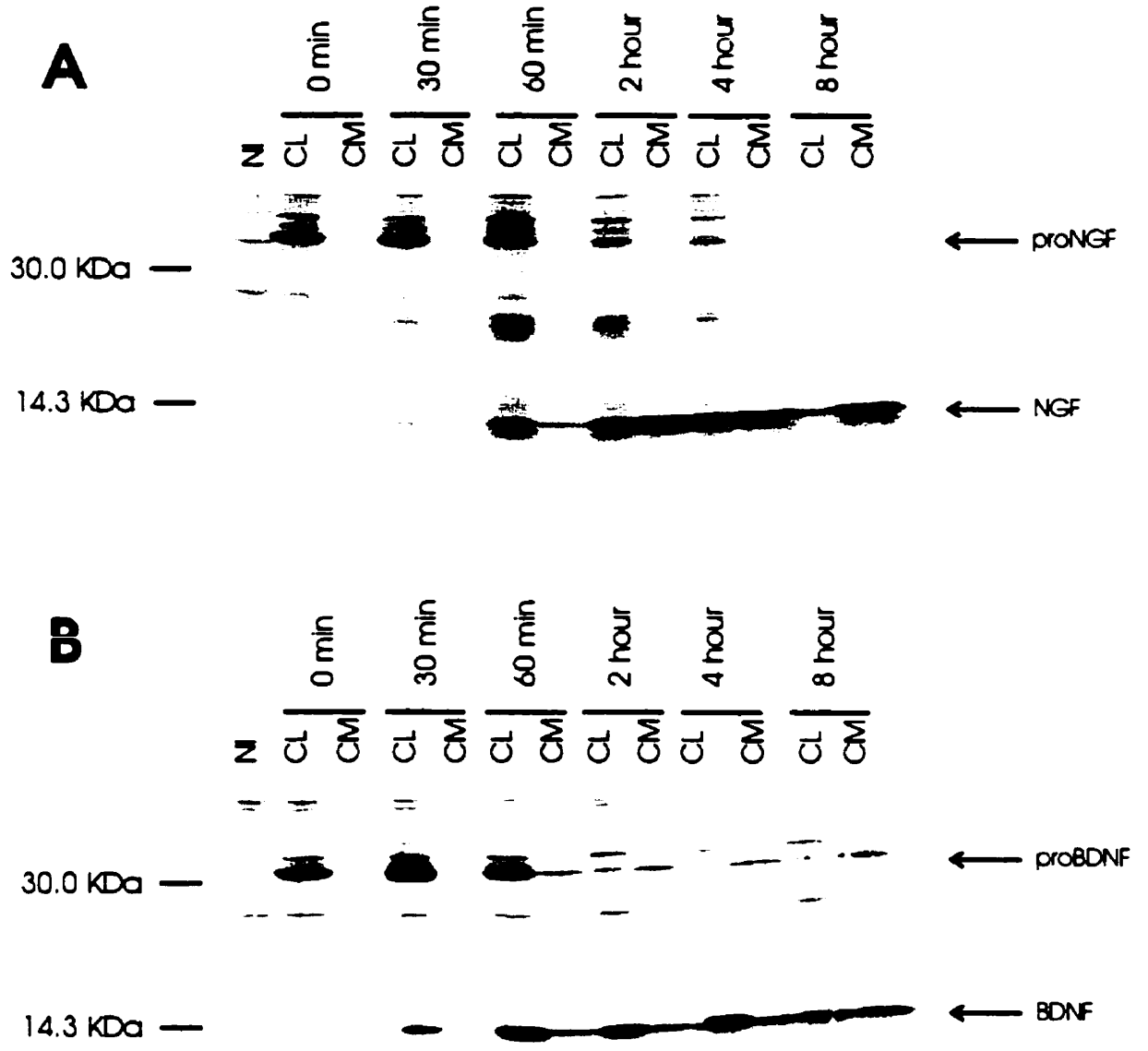

Figure 3. Pulse-chase metabolic labeling of pro-NGF (*A*) and pro-BDNF (*B*) production and processing in VV-infected AtT 20 cell cultures. Methods were identical to those described in the legend to Figure 1. NGF and BDNF and their precursors were measured in conditioned medium (*CM*) and in cell lysates (*CL*). *NI* is a sample of *CL* precipitated with nonimmune serum.

We performed metabolic labeling and SDS-PAGE analyses to determine whether pro-BDNF- and pro-NGF–GFP-labeled constructs were appropriately translated and processed in these experiments. However, these experiments were unsuccessful in AtT 20 cells because of low transfection efficiency. We repeated the experiments in COS 7 cells and found that both pro-NGF–GFP and pro-BDNF–GFP were processed appropriately, without cleavage of the GFP tag, as reported previously (Haubensak et al., 1998) (Fig. 7*C*). We also determined that medium conditioned by COS 7 cells that had been transfected with pro-NGF–GFP and pro-BDNF–GFP was fully active in inducing Trk A and Trk B autophosphorylation, respectively, in NIH 3T3 cells engineered to express the receptors (Fig. 7*D*). These data indicate that GFP-tagged pro-BDNF and pro-NGF are processed appropriately and that conditioned media containing the precursor and mature forms of the proteins can activate their cognate receptors. Data monitoring the distribution of the neurotrophin–GFP fusion proteins further confirm our VV data indicating clear differences in the sorting and intracellular distribution of NGF and BDNF.

Depolarization-induced release of BDNF from hippocampal neurons

If BDNF is in the regulated secretory pathway, depolarization should promote its release. Figure 8 shows that BDNF levels in conditioned medium nearly doubled when hippocampal neurons

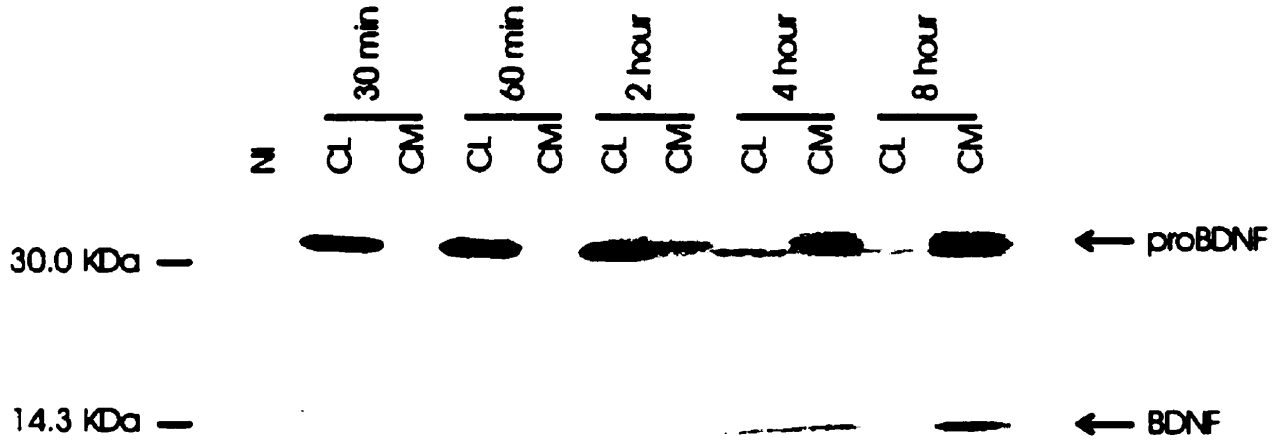

Figure 4. Pulse–chase metabolic labeling of primary rat Schwann cells infected with VV encoding pro-BDNF. Methods were identical to those described in the legend to Figure 1. BDNF and its precursor were measured in cell lysates (*CL*) and conditioned media (*CM*). *NI* is a *CL* sample precipitated with nonimmune serum.

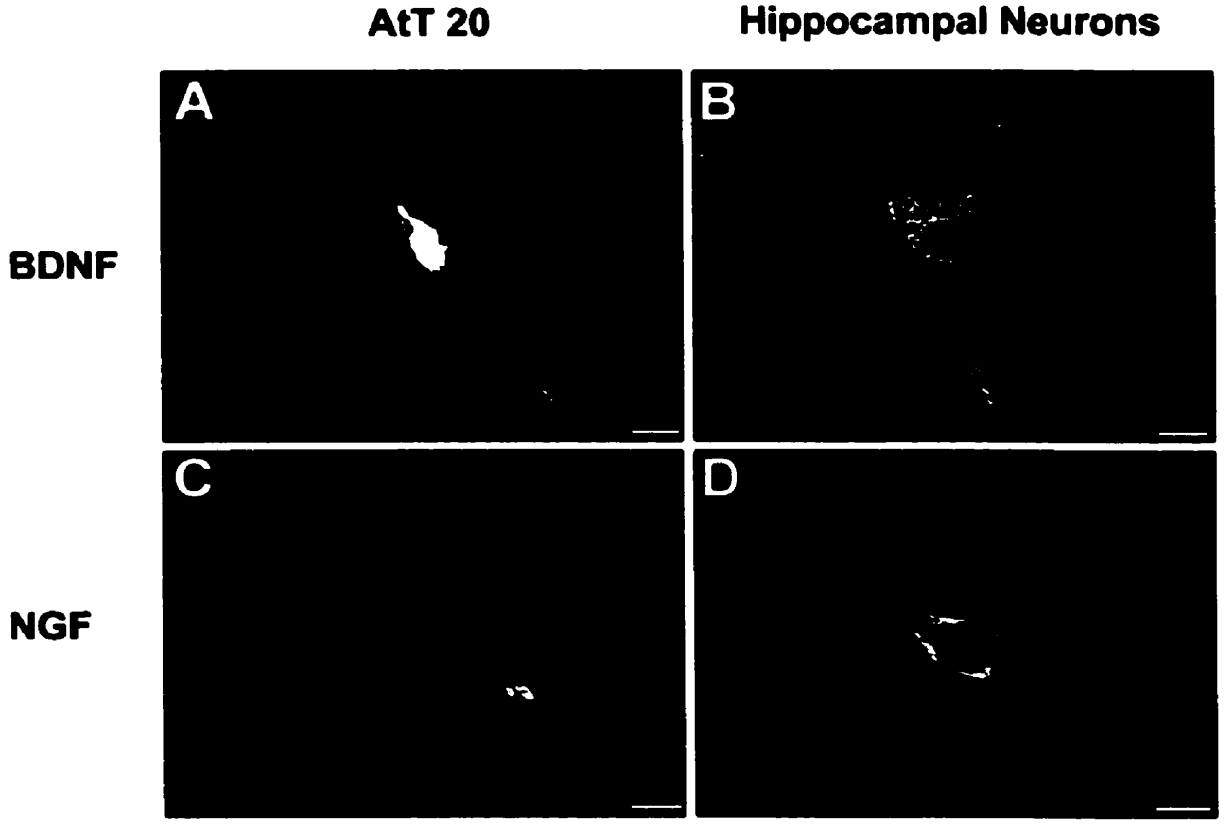

Figure 5. Confocal microscopy of AtT 20 cells (*A*, *C*) and hippocampal neurons (*B*, *D*) infected with VV encoding pro-NGF (*C*, *D*) or pro-BDNF (*A*, *B*). Cells were infected for 1 hr and postincubated in the absence of virus for another 8 hr. The cultures were fixed and treated with antibodies against NGF or BDNF, followed by CY3-conjugated goat anti-rabbit IgG. Scale bar, 10 μm.

were exposed to KCl; however, depolarization had no effect on NGF release. Depolarization did not promote the release of pro-BDNF or pro-NGF under these experimental conditions (data not shown). To be certain that infecting hippocampal neurons with the NGF-coding virus had not altered the regulated secretory pathway of hippocampal neurons, we monitored the effects of KCl depolarization on the release of endogenously produced secretogranin II, which is present in the regulated pathway. Figure 9 shows that KCl treatment effectively promoted secretogranin II release in cells infected with pro-NGF encoding VV.

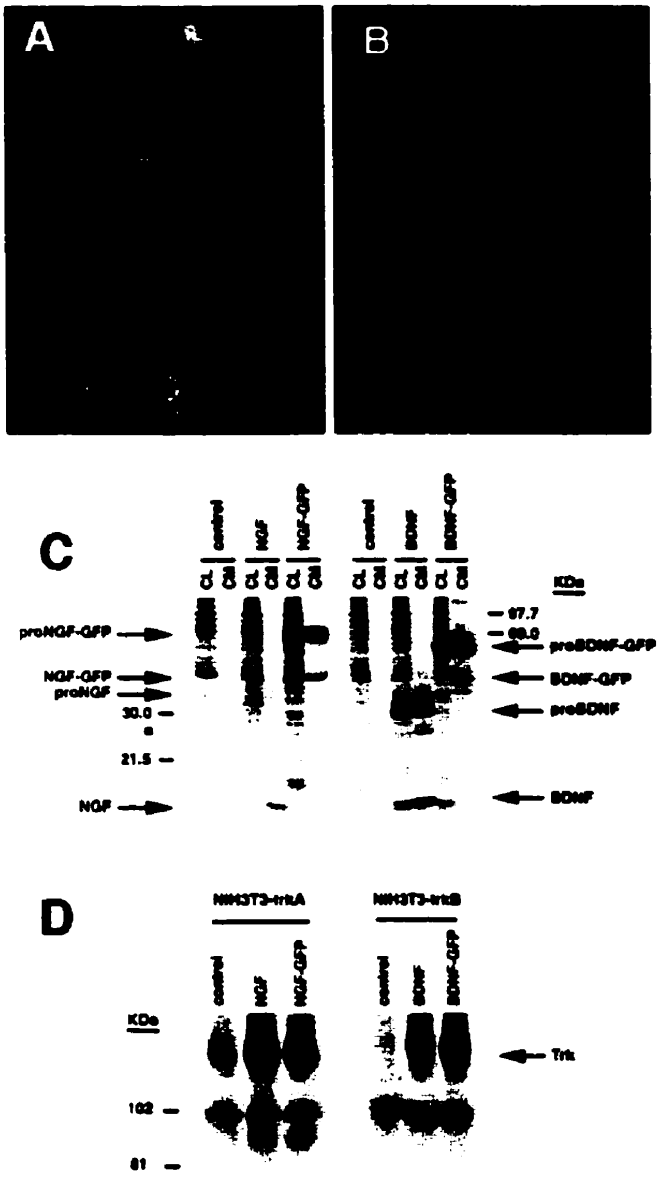

Figure 7. Expression of pro-neurotrophin–GFP fusion proteins in AtT 20 cells. Cells were plated on poly-L-lysine-coated coverslips and transfected using lipofectamine with cDNAs encoding either (*A*) pro-NGF–GFP or (*B*) pro-BDNF–GFP. Three days later, the cells were analyzed by fluorescence microscopy. *C*, Immunoprecipitation and SDS-PAGE of metabolically labeled GFP fusion proteins from transfected COS 7 cells. *D*, Conditioned medium from pro-NGF–GFP or pro-BDNF–GFP expressing COS 7 cells activate Trk A and Trk B phosphorylation, respectively, in NIH 3T3 cells engineered to overexpress either receptor.

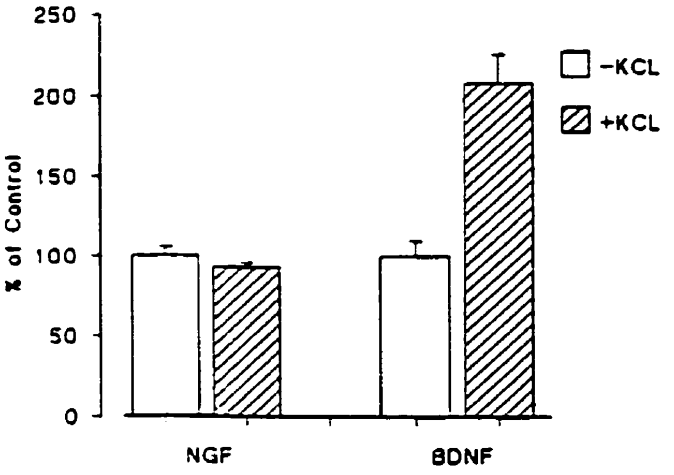

Figure 8. KCl-induced release of BDNF but not NGF from hippocampal neurons. Hippocampal neurons from E18 mice were cultured for 7 d and infected for 1 hr with VV encoding pro-NGF or pro-BDNF. After 10 hr in medium without virus, the cells were labeled for 30 min with [^{35}S] Cys-Met, incubated in medium without radiolabel for 4 hr, and treated with medium with or without KCl and $CaCl_2$ for 15 min. Conditioned media were immunoprecipitated with antibodies to NGF or BDNF and electrophoresed on a SDS gel. Results were analyzed on a phosphorimager and are an average (±SEM) of three independent experiments.

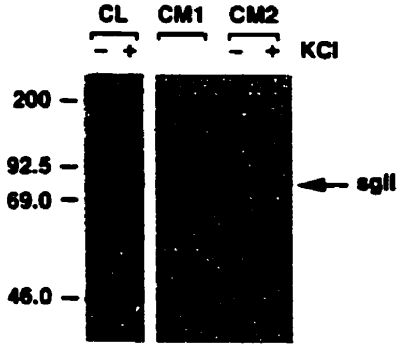

Figure 9. Release of secretogranin II (*sgII*) from hippocampal neurons infected with VV coding for pro-NGF. Eight hours after neurons were exposed to VV, the cells were pulsed for 30 min with medium containing [^{35}S] Cys-Met. The cells were chased for an additional 4 hr, after which samples of conditioned medium were analyzed (*CM1*), and again 30 min later after the addition (+) or in the absence (−) of KCl (50 mM) added to the culture medium (*CM2*).

whereas cold block totally inhibited the generation of mature BDNF from the BDNF precursor. Therefore pro-NGF, but not pro-BDNF, is cleaved within the TGN, probably by furin.

We also compared pro-NGF and pro-BDNF processing in the presence of α1-PDX, an α1-anti-trypsin derivative that selectively interferes with furin's ability to process precursor proteins within the TGN (Anderson et al., 1993; Watanabe et al., 1995; Vollenweider et al., 1996). In these studies, we monitored neurotrophin processing in AtT 20 cells coinfected with VVs encoding either pro-NGF or pro-BDNF with or without VVs coding for α1-PDX. Figure 11 shows that α1-PDX had no detectable effect on pro-BDNF processing. However, α1-PDX did increase the amount of pro-NGF released constitutively into conditioned medium, a result that was similar to those obtained when we monitored pro-BDNF processing in hippocampal neurons and AtT 20 cells (Figs. 1, 3). Identical results (data not shown) were obtained when we infected neurotrophin-encoding viruses into a stably transfected AtT 20 cell line overexpressing α1-PDX (Benjannet et al., 1997).

The finding that α1-PDX caused the constitutive release of pro-NGF into conditioned medium suggested to us that α1-PDX might be altering sorting of NGF within the cell, an idea confirmed by immunocytochemistry. Figure 12*B* shows that in AtT 20 cells that stably overexpress α1-PDX, pro-NGF encoding viruses cause the accumulation of NGF immunoreactivity in punc-

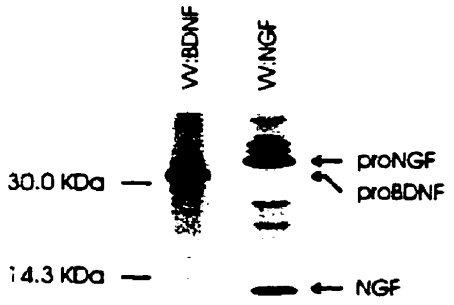

Figure 10. Cold-block experiments. Hippocampal neurons were infected with VV encoding pro-NGF or pro-BDNF for 1 hr, and metabolically labeled at 20°C for 3 hr. Cell lysates were prepared, immunoprecipitated with antibodies to NGF or BDNF, and electrophoresed on SDS gels.

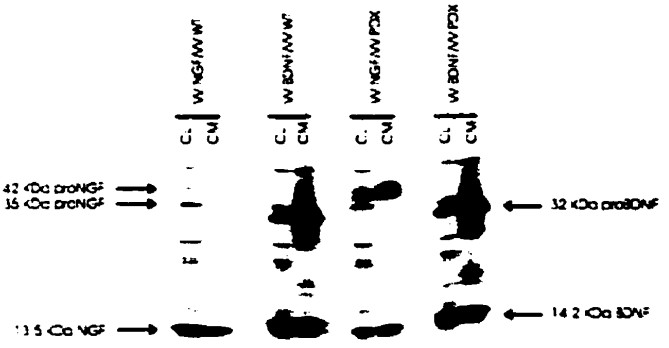

Figure 11. The effects of α1-PDX on pro-NGF processing in AtT 20 cells. AtT 20 cells were infected for 2 hr with VV encoding pro-NGF or pro-BDNF with or without VV encoding the furin inhibitor α1-PDX. Cells were incubated in virus-free medium for 10 hr and metabolically labeled for 3 hr. Cell lysates (*CL*) and conditioned media (*CM*) were collected, immunoprecipitated, and analyzed by SDS gel electrophoresis.

tate vesicles within the cell cytoplasm and tips of cell processes that does not occur in the absence of α1-PDX. Figure 12*C* shows that at least some of the NGF in these cells can be released into conditioned medium in response to extracellular cAMP, which is consistent with the protein being sorted to the regulated secretory pathway. Taken together, these data suggest that α1-PDX, which partially inhibits the processing of pro-NGF in the TGN, targets some NGF processing to the regulated secretory pathway, where its release can be promoted by extracellular cues.

Finally, Edwards et al. (1988) reported that cAMP causes the release of NGF from VV-infected AtT 20 cells, data that led them to suggest that NGF is processed and released by the regulated secretory pathway. In that study, cells were infected with an MOI of 10–20, as opposed to 1 MOI in our study. The probable explanation for differences in their results and ours is presented in Figure 13. Immunocytochemical data show that increasing levels of viral infection shifts the intracellular distribution of NGF from a diffuse to a punctate pattern (Fig. 13*a*). Furthermore, cells receiving 1 MOI do not release NGF in response to cAMP, but cAMP-induced NGF release is seen when cells are exposed to 5 and 10 MOI (Fig. 13*b*), data that agree with those presented by Edwards et al. (1988). (In the figure, compare *CM3* in the presence and absence of cAMP). In contrast, reducing by up to 50-fold the MOI of VV coding for BDNF had no effect on the punctate localization of BDNF in infected cells (data not shown).

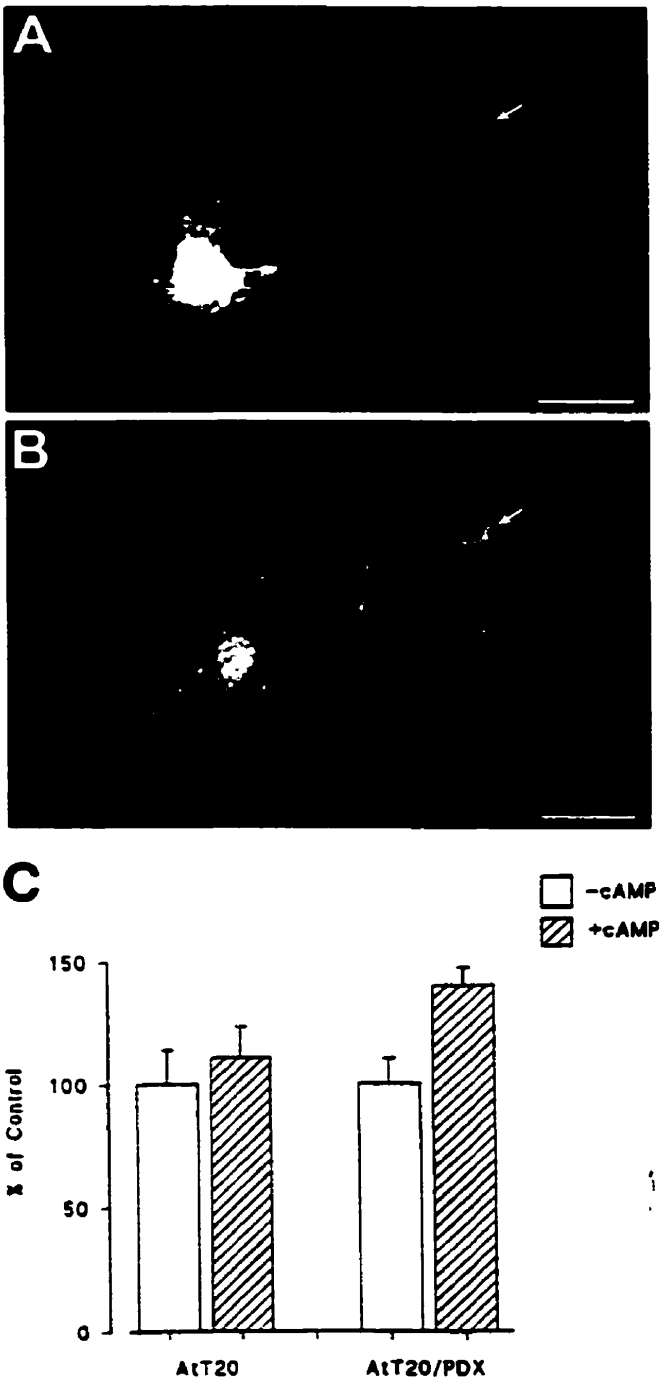

Figure 12. The effects of α1-PDX on NGF sorting in AtT 20 cells. AtT 20 cells (*A*) or AtT 20 cells that stably express α1-PDX (*B*) were infected for 1 hr with VV encoding pro-NGF, postinfected for 10 hr, and prepared for immunocytochemistry. In *C*, AtT 20 cells with or without stably expressed α1-PDX were infected with VV encoding pro-NGF for 1 hr, postinfected for 10 hr in control medium, pulsed with medium containing [^{35}S] Cys-Met for 2 hr, chased for 3 hr, and treated with medium with or without 5 mM cAMP for 3 hr. Cell lysates and conditioned media were immunoprecipitated and analyzed by SDS-PAGE. Results were analyzed on a phosphorimager and report an average (±SEM) of three independent experiments.

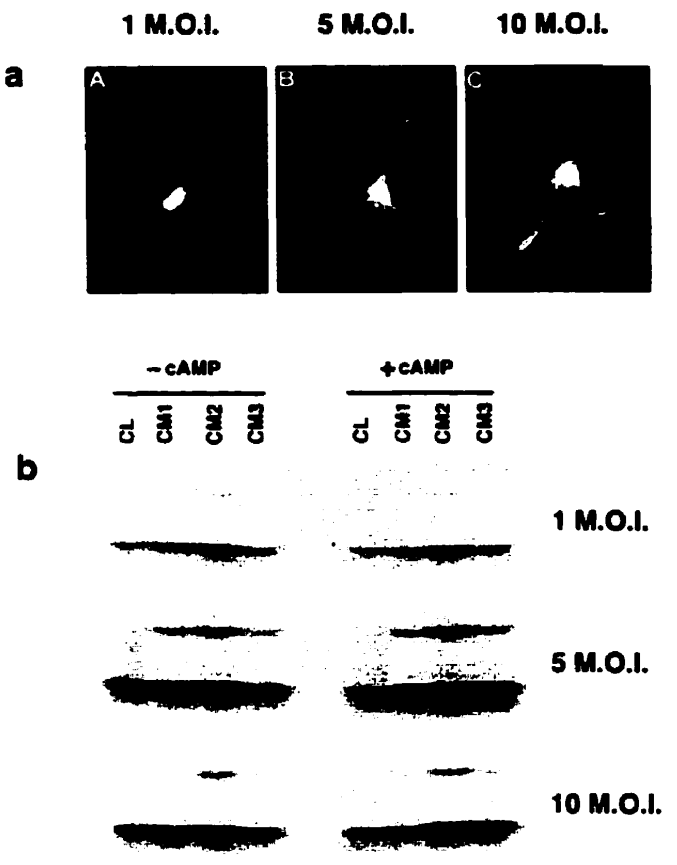

Figure 13. Overexpression of NGF results in missorting of NGF from the constitutive to the regulated secretory pathway. *a*, AtT 20 cells were infected for 1 hr with 1 (*A*), 5 (*B*), or 10 (*C*) MOI of VV coding for pro-NGF, postinfected for 8 hr, fixed, and prepared for immunocytochemistry using an antibody to NGF followed by a CY3-conjugated secondary antibody. Cells were analyzed by confocal microscopy. *b*, AtT 20 cells were infected with 1 (*A*), 5 (*B*), or 10 (*C*) MOI for 1 hr followed by a 4 hr postinfection and 3 hr incubation in medium containing [^{35}S] Translabel. Conditioned media were collected (*CM1*), the cells were chased for 3 hr, and media was again collected (*CM2*). 8-bromo-cAMP (5 mM) was then added to some cultures, and cells were incubated for an additional 3 hr, after which media were collected (*CM3*) and the cells were lysed (*CL*). NGF was immunoprecipitated from all samples, and the precipitate was analyzed by SDS-PAGE. Comparison of *CM3* samples shows that NGF release can be stimulated by cAMP from cells infected with 5 or 10 MOI but not from cells receiving 1 MOI.

Therefore, sorting of BDNF to the regulated pathway is likely not attributable to concentration effects arising from the level of viral infection.

DISCUSSION

Pulse–chase studies show that hippocampal neurons and AtT 20 cells retain more newly synthesized BDNF than they release. BDNF immunoreactivity is evident in punctate, vesicle-like structures distributed throughout the cell cytoplasm, including in the tips of cell processes, and cell depolarization induces BDNF release. Thus, BDNF appears to sort primarily to the regulated secretory pathway. In contrast, hippocampal neurons and AtT 20 cells release more NGF than they retain, NGF immunoreactivity is distributed diffusely within the perinuclear cytoplasm, presumably within the endoplasmic reticulum and Golgi apparatus, and depolarization fails to promote NGF's release into conditioned medium. Thus, NGF appears to be processed and released in the constitutive pathway. These results are not unique to the Vaccinia virus expression system because NGF and BDNF were differentially distributed as well within cells transfected with cDNAs coding for GFP–pro-neurotrophin fusion proteins.

Furin appears to cleave pro-NGF in the cells we tested. Pro-NGF processing is unaffected by cold-block conditions that inhibit the exit of proteins from the TGN (Matlin and Simons, 1983), suggesting that NGF processing occurs within the TGN. In contrast, cold block totally inhibited the processing of pro-BDNF. Pro-BDNF processing likely occurs in immature secretory vesicles after they bud from the TGN, which is an early step in the regulated secretory pathway. Also, α1-PDX, a competitive inhibitor of furin, increased levels of NGF precursor in conditioned medium, which is consistent with its inhibiting pro-NGF processing within the TGN. α1-PDX did not affect pro-BDNF processing. In the presence of α1-PDX, NGF immunoreactivity appeared in punctate structures similar to those in cells infected with pro-BDNF encoding viruses, and some NGF precursor was constitutively secreted. Furthermore, depolarization released small amounts of NGF into conditioned medium. These results are similar to those obtained with pro-BDNF in the absence of α1-PDX. Thus, inhibiting furin activity induces some pro-NGF to be sorted to the regulated secretory pathway. Similar effects have been observed for pro-opiomelanocortin (Benjannet et al., 1997).

Cleavage by furin or furin-like enzymes within the TGN may be one factor determining whether neurotrophins are sorted into the constitutive or regulated secretory pathways. Studies with the precursor for egg-laying hormone (pro-ELH) in mollusks may explain how this mechanism could work. Pro-ELH contains bioactive peptides on both the C- and amino-terminal sides of a furin cleavage site (Sossin et al., 1990). The C-terminal side of the precursor is sorted into the regulated secretory pathway after furin cleavage, and the amino-terminal side is released constitutively, degraded, or sorted into a separate regulated secretory pathway (Jung and Scheller, 1991). In cells with low furin levels, the precursor avoids cleavage, and both sides of the precursor are sorted into the same regulated secretory vesicles (Klumperman et al., 1996). Thus, sorting of the amino-terminal active peptides into dense-core secretory vesicles occurs only in the absence of furin cleavage, and different cells with different amounts of furin sort the same neuropeptide differently.

A similar situation may occur with neurotrophin processing. Sorting into the regulated pathway may require signals within the pro-domain or near the consensus cleavage site. When cleaved by furin, the NGF precursor may lose these signals, and the mature cleaved protein is sorted into the constitutive pathway for release. In that regard, inserting furin-sensitive cleavage sites into pro-insulin (Yanagita et al., 1992) and pro-renin (Oda et al., 1991), which are normally processed by the regulated pathway, redirects these proteins into the constitutive pathway. In the absence of furin cleavage, the precursor remains intact, and sorting signals that direct the protein to the regulated pathway become functional. This may explain why pro-BDNF, which likely eludes furin cleavage in the TGN, is targeted to the regulated pathway where its processing appears to occur in secretory granules.

Differences between pro-BDNF and pro-NGF processing may therefore arise because of the furin sensitivity of their pro-protein processing cleavage sites. In pro-NGF (Arg-Ser-Lys-Arg ↓ Ser) the site is highly suited to furin processing, whereas

the analogous site in pro-BDNF (Arg-Val-Arg-Arg ↓ His) is less suitable because of the replacement of Ser in the +1 position (P1) of NGF with His in the P1 of BDNF (Seidah et al., 1996a). Sequences with His at P1 show reduced sensitivity to furin-mediated cleavage (Ogi et al., 1990; Matthews et al., 1994).

Our results are consistent with this model. In constitutively secreting cells with moderate levels of furin-like enzymes, such as fibroblasts and Schwann cells (M. Marcinkiewicz and N. G. Seidah, unpublished observations), pro-NGF and pro-BDNF are cleaved, with only low levels of unprocessed precursor secreted constitutively. In AtT 20 cells and hippocampal neurons with lower levels of furin (Seidah et al., 1994), pro-NGF is cleaved efficiently, and NGF is released constitutively. When furin cleavage is inhibited with α1-PDX, pro-NGF is not cleaved efficiently, some sorting occurs into the regulated secretory pathway, where some of the protein can be released by depolarization, and some unprocessed precursor is secreted constitutively. In contrast, pro-BDNF avoids furin cleavage and is sorted into the regulated secretory pathway, and some unprocessed precursor is secreted constitutively into conditioned medium. Constitutive release of other precursors normally processed in the regulated pathway has also been reported (Kelly et al., 1983; Moore et al., 1983; Brechler et al., 1996)

An increasing number of reports suggest that BDNF, but not NGF, is anterogradely transported within axons in brain neurons to carry out a number of physiological actions [Altar et al. (1997); Fawcett et al. (1998); for review, see Altar and DiStefano (1998)]. Also, BDNF is enriched in a microvesicular fraction of rat brain synaptosomes along with synaptotagmin, a protein associated with synaptic and large dense-core vesicles in nerve terminals (Fawcett et al., 1997). BDNF immunoreactivity is also present in mossy fiber terminals in the hippocampus (Conner et al., 1997; Fawcett et al., 1997) and in large dense-core vesicles of axon terminals in lamina II of lumbar spinal cord (Michael et al., 1997). BDNF can be transported anterogradely in neurons within the visual system (von Bartheld et al., 1996) and released by depolarization in a calcium-dependent mechanism from virus-infected hippocampal neurons (Goodman et al., 1996). Taken together, these data are consistent with BDNF being packaged within secretory vesicles of the regulated pathway. Presumably these granules are targeted to axons, although transport may occur to other parts of the cell as well.

Fewer studies have monitored the processing of NGF in cells containing the regulated pathway. Edwards et al. (1988) reported that AtT 20 cells secrete VV-encoded NGF in response to cAMP, suggesting regulated release. In that study, they infected cells with an MOI of 10–20 as opposed to an MOI of 1 in our study, which explains why their results and ours differ (Fig. 13). Overloading the furin pathway beyond its capacity may drive NGF into the regulated pathway, as does inhibiting furin with α1-PDX (Figs. 11, 12). In a similar manner, overexpressing β2-microglobulin in pancreatic β cells drives the protein from the constitutive pathway into secretory vesicles of the regulated pathway (Allison et al., 1991). In contrast, reducing infectivity from 1 to 0.02 MOI had no effect on the sorting of BDNF, although we cannot rule out the possibility that local concentration effects contributed to BDNF's sorting into the regulated pathway.

Heymach et al. (1996) reported that AtT 20 cells release NGF as well as BDNF and NT-3 in response to secretagogues. It may be that in those studies the level of NGF production was above the threshold levels in which NGF is shunted from the constitutive pathway into the regulated pathway. Heymach et al. (1996) used transfection instead of infection, expressed the neurotrophins using a different promoter, and treated cells with secretagogues for a longer time, which may further explain why their conclusions differ from ours. Blochl and Thoenen (1995) hypothesized that there is both constitutive and regulated sodium-dependent release of NGF from neurons. They suggest that release occurs independent of extracellular calcium, which is essential for protein release in the regulated pathway (DeCamilli and Jahn, 1990), including the release of BDNF (Goodman et al., 1996). Canossa et al. (1997) extended these findings by reporting that neurotrophins can also induce neurotrophin release from neurons (also see Kruttgen et al., 1998). These conflicting data need to be investigated further.

Our results provide a mechanism whereby neurotrophins in brain neurons can act either as survival factors or as neuropeptides. When neurotrophins are cleaved by furin, the bioactive peptide may be released constitutively to promote neuronal survival. Perhaps this explains how hippocampal neurons constantly provide NGF to innervating cholinergic neurons in the basal forebrain. In contrast, BDNF may avoid furin cleavage and be sorted into the regulated secretory pathway where it is processed by PC1 (Seidah et al., 1996a) and released in an activity-dependent manner similar to other neuropeptides. This may be the mechanism that allows BDNF to alter synaptic transmission, connectivity, and synaptic plasticity in an activity-dependent manner (Ghosh, 1996). Also, perhaps neurons modulate the physiological fates of neurotrophins by regulating furin levels and thus the intracellular sorting of the neurotrophins they produce.

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Neurotrophin-3 Sorts to the Constitutive Secretory Pathway of Hippocampal Neurons and Is Diverted to the Regulated Secretory Pathway by Coexpression with Brain-Derived Neurotrophic Factor

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Hippocampal neurons release nerve growth factor (NGF) through the constitutive secretory pathway, thus allowing the protein to be continuously available for promoting nerve cell survival. In contrast, hippocampal neurons use the regulated secretory pathway to process brain-derived neurotrophic factor (BDNF), which alters synaptic activity when released acutely from dense-core vesicles. Thus, understanding how neurons sort and deliver neurotrophins may provide clues to their functions in brain. In this study, we monitored the processing and delivery of neurotrophin-3 (NT-3). Pulse–chase studies, immunocytochemistry, and secretagogue-induced release experiments were performed on cultured hippocampal neurons and AtT-20 cells infected with vaccinia viruses encoding the NT-3 precursor (pro-NT-3). Results show that most newly synthesized NT-3 is released through the constitutive secretory pathway as a result of furin-mediated endoproteolytic cleavage of pro-NT-3 in the *trans*-Golgi network. Pro-NT-3 can also be diverted into the regulated secretory pathway when cells are treated with α1-PDX, a selective inhibitor of furin-like enzymes, or when pro-NT-3 expression is increased by transient transfection methods. In cells coinfected with viruses coding for pro-NT-3 and pro-BDNF, NT-3 is sorted into the regulated pathway, stored in secretory granules, and released in response to extracellular cues together with BDNF, apparently as a result of heterodimerization, as suggested by coimmunoprecipitation data. Taken together, these data show that sorting of the NT-3 precursor can occur in both the constitutive and regulated secretory pathways, which is consistent with NT-3 having both survival-promoting and synapse-altering functions.

Key words: neurotrophin; NT-3; BDNF; constitutive pathway; regulated secretory pathway; heterodimer

Neurotrophins are synthesized as high molecular weight precursors containing a prodomain linked to the amino terminus of the mature protein. The two components are separated by endoproteolytic cleavage within either the constitutive or regulated secretory pathways. Understanding how neurotrophins are processed in these pathways may help explain their physiological functions.

Hippocampal neurons and AtT-20 cells in culture process nerve growth factor (NGF) within the constitutive secretory pathway (Mowla et al., 1999). The NGF precursor is cleaved within the *trans*-Golgi network (TGN) by the endoprotease furin, a member of the subtilisin/kexin-like family of proteases (for review see Seidah et al., 1998; Zhou et al., 1999). Constitutive release of NGF occurs soon after the molecule is synthesized. Thus, as a result of being processed in the constitutive pathway, NGF is continuously available to cells that require it, which is consistent with hippocampal neurons producing NGF as an apoptosis-inhibiting survival factor for basal forebrain cholinergic neurons (for review, see Yuen et al., 1996).

In contrast, hippocampal neurons process brain-derived neurotrophic factor (BDNF) within the regulated secretory pathway (Goodman et al., 1996; Mowla et al., 1999). Intact pro-BDNF is shunted from the TGN into immature secretory granules where it is likely cleaved by prohormone convertase 1 (PC1), another member of the subtilisin/kexin-like endoprotease family (Seidah et al., 1996b; Mowla et al., 1999). BDNF is stored within dense-core vesicles (Fawcett et al., 1997; Michael et al., 1997) (for review, see Altar and DiStefano, 1998), and once released, presumably in response to extracellular cues, it can induce changes in neuronal structure (Ventimiglia et al., 1995; Fawcett et al., 2000), membrane depolarization (Kafitz et al., 1999), and changes in synaptic function (for review, see McAllister et al., 1999). Likewise, neurotrophin-3 (NT-3) may regulate neuronal depolarization (Kafitz et al., 1999) and synaptic plasticity (Kang and Schuman, 1995), but as yet little is known about its intracellular sorting *in vivo*.

Neurotrophins are normally synthesized as noncovalently linked homodimers consisting of two identical chains. When different neurotrophins are coexpressed within the same cell, however, they also form heterodimers. Heterodimers of BDNF and NT-3 are stable, whereas heterodimers involving NGF are not (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Heymach and Shooter, 1995; Robinson et

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al., 1995). BDNF/NT-3 heterodimers can induce autophosphorylation of Trk receptors, promote the survival of sympathetic neurons *in vitro*, and induce dopamine uptake in cultures of substantia nigra neurons (Arakawa et al., 1994; Philo et al., 1994). However, the physiological functions of heterodimers remain unknown.

In this study, we monitored the sorting of NT-3 by infecting hippocampal neurons and AtT-20 cells with vaccinia viruses (VVs) encoding pro-NT-3. Pulse–chase studies and immunocytochemistry show that processed NT-3 is primarily released through the constitutive secretory pathway. Inhibition of furin-mediated cleavage or overexpression of pro-NT-3 shifts pro-NT-3 sorting into the regulated secretory pathway. NT-3 is also sorted to the regulated secretory pathway when it is coexpressed with BDNF. Immunoprecipitation data suggest that the transfer of NT-3 to the regulated pathway occurs as a result of NT-3 and BDNF heterodimerizing. Thus, either secretory pathway can sort NT-3.

Some of these results have been published previously in abstract form (Farhadi et al., 1998).

MATERIALS AND METHODS

Cell cultures. Hippocampal neurons were prepared according to the method of Banker and Cowan (1977) as modified by Brewer et al. (1993). Briefly, the hippocampus was dissected from embryonic day 18 (E18) mice (Charles River, Montreal, Quebec, Canada), exposed to trypsin, dissociated mechanically, and grown in 60 mm collagen/poly-L-lysine-coated dishes. Cells from two litters of mice were plated into six dishes. Cultures were maintained in serum-free Neurobasal medium (Life Technologies, Gaithersburg, MD) containing 0.5 mM glutamine and 1× B27 supplement (Life Technologies). AtT-20 cells and COS-1 cells were cultured as reported previously (Seidah et al., 1996a). AtT-20 cells are a neuroendocrine cell line that has been used extensively for studying the regulated secretory pathway (Moore et al., 1983). We also used an AtT-20 cell line stably transfected with α1-PDX cDNA that has been described previously (Benjannet et al., 1997). Special care was taken to ensure that cells were distributed in equal numbers in dishes that were to be used for group comparisons.

VV recombinants and infections. Purified recombinant VVs containing the full-length coding regions of mouse pro-NGF, human pro-BDNF, and human pro-NT-3 (generously provided by Regeneron Pharmaceuticals) were constructed as described previously (Seidah et al., 1996a,b). VVs coding for α1-PDX were kindly provided by Dr. Gary Thomas (Vollum Institute, Portland, OR). Separate plates of cells were infected as described previously (Seidah et al., 1996a) with VV encoding pro-NGF, pro-BDNF, or pro-NT-3, generally at a multiplicity of infection (MOI) of 1. We incubated the cells for 8–10 hr in virus-free medium before metabolic labeling. In some experiments, plates were coinfected with pro-BDNF and either pro-NT-3 or pro-NGF, using an MOI of 0.5 for each. Under our experimental conditions, there was no evidence of cell death after exposure to VVs for the times indicated.

Construction of expression vectors and transfections. In some experiments, we transfected cells with DNA using the lipofectamine reagent (Life Technologies). cDNAs corresponding to the full-length coding regions of human pro-BDNF and human pro-NT-3 were subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA). AtT-20 cells growing on poly-L-lysine-coated coverslips were transfected using lipofectamine with a constant amount of DNA (2 μg DNA/2 ml medium) that was composed of expression vector alone (with no insert) together with 0.1, 0.5, 1, or 2 μg of pro-BDNF or pro-NT-3 plasmid DNA. After 5 hr of incubation, the transfection medium was diluted 1:1 with DMEM/20% fetal calf serum, and after 2 d, coverslips were processed for immunostaining (see below).

Metabolic labeling and immunoprecipitation. For pulse–chase experiments, we incubated infected cells with 1.5 ml of Cys-Met-free DMEM containing 10% FCS and 0.5 mCi/ml [^{35}S] Translabel (ICN Biochemicals, Costa Mesa, CA) (70% methionine, 30% cysteine) for 30 min. Pro-BDNF contains eight methionines as compared with three in pro-NT-3, and mature BDNF contains three methionines as compared with none in mature NT-3. These differences, together with higher concentrations of methionine than cysteine in the Translabel, explain why, in Figures showing the results of metabolic labeling experiments, pro-BDNF and mature BDNF label more heavily than pro-NT-3 and NT-3. For the chase periods, cells were washed, and the medium was replaced with an equal volume of DMEM containing 10% FCS plus twofold excess concentrations of nonradioactive cysteine and methionine for the times indicated. In all experiments, conditioned media and cell lysates were brought to final volumes of 1.5 ml, 750 μl of which was subjected to immunoprecipitation. Samples immunoprecipitated with nonimmune rabbit IgG showed no bands corresponding to standards of neurotrophin precursors or products.

Immunoprecipitations were performed as described previously (Seidah et al., 1996a). For NT-3, we used an affinity-purified rabbit anti-NGF IgG that recognizes NT-3 (Murphy et al., 1993; Seidah et al., 1996a). BDNF immunoprecipitations were performed using an antibody kindly supplied by Amgen and characterized previously (Fawcett et al., 1997; Yan et al., 1997). Cell lysates and conditioned media were analyzed by electrophoresis on a 13–22% SDS-PAGE. Gels were fixed in 40% methanol and 10% acetic acid, treated with ENHANCE (Dupont NEN, Boston, MA), and washed in 10% glycerol, all for 1 hr. Dried gels were analyzed by a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA), and radioactivity in each band was quantitated using the ImageQuant program. Levels of radioactivity were within the linear range of the device. Statistical significance was determined using the Student's *t* test on a minimum of triplicate experiments.

To monitor the effects of depolarization on neurotrophin release, we infected hippocampal neurons with recombinant viruses, metabolically labeled the cells for 30 min, and washed and incubated the cells in medium containing excess nonradioactive methionine and cysteine for 4 hr. The cells were exposed to tissue culture medium supplemented with or without KCl (56 mM) and $CaCl_2$ (5.8 mM) for 15 min. Conditioned media and cell lysates were collected, immunoprecipitated, and fractionated by SDS-PAGE. Neurotrophin levels were estimated and compared by PhosphorImager analysis. In a previous control experiment (Mowla et al., 1999), we confirmed that KCl induces the release of endogenous secretogranin II in cultures of VV:NGF-infected hippocampal neurons to confirm that the regulated secretory pathway is fully functional in cells infected with VV constructs (Mowla et al., 1999).

Immunocytochemistry and confocal microscopy. We visualized VV-infected AtT-20 cells and primary cultures of hippocampal neurons as well as controls consisting of uninfected cells or cells infected with wild-type VVs. Cells were rinsed with PBS, fixed for 25 min in 4% paraformaldehyde/15% picric acid in 0.1 M phosphate buffer, pH 7.4, and incubated in PBS containing 20% horse serum for 30 min to reduce nonspecific binding. The cells were incubated with 1 μg/ml of affinity-purified anti-NT-3 (Chemicon, Temecula, CA) in PBS/0.2% Triton X-100 overnight at 4°C, washed three times with PBS/0.05% Tween-20 (5 min each), and incubated for 1 hr with CY3-conjugated goat anti-rabbit antibody (Jackson Laboratory, Bar Harbor, ME) diluted 1:2000 in PBS/0.05% Tween-20 containing 10% goat serum. Cells were washed three times in PBS and mounted in a Tris-buffered glycerol mounting medium (Sigma, St. Louis, MO). In control experiments, the anti-NT-3 antibody showed no detectable cross-reactivity by immunocytochemistry with either NGF or BDNF (data not shown).

Double-label immunocytochemistry was also performed on VV-infected AtT-20 cells to compare the distribution of NT-3 and NT-3/BDNF with that of TGN38, a marker of the TGN (Luzio et al., 1990), and ACTH, which is packaged in secretory vesicles. Antibody to TGN38 raised in guinea pig (kindly provided by Drs. G. Banting and W. Garten) was used at a 1:50 dilution and visualized using an FITC-conjugated secondary antibody raised in goat (Jackson Laboratory) diluted 1:50 in PBS containing 10% goat serum. ACTH was localized with a monoclonal antibody (Cortex Biochem) at a dilution of 1:1000, visualized with a CY2-conjugated goat anti-mouse secondary antibody (Jackson Laboratory) diluted 1:1000 in PBS containing 10% goat serum.

Cells were analyzed by confocal laser scanning microscopy using a Zeiss LSM 410 inverted confocal microscope and a 63×, 1.4 NA objective. Cells were excited at 543 nm and imaged on a photomultiplier after passage through FT 590 and LP 590 filter sets. The confocal images represent one confocal level (a depth of ~1 μm) that includes the cell nucleus along with as many cell processes as were possible to capture, the goal being to evaluate the distribution of secretory vesicles. No differences were evident in the distribution of NT-3 or NT-3/BDNF immunoreactivity when we scanned at various levels below or above the nucleus.

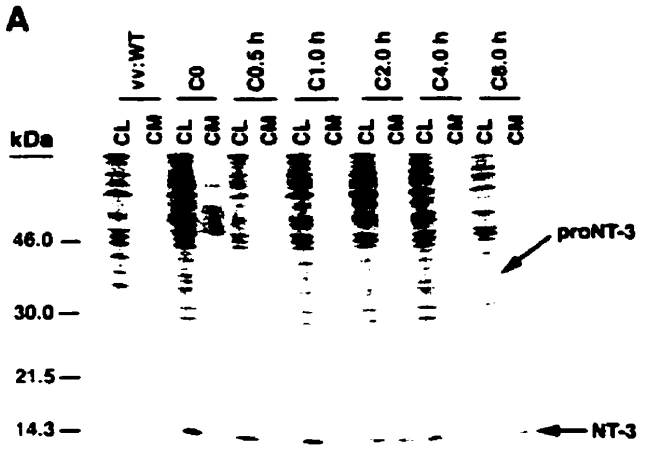

Figure 1. Pulse–chase metabolic labeling of pro-NT-3 in primary cultures of hippocampal neurons (*A*) and AtT-20 cells (*B*). Cells were infected with VV encoding the NT-3 precursor for 1 hr and postincubated in fresh medium without virus for 8 hr. Cells were then exposed to medium containing [^{35}S] Cys-Met for 30 min and chased for 0, 0.5, 1, 2, 4, and 8 hr. Identical volumes (750 μl) of cell lysates (*CL*) and conditioned media (*CM*) were incubated with antibodies to NGF, which immunoprecipitate NT-3, and electrophoresed on 13–22% SDS gradient gels.

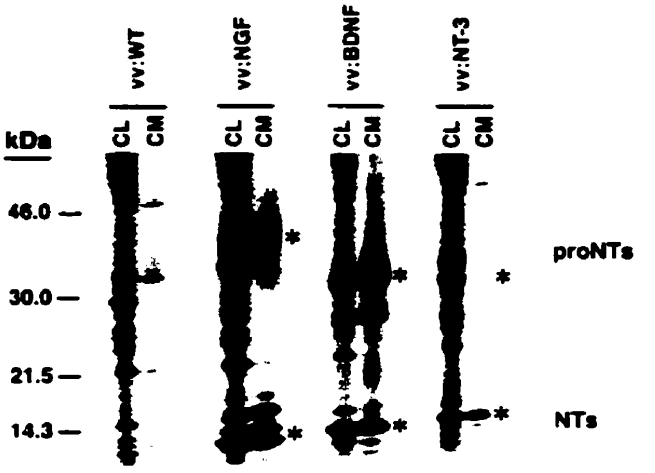

Figure 2. Differential processing of neurotrophin precursors in COS-1 cells, which contain only the constitutive secretory pathway. Cells were infected at an MOI of 1 with either wild-type VV (*vv:WT*) or VV encoding pro-NGF, pro-BDNF, or pro-NT-3. The cells were postincubated in the absence of virus for another 8 hr and metabolically labeled for 3 hr. Identical volumes of cell lysates (*CL*) and conditioned media (*CM*) from *vv:WT*-, *vv:NGF*-, and *vv:NT-3*-infected cells were immunoprecipitated with an NGF antibody that recognizes both NGF and NT-3. *vv:BDNF*-infected cells were immunoprecipitated with a BDNF-specific antibody.

RESULTS

NT-3 is released constitutively from hippocampal neurons and AtT-20 cells

To determine the pathway by which NT-3 is processed and released, we did the following: (1) measured the retention or release of processed NT-3 from virally infected cells after pulse–chase labeling; (2) determined whether agents that promote vesicle exocytosis promote the release of NT-3; and (3) used immunocytochemistry to visualize the intracellular localization of NT-3 in virally infected cells.

Figure 1 shows the results of 30 min pulse–chase studies performed over 8 hr to monitor the processing of pro-NT-3 in primary cultures of hippocampal neurons (Fig. 1*A*) and in AtT-20 cells (Fig. 1*B*). In both cell types, pro-NT-3 (33.5 kDa) is processed to mature NT-3 (14.5 kDa). The precursor is detectable in cell lysates at the start of the chase period, and levels decrease thereafter; by 8 hr of chase, the precursor is barely detectable. Intact pro-NT-3 is not detectable in conditioned medium at any time point in either cell type. Mature NT-3 is visible in cell lysates at the beginning of the chase period, but over time, levels in cell lysates decrease. Over the same time period, NT-3 levels in conditioned medium increase, and by 2 hr they exceed those in cell lysates. Therefore, most newly processed NT-3 is rapidly released from both hippocampal neurons and AtT-20 cells.

We were surprised that levels of pro-NT-3 were so low in these cells and that processed NT-3 was evident immediately after the 30 min pulse period. In our previous studies, levels of pro-NGF and pro-BDNF were much higher at the beginning of the chase period, attributable in part to increased labeling efficiency (see Materials and Methods). Also, significant processing of pro-NGF and pro-BDNF was not evident until 30 min after the chase began. To test whether these differences were caused by pro-NT-3 being processed more efficiently in the constitutive pathway than either pro-NGF or pro-BDNF, we compared precursor processing in virally infected COS-1 cells, a cell line that contains only the constitutive secretory pathway. Results show that abundant amounts of processed NGF and BDNF are detectable in cell lysates and conditioned medium at the end of the test period (Fig. 2). As well, significant levels of unprocessed pro-NGF and pro-BDNF are detectable in cell lysates and in conditioned medium, which suggests that some precursor escapes proteolysis, perhaps by overwhelming the processing capacity of the cell (Mowla et al., 1999). In contrast, pro-NT-3 is detectable in cell lysates but not in conditioned medium, whereas processed NT-3 is evident in both. One explanation for these data is that pro-NT-3 is more efficiently processed within the constitutive secretory pathway than either pro-NGF or pro-BDNF.

We used pulse–chase labeling methods similar to those used in Figure 1 to determine whether the endoprotease furin, which is present in the constitutive pathway, is involved in pro-NT-3 processing in AtT-20 cells. For this experiment, we used AtT-20

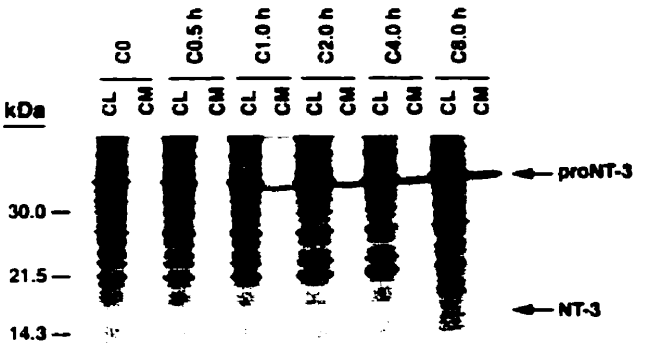

Figure 3. Inhibition of pro-NT-3 processing in AtT-20 cells expressing α1-PDX. AtT-20 cells stably expressing the furin inhibitor α1-PDX were infected for 30 min with VV encoding pro-NT-3. Cells were then incubated in virus-free medium for 8 hr, metabolically labeled for 30 min, and chased for up to 8 hr. Cell lysates (*CL*) and conditioned media (*CM*) were immunoprecipitated and analyzed by SDS-PAGE.

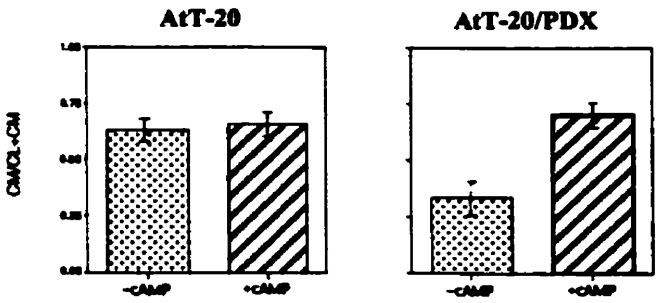

Figure 4. cAMP-induced release of NT-3 from AtT-20 cells expressing α1-PDX. Cells were infected with VV:pro-NT-3 for 1 hr, incubated in virus-free medium for 8 hr, metabolically labeled for 3 hr, chased for 3 hr, and treated for 3 hr with medium with or without 5 mM cAMP. CL and CM were immunoprecipitated and the amount of processed, mature NT-3 was analyzed by SDS-PAGE. Analysis was performed on a PhosphorImager, and values represent an average (±SEM) of three independent experiments.

Figure 5. Confocal microscopy of AtT-20 (*A*) and AtT-20/α1-PDX cells (*B*) infected with VV encoding pro-NT-3. Cells were infected for 1 hr and postincubated in the absence of virus for another 8 hr. The cultures were fixed and treated with antibodies against NT-3 (Chemicon), followed by CY3-conjugated goat anti-rabbit IgG. Scale bar, 10 μm. Photomicrographs were obtained by overlaying confocal fluorescence images over transmitted light images. Scale bar, 10 μm.

cells that have been stably transfected with α1-PDX, an α1-antitrypsin structural variant that selectively inhibits furin-mediated cleavage of precursor proteins in the TGN (Anderson et al., 1993; Watanabe et al., 1995; Vollenweider et al., 1996; Benjannet et al., 1997). In cells expressing α1-PDX (Fig. 3), significant levels of pro-NT-3 are released into conditioned medium over the 8 hr chase period, but levels of processed NT-3 are markedly reduced (compare Fig. 3 with Fig. 1*B*). Therefore, inhibition of furin-mediated cleavage prevents the processing of pro-NT3 and the efficient generation of mature product.

The finding that α1-PDX prevents the processing of pro-NT-3 and induces its release into conditioned medium is identical to that obtained previously with pro-NGF (Mowla et al., 1999). In that study, we determined that inhibiting furin cleavage with α1-PDX caused the shunting of pro-NGF from the constitutive to the regulated secretory pathway (Mowla et al., 1999). We also observed that some pro-BDNF is constitutively released in the course of its processing within the regulated secretory pathway (Mowla et al., 1999). Together, these results suggest that inhibiting furin-mediated cleavage of pro-NT-3 may also direct the precursor from the constitutive to the regulated secretory pathway.

To test this idea, we monitored the effects of cAMP on NT-3 release from AtT-20 cells in the presence or absence of α1-PDX. We also used immunocytochemistry to analyze the distribution of NT-3 under both experimental conditions. Results show that cAMP stimulates the release of NT-3 from AtT-20 cells in the presence (Fig. 4, *right panel*) but not in the absence (*left panel*) of α1-PDX. This result is consistent with immunocytochemical data (Fig. 5) showing that in the absence of α1-PDX (Fig. 5*A*), NT-3 immunoreactivity is distributed in the perinuclear cytoplasm and not in cell processes, an appearance identical to that previously reported for NGF (Mowla et al., 1999). However, in the presence of α1-PDX (Fig. 5*B*), punctate NT-3 immunoreactivity is evident throughout the cytoplasm and in the tips of cell processes, which is consistent with earlier results monitoring the distribution of BDNF in wild-type cells and NGF in α1-PDX-treated cells (Mowla et al., 1999). Thus NT-3, which is normally processed and released from the constitutive pathway, can be rerouted to the regulated secretory pathway when furin-mediated cleavage within the TGN is inhibited.

In earlier studies, Heymach et al. (1996) and Moller et al. (1998) showed that NT-3 is packaged in dense-core vesicles of AtT-20 cells and PC12 cells and released in response to secretagogues or cell depolarization. To explain why their results differ from ours, we investigated whether elevating levels of pro-NT-3 expression could redirect NT-3 from the constitutive to the regulated secretory pathway, as has been shown previously for pro-NGF (Mowla et al., 1999). Initially, we determined that infecting AtT-20 cells with 5, 10, and 25 MOIs of VV encoding pro-NT-3 resulted in only a small amount of pro-NT-3 being redirected into the regulated secretory pathway, as determined by the ability of cAMP to induce NT-3 release (data not shown). Similar infection levels were extremely effective in rerouting NGF from the constitutive to regulated secretory pathway (Mowla et al., 1999). We suspect that the differences are attributable to pro-NT-3 being more efficiently processed than pro-NGF within the constitutive secretory pathway, as shown in Figure 2. To achieve higher intracellular concentrations of pro-NT-3, we opted for a lipofectamine transfection method similar to that used by Heymach et al. (1996) and Moller et al. (1998).

Lipofectamine transfection of pro-NT-3 DNA (0.1 μg with 1.9

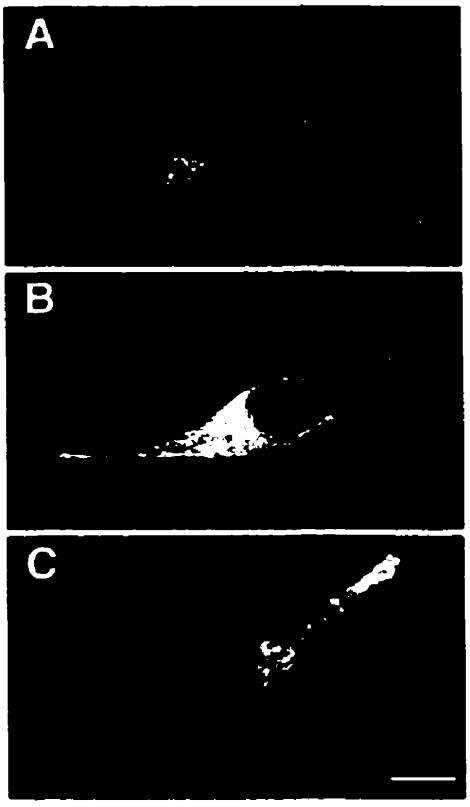

Figure 6. Overexpression of NT-3 results in rerouting from the constitutive to the regulated secretory pathway. AtT-20 cells were transfected with a construct encoding either pro-NT-3 (*A*, *B*) or pro-BDNF (*C*), and immunocytochemistry using NT-3 (*A*, *B*) and BDNF (*C*) antibodies was performed as described in Figure 5. In *A*, cells were lipofectamine-transfected with 0.1 μg of pro-NT-3 DNA (and 1.9 μg vector DNA). In *B*, cells were transfected with 2 μg pro-NT-3 DNA. In *C*, cells were transfected with 0.1 μg of pro-BDNF (and 1.9 μg vector DNA). Scale bar, 10 μm.

μg vector DNA/2 ml of culture medium) resulted in NT-3 immunoreactivity in AtT-20 cells that is diffusely distributed in the perinuclear cytoplasm (Fig. 6*A*), as seen earlier (compare with Fig. 5*A*). However, when we transfected 0.5 or 1 μg of pro-NT-3 DNA (with vector DNA to a total of 2 μg/2 ml of culture medium), NT-3 immunoreactivity is evident in punctate structures that extend into cell processes, which is consistent with the appearance of secretory proteins processed in the regulated secretory pathway (Fig. 6*B*). For comparison, we show in Figure 6*C* the punctate localization of BDNF immunoreactivity in cells exposed to only 0.1 μg of pro-BDNF with 1.9 μg of vector DNA/2 ml of culture medium. Pro-BDNF is processed within the regulated secretory pathway (Mowla et al., 1999). Thus, cells transfected with high concentrations of pro-NT-3 DNA process the protein in the regulated secretory pathway, which likely explains why our results with VV infection methods differ from those of Heymach et al. (1996) and Moller et al. (1998) using lipofectamine transfection.

NT-3 is sorted to the regulated secretory pathway when coexpressed with pro-BDNF

Because NT-3 is processed in the constitutive pathway and BDNF is processed in the regulated pathway (Mowla et al., 1999), we questioned what would happen if we coexpressed precursors to the two proteins within the same cell. Others have shown that neurotrophins readily heterodimerize (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Philo et al., 1994; Heymach and Shooter, 1995; Robinson et al., 1995; Treanor et al., 1995), but nothing is known about the mechanisms regulating the processing, sorting, and release of heterodimers within cells.

For these studies, we first analyzed the specificity of our antibodies, because previous studies have shown that antibodies to one neurotrophin can cross-react on Western blots with the other (Murphy et al., 1993). In these studies we infected AtT-20 cells for 1 hr with 1 MOI of VV coding for either pro-NT-3 or pro-BDNF, incubated the cells for 8 hr without virus, metabolically labeled the cells for 3 hr, and immunopreciptated cell lysates and conditioned media with antibodies to NGF (for NT-3) or BDNF. In some experiments, we coinfected cells with 0.5 MOI of VV coding for pro-NT-3 and pro-BDNF to ensure that the total level of viral infection (1 MOI) was held constant.

Figure 7 (*left side*) shows that antibody to NGF immunoprecipitates NT-3 but not BDNF or pro-BDNF. Similarly, antibody to BDNF (Fig. 7, *right side*) immunoprecipitates BDNF and pro-BDNF but not NT-3. In contrast, in cells coinfected with VV coding for pro-NT-3 and pro-BDNF, antibody to NGF immunoprecipitates in conditioned medium NT-3 as well as a protein migrating in a position identical to that of pro-BDNF (Fig. 7, *left side*). We interpret this result to mean that NT-3 and pro-NT3 are associating with the BDNF precursor. In cell lysates as well as conditioned medium, the NGF antibody immunoprecipitates a doublet consisting of a higher molecular weight band (probably arising from precipitation of NT-3 alone and with BDNF) as well as a lower molecular weight band (BDNF) that precipitates because of association with NT-3. The *right side* of Figure 7 shows that antibodies to BDNF precipitate in cell lysates, and to a lesser extent in conditioned medium, a doublet consisting of a lower band (probably arising from precipitation of the BDNF alone or with NT-3) as well as an upper band (NT-3). Results identical to those shown in Figure 7 were obtained when the same experiments were performed in cultures of hippocampal neurons (data not shown).

In short, NT-3-specific antibodies precipitate BDNF, and BDNF-specific antibodies precipitate NT-3 only when the two neurotrophins are coexpressed within cells. We strongly suspect that these two independent but mutually consistent results arise from NT-3 and BDNF forming heterodimers intracellularly. Immunoprecipitation methods similar to ours were used previously by Jungbluth et al. (1994) and Heymach and Shooter (1995) to characterize neurotrophin heterodimers, including NT-3/BDNF. It should be noted that coinfection with wild-type and BDNF-encoding VV and coinfection with VV:pro-NGF and VV:pro-BDNF did not result in coprecipitated neurotrophins (data not shown), probably because of the relative instability of NGF/BDNF heterodimers, as reported previously (Radziejewski and Robinson, 1993; Arakawa et al., 1994).

Evidence in support of the idea that coexpression of NT-3 with BDNF results in some NT-3 being shunted from the constitutive to the regulated secretory pathway is shown in Figure 8. The bar graph (Fig. 8*A*) shows the amount of processed NT-3 in cell lysates as a function of the total amount of NT-3 present in cell lysates and conditioned media. Results indicate that more NT-3 is retained within cells when pro-NT-3 is coexpressed with pro-BDNF-encoding virus as compared with wild type. This result suggests that NT-3 and BDNF are noncovalently associated

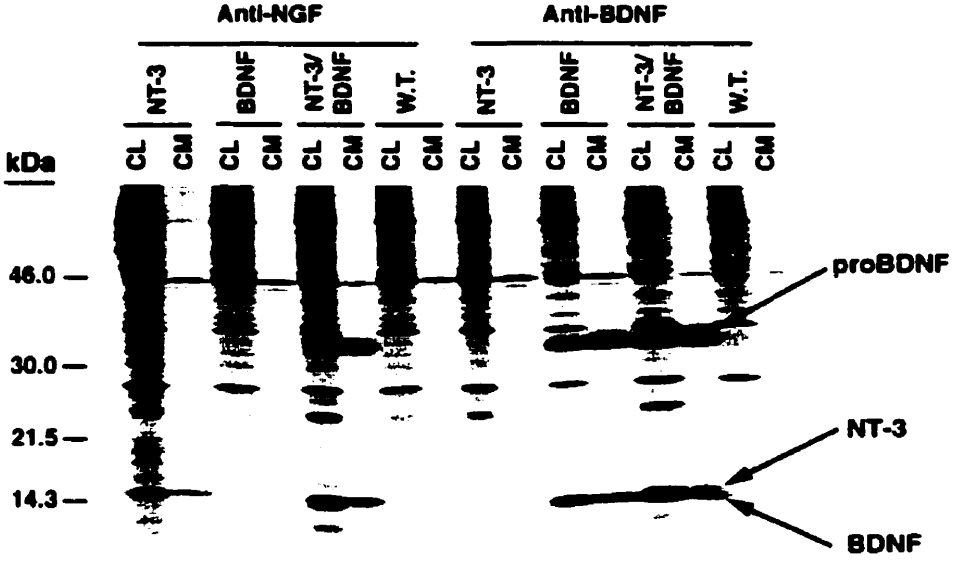

Figure 7. Immunoprecipitation of cell extracts and conditioned medium from cells infected with 1 MOI of wild type (*W.T.*) VV, VV:pro-NT-3, VV:pro-NGF, VV:pro-BDNF, alone, or coinfected with 0.5 MOI of pro-NT-3 and pro-BDNF. AtT-20 cells were infected for 1 hr with the viruses indicated, postincubated for 8 hr, and metabolically labeled for 3 hr. Cell lysates and conditioned media were immunoprecipitated with either an anti-NGF antibody (*left side*) or a BDNF antibody (*right side*).

Figure 8. NT-3/BDNF is retained in hippocampal neurons. *A*, The methodology in Figure 1*A* (involving a 4 hr chase only) was repeated three times with cells infected with either VV:pro-NT-3/VV:WT or VV:pro-NT-3/VV:pro-BDNF. The NGF antibody was used for immunoprecipitations. Results were analyzed on a PhosphorImager and are an average (±SEM) of the ratio of mature NT-3 in cell lysates (*CL*) over the total amount of NT-3 in CL + conditioned medium (*CM*). *B*, A representative SDS gel from the experiments in *A* showing the NT-3/BDNF heterodimer in the cell lysate and conditioned medium.

through heterodimerization and that association of the two leads to intracellular retention of NT-3, perhaps within dense-core vesicles. Examination of a typical SDS gel used in this analysis (Fig. 8*B*) shows that NT-3 and BDNF, which are coprecipitated by the NGF (NT-3-reactive) antibody, are evident within the cell lysate and conditioned medium, suggesting that coinfected cells synthesize and release the NT-3 and BDNF together. In companion experiments, coinfection with VV:pro-BDNF and VV:pro-NGF had no effect on NGF retention as compared with the wild-type coinfection control (data not shown).

The idea that NT-3 is diverted into the regulated secretory pathway is further confirmed by secretion data presented in Figure 9. When pro-NT-3 is coexpressed with pro-BDNF, processed NT-3 can be released from AtT-20 cells (Fig. 9*A*) in response to cAMP and from hippocampal neurons by KCl depolarization (Fig. 9*B*). Thus, release of NT-3 that is coexpressed (and presumably dimerized) with BDNF appears to be regulated by the same extracellular signals that regulate the release of homodimeric BDNF (Mowla et al., 1999). Cell lysates and conditioned media immunoprecipitated with NGF antibodies contained both NT-3 and BDNF, further indicating that the two neurotrophins are synthesized and released together in our culture system.

Finally, we used immunocytochemistry and confocal microscopy to compare the intracellular distributions of NT-3 and BDNF when they are singly expressed or coexpressed in hippocampal neurons. Figure 10 shows that in hippocampal neurons infected with 1 MOI of VV:pro-NT-3, NT-3 immunoreactivity is distributed in the perinuclear cytoplasm (Fig. 10*A*), as was seen in AtT-20 cells (Fig. 5). However, in cells coinfected with 0.5 MOI of VV encoding pro-NT-3 and pro-BDNF, NT-3 immunoreactivity (Fig. 10*C*) is localized in punctate structures distributed throughout the cell cytoplasm and in the tips of cell processes, an appearance similar to that of BDNF immunoreactivity in cells infected with pro-BDNF alone (Fig. 10*B*). In control studies, coexpression of pro-NT-3 and pro-NGF did not change the distribution of NT-3, as assessed by immunocytochemistry (data not shown). Furthermore, coexpression of pro-BDNF and pro-NGF did not divert NGF from the constitutive to the regulated pathway as assessed by either immunoprecipitation or immunocytochemistry (data not shown).

Figure 11 compares the distribution in virally infected AtT-20 cells of NT-3 alone and NT-3 coexpressed with BDNF with that of endogenous TGN38, a Golgi marker, and endogenous ACTH, which is packaged within secretory granules of AtT-20 cells. In cells infected with VV:pro-NT-3 alone, immunoreactivity for NT-3 (Fig. 11*A*) and TGN38 (Fig. 11*B*) colocalize in the perinuclear cytoplasm (Fig. 11*C*). In contrast, in cells coinfected with VV encoding pro-NT-3 and pro-BDNF, NT-3 immunoreactivity (Fig. 11*D*) is localized in the perinuclear cytoplasm as well as in punctate structures within the cytoplasm and tips of cell processes. In the same cells, ACTH immunoreactivity is distributed in punctate structures within cell processes (Fig. 11*E*). In a subpopulation of vesicles, NT-3 and ACTH immunoreactivity colocalize (Fig. 11*F*).

DISCUSSION

Data in this study show that NT-3 is processed and released from the constitutive secretory pathway of hippocampal neurons and

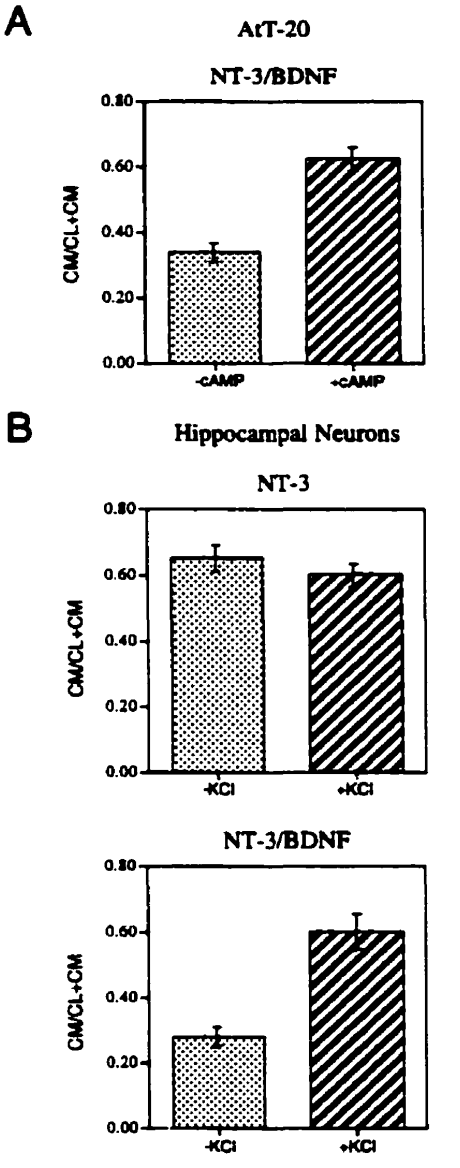

Figure 9. Secretagogue-induced release of NT-3/BDNF but not NT-3. *A*, AtT-20 cells coinfected with VV encoding pro-NT-3 and VV encoding pro-BDNF were processed using the methodology described in Figure 4. *B*, Hippocampal neurons from E18 mice were cultured for 7 d and infected for 1 hr with either (1) VV encoding pro-NT-3 or (2) VV encoding pro-NT-3 and VV encoding pro-BDNF. After 8 hr in medium without virus, the cells were labeled for 30 min with [^{35}S] Cys-Met, incubated in medium without radiolabel for 4 hr, and treated with medium with or without KCl and $CaCl_2$ for 15 min. Cell lysates and conditioned media were immunoprecipitated with the antibody to NGF and electrophoresed on an SDS gel. Results were analyzed on a PhosphorImager and are an average (±SEM) of three independent experiments.

AtT-20 cells. Pulse–chase experiments reveal that the NT-3 precursor is rapidly cleaved in cells infected with VV encoding the NT-3 precursor (Fig. 1), with processed NT-3 being evident 30 min after cells are exposed to medium containing radiolabeled amino acids. Over the next 8 hr, most processed NT-3 is released into conditioned medium, a result similar to that reported previ-

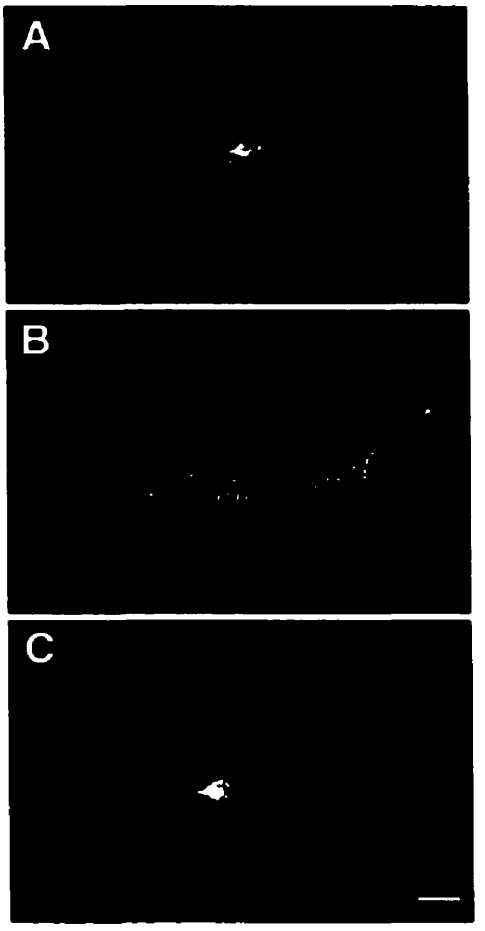

Figure 10. Confocal microscopy of hippocampal neurons infected with pro-NTs. Hippocampal neurons were infected with 1 MOI VV encoding either pro-NT-3 (*A*) or pro-BDNF (*B*); in *C*, the cells were coinfected with 0.5 MOI each of VV:pro-NT-3 and VV:pro-BDNF. Immunocytochemistry was performed with the NT-3 antibody in *A* and *C* and the BDNF antibody in *B*. Scale bar, 10 μm.

ously for NGF (Mowla et al., 1999). Immunocytochemical analysis shows that NT-3 is diffusely distributed within the perinuclear cytoplasm (Figs. 5, 10, 11), and colocalizes with TGN38, a marker of the *trans*-Golgi network (Figs. 11*A–C*). Secretagogues (Figs. 4, 9*A*) and KCl-induced depolarization (Fig. 9*B*) do not induce the release of radiolabeled NT-3 from virus-infected AtT-20 cells or hippocampal neurons, respectively. Thus, NT-3 release is constant and not dependent on extracellular cues, which is similar to the results we obtained for NGF (Mowla et al., 1999).

Pro-NGF, pro-BDNF, and pro-NT-3 were cleaved in COS-1 cells, which is a constitutively secreting cell line that does not have a regulated pathway. Most pro-BDNF and some pro-NGF (but not pro-NT-3) were released into conditioned medium (Fig. 2). This result could be attributable to pro-BDNF and to a lesser degree pro-NGF not being cleaved as effectively as pro-NT-3 by furin or furin-like enzymes within the TGN. The consensus cleavage site of pro-NT-3 (Arg-Arg-Lys-Arg ↓ Tyr) is ideally suited for furin-mediated processing, probably even more so than that of pro-NGF (Arg-Ser-Lys-Arg ↓ Ser) (Decroly et al., 1994). Basic residues at positions -4, -2, -1 are conducive to processing in the constitutive secretory pathway (Watanabe et al., 1992). How-

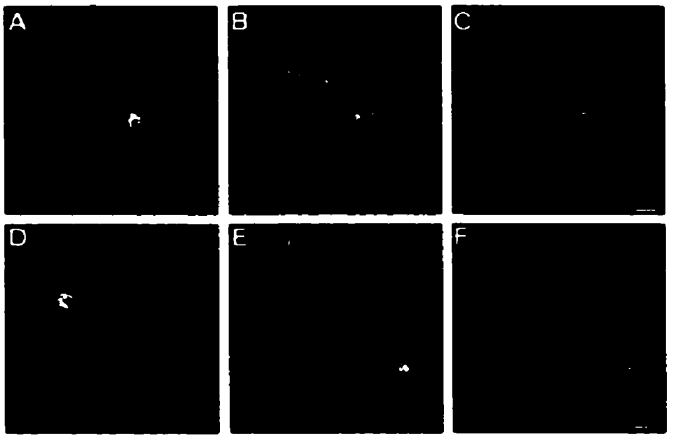

Figure 11. Double-label immunocytochemistry comparing the distribution in infected AtT-20 cells of NT-3 and NT-3/BDNF with that of endogenous TGN38 and ACTH. NT-3 immunoreactivity (*A*) colocalizes with TGN38 (*B*) in the perinuclear region as seen in *C* (NT-3 in *red* and TGN38 in *green*). NT-3 immunoreactivity in cells coinfected with pro-NT-3 and pro-BDNF (*D*) colocalizes with ACTH (*E*) primarily in the tip of the cellular process as seen in *F* (NT-3 in *red* and ACTH in *green*). Scale bar, 10 μm.

ever, the presence of an additional Arg residue at position -3 in pro-NT-3 may enhance even more the ability of furin to cleave the protein at this site.

Although it has yet to be shown directly with neurotrophin precursors, several lines of evidence suggest that sensitivity to furin-mediated cleavage within the TGN is an important factor in determining whether a protein is sorted to the constitutive or regulated secretory pathway (Brechler et al., 1996). In this study, blocking furin activity with α1-PDX inhibited pro-NT-3 processing in the constitutive pathway and resulted in the constitutive release of unprocessed pro-NT-3 (Fig. 3). In addition, α1-PDX treatment caused a shift in the appearance of NT-3 immunoreactivity from diffuse and perinuclear to punctate and distributed throughout the cell cytoplasm, including in the tips of cell processes (Fig. 5). Also, processed NT-3 was released by cAMP treatment (Fig. 4), which is characteristic of proteins within the regulated secretory pathway. Insertion of furin-sensitive cleavage sites into precursors that are normally processed in the regulated secretory pathway favors release through the constitutive secretory pathway (Oda et al., 1991; Yanagita et al., 1992), which is further evidence of the importance of furin sensitivity in the sorting decision (Jung and Scheller, 1991; Brechler et al., 1996; Mowla et al., 1999).

Increasing the MOI of vaccinia virus also shifted pro-NGF from the constitutive to the regulated secretory pathway (Edwards et al., 1988; Mowla et al., 1999), probably by saturating the ability of furin to cleave pro-NGF as a substrate. However, increasing MOI had little effect on the subcellular localization of NT-3 (data not shown), probably because pro-NT-3 is especially well cleaved in the TGN by furin. In contrast, lipofectamine transfection methods (Fig. 6) resulted in NT-3 immunoreactivity becoming localized within punctate structures throughout the cytoplasm and in the tips of cell processes. Lipofectamine transfection may yield higher intracellular concentrations of neurotrophin precursors in the comparatively small number of cells that are transformed (<10%) when compared with vaccinia virus infection (>90% cells infected). These differences in intracellular levels of the protein can be inferred by comparing NT-3 immunoreactivity in lipofectamine-transfected cells (Fig. 6*B*) with that of vaccinia virus-infected cells (Fig. 11*A*). The differences probably explain why Heymach et al. (1996) and Moller et al. (1998) detected NT-3 within the regulated secretory pathway of AtT-20 and PC12 cells. Similarly, increasing expression levels altered the intracellular distribution of β2-microglobulin in pancreatic β cells of transgenic mice (Allison et al., 1991).

Exogenously applied NT-3 is as effective as BDNF in potentiating synaptic efficacy in hippocampal CA1 neurons, which suggests that NT-3, like BDNF, may normally access the synapse through its activity-dependent release from presynaptic neurons (Kang and Schuman, 1995). However, removing endogenous NT-3 has no effect on long-term potentiation (LTP) in mouse hippocampal CA1 neurons (Chen et al., 1999; Ma et al., 1999). In contrast, blocking endogenous BDNF reduces LTP (Korte et al., 1995). Thus, exogenously applied NT-3 may mimic the effects of BDNF through mechanisms that are unrelated to the way NT-3 normally accesses the synapse (Chen et al., 1999).

Neurotrophin heterodimers have not yet been detected *in vivo*, even with antibodies that recognize them specifically (Kolbeck et al., 1999). However, neurotrophins can form stable heterodimers either when renatured together or coexpressed in cells (Radziejewski and Robinson, 1993; Arakawa et al., 1994; Jungbluth et al., 1994; Philo et al., 1994; Heymach and Shooter, 1995; Robinson et al., 1995; Treanor et al., 1995). Thus, cells that coexpress neurotrophins such as hippocampal neurons (Maisonpierre et al., 1990; Schecterson and Bothwell, 1992) could produce heterodimeric forms of these proteins. The NT-3/BDNF heterodimer is especially stable (Arakawa et al., 1994), which has allowed its crystal structure to be resolved (Robinson et al., 1995).

In our experiments, coexpressing pro-NT-3 and pro-BDNF resulted in the retention of NT-3 within vesicle-like structures that were distributed throughout the cytoplasm and within cellular processes of both AtT-20 cells and hippocampal neurons, a distribution that was identical to that of BDNF (Mowla et al., 1999). NT-3 was also released together with BDNF in response to secretagogues or depolarization. These data together with our coimmunoprecipitation studies strongly suggest that in our experimental system, NT-3 and BDNF heterodimerize, as shown previously by others (Philo et al., 1994; Heymach and Shooter, 1995). However, confirming that idea will require isolating the heterodimers to purity and characterizing them chemically, which we have not done.

As yet, we do not know how NT-3/BDNF heterodimers form. Protein dimerization normally occurs between monomers of unprocessed precursors in the endoplasmic reticulum (Danielsen, 1990; Zhu et al., 1996), which would yield pro-NT-3/pro-BDNF heterodimers. However, heterodimers could also exist between NT-3, which is efficiently processed in the TGN, and pro-BDNF, which is processed in immature secretory granules. Indeed, in our coimmunoprecipitation experiments, we detect within conditioned medium pro-BDNF along with mature NT-3 and BDNF. Further processing of the NT-3/pro-BDNF heterodimer likely occurs in immature secretory granules to yield heterodimers of mature forms of NT-3 and BDNF.

Others have noted previously that heterodimerization can alter the intracellular trafficking of proteins. For example, the common α-subunit of lutropin, follitropin, and chorionic gonadotropin is constitutively secreted when produced alone; however, heterodimerization with the appropriate β-subunit causes the heterodimer to be sorted and released by the regulated secretory

pathway (Blomquist and Baenziger, 1992; Bielinska et al., 1994). Apparently, a single chain of a protein destined for the regulated secretory pathway contains sufficient information to reroute the entire heterodimeric complex.

The finding that pro-NT-3 can be released from either the constitutive or regulated secretory pathways suggests that NT-3 could have multiple functions. The release of homodimeric NT-3 from the constitutive secretory pathway would allow NT-3 to be constantly available as a differentiation and survival-promoting factor for neurons, which appears to be its role during development (for review, see Chalazonitis, 1996). In contrast, when NT-3 heterodimerizes with BDNF, as may occur when both are highly expressed in adult hippocampus (Maisonpierre et al., 1990), NT-3 is sorted to the regulated secretory pathway, where the NT-3/BDNF heterodimer is packaged within vesicles that are released in response to activity. Under these conditions, NT-3 acting at TrkC receptors or NT-3/BDNF heterodimers acting in concert at TrkC and TrkB receptors (Philo et al., 1994) could regulate synaptic transmission and plasticity. Thus, understanding how NT-3 is processed in specific populations of neurons at different times of development could reveal much about its physiological functions.

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