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Sorting and Activity-Dependent Secretion of BDNF Require Interaction of a Specific Motif with the Sorting Receptor Carboxypeptidase E

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Summary

Activity-dependent secretion of BDNF is important in mediating synaptic plasticity, but how it is achieved is unclear. Here we uncover a sorting motif receptor-mediated mechanism for regulated secretion of BDNF. X-ray crystal structure analysis revealed a putative sorting motif, $I_{16}E_{18}I_{105}D_{106}$, in BDNF, which when mutated at the acidic residues resulted in missorting of proBDNF to the constitutive pathway in AtT-20 cells. A V20E mutation to complete a similar motif in NGF redirected a significant proportion of it from the constitutive to the regulated pathway. Modeling and binding studies showed interaction of the acidic residues in the BDNF motif with two basic residues in the sorting receptor, carboxypeptidase E (CPE). ^{35}S labeling experiments demonstrated that activity-dependent secretion of BDNF from cortical neurons was obliterated in CPE knockout mice. Thus, we have identified a mechanism whereby a specific motif $I_{16}E_{18}I_{105}D_{106}$ interacts with CPE to sort proBDNF into regulated pathway vesicles for activity-dependent secretion.

Introduction

Neurotrophins, a family of secretory neurotrophic factors that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 (NT-3, NT-4/5), are well known for their ability to promote neuronal survival and differentiation during development and to maintain the viability of neurons in adulthood (Huang and Reichardt, 2001; Levi-Montalcini, 1987; Lewin and Barde, 1996). An emerging concept, based on numerous studies in recent years, is that neurotrophins, particularly BDNF, could elicit rapid and acute regulation of synaptic transmission and plasticity in the central nervous system (Lu, 2003; Poo, 2001; Thoenen, 2000). BDNF also plays a long-term regulatory role in the development and function of synapses (Korte et al., 1998; Lu, 2004; Mizuno et al., 2000).

A remarkable feature is that, despite its diffusible nature, BDNF is able to confine its regulation to a specific synapse without affecting the neighboring synapses (Lu, 2003). An important mechanism to ensure synapse-specific regulation by BDNF is local and activity-dependent secretion of the protein. Indeed, activity-dependent secretion of BDNF has been observed in a number of neuronal populations (Balkowiec and Katz, 2000; Lever et al., 2001; Luo et al., 2001; Michael et al., 1997). Although the neurotrophins share homologous sequences and are primarily transported into the constitutive pathway in hippocampal neurons, Murphy and others showed that BDNF is sorted into the regulated pathway (Farhadi et al., 2000; Goodman et al., 1996; Mowla et al., 1999). The physiological significance of activity-dependent secretion of BDNF has been highlighted by a number of recent studies showing that a single nucleotide change in the pro region of BDNF (val66met) results in marked impairments in dendritic trafficking and regulated secretion of BDNF (Chen et al., 2004; Egan et al., 2003). Human subjects carrying the val66met polymorphism in the BDNF gene exhibit impairments in hippocampal function and hippocampal-specific short-term memory (Egan et al., 2003). Failure to sort BDNF to the regulated secretory pathway may result in unregulated secretion of this neurotrophin, leading to impairments in memory function. Although the importance of activity-dependent secretion of BDNF is well appreciated, its underlying molecular mechanisms remain unknown.

All neurotrophins are synthesized as precursors that are proteolytically processed, either intracellularly by furin or prohormone convertases (Edwards et al., 1988; Seidah et al., 1996) or extracellularly by plasmin or metalloproteases MPP3 or MPP7 (Lee et al., 2001; Smith et al., 1995), at highly conserved dibasic amino acid cleavage sites to yield mature neurotrophins. Precursors such as proNGF, proNT3, and proNT4 could be cleaved within the trans-Golgi network (TGN) by the endoprotease furin, packaged primarily into constitutive vesicles, and then secreted continuously into the extracellular environment (Gu et al., 2001; Hibbert et al., 2003; Mowla et al., 2001). In contrast, the precursor of BDNF is predominantly sorted away from other proteins at the TGN, packaged into regulated secretory pathway vesicles, processed, and then secreted in an activity-dependent manner (Goodman et al., 1996; Haubensak et al., 1998; Mowla et al., 1999).

Although the mechanism by which BDNF is sorted to the regulated secretory pathway for secretion is unknown, over the last few years, studies on prohormone/proneuropeptide trafficking have provided evidence that the sorting of these molecules to the regulated secretory pathway involves a receptor-mediated mechanism (Cool et al., 1997; Loh et al., 2004; Zhang et al., 1999), similar to the sorting of lysosomal enzymes to lysosomes (Bicknell et al., 2001). A sorting signal motif necessary and sufficient for targeting the prohormone to the regulated secretory pathway has been identified for pro-opiomelanocortin (POMC) (Cool et al., 1995). This motif consists of an amphipathic loop (residues 8–20) with two acidic residues (Asp_{10} and Glu_{14}) and two aliphatic hydrophobic residues (Leu_{11} and Leu_{18}) exposed on the surface. A similar sorting signal motif was shown

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to be critical for sorting proinsulin into the regulated secretory pathway in a pancreatic islet cell line, INS-1 cells (Dhanvantari et al., 2003). These sorting motifs interact with membrane carboxypeptidase E (CPE), which acts as a sorting or retention receptor to target these prohormones to the regulated secretory pathway (Dhanvantari et al., 2003; Normant and Loh, 1998; Zhang et al., 1999). In this study, we hypothesize that proBDNF is sorted by a similar mechanism.

To identify a sorting motif in BDNF, we carried out an analysis of the X-ray crystal structure of BDNF using the insulin sorting motif as a template. We found a predicted sorting motif in BDNF and tested its role in directing BDNF to the regulated secretory pathway. We present evidence from pulse-chase secretion studies and immunocytochemistry in AtT-20 cells, pituitary cells, and hippocampal and cortical neurons from wild-type and CPE knockout (KO) mice, showing that proBDNF is sorted by a sorting signal-CPE mediated mechanism to vesicles for activity-dependent secretion.

Results

X-Ray Crystal Structure of BDNF Reveals a Putative Sorting Motif

High-resolution X-ray crystallographic structures of BDNF, NGF, and insulin were superimposed to determine whether the BDNF molecule contains a sorting motif for the regulated secretory pathway, similar to that of insulin. All acidic and aliphatic hydrophobic residues in BDNF and NGF with C_α atoms lower than 2.0 Å root mean squared deviation (RMSD) value relative to insulin were selected. Based on the insulin and POMC sorting motifs, we searched for a three-dimensional structure with two acidic residues 9–15 Å apart and an aliphatic hydrophobic residue in close proximity (5–7 Å) to each of the acidic residues (Dhanvantari et al., 2003; Zhang et al., 1999). A putative sorting motif with these parameters was identified in the BDNF. The motif consisted of residues I_{16}, E_{18}, I_{105}, and D_{106} with their side chains exposed on the surface of BDNF (Figure 1A). From the X-ray structure of BDNF, the distance between the C_α atoms of the two acidic residues E_{18} and D_{106} was 8.99 Å, and the distances between the C_α atoms of acidic and neighboring hydrophobic residues I_{16} and E_{18} and I_{105} and D_{106} were 6.34Å and 5.50Å, respectively. These distances are similar to that found in insulin (Figure 1B). The RMSD value of the four C_α atoms in the putative sorting motif between insulin and BDNF was 1.73 Å, indicating a high degree of configurational similarity.

NGF has a very similar crystal structure to BDNF (Figure 1A). A partial sorting motif was found in NGF, in which the residue at position 20, equivalent to E_{18} in BDNF, is an uncharged residue: valine. This difference may contribute to the fact that NGF is not efficiently sorted to the regulated secretory pathway.

To determine if the putative sorting signal motif identified in BDNF could interact with the sorting receptor, CPE, molecular modeling was carried out. The BDNF sorting motif was manually docked to the CPE sorting signal binding domain ($CPE_{254–273}$). As shown in Figure 1C, the molecular distances were appropriate for interaction of the side chains of the two acidic residues, Glu_{18} and Asp_{106} (E_{18} and D_{106}), of BDNF with the side chains of the basic residues, Arg_{255} and Lys_{260} (R_{255} and K_{260}), in the sorting signal binding domain of CPE. Thus, from the molecular modeling studies, it is predicted that the acidic residues of the BDNF sorting motif would be able to interact with CPE to effect sorting to the regulated secretory pathway. The following studies tested this hypothesis.

Mutation of the Putative Sorting Motif Results in Missorting of BDNF

Site-directed mutagenesis of the putative sorting motif at the two acidic residues was carried out to determine their role in targeting BDNF for regulated secretion. A recombinant mammalian expression vector encoding mutant proBDNF (mutBDNF) with the two acidic residues substituted for alanine (IAIA) so as to eliminate the charge was generated. AtT-20 cells were transfected with the IAIA mutBDNF or wild-type proBDNF (wtBDNF). The cells were metabolically pulse labeled with ^{35}S Met/Cys and chased in the absence and presence of 50 mM K^+ to study basal and stimulated secretion, respectively. Newly synthesized BDNF secreted into the medium was immunoprecipitated and analyzed by SDS-PAGE. ^{35}S-BDNF secreted was expressed as a percentage of total labeling in the media plus cell extract at the end of the experiment. Both wt and mutant proBDNF ($\sim$34 kDa) and mature BDNF ($\sim$14 kDa) were detected in the cell extract after a 30 min pulse, and their levels of labeling were very similar (Figure 2A). To determine the specificity of the anti-BDNF antibody, we immunoprecipitated the secretion media from ^{35}S-labeled cells. Anti-BDNF IgY, but not preimmune chicken IgY, precipitated both pro- and mature BDNF (Figure 2B, lane 3 and lane 1). Both bands were eliminated when anti-BDNF IgY were used together with 2.5 μg recombinant human BDNF (rhBDNF, Promega) (Figure 2B, lane 2).

In cells expressing wtBDNF, depolarization with 50 mM K^+ for 30 min resulted in a 3.1-fold increase in the secretion of BDNF (pro plus mature, 42.7% $\pm$ 7.0% with stimulation; Figure 2C, lane S, and Figure 2D) versus 13.7% $\pm$ 1.2%, in the previous 30 min basal secretion (Figure 2C, lane B, and Figure 2D; $p < 0.02$). In a parallel experiment, we show no difference in the basal secretion between the first (Figure 2C, lower panel, lane B1) and the second 30 min chase period without 50 mM K^+ (Figure 2C, lower panel, lane B2). In contrast, cells transfected with mutBDNF failed to show activity-dependent secretion (Figures 2C and 2D). The total amount of mutant proBDNF plus mature mutBDNF secreted was 15.2% $\pm$ 2.9% in depolarizing medium and 17.8% $\pm$ 2.0% in basal medium ($p > 0.05$). Instead, these cells showed an increase in basal release (17.8% $\pm$ 2.0%) as compared to that in cells transfected with wtBDNF (13.7% $\pm$ 1.2%, $p < 0.05$), indicating increased constitutive secretion of mutBDNF. Taken together, these results indicate that the two acidic residues in the sorting motif are essential for directing BDNF molecules to the regulated secretory pathway in AtT-20 cells.

In addition, we observed a qualitative difference in the forms of wtBDNF versus mutBDNF released with stimulation. More mature wtBDNF (25.0% $\pm$ 4.6% of

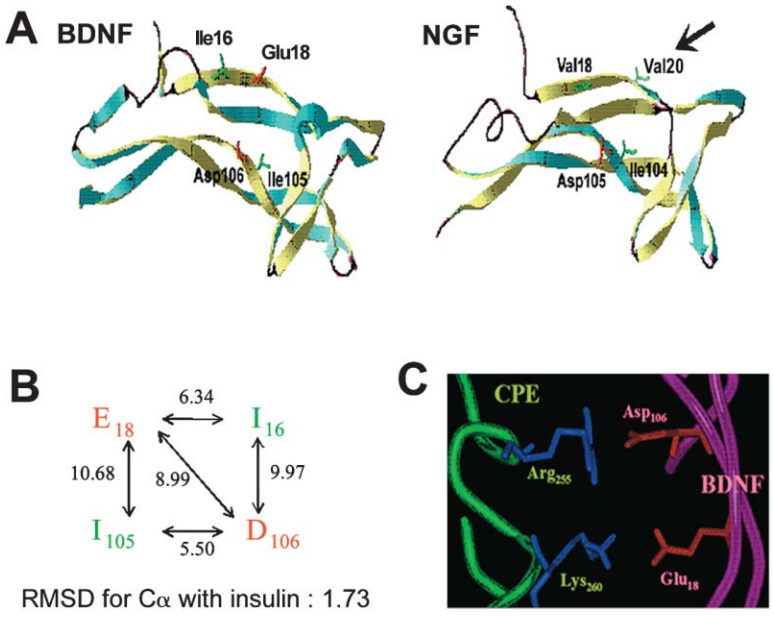


Figure 1. X-Ray Crystal Structure Analysis of BDNF and NGF

(A) The X-ray crystal structures of BDNF (PDB: 1BND) and NGF (PDB: 1BET) were visualized and arranged using Swiss PdbViewer, version 3.7b2 (http://us.expasy.org/spdbv/). Two aliphatic hydrophobic (Ile_{16} and Ile_{105}) and two acidic (Glu_{18} and Asp_{106}) residues with lower than 2.0 Å RMSD value were selected. The left structure is BDNF with the 4 residues of the sorting signal motif indicated, I_{16}, E_{18}, I_{105}, and D_{106}. The right structure is NGF, showing 3 of the 4 residues of the sorting signal motif, V_{18}, I_{104}, and D_{105}. Acidic residues are depicted in red, and the hydrophobic residues are depicted in green. The arrow shows the presence of the uncharged V_{20} in NGF in the corresponding position of the acidic E_{18} of BDNF.

(B) The sorting signal motif of BDNF. The side chain distances of the residues forming the sorting signal motif of BDNF are displayed. The average RMSD value of the four C_α atoms for the acidic and aliphatic hydrophobic residues between the BDNF and insulin motifs is 1.73.

(C) Predicted interaction between BDNF and CPE. Molecular models of the BDNF sorting motif can be manually docked to visualize the interaction of the CPE sorting signal binding domain ($CPE_{254–273}$). The model shows the complementarity of the two acidic residue side chains of BDNF (Glu_{18} and Asp_{106}) to the basic residues of Arg_{255} and Lys_{260} in the sorting signal binding determinant of CPE.

total) was secreted compared to mature mutBDNF (6.6% ±2.4% of total, $p < 0.02$; Figure 2C, lanes S). Moreover, the ratio of proBDNF:BDNF secreted was greater in cells transfected with mutBDNF than cells with wtBDNF (see Figure 2C). The greater extent of processing of wt proBDNF is consistent with more efficient processing of proBDNF to BDNF in the granules of the regulated secretory pathway, where the appropriate converting enzymes are located. Furthermore, since the sorting signal resides in mature BDNF, sorting of proBDNF/BDNF is independent of processing.

The subcellular localization of BDNF was examined by fluorescence immunocytochemistry and confocal microscopy. AtT-20 cells expressing wtBDNF showed punctate immunostaining of BDNF along and at the tips of the cell processes (Figure 3A), similar to POMC, an endogenous secretory granule marker (Figure 3B). In these cells, colocalization of BDNF with POMC was evi-

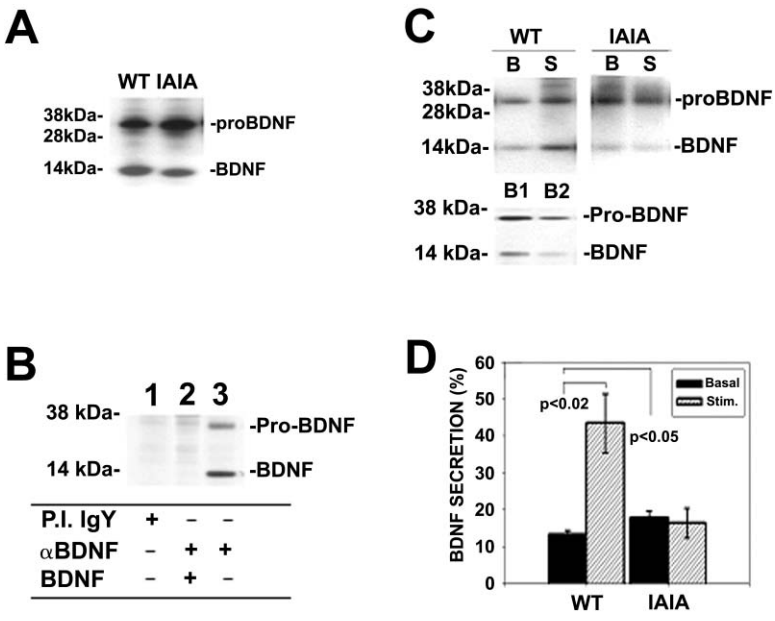


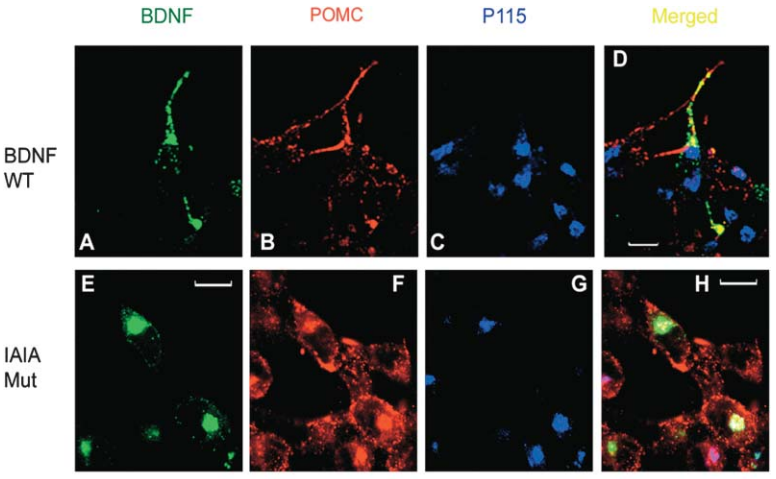
Figure 2. Analysis of ^{35}S-Labeled ProBDNF/BDNF in Cells and Secretion Media

(A) Radiograms of wild-type BDNF (wt) and IAIA mutBDNF (IAIA) in transfected AtT-20 cells which were pulse labeled with [^{35}S]Met/Cys for 30 min. The cell extracts were immunoprecipitated with BDNF antibody, and the immunoprecipitates were run on SDS-PAGE.

(B) Antibody control experiments. Radiogram of [^{35}S]BDNF secreted from AtT-20 cells transfected with wt BDNF. The secretion medium was immunoprecipitated with P.I. (preimmune) chicken IgY (lane 1), anti-BDNF IgY in the presence of BDNF (lane 2), or anti-BDNF IgY (lane 3).

(C) Radiograms of [^{35}S]BDNF secreted from AtT-20 cells transfected with wt or IAIA mutant BDNF. Lanes B show BDNF or mutBDNF (IAIA) secreted in the basal media, and lanes S show stimulated secretion of wt BDNF or mutBDNF (IAIA) induced by 50 mM KCl. (Lower panel) Parallel experiment showing [^{35}S]wtBDNF secreted in the first 30 min (B1) and second 30 min basal media (B2).

(D) Bar graph of basal secretion (solid bar) and stimulated secretion (striped bar) of wt or IAIA BDNF from AtT-20 cells. The intensity of the bands corresponding to proBDNF and BDNF in each sample was quantitated, and proBDNF/BDNF secreted was expressed as a percentage of the total labeling (all the media plus cell lysate collected at the end the experiment). The mean percent and standard error was calculated from four separate experiments.



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	Exp.1	Exp.2	Exp.3	M±SE %	P value
BDNF-WT	52/55 (94.5)	50/53 (94.3)	56/65 (91.3)	91.7± 2.8	
IAIA-Mut	4/20 (20.0)	4/27 (14.8)	5/29 (17.2)	17.4 ± 1.4	<0.02

Figure 3. Immunocytochemical Localization of BDNF in AtT-20 Cells

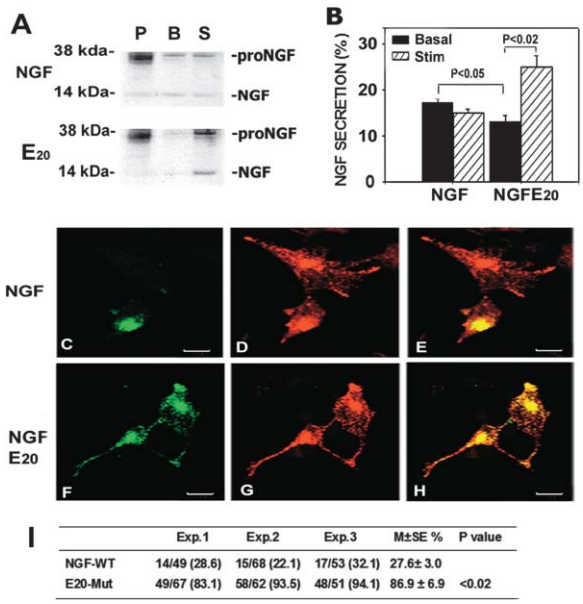
The cells transfected with BDNF wild-type (wt, [A–D]) and IAIA mutBDNF (IAIA Mut., [E–H]) were incubated with anti-BDNF (A and E), DP4 anti-POMC (B and F), and p115, a Golgi marker (C and G), followed by incubation with the appropriate fluorescent labeled secondary antibodies. BDNF appears green, POMC appears red, and p115 appears blue. The colocalization of BDNF on the merged images appears as yellow punctate staining in the cell processes (D). Cells transfected with mutBDNF showed no BDNF punctate staining but colocalization with P115 (G and H). Scale bars, 10 μm. (I) Quantification of colocalization of BDNF with the regulated secretory granule marker POMC by immunocytochemistry and confocal microscopy. The fractions represent the number of cells showing colocalization of BDNF with POMC in punctate granules along the cell processes over the total number of cells with cell processes examined. Numbers in parentheses are percentages. The mean and standard error of the mean (SE) were calculated from three separate experiments.

dent from the yellow staining in the merged images (Figure 3D), indicating that BDNF was packaged into secretory granules of the regulated secretory pathway. Quantification revealed that, out of 488 BDNF immunopositive cells examined, 82.3% showed BDNF staining in cell processes. Among these cells, 91.7% ± 2.8% exhibited colocalization of wtBDNF and POMC (Figure 3I). Thus overall, 76% of the total BDNF-immunopositive cells had processes showing punctate immunostaining of BDNF colocalized with POMC. In contrast, mutBDNF (IAIA) immunostaining was primarily in the perinuclear region (Figure 3E) and colocalized with p115, a Golgi marker (Figures 3G and 3H). Out of 690 BDNF-immunopositive cells examined, 89% showed no immunoreactive mutBDNF in the cell processes, but only in the cell body. In cells that did show staining of mutBDNF in cell processes, only 17.4% ± 1.4% of them showed colocalization of mutBDNF with POMC (Figure 3I). Thus, only 1.9% of total immunopositive cells showed punctate colocalization of mutBDNF with POMC in cell processes. These data indicate that mutation in the sorting motif resulted in a failure to sort mutBDNF into regulated secretory pathway granules.

Substitution of Val_{20} with Glu_{20} Redirected NGF to the Regulated Secretory Pathway

To further test the functional role of the sorting motif, we substituted Val_{20} (V_{20}) (Figure 1A) with an acidic residue, Glu_{20} (E_{20}), in the partial sorting motif of NGF and examined whether this substitution could redirect the constitutively secreted NGF to the regulated secretory pathway. An expression vector encoding the E_{20} mutation of proNGF ($NGFE_{20}$) was generated and transfected into AtT-20 cells. The cells were labeled with ^{35}S Met/Cys and then chased for 2 hr. Quantitative analysis indicated that this substitution resulted in a decrease in basal secretion. The NGF (proNGF/NGF) basal secretion was 16.5% ± 1.0% in wild-type versus 13.0% ± 1.0% in $NGFE_{20}$ mutant ($p < 0.05$; Figure 4A, lane B, and Figure 4B). More importantly, while high K^+ depolarization failed to induce secretion in cells expressing wild-type proNGF (14.8% ± 1.4% versus 16.5% ± 1.0%, $p > 0.05$), remarkable activity-dependent secretion was observed in cells expressing $NGFE_{20}$ (Figures 4A and 4B; basal, 13.0% ±1.0%; with depolarization, 24.7% ±2.4%; $p < 0.02$). The secretion of $NGFE_{20}$ was stimulated 1.9-fold with depolarization.

Fluorescent immunocytochemistry of AtT-20 cells expressing mutant $NGFE_{20}$ showed strong punctate immunostaining of $NGFE_{20}$ along the cell processes that colocalized with POMC (Figures 4F–4H), indicating packaging into granules of the regulated secretory pathway. In contrast, the distribution of transfected NGF was primarily in the perinuclear region (Figure 4C), characteristic of localization in the Golgi. Very little colocalization of NGF with POMC was observed in the cell processes (Figures 4D and 4E). Quantification of 724 cells expressing NGF indicated that 21% of them had NGF staining in the cell processes while the other 79% showed NGF immunostaining only in the cell body. Of the cells having stained processes, only 27.6% exhibited punctate colocalization of NGF with POMC (Figure 4I). Thus, only 5.7% of total NGF-immunopositive cells showed punctate colocalization of NGF with POMC in the cell processes. In contrast, 49.6% of the 682 cells expressing mutant $NGFE_{20}$ exhibited immunostaining in the cell processes. The number of cells showing punctate colocalization of $NGFE_{20}$ with POMC in the cell processes was significantly higher (86.9% ± 2.5%) than that in cells expressing NGF (27.6% ± 3.0%, $p < 0.02$; Figure 4I). Overall, 43% of total mutant $NGFE_{20}$-immunopositive cells showed colocalization of mutant $NGFE_{20}$ with POMC in the cell processes versus 5.7% for NGF. These results, taken together with the secretion data, indicate that, by introducing an acidic residue to position 20, the partial motif of NGF was transformed to a complete motif. Such



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	Exp.1	Exp.2	Exp.3	M±SE %	P value
NGF-WT	14/49 (28.6)	15/68 (22.1)	17/53 (32.1)	27.6± 3.0	
E20-Mut	49/67 (83.1)	58/62 (93.5)	48/51 (94.1)	86.9 ± 6.9	<0.02

Figure 4. Secretion and Immunocytochemical Localization of NGF

(A) Radiogram of wild-type NGF and mutNGF (E_{20}) in transfected AtT-20 cells that were pulse labeled with [^{35}S]Met/Cys for 30 min. The cell extracts and media were immunoprecipitated with NGF antibody, and the immunoprecipitates were ran on SDS-PAGE. The lanes show proNGF/NGF in cells after pulse (P, 1/5 of sample was used); basal release media (B); stimulation media (S).

(B) Bar graph of basal (solid bars) and stimulated (striped bars) secretion of proNGF/NGF and proNGF$_{20}$/NGF$_{20}$ from transfected cells. Secretion was expressed as the percentage of total labeling. The mean percent and standard error (SE) were calculated from three separate experiments.

(C–H) Immunocytochemical localization of NGF in AtT-20 cells. The cells transfected with NGF and NGFE$_{20}$ mutant were labeled with rat anti-NGF (C and F) or rabbit anti-POMC antibody (D and G) and detected with fluorescence secondary antibodies. Cells transfected with NGF showed staining primarily in the perinuclear region and did not colocalize with POMC (C and E). The merged image of NGFE$_{20}$-expressing cells (H) showed colocalization of NGF with POMC in the cell body and throughout the cell processes. Scale bar, 10 μm.

(I) Quantification of colocalization of NGF with regulated secretory granule marker POMC by immunocytochemistry and confocal microscopy. The fractions represent the number of cells showing colocalization of NGF with POMC in punctate granules along the cell processes over the total number of cells with cell processes. Numbers in parentheses are percentages, and the mean and standard error of the mean (SE) were calculated from three separate experiments.

a small change can then redirect NGF to the regulated secretory pathway, emphasizing an important role of these motifs in sorting.

BDNF Binds Specifically to Membrane CPE

Our molecular modeling studies predicted that the sorting signal motif of BDNF can interact with the sorting receptor, membrane CPE (Figure 1C). We therefore investigated the interaction of mature BDNF and NGF with CPE using bovine pituitary secretory granule membranes, which are a rich source of membrane CPE. We have previously shown that CPE in solution does not bind well to the sorting signal in N-POMC. Binding is most efficient when CPE is associated with membrane (Zhang et al., 1999). Figure 5A shows that ^{125}I-labeled BDNF bound to these membranes at a level three times greater than NGF, which does not have the sorting signal motif. Moreover, the binding was displaced completely ($p < 0.02$) by N-POMC$_{1–26}$ peptide, which contains the sorting motif of POMC, shown previously to interact with CPE to effect sorting to the regulated secretory pathway. β-endorphin$_{1–27}$, a control peptide without the sorting motif, did not displace the BDNF binding. Furthermore, neither N-POMC$_{1–26}$ nor β-endorphin$_{1–27}$, displaced the low amount of NGF binding observed.

The binding study was also performed with the membranes from sf9 cells expressing recombinant CPE (Figure 5B) and with control sf9 cell membranes (from mock-infected cells) as background for subtraction. The results are similar to those obtained for pituitary secretory granule membranes. These results indicate that BDNF bound specifically to CPE from two different membrane sources.

BDNF Is Constitutively Secreted from Pituitary Cells of CPE Knockout Mice

To assess if CPE is necessary for the sorting of BDNF in vivo, we studied proBDNF sorting in anterior pituitary cells of wild-type and CPE KO (*Cpe*$^{-/-}$) mice (Cawley et al., 2004). Anterior pituitary cells were chosen since it has been shown that the sorting motif of BDNF is required for activity-dependent secretion of BDNF from AtT-20 cells, a mouse anterior pituitary tumor cell line (Figure 2). Figure 6A shows that CPE is absent in the pituitary, cortex, and hippocampus of *Cpe*$^{-/-}$ mice compared to wild-type littermates. Pulse-chase studies on primary pituitary cells of wild-type mice transfected with BDNF showed a 3.3-fold increase in secretion of proBDNF/BDNF in response to high K^+ depolarization, relative to basal secretion (18.9% ± 1.5% versus 5.8% ± 0.1%, respectively, $p < 0.002$; Figures 6B and 6C). In contrast, there was no stimulated secretion of proBDNF/BDNF from pituitary cells of *Cpe*$^{-/-}$ mice (12.4% ± 2.5% versus 12.2% ± 1.9%). Additionally, basal secretion was significantly higher in the cells of *Cpe*$^{-/-}$ mice (12.2% ± 1.9%) compared to wild-type mice (5.8% ± 0.1%, $p < 0.05$; Figure 6C), indicative of missorting of BDNF to the constitutive pathway in the absence of CPE.

Missorting of BDNF in Cortical and Hippocampal Neurons of CPE Knockout Mice

To identify the sorting signal motif, we initially used endocrine cells (AtT-20 and pituitary cells), which do not express BDNF, as a model, so as to avoid having to tag the BDNF, which could interfere with sorting. However, it was important to verify that the sorting signal-CPE dependent mechanism also operates in neurons that do synthesize BDNF. We therefore carried out pulse-chase studies in embryonic cortical neurons of *Cpe*$^{-/-}$ mice and their wild-type littermates to test the role of CPE in regulating activity-dependent secretion of BDNF.

Figure 7A shows stimulated release of ^{35}S-labeled endogenous proBDNF/BDNF from cortical neurons of wt but not *Cpe*$^{-/-}$ mice. In a parallel experiment, we show no significant difference in the basal secretion between first hour (B1) and second (B2) hour of incubation without 50 mM K^+ (Figure 7A, middle panel). The identification of the pro- and mature BDNF bands were verified by

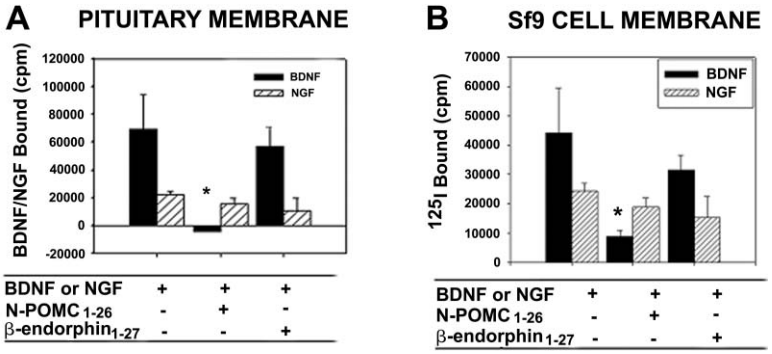

Figure 5. BDNF Binds to CPE In Vitro

[^{125}I]BDNF and [^{125}I]NGF were incubated with CPE-rich secretory granule membranes (A) or membranes from sf9 cells expressing recombinant CPE (B) in the binding buffer at pH 6.5 for 1 hr at 4°C. The incubates were centrifuged, and the recovered membranes were counted. Bar graphs show the mean and the standard error of the mean of CPM of BDNF and NGF bound to membranes in the presence and absence of N-POMC$_{1-26}$. The middle panel shows that BDNF binding was displaced in the presence of POMC$_{1-26}$ peptide (*$p < 0.02$). The right panel shows that, in the presence β-endorphin$_{1-27}$, a control peptide without binding motif, the bindings were not displaced.

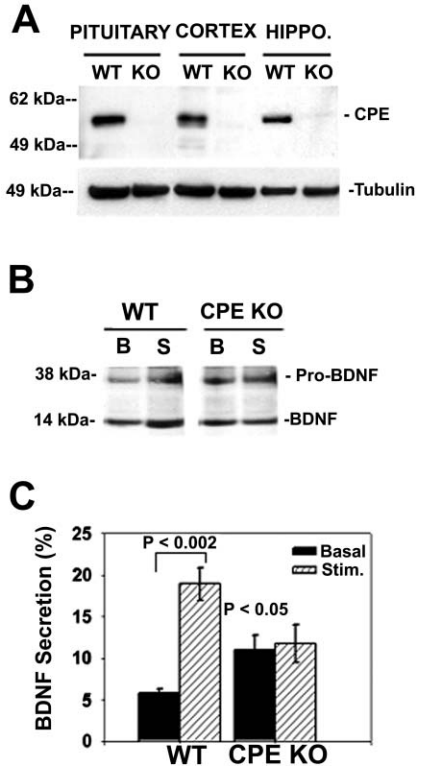

Figure 6. Analysis of BDNF Secretion from Anterior Pituitary Cells of CPE Knockout Mice

(A) The upper panel shows Western blotting of CPE in anterior pituitary, cortex, and hippocampus (hippo.) from CPE KO mice and their wild-type littermates (wt). Ten micrograms of protein were loaded in each lane. The lower panel shows tubulin expression in the same blot after stripping.

(B) Radiogram of proBDNF/BDNF secreted from primary anterior pituitary cells transfected with wtBDNF construct. The cells of wt and CPE KO mice were labeled with [^{35}S]Met/Cys and then stimulated to secrete with 50 mM K^+. Secretion of proBDNF (~34 kDa) and BDNF (~14 kDa) were detected in basal release media (B) and stimulation media (S).

(C) Quantification of activity-dependent secretion (striped bar) or basal secretion (solid bar) of proBDNF/BDNF. ProBDNF/BDNF secreted into the media were expressed as the percent of the total labeling. The mean percent and the standard error of the mean were calculated from three separate experiments. Stimulated release was demonstrated in the wt cells ($p < 0.02$), while higher basal secretion was seen in CPE KO cells versus wt mice ($p < 0.05$).

immunoprecipitation of the ^{35}S-labeled cell lysate with anti-BDNF chicken IgY, preimmune (P.I.) IgY, and anti-BDNF in the presence of rhBDNF. Anti-BDNF, but not the P.I. IgY, precipitated both pro- and mature BDNF (Figure 7A, lower panel, lanes 3 and 1). Both bands were eliminated when anti-BDNF was used together with rhBDNF (Figure 7A, lower panel, lane 2). Figure 7B shows that endogenous proBDNF/BDNF secretion was stimulated 2.7-fold (20.3% ± 3.2% versus 7.6% ± 0.8% basal release, $p < 0.02$) in wild-type neurons. However, no activity-dependent secretion of proBDNF/BDNF was seen in *Cpe*$^{-/-}$ neurons (12.4% ± 2.0% basal release versus 10.3% ± 1.8%, with stimulation; Figure 7B). Instead, higher basal release of proBDNF/BDNF was found in *Cpe*$^{-/-}$ neurons compared to neurons from wild-type mice (12.4% ± 2.0% versus 7.6% ± 0.8%, respectively, $p < 0.05$; Figure 7B). These results indicate that sorting and activity-dependent secretion of endogenous proBDNF/BDNF requires CPE. Furthermore, analysis of the mature BDNF and proBDNF secreted revealed a ratio of 1.8:1 (13.2% ± 1.6% BDNF versus 7.3% ± 1.4% proBDNF) in the stimulation medium of wild-type neurons, compared to a ratio of 1:1 (5.2% ± 0.8% BDNF versus 5.1% ± 0.9% proBDNF) from *Cpe*$^{-/-}$ neurons, indicating less processing in this pathway due to more rapid release and deficiency of the appropriate processing enzyme found in secretory granules of the regulated secretory pathway.

The dependence of CPE for the sorting of BDNF was also investigated in hippocampal neurons. A morphological approach was used, since the *Cpe*$^{-/-}$ embryonic hippocampal neurons were difficult to obtain in the large quantity needed for secretion studies (see Experimental Procedures). Primary cultures of the hippocampal neurons from wild-type and *Cpe*$^{-/-}$ mice after infection with a viral vector encoding wtBDNF-EGFP fusion protein were examined by fluorescence confocal microscopy. BDNF-EGFP was distributed in the cell body and extended to the distal processes in wild-type neurons (Figures 7C and 7D). BDNF-EGFP punctate fluorescence which colocalized with secretogranin II (SecII), a secretory vesicle marker in neurons, was visible in the neurites of these neurons (Figures 7E and 7F). However, in neurons from *Cpe*$^{-/-}$ mice, BDNF-EGFP was localized primarily in the cell body (Figure 7G), with some diffuse and weak staining in the neurites (Figures 7G and 7H). The *Cpe*$^{-/-}$ neurons have normal neurites, as evidenced by the punctate SecII staining (Figure 7I). However, the

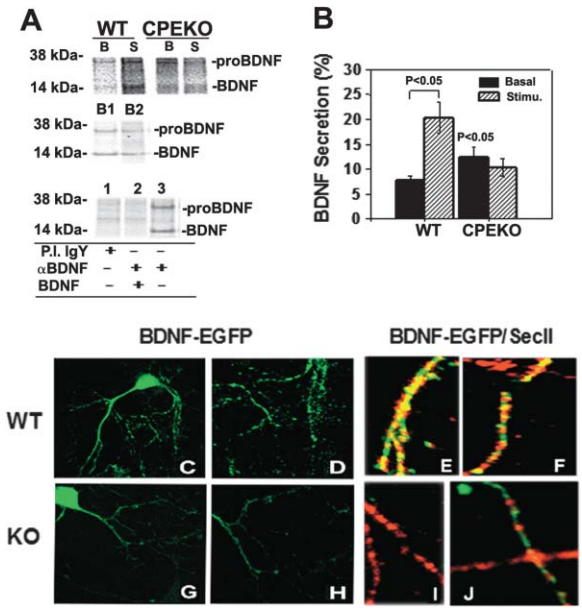

Figure 7. Analysis of Secretion and Localization of BDNF in Neurons of CPE KO Mice

(A) Radiograms of proBDNF/BDNF secreted from embryonic (E16) cortical neurons. (Upper panel) Cortical neurons from wt or CPE KO mice were labeled with [^{35}S]Met/Cys and then stimulated to secrete with 50 mM K^+. ProBDNF/BDNF were detected in basal release media (B) and stimulation media (S). (Middle panel) Parallel experiment showing [^{35}S]wt proBDNF/BDNF secreted in the first hr basal media (B1) and second hr basal media (B2) from wt neurons. (Lower panel) Antibody control experiments from wt neurons. The ^{35}S-labeled cell lysates were immunoprecipitated with preimmune chicken IgY (P.I. IgY, lane 1), anti-BDNF IgY in the presence of rhBDNF (lane 2), or anti-BDNF IgY (lane 3). (B) Quantification of activity-dependent secretion and basal secretion of proBDNF/BDNF from wt and CPE KO cortical neurons. ProBDNF/BDNF secreted into the media were expressed as the percentage of the total labeling (media plus cell lysate collected at the end the experiment). The mean percent and the standard errors of the mean were calculated from three separate experiments. Each experiment contained neurons from two to four cortices pooled. (C–J) Cellular localization of BDNF in embryonic (E16) hippocampal neurons infected with a viral vector encoding proBDNF-EGFP fusion protein. (C and D) wt neurons showing punctate distribution of BDNF-EGFP in neurites. (E and F) Colocalization of BDNF-EGFP with SecII, a secretory vesicle marker in neurons. (G and H) *Cpe*$^{-/-}$ neurons from CPE KO mice showing weak diffuse distribution of BDNF-EGFP in neurites. (I and J) Neurites from *Cpe*$^{-/-}$ neurons showing punctate SecII immunostaining (I) and lack of colocalization of BDNF-EGFP with SecII (J). Magnifications, (C and G) 100×; (D and H) 200×; and 800× for all the cell processes of wt (E and F) and of CPE KO neurons (I and J).

diffuse BDNF-EGFP fluorescence did not colocalize with the SecII containing secretory granules (Figure 7J). The lack of robust punctate green fluorescence that colocalized with SecII in the neurites of *Cpe*$^{-/-}$ neurons is consistent with little or no sorting of BDNF-EGFP into the regulated secretory pathway in the absence of CPE. Taken together with the secretion data, the sorting of BDNF to the regulated secretory pathway in cortical and hippocampal neurons is dependent on CPE. By extrapolation, therefore, the highly specific conformation-dependent BDNF sorting signal motif ($I_{16}E_{18}I_{105}D_{106}$) identified using model cell systems also functions in neurons to interact with CPE to effect sorting of BDNF to the regulated secretory pathway.

Discussion

Sorting of ProBDNF Requires Interaction of a Sorting Motif with CPE

Activity-dependent secretion is important for mediating various forms of synaptic plasticity, such as hippocampal long-term potentiation (LTP) (Katz and Shatz, 1996). BDNF secreted in response to transient or sustained neuronal activity is likely to participate in acute modulation of synaptic transmission, as well as long-term regulation of synaptic structure and functions, respectively (Lu, 2004). Correct trafficking and sorting of BDNF into vesicles of the regulated secretory pathway is therefore critical for proper processing and activity-dependent secretion of BDNF. In this study, we have uncovered a mechanism for sorting BDNF to the regulated secretory pathway. We employed a highly sensitive, quantitative radioactive labeling pulse-chase paradigm to track synthesis, trafficking, and secretion of proBDNF and therefore avoided the need of overexpression of proBDNF for detection, as in other studies (Heymach et al., 1996; Hibbert et al., 2003; Moller et al., 1998; Wang et al., 2004; Wu et al., 2004), which could interfere with sorting. We showed that mature BDNF contains a three-dimensional sorting signal motif, consisting of $I_{16}E_{18}I_{105}D_{106}$, which was necessary for the sorting of proBDNF to the regulated secretory pathway. The precise configuration required for this motif to function was clearly demonstrated by the mutagenesis data where substitution of the two acidic residues (E_{18} and D_{106}) with alanine in the BDNF motif resulted in missorting of proBDNF to the constitutive pathway. Mutation of all four residues of the predicted sorting motif also resulted in obliteration of regulated secretion of BDNF (unpublished data). As shown previously for proinsulin, the hydrophobic residues are required to provide the environment to allow the acidic residues to be charged (Dhanvantari et al., 2003). Additionally, we showed in that study that such a sorting motif can also act as a retention motif in pancreatic β cells to prevent constitutive-like secretion of proinsulin from the immature granule.

Our studies and those of others have shown that NGF was largely secreted constitutively (Mowla et al., 1999), although some regulated secretion has been reported in cortical neurons that have been either transfected with high doses of DNA or infected with virus carrying NGF (Griesbeck et al., 1999; Heymach et al., 1996; Wu et al., 2004). When we introduced an acidic residue to replace V_{20} in the NGF molecule so as to provide the same configuration and parameters of a typical sorting motif—two acidic and two aliphatic hydrophobic residues (see Figure 1A)—a significant proportion of NGF was redirected to the regulated secretory pathway, bringing about activity-dependent secretion of this mutant NGF, thus providing proof of concept of the sorting motif identified in BDNF.

The above findings emphasize the importance of the acidic residues needed to interact with the sorting receptor CPE at two basic residues, R_{255} and K_{260}, known to be critical for the interaction of a similar sorting motif

in proinsulin to effect sorting to the regulated secretory pathway (Dhanvantari et al., 2003). Indeed, the sorting motif of BDNF, but not NGF, which lacks the sorting signal motif, was shown to interact specifically with CPE in secretory granule membranes. The importance of CPE in the sorting of proBDNF in vivo was demonstrated by the obliteration of activity-dependent secretion of BDNF in primary cultures of pituitary cells and neurons from *Cpe*$^{-/-}$ mice. Thus, the mechanism of sorting proBDNF to the regulated secretory pathway involves the interaction of a sorting motif $I_{16}E_{18}I_{105}D_{106}$ with the sorting receptor, CPE. Previous studies demonstrated that a val66met mutation in the pro region caused significant impairments in dendritic trafficking and activity-dependent secretion of BDNF (Chen et al., 2004; Egan et al., 2003). Although these results point to the importance of the pro region, it is unclear whether the pro region is directly involved in sorting proBDNF to the regulated secretory pathway or whether val66met mutation resulted in a defect in trafficking of the molecule to dendritic spines, where the majority of regulated secretion occurs (Lu, 2003). The fact that increased constitutive secretion was not observed in val66met mutant proBDNF supports the notion that a defect in trafficking, rather than sorting, is the reason for impairment of activity-dependent secretion. Alternatively, the pro and mature regions may act synergistically or independently in sorting BDNF to the regulated secretory pathway. It is possible that in certain cell types or subcellular compartments, proBDNF is converted to mature BDNF intracellularly (in TGN), and therefore sorting by the motif in the mature BDNF becomes essential. In other cells/compartments, proBDNF is the major form, and the pro region may participate in sorting to ensure activity-dependent secretion.

Incomplete Processing of ProBDNF in Cortical Neurons: By Design or By Default?

Given the recent evidence suggesting that proBDNF or proNGF elicits its action through p75 neurotrophin receptor (Chao and Bothwell, 2002; Pang et al., 2004) while mature BDNF binds TrkB (Mowla et al., 2001; Patapoutian and Reichardt, 2001), analysis of the processing and forms of BDNF secreted from mouse cortical neurons would provide insight into the various functions of pro/mature BDNF at the synapse. Our data revealed that in cortical neurons (1) there is incomplete processing of endogenous proBDNF, even after 4 hr of chase following pulse labeling, indicating that the processing of proBDNF is slow in these neurons; (2) although there was twice as much mature BDNF than proBDNF secreted with stimulation, there was nevertheless a substantial amount of pro form cosecreted with the mature form. While stimulated release of proBDNF was shown in transfected or virus-infected hippocampal neurons (Farhadi et al., 2000; Heymach et al., 1996; Mowla et al., 2001), the caveat existed that the proBDNF secreted may have been due to overexpression. Our verification of significant release of endogenous proBDNF from the cortical neurons has profound implications on synaptic modulation and neuronal survival. The ratio of proBDNF versus mature BDNF released can be regulated at different levels, for example, by the expression of different levels of the intracellular processing enzymes in various neurons. Additionally, sustained neuronal activity would lead to an elevated secretion of newly synthesized BDNF, which would likely include a higher amount of proBDNF, since there would be less time for complete processing to occur. Extracellular processing of the proBDNF, therefore, becomes an important mechanism for up- as well as downregulation of synaptic efficacy by BDNF (Lu, 2003). Candidate extracellular proteases such as plasmin have been shown to cleave proBDNF correctly (Lee et al., 2001). Altering the processing of secreted proBDNF through regulating the activity of extracellular proteases could represent a novel mechanism by which the function of various synapses can be dictated. Indeed, preliminary experiments demonstrated that tissue plasminogen activator, by activating plasmin, converted proBDNF to mature BDNF in the hippocampus, and such conversion is required for the expression of late-phase LTP (L-LTP) (Pang et al., 2004). In contrast, activation of p75 neurotrophin receptor, possibly by proBDNF, appears to be required for the expression of long-term depression (LTD) in the hippocampus (unpublished data).

In conclusion, our present study has uncovered a sorting motif receptor-mediated mechanism for sorting proBDNF to the regulated secretory pathway in cortical and hippocampal neurons necessary for activity-dependent secretion of BDNF. We have also demonstrated that active sorting of endogenous proBDNF to the regulated secretory pathway, versus entering the constitutive pathway by default, has a major impact on the processing and hence the ratio of proBDNF:BDNF secreted from these two different secretory pathways in neurons. This in turn dictates the function, since the two forms, pro- and mature BDNF, bind very different receptors with opposing effects.

Experimental Procedures

Molecular Modeling of BDNF and CPE

The X-ray structures of BDNF (PDB code: 1BND) (Robinson et al., 1995), NGF (PDB code: 1BET) (McDonald and Blundell, 1991), and insulin (PDB code: 7INS) (Balschmidt et al., 1991) were downloaded from the Protein Data Bank (PDB) website (www.rcsb.org) and superimposed. Four residues (L_{16} and E_{17} in A chain and E_{13} and L_{17} in B chain) were previously identified as the sorting signal motif in insulin (Dhanvantari et al., 2003). The RMSD values of the C_α atoms for all acidic and aliphatic hydrophobic residues in BDNF and NGF were compared to that in the sorting motif in the X-ray crystal structure of insulin, using the SYBYL molecular modeling system (Tripos Inc., St. Louis, MO). Four residues, i.e., two aliphatic hydrophobic and two acidic residues, with lower than 2.0 Å RMSD value were selected. Low-energy conformers of CPE were generated using a combination of molecular dynamics and mechanics (Discover, MSI Inc., San Diego, CA) as described previously (Zhang et al., 2003). The lowest energy conformer of CPE was manually docked with BDNF in the conformation of the X-ray crystal structure, aligning the Arg_{255} and Lys_{260} of CPE and the Glu_{18} and Asp_{106} of BDNF. Molecular mechanics were used to optimize the interaction between the two structures.

Generation of BDNF and NGF Mutant Constructs

The full-length coding region of human proBDNF was excised at the EcoRI and BamH1 restriction sites of pro-hBDNF/pBluescript KS (-) plasmid and subcloned into a mammalian expression vector, pcDNA 3.1 (Invitrogen, Carlsbad, CA) to generate proBDNF/pcDNA3.1. A mutant proBDNF with substitution of residues of E_{18} and D_{106} to A_{18} and A_{106} (IAIA) was generated from this construct

using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two pairs of primers were used: A_{18}-5′ GCGTGTGTGACAGTATTAGTGC GTGGGTAACGGCG; A_{18}-3′ CGCCGTTACCCACGCACTAATACTGT CACACACGC; A_{106}-5′ GGCTGGCGATTCATAAGGATAGCCACTTCT TGTGTATGTACATTGACC; and A_{106}-3′ GGTCAATGTACATACACAA GAAGTGGCTATCCTTATGAATCGCCAGCC.

The cDNA encoding full-length proNGF was excised from rNGF/pBS using EcoRI and BamH1 restriction sites and was subcloned into pcDNA3.1 (proNGF/pcDNA3.1). This cDNA was used as a template to generate an E_{20} mutation, so as to substitute a noncharged residue (valine, V_{20}) with an acidic residue (glutamic acid, E) to create a sorting motif similar to that described for BDNF. The primers used were as follows: E_{20}-5′ GACAGTGTCAGTGAGTGGGTTGGAGATAA GACC; E_{20}-3′ GGTCTTATCTCCAACCCACTCACTGACACTGTC.

All the constructs were sequenced to confirm their orientation and structure.

Cell Culture and Transfection of AtT-20 Cells

A mouse corticotroph cell line, AtT-20 cell (A.T.C.C. Manassas, VA), was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (Invitrogen). The cells were grown to ~80% confluence and transfected with the cDNA constructs. To avoid overexpression of the protein, which may interfere with sorting, we titrated the dose of DNA to determine the minimal optimum dose necessary to give detectable radiolabeled BDNF bands using a pulse-chase paradigm. Cells were transfected at a concentration of 4 μg of DNA/10 ml medium for a 10 cm^2 dish in the presence of Lipofectamine 2000, according to the manufacturer's protocol (Invitrogen). Subsequently, the cells were assessed for synthesis, secretion, and subcellular localization of neurotrophins.

Intracellular Localization of Neurotrophins in AtT-20 Cells

For immunocytochemical studies, AtT-20 cells were grown to ~50% confluence. After overnight transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100/PBS. The cells were incubated with two or more of the following antibodies: chicken anti-BDNF IgY antibody, preimmune IgY, and rat anti-NGF antibody (all from Promega, Madison, WI); rabbit anti-DP4 antibody for endogenous POMC, a known regulated secretory granule marker (Cool et al., 1997); and p115 monoclonal antibody for Golgi marker (Transduction Laboratories, Lexington, KY). Fluorescence-conjugated corresponding goat secondary antibodies (Molecular Probes, Eugene, OR) were used at 1:1000 dilution. All the images were captured with a Bio-Rad MRC-1000 confocal microscope, analyzed with LaserSharp software, and processed with Adobe Photoshop 6.0. To quantify the cells showing colocalization, cells with overlapping neurotrophin and POMC punctate staining in the processes were recorded and expressed as a percentage of the total number of cells with processes examined, in three separate experiments.

Metabolic Labeling and Secretion Assay of Neurotrophins

To determine the secretion pathway of wt and mutant BDNF or NGF, a modified pulse-chase paradigm was used (Shen et al., 1999). Briefly, transfected AtT-20 cells or primary culture of pituitary cells or neurons were preincubated with Met/Cys-free media for 30 min and metabolically labeled for 30 min with 200 μCi/ml of [^{35}S]Met/Cys labeling mix (Amersham Pharmacia Biotech, Arlington Height, IL). The cells were rinsed with 5 ml of PBS (pH 7.4). One dish of cells was extracted with lysis buffer (1% Triton X-100/PBS). Media were collected from a second dish after 1 hr (for BDNF) or 2 hr chase (for NGF), followed by a 30 min basal release and a 30 min stimulation with depolarizing medium (DMEM with 50 mM KCL, Invitrogen) sequentially. At the end of the secretion study, the cells were extracted with lysis buffer. For cortical neurons, the pulse-labeling time was extended to 1 hr, followed by a 2 hr chase, a 1 hr basal release, and a 1 hr stimulation. All of the samples collected were immediately treated with 1x Complete Inhibitor Cocktail (Roche, Indianapolis, IN) to prevent protein degradation prior to analysis.

Immunoprecipitation and Quantification of Neurotrophins

Samples of cell extracts or media were incubated with chicken anti-BDNF IgY (5 μg) overnight at 4°C, followed by 1 hr incubation with agarose immobilized anti-chicken antibody (both from Promega), to pull down the immunocomplex. Purified rabbit anti-NGF IgG and protein A-plus Sepharose beads (BD Biosciences, Bedford, MA) were used to immunoprecipitate NGF. Immunoprecipitates were analyzed by electrophoresis on 12% SDS-PAGE and transferred onto nitrocellulose membrane. The radiolabeled signal on the membrane was recorded and read by a phosphorimage system (Molecular Dynamics, Sunnyvale, CA). The level of radiolabeled signal in the band corresponding to the neurotrophin was quantified using ImageQuant 2 (Molecular Dynamics). Following background subtraction, radioactivity in each band was expressed as the percentage of total labeling, which included labeled BDNF from all the media plus cell lysate extracted at the end of the secretion assay. Due to the lower number of Met/Cys residues in mature neurotrophin versus the precursor (9 versus 17 in proBDNF), a correction factor of 1.89 was used for mature BDNF to bring it up to the molar amount of the pro form to allow comparison of the ratio of proBDNF:BDNF and quantification of the two forms on a molar basis. Likewise, a correction factor of 1.7 was used in the calculation of mature NGF. Statistical significance was determined for a minimum of three experiments using the Student's t test.

For antibody control experiments, AtT-20 cells were transfected with wt BDNF, pulse labeled with [^{35}S]Met/Cys for 30 min, and chased for 1 hr. The secretion medium was immunoprecipitated with preimmune chicken IgY (P.I. IgY, 5 μg); anti-BDNF IgY (5 μg) in the presence of 2.5 μg rhBDNF (Promega); or anti-BDNF IgY (5 μg).

In Vitro Binding Assay of BDNF or NGF to CPE

Secretory granule membranes were prepared from bovine pituitary intermediate lobe, as previously described (Cool and Loh, 1998). Membranes from sf9 cells expressing recombinant CPE using the baculovirus expression system were isolated as described in Zhang et al., 1999. Ten micrograms of membrane protein were incubated with [^{125}I]BDNF (NEN Life Science Products, Inc., Boston, MA) or [^{125}I]NGF (Amersham Pharmacia Biotech) at a final concentration of 2 pM (~150,000 cpm) at pH 6.5. Each assay was done in duplicate. Specific binding was calculated by subtracting the nonspecific binding measured in tubes without membrane for assays with granule membrane. Control sf9 cell membranes without CPE (mock-infected cells) were used for background subtraction from the binding of sf9 cell membranes with CPE. To determine the specificity of BDNF and NGF binding to CPE, binding of these molecules was carried out in the absence or presence of a 10 μM concentration of a peptide that contains the sorting signal that binds CPE (N-$POMC_{1-26}$) and another that does not (β-$endorphin_{1-27}$), as a negative control (both peptides were from Phoenix Pharmaceuticals, Belmont, CA). The means ± SEM were obtained from at least three experiments.

Western Blotting of Tissues from CPE Knockout Mice

Cpe$^{-/-}$ mice were generated in our laboratory. These mice are infertile, and homozygotes were generated by mating two heterozygotes (*Cpe*$^{+/-}$). Anterior pituitary, cortex, and hippocampus were isolated from 21-week-old CPE KO mice and their wild-type littermates. The tissues were extracted with lysis buffer and the protein concentration was determined. Ten micrograms of each sample were denatured with sample buffer, fractionated by electrophoresis on 12% SDS-PAGE, and blotted onto nitrocellulose membrane. CPE was detected with a primary antibody against the C terminus of CPE (CPH 7-8) (Normant and Loh, 1998) and an HRP-conjugated secondary antibody followed by visualization using an ECL system (Amersham). The blot was stripped and reprobed with a mouse anti-tubulin antibody (1:10,000, BD Bioscience) to verify equal loading of the samples.

Primary Cultures of Anterior Pituitary and Cortical Neurons for Secretion Studies

The anterior pituitary was isolated from four adult (45 weeks) CPE KO mice and four wild-type littermates and prepared by enzymatic dispersion as described previously (Loh, 1988). The cells were grown for 5 days, then transfected with wtBDNF/pcDNA3.1 (2 μg/5 ml for 6 cm^2 dish), and BDNF secretion was studied using the pulse-chase paradigm described above. Embryonic cortical neurons were isolated as described previously, with modification (Du et al., 2003),

from the entire litter of embryonic day 16 (E16) pups derived from mating two heterozygote (*Cpe*$^{+/-}$) mice. Cells from each embryo were handled individually and mechanically dissociated and then were plated in a separate poly-D-lysine/laminin-treated dish. Cells belonging to wild-type or *Cpe*$^{-/-}$ pups were identified after genotyping. As expected from the genetics, there were only one or two pups from each litter that were *Cpe*$^{-/-}$. The cells were grown in culture medium (DMEM supplemented with 10% FBS and antibiotic). The next day, the medium was replaced with neurobasal medium supplemented with B27 (Invitrogen). Embryonic cortical neurons were kept in culture for 7 days, and activity-dependent secretion of endogenous BDNF was determined as described above.

Fluorescence Imaging of Hippocampal Neurons

Primary cultures of hippocampal neurons (E16) from wild-type and *Cpe*$^{-/-}$ mice were prepared as described above for cortical neurons. Hippocampal neurons were plated on poly-D-lysine/laminin-treated coverslips. Neurons 12 DIV from *Cpe*$^{-/-}$ or wild-type mice were infected with BDNF-EGFP coding sindbis virus, with an MOI to achieve a 10% infection rate. After overnight incubation, the media containing the viruses were removed and replaced with fresh media. After another 24 hr, the expression of BDNF-EGFP was captured using a Bio-Rad MRC 1000 confocal microscope. BDNF-EGFP coding sindbis virus was constructed as described previously (Egan et al., 2003), except that a less toxic SINrep (nsP2S) sindbis vector was used (Kim et al., 2004). In colocalization studies of BDNF-EGFP with a secretory vesicle marker, SecII, BDNF-EGFP-infected cells were fixed, permeabilized, and incubated with SecII polyclonal antibody (gift from Dr. T. Watanabe) at 1:250 dilution, followed by a fluorescent conjugated secondary antibody, and then the images were captured, as described above.

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References

Balkowiec, A., and Katz, D.M. (2000). Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. J. Neurosci. *20*, 7417–7423.

Balschmidt, P., Hansen, F.B., Dodson, E.J., Dodson, G.G., and Korber, F. (1991). Structure of porcine insulin cocrystallized with clupeine Z. Acta Crystallogr. B *47*, 975–986.

Bicknell, A.B., Lomthaisong, K., Woods, R.J., Hutchinson, E.G., Bennett, H.P., Gladwell, R.T., and Lowry, P.J. (2001). Characterization of a serine protease that cleaves pro-gamma-melanotropin at the adrenal to stimulate growth. Cell *105*, 903–912.

Cawley, N.X., Zhou, J., Hill, J.M., Abebe, D., Romboz, S., Yanik, T., Rodriguiz, R.M., Wetsel, W.C., and Loh, Y.P. (2004). The carboxypeptidase e knockout mouse exhibits endocrinological and behavioral deficits. Endocrinology *145*, 5807–5819.

Chao, M.V., and Bothwell, M. (2002). Neurotrophins: to cleave or not to cleave. Neuron *33*, 9–12.

Chen, Z.Y., Patel, P.D., Sant, G., Meng, C.X., Teng, K.K., Hempstead, B.L., and Lee, F.S. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. J. Neurosci. *24*, 4401–4411.

Cool, D.R., and Loh, Y.P. (1998). Carboxypeptidase E is a sorting receptor for prohormones: binding and kinetic studies. Mol. Cell. Endocrinol. *139*, 7–13.

Cool, D.R., Fenger, M., Snell, C.R., and Loh, Y.P. (1995). Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway. J. Biol. Chem. *270*, 8723–8729.

Cool, D.R., Normant, E., Shen, F., Chen, H.C., Pannell, L., Zhang, Y., and Loh, Y.P. (1997). Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe(fat) mice. Cell *88*, 73–83.

Dhanvantari, S., Shen, F.S., Adams, T., Snell, C.R., Zhang, C., Mackin, R.B., Morris, S.J., and Loh, Y.P. (2003). Disruption of a receptor-mediated mechanism for intracellular sorting of proinsulin in familial hyperproinsulinemia. Mol. Endocrinol. *17*, 1856–1867.

Du, J., Feng, L., Zaitsev, E., Je, H.S., Liu, X.W., and Lu, B. (2003). Regulation of TrkB receptor tyrosine kinase and its internalization by neuronal activity and Ca2+ influx. J. Cell Biol. *163*, 385–395.

Edwards, R.H., Selby, M.J., Garcia, P.D., and Rutter, W.J. (1988). Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. J. Biol. Chem. *263*, 6810–6815.

Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M., et al. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*, 257–269.

Farhadi, H.F., Mowla, S.J., Petrecca, K., Morris, S.J., Seidah, N.G., and Murphy, R.A. (2000). 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. J. Neurosci. *20*, 4059–4068.

Goodman, L.J., Valverde, J., Lim, F., Geschwind, M.D., Federoff, H.J., Geller, A.I., and Hefti, F. (1996). Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol. Cell. Neurosci. *7*, 222–238.

Griesbeck, O., Canossa, M., Campana, G., Gartner, A., Hoener, M.C., Nawa, H., Kolbeck, R., and Thoenen, H. (1999). Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc. Res. Tech. *45*, 262–275.

Gu, F., Crump, C.M., and Thomas, G. (2001). Trans-Golgi network sorting. Cell. Mol. Life Sci. *58*, 1067–1084.

Haubensak, W., Narz, F., Heumann, R., and Lessmann, V. (1998). BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. J. Cell Sci. *111*, 1483–1493.

Heymach, J.V., Jr., Kruttgen, A., Suter, U., and Shooter, E.M. (1996). The regulated secretion and vectorial targeting of neurotrophins in neuroendocrine and epithelial cells. J. Biol. Chem. *271*, 25430–25437.

Hibbert, A.P., Morris, S.J., Seidah, N.G., and Murphy, R.A. (2003). Neurotrophin-4, alone or heterodimerized with brain-derived neurotrophic factor, is sorted to the constitutive secretory pathway. J. Biol. Chem. *278*, 48129–48136.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. *24*, 677–736.

Katz, L.C., and Shatz, C.J. (1996). Synaptic activity and the construction of cortical circuits. Science *274*, 1133–1138.

Kim, J., Dittgen, T., Nimmerjahn, A., Waters, J., Pawlak, V., Helmchen, F., Schlesinger, S., Seeburg, P.H., and Osten, P. (2004). Sindbis vector SINrep(nsP2S726): a tool for rapid heterologous expression with attenuated cytotoxicity in neurons. J. Neurosci. Methods *133*, 81–90.

Korte, M., Kang, H., Bonhoeffer, T., and Schuman, E. (1998). A role for BDNF in the late-phase of hippocampal long-term potentiation. Neuropharmacology *37*, 553–559.

Lee, R., Kermani, P., Teng, K.K., and Hempstead, B.L. (2001). Regulation of cell survival by secreted proneurotrophins. Science *294*, 1945–1948.

Lever, I.J., Bradbury, E.J., Cunningham, J.R., Adelson, D.W., Jones, M.G., McMahon, S.B., Marvizon, J.C., and Malcangio, M. (2001). Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. J. Neurosci. *21*, 4469–4477.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*, 1154–1162.

Lewin, G.R., and Barde, Y.A. (1996). Physiology of the neurotrophins. Annu. Rev. Neurosci. *19*, 289–317.

Loh, Y.P. (1988). The effect of pepstatin A, an inhibitor of the pro-opiomelanocortin (POMC)-converting enzyme, on POMC processing in mouse intermediate pituitary. FEBS Lett. *238*, 142–146.

Loh, Y.P., Kim, T., Rodriguez, Y.M., and Cawley, N.X. (2004). Secretory granule biogenesis and neuropeptide sorting to the regulated secretory pathway in neuroendocrine cells. J. Mol. Neurosci. *22*, 63–71.

Lu, B. (2003). BDNF and activity-dependent synaptic modulation. Learn. Mem. *10*, 86–98.

Lu, B. (2004). Acute and long-term synaptic modulation by neurotrophins. Prog. Brain Res. *146*, 137–150.

Luo, X.G., Rush, R.A., and Zhou, X.F. (2001). Ultrastructural localization of brain-derived neurotrophic factor in rat primary sensory neurons. Neurosci. Res. *39*, 377–384.

McDonald, N.Q., and Blundell, T.L. (1991). Crystallization and characterization of the high molecular weight form of nerve growth factor (7 S NGF). J. Mol. Biol. *219*, 595–601.

Michael, G.J., Averill, S., Nitkunan, A., Rattray, M., Bennett, D.L., Yan, Q., and Priestley, J.V. (1997). Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. J. Neurosci. *17*, 8476–8490.

Mizuno, M., Yamada, K., Olariu, A., Nawa, H., and Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. J. Neurosci. *20*, 7116–7121.

Moller, J.C., Kruttgen, A., Heymach, J.V., Jr., Ghori, N., and Shooter, E.M. (1998). Subcellular localization of epitope-tagged neurotrophins in neuroendocrine cells. J. Neurosci. Res. *51*, 463–472.

Mowla, S.J., Pareek, S., Farhadi, H.F., Petrecca, K., Fawcett, J.P., Seidah, N.G., Morris, S.J., Sossin, W.S., and Murphy, R.A. (1999). Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J. Neurosci. *19*, 2069–2080.

Mowla, S.J., Farhadi, H.F., Pareek, S., Atwal, J.K., Morris, S.J., Seidah, N.G., and Murphy, R.A. (2001). Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J. Biol. Chem. *276*, 12660–12666.

Normant, E., and Loh, Y.P. (1998). Depletion of carboxypeptidase E, a regulated secretory pathway sorting receptor, causes misrouting and constitutive secretion of proinsulin and proenkephalin, but not chromogranin A. Endocrinology *139*, 2137–2145.

Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L., and Lu, B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science *306*, 487–491.

Patapoutian, A., and Reichardt, L.F. (2001). Trk receptors: mediators of neurotrophin action. Curr. Opin. Neurobiol. *11*, 272–280.

Poo, M.M. (2001). Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. *2*, 24–32.

Robinson, R.C., Radziejewski, C., Stuart, D.I., and Jones, E.Y. (1995). Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry *34*, 4139–4146.

Seidah, N.G., Benjannet, S., Pareek, S., Chretien, M., and Murphy, R.A. (1996). Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett. *379*, 247–250.

Shen, F.S., Aguilera, G., and Loh, Y.P. (1999). Altered biosynthesis and secretion of pro-opiomelanocortin in the intermediate and anterior pituitary of carboxypeptidase E-deficient, Cpe(fat)/ Cpe(fat)-mice. Neuropeptides *33*, 276–280.

Smith, M.M., Shi, L., and Navre, M. (1995). Rapid identification of highly active and selective substrates for stromelysin and matrilysin using bacteriophage peptide display libraries. J. Biol. Chem. *270*, 6440–6449.

Thoenen, H. (2000). Neurotrophins and activity-dependent plasticity. Prog. Brain Res. *128*, 183–191.

Wang, L.C., Meijer, H.K., Humbel, B.M., Jenks, B.G., and Roubos, E.W. (2004). Activity-dependent dynamics of coexisting brain-derived neurotrophic factor, pro-opiomelanocortin and alpha-melanophore-stimulating hormone in melanotrope cells of Xenopus laevis. J. Neuroendocrinol. *16*, 19–25.

Wu, Y.J., Kruttgen, A., Moller, J.C., Shine, D., Chan, J.R., Shooter, E.M., and Cosgaya, J.M. (2004). Nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 are sorted to dense-core vesicles and released via the regulated pathway in primary rat cortical neurons. J. Neurosci. Res. *75*, 825–834.

Zhang, C.F., Snell, C.R., and Loh, Y.P. (1999). Identification of a novel prohormone sorting signal-binding site on carboxypeptidase E, a regulated secretory pathway-sorting receptor. Mol. Endocrinol. *13*, 527–536.

Zhang, C.F., Dhanvantari, S., Lou, H., and Loh, Y.P. (2003). Sorting of carboxypeptidase E to the regulated secretory pathway requires interaction of its transmembrane domain with lipid rafts. Biochem. J. *369*, 453–460.