DR. AGUSTIN ANASTASIA (Orcid ID : 0000-0001-7238-9041)

Received Date : 29-Dec-2016

Revised Date : 08-Feb-2017

Accepted Date : 13-Feb-2017

Article type : Review

Title: Growth factors and hormones pro-peptides: the unexpected adventures of the BDNF prodomain (pBDNF)

Authors: Juan Pablo Zanin[1], Nicolás Unsain[2], Agustin Anastasia[2*].

Affiliations: [1] Department of Biological Sciences, Rutgers University, Newark, NJ 07102, United States, [2] Instituto de Investigación Médica Mercedes y Martin Ferreyra, (INIMEC-CONICET- Universidad Nacional de Córdoba). Friuli 2434, Córdoba, Córdoba 5016. Argentina.

*** Corresponding author:** Agustin Anastasia (aanastasia@immf.uncor.edu).

Running title (45 characters): Growth factors prodomains

Keywords (6): pBDNF, proBDNF, Active prodomains, Biosynthetic pathway, Precursor cleavage peptides, Val66Met polymorphism.

ARRIVE guidelines have been followed:
Yes
=> if No, skip complete sentence

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jnc.13993

=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines."

Acknowledgements: We would like to acknowledge the support of the International Society for Neurochemistry (ISN) and Committee for Aid and Education in Neurochemistry (CAEN) Return Home Award (AA and NU), International Brain Research Organization (IBRO) Return Home Fellowships (AA), NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (AA), and the PICT-FONCyT grants from the Argentinean Ministry of Science and Technology (MINCyT) (AA and NU).

The authors have no conflicts of interest to declare.

Abbreviations

proBDNF: pro-protein of the neurotrophin brain-derived neurotrophic factor

pBDNF: BDNF prodomain

mBDNF: mature BDNF

tPA: tissue plasminogen activator

MMPs: matrix metalloproteinases

NGF: nerve growth factor

NT-3: Neurotrophin 3

SNP: single nucleotide polymorphism

Val66Met: valine (Val) to methionine (Met) substitution at codon 66

VWF: von Willebrand factor

TGFβ: Transforming growth factor β

BMP: Bone morphogenetic protein

PDGF: platelet-derived growth factor

P75NTR: p75 neurotrophin receptor

LTD: long-term depression

SorCS2: Vps10p-domain sorting receptor 2

POMC: preproopiomelanocortin

GPR146: G-protein coupled receptor 146
NMR: nuclear magnetic resonance
CD: circular dichroism
NMDA: N-methyl-D-aspartate receptor
TrkB: tropomyosin receptor kinase B
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
GDNF: glial cell line-derived neurotrophic factor
DNSP: dopamine neuron stimulating peptide
JNK: c-Jun N-terminal kinase
NR2B: N-methyl D-aspartate receptor subtype 2B
RhoA: Ras homolog
Rock: Rho-associated, coiled-coil containing protein kinase
PTEN: Phosphatase and tensin homolog
JAK: Janus kinase
STAT: Signal transducer and activator of transcription
ICER: Inducible cAMP early repressor
PI4K: Phosphatidylinositol 4-kinase

Abstract

Most growth factors and hormones are synthesized as pre-pro-proteins which are processed to the biologically active mature protein. The pre- and prodomains are cleaved from the precursor protein in the secretory pathway or, in some cases, extracellularly. The canonical functions of these prodomains are to assist in folding and stabilization of the mature domain, to direct intra and extracellular localization, to facilitate storage, and to regulate bioavailability of their mature counterpart. Recently, exciting evidence has revealed that prodomains of certain growth factors, after cleaved from the precursor pro-protein, can act as independent active signaling molecules. In this review, we discuss the various classical functions of prodomains, and the biological consequences of these pro-peptides acting as ligands. We will focus our attention on

the BDNF prodomain (pBDNF), which has been recently described as a novel secreted ligand influencing neuronal morphology and physiology.

Introduction

Most growth factors and hormones are synthetized as pre-pro-proteins. These prefixes are used to describe the intermediate processed forms of a protein before it is active and at the appropriate location. The prefix “pre” indicates the presence of an N-terminal sequence that targets the precursor pro-protein to the secretory pathway or an intracellular compartment. For this reason, this sequence is referred to as “signal peptide” or “signal sequence”. Signal peptides, which are typically from 5 to 50 residues, are cleaved by signal peptidases and quickly degraded. Later, it was discovered that many proteins require additional proteolytic processing to become functional. This additional step involves cleavage of a subsequent N-terminal peptide, which is indicated by the prefix "pro" accounting for prodomain (or pro-peptide; example in Figure 1A). The processing of pro-proteins to remove their prodomains requires enzymes including pro-protein convertases (or proconvertases), which are present in the Golgi apparatus or in secretory vesicles, or extracellular enzymes such as matrix metalloproteinases among others. For example, the pro-protein of the neurotrophin brain-derived neurotrophic factor (proBDNF), can be cleaved by furin and other proconvertases in the trans-Golgi network or secretory vesicles (Teng *et al.* 2010, Mowla *et al.* 2001). In addition, proBDNF can be processed extracellularly by plasmin generated by tissue plasminogen activator (tPA) or by selective matrix metalloproteinases (MMPs) including MMP3, MMP7 (Lee *et al.* 2001) and MMP9 (Mizoguchi *et al.* 2011) to release the prodomain (pBDNF) and mature BDNF (mBDNF) (reviewed in Mizui *et al.* 2016, Hempstead 2015) (Figure 1A). In many cases more than one biologically active cleavage product is generated from one pro-protein precursor. Prodomains were thought to merely assist mature domains during biosynthesis (see below in “established prodomains functions”). However, recent evidence demonstrates that prodomains can be independent active peptides which often display different biological activities than other cleavage products from the precursors. Discussing the recent literature, this review highlights new

biological functions of growth factor and hormone prodomains beyond the classical roles in the biosynthetic pathway.

Established prodomains functions

In general, prodomains can (1) promote protein stabilization and proper folding, (2) direct intracellular trafficking, (3) participate in protein storage, (4) regulate extracellular distribution, or (5) shield mature domains from receptors to limit bioavailability. These biological roles are discussed below.

(1) Many proteins fold poorly or are misfolded in the absence of their prodomains. For that reason, they are often referred to as "intramolecular chaperones" (Inouye 1991). *In vitro* folding studies suggested that prodomains stabilize folding intermediate states. Prodomains presumably work as scaffold peptides which guide the intermediate, low energy states, to the native active conformation (Rattenholl *et al.* 2001b). As an example, the nerve growth factor (NGF) prodomain was found to be essential for the proper folding of active mature NGF (mNGF). Mutagenesis of a critical region comprising amino acids 67 to 94 (27 residues) within the NGF prodomain sequence showed impaired expression of biologically active mNGF (Suter *et al.* 1991). Moreover, the NGF prodomain has been shown to enhance mNGF folding yields and kinetics, and to assist in its disulfide bond formation (Rattenholl et al. 2001b). The same study suggested that the NGF prodomain function would be to facilitate the formation of the characteristic cysteine knot of neurotrophins (He & Garcia 2004, Gong *et al.* 2008, Feng *et al.* 2010, Wiesmann *et al.* 1999) and/or would passively confer solubility to the folding intermediates shielding hydrophobic patches to enhance folding yields. The NGF prodomain also augments the yield and rate of refolding of mNGF from *Escherichia coli* inclusion bodies (Rattenholl *et al.* 2001a). In addition, the mNGF domain can in turn stabilize the structure of its prodomain (Kliemannel *et al.* 2004). Prodomain-dependent folding of the mature sequences can occur in "trans". For instance, the neurotrophin 3 (NT-3) prodomain can promote structure and folding of mNGF (Hauburger *et al.* 2007). The relevance of this prodomain interchangeability remains to be clarified *in vivo* and would only be biologically relevant when these two proteins are co-expressed in the

same local. The prodomain-dependent folding in "trans" suggests that the prodomains' chaperone activity is conserved in the neurotrophin family.

(2) Prodomains might interact with chaperones to direct their intracellular trafficking to their final subcellular destination. A very well documented example of this process is the interaction of the BDNF prodomain with the Vps10p domain protein sortilin in the trafficking of its precursor proBDNF. Chen and collaborators have found that sortilin interacts specifically with residues 44 to 103 of the prodomain (Chen *et al.* 2005). Utilizing different mutants for the transmembrane and intracellular domains of sortilin they showed that proBDNF was misslocalized as it was more diffusely distributed in the cell body with minimal staining in the processes. Moreover, mutant-sortilin transfected cells exhibited decreased colocalization of proBDNF with the secretory granule maker SecII (Chen et al. 2005). This sortilin-proBDNF interaction was shown to regulate activity-dependent secretion of mBDNF (Chen et al. 2005). Other proteins such as the somatostatin and myeloperoxidase require their prodomains for targeting of the mature protein to the regulated secretory pathway (Sevarino *et al.* 1989, Andersson *et al.* 1998).

A common single nucleotide polymorphism (SNP) in the human BDNF gene leads to a nucleotide change from a guanine to an adenine at position 196 (G196A) that results in a valine (Val) to methionine (Met) substitution at codon 66 (Val66Met) in the middle of the pBDNF sequence (Figure 1A). This SNP is observed in more than 25% of the human population (Database of Single Nucleotide Polymorphisms-National Center for Biotechnology Information, National Library of Medicine, 2012), and is associated with alterations in fear memory regulation and enhanced risk for depression and anxiety disorders as well as with some neurodegenerative disorders in humans (Hajek *et al.* 2012, Egan *et al.* 2003, Frielingsdorf *et al.* 2010, Soliman *et al.* 2010, Verhagen *et al.* 2010, Martinowich *et al.* 2007, Dincheva *et al.* 2012). Interestingly, the Met proBDNF variant exhibits decreased binding to sortilin, altered intracellular trafficking, and a reduction in the activity-dependent secretion of mBDNF (Egan et al. 2003, Chen et al. 2005, Chen *et al.* 2004). Moreover, knock-in mice that express the Val66Met SNP recapitulates specific phenotypic properties of the human polymorphism (Chen *et al.* 2006). Thus, their proposed mechanism by which Met allele mediates central nervous

system effects is indirect, by a reduction in activity-dependent release of mBDNF leading to altered synaptic plasticity. A complementary mechanism for the deleterious function of the Met allele has been reported (Anastasia *et al.* 2013) and will be discussed later in this review.

(3) Prodomains are thought to optimize storage space in granules and vesicles by promoting multimerization and aggregation of the pro-proteins and/or mature peptides. Moreover, this aggregation might prevent proteolytic enzymes access and augment proteins half-life. For instance, the prodomain of von Willebrand factor (VWF), a glycoprotein that participates in wound healing and coagulation, is needed for the proper processing and storage of the mature protein. The VWF prodomain is required for multimerization of the VWF and to target this factor to storage granules, while the mature VWF expressed alone is not stored. However, the expression of the mature and prodomain together either in cis (in one polypeptide) or in trans (in two separate polypeptides) results in the granular storage of both moieties (Haberichter 2015).

(4-5) Transforming growth factor β (TGFβ) family members are also synthetized as precursors (proTGFβs), with an N-terminal prodomain and a mature domain, which are processed intracellularly by furin and proconvertases. Interestingly, the prodomains continue to interact with their mature domains after cleavage of the precursor, and even after secretion. This interaction is noncovalent and maintains the molecules in a conformation capable of dimerization. For many TGFβ family members the mature-prodomain association persists in the extracellular matrix where the prodomain interacts with some of its components such as fibrillin and perlecan. The interaction of prodomains with the matrix components assists the mature TGFβ domains to localize near their target cells. Another consequence of this prodomain-mature domain interaction is to confer latency of some TGFβ family members. The prodomains of TGFβ1, TGFβ2, TGFβ3, myostatin, growth differentiation factor 11 (GDF11) and bone morphogenetic protein 10 (BMP10) bind with enough affinity to their mature domains that are capable of suppressing their biological activity, adding a step at which the ligand's availability can be regulated. On the other hand, the prodomains of other BMPs do not bind strongly enough to prevent the mature domain from interacting with their receptors and trigger signaling (Gregory *et al.* 2005, Sengle *et al.* 2011, Bidart *et al.*

2012). Recently it has been proposed that there is an “open-armed” structure that does not preclude the activity of BMP9 interacting with its prodomain, while the “cross-armed” feature observed in TGFβ1-prodomain complex confers the latent conformation (Mi *et al.* 2015). The authors also suggest that interactors in the extracellular matrix stabilize a cross-armed conformation and regulate the transition between cross-armed (latent) and open-armed (nonlatent) conformations of some TGFβ family members (Mi et al. 2015). Another mechanism has been found for the release of mature domains of TGFβs proteins from the inhibitory interaction with prodomains: activation of TGFβ1 requires the binding of integrin to an arginine/glycine/aspartic acid (RGD) sequence within the prodomain sequence and exertion of force on this domain to disengage both peptides and release the active mature protein (Shi *et al.* 2011). Besides these proposed mechanisms, activation of other TGFβ family members depend on the cell type and context, but always requires targeting of their prodomains. In all, prodomains control many aspects of TGFβ family biology as they regulate the activation of these multi-functional cytokines. A recent structural study demonstrates that also platelet-derived growth factor (PDGF) stably binds to its prodomain. The regions of interaction between the mature and prodomain are those that mediate the interface with its receptor (Shim *et al.* 2010). Thus, this report suggests that the PDGF prodomain-mature domain complex may prevent receptor activation, and implies that the inhibitory-effects of prodomains can be conserved (to some degree).

Prodomains as part of active pro-protein precursors.

Besides the classic prodomain functions described above, prodomains can also be part of biologically active precursors. Some precursors have biological functions and act via receptors that are different from those of their cleaved mature domains. These active pro-proteins include the precursor of cerebellin, the family of chromogranins/secretogranins, proapolipoprotein (apo) A1, procorticotrophin-releasing hormone, progastrin, prograstrin-releasing peptide, parathyroid hormone (PTH)-related protein, proenkephalin and the proneurotrophins (reviewed in Dicou 2008). The proneurotrophins, from which we have already described some aspects of proNGF and proBDNF above, is a family of precursors that were discovered to signal independently

and with distinct functions from their mature counterparts (Lee et al. 2001). While mature neurotrophins bind a tropomyosin-related kinase (Trk) receptor to promote neuronal survival, differentiation, neurogenesis and synaptic plasticity, the proneurotrophins proNGF and proBDNF, interact with a receptor complex of p75 neurotrophin receptor (p75NTR) and sortilin (a Vps10p-domain sorting receptor) to induce apoptosis and morphological changes in cell lines, smooth muscle cells, Schwann cells, and primary neurons in culture (Lee et al. 2001, Nykjaer *et al.* 2004, Teng *et al.* 2005, Ibanez 2002, Song *et al.* 2010, Willnow *et al.* 2008, Jansen *et al.* 2007, Je *et al.* 2012). Likewise, the unprocessed neurotrophin 3 (proNT3) triggers apoptosis in inner ear neurons and sympathetic neurons which co-express p75NTR and sortilin (Tauris *et al.* 2011, Yano *et al.* 2009). Application of proBDNF to p75NTR-expressing hippocampal or entorhinal slices enhanced long-term depression (LTD) (Woo et al., 2005) and suppressed persistent firing (Gibon *et al.* 2015), respectively. These results suggest that proBDNF can also act as an endogenous ligand to directly regulate LTD and to control neuron excitability. Recently, Yang and collaborators showed that a cleavage-resistant proBDNF knock-in mouse displays decreased hippocampal dendritic complexity, reduced spine density and enhanced LTD in the CA1 region of the hippocampus (Yang *et al.* 2014). These results confirm previous observations by Woo and collaborators (Woo *et al.* 2005), and suggest that proBDNF acts *in vivo* regulating hippocampal structure and plasticity. ProBDNF functions are summarized in Table 1. A cleavage-resistant proNGF knock-in mouse exhibits cardiac microvascular endothelial activation, a decrease in pericyte process length, and increased vascular permeability, leading to lethal cardiomyopathy in adulthood (Siao *et al.* 2012). Genetic approaches were conducted by Yang and collaborators and by Siao and collaborators to confirm that the observed phenotypes of the cleavage-resistant proBDNF and proNGF mouse lines were a gain of function of the precursors and not a consequence of the expected decrease in the mature forms (Siao et al. 2012, Yang et al. 2014). Interestingly, recent reports have shown that proBDNF (Anastasia et al. 2013, Sun *et al.* 2012) as well as proNGF (Deinhardt *et al.* 2011) induce growth cone retraction in cultured neurons by interacting with p75NTR and the sortilin family member Vps10p-domain sorting receptor 2 (SorCS2).

Multiple active cleavage products from a pro-protein precursor

Pre-pro-proteins can give rise to multiple active products generated after processing. Some interesting examples includes the insulin precursor preproinsulin and the proopiomelanocortin (POMC) precursor pre-POMC. Preproinsulin is a single polypeptide synthetized in pancreatic β-cells that holds a signal peptide (amino acid 1-24), followed by the insulin B chain (amino acids 25-54), a prodomain named C peptide (amino acids 57-87) and finally by insulin A chain (amino acids 90-110). After removal of the signal peptide, proinsulin undergoes cleavage by proconvertases and carboxypeptidase E to release the A and the B chains which are linked by 2 disulfide bonds to form mature insulin, and the C peptide (Bell *et al.* 1980, Steiner & Oyer 1967). The C peptide participates in the assembly and processing of insulin as a prodomain, but it has been shown to be a bioactive peptide on its own. Recent evidence suggests that the G-protein coupled receptor 146 (GPR146) is the C peptide receptor (Yosten *et al.* 2013). In particular, the C peptide promotes some anti-inflammatory, anti-oxidant and cell protective mechanisms (reviewed in Wahren & Larsson 2015). Pre-POMC is a hormone precursor that undergoes several tissue-specific processing via cleavage by proconvertases to give rise to multiple active peptides (Raffin-Sanson *et al.* 2003). Thus, it is considered a polyprotein. The active cleavage products include the N-terminal peptide of POMC, α- β- γ-melanotropin, corticotropin, corticotropin-like intermediate peptide, β- and γ-lipotropin, β-endorphin and met-enkephalin. The functions of these independent peptides range from pigmentation (melanotropins), production and secretion of cortisol (corticotropin), to activation of opioid receptors (β-endorphin and met-enkephalin) (reviewed in Cawley *et al.* 2016, Chretien & Mbikay 2016).

The origin and selective pressures that give rise to all these important peptides in one gene and under the control of one set of promoters remains to be elucidated. The finding of multiple active cleavage products from a pro-protein precursor like POMC challenges the field to begin to explore other cleaved peptides from hormones and growth factors which were thought (with little or no evidence) to be degraded or non-functional after cleavage from a known functional domain.

A prodomain as an independent signaling ligand: pBDNF

A previous study failed to detect the isolated BDNF prodomain (pBDNF) (Yang *et al.* 2009); thus, it was thought to be rapidly degraded after processing of the proBDNF precursor. However, Dieni and collaborators found for the first time the isolated pBDNF in brain tissue (Dieni *et al.* 2012). They suggested that pBDNF was not previously found because it was washed away from the western blot membranes after transference. They found that by fixing the transferred membranes with glutaraldehyde, they were able to reliably detect the pBDNF. In this context, it has been well-established that fixatives crosslink peptides to the transfer membrane and thus allow the retention and detection of some low molecular weight proteins (Karey & Sirbasku 1989, Kurien & Scofield 2009). Dieni and collaborators also showed that BDNF isoforms including pBDNF are trafficked and stored in dense core vesicles like other growth factors and hormones (Dieni et al. 2012). During late embryonic and early postnatal mice development, the expression of pBDNF in the hippocampus is negligible. However, the pBDNF is detected at postnatal day 5 (P5), and its expression increases significantly at 1 month plateauing in adult mice (3–9 months) (Anastasia et al. 2013). This is in agreement with previous findings that mBDNF and pBDNF are the most abundant moieties during adulthood compared to proBDNF which is abundant during development (Dieni et al. 2012, Yang et al. 2009, Yang et al. 2014, Anastasia et al. 2013, Rauskolb *et al.* 2010). Subsequently, others have also detected pBDNF in brain tissues utilizing the same fixation technique and independent antibodies (Mizui *et al.* 2015, Lee *et al.* 2015, Guo *et al.* 2016, Yang *et al.* 2016, Lim *et al.* 2015).

After Dieni and collaborators found the isolated pBDNF, we asked for the first time if this peptide could function as an independent signaling molecule (Anastasia et al. 2013). First, we showed that pBDNF is secreted in an activity dependent manner from hippocampal neurons in culture (Anastasia et al. 2013), similar to mBDNF (Goodman *et al.* 1996) and proBDNF (Yang et al. 2009, Nagappan *et al.* 2009). Inhibition of extracellular cleavage of secreted proBDNF using α-2 anti-plasmin and an inhibitor of numerous MMPs did not significantly change levels of secreted prodomain from

neuronal culture, suggesting that most of the cleavage of proBDNF to pBDNF and mBDNF might occur in intracellular compartments (Anastasia et al. 2013).

The high sequence conservation (pBDNF sequence is highly conserved in more than 70 species examined to date) and the fact that pBDNF can be secreted from neurons prompted to ask whether this protein can act as an active ligand. As discussed previously there is a human SNP in BDNF leading to a Val66Met substitution within the pBDNF sequence and highly correlated with the occurrence of neuropsychiatric disorders. Thus, it was attractive to evaluate if the Val and/or the Met-pBDNFs were active, and if they stimulated different biological functions. Up to date, three studies have addressed these questions. The first study (1) found that only the Met variant of pBDNF acutely alters neuronal morphology as this ligand induces growth cone retraction and collapse. In this study the Val-pBDNF was inert as it did not affect growth cone shape (Anastasia et al. 2013). The second study (2) showed that the Val-pBDNF facilitate hippocampal long-term depression, while the Met-pBDNF variant prevented this effect (Mizui et al. 2015). The third report (3) found that the Val-pBDNF reduces dendritic spine density in hippocampal neurons (Guo et al. 2016). The key findings of these three publications are analyzed below and summarized in Table 1.

(1) The proper development and establishment of functional neuronal networks rely, in part, on the emergence, path finding and retractions of neuronal processes. Growth cones at the tip of growing neurites are actin-rich structures which can sense the environment to seek for their synaptic target. Disruption of the growth cone's normal pathfinding can lead to altered circuits that can ultimately affect human behavior, including pathological conditions such as neuropsychiatric disorders. Anastasia and collaborators found for the first time that the pBDNF is an active independent signaling molecule: the Met variant of pBDNF (10ng/ml) is capable of altering the normal development of growth cones in cultured neurons inducing freezing, retraction and collapse of these structures (Figure 1B, C). This effect is mediated by Met-pBDNF engaging to a complex of two unrelated receptors: p75NTR and the SorCS2 (Anastasia et al. 2013). Interestingly, both pBDNF variants appear to be intrinsically disordered proteins with some transient structural features as detected using nuclear magnetic resonance (NMR) and circular dichroism (CD). However, both variants have specific

conformational changes due to the substitution. In the residues neighboring the Val66Met substitution the Val-pBDNF has more tendency to β-structure while the Met variant is more helical (represented in Figure 1B, C). These structural difference results in a differential interaction with the SorCS2 receptor which can explain their difference in activity. Other consequences of the Val66Met substitution on the biology of pBDNF have yet to be unveiled and may include differences in bio-availability, in intracellular trafficking, and/or in aggregation. If corroborated *in vivo*, Met-pBDNF-induced growth cone collapse during brain development could give rise to changes in circuitry that can explain why the Val66Met SNP carriers are more prone to developing neuropsychiatric disorders. Therefore, it would be an additional molecular explanation for the Val66Met phenotypes together with the loss function of mBDNF proposed earlier (Chen et al. 2006). The Val-pBDNF was inert in growth cones even at higher concentrations (Anastasia et al. 2013) and the role of this variant in developing neurons requires further studies. A question that arises from this study is how p75NTR and SorCS2 receptors interact to transduce the Met-pBDNF signal. It has been shown that p75NTR and SorCS2 cooperate to transduce proNGF signaling (Deinhardt et al. 2011). In that case, proNGF prodomain binds to SorCS2 while the mature moiety of the precursor binds to p75NTR forming a trimeric signaling unit. The expression of p75NTR is necessary for triggering pBDNF signaling but this ligand only interacts with SorCS2 (Anastasia et al. 2013). It has been shown that P75NTR and SorCS2 complexes are present even in the absence of ligands (Deinhardt et al. 2011, Glerup *et al.* 2014). Thus, we hypothesize that the Met-pBDNF/SorCS2 complex somehow activates p75NTR to induce growth cone retraction, but further experiments are required to test this possibility.

(2) Another function for pBDNF was shown by Mizui and collaborators in that they demonstrated that the addition of Val-pBDNF (10ng/ml) facilitates long-term depression (LTD) induction in hippocampal slices (Mizui et al. 2015). This effect requires the activation of N-methyl-D-aspartate (NMDA) receptors containing the GluN2B subunit and the p75NTR receptor, but not the tropomyosin receptor kinase B (TrkB). The endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is an important event in the expression of LTD and is triggered by NMDA receptor activation (Carroll *et al.* 2001). The authors proposed that the Val-

pBDNF increases GluN2B expression at the cell surface which then promotes the internalization of the AMPA receptor's GluA1 and GluA2 subunits, thus, resulting in LTD facilitation (Figure 1D). Moreover, they demonstrated that NMDA and the Val-pBDNF act additively to induce GluA2 internalization and LTD facilitation and that blocking the NMDA receptor prevented the ligand's effect. These results suggest that Val-pBDNF is upstream of the NMDA receptor on the signaling cascade inducing this neuroplasticity effect. Interestingly, the Met-pBDNF failed to induce LTD facilitation, and also prevented the NMDA-induced AMPA receptors internalization (Figure 1E). These differences between the variants open the question of how the Val version is active while the Met-pBDNF is inert, and which is/are the co-receptor/s involved, other than p75NTR.

(3) Guo and collaborators tested the Val-pBDNF in mature primary hippocampal neurons and showed that this ligand induces a reduction of dendritic spines density (Figure 1F) (Guo et al. 2016). Notably, this effect was evident 24 hours after the administration of the ligand, but not earlier. They did not test the effects of the Met-pBDNF on dendritic spines. Later on, the authors performed yeast two-hybrid, cross-linking and pull downs followed by mass spectrometry, and protein-protein interaction chips to shed light on the mechanism underlying this morphological effect. Surprisingly, they could not detect pBDNF interactions with SorCS2 which is the only reported receptor for the isolated prodomain demonstrated by co-immunoprecipitation and nuclear magnetic resonance (Anastasia et al. 2013). The p75NTR receptor, which is somehow required to trigger the pBDNF effects (Anastasia et al. 2013, Mizui et al. 2015), does not interact directly with pBDNF (Anastasia et al. 2013). Using those unbiased methods, Guo and collaborators supported this observation. Unfortunately, the interaction screening data is not available in their report. They propose that the mechanism for the Val-pBDNF effect on dendritic spines includes a mitochondrial pathway. In particular, they showed that the Val-pBDNF induces mitochondrial elongation, release of cytochrome c to the cytosol, and finally caspase 3 activation which alters dendritic spine number. It is important to mention that Guo and collaborators utilized 50 ng/ml of recombinant Val-pBDNF for their studies. Anastasia and collaborators showed that 50 ng/ml of the Val-pBDNF remains inactive without showing significant growth cone retraction (Anastasia et al. 2013).

One of the major challenges in the study of prodomain biology is the difficulty of generating antibodies that exclusively recognize the pro sequence, but not the precursor pro-protein which obviously includes the prodomain. Previous studies have used a monoclonal antibody (mAb287, available at GeneCopoeia, Rockville, MD, USA) (Yang et al. 2009, Anastasia et al. 2013, Mizui et al. 2015), or a polyclonal (ANT-006 from Alomone Labs, Jerusalem, Israel) (Dieni et al. 2012) to detect pBDNF, although both antibodies were raised against proBDNF. It's important to mention that different batches of the Alomone proBDNF antibody have shown variability in detecting pBDNF as it has successfully detected pBDNF in a study (Dieni et al. 2012), but it detected an unspecific 18-19 kDa band in another work (Anastasia et al. 2013). Guo and collaborators generated an antibody specifically against pBDNF which recognizes an epitope in generated after furin processing of proBDNF (Guo et al. 2016). The authors claim that this antibody does not recognize proBDNF. Using this antibody they confirmed previously reported data showing the detection of pBDNF in hippocampal neurons and the release of pBDNF in an activity dependent manner.

In addition to these three initial publications, the biological actions of pBDNF have been recently studied by others. Increased pBDNF levels have been correlated with stress resilience (Yang et al. 2016), and with nicotine withdrawal-induced anxiety (Lee et al. 2015). Interestingly, there is a synergistic interaction between the amyloid-β and Met-pBDNF in the induction of toxicity of SH-SY5Y human neuroblastoma cell lines (Lim et al. 2015). However, these experiments were performed with very high Met-pBDNF concentrations (200ng/ml), most likely out of the physiological range. Lim and collaborators also showed that pBDNF levels are significantly increased in a subset of post-mortem hippocampal lysates from advanced Alzheimer's disease patients (Lim et al. 2015). These interesting results suggest, for the first time, that pBDNF could be a therapeutic target involved in the pathogenesis of the disease.

In addition to pBDNF, the only other neurotrophin prodomain that has been evaluated for biological activity is the NGF prodomain. The NGF prodomain has been shown to induce death of PC12 cells (Armugam *et al.* 2012). However, the NGF prodomain was inert on the induction of LTD (Mizui et al. 2015) and did not affect dendritic spines density in primary neuronal cultures (Guo et al. 2016). The significance

of these findings is relative as the endogenous NGF prodomain has not been found yet in neural tissues or primary neurons. Apart from the neurotrophin family, another growth factor prodomain has been tested for activity: fragments of the glial cell line-derived neurotrophic factor (GDNF) prodomain, carrying the term dopamine neuron stimulating peptides (DNSP), has also been shown to be active as they display robust neurotrophic actions similar to mature GDNF (Bradley *et al.* 2010, Stenslik *et al.* 2015). DNPSs and mature GDNF promote similar actions in neurons, in contrast to pBDNF and mBDNF which display opposing effects.

Conclusions and perspectives

All these reports together support the idea that several growth factors and hormones prodomains have novel and overlooked physiological and/or pathological functions. In general, prodomains were thought to be transient species, which were quickly degraded after cleavage of their respective precursor pro-proteins. However, recent reports have demonstrated that prodomains and mature domains co-exist and both peptides can be secreted to the extracellular space. Interestingly, some of these prodomains are part of active precursors while some have independent (even antagonistic) functions. The BDNF pro-peptide (pBDNF) was undetected for some time despite efforts developed by several groups (Yang et al. 2009 and unpublished experiments). The combination of specific antibodies and crosslinking of the proteins to the transfer membrane after western blotting allowed the detection of significant amounts of pBDNF in neuronal tissues and primary neurons. In the future, other isolated growth factors and hormones prodomains may be discovered in the nervous system using this biochemical technique together with new specific antibodies.

BDNF is one of the most widely expressed trophic factors in the nervous system (Conner *et al.* 1997). Its functions ranges from survival and differentiation of neural stem cell, neuron survival, axon-dendrite differentiation, synapse formation and maintenance, to development and regulation of neural circuits (Park & Poo 2013). BDNF is also expressed and active in non-neuronal cells such as endothelial cells, smooth muscle cells, immune cells, and epithelial cells in different organs (Kermani & Hempstead 2007,

Anastasia *et al.* 2014). As pBDNF and mBDNF are stored and secreted from the same dense core vesicles (Dieni et al. 2012), we speculate that pBDNF is a potential active ligand in every cell and tissue where mBDNF has been shown to be released. Thus, it is very important to study the presence of this isolated prodomain and new potential local actions in these scenarios. Moreover, functions previously attributed to proBDNF should be reconsidered and/or reinterpreted in light of the existence of pBDNF, which is more abundant than the precursor during adulthood.

The main question that arises from active prodomains is why two or more proteins with different or even opposing effects are synthetized from the same gene or exon. A possible answer is that the biological functions regulated by these gene products are extremely important for the homeostasis of the nervous system. Thus, different peptides with different functions can precisely regulate the fine-tuning of the system. We propose that for some hormones and growth factors the nomenclature "prodomain" and "mature domain" should be revisited because fragments that were considered merely to aid the synthetizes of an active domains are bioactive by themselves.

Figure legend

Figure1: BDNF precursor (proBDNF) structure and BDNF prodomain (pBDNF) effects on neurons. (A) Pre-pro-BDNF domains. The prefix "pre" indicates the presence of signal peptide (SP). The prefix "pro" points the prodomain (also named or pro-peptide) which is also cleaved enzymatically. BDNF prodomain: pBDNF. Mature BDNF: mBDNF. There is a human single nucleotide polymorphism inducing a substitution of a valine in position 66 for a methionine within the prodomain sequence (Val66Met). N- = amino-terminus; C- = carboxyl-terminus. (B-F) Three independent laboratories have studied pBDNF activity. (B-C) The first study found that only the Met variant of pBDNF acutely alters neuronal morphology inducing changes in neuron growth cones (correlated with a decrease in Rac activity), while the Val-pBDNF was inert (Anastasia et al. 2013). The Val-pBDNF is drawn with tendency to β-structure, while the Met version with some helical structure. (D-E) The second study showed that the Val-pBDNF facilitate

hippocampal long-term depression (LTD) by inducing an increase in surface expression of the GluN2B subunit of the NMDA receptor and the consequent AMPA internalization. The Met variant prevented this effect (Mizui et al. 2015). (F) The third report found that the Val-pBDNF reduces dendritic spine density in hippocampal neurons through a pathway involving mitochondrial release of cytochrome c (CytoC) and caspase 3 (Casp3) activation (Guo et al. 2016).

Table 1

Ligand	Action	Target	Ligand source	Proposed mechanism	Reference
proBDNF (precursor)	Neuronal death	Primary sympathetic superior cervical ganglia neurons (*in vitro*)	Recombinant protein addition (4ng/ml)	Activation of a p75NTR and sortilin complex	(Teng et al. 2005)
		PC12 cells (in vitro) and sensory dorsal root ganglia neurons (after axotomy, *in vivo*)	Recombinant protein addition (*in vitro* 10ng/ml, *in vivo* 10μg placed on the sciatic nerve lesion site)	P75NTR and sortilin are suggested to be involved.	(Fan *et al.* 2008)
		Primary cerebellar granule neurons (*in vitro*)	Recombinant protein addition (10-100ng/ml)	P75NTR and JNK are required. Rac1 and caspase 3 activation are suggested to be involved.	(Koshimizu *et al.* 2009, Koshimizu *et al.* 2010)
		Primary motoneuron (*in vitro*)	Muscle conditioned media	P75NTR and sortilin are required. Caspase activation is suggested to be involved.	(Taylor *et al.* 2012)
	Facilitation of long-term depression	Hippocampal slices (*ex vivo*)	Recombinant protein addition (2ng/ml)	Activation of P75NTR which potentiates the NR2B component of the NMDA currents	(Woo et al. 2005)
			Endogenous expression (cleavage resistant proBDNF knock-in mice)		(Yang et al. 2014)
	Reduction in neurite growth/complexity and dendritic spine density	Primary basal forebrain cholinergic neurons, primary hippocampal neurons (*in vitro*)	Recombinant protein addition (10ng/ml)		(Koshimizu et al. 2009)
		Hippocampal neurons (*in vivo*)	Endogenous expression (cleavage resistant proBDNF knock-in mice)	P75NTR is required.	(Yang et al. 2014)
	Gabaergic synaptic activity modulation	Hippocampal and cortical slices (*ex vivo*), and primary hippocampal neurons (*in vitro*)	Recombinant protein addition (*ex vivo* 10ng/ml, *in vitro* 25ng/ml) and in utero electroporation	RhoA-Rock-PTEN pathway and the JAK-STAT-ICER pathway. P75NTR is required for some effects.	(Langlois *et al.* 2013, Riffault *et al.* 2014, Riffault *et al.* 2016)
	Attenuation of synaptic transmission	Hippocampal slices (*ex vivo*)	Recombinant protein addition (50ng/ml)		(Koshimizu et al. 2009)
			Endogenous expression (cleavage resistant proBDNF knock-in mice)		(Yang et al. 2014)
	Control of cortical persistent firing	Entorhinal slices (*ex vivo*)	Recombinant protein addition (2ng/ml)	P75NTR-Rac1-PI4K pathway.	(Gibon et al. 2015)
pBDNF	Growth cone	Primary hippocampal	Recombinant protein	Differential interaction	(Anastasia

(prodomain)	retraction (only the Met variant, Val variant was inert)	neurons (*in vitro*)	addition (10ng/ml)	with the SorCS2 receptor and Rac1 inactivation. P75NTR is required.	et al. 2013)
	Facilitation of long-term depression (only Val variant; Met variant prevented the effect)	Hippocampal slices (*ex vivo*)	Recombinant protein addition (10ng/ml)	Increase surface expression of GluN2B and AMPA subunits internalization. P75NTR is required.	(Mizui et al. 2015)
	Reduction in dendritic spine density (only Val variant; Met variant was not tested)	Primary hippocampal neurons (*in vitro*)	Recombinant protein addition (50ng/ml)	Release of cytochrome c and caspase 3 activation	(Guo et al. 2016)

Table title and legend

Table 1: Summary of the BDNF precursor (proBDNF) and the isolated BDNF prodomain (pBDNF) actions. Given the breadth of this field, it is difficult to include all contributions, and oversights are unintended.

References

Anastasia, A., Deinhardt, K., Chao, M. V., Will, N. E., Irmady, K., Lee, F. S., Hempstead, B. L. and Bracken, C. (2013) Val66Met polymorphism of BDNF alters prodomain structure to induce neuronal growth cone retraction. *Nature communications,* **4,** 2490.

Anastasia, A., Deinhardt, K., Wang, S. et al. (2014) Trkb signaling in pericytes is required for cardiac microvessel stabilization. *PLoS One,* **9,** e87406.

Andersson, E., Hellman, L., Gullberg, U. and Olsson, I. (1998) The role of the propeptide for processing and sorting of human myeloperoxidase. *The Journal of biological chemistry,* **273,** 4747-4753.

Armugam, A., Koh, D. C., Ching, C. S., Chandrasekaran, K., Kaur, P. and Jeyaseelan, K. (2012) Pro-domain in precursor nerve growth factor mediates cell death. *Neurochem.Int.,* **60,** 852-863.

Bell, G. I., Pictet, R. L., Rutter, W. J., Cordell, B., Tischer, E. and Goodman, H. M. (1980) Sequence of the human insulin gene. *Nature,* **284,** 26-32.

Bidart, M., Ricard, N., Levet, S. et al. (2012) BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. *Cell Mol Life Sci,* **69,** 313-324.

Bradley, L. H., Fuqua, J., Richardson, A. et al. (2010) Dopamine neuron stimulating actions of a GDNF propeptide. *PLoS One,* **5,** e9752.

Carroll, R. C., Beattie, E. C., von Zastrow, M. and Malenka, R. C. (2001) Role of AMPA receptor endocytosis in synaptic plasticity. *Nature reviews. Neuroscience,* **2,** 315-324.

Cawley, N. X., Li, Z. and Loh, Y. P. (2016) 60 YEARS OF POMC: Biosynthesis, trafficking, and secretion of pro-opiomelanocortin-derived peptides. *J Mol Endocrinol,* **56,** T77-97.

Chen, Z. Y., Ieraci, A., Teng, H., Dall, H., Meng, C. X., Herrera, D. G., Nykjaer, A., Hempstead, B. L. and Lee, F. S. (2005) Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *J Neurosci.,* **25,** 6156-6166.

Chen, Z. Y., Jing, D., Bath, K. G. et al. (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science,* **314,** 140-143.

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.

Chretien, M. and Mbikay, M. (2016) 60 YEARS OF POMC: From the prohormone theory to pro-opiomelanocortin and to proprotein convertases (PCSK1 to PCSK9). *J Mol Endocrinol,* **56,** T49-62.

Conner, J. M., Lauterborn, J. C., Yan, Q., Gall, C. M. and Varon, S. (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **17,** 2295-2313.

Database of Single Nucleotide Polymorphisms (dbSNP). National Center for Biotechnology Information, National Library of Medicine. http://www.ncbi.nlm.nih.gov/SNP/.(2012)

Deinhardt, K., Kim, T., Spellman, D. S., Mains, R. E., Eipper, B. A., Neubert, T. A., Chao, M. V. and Hempstead, B. L. (2011) Neuronal growth cone retraction relies on proneurotrophin receptor signaling through Rac. *Sci Signal.,* **4,** ra82.

Dicou, E. (2008) Biologically active, non membrane-anchored precursors: an overview. *FEBS J,* **275,** 1960-1975.

Dieni, S., Matsumoto, T., Dekkers, M. et al. (2012) BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. *J Cell Biol.,* **196,** 775-788.

Dincheva, I., Glatt, C. E. and Lee, F. S. (2012) Impact of the BDNF Val66Met Polymorphism on Cognition: Implications for Behavioral Genetics. *Neuroscientist.*

Egan, M. F., Kojima, M., Callicott, J. H. et al. (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell,* **112,** 257-269.

Fan, Y. J., Wu, L. L., Li, H. Y., Wang, Y. J. and Zhou, X. F. (2008) Differential effects of pro-BDNF on sensory neurons after sciatic nerve transection in neonatal rats. *Eur.J Neurosci.,* **27,** 2380-2390.

Feng, D., Kim, T., Ozkan, E., Light, M., Torkin, R., Teng, K. K., Hempstead, B. L. and Garcia, K. C. (2010) Molecular and structural insight into proNGF engagement of p75NTR and sortilin. *J.Mol.Biol.,* **396,** 967-984.

Frielingsdorf, H., Bath, K. G., Soliman, F., Difede, J., Casey, B. J. and Lee, F. S. (2010) Variant brain-derived neurotrophic factor Val66Met endophenotypes: implications for posttraumatic stress disorder. *Ann N.Y.Acad Sci,* **1208,** 150-157.

Gibon, J., Buckley, S. M., Unsain, N., Kaartinen, V., Seguela, P. and Barker, P. A. (2015) proBDNF and p75NTR Control Excitability and Persistent Firing of Cortical Pyramidal Neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **35,** 9741-9753.

Glerup, S., Olsen, D., Vaegter, C. B. et al. (2014) SorCS2 regulates dopaminergic wiring and is processed into an apoptotic two-chain receptor in peripheral glia. *Neuron,* **82,** 1074-1087.

Gong, Y., Cao, P., Yu, H. J. and Jiang, T. (2008) Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. *Nature,* **454,** 789-793.

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.

Gregory, K. E., Ono, R. N., Charbonneau, N. L., Kuo, C. L., Keene, D. R., Bachinger, H. P. and Sakai, L. Y. (2005) The prodomain of BMP-7 targets the BMP-7 complex to the extracellular matrix. *The Journal of biological chemistry,* **280,** 27970-27980.

Guo, J., Ji, Y., Ding, Y., Jiang, W., Sun, Y., Lu, B. and Nagappan, G. (2016) BDNF pro-peptide regulates dendritic spines via caspase-3. *Cell death & disease,* **7,** e2264.

Haberichter, S. L. (2015) von Willebrand factor propeptide: biology and clinical utility. *Blood,* **126,** 1753-1761.

Hajek, T., Kopecek, M. and Hoschl, C. (2012) Reduced hippocampal volumes in healthy carriers of brain-derived neurotrophic factor Val66Met polymorphism: meta-analysis. *World J Biol.Psychiatry,* **13,** 178-187.

Hauburger, A., Kliemannel, M., Madsen, P., Rudolph, R. and Schwarz, E. (2007) Oxidative folding of nerve growth factor can be mediated by the pro-peptide of neurotrophin-3. *FEBS Lett,* **581,** 4159-4164.

He, X. L. and Garcia, K. C. (2004) Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science,* **304,** 870-875.

Hempstead, B. L. (2015) Brain-Derived Neurotrophic Factor: Three Ligands, Many Actions. *Trans Am Clin Climatol Assoc,* **126,** 9-19.

Ibanez, C. F. (2002) Jekyll-Hyde neurotrophins: the story of proNGF. *Trends Neurosci.,* **25,** 284-286.

Inouye, M. (1991) Intramolecular chaperone: the role of the pro-peptide in protein folding. *Enzyme,* **45,** 314-321.

Jansen, P., Giehl, K., Nyengaard, J. R. et al. (2007) Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. *Nat Neurosci,* **10,** 1449-1457.

Je, H. S., Yang, F., Ji, Y., Nagappan, G., Hempstead, B. L. and Lu, B. (2012) Role of pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF conversion in activity-dependent competition at developing neuromuscular synapses. *Proceedings of the National Academy of Sciences of the United States of America,* **109,** 15924-15929.

Karey, K. P. and Sirbasku, D. A. (1989) Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for western blot analysis. *Anal.Biochem.,* **178,** 255-259.

Kermani, P. and Hempstead, B. (2007) Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. *Trends Cardiovasc Med,* **17,** 140-143.

Kliemannel, M., Rattenholl, A., Golbik, R., Balbach, J., Lilie, H., Rudolph, R. and Schwarz, E. (2004) The mature part of proNGF induces the structure of its pro-peptide. *FEBS Lett,* **566,** 207-212.

Koshimizu, H., Hazama, S., Hara, T., Ogura, A. and Kojima, M. (2010) Distinct signaling pathways of precursor BDNF and mature BDNF in cultured cerebellar granule neurons. *Neurosci Lett,* **473,** 229-232.

Koshimizu, H., Kiyosue, K., Hara, T. et al. (2009) Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. *Mol Brain,* **2,** 27.

Kurien, B. T. and Scofield, R. H. (2009) A brief review of other notable protein detection methods on blots. *Methods Mol Biol.,* **536,** 557-571.

Langlois, A., Diabira, D., Ferrand, N., Porcher, C. and Gaiarsa, J. L. (2013) NMDA-dependent switch of proBDNF actions on developing GABAergic synapses. *Cereb Cortex,* **23,** 1085-1096.

Lee, B. G., Anastasia, A., Hempstead, B. L., Lee, F. S. and Blendy, J. A. (2015) Effects of the BDNF Val66Met Polymorphism on Anxiety-Like Behavior Following Nicotine Withdrawal in Mice. *Nicotine Tob Res,* **17,** 1428-1435.

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

Lim, J. Y., Reighard, C. P. and Crowther, D. C. (2015) The pro-domains of neurotrophins, including BDNF, are linked to Alzheimer's disease through a toxic synergy with Abeta. *Hum Mol Genet,* **24,** 3929-3938.

Martinowich, K., Manji, H. and Lu, B. (2007) New insights into BDNF function in depression and anxiety. *Nat.Neurosci.,* **10,** 1089-1093.

Mi, L. Z., Brown, C. T., Gao, Y., Tian, Y., Le, V. Q., Walz, T. and Springer, T. A. (2015) Structure of bone morphogenetic protein 9 procomplex. *Proceedings of the National Academy of Sciences of the United States of America,* **112,** 3710-3715.

Mizoguchi, H., Nakade, J., Tachibana, M. et al. (2011) Matrix metalloproteinase-9 contributes to kindled seizure development in pentylenetetrazole-treated mice by converting pro-BDNF to mature BDNF in the hippocampus. *J Neurosci.,* **31,** 12963-12971.

Mizui, T., Ishikawa, Y., Kumanogoh, H. and Kojima, M. (2016) Neurobiological actions by three distinct subtypes of brain-derived neurotrophic factor: Multi-ligand model of growth factor signaling. *Pharmacol Res,* **105,** 93-98.

Mizui, T., Ishikawa, Y., Kumanogoh, H. et al. (2015) BDNF pro-peptide actions facilitate hippocampal LTD and are altered by the common BDNF polymorphism Val66Met. *Proceedings of the National Academy of Sciences of the United States of America,* **112,** E3067-3074.

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.

Nagappan, G., Zaitsev, E., Senatorov, V. V., Jr., Yang, J., Hempstead, B. L. and Lu, B. (2009) Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. *Proc.Natl.Acad Sci U.S.A,* **106,** 1267-1272.

Nykjaer, A., Lee, R., Teng, K. K. et al. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature,* **427,** 843-848.

Park, H. and Poo, M. M. (2013) Neurotrophin regulation of neural circuit development and function. *Nature reviews. Neuroscience,* **14,** 7-23.

Raffin-Sanson, M. L., de Keyzer, Y. and Bertagna, X. (2003) Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Eur J Endocrinol,* **149,** 79-90.

Rattenholl, A., Lilie, H., Grossmann, A., Stern, A., Schwarz, E. and Rudolph, R. (2001a) The pro-sequence facilitates folding of human nerve growth factor from Escherichia coli inclusion bodies. *Eur J Biochem,* **268,** 3296-3303.

Rattenholl, A., Ruoppolo, M., Flagiello, A., Monti, M., Vinci, F., Marino, G., Lilie, H., Schwarz, E. and Rudolph, R. (2001b) Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. *Journal of molecular biology,* **305,** 523-533.

Rauskolb, S., Zagrebelsky, M., Dreznjak, A. et al. (2010) Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. *J Neurosci.,* **30,** 1739-1749.

Riffault, B., Kourdougli, N., Dumon, C. et al. (2016) Pro-Brain-Derived Neurotrophic Factor (proBDNF)-Mediated p75NTR Activation Promotes Depolarizing Actions of GABA and Increases Susceptibility to Epileptic Seizures. *Cereb Cortex*.

Riffault, B., Medina, I., Dumon, C., Thalman, C., Ferrand, N., Friedel, P., Gaiarsa, J. L. and Porcher, C. (2014) Pro-brain-derived neurotrophic factor inhibits GABAergic neurotransmission by activating endocytosis and repression of GABAA receptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **34,** 13516-13534.

Sengle, G., Ono, R. N., Sasaki, T. and Sakai, L. Y. (2011) Prodomains of transforming growth factor beta (TGFbeta) superfamily members specify different functions: extracellular matrix interactions and growth factor bioavailability. *The Journal of biological chemistry,* **286,** 5087-5099.

Sevarino, K. A., Stork, P., Ventimiglia, R., Mandel, G. and Goodman, R. H. (1989) Amino-terminal sequences of prosomatostatin direct intracellular targeting but not processing specificity. *Cell,* **57,** 11-19.

Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T. and Springer, T. A. (2011) Latent TGF-beta structure and activation. *Nature,* **474,** 343-349.

Shim, A. H., Liu, H., Focia, P. J., Chen, X., Lin, P. C. and He, X. (2010) Structures of a platelet-derived growth factor/propeptide complex and a platelet-derived growth factor/receptor complex. *Proceedings of the National Academy of Sciences of the United States of America,* **107,** 11307-11312.

Siao, C. J., Lorentz, C. U., Kermani, P. et al. (2012) ProNGF, a cytokine induced after myocardial infarction in humans, targets pericytes to promote microvascular damage and activation. *The Journal of experimental medicine,* **209,** 2291-2305.

Soliman, F., Glatt, C. E., Bath, K. G. et al. (2010) A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science,* **327,** 863-866.

Song, W., Volosin, M., Cragnolini, A. B., Hempstead, B. L. and Friedman, W. J. (2010) ProNGF induces PTEN via p75NTR to suppress Trk-mediated survival signaling in brain neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **30,** 15608-15615.

Steiner, D. F. and Oyer, P. E. (1967) The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. *Proceedings of the National Academy of Sciences of the United States of America,* **57,** 473-480.

Stenslik, M. J., Potts, L. F., Sonne, J. W. et al. (2015) Methodology and effects of repeated intranasal delivery of DNSP-11 in a rat model of Parkinson's disease. *J Neurosci Methods,* **251,** 120-129.

Sun, Y., Lim, Y., Li, F., Liu, S., Lu, J. J., Haberberger, R., Zhong, J. H. and Zhou, X. F. (2012) ProBDNF Collapses Neurite Outgrowth of Primary Neurons by Activating RhoA. *PLoS.One.,* **7,** e35883.

Suter, U., Heymach, J. V., Jr. and Shooter, E. M. (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. *Embo j,* **10,** 2395-2400.

Tauris, J., Gustafsen, C., Christensen, E. I. et al. (2011) Proneurotrophin-3 may induce Sortilin-dependent death in inner ear neurons. *Eur J Neurosci,* **33,** 622-631.

Taylor, A. R., Gifondorwa, D. J., Robinson, M. B., Strupe, J. L., Prevette, D., Johnson, J. E., Hempstead, B., Oppenheim, R. W. and Milligan, C. E. (2012) Motoneuron programmed cell death in response to proBDNF. *Dev Neurobiol,* **72,** 699-712.

Teng, H. K., Teng, K. K., Lee, R. et al. (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J.Neurosci.,* **25,** 5455-5463.

Teng, K. K., Felice, S., Kim, T. and Hempstead, B. L. (2010) Understanding proneurotrophin actions: Recent advances and challenges. *Dev.Neurobiol.,* **70,** 350-359.

Verhagen, M., van der, M. A., van Deurzen, P. A., Janzing, J. G., rias-Vasquez, A., Buitelaar, J. K. and Franke, B. (2010) Meta-analysis of the BDNF Val66Met polymorphism in major depressive disorder: effects of gender and ethnicity. *Mol Psychiatry,* **15,** 260-271.

Wahren, J. and Larsson, C. (2015) C-peptide: new findings and therapeutic possibilities. *Diabetes Res Clin Pract,* **107,** 309-319.

Wiesmann, C., Ultsch, M. H., Bass, S. H. and de Vos, A. M. (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature,* **401,** 184-188.

Willnow, T. E., Petersen, C. M. and Nykjaer, A. (2008) VPS10P-domain receptors - regulators of neuronal viability and function. *Nat.Rev.Neurosci.,* **9,** 899-909.

Woo, N. H., Teng, H. K., Siao, C. J., Chiaruttini, C., Pang, P. T., Milner, T. A., Hempstead, B. L. and Lu, B. (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nat Neurosci,* **8,** 1069-1077.

Yang, B., Yang, C., Ren, Q., Zhang, J. C., Chen, Q. X., Shirayama, Y. and Hashimoto, K. (2016) Regional differences in the expression of brain-derived neurotrophic factor (BDNF) pro-peptide, proBDNF and preproBDNF in the brain confer stress resilience. *Eur Arch Psychiatry Clin Neurosci,* **266,** 765-769.

Yang, J., Harte-Hargrove, L. C., Siao, C. J. et al. (2014) proBDNF negatively regulates neuronal remodeling, synaptic transmission, and synaptic plasticity in hippocampus. *Cell Rep,* **7,** 796-806.

Yang, J., Siao, C. J., Nagappan, G. et al. (2009) Neuronal release of proBDNF. *Nat.Neurosci.,* **12,** 113-115.

Yano, H., Torkin, R., Martin, L. A., Chao, M. V. and Teng, K. K. (2009) Proneurotrophin-3 is a neuronal apoptotic ligand: evidence for retrograde-directed cell killing. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* **29,** 14790-14802.

Yosten, G. L., Kolar, G. R., Redlinger, L. J. and Samson, W. K. (2013) Evidence for an interaction between proinsulin C-peptide and GPR146. *J Endocrinol,* **218,** B1-8.