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Retrograde-Induced Cell Death:
Role of proNGF

Tânia Marisa Melim Perestrelo

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Ramiro Almeida (Centro de Neurociências e Biologia Celular) e do Professor Doutor Carlos Duarte (Centro de Neurociências e Biologia Celular)

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Agradecimentos

Gostaria de começar por agradecer a toda a minha família, especialmente aos meus pais e à minha irmã por toda a confiança em mim depositada, carinho e acima de tudo incentivo! Obrigada pela vossa luta diária com o intuito de eu conseguir alcançar os meus sonhos!

Um muito obrigado a todos os meus amigos da Madeira, Monte (da Caparica) e de Coimbra por toda a boa disposição, pela imensa força que me transmitiram ao longo deste ano e por toda a ajuda prestada. Contudo, não poderia deixar de fazer um agradecimento especial à Sofia, Filipa e Maria Joana porque, tenho a certeza, que sem a vossa ajuda esta tese não seria o que é hoje!

Gostaria igualmente de agradecer a todos os membros do laboratório por toda a ajuda e, por tornarem o laboratório num sítio tão agradável. Um obrigado especial ao grupo RDA por todo o incentivo, boa disposição e ajuda!

Agradeço ao Professor Carlos Duarte e à Professora Ana Luísa Carvalho pela disponibilidade e pelos conhecimentos transmitidos.

Um agradecimento especial ao Doutor Ramiro Almeida pela excelente orientação. Muito obrigada pela ajuda, preocupação, por todo o conhecimento transmitido e acima de tudo pelo entusiasmo com que encontra sempre o lado positivo de cada experiência.

Finalmente, ao Mário, um muitíssimo obrigado por estares sempre presente!

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Abbreviations

5-FDU	5-fluoro-2'deoxiuridina
AD	Alzheimer disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ATF-6	Activating transcription factor-6
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BF	Basal forebrain
BH domain	Bcl-2 homology
BSA	Bovine serum albumin
CAD	Caspases-activated DNase
CLAP	Chymostatin, pepstatin, antipain and leupeptin
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle's Medium
DRG	Dorsal root ganglion
ECF™	Enhanced Chemifluorecense substrate
ER	Endoplasmic reticulum
FADD	Fas-associated protein with a death domain
FasL	Fas ligand
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAP	Inhibitor of apoptosis proteins
ICAD	Inhibitor of caspases-activated DNase
IP_3R	Inositol triphosphate receptor
IgG	Immunoglobulin G
IRE1	Inositol-requiring protein-1
JNK	C-jun N-terminal kinase
LTD	Long term depression
LTP	Long term potentiation
mBDNF	Mature brain-derived neurotrophic factor

mNGF	Mature nerve growth factor
MMP-9	Matrix metalloproteinase 9
mPTP	Mitochondrial permeability transition pore
Na_2HPO_4	Sodium phosphate dibasic solution
NaCl	Sodium Chloride
NGF	Nerve Growth factor
NF-kB	Nuclear factor-kappaB
NMDA	N-methyl-D-aspartic acid
NRH2	Neurotrophin receptor homolog 2
NRIF	Neurotrophin receptor interacting factor
NTFs	Neurotrophins
NT3	Neurotrophin 3
NT4	Neurotrophin 4
$p75^{NTR}$	75 kDa neurotrophin receptor
PCD	Programmed cell death
PDMS	Poly-dimethylsiloxane mold chamber
PERK	Protein kinase RNA-like ER Kinase
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
proNGF	Pro-nerve growth factor
proNT3	Pro-neurotrophin 3
SCG	Superior cervical ganglia
SDS	Sodium dodecyl sulfate
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
Smac	Second mitochondrial activator of caspases
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGS	Tris-glycine-SDS
TIMP-1	Tissue inhibitor metalloproteinase 1
TNF	Tumor necrosis factor
TNF-R	TNF receptor
tPA	Tissue plasminogen activator
TRADD	TNF-receptor associated via death domain
Trk	Tropomyosin-related kinase

UPR Unfolded protein response

UPS Ubiquitin proteasome system

Abstract

Proneurotrophins are believed to play an important biological role in the nervous system. At first, proneurotrophins were thought to be simple inactive precursors, only responsible for promoting the folding of the mature domain and the regulation of the neurotrophin secretory pathway. However, recent evidences showed that proneurotrophins can be secreted to the extracellular space, binding with high affinity to the $p75^{NTR}$-sortilin receptor complex and inducing cell death in different neuronal populations.

The release of proNGF is known to play a pivotal role in neuronal death. There are evidences suggesting that upon injury there is an increase of proNGF levels in the extracellular environment, which might lead to the activation of the apoptotic machinery in smooth muscle cells, oligodendrocytes and neurons. However, neurons are highly polarized cells characterized by a complex network of processes and the spatial and temporal action of proneurotrophins is still not fully understood.

In the present work we investigated the localized action of proNGF on DRG neurons. Using microfluidic chambers, small devices capable of physically and fluidically isolate neuronal subcompartments, proNGF elicited axonal fragmentation when added specifically to the axons. Moreover, the axonal degeneration induced by proNGF override the survival factors present in the cell body. These results present the first evidence that a local insult with proNGF can induce axonal degeneration, raising the possibility that a retrograde death signal might be generated in distal axons.

Keywords: proNGF, axonal degeneration, neuronal cell death, proNGF expression, microfluidic chambers.

Resumo

Acredita-se que as proneurotrofinas têm um importante papel biológico no sistema nervoso. Inicialmente, as proneurotrofinas eram consideradas simples precursores inactivos, responsáveis apenas por promover o *folding* do domínio maduro e pela regulação da via de secreção das neurotrofinas. Contudo, evidências recentes demonstram que as proneurotrofinas podem ser secretadas para o espaço extracelular, ligando-se com grande afinidade ao complexo de receptores $p75^{NTR}$-sortilina e induzindo morte celular em diferentes populações neuronais.

Sabe-se que a libertação do proNGF tem um papel central na morte neuronal. Diferentes indícios sugerem que após um insulto existe um aumento dos níveis de proNGF no meio extracelular, o que pode levar à activação da maquinaria apoptótica em células do músculo liso, oligodendrócitos e neurónios. Contudo, os neurónios são células altamente polarizadas, caracterizadas por uma rede complexa de prolongamentos e a acção temporal e espacial das proneurotrofinas não é totalmente compreendida.

No presente trabalho investigou-se a acção localizada do proNGF em neurónios de *DRG*. Utilizando câmaras microfluídicas, pequenos dispositivos capazes de isolar fisicamente e fluidicamente subcompartimentos neuronais, o proNGF induziu fragmentação axonal quando especificamente adicionado ao compartimento axonal. Além disso, a degeneração axonal induzida pelo proNGF sobrepôs-se aos factores de sobrevivência presentes no corpo celular. Estes resultados constituem a primeira

evidência de que um insulto localizado com proNGF pode induzir degeneração axonal, levantando à hipótese de que um sinal de morte retrógrado possa ser gerado nos axónios distais.

Palavras chave: proNGF, degeneração axonal, morte neuronal, expressão de proNGF, câmaras microfluídicas

Chapter 1

Introduction

1.1. Cell Death Mechanisms

Cell death mechanisms are cellular responses that have the crucial role of regulating tissue development and homeostasis by eliminating unwanted or damaged cells. The balance between cell proliferation and cell death is responsible for regulating and controlling the number of cells in an organism (Degterev and Yuan, 2008).

Cell death has historically been subdivided into regulated (programmed cell death) and unregulated mechanisms. The ability to undergo programmed cell death (PCD) is a built-in latent capacity in all cells of multicellular organisms. Defects in cell death pathways are associated with a wide variety of illnesses, including autoimmune diseases, neurodegenerative disorders and cancer (Pollard and Earnshaw, 2008).

The term PCD, first introduced by Lockshin and colleagues in 1964, describes a genetically controlled form of cell death, mediated by an intracellular program (Lockshin and Zakeri, 2001). Although over the years PCD has often been equated with apoptosis, it is important to remind that there are also non-apoptotic forms of PCD such as autophagy, anoikis and pyroptosis (Bergsbaken et al., 2009).

1.1.1. Apoptosis

The first form of PCD to be characterized was apoptosis. Although it was first referred in 1972 by Kerr and colleagues, in 1885 Flemming had already used the term chromatolysis to describe the chemical changes within the cells during cell death as a part of physiological function (Clarke and Clarke, 1996).

Apoptosis is the most well-defined type of cell death pathway, consisting on a two-stage process divided in latent and execution phases (Pollard and Earnshaw, 2008). The latent

phase, triggered by pro-apoptotic signals, is characterized by the actively preparations for death, though the cells still looks morphologically normal. According to Mignotte and colleagues, this phase can be subdivided into two stages: condemned and committed (Kroemer et al., 1995). These stages occur sequentially and lead the cells towards death, but during the condemned stage the cells can still be rescued if exposed to survival factors. Conversely, no rescue is possible when the committed stage takes place.

Ultimately, the cells enter the execution phase of apoptosis. In this phase, the activated apoptotic machinery leads to dramatic morphologic and physiological changes: intercellular junction's loss, cytoplasm shrinkage, dramatic changes in cytoplasmic motility, plasma membrane asymmetry loss (phosphatidylserine is flipped onto the cellular surface), enlargement of the nucleolus and formation of aggregates (subjected to cleavage by endonucleases) as a result of nuclear chromatin condensation near the nuclear membrane. These changes lead to the formation of apoptotic bodies, structures that are surrounded by an intact plasma membrane and contain remnants of the nucleus, mitochondria and other organelles. To finalize the process, apoptotic bodies are engulfed by phagocytic cells through recognition of cell surface markers. A central characteristic of this recognition is the absence of inflammatory response, since it could have deleterious effects on the surrounding cells (Blank and Shiloh, 2007)

1.1.1.1. Signals and pathways of apoptosis

In mammalian cells, the apoptotic response leading to cell death is tightly regulated and can be mediated by an intrinsic or an extrinsic pathway, depending on the death stimulus' origin (Pollard and Earnshaw, 2008).

The intrinsic pathway involves central death machinery located at mitochondria and endoplasmic reticulum (ER)(Movassagh and Foo, 2008). It can be activated by a wide variety of extracellular and intracellular stimuli including growth factors deprivation, toxins, radiation, hypoxia, oxidative stress, cytoskeleton damage, DNA damage and excessive activation of factors that promote cell-cycle progression (Ghiotto et al., 2010).

The mitochondrial events inducing cell death are regulated by the Bcl-2 family of proteins, which form a complex network of checks and balances crucial on cell fate control (Breckenridge and Xue, 2004). These proteins are characterized by the presence of one to four conserved motifs, the Bcl-2 homology (BH) domains, and can be grouped into three subfamilies: Bcl-2 killers, Bcl-2 regulators and Bcl-2 protectors (Walensky, 2006) (Figure 1).

Bax and Bak, two of the killer proteins, are essential for the activation of this pathway. During a death stimuli, Bax and Bak are deeply inserted into the mitochondrial outer membrane and form oligomers, which possibly leads to membrane pore formation, consequently inducing mitochondrial membrane permeabilization (Zamzami and Kroemer, 2003, Breckenridge and Xue, 2004). This event, in turn, enables the release of pro-apoptotic factors from the mitochondrial intermembrane such as cytochrome c, Smac (second mitochondrial activator of caspases) and endonucleases G to the cytoplasm (Ghiotto et al., 2010). The release of cytochrome c triggers a series of events centered on caspases activity (Figure 2).

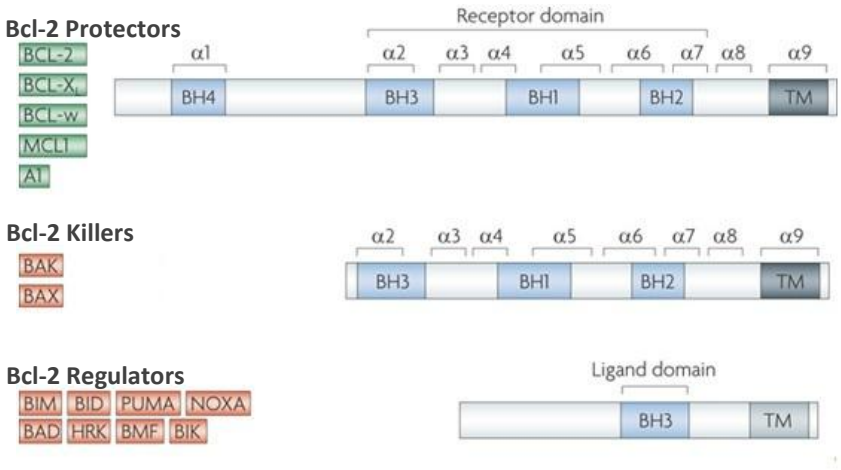


Figure 1 – The Bcl-2 protein family. This family presents homology in specific regions, called Bcl-2 homology domains and is divided in: (1) Bcl-2 protectors, anti-apoptotic proteins that keep mitochondria integrity, regulate the behavior of Bcl-2 killers and inhibit caspases activation, protecting cells from apoptosis. Bcl-2 protectors share the four BH domains and the three-dimensional fold (Degterev et al., 2008); (2) Bcl-2 killers, pro-apoptotic proteins, that share three BH domains (BH1, BH2 and BH3) and are essential for the intrinsic pathway; (3) Bcl-2 regulators, also called BH3 only, share only the BH3 domain. Bcl-2 regulators are pro-apoptotic proteins that appear to function as death promoters by interfering with the Bcl-2 protectors or activating the Bcl-2 killers and amplifying the cell death signal (Degterev et al., 2008). BH1/BH2/BH3/BH4 – Bcl-2 homology-1/2/3/4 domain; TM – transmembrane domain (adapted from (Lessene et al., 2008).

In the cytoplasm, cytochrome c binds the scaffolding protein Apaf-1. In the absence of cytochrome c, Apaf-1 exists in an autoinhibited monomeric state, promoted by its C-terminal portion. After cytochrome c binding, there is a conformational change which induces dATP/ATP hydrolysis and formation of an Apaf-1 heptamer-cytochrome c complex, the apoptosome (Ledgerwood and Morison, 2009). In this state, Apaf-1 caspase recruitment domains are free to bind procaspase-9 monomers promoting its activation, which then leads to procaspase-3 zymogen cleavage (Riedl and Shi, 2004). In its active form, this caspase cleaves other effector caspases, thus amplifying the cell death cascade. Effector caspases cleave the BH3 domain of Bid, a Bcl-2 regulator protein, and this protein induces the activation of other Bcl-2 killer proteins like Bax

and Bad in a positive feedback loop, thereby promoting the release of more cytochrome c and Smac and enhancing caspase-9 activation (Pollard and Earnshaw, 2008) (Figure 2).

In addition to mitochondria, the ER also appears to participate in the initiation of apoptosis. Its participation in this process occurs when this organelle is extensively damaged and takes place via two different mechanisms: calcium signaling and unfolded protein response (UPR)(Movassagh and Foo, 2008).

The calcium present in the ER is taken up from the cytoplasm by the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and released through the inositol triphosphate receptor (IP_3R) (Szegezdi et al., 2009) The disturbance of calcium levels can promote the accumulation of unfolded or misfolded proteins in the ER. The continuous accumulation of this proteins leads to ER stress and UPR activation, which induces key proteins involved in chaperoning, protein folding, and degradation pathways, in an attempt to restore homeostasis (Szegezdi et al., 2009). However, when ER stress is insurmountable, apoptosis is activated. In addition to UPR activation, calcium homeostasis disturbances can trigger apoptosis by direct induction of mitochondrial membrane permeabilization and mitochondrial apoptogens release (Movassagh and Foo, 2008, Szegezdi et al., 2009).

The extrinsic pathway is another mechanism that can activate apoptotic response. This pathway is triggered by extracellular ligands, such as Fas ligand (FasL) and tumor necrosis factor (TNF), which are expressed on the surface of other cells. These ligands bind to cell surface molecules, termed death receptors, activating them and turning on a pathway that leads to apoptotic death (Ashkenazi and Dixit, 1998).

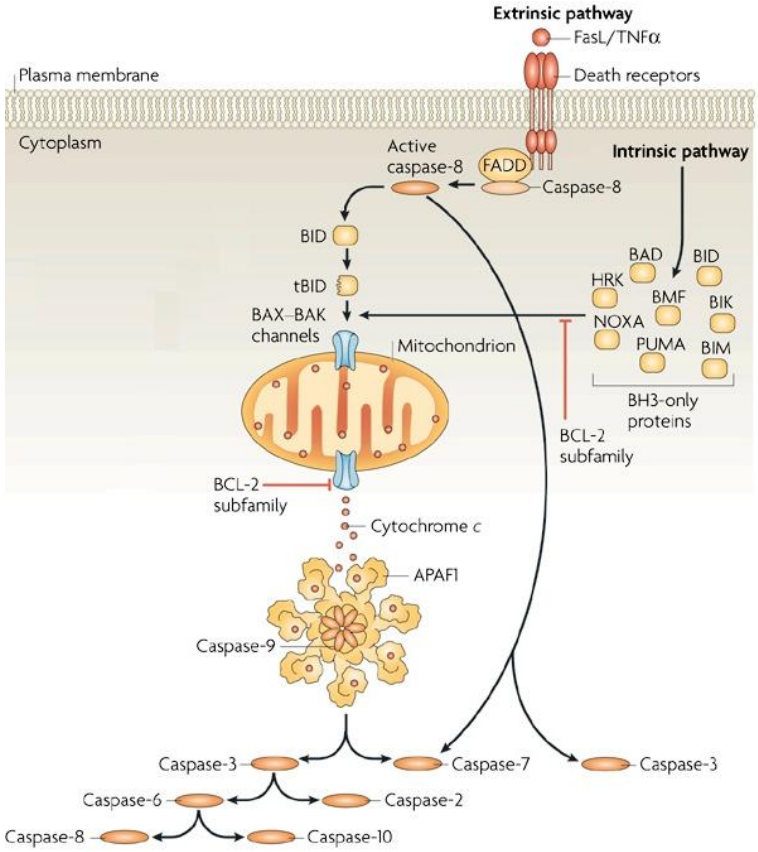


Figure 2 – Intrinsic versus extrinsic apoptotic pathways. The intrinsic pathway is activated by diverse stimuli that lead to cell damage. This activates one or more members of Bcl-2 regulator proteins that, above a crucial threshold, can overcome the inhibitory effect of the Bcl-2 protector proteins and promote the assembly of the Bcl-2 killer proteins, BAK-BAX oligomers, within mitochondrial outer membranes. These oligomers promote cytochrome c release to the cytoplasm which, in turn, induces the apoptosome formation. The initiator caspase of the intrinsic pathway, procaspase-9, binds to the apoptosome, gets activated and cleaves the effector caspase-3, promoting further caspases activation. On the other hand, the extrinsic pathway is initiated by binding of extracellular death ligands to transmembrane death receptor which promotes the assembly of a complex formed by the death receptor and the adaptor FADD. This complex creates a platform that brings together molecules of procaspase-8, the initiator caspase of the extrinsic pathway, and enables their autoprocessing and activation. Active caspase-8 then activates caspase-3 and caspase-7, promoting further caspases activation which culminates in cell death. In some situations, the two pathways are interconnected by tBID that is formed when BID, a Bcl-2 regulator protein, is cleaved by active caspase-8. FADD - Fas-associated death-domain protein; tBID – truncated BID (adapted from (Taylor et al., 2008).

Fas and TNF-receptor (TNF-R) are death receptors, members of the tumor necrosis factor receptor family. They have a single transmembrane domain and a cytoplasmic domain that contains a death domain of about 80 residues, shared by all of the death receptors (Lodish et al., 2008). When the ligand (FasL or TNF) binds to the death receptor (Fas or TNF-R, respectively), a signal in the intracellular death domain of the receptor is activated, possibly by death receptor trimers stabilization or by a conformational change (Van Herreweghe et al., 2010). Fas activation allows the binding of this ligand to an adapter protein called Fas-associated protein with a death domain (FADD) and the Fas-FADD complex thus formed binds procaspase-8 monomers promoting its dimerization and catalytic activity acquisition (Ashkenazi and Dixit, 1998). On the other hand, TNF-R binds first to TNF-receptor associated via death domain (TRADD) and only then to FADD. The complex formed binds to procaspase-8 monomers and promotes its activation, similarly to what happens after Fas binding (Van Herreweghe et al., 2010). Once activated, caspase-8 (also called FLICE) promotes amplification of the death cascade by activation of downstream effector caspases (Figure 2). Although mitochondrial membrane permeabilization is not required for the extrinsic apoptosis pathway progress, it significantly amplifies the extracellular death signal received (Blank and Shiloh, 2007).

Recent data showed that TNF signaling depends on the balance between the nuclear factor-kappaB (NF-kB) and the C-jun N-terminal kinase (JNK) signaling (Muppidi et al., 2004). On the one hand, NF-kB promotes cell survival by inducing c-$FLIP_L$ synthesis and controlling the duration of JNK activation, through induction of genes encoding antioxidant proteins (Chang et al., 2006). On the other hand, JNK leads to apoptosis since it directly antagonize a critical NF-kB dependent antiapoptotic step (Kamata et al., 2005). The activated JNK induce phosphorylation and activation of E3

ubiquitin ligase ITCH promoting c-$FLIP_L$ degradation through proteosome and consequently caspase-8 activation (Chang et al., 2006).

One of the hallmarks of the terminal stages of apoptosis is the breakdown of DNA. This process can occur through the activity of cell autonomous endonucleases, such as caspases-activated DNase (CAD), that degrades the DNA from within the dying cell and is normally present in a complex with its own inhibitor, ICAD, which guarantees that only inactive CAD is synthesized in healthy cells. When stored in the nucleus, CAD is inhibited by ICAD and only becomes activated when ICAD is cleaved by caspases. Another proteins involved in DNA breakdown are waste management nucleases, such as DNase II, responsible for cleaning up the debris after cell death (Samejima and Earnshaw, 2005).

Over the last years, a novel apoptotic cell death mechanism has been described. The induction of this mechanism is caspase-independent but can occur in response to the majority of intrinsic apoptotic cues that promote mitochondria outer membrane permeabilization (Lin et al., 2010). This apoptotic mechanism has been attributed to two mitochondrial proteins, endonuclease G and apoptosis-inducing factor (AIF) (Cande et al., 2002). These proteins are translocated to the nucleus upon mitochondria outer membrane permeabilization and then trigger chromosome condensation and DNA fragmentation leading to cell death. Besides AIF's role in the nucleus, some evidence show that it can also promote externalization of phosphatidyl serine cell surface and induce the loss of mitochondrial transmembrane potential (Joza et al., 2009).

1.1.2. Necrosis

Necrosis, also called accidental cell death, has traditionally been considered a passive event, in which cell death is caused by irreversible damages due to severe structural or chemical insults. Examples of such insults include changes in temperature or pH and physical trauma, which lead to a loss of homeostatic control, causing an influx of ions into the cell, massive oxidative stress and mitochondrial dysfunction. This eventually results in loss of membrane integrity, ATP depletion and a generalized process of autodigestion and dissolution. This process culminates in the plasma membrane permeabilization and spilling of the cytoplasmic contents out into the surroundings, which induces an inflammatory response with recruitment of macrophages and the consequent damage within healthy surrounding tissues (Degterev and Yuan, 2008). However, accumulating evidence show that necrotic cell death is, sometimes, as well controlled and programmed as apoptosis.

Although necrosis route predominates when and where ATP is depleted, different cellular stimuli, such as ischemia and hypoxia, have been shown to induce a necrotic process that follows defined steps and signaling events, suggesting that necrosis is another form of PCD (Krysko et al., 2008, Moquin and Chan, 2010). In programmed necrosis, besides mitochondria activation, other organelles such as lysosomes and ER are also activated. In addition, proteases (other than caspases) such as cathepsin (originated from lysosomes) and calpains (originated from ER) are activated (Krantic et al., 2005). So, although necrosis has been considered for a long time as an alternative of apoptosis, recent data indicate that there are many examples where necrosis can be a normal physiological and regulated event.

1.1.3. Cell death mechanisms in neurons

Neurons may die as a normal physiological process during development or as result of pathological process such as neurodegenerative disorders. During brain development PCD regulates the number and maintenance of neurons in the nervous system once the production of excess neuron cells ensures that a sufficient number of axons reach their targets (Pollard and Earnshaw, 2008). PCD occurs in approximately all types of developing neurons and can take place at any stage of differentiation. This loss of neurons is important to establish optimal levels of connectivity between neuronal populations, to eliminate aberrant cells or connections and to regulate the size of progenitor populations (Squire et al., 2008).

The best studied type of PCD in developing neurons regards the phase when neurons establish connections with targets and afferents. In 1940, Viktor Hamburger and Rita Levi-Montalcini postulated that target-derived signals (later identified as nerve growth factor) act to regulate the number of sensory and sympathetic neurons that survive in embryonic development. Embryonic ganglia have more neurons than are required but these developing neurons prevail and survive only when make successful connections to other neurons or muscles. Besides, internalization of target-derived trophic molecules at the nerve terminal is also required for neuron survival (Davies, 2003).

The neuron fate decision is controlled by both intrinsic and extrinsic signals. In addition to synaptic transmission and physiological activity, neurotrophins (NTFs) can act as extracellular signals, creating paracrine as well as autocrine loops of synaptic signaling. When present in sufficient amounts, NTFs result in intracellular signaling that inhibits the activity or expression of pro-apoptotic genes and induces the activity or expression of anti-apoptotic genes (Deshmukh and Johnson, 1997).

The endosome binding hypothesis postulates retrograde transport of exogenous NTFs to the cell body. Different NTFs bind preferentially to a specific tropomyosin-related kinase (Trk) receptor in the presynaptic surface membrane of nerve terminals, promoting internalization of the ligand-receptor complex. After its axonal transport along microtubules, the endosome with Trk-NTF complex initiates a signal transduction cascade at cell body, changing proapoptotic and antiapoptotic gene expression (Mok et al., 2009).

In some neurons, NTFs can also induce death by binding 75 kDa neurotrophin receptor ($p75^{NTR}$), which is structurally related to members of the TNF-R family. $p75^{NTR}$ can activate a PCD signaling via the JNK or caspase pathways.

An interesting finding is that during neuronal development central nervous system (CNS) is not as sensitive to the loss of NTFs as peripheral nervous system (PNS). One possible explanation is the higher complexity and number of synaptic relationship established by CNS, which could have synergistic and compensatory effects compared to PNS neurons (Squire et al., 2008).

During adult life, insults such as cell over-stimulation by excess of glutamate promote programmed necrosis activation (Christofferson et al., 2010), while accumulation of cytotoxic proteins, characteristic of neurodegenerative diseases (e.g. synuclein, parkin, amyloid-β), can promote death through the increase of oxidative stress, excitotoxicity, misfolded protein aggregation and mitochondrial dysfunction, which converge in apoptosis (Cordeiro et al., 2004, Lindholm et al., 2006, Kvam et al., 2009). It has been recently shown that in neurodegenerative diseases, such as Alzheimer, there is a change in proneurotrophins levels that can also be responsible for promoting apoptosis (Fahnestock et al., 2001).

The release of proneurotrophins plays an important role in nervous system. These proteins were shown to be involved in long term depression and neuronal cell death in hippocampal neurons (Nykjaer et al., 2004, Woo et al., 2005). Upon injury and neurodegenerative disorders, proneurotrophins levels in the extracellular space increase leading to cell death in different neuronal populations through activation of apoptotic machinery (Fahnestock et al., 2001, Harrington et al., 2004) (this topic will be discussed with further detail in the following section).

1.2 Proneurotrophins

NTFs are a family of soluble, basic growth factor proteins required for differentiation, neuronal survival and synaptogenesis (Lu et al., 2005). There are four NTFs known to be expressed in the mammalian brain: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4) (Hennigan et al, 2007). All NTFs are initially synthesized, both by neuronal and non-neuronal cell types, as larger precursor proteins with approximately 250 amino acids long, called proneurotrophins (Lu et al., 2005). These precursor proteins have an N-terminal domain that can be enzymatically cleaved originating the mature neurotrophins. Although NTFs have been discovered and described by a group of embryologists in the early 1950s, until recently proneurotrophins were described as inactive precursors, with its prodomain being responsible only for promoting the folding of the mature domain and regulation of NTFs secretory pathways. Only in 2001 it was discovered that proneurotrophins are biological active (Lee et al., 2001). In addition, both mature and precursor forms of NTFs are implied in various aspects of the nervous system, such as synaptic plasticity and neuronal survival (Schweigreiter, 2006).

NTFs are synthesized as preproneurotrophins into the ER and are transformed to proneurotrophins into the ER lumen (Lessmann and Brigadski, 2009). The proneurotrophins contain a signal peptide, sites for glycosylation, and pairs of basic amino acids that are recognized by processing enzymes (Chao, 2003) and trafficked via transport vesicles to the Golgi apparatus, where they undergo posttranslational modifications. The proneurotrophins can then be cleaved at a dibasic cleavage site in the middle of the precursor protein by the calcium-dependent serine protease furin and other members of the prohormone convertase. Then, neurotrophins, or its precursors, end up into two distinct types of vesicles in the trans-Golgi network: vesicles of the constitutive pathway of secretion or secretory granules of the regulated pathway of secretion. In the constitutive pathway, the vesicles release the cargo by default when they reach the plasma membrane whereas in the regulated pathway, the secretory granules are released by a calcium-dependent exocytosis. The information for proteolytic cleavage and sorting of neurotrophins are in a large part encoded by their prodomain (Lessmann and Brigadski, 2009).

The BDNF is synthesized, as the other neurotrophins, in a precursor form and is trafficked either to the regulated secretory pathway by chaperone proteins, such as sortilin, or to the constitutive pathway (Figure 3). However, it is not clear how efficient is the intracellular cleavage of proBDNF and how much proBDNF is secreted by neurons. Although proBDNF secretion was at first questioned (Matsumoto et al., 2008), recent evidence by Hempstead and colleagues show that both proBDNF and mature BDNF (mBDNF) can be targeted and released in the CNS (Yang et al., 2009) (Figure 4).

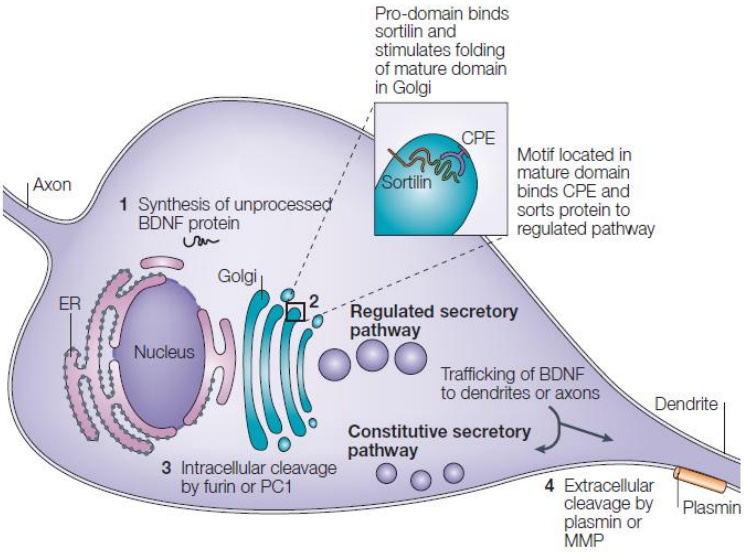


Figure 3 – Schematic representation of the synthesis and sorting of BDNF in a typical neuron. BDNF is synthesized in the unprocessed form in the endoplasmic reticulum. Then, the proBDNF goes to the Golgi and binds to intracellular sortilin which facilitates the correct folding of the mature domain. The sorting of BDNF to large dense core vesicles, components of the regulated secretory pathway is promoted by the mature domain motif of BDNF that binds to CPE. In the absence of this motif, BDNF is sorted into the constitutive pathway. The transport of BDNF to dendrites or axons is dependent of the size of its 3'UTR mRNA. CPE - Carboxypeptidase E; PC1 – Protein convertase 1 (adapted from Lu et al., 2005).

The NGF is a glycoprotein with 118 amino acids and consists of three subunits. The α_2 subunit appears to be inactive while β subunit is responsible for the NGF biological activity and Υ_2 subunit is an extremely specific active protease capable to process NGF precursor to its mature form. Both precursor and mature NGF (mNGF) exist in their active forms as homodimers (Buttigieg et al., 2007).

Conflicting data have been published about where proNGF is cleaved and converted to the mature form. According to some authors, NGF is converted to the mature form inside the neurons through intracellular proteases, including furin (Lim et al., 2007), is predominantly released in vesicles in a constitutive pathway manner (Brigadski et al.,

2005) and its precursor form is only released under an injury or ischemia (Volosin et al., 2006, Friedman, 2010). However, Cuello and colleagues provided both in vitro and in vivo evidence that NGF is released in the CNS to the extracellular environment in its precursor form and its maturation and degradation occurs there with the involvement of a complex protease cascade (Bruno and Cuello, 2006) (Figure 5).

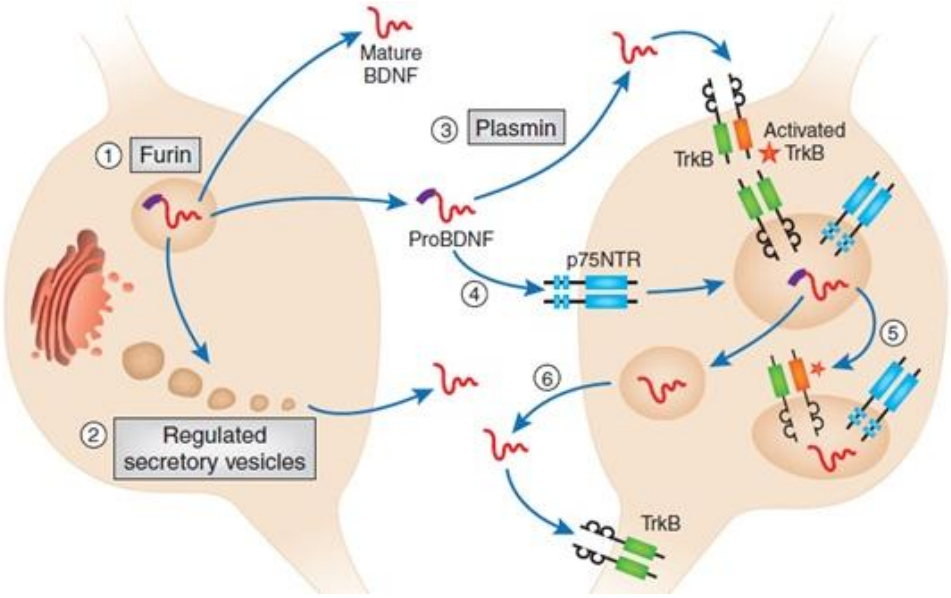


Figure 4 – Schematic representation of proBDNF processe to mature BDNF by several cellular mechanisms. The proneurotrophins can be cleaved intracellularly followed by secretion; secreted followed by extracellular cleavage, or secreted without subsequent cleavage. ProBDNF can be cleaved intracellularly by furin or by proconvertase enzymes, in regulated secretory vesicles. However, if proBDNF gets to extracellular space, it can be processed by plasmin and the mature BDNF formed can then activate cell surface TrkB receptors or, alternatively, proBDNF can bind to $p75^{NTR}$ and become endocytosed followed by cleavage, which can produce mature BDNF that either activates TrkB within endosomes or is recycled to the cell surface (adapted from Barker, 2009).

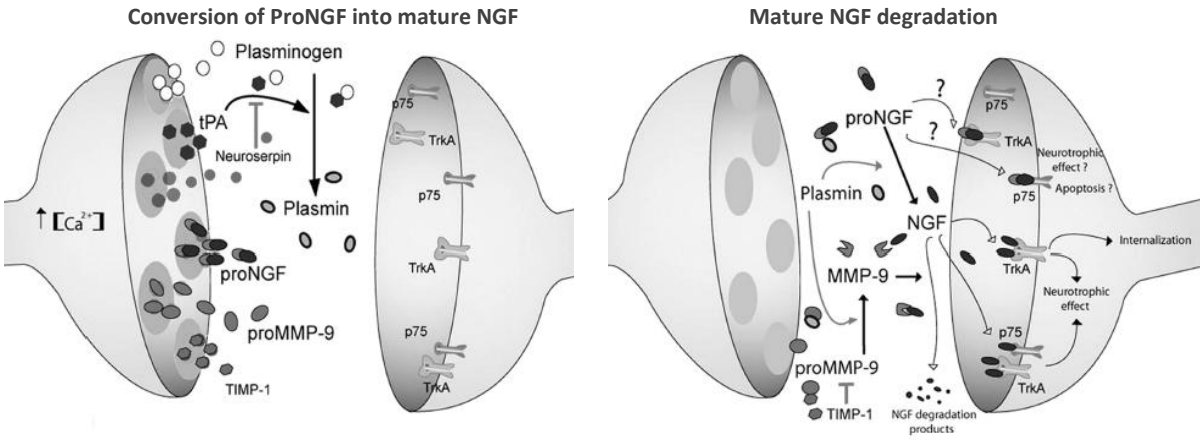


Figure 5 – Schematic representation of the protease cascade responsible for the conversion of proNGF into mature NGF and its subsequent degradation within extracellular space. After neuronal stimulation proNGF, plasminogen, tPA, neuroserpin, proMMP-9 and TIMP-1 are coordinately released into the extracellular space. tPA promotes the conversion of plasminogen to plasmin, mechanism regulated by neuroserpin. Plasmin in turn, converts proNGF into mature NGF and pro-MMP-9 into active MMP-9. The mature NGF that does not bound to receptors or is internalized is degraded in the extracellular space by activated MMP-9. This process is mediated by MMP-9 activation and is regulated by TIMP-1. MMP-9 – matrix metalloproteinase 9; proMMP-9 – inactive matrix metalloproteinase 9; TIMP-1 – tissue inhibitor metalloproteinase 1; tPA – tissue plasminogen activator (adapted from Bruno et al., 2006).

1.2.1 Proneurotrophin receptors

In 2001 it was first referred by Hempstead and colleagues that the proneurotrophins are secreted and cleaved extracellularly, promoting biological activity mediated by proneurotrophin receptor binding (Lee et al., 2001). Thereafter, several studies have described that proNGF and proBDNF have the capacity to selectively activate $p75^{NTR}$-sortilin receptor complex with high affinity (Nykjaer et al., 2004).

The $p75^{NTR}$ receptor is synthesized as a soluble precursor with a signal peptide that is cleaved when the receptor is inserted into the membrane (Schor, 2005). The mature receptor is composed of three main parts: the extracellular domain with four ligand-binding sites formed by cysteine-rich extracellular repeats; a single domain that crosses the plasma membrane and an intracellular TNF-like death domain (Schor, 2005, Diarra et al., 2009). There are several truncated isoforms of $p75^{NTR}$ resulting from alternative splicing and post-synthetic proteolysis (Diarra et al., 2009). These isoforms are responsible for binding the unprocessed and processed neurotrophins. Growing evidence have shown that this receptor plays an important role in controlling neuronal survival or cell apoptosis within the nervous system in development and in pathological or injury conditions (Kenchappa et al., 2006).

Sortilin is a member of the Vps10-domain transmembrane receptors family and is mainly expressed in brain, spinal cord and muscle. The sortilin receptor is synthesized with an N-terminal propeptide that hinders the ligands from accessing to the binding site. So, for sortilin full activity the cleavage of this propeptide is required (Paiardini et al., 2008). This receptor is predominantly localized intracellularly, particularly in the trans-Golgi network, endosomes and lysosomes. Less than 10% of the total sortilin pool is found on the plasma membrane (Willnow et al., 2008).

Petersen and colleagues described for the first time that sortilin is an important proneurotrophin receptor that targets the prodomain of proNGF with high affinity while the mature domain binds to $p75^{NTR}$ (Nykjaer et al., 2004). Sortilin acts as a coreceptor functioning in concert with $p75^{NTR}$, creating heterotrimeric complexes. Recent studies indicate that sortilin is a required element for transmitting proNGF and proBDNF-dependent cell death signals via $p75^{NTR}$ (Jansen et al., 2007). The sequence ^{163}RIFRSSDFAKNF^{174} is important in the binding of sortilin to proneurotrophins and

the amino acids Arg^{163}, Phe^{165}, Arg^{166} and Phe^{170} are critical for this interaction, but not for sortilin-$p75^{NTR}$ binding (Serup Andersen et al., 2010). Recent data showed that neurotrophin receptor homolog 2 (NRH2) is expressed in regions where $p75^{NTR}$ and sortilin are coexpressed and that expression promotes sortilin relocation to the cell surface, leading to the formation of $p75^{NTR}$-sortilin receptor complex and consequently to a high sensitivity of neurons to the apoptotic actions of proneurotrophins (Kim and Hempstead, 2009, Teng et al., 2010).

In the absence of sortilin, proNGF can remain in its immature form in the extracellular space and bind with low affinity to $p75^{NTR}$ or TrkA receptors (Fahnestock et al., 2004) or, in contrary, it can originate mNGF. mNGF is then able to bind with high affinity to TrkA, mediating survival signals (Nykjaer et al., 2004).

Although the binding of proneurotrophins to Trk receptors is not consensual in the literature, several studies show that proNGF and proBDNF can bind with low affinity to Trk receptors, TrkA and TrkB respectively (Fayard et al., 2005). Trk receptors are composed by immunoglobulin-C2 domains and repeats rich in leucine and cysteine residues in the extracellular domain, a tyrosine kinase domain and a short cytoplasmic tail (Yano et al., 2009). These receptors are responsible for differentiation and survival signals. They show different binding specificity that is dependent of Trk receptor dimerization, its structural modification or its association with $p75^{NTR}$. In this case, $p75^{NTR}$ acts as a coreceptor, increasing the affinity of Trk receptors for neurotrophins (Chao, 2003).

Like proNGF, proBDNF can also bind with low affinity to TrkB and $p75^{NTR}$ or with high affinity to $p75^{NTR}$-sortilin receptor complex. The binding to TrkB and $p75^{NTR}$

occurs through the proneurotrophin mature domain whereas proneurotrophins bind to sortilin through its prodomain (Fayard et al., 2005).

It is established that all interactions of proneurotrophins with their receptors share some homology. Krüttgen and colleagues reported that all proneurotrophins might bind to Trk inducing some response (Fayard et al., 2005) and, in 2008, Barker and colleagues showed that proNGF can induce TrkA activation through its endocytosis and cleavage by furin-like enzymes (Boutilier et al., 2008). Nonetheless, recent data indicate that the ratio between Trk and $p75^{NTR}$ is responsible for the role of proneurotrophins in cell fate (discussed later).

1.2.2. Proneurotrophin in Long Term Depression

Persistent modifications of synapses, also called long-term synaptic plasticity, are thought to be an essential mechanism for information storage and processing in the brain. The proBDNF, in contrary to proNGF, has been implicated in activity-dependent synaptic plasticity, more precisely in the regulation of hippocampal long-term depression (LTD) through activation of $p75^{NTR}$ (Rosch et al., 2005, Woo et al., 2005).

The LTD is characterized by weakness of synaptic connections. Homosynaptic LTD requires simultaneous pre- and postsynaptic activity and is induced by low-frequency stimulation. For LTD to occur, the activation of N-methyl-D-aspartic acid (NMDA) receptors is required, resulting in a small rise of calcium into the postsynaptic cell. This has as consequence the activation of calcium-dependent phosphatases that cleave the phosphate group of target proteins, such as the GLUR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Thus, LTD promotes alteration of AMPA receptor expression in synapses due to dephosphorylation of Ser^{845} on the C-

terminal of the GluR1 subunit. The overall result is the internalization of AMPA receptors from the postsynaptic cell membrane (Purves et al., 2008).

It has been demonstrated that proBDNF is an endogenous ligand to $p75^{NTR}$ and this interaction is important for expression of LTD in hippocampal neurons (Rosch et al., 2005, Woo et al., 2005). As Wang and colleagues have already verified in hippocampal slices, the expression of NR2B subtype of NMDA receptor is involved in LTD while NR2A subunit of NMDA receptor is involved in long term potentiation (LTP) (Liu et al., 2008). Thereby, activation of $p75^{NTR}$ by proBDNF enhances an NR2B-mediated current which induces LTD through NR2B upregulation (Woo et al., 2005). However, the exactly signals through which proBDNF-$p75^{NTR}$ complex regulates NR2B expression remain to be described. Double staining conducted in cultured hippocampal neurons by Lu and colleagues showed that $p75^{NTR}$ is localized in dendritic spines (Woo et al., 2005). This result leads to the hypothesis that proBDNF regulates hippocampal LTD by acting directly on $p75^{NTR}$ in the dendritic spines of CA1 neurons.

Presynaptic structure can be changed as a result of positive and negative signals generated by postsynaptic cells. Lu and colleagues have demonstrated that proBDNF can also induce depression and retraction of presynaptic terminals in developing neuromuscular synapses in Xenopus laevis though $p75^{NTR}$. This is a result of proBDNF postsynaptic release that, in turn, acts in the presynaptic terminals (Yang et al., 2009).

Besides LTD and presynaptic retraction, Kojima and colleagues have shown that proBDNF can also be responsible for the inhibition of neurite outgrowth in basal forebrain cholinergic neurons and for reduction of dendritic spine density in mature hippocampal neurons without affecting the survival of these neurons (Koshimizu et al., 2009).

1.2.3. Proneurotrophins and cell death

CNS and PNS neurons respond to several factors in their environment that influence their death. Among these factors, recent data have shown that proneurotrophins exert pro-apoptotic activity via $p75^{NTR}$. It has been described in vitro that $p75^{NTR}$-mediated cell death can be triggered through both precursor and mature forms of neurotrophins. Nonetheless, in order to obtain an equivalent effect, higher concentrations of mature ligands are needed when compared to the precursor forms (Teng et al., 2010).

It is important to notice that there are contradictory evidence about the role of proneurotrophins in the nervous system. Some studies show that proneutrotrophins induce neuronal death while others demonstrate that proneurotrophins can exert neurotrophic besides the neurotoxic effects. However, it is consensual that the cleavage of proneurotrophin and the change in Trk – $p75^{NTR}$ receptors ratio are critical steps in determining neuronal fate (Bruno and Cuello, 2006, Koshimizu et al., 2009) (Figure 6).

Accumulating evidence show that $p75^{NTR}$-sortilin complex can induce apoptosis, both in vitro and in vivo, when bound to proneurotrophins in neuron and non-neuron cells such as smooth muscle cells and oligodendrocytes (Beattie et al., 2002, Nykjaer et al., 2004). Yoon and colleagues have reported that $p75^{NTR}$ promotes apoptosis by Rac GTPase, a key adaptor protein, and JNK activation (Harrington et al., 2002). However, it has been recently shown that $p75^{NTR}$ can also promote phosphorylation of the Bcl-2 regulator proteins Bad and BimEL, thus activating the intrinsic caspases pathway (Troy et al., 2002, Bhakar et al., 2003, Becker et al., 2004, Friedman, 2010). Nevertheless, some data suggest that JNK activation by $p75^{NTR}$ in sympathetic neurons can also be mediated by the neurotrophin receptor interacting factor (NRIF). In this case, the $p75^{NTR}$ intracellular domain is cleaved by Υ-secretase resulting in ubiquitination and a

consequent translocation of NRIF, a DNA binding protein, to the nucleus (Linggi et al., 2005, Kenchappa et al., 2006).

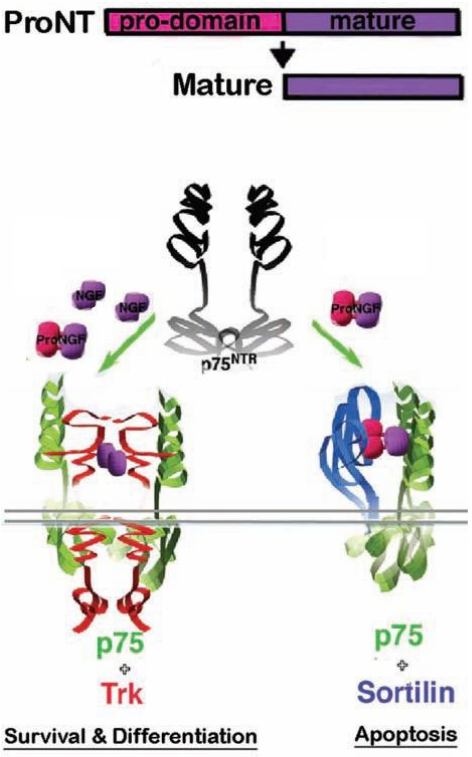


Figure 6: Schematic diagram illustrating the interactions between the proforme and mature form of neurotrophins to neurotrophins receptors. While neurotrophins bind only with Trk-p75^{NTR} receptors, their precursor form, such as proNGF and proBDNF, can bind with p75^{NTR}-sortilin receptors or with p75^{NTR}-Trk receptors. The interaction of Trk with p75^{NTR} enhances the responsiveness to mature neurotrophin, promoting neuronal survival and differentiation. In contrast, when proneurotrophins bind to p75^{NTR}-sortilin receptors it induces cell death. Mature NT – mature neurotrophin; ProNT – proneurotrophin; p75 - 75 kDa neurotrophin receptor; Trk - tropomyosin-related kinase (adapted from Friedman, 2010).

As mentioned before, some authors have shown that proBDNF is constitutively secreted from cultured cortical neurons and that not all proBDNF is converted to the mature form (Teng et al., 2005). In the precursor form, proBDNF might bind to sortilin coreceptor becoming stabilized and protected from proteolytic degradation. Thus, the binding of proBDNF to p75^{NTR}-sortilin complex can induce glia, sympathetic neuron

and also cerebellar granule neurons apoptosis by $p75^{NTR}$-Rac-GTPase-JNK-caspase-3 pathway (Koshimizu et al., 2010, Teng et al., 2010).

1.2.3.1. Proneurotrophins and neurodegeneration

During aging, proNGF is highly elevated in areas vulnerable to age-related neurodegeneration in CNS and PNS. Susceptible subpopulations include NGF-responsive cholinergic neurons of the basal forebrain (BF) nuclei and of superior cervical ganglia (SCG) (Gatzinsky et al., 2004, Smith et al., 2004). BF neurons provide the major source of cholinergic innervation to the cerebral cortex and their degeneration plays a key role in memory and cognition in Alzheimer disease (AD) (Mufson et al., 2003).

AD is a progressive degenerative disease, pathologically characterized by the presence of senile plaques and intracellular neurofibrillary tangles. Severe degeneration of synapses and neuritis, cell death and loss of neurons in BF, hippocampus and cortex are some of the AD hallmarks (Mann, 1991, Michalski and Fahnestock, 2003).

As AD progresses, the levels of proNGF, expressed particularly in glial cells as well as in cortical and hippocampal neurons, increase in a stage-dependent manner, although NGF mRNA expression is not altered (Fahnestock et al., 1996, Fahnestock et al., 2001, PENG et al., 2004). Recent data showed that this might be due to a diminished conversion of the NGF precursor molecule to its mature form in AD as well as an increased in mature NGF degradation (Bruno and Cuello, 2006). The levels of both plasminogen and tPA (Figure 5) decrease, which impairs the efficient conversion of proNGF to mNGF. Moreover, the protein levels and the enzymatic activity of matrix

metalloproteinase 9 (MMP-9) (Figure 5) increase, promoting mNGF degradation. These deregulation in mNGF trophic supply leads to cholinergic atrophy.

However, unlike proNGF, the levels of proBDNF in pre-clinical stages of AD are decreased as well as its mature form (Michalski and Fahnestock, 2003, PENG et al., 2004). This further suggests that decreased BDNF and proBDNF also play a crucial role in cell atrophy, cell loss and synaptic dysfunction observed in the cognitive memory systems affected in AD (PENG et al., 2004).

In addition to the changes on proneurotrophins levels there is a decrease in TrkA levels in AD (Counts et al., 2004). However, $p75^{NTR}$ and sortilin receptor levels in cortex remain stable (Counts et al., 2004, Mufson et al., 2010). It is considered that TrkA reduction may play a key role in the loss of cholinergic NB neurons observed in later stages of AD since it makes the neurons more permissive to $p75^{NTR}$-mediated signaling death (Counts et al., 2004). However, the mechanisms underlying the reduction of cortical TrkA protein in AD are unclear.

1.2.3.2. Proneurotrophins and injury

Several groups have reported that proNGF is increased under pathological conditions in the extracellular environment, which indicates that it might have an important role in promoting cell death under these conditions. However, some authors have shown that proNGF is induced and secreted in vivo only after an injury or seizure (Harrington et al., 2004, Volosin et al., 2008) while others have reported that proNGF is always secreted and that after an injury or seizure there is a decrease of proNGF cleavage, which promotes the increase of proNGF levels. Besides the increase of proNGF after an injury, there is also stimulation of $p75^{NTR}$ expression in neurons, oligodendrocytes or

Schwann cells (Beattie et al., 2002). This indicates that cell death due to injury may be a result of alterations in $p75^{NTR}$ levels combined with increased levels of proNGF in the extracellular environment (Harrington et al., 2004).

Yoon and colleagues have reported that the binding of proNGF to $p75^{NTR}$ is required for the death of oligodendrocytes following spinal cord injury (Beattie et al., 2002). These authors suggest that proNGF is responsible for eliminating the damaged cells through apoptotic machinery activation. However, the death of oligodendrocytes can contribute to chronic demyelinition and consequently spinal cord dysfunction.

In the case of seizures, there are many potential mechanisms through which neurons may die, such as proneurotrophin-induced of apoptosis. It has been reported that (i) seizures promote an increase in BDNF and NGF mRNAs in hippocampal neurons; (ii) proNGF and proBDNF are released to the extracellular space in hippocampal neurons and hippocampal astrocytes; (iii) proNGF and proBDNF can induce NRIF $p75^{NTR}$ interaction; (iv) proNGF and proBDNF are responsible for neuronal loss after seizures in hippocampus (Volosin et al., 2008, Friedman, 2010).

In spite of this lack of information the understanding about roles of proneurotrophins in the nervous system has been increasing. However, the release of proneurotrophins to the extracellular environment in physiological conditions, its binding to Trk receptors and their role in survival are not consensual in the literature.

1.3. Objectives

The objective of this work is to determine the role of proNGF in retrograde death signaling. NGF axon withdrawal has been recently reported to induce a retrograde

apoptotic signaling that is sufficient to suppress NGF-induced survival signals in the cell body (Mok et al., 2009). Moreover, pro-NT3 has been reported to induce cell death by a retrograde transport mechanism (Yano et al., 2009). However, the role of proNGF in cell death is not consensual and in all studies performed until now the entire neuron is stimulated with proNGF, not addressing the possibility that proNGF can induce a retrograde apoptotic signaling pathway.

In order to accomplish our goal, we first aim to produce and purify biological active proNGF using a mammalian expressing system. Then, we aim to characterize the effect of proNGF when applied locally to axons. Taking advantage of the fluidic properties and the axonal isolation of microfluidic chambers, we propose to specifically stimulate axons with proNGF and determine if the localized action of proNGF induces neuronal cell death and/or axonal degeneration.

Chapter 2

Materials and Methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), G418 disulfate salt, protease inhibitors chymostatin, pepstatin, antipain and leupeptin (CLAP), ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), IGEPAL, 5-fluoro-2'deoxiuridina (5-FDU) and paraformaldehyde were all acquired from Sigma-Aldrich Química S.A. (Sintra, Portugal). Fetal Bovine Serum (FBS), trypsin, B27 supplement, NGF 2.5S, sodium pyruvate, glutamine, goat serum, neurobasal medium and penicillin-streptomycin were purchased from Gibco Invitrogen Corporation (Barcelona, Spain). Sodium dodecyl sulfate (SDS), SDS-polyacrilamide gel electrophoresis buffer (10x Tris/Glycine/SDS stock) and Precision Plus Protein^{TM} Standards All Blue were obtained from BioRad Laboratories, Inc (Amadora, Portugal). PVDF (polyvinylidene difluoride) and Poly-D-lysine were acquired from Millipore (Madrid, Spain). Ni Sepharose^{TM} High Performance and Enhanced Chemifluorecense substrate (ECF^{TM}) were purchased from GE Healthcare (Carnaxide, Portugal). BCA Protein Assay kit was obtained from Thermo Scientific (Rockford, USA). Triton X-100 and bovine serum albumin (BSA) were acquired from Calbiochem, as part of Merck Scientific (Darmstadt, Germany). Mounting media ProLong Gold antifade reagent with DAPI was purchased from Molecular Probes®, as part of Invitrogen Life Technologies (Barcelona, Spain). Collagen was obtained from Roche Diagnostics (Amadora, Portugal). ProNGF (Mut-mouse) was acquired from Alomone Labs (Jerusalem, Israel). All other reagents were purchased from Sigma (Sintra, Portugal), Fisher Scientific (Illinois, USA), Panreac Quimica Sau (Barcelona, Spain), Merck (Darmstadt, Germany) and from BioRad Laboratories, Inc (Amadora, Portugal).

PDL was prepared in 166 mM borate buffer solution (10.3 g/mL boric acid, pH 8.2), collagen was prepared in 0.2 % (v/v) acetic acid, phenylmethanesulphonyl fluoride

(PMSF) was prepared in dimethyl sulfoxide (DMSO) and stock solution of NGF were made in phosphate buffered saline (PBS) with 0.1% BSA. All other solutions were aqueous.

2.1.1 Antibodies

Anti-NGF polyclonal antibody (M-20: sc-549) (rabbit) and anti-His Tag polyclonal antibody (#2365) (rabbit) used for Western Blot (1:1000 dilution), were purchased from Santa Cruz Biotechnology (Maia, Portugal) and Cell Signaling (Carnaxide, Portugal), respectively. The secondary anti-rabbit immunoglobulin G (IgG) alkaline phosphatase linked whole antibody (from goat) (1:10000 dilution) was acquired from GE Healthcare (Carnaxide, Portugal).

The antibodies listed below were used in immunocytochemistry. Anti-ß-Tubulin I monoclonal antibody (T7816) (mouse) (1:1000 dilution) was acquired from Sigma-Aldrich Química S.A. (Sintra, Portugal) and anti-MAP2 polyclonal antibody (ab5392) (chicken) (1:50 dilution) was purchased from Abcam (Cambridge, England). TexasRed goat anti-mouse igG (T862) (1:200 dilution), Alexa 488 goat anti-chicken (A-11039) (1:1000 dilution) and Alexa 488 rabbit anti-mouse (A-11059) (1:200 dilution) secondary antibodies were obtained from Molecular Probes®, as part of Invitrogen Life Technologies (Barcelona, Spain).

2.2. Cell's transfection

2.2.1. Cell lines

COS-7 cells and Human Embryonic Kidney (HEK) 293T cells were grown in DMEM (D7777) supplemented with 10 % (v/v) FBS (heat inactivated) and 1:100 penicillin-streptomycin. Cells were maintained at 37 ˚C in a 5 % CO_2 humidified atmosphere.

2.2.2. Expression plasmids

Eucaryotic expression pEGFP plasmid was a kind gift from Dr. Francis Lee, Weill Corner Medical College of Cornell University (NY, USA). The cDNA for proNGF was cloned from a mouse brain cDNA library by reverse transcriptase polymerase chain reaction (RT-PCR). A six histidine tag (6xHis) was added at the C-terminus for posterior Ni-bead affinity purification and the resulting fragment was then inserted into pCDNA3.1. The furin-resistant proNGF sequence was generated as described before (Lee et al., 2001) and sequenced for confirmation (STABvida).

2.2.3. COS-7 stable cell lines

On the day prior to transfection, COS-7 cells were seeded in a 10 cm diameter culture dish. Stable cell lines were generated using a calcium-phosphate method, based on Chen and colleagues (Jiang et al., 2004), in cells with 70-80 % confluence. Briefly, a solution of 2 M $CaCl_2$ and 48 µg of DNA (pcDNA 3.1 proNGF) were diluted in ddH_2O. This $CaCl_2$/DNA solution was added drop-wised to an equal volume of 2x HEPES

buffered solution (HBS) (pH=7.05) (280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4). The solution was incubated at room temperature for 30 min, with vortex for a few seconds every 5 min. Finally, the solution was added to the cell plate and cells were then returned to the 37 ˚C incubator (5 % CO_2). 24 h after transfection, transfection medium was replaced for DMEM supplemented with 10 % FBS and 1:100 penicillin/streptomycin. 48 h after COS-7 transfection, G418 antibiotic was added at a concentration of 0.4 mg/ml for positive clone selection.

2.2.4. HEK 293T transient transfection cells

One day prior to transfection, confluent HEK 293T cells were seeded in a 10 cm diameter culture dish. Transient transfection was carried out in 70-80 % confluent cells using the calcium-phosphate method described above with some modified steps: the desired amount of plasmid DNA was different between experiments (specified in each result); 24 h after transfection, transfection medium was replaced for DMEM supplemented with 0.05% FBS and 1:100 penicillin/streptomycin and expression was left to occur for 24 h.

2.3. Protein concentration

Medium and extracts of transfected HEK 293T cells and total extracts of COS-7 proNGF stable cell lines were prepared for analysis of proNGF presence by Western Blot. In HEK 293T transfected cells, medium was removed and protease inhibitors (0.1 mM PMSF and 1 µg/ml of CLAP) were immediately added. Medium was cleared of its cellular debris by centrifugation (15000 x *g*, 10 min, 4 ˚C) or with a 0.45 µm filter.

Afterwards, total extracts of HEK 293T transfected cells and COS-7 selected clones were prepared: ice-cold 1.5 ml of lysis solution (50 mM Tris-HCl pH 7.05, 150 mM NaCl, 0.1 % (v/v) IGEPAL), supplemented immediately before use with protease inhibitors (0.1 mM PMSF and 1 µg/ml of CLAP), was added to cells, which were then scraped. The lysates obtained were sonicated in an ultrasonic probe with 5 pulses of 5 s each (15 s between sonication pulses) and samples were then centrifuged (15000 x *g*, 10 min, 4 ˚C) for insoluble fraction clearance. The above procedures were all carried on ice. Protein concentration of cell's extract and medium were then assessed by the bicinchoninic acid (BCA) method using the BCA protein kit, according to manufacturer recommendations. Samples of COS-7 selected clones were then denaturated in 1 x concentrated sample buffer at 95 ˚C for 5 min and left in ice for 5 min.

After analysis of the protein concentration in medium and total extracts of HEK 293T transfected cells, samples were incubated with 125 µL of Ni Sepharose^{TM} High Performance beads slurry overnight, at 4˚C. Beads were then centrifuged (2700 x *g*, 10 min, 4 ˚C), washed once with 5 column volumes of wash buffer solution (50 mM sodium phosphate and 500 mM NaCl, pH 7.4) and centrifuged again. Elution was completed by the addition of 1 column volume of 1x concentrated sample buffer to the beads and its denaturation at 95 ˚C for 5 min. Samples were left in ice for 5 min and finally a centrifugation at 13336 x *g*, 1min to collect the supernatant.

As a control, the above procedures were also carried in non-transfected HEK 293T and COS-7 cells and in HEK 293T transfected with pEGFP plasmids.

2.4. Electrophoresis and Western blot

Samples destined for Western blot analysis were resolved in tris-glycine-SDS (TGS) buffer in 12 % (v/v) polyacrylamide gels 1.5 mm thick at 120 V. Each well was loaded with 40 µl of sample with different protein amounts between experiments. Proteins were electrotransfered onto a PVDF membrane for 3 h at 250 mA at 4 ˚C. Membranes were washed once with TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) with 0.1 % (v/v) Tween 20 (TBS-T) and non-specific binding sites were blocked with TBS-T containing 5 % non-fat dry milk for 1 h at room temperature. Membranes were washed with TBS-T (3 times, 10 min each) and then incubated overnight at 4 ˚C with primary antibodies diluted in TBS-T containing 5 % BSA. Membranes were then washed with TBS-T (3 times, 15 min each) and incubated 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit secondary antibody diluted 1:10000 in TBS-T with 0.5 % non-fat dry milk. Membranes were again washed with TBS-T (3 times, 15 min each) and proteins were detected by incubation with ECF substrate in a maximum period of 5 min. Membrane's scanning was performed on Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, United Kingdom).

2.5. Primary cell cultures

2.5.1. Preparation of microfluidic devices

The microfluidic device is composed of poly-dimethylsiloxane mold chamber (PDMS) attached to a glass coverslip providing a surface for cellular adhesion. The glass slices (25 x 25 mm, Assistant, USA) were cleaned with 65 % nitric acid for 24 h in constant

agitation. After that, glass slices were washed also in constant agitation with mQ H_2O (5 times, 30 min each), rinsed in pure ethanol (2 times, 30 min each) and then placed in an oven at 50 ˚C until dry (15 min to 20 min, approximately). Finally, glass slides were exposed to UV light for 15 min to guarantee their sterilization.

The following steps were performed under sterile conditions. The microfluidic device was always constructed in the plating day. Glass slides were placed against a 10 cm diameter dish and coated with PDL (0.1 mg/ml) overnight at 37 ˚C. Glass slides were then washed three times with mQ H_2O and coated with collagen (20 µg/ml) for 1 h at room temperature. Three further washes with mQ H_2O were done. As soon as glass slides were dry, chambers were assembled on coated slides and neurobasal medium was applied to the reservoirs of the somal compartment. Chambers were placed at 37 ˚C for 15 min to make sure that microgrooves were completely filled. Neurobasal medium was then applied to the reservoirs of axonal compartment and the microfluidic device was left in the incubator until cell plating.

2.5.2. Culture of embryonic rat DRG neurons

Primary cell cultures of rat dorsal root ganglion (DRG) neurons were prepared from E 14 Wistar rat embryos. DRG neurons were dissected in ice-cold Hank's balanced salt solution (HBSS) (137 mM NaCl, 0.34 mM $Na_2HPO_4 \cdot H_2O$, 4.16 mM $NaHCO_3$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001 % phenol red), incubated with 0.25 % trypsin and 10 µg/ml of deoxyribonuclease I in HBSS for 15 min at 37 ˚C. After centrifugation at 170 x *g*, 1 min, medium was removed and the remaining trypsin was blocked with DMEM containing 10 % of FBS and 5 µg/ml of deoxyribonuclease. Cells were then centrifuged (170 x *g*, 1 min),

resuspended in DRG medium (Neurobasal medium supplemented with 2% B27, 2mM glutamine, 0.7 mM sodium pyruvate, 0.3 % glucose, 12.5 U/mL penicillin and 12.5 ug/mL streptomycin) and mechanically dissociated with a P1000-pipette followed by a Pasteur pipette, until a single cell suspension was formed. After that, the cell suspension was plated in a 60 mm tissue culture dish for 30 min at 37 ˚C in order to eliminate the fibroblasts and glial cells. Finally, the cell suspension was diluted in DRG medium supplemented with 50 ng/mL NGF and 10 µM 5-FDU and 50000 cells were plated per well of a 24 multiwell plate. In microfluidic chambers 6 µl of a cell suspension at 8 x 10^6 cells/ml was added to the somal side.

At DIV 2, 100 µl of DRG medium supplemented with 100 ng/ml NGF were added to microfluidic chambers and 100 µl of medium were replaced in 24 multiwell plates to DRG medium supplemented with 100 ng/ml NGF.

2.5.3. proNGF stimulation

Primary DRG cultures were grown in microfluidic chambers for 8 days and the axonal compartment was then carefully washed three times with Neurobasal medium to remove all NGF that could be present. DRG medium with 50 ng/ml proNGF was then added to the axonal compartment for 48 h. In order to prevent the diffusion of applied proNGF from the axonal to the somal side, a minimum volume difference between somal and axonal compartments (about 50 µL) was maintained. However, this difference is only maintained up to 20 h (Taylor et al., 2005). As a result, complete DRG medium was added to the somal side in intervals not exceeding 20 h. The slightly higher volume on the somal side allows chemical microenvironments to be isolated and causes a slow

flow of liquid only from somal to axonal compartment owing to the high fluidic resistance of the microgrooves.

2.5.4. Immunocytochemistry

DRG neurons were pre-fixed in 1 % paraformaldehyde (in cytoskeleton buffer (60 mM PIPES at pH7.0, 27 mM HEPES, 10 mM EGTA and 4 mM $MgSO_4$) with 4% sucrose) for 15 min at 37 ˚C in order to reduce the aggressive effect of paraformaldehyde. After that, DRG neurons were fixed in 4 % paraformaldehyde for 15 min, 37 ˚C. Cells were washed with ice-cold tris-buffered saline (TBS) (3 times, 5 min each) and permeabilized utilizing TBS with 0.25 % Triton X-100 for 5 min at room temperature. Cells were then washed once with TBS for 5 min and incubated for 30 min at room temperature in blocking solution (5 % of goat serum and 5% of FBS in TBS) to block non-specific binding sites. Primary antibodies were incubated overnight at 4 ˚C in blocking solution. After primary antibodies' incubation, cells were washed with TBS (3 times, 5 min each) to remove non-specific primary antibody binding. Secondary antibodies in blocking solution were then added and incubated 1 h at room temperature. After that, cells were washed using TBS with 0.1% Triton X-100 (2 times, 5 min each) followed by one wash in TBS, 5 min. Finally, glass slides were rinsed in mQ H_2O and mounted in prolong mounting media with DAPI. The procedure described above was performed with the microfluidic chamber device assembled to the coverslip; only after the last wash with TBS the chamber was carefully removed. DRG neurons used to nuclear morphology analysis were pre-fixated and fixated as described above. Cells were then washed with TBS (3 times, 5 min each) and glass slides were rinsed in mQ H_2O and mounted in

prolong mounting media with DAPI. All preparations were kept at 4 ˚C, protected from light until microscopy acquisition.

In DiI experiments, 48 h before immunocytochemistry, 1:750 DiI was added to the axonal compartment only. In order to prevent DiI diffusion from the axonal to the somal side, a minimum volume difference between somal and axonal compartments (about 50 µL) was maintained and complete DRG medium was added to the somal side in intervals not exceeding 20 h.

2.5.5. Immunofluorescence data acquisition and analysis

Cell viability analysis was done using an inverted Zeiss Axiovert 200 fluorescence microscope. Images were acquired with a Plan-Neofluar 10x or 20x (numerical aperture 0.3 and 0.5, respectively) and taken utilizing an AxioCam HRm camera and AxioVision 4.8 software. The experiments were done in duplicate and 400 – 600 cells were counted per coverslip after randomly selection of at least 8 different optical fields.

Images that cover the entire microfluidic chamber (both somal and axonal side) were taken utilizing a confocal microscope Zeiss LSM 510 Meta and LSM 510 software. Single images were obtained with an EC-PlanNeoFluar 20x oil objective with a 0.5 numerical aperture and then aligned using the LSM 510 software to obtain a multi-field final image.

2.5.5.1. Axonal degeneration

Axonal degeneration experiments were done utilizing an inverted Zeiss Axiovert 200 fluorescence microscope and a Plan-Neofluar 20 x objective (numerical aperture 0.5). Images were acquired in the axonal side selecting distal regions from the microgrooves entrances that were not too close of the channel wall. Images were taken utilizing an AxioCam HRm camera and AxioVision 4.8 software, with conserved exposure times between single experiments.

Axonal degeneration was quantified using a protocol modified from that described previously (Sasaki et al., 2009). Degenerated axons were analyzed with Image J 1.43 software. Total axonal area was determined through a binary application measure in exported 16-bit images. Brightness and contrast for each image was then achieved and an average value was calculated in order to apply the same mean of brightness and contrast to all images from the same independent experiment. After that, images were binarized such that regions corresponding to axons were altered to black and all other regions were altered to white. Degenerating axons have a particular structure due to their beading and fragmentation while healthy axons show continuous tracts. Therefore, each particle was defined either intact or fragmented based on its circularity index (image J tool) that is characterized as $circularity = area/(\pi \times radius^2)$ where area and radius are measured in μm^2 and µm, respectively. Fragments were detected using the particle analyzer tool of Image J with circularity limits between 0.2 and 1. Per condition particle number, area, and average size were measured and the degeneration index (DI), ratio of fragmented axonal area to total axonal area were determined. The values obtained per image were normalized against the mean of control. The number of independent experiments and the total number of images analyzed are indicated in the graphs.

2.5.6. Statistical analysis

All the statistical data analyses were performed in Graph Pad Prism 5 software and the results are presented as normalized means ± SEM of independent experiments. Statistical significance was evaluated by student's t-test with confidence intervals of 95 % and $P<0.05$ was considered significant.

Chapter 3

Results and Discussion

3.1. proNGF production and concentration

Proneurotrophins are neurotrophin precursors that are involved in regulation of cell survival (Lee et al., 2001). As described in section 1.2, neurotrophins have been shown to regulate cell survival and lately synaptic plasticity, memory and behavior (Lu et al., 2005) while proneurotrophins have recently been described as key players in apoptosis mechanisms (Lee et al., 2001).

proNGF is produced intracellularly and released to the extracellular space not only as mature NGF but also as proNGF (Fahnestock et al., 2001, Bruno and Cuello, 2006). proNGF is encoded by a single gene that can undergo alternative splicing when transcribed, leading to the translation of a 34 kDa or 27 kDa protein (pre-proNGF), depending on the initiation site. The removal of the signal sequence in endoplasmic reticulum leads to the formation of a 32 kDa or 25 kDa proNGF (Fahnestock et al., 2004). This protein contains three glycosylation sites, two of them in the prodomain region, which are important to protein stability and efficient expression.

Since our main goal was to evaluate the effect of proNGF when applied specifically to axons, we tried to express proNGF in a heterologous system in order to produce and purify biological active proNGF. Since post-translation mechanisms in bacteria are different from mammalian and could lead to the production of non-active proNGF, we tried to express proNGF in mammalian cells. However, furin and other convertases that are present in all mammalian cells are able to process intracellularly proNGF into NGF at a highly conserved dibasic amino acid cleavage site (Pagadala et al., 2006). Consequently, a mutated cleavage resistant form of proNGF was designed, in order to prevent cleavage by furin when produced in mammalian systems (a methodology already described by Hempstead and colleagues (Lee et al., 2001)).

We first started to optimize HEK 293T cells transfection using a calcium phosphate method. We tested different amounts of DNA by transiently transfecting HEK 293T with 4 µg, 8 µg and 12 µg (per well in a 6 multiwell plate) of pEGFP-C1 (Figure 7). After transient transfection, medium was replaced to DMEM with 10 % FBS and protein expression was allowed to occur for 24 h. The results obtained in Figure 7 evidence that the DNA amount is correlated with protein expression, and 12 µg of DNA increased expression considerably without promoting a significant enhancement in cell toxicity. So, we used this DNA ratio in subsequent experiments to express proNGF.

Since the proNGF construct used does not have a fluorescent tag, we used pEGFP-C1 as a transfection control. Although the vector backbones of pEGFP-C1 and pCDNA 3.1-proNGF are different, the promoters are the same and both plasmids have a polyadenilation signal, important to the exportation of mRNA into the cytoplasm and also its stability. However, it should be noted that expression of EGFP protein does not necessarily mean that proNGF is also being expressed.

24 h after transfection, medium was replaced by DMEM with 0.05% FBS. This change improves cell viability and more importantly allows the reduction of the total amount of proteins present in the medium, thus reducing the level of contaminants. Protein expression was then allowed to occur for 24 h and cells were visualized using a fluorescence microscope (Figure 8A). The images show that EGFP, the positive transfection control, was successfully expressed in HEK 293T cells. Also, careful morphological analysis show that cells transfected with pCDNA 3.1-proNGF are similar to the cells transfected with pEGFP-C1 and not to non transfected cells, which suggests that proNGF transfection was also successful. Since the level of expression in mammalian systems is generally low, transfected cell media was concentrated using Ni affinity chromatography to assess proNGF expression (Figure 8B). In addition, to

overcome the possibility that proNGF could not be secreted to the extracellular space (medium), we did total extracts of HEK 293T cells transfected with pCDNA 3.1-proNGF and checked for proNGF presence by Western Blot (Figure 8C).

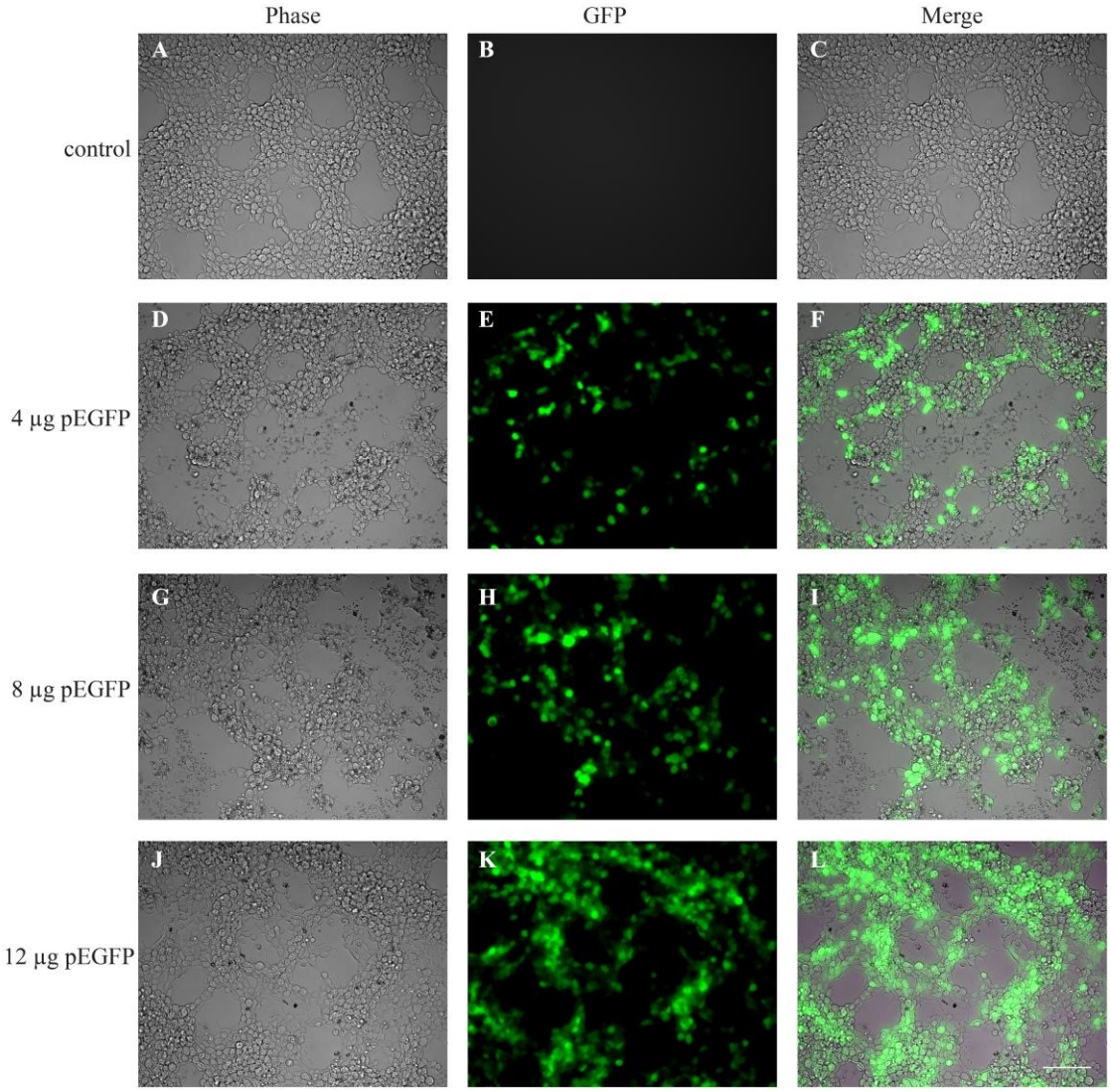


Figure 7 – Optimization of HEK 293T transfection. HEK 293T cells were transiently transfected with 0 µg **(A, B, C)**, 4 µg **(D, E, F)**, 8 µg **(G, H, I)** and 12 µg **(J, K, L)** of pEGFP-C1. Transfection medium was replaced 24 h after transfection and EGFP expression was allowed to occur for an extra 24h. Comparison between EGFP signal and different DNA amounts suggests a correlation between the amount of DNA and protein expression; a greater DNA amount increased the protein expression. Phase and immunofluorescent images of HEK 293T cells were acquired 48 h after transfection. Images were taken using a Zeiss Axiovert 200 fluorescence microscope with a Plan-Neofluar 20x objective. ctr – not transfected cells (control). The scale bar is 100 µm.

In wells loaded with samples of HEK 293T cells transfected with pCDNA 3.1-proNGF (Figure 8B, C), the presence of two specific bands was expected. These should appear close together, at approximately 30 kDa, due to the already described glycosilation of proNGF isoforms (32 kDa and 25 kDa). However, it is important to notice that in total extracts of HEK 293T transfected with pCDNA 3.1-proNGF, bands of low molecular weight could also appear if the post-translation proNGF mechanisms were not completed.

However, these results (Figure 8B, C) show that there are no differences between the band pattern in media and cytosolic fraction of HEK 293T non-transfected cells, HEK 293T cells transfected with pEGFP-C1 and HEK 293T cells transfected with pCDNA 3.1-proNGF. Moreover, proNGF does not seem to be cleaved since there is no band at approximately 13 kDa (mature NGF). Since the antibody used to detect proNGF expression is directed against the N-terminal of mature NGF, we speculated there could be a difference in the tridimensional structure between mature NGF and proNGF, which would not allow proNGF detection. The proNGF construct used to transfect cells has a Histidine tag at the N-terminus to allow for affinity purification and detection. We analyzed by Western Blot the same samples of medium and cells extracts, described above, with an antibody directed against the C-terminal, an anti-His tag (Figure 8D, E). We found no differences in HEK 293T cells transfected with proNGF when compared to control (EGFP) transfected cells, confirming that proNGF was not successfully expressed and/or concentrated. The fact that we could not detect proNGF suggests that HEK 293T cells are not expressing proNGF in sufficient amounts or the concentration step was not successful.

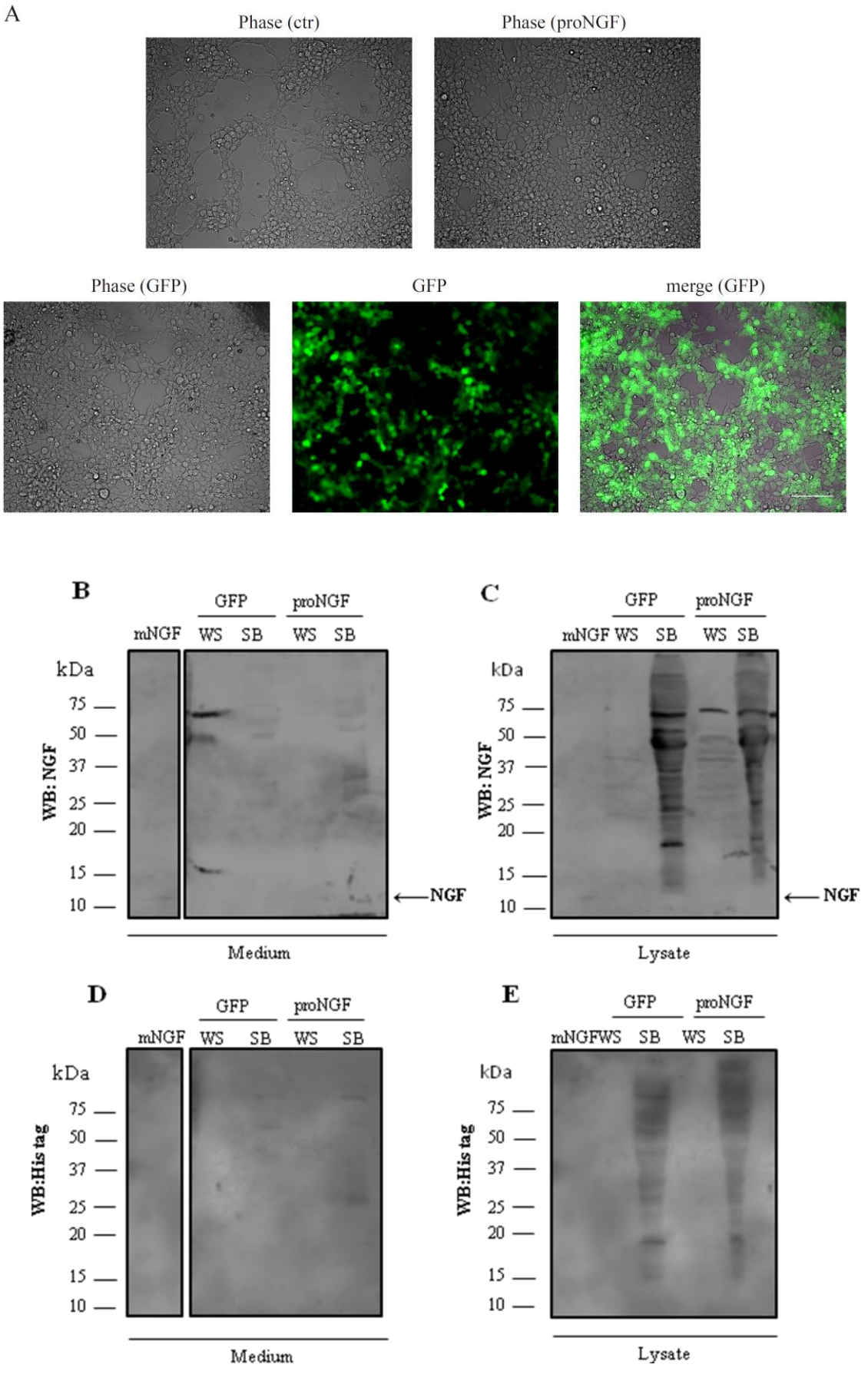
To address these two scenarios, we decided to purify proNGF from a different expression system: a mammalian stable cell line expressing proNGF, an experimental

approach successfully used before by other groups (Lee et al., 2001, Beattie et al., 2002). We transfected COS-7 cells with pCDNA 3.1-proNGF with a neomycin resistance gene and selected for positive clones. A dose-response curve was performed and a concentration of 0.4 mg/ml G418 was determined as the one optimal for selection (data not shown).

To test whether proNGF was being produced, proNGF selected clones were cultured in DMEM with 0.05% FBS and 0.4 mg/ml G418, and protein expression was allowed to occur for 72 h, proNGF expression was screened by Western Blot (Figure 9). Total cell extracts were used to maximize the detection levels. proNGF was not detected in any of the clones tested, since no differences are found between bands when comparing Cos-7 proNGF clones and Cos-7 non transfected cells. In addition, no bands of low molecular weight appeared, meaning that proNGF is not being cleaved to its mature form (NGF). Therefore, it is likely that expression of proNGF was unsuccessful.

Our results contrast with those obtained by Hempstead and colleagues that successfully purified biological active proNGF from a HEK 293 stable cell line (Lee et al., 2001). One possible explanation is that the selected COS-7 have acquired natural resistance to the G418 due to their exposure to toxic G418 concentrations that lead to the appearance of spontaneous G418 resistant mutations. However, we find this unlikely and as speculated before proNGF is most likely being expressed at a very low level, which we cannot detect and therefore purify.

Although it is not evident why we did not succeed in detecting proNGF, we can speculate some possibilities. Firstly, it is important to keep in mind that the lack of detection does not necessarily imply absence of proNGF expression. As described before, the proNGF mutant construct used is based in a furin-resistant sequence, which


A
Phase (ctr)
Phase (proNGF)
Phase (GFP)
GFP
merge (GFP)
B
GFP
proNGF
mNGF
WS
SB
WS
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kDa
75
50
37
25
20
15
10
WB: NGF
NGF
Medium
C
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proNGF
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Figure 8 – Expression and concentration of proNGF in HEK 293T cells. HEK 293T cells were transfected with 144 µg of pCDNA 3.1-proNGF or pEGFP-C1. Medium was changed 24 h after transfection and protein expression was allowed to occur for an extra 24 h. **(A)** 48 h after transfection, phase and immunofluorescent images of HEK 293T cells were acquired. GFP transfection was used as a control. Cell medium **(B, D)** and cell lysates **(C, E)** were concentrated overnight with Ni Sepharose™ High Performance beads slurry and elution was performed firstly with wash solution (WS) and then 1x concentrated sample buffer (SB). The resulting fractions were analyzed by immunoblot with antibodies against proNGF **(B,C)** and against the Histidine tail **(D,E)**. No differences are found between bands when comparing HEK 293 T cells transfected with pCDNA 3.1-proNGF and HEK 293T cells transfected with pEGFP-C1 using anti-NGF or anti-His tag, which suggests that expression or concentration of proNGF was not achieved. Images were taken using a Zeiss Axiovert 200 fluorescence microscope with a Plan-Neofluar 20x objective. The scale bar is 20 µm. 40 µg of protein was loaded per lane and 10 ng/mL of mature recombinant NGF (mNGF) was included as a positive control.

is the main protease responsible for the processing of proNGF into mature NGF (Pagadala et al., 2006).

Nevertheless, there are others convertases that also cleave proNGF into NGF, such as PACE4 and PC and so could be responsible for proNGF cleavage (Seidah et al., 1996, Pagadala et al., 2006). In addition, mutations of proNGF only in the furin cleavage site result in a form of proNGF that is only partially stable because they lead to the expression of proNGF, mature NGF and intermediate processed forms of proNGF (Pagadala et al., 2006). This could only be overcomed by the introduction of additional mutations, namely in the two dibasic sites recognized by PACE4 and PC2, what would significantly increase the stability of proNGF (Pagadala et al., 2006). Moreover, furin-resistant proNGF protein has also been described to be sensitive to cleavage by extracellular proteases, such as metalloproteinase-7 and plasmin (Lee et al., 2001). Consequently, it is plausible to hypothesize that both expression systems used (transiently transfection or stable transfection) could be producing proNGF but the

presence of PACE4 and PC2 convertases could lead to the formation of different isoforms of proNGF and NGF as described above. Although we have used an antibody against the mature form of NGF, which is expected to bind all possible transcripts described before, it is important to emphasize that most probably proNGF transcripts (proNGF, NGF and different isoforms of proNGF) have low expression levels under our experimental conditions, and so, unable to be detected. Taking this into consideration, future studies should be performed using a proNGF construct with the three mutations, in order to discard the possibility of proNGF cleavage.

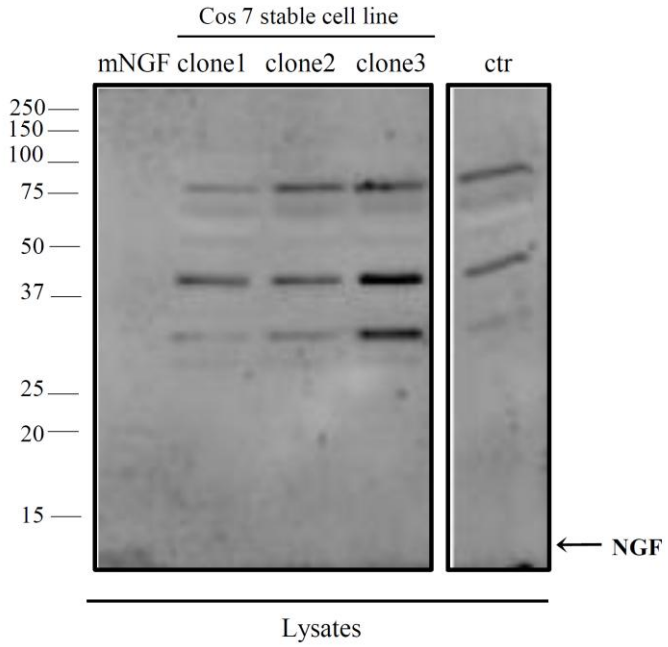

Figure 9 – proNGF expression and concentration in Cos-7 stable cell line clones. Cos-7 stable cell line clones were generated as described in material and methods section. Cells were maintained in selection media and for proNGF expression cells were plated in a 6 multiwell plate and medium changed to DMEM, with 0.05 % FBS and 1:100 penicillin/streptomycin. Expression of proNGF was allowed to occur for 72 h. Total cell extracts were prepared and Western Blot analysis for proNGF presence was performed with the anti-NGF antibody. No differences are found between bands when comparing Cos-7 proNGF clones and Cos-7 non transfected cells. Therefore, expression of proNGF was possibly not successful. 20 µg of protein was loaded per lane and 10 ng/mL of mature recombinant NGF (mNGF) was included as a positive control. ctr – not transfected cells (control).

Since we were unable to purify proNGF from mammalian expression systems we then decided to use commercially available recombinant proNGF generated in *E. coli*. In the course of this work a study was published showing that commercial proNGF induces death of retinal ganglion cells *in vivo* (Lebrun-Julien et al., 2010), suggesting that recombinant proNGF is biologically active.

3.2. proNGF induces axonal degeneration

Accumulating evidences show that proNGF is increased in the extracellular environment under pathological conditions, such as brain and spinal cord injuries. This increase has been reported to be responsible for the activation of cell death cascades (Beattie et al., 2002, Harrington et al., 2004). Since neurons are highly polarized cells, it is crucial to determine the specific location where proNGF exerts its effect. Taking into consideration that neuronal cell death is often preceded by axonal degeneration, and retrograde signaling has been suggested as a possible mechanism for the physiological role of neurotrophins, we asked if proNGF could exert its actions when applied locally to axons. However, it has been technically challenging to manipulate axons independently from the cell body due to the lack of reliable systems. Jeon and colleagues have recently developed a highly useful microfluidic platform to isolate axons from somata and dendrites (Taylor et al., 2005). In the next section I will describe in detail these devices.

3.2.1. Microfluidic devices for cell culture

Microfluidics refers to devices and methods that allow the manipulation of fluid flows at the micrometer scale in tiny artificial microsystems (Pihl et al., 2005). These

microsystems are able to transport small volumes of fluid (10^{-6} to 10^{-15} litres), promoting a high fluidic resistance that can counteract particle diffusion (Taylor et al., 2005, Weibel and Whitesides, 2006). The precise control of fluid dynamics allows users to know the exact content of individual compartments. In addition, these devices require small quantities of samples, reagents and cells, which are very important when working with biological systems.

Microfluidic chambers are small multicompartment devices that have the advantage to precisely control, monitor and manipulate cellular microenvironments. In recent years, the microfluidic field has grown and microfluidic devices are now emerging as powerful tools in a variety of areas such as drug discovery and cell biology (Pihl et al., 2005, Sekhon and Kamboj, 2010).

The first report of the application of PDMS microfluidic devices in neurobiology has less than a decade (Taylor et al., 2003). However, these microsystems have been employed in a variety of studies such as localization and identification of mRNAs in axons (Taylor et al., 2005), neuron-to-cell spread and viral transport in axons (Liu et al., 2008), synapse-to-nucleus signaling (Taylor et al., 2010), intracellular pH regulation in neuronal soma and neuritis (Vitzthum et al., 2010), axonal navigation and network formation (Millet et al., 2010) and multi-compartment neuron-glia co-cultures (Park et al., 2009). Therefore, microfluidic devices arise as an important support to unravel many of the open questions in neurobiology.

Each microfluidic device is composed of a molded PDMS (Figure 10A) placed against a coated-glass coverslip, to which neurons adhere. PDMS devices are highly permeable to carbon dioxide and oxygen, thermally stable, physiologically and chemically inert, optically transparent, easily fabricated and most relevant, biocompatible. Therefore,

PDMS has been shown to be the most appropriate material to fabricate the micro-devices used in cell growth (Whitesides, 2006, Wang et al., 2009).

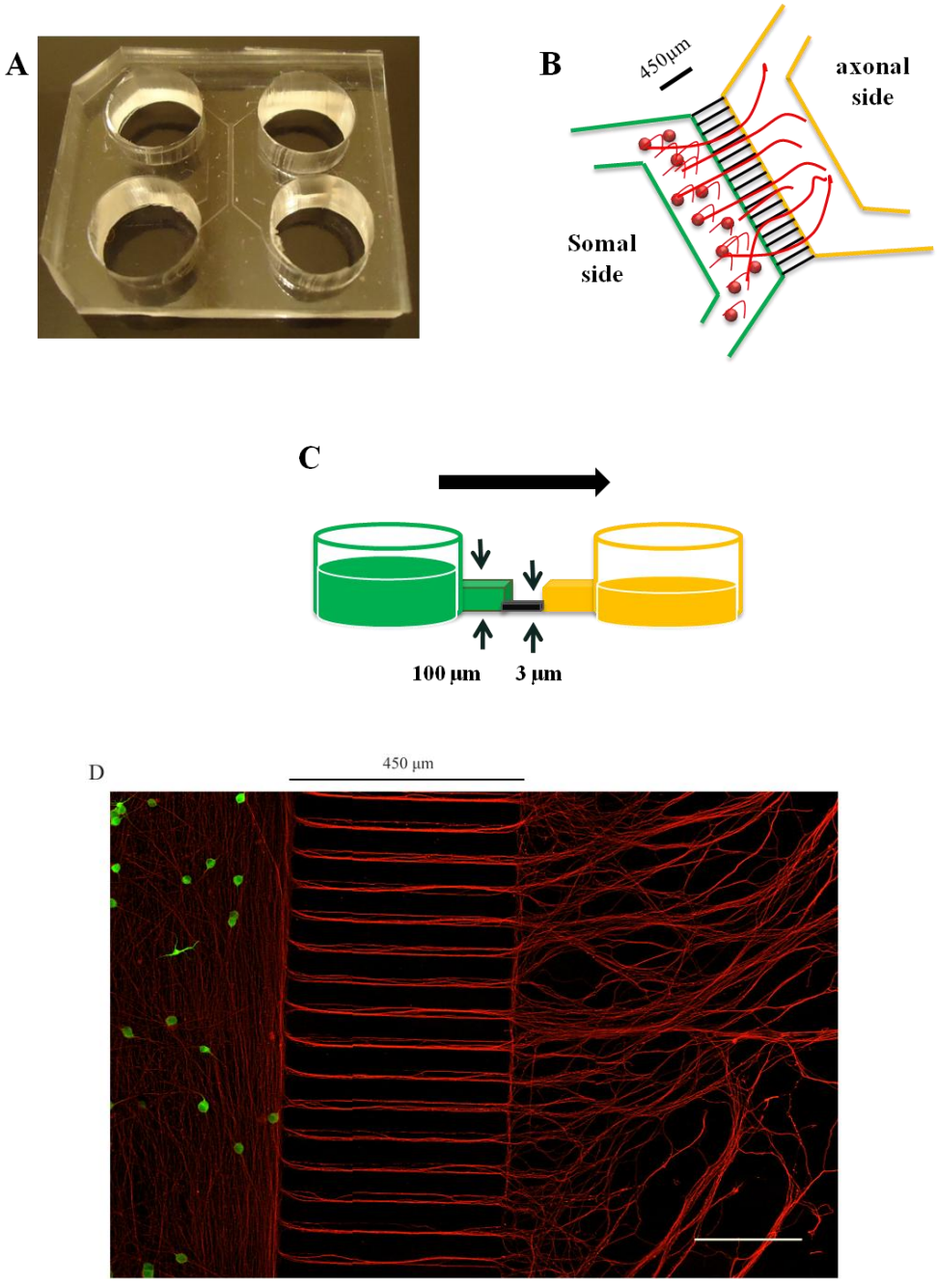

Figure 10 – Culture of rat DRG neurons in microfluidic chambers. (A) These small devices (20 mm × 25 mm) consist of a molded polydimethylsiloxane (PDMS) chamber placed against a glass coverslip. **(B)** Chamber top view. The somal side (green) and the axonal side (yellow) are separated by a set of microgrooves (black) (450 µm long, 10 µm wide). In each side a compartment (1.5 mm wide, 7 mm long) is connected by two reservoirs. Neurons are plated in the somal compartment, and randomly growing axons (red) are guided to the axonal side

through the microgrooves. **(C)** Chamber side view. Microgrooves' height (3 μm) is lower than compartments' height (100 μm). If a volume difference is maintained (higher green volume in the somal side), microgrooves' high fluidic resistance guarantees fluidic isolation for 20 h (Taylor et al., 2005). **(D)** Primary cultures of rat embryonic DRG neurons grown in microfluidic devices were immunostained at DIV6 against β-Tubulin I and MAP2. Contiguous images were taken from a random area of the microfluidic chamber using a Zeiss LSM 510 confocal microscope with an EC-PlanNeofluar 40× objective and assembled into a single image using the LSM 510 software. The scale bar is 200 μm.

The microfluidic chambers used are composed of two symmetric chambers, connected by arrays of microgrooves. These chambers physically isolate the somal compartment from the axonal side (Figure 10B). A small volume of cell suspension is loaded into the somal side (green) where the cell bodies adhere and grow. Randomly, growing neuritis pass through the microgrooves and achieve the axonal compartment (yellow), without any contamination of soma and dendrites (Figure 10B). This occurs because the microgrooves are long enough to prevent dendrites from reaching the other side and too narrow so that cell bodies entrance and passage through them is completely blocked (10μm wide, 3μm high). Moreover, the maintenance of a minimal volume difference between the somal and axonal compartments induces the formation of an unidirectional flow and allows the fluidic isolation of chemical microenvironments (Figure 10C).

3.2.1.1. Growth of embryonic DRG rat neurons in microfluidic chambers

DRG neurons have two main functions: stimulus transduction and transmission of the encoded information to the central nervous system (Julius and Basbaum, 2001). DRG neurons are a good *in vitro* model because they express $p75^{NTR}$ and sortilin receptors (complex responsible for proNGF response) (Arnett et al., 2007), their axons have

central and peripheral processes but only the terminal of the latter nerves are sensitive to natural stimuli and they are capable of regenerating after an injury (Hannila and Filbin, 2008). Therefore, to determine the effect of proNGF when it is locally applied to axons we used primary cultures of rat embryonic DRG neurons as an *in vitro* model.

We started by optimizing long-term primary cultures of DRG neurons in microfluidic devices. Despite the difficulties to successfully culture CNS neurons in microfluidic chambers, researchers have been able to successfully culture PNS neurons, such as chick and rat DRG neurons (Park et al., 2009, Rivieccio et al., 2009). In order to establish a primary cell culture of DRG neurons grown in microfluidic chambers, DIV 6 cultured DRG neurons were immunostained against β-Tubulin I and MAP2, a random area of the microfluidic device was chosen and contiguous images were taken to generate a single image (Figure 10D). The somal compartment (left side), where neuronal cell bodies are located, and the axonal compartment (right side) where only axons are present can be distinguished. The two compartments are isolated by a set of microgrooves that provides a barrier and a passageway that allows the isolation of cell bodies (in the somal compartment) and the growth of axons into the axonal compartment.

We optimized a protocol to maintain long-term cultures of DRG neurons in microfluidic chambers. Our results show that neurons grow, develop and construct a complex and normal network of neuritis (Figure 11A). Moreover, DRG neurons viability inside the microfluidic chambers is not affected when compared to control cultures, DRG neurons cultured in a 24 multiwell plate (Figure 11B, C). In addition, axons are isolated from the cell body and dendrites and, after reaching the axonal side, they spread in every direction covering the whole surface of the axonal compartment and no axon bundles are formed (Figure 12), which reveals that fluidicaly isolated axons are healthy.

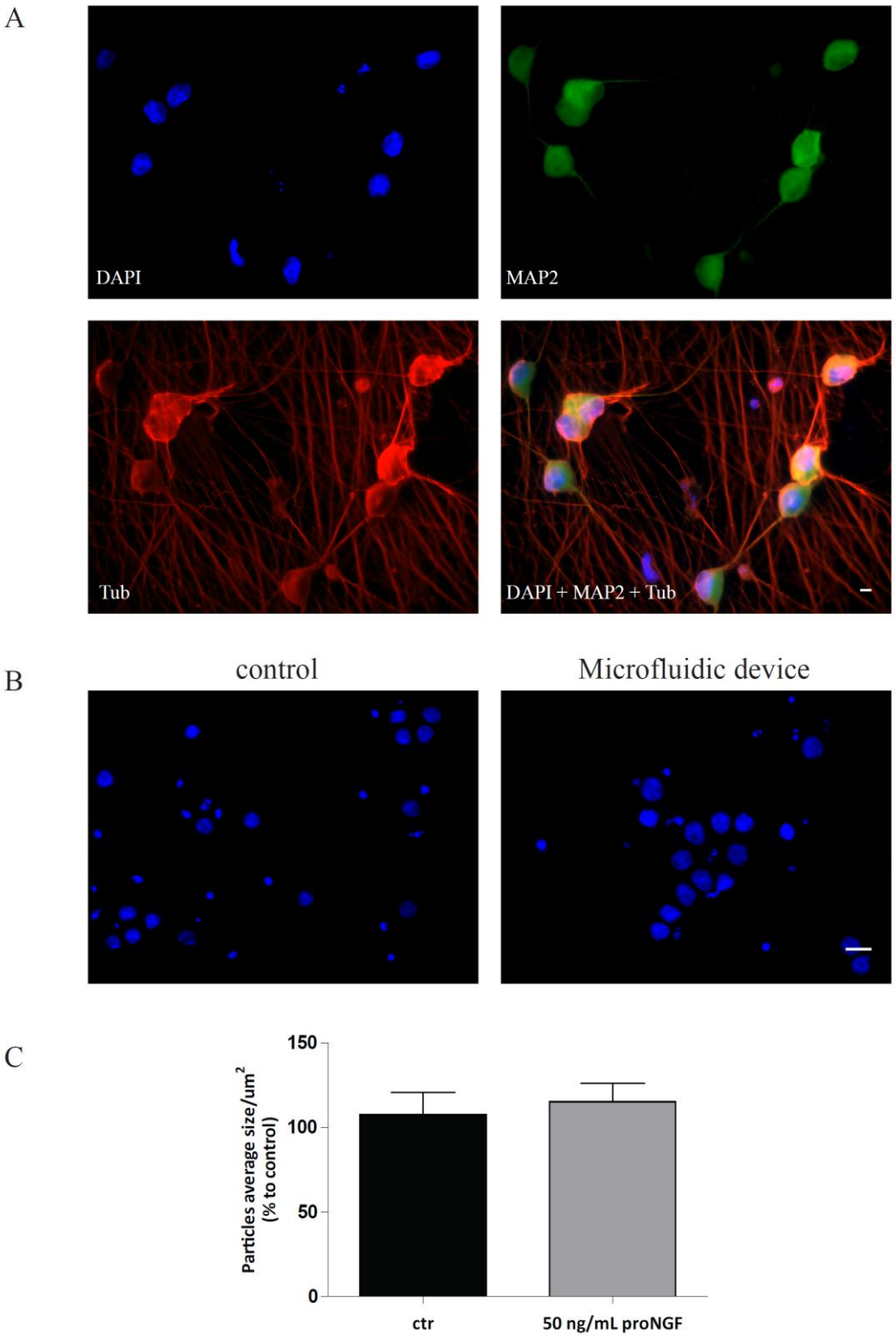

Figure 11 – Viability of DRG neurons cultured in microfluidic chambers is not altered. Primary cultures of rat embryonic DRG neurons at DIV 6 were immunostained against β-Tubulin I and MAP2 and then mounted with DAPI. **(A)** No neurite fragmentation is observed in DRG neurons cultured in microfluidic chambers (images were taken from the somal compartment using a Zeiss Axiovert 200 fluorescent microscope (EC-PlanNeofluar 63x

objective). **(B)** To examine DRG neurons viability, primary cultures of rat embryonic DRG neurons grown in microfluidic devices (DIV 8) and in multi-wells (DIV 8) were fixed and mounted with DAPI. Analysis of DRG cell death was assessed taking in account fragmented nuclei and condensed chromatin. 400 – 600 cells were counted per experiment after random selection of at least 8 different optical fields. Preparations were examined with a Zeiss Axiovert 200 fluorescence microscope using a Plan-Neofluar 20x objective. The scale bar is 20 µm. **(C)** Quantification of results shown in A. No significant differences were found in neuronal viability in cells cultured inside microfluidic chambers when compared to controls. Bars represent the mean ± SEM from two independent experiments. Statistical analysis was performed with unpaired student's t-test.

The microgrooves height (3 µm) is responsible to trap neuronal cell bodies in the somal compartment because cell bodies are known to vary from 4 µm to 100 µm in diameter (Figure 12A). Importantly, when cell suspension is added, the axonal reservoirs are filled with medium that leads to a continuous unidirectional flow (from axonal to somal side) and avoids the diffusion of suspended components from the somal to the axonal side.

On the other hand, dendrites are thinner than cell bodies and can therefore easily enter the microgrooves. However, they grow slowly and they are smaller than axons. According to what has been observed, DRG dendrites cannot reach the axonal compartment since microgrooves are much longer (450 µm of length) than dendrites'maximum lenght (Figure 12B).

Microfluidic chambers, as described above, also guarantee fluidic isolation. In these devices, the fluidic flow is laminar, consequently, diffusion is the only mechanism to mix solutes that are located at different compartments. Since diffusion is a slow process, microfluidic channels are capable of creating a high fluidic resistance between compartments and a cross sectional gradient in the presence of a hydrostatic pressure

(established with a higher volume on one of the compartments) which lead to fluidic integrity (Taylor et al., 2003, Taylor et al., 2005).

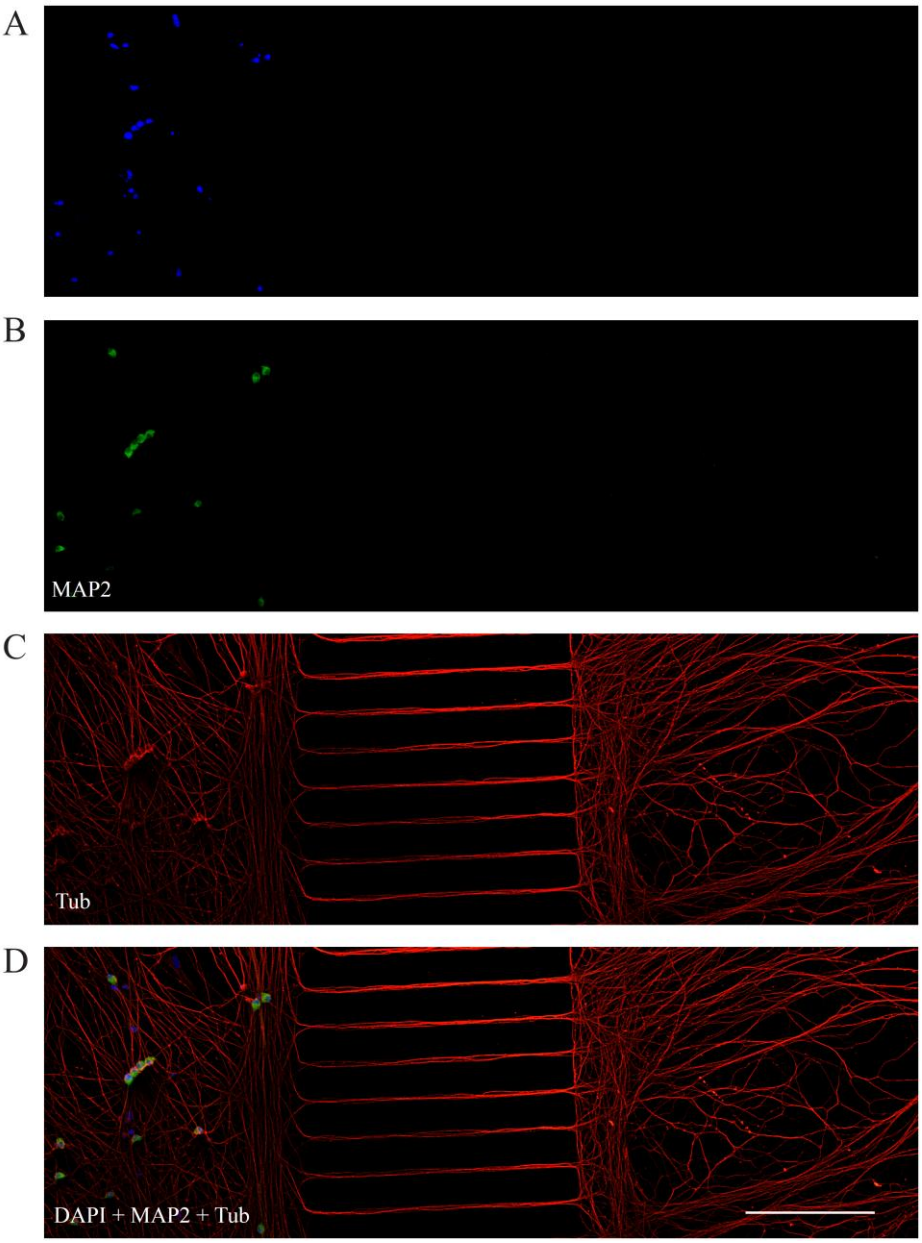

Figure 12 – Isolation of pure axonal populations. Primary cultures of rat embryonic DRG neurons were plated in the somal compartment of microfluidic chambers and allowed to develop for 6 days. Cells were immunostained against **(A)** DNA, **(B)** MAP2 (dendrites) and **(C)** β-Tubulin I (axons). Continuous images were taken from a random area using a Zeiss LSM 510 Meta confocal microscope with an EC PlanNeofluar 20 x objective, and **(D)** assembled into a single image using a LSM software. Cell bodies are unable to pass through the microgrooves

duo to their size, while dendrites are not long enough to pass the microgrooves and reach the axonal compartment. As a result only axons are capable to cross the microgrooves and reach the axonal compartment making these chambers a powerful tool to compartmentalize and isolate axons in living cells. The scale bar is 200 μm.

To demonstrate fluidic integrity, we added DiI to the axonal compartment of a microfluidic chamber for 48 h, with a volume difference of 50 μL between compartments. However, this difference is maintained up to 20 h (Taylor et al., 2005). As a result, medium was added to the somal side in intervals not exceeding 20 h. After 48 h, only neurons that had axons in the axonal compartment are immunostained (Figure 13). These results constitute strong evidence that a particular drug can be applied in one of the compartments in order to produce a localized effect.

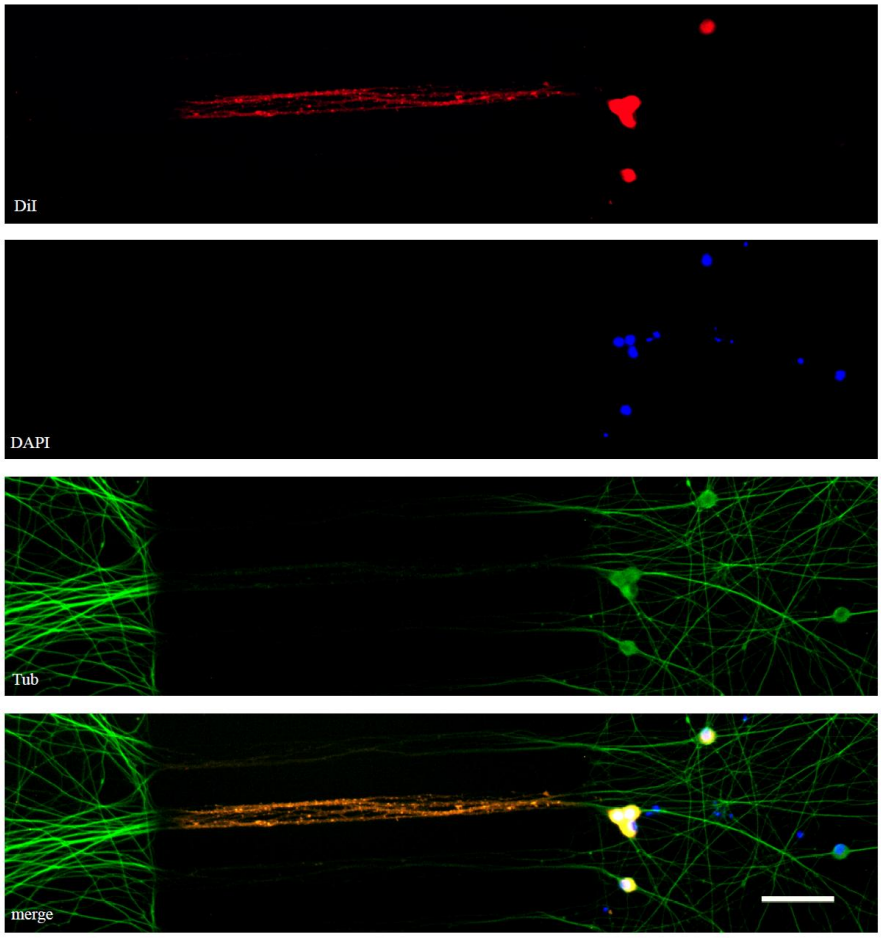

Figure 13 – Fluidic isolation of microfluidic chambers. Rat embryonic DRG neurons were grown in microfluidic chambers. At DIV7 DiI (1:750) was added to the axonal compartment only and 48 h later the cells were labeled for β-Tubulin I and DNA. Only the neurons that have extended axons into the axonal compartment are stained with DiI which demonstrates fluidic isolation. Therefore, axonal or somatic microenvironments can be independently manipulated. Images were taken using a Zeiss Axiovert 200 fluorescence microscope with a Plan-Neofluar 20x objective. The scale bar is 50 μm.

3.2.2. Local proNGF stimulation induces axonal degeneration

NGF and proNGF are important regulators of cell fate. Recent findings have reported that cells are capable of releasing both proNGF and NGF molecules to the extracellular space (Lee et al., 2001). The different neuronal fates induced by these two molecules are emphasized by a recent study that identifies clear differences between the mRNA profiles induced by each of them and the influence in gene expression when proNGF and NGF coexist (D'Onofrio et al., 2011). Therefore, experimental evidences support a key role for proNGF/NGF ratio in cell fate *in vivo*.

The increase of proNGF/NGF ratio has been described as a cause of cell death. proNGF is cleaved to mature NGF by different intracellular and extracellular proteases (Lee et al., 2001, Bruno and Cuello, 2006). When released to the extracellular space, its conversion to mature NGF is coordinated by the action of zymogens, convertases and endogenous regulators (Bruno and Cuello, 2006). The failure in this regulation is one of the possible mechanisms responsible for the proNGF-mediated cell death.

Sortilin is essential for proNGF-induced death signals via $p75^{NTR}$. Approximately 90 % of sortilin resides in intracellular membranes, predominantly in the Golgi apparatus, endosomes and lysosomes, and the remaining sortilin is localized on the cell surface (Nykjaer et al., 2004, Mari et al., 2008). A recent study performed by Hempstead and

colleagues (Kim and Hempstead, 2009) suggests that NRH2, a $p75^{NTR}$ homologue, regulates the expression of sortilin on the neuronal cell surface by binding to sortilin and thus preventing its lysosomal degradation. Consequently, sortilin is free to bind to $p75^{NTR}$ and then to proNGF, thus inducing cell death.

The mechanism by which $p75^{NTR}$-sortilin complex transmits the apoptotic signal into cells is not entirely understood. Sortilin is responsible for targeting the prodomain of proNGF with high affinity, serving as a co-receptor. Oppositely, $p75^{NTR}$ targets the mature domain of proNGF with high affinity (Nykjaer et al., 2004). The binding of proNGF to both $p75^{NTR}$ and sortilin receptors induces *phosphatase and tensin homolog deleted on chromosome 10* (*PTEN*) upregulation and leads to the cleavage of the $p75^{NTR}$ intracellular domain by *γ-secretase*. This cleavage induces nuclear translocation of the DNA-binding protein neurotrophin receptor-inducing factor (NRIF) and c-Jun-N-terminal kinase (JNK) activation which leads to the initiation of the apoptotic cascade (Kenchappa et al., 2006, Song et al., 2010).

Neuronal cell death is often preceded by axonal degeneration. Recent results performed by Zhou and colleagues demonstrate that in the presence of proNGF, cortical neurons from adult mice show a decreased in viability and inhibition of neurite growth (Wang et al., 2010). In addition, proNGF is increased in aged sympathetic neurons and in their targets, which suggests an effect of proNGF in the neuron cell body as well as in axons (Bierl and Isaacson, 2007). Moreover, mature NGF is suggested to activate Trk A receptors in the axons, where it can induce a response through a retrograde signaling (Mok et al., 2009, Niewiadomska et al., 2011). Taking these studies into consideration we hypothesized that proNGF could exert its action when locally applied to axons.

To test whether proNGF can elicit an effect on distal axons, primary cultures of rat embryonic DRG neurons were stimulated at DIV 8. This time point was chosen because DRG neurons have been described to express sortilin and $p75^{NTR}$ in cell surface at post-natal day 1 (Kim and Hempstead, 2009). However, it is important to take into consideration the need to exclude the effect of mature NGF in axons. Therefore, conditioned media was removed from the axonal compartment and proNGF was selectively applied to this compartment for 48 h, while neuronal cell bodies continued to be kept in trophic medium. After stimulation of axons with proNGF, axonal fragmentation was observed (Figure 14). The results obtained show an increase in the number of particles per axonal area (170 %, $p < 0.05$) when proNGF is applied to the axonal compartment of microfluidic chambers (Figure 14). In contrast, the average size of each particle is not altered when compared to control (Figure 14C), indicating that proNGF is able to induce axonal fragmentation without an increase of the individual particles size.

To further validate our results we determined the degeneration index (Figure 15), which corresponds to the ratio between fragmented axon area and total axon area in each field. The results evidence that proNGF can induce a pronounced increase of axonal degeneration when it is present exclusively at the axonal compartment (187 %, $p < 0.05$), when compared to control (Figure 15B). The effect of proNGF was quantified using an image analysis algorithm that allows the distinction between fragmented and intact axonal segments as these changes disrupt axon uniformity and result in isolated round particles.

Here we observed that proNGF is an axonal degeneration-inducing ligand for DRG neurons. Neurons seem to have two programs of "self-destruction": an intracellular death program (apoptosis) activated upon an injury or an infection; and a selective

axonal death program, activated when the axons are severed. However, these programs seem to be connected, as axonal degeneration precedes and sometimes causes neuronal death (Coleman, 2005).

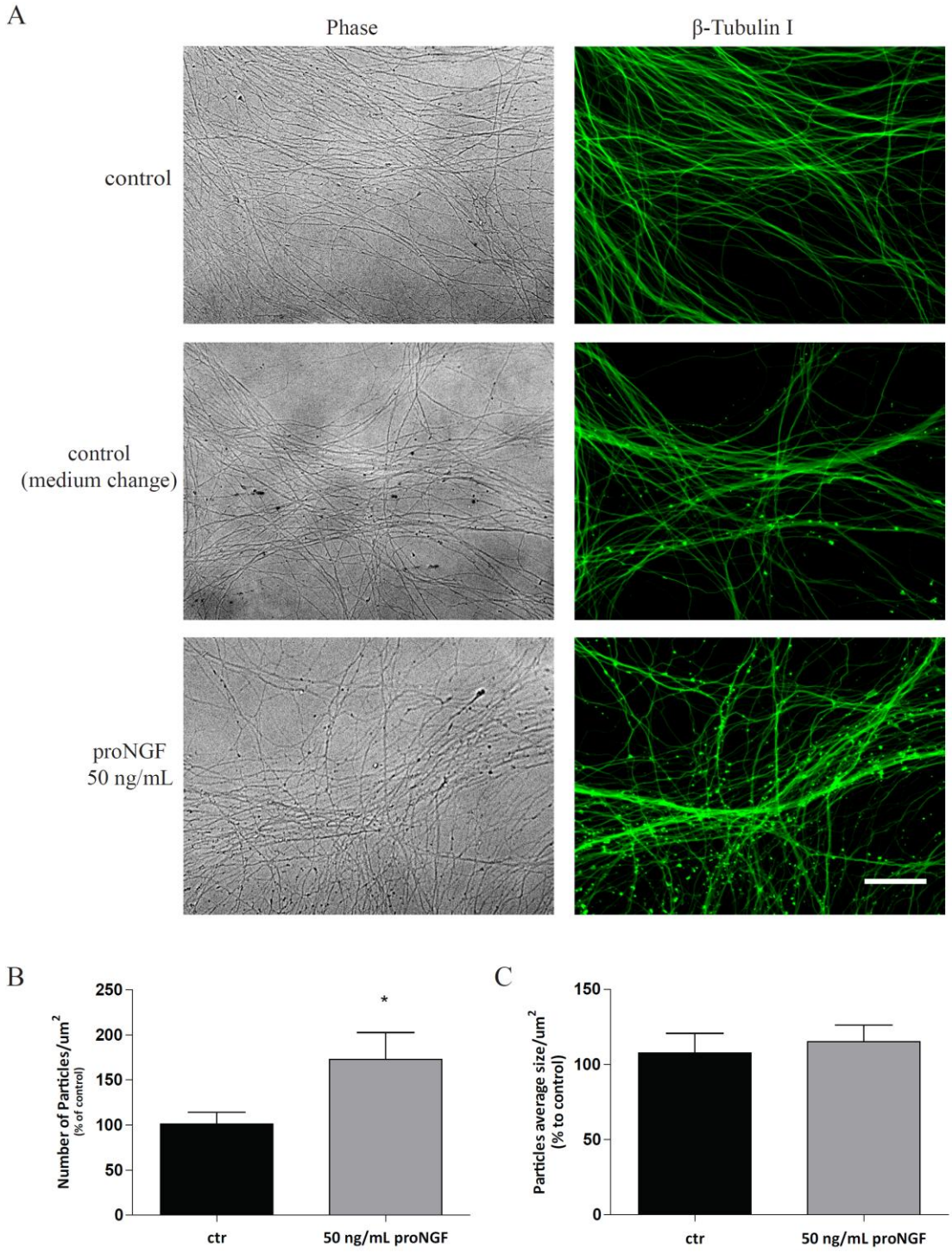

Figure 14 – proNGF induces axonal fragmentation. (A) Primary cultures of rat embryonic DRG neurons grown in microfluidic chambers were stimulated at DIV 8 in fresh cultured

medium with either 50 ng/ml proNGF or vehicle (control). The stimuli were performed in the axonal compartment only and a volume difference was maintained throughout the experiment to guarantee axonal fluidic isolation. Cells were immunostained against β-Tubulin I and random images from the axonal compartment were taken with a Zeiss Axiovert 200 fluorescence microscope with a Plan-Neofluar 20x objective. The scale bar is 50 µm. Representative images of axons without manipulation (control), addition of DRG medium (vehicle) and addition of DRG medium with 50 ng/ml proNGF. The number of particles **(B)** and their average size **(C)** were measured using Image J 1.42 software in randomly selected images. Results indicate that local application of proNGF induces a significant increase in the number of particles while the average size of individual particles is not altered. Values were normalized to the control mean. About 40 images were analyzed per condition. Bars represent the mean ± SEM of three independent experiments (* represents $p < 0.05$ using students' t-test with confidence intervals of 95 %).

Elimination of axons seems to occur by two different processes: retraction, a small axonal elimination, and degeneration, where axon large-scale eliminations appear to occur. Axonal degeneration is characterized by sequential morphological changes, which begin in axonal fragmentation and culminate in degeneration of intact axons (Luo and O'Leary, 2005). This event can result from a diminished trophic support, direct induction of an intrinsic program for degeneration or activation of a default degradation program owing to its diminished repression (Luo and O'Leary, 2005).

Axonal degeneration seems to be one of the keys to remove axons from incorrect or inhospitable environments. The use of a huge number of models has provided evidence that axonal degeneration leads or is caused by alterations in axonal transport, which might cause defects in the degradative pathway, impairment of organelle motility and neurotrophic signaling and elevated stress-signaling (Perlson et al., 2010).

Wallerian degeneration is a slow axonal self-destruction program that follows axonal injury that has been used as a model for axonal degeneration (Finn et al., 2000, Luo and

O'Leary, 2005). Researchers have been using this model to show that a blockade of axonal transport triggers a proactive axon death programme (Raff et al., 2002, Luo and O'Leary, 2005). Interestingly, in motor axon injury it takes 20 h for axons to undergo Wallerian degeneration. 44 h after the injury or axonal transport blockade process, some axons are completely fragmented while others are partially fragmented and some remain entirely linear. Finally, at a later point the cell body also degenerates (Conforti et al., 2007). At the molecular level, Wallerian degeneration does not seem to induce apoptotic pathways since mutations in genes involved in the induction of this type of death do not prevent axon degeneration in injury (Finn et al., 2000). However, axonal degeneration seems to be involved in an active and regulated program (Luo and O'Leary, 2005).

Nevertheless, axonal transport is a complex bidirectional process that involves motor proteins and signaling between soma and axons. The disruption of the retrograde transport, for example, leads to the impairment of neurotrophin signaling endosomes transport, which has been shown to cause neurodegeneration in both mice and human diseases (Cosker et al., 2008). Moreover, some studies show that neurotrophin deprivation can mimic, at least morphologically, the Wallerian degeneration (Finn et al., 2000) and both models of axonal degeneration seem to have a caspase-independent signaling (Raff et al., 2002).

Further research in axonal degeneration has evidenced that several pathways contribute to this mechanism. Thus, the balance of signaling along the axon, from survival to stress signals, seems to be a crucial component of rapidly progressive axonal degeneration and consequently neuronal cell death (Perlson et al., 2009, Perlson et al., 2010).

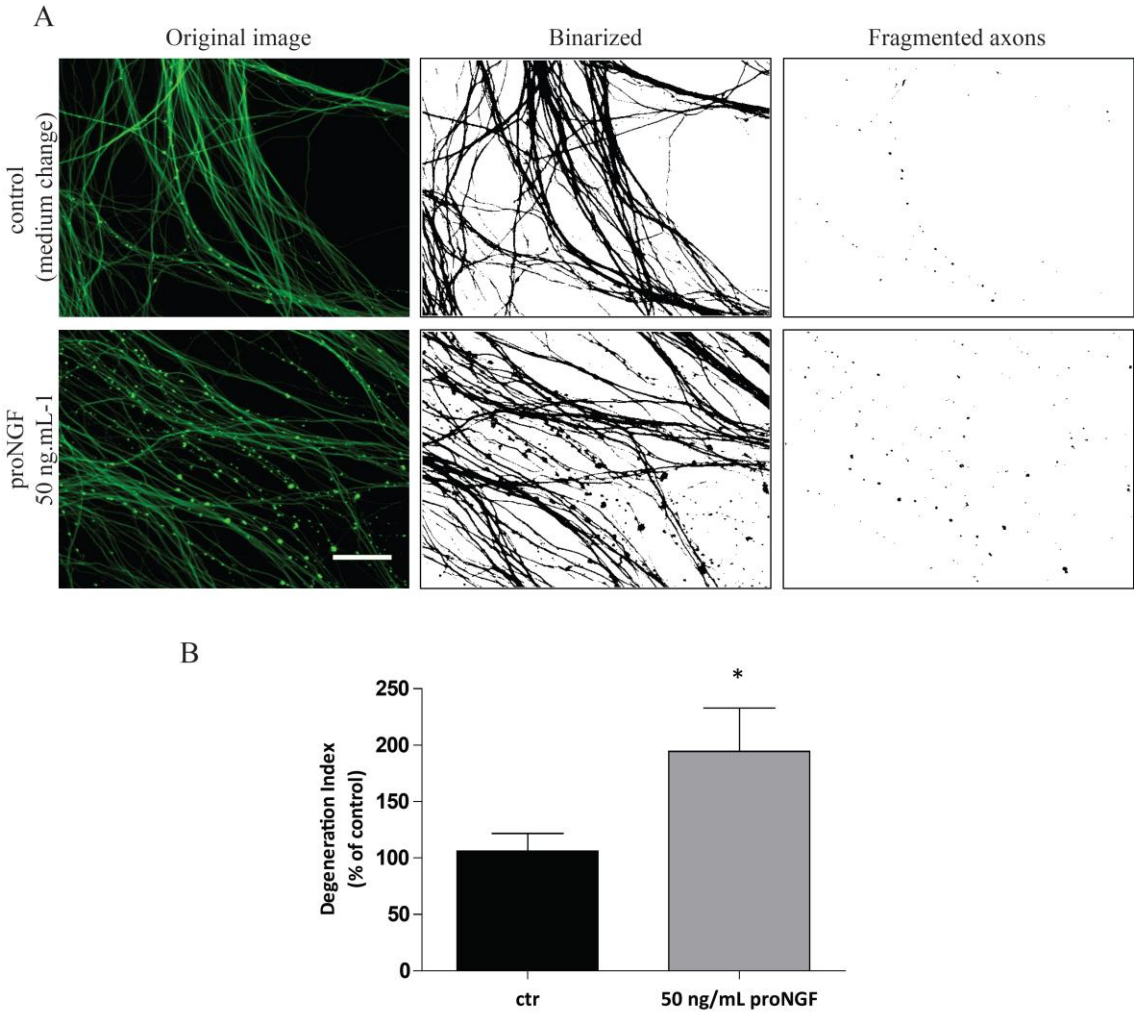

Figure 15 – Local application of proNGF induces axonal degeneration. (A) Primary cultures of rat embryonic DRG neurons grown in microfluidic chambers were stimulated at DIV 8 in fresh cultured medium with either 50 ng/ml proNGF or vehicle (control). The stimuli were performed only in the axonal compartment and a volume difference was maintained throughout the experiment to guarantee axonal fluidic isolation. Cells were immunostained against β-Tubulin I and random images from the axonal compartment were taken with a Zeiss Axiovert 200 fluorescence microscope with a Plan-Neofluar 20x objective. Representative pictures of original images (left panel), binarized images (central panel) and fragmented axons (right panel) from axonal compartment. The scale bar is 50 µm. **(B)** The fragmented axonal area and total axonal area were measured using Image J 1.42 software in randomly selected images and degeneration index (ratio of fragmented axonal area to total axonal) was calculated. Results show that local application of proNGF induces a pronounced increase of axonal degeneration.

Values were normalized to the control mean. About 40 images were analyzed per condition. Bars represent the mean ± SEM of three independent experiments (* represents $p < 0.05$ using students' t-test with confidence intervals of 95 %).

Using SCG and DRG neurons, it was shown that pro-NT3 can induce neuronal death when exclusively present at distal axons, which is not reversed with the direct supply of NGF to the cell body (Yano et al., 2009). In our results it is interesting to notice that, when proNGF was present, NGF in the cell body did not prevent axonal degeneration. In addition, a tandem-repeated swelling on individual axons seems to occur, which suggests a multifocal block of axonal transport that is correlated with neurodegenerative disorders (Coleman, 2005). Moreover, proNGF has been demonstrated to be secreted *in vivo* from target tissues in physiological (Bruno and Cuello, 2006) and pathological conditions (Bierl and Isaacson, 2007, Domeniconi et al., 2007). According to these studies and the results shown here, we can assume that even in the presence of trophic support in cell body, the pathways activated by the increase of proneurotrophin levels in axons override the survival factors present in the cell body. Moreover, it is reasonable to speculate that the axonal degeneration could be due to proNGF insult rather than to trophic support deprivation of axons. Consequently, proNGF seems to have a role in axon elimination as an inducer of pathways that lead to axonal degeneration. Therefore, whether proNGF recruits distinct cellular components from NGF-withdrawal is an intriguing possibility. However, it is important to keep in mind that although the methodology used regarding what happens in sympathetic neurons *in vivo* (cell body is not usually deprived of trophic factors), in a real situation NGF and proNGF molecules coexist in the presynaptic region and the ratio between them is probably the key to cell response.

Human genetic studies have shown that neurons are highly vulnerable to disruption of the retrograde axonal transport (Perlson et al., 2010). Given the importance of the cytoskeleton in maintaining axon integrity, disruption of the anterograde and retrograde motor complexes is also an important key in axonal degeneration since they lead to defects in both supply and clearance of intracellular components (Perlson et al., 2009). During neurotrophin deprivation, neurofilament degradation and microtubule fragmentation have shown to be co-related with axonal degeneration (Zhai et al., 2003). Importantly, in our study NGF support in the cell body was not altered, indicating that the significant axonal degeneration shown here seems to be a result of a direct induction of an intrinsic program for degeneration initiated at distal axons. Moreover, recently, neurotrophin deprivation has shown to be related with $p75^{NTR}$ apoptotic signaling, including activation of JNK and the caspase cascade in a mouse model of amyotrophic lateral sclerosis (Perlson et al., 2009) and in sympathetic neurons (Mok et al., 2009). Interestingly, in our results NGF-withdrawal clearly increases axonal fragmentation when compared with control non-manipulated DRG neurons. However, proNGF induces higher levels of degeneration, which clearly suggest that proNGF is a death ligand and that this signaling pathway can be activated through $p75^{NTR}$, as it occurs in neurotrophin deprivation. To our knowledge, this study is the first to report that proNGF induces axonal degeneration when locally applied to axons overrinding the survival-promoting actions of trophic factors present in the cell body. In order to further extend our findings it will be extremely important to identify the molecules and/or signaling pathways that mediate this mechanism.

Chapter 4

Closing remarks

4.1. Conclusion

In this study, proNGF production in mammalian systems faced several difficulties. These problems seem to result from the combination of low proNGF expression and its degradation. In future experiments the combination of a higher expression of proNGF and the inhibition of its degradation by proteases will be crucial to understand the reasons why proNGF production was not achieved. It will be important to test the mutant proNGF construct with three mutation sites. This would be a nice attempt to address the issue of proNGF cleavage by proteases other than furin. In addition, to overcome the difficulties found in producing proNGF in mammalian systems, a baculovirus expression system in *Spodoptera frugiperda* SF9 insect cells could also be used.

In the second part of this project, using fluidically isolated axons, proNGF was found to induce significant axonal degeneration. Moreover, trophic factors present in cell bodies did not prevent proNGF-induced axonal degeneration, which suggests that proNGF initiates a signaling mechanism in axons that overrides the survival factors present in the cell body.

Our main goal was to identify the role of proNGF in retrograde death signaling. Recently, proNGF has been described to have an opposite role of mature NGF (Lee et al., 2001) and reported to induce cell survival only when it undergoes endocytosis and intracellular proteolysis by furin-like enzymes, which leads to its conversion to mature NGF (Boutilier et al., 2008). Probably, the differences in the proNGF's effects in cells are due to the cleavage of proNGF to its mature form.

The increase of proNGF/NGF ratio has been described to play a critical role in cell death (Lee et al., 2001, D'Onofrio et al., 2011). Application of proNGF in CNS and PNS

neurons promotes death through activation of $p75^{NTR}$ signaling cascade (Beattie et al., 2002, Harrington et al., 2004). Moreover, several models of injury and neurodegenerative disorders have point out the key role of neurotrophin absence in axons that lead to axonal degeneration (Finn et al., 2000, Raff et al., 2002, Zhai et al., 2003). However, i*n vivo*, the total absence of neurotrophin support do not correspond to the reality, instead, the proNGF/NGF ratio seems to increase in injury and in some neurodegenerative disorders (Bruno and Cuello, 2006). The present study demonstrates that proNGF acts locally and induces a significantly higher degeneration than NGF withdrawal.

Moreover, proNGF is released to the extracellular environment in injury conditions (Domeniconi et al., 2007) or in neurodegenerative disorders (Fahnestock et al., 2001, Harrington et al., 2004). In these conditions, proNGF is frequently in contact with axons and cell bodies are not usually deprived of trophic factors. We demonstrated that the effect of proNGF in axons override the survival factors present in cell body. Our results are more likely to mimic what happens in neurons since in some injury models only axons are in contact with proNGF.

Recently, $p75^{NTR}$ apoptotic signaling has been reported to induce degeneration upon neurotrophin deprivation in a mouse model of amyotrophic lateral sclerosis (Perlson et al., 2009). In addition, neurotrophin deprivation in axons has also been correlated with cell death in sympathetic neurons (Mok et al., 2009). Moreover, proNT-3 has been shown to induce neuronal death when specifically applied to axons (Yano et al., 2009). Taking these studies and our results into consideration, we can speculate that proNGF might induce a retrograde signaling that leads to axonal degeneration and also to cell death.

4.2. Future Perspectives

Our results show that proNGF induces axonal degeneration when applied specifically to axons. Axonal degeneration is often preceded or caused by cell death. In order to establish a link with the recent studies that describe proNGF as an apoptotic molecule (Lee et al., 2001), it will be important to evaluate the effect of proNGF on the survival rate of DRG neurons. However, not all neurons project axons to the axonal compartment of microfluidic chambers. Taking advantage of the fluidic isolation in these chambers, we propose to add fluorescent microspheres to the axonal compartment. Only the neurons that extend axons to the opposite compartment will uptake the microspheres and will be analyzed for analysis, which will allow us to evaluate only the survival rate of cells that have axons projected to the axonal compartment. To clearly determine if the axonal degeneration occurs before or after cell death, a time curse will be crucial to establish the time needed for proNGF to induce retrograde cell death. Taking into account that proNGF has been reported to induce to cell death in pathological conditions and these pathologies are also associated with axonal degeneration (Beattie et al., 2002, Jansen et al., 2007), we expect that the degeneration of axons observed in the presence of proNGF occurs before cell death.

If, in fact, proNGF induces cell death, several questions then can be raised: which are the pathways that lead to cell death? Are they also responsible for axonal degeneration? proNGF has been described to bind $p75^{NTR}$ sortilin complex (Nykjaer et al., 2004) and $p75^{NTR}$ has been reported to induce cell death through the MAP kinase family member JNK (Kenchappa et al., 2006). In addition, this pathway has been shown to be involved in pro-NT3-induced cell death (Yano et al., 2009) and in NGF withdrawal-induced cell death (Mok et al., 2009), which makes JNK an ideal candidate to test. Moreover, recent studies have described that inhibition of JNK inhibits degeneration of axons in *ex vivo*

and *in vitro* animal model systems (Barrientos et al., 2011). To test the hypothesis that cell death and axonal degeneration induced by proNGF occurs through JNK pathway activation we propose to analyze by immunocytochemistry the activation of phosphorylated JNK in DRG axons when exposed to proNGF, in the presence or absence of a JNK inhibitor (only in the axonal compartment). If JNK pathway is responsible for cell death induced by proNGF, it is expected that in the presence of the JNK inhibitor proNGF is not able to induce axonal degeneration and cell death.

It will be interesting to verify whether the induction of axonal degeneration by proNGF is due to $p75^{NTR}$ sortilin complex. To address this question, we propose to stimulate DRG axons with proNGF in the presence of TAT-Pep5 (an intracellular inhibitor of p75) and/or neurotensin (a sortilin antagonist) and determine the degeneration index. If the axonal degeneration pathway induced by proNGF is due to $p75^{NTR}$ sortilin complex binding, disruption of this association will prevent axonal degeneration.

Axonal degeneration has also been related with the activation of mitochondrial permeability transition pore (mPTP) (Barrientos et al., 2011). Mitochondria are extremely important in the maintenance of anterograde and retrograde axonal transport. Moreover, addition of the calcium chelator EGTA had a protective effect on injured axons (Zhai et al., 2003), which suggests that axonal degeneration has a calcium-dependent step. To test the hypothesis that proNGF induction of axonal degeneration is linked to the mPTP, it will be important to analyze the proNGF-induced axonal degeneration in the presence or absence of CsA, an inhibitor of CypD (mPTP regulatory protein) and FK506 (inhibits calcineurin). If mPTP has a role in axonal degeneration, it is expected that CsA and FK506 will be protect axons from proNGF-induced degeneration. If axonal degeneration does not occur in the presence of CsA and FK506 in the axonal compartment it will be extremely important to determine if they can also

prevent neuronal cell death. This experiment will be extremely important to understand the pathways responsible for the interaction between axonal degeneration and cell death.

Degeneration of sympathetic axons has also been reported to operate in an apoptosis-independent program. NGF withdrawal-induced axonal degeneration is dependent on the activity of the ubiquitin-proteasome system (UPS) (Zhai et al., 2003). MG132, a reversible inhibitor, of the proteosome did not prevent the appearance of apoptotic cell bodies but the majority of axons were intact. To test the hypothesis that axonal degeneration induced by proNGF is an apoptosis independent program associated with UPS, we propose to analyze by immunocytochemistry the degeneration index of DRG neurons when exposed to proNGF in the presence or absence of MG132 (in the axonal compartment only). If the UPS is the pathway responsible for proNGF-induced axonal degeneration, it is expected that in the presence of MG132 the axonal degeneration, but not cell death, is prevented.

When deregulated, axonal degeneration can lead to pathological processes. A more extensive knowledge of proNGF signaling pathways will allow a better understanding of the mechanisms that lead to axonal degeneration and cell death and will possibly contribute to the development of new strategies designed to minimize the deleterious effects of proNGF under injury conditions.

References

Arnett MG, J.M. R, Wright DE (2007) proNGF, sortilin, and p75NTR: potential mediators of injury-induced apoptosis in the mouse dorsal root ganglion. Brain Res 1183:32-42.

Ashkenazi A, Dixit VM (1998) Death receptors: signaling and modulation. Science 281:1305-1308.

Barrientos SA, Martinez NW, Yoo S, Jara JS, Zamorano S, Hetz C, Twiss JL, Alvarez J, Court FA (2011) Axonal Degeneration Is Mediated by the Mitochondrial Permeability Transition Pore. The Journal of Neuroscience 31:966-978.

Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Bresnahan JC, Hempstead BL, Yoon SO (2002) ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron 36:375-386.

Becker EB, Howell J, Kodama Y, Barker PA, Bonni A (2004) Characterization of the c-Jun N-terminal kinase-BimEL signaling pathway in neuronal apoptosis. J Neurosci 24:8762-8770.

Bergsbaken T, Fink SL, Cookson BT (2009) Pyroptosis: host cell death and inflammation. Nat Rev Microbiol 7:99-109.

Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EB, Said F, Bonni A, Barker PA (2003) Apoptosis induced by p75NTR overexpression requires Jun kinase-dependent phosphorylation of Bad. J Neurosci 23:11373-11381.

Bierl MA, Isaacson LG (2007) Increased NGF proforms in aged sympathetic neurons and their targets. Neurobiol Aging 28:122-134.

Blank M, Shiloh Y (2007) Programs for cell death: apoptosis is only one way to go. Cell Cycle 6:686-695.

Boutilier J, Ceni C, Pagdala PC, Forgie A, Neet KE, Barker PA (2008) Proneurotrophins require endocytosis and intracellular proteolysis to induce TrkA activation. J Biol Chem 283:12709-12716.

Breckenridge DG, Xue D (2004) Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. Curr Opin Cell Biol 16:647-652.

Brigadski T, Hartmann M, Lessmann V (2005) Differential vesicular targeting and time course of synaptic secretion of the mammalian neurotrophins. J Neurosci 25:7601-7614.

Bruno MA, Cuello AC (2006) Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. Proc Natl Acad Sci U S A 103:6735-6740.

Buttigieg H, Kawaja MD, Fahnestock M (2007) Neurotrophic activity of proNGF in vivo. Exp Neurol 204:832-835.

Cande C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215-222.

Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299-309.

Clarke PG, Clarke S (1996) Nineteenth century research on naturally occurring cell death and related phenomena. Anat Embryol (Berl) 193:81-99.

Coleman M (2005) Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci 6:889-898.

Conforti L, Adalbert R, Coleman MP (2007) Neuronal death: where does the end begin? Trends Neurosci 30:159-166.

Cordeiro MF, Guo L, Luong V, Harding G, Wang W, Jones HE, Moss SE, Sillito AM, Fitzke FW (2004) Real-time imaging of single nerve cell apoptosis in retinal neurodegeneration. Proc Natl Acad Sci U S A 101:13352-13356.

Cosker KE, Courchesne SL, Segal RA (2008) Action in the axon: generation and transport of signaling endosomes. Curr Opin Neurobiol 18:270-275.

Counts SE, Nadeem M, Wuu J, Ginsberg SD, Saragovi HU, Mufson EJ (2004) Reduction of cortical TrkA but not p75(NTR) protein in early-stage Alzheimer's disease. Ann Neurol 56:520-531.

D'Onofrio M, Paoletti F, Arisi I, Brandi R, Malerba F, Fasulo L, Cattaneo A (2011) NGF and proNGF regulate functionally distinct mRNAs in PC12 cells: an early gene expression profiling. PLoS One 6:e20839.

Davies AM (2003) Regulation of neuronal survival and death by extracellular signals during development. EMBO J 22:2537-2545.

Degterev A, Yuan J (2008) Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9:378-390.

Deshmukh M, Johnson EM, Jr. (1997) Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. Mol Pharmacol 51:897-906.

Diarra A, Geetha T, Potter P, Babu JR (2009) Signaling of the neurotrophin receptor p75 in relation to Alzheimer's disease. Biochem Biophys Res Commun 390:352-356.

Domeniconi M, Hempstead BL, Chao MV (2007) Pro-NGF secreted by astrocytes promotes motor neuron cell death. Mol Cell Neurosci 34:271-279.

Fahnestock M, Michalski B, Xu B, Coughlin MD (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 18:210-220.

Fahnestock M, Scott SA, Jette N, Weingartner JA, Crutcher KA (1996) Nerve growth factor mRNA and protein levels measured in the same tissue from normal and Alzheimer's disease parietal cortex. Brain Res Mol Brain Res 42:175-178.

Fahnestock M, Yu G, Coughlin MD (2004) ProNGF: a neurotrophic or an apoptotic molecule? Prog Brain Res 146:101 - 110.

Fayard B, Loeffler S, Weis J, Vogelin E, Kruttgen A (2005) The secreted brain-derived neurotrophic factor precursor pro-BDNF binds to TrkB and p75NTR but not to TrkA or TrkC. J Neurosci Res 80:18-28.

Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC (2000) Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci 20:1333-1341.

Friedman WJ (2010) Proneurotrophins, seizures, and neuronal apoptosis. Neuroscientist 16:244-252.

Gatzinsky KP, Thrasivoulou C, Campioni-Noack M, Underwood C, Cowen T (2004) The role of NGF uptake in selective vulnerability to cell death in ageing sympathetic neurons. Eur J Neurosci 20:2848-2856.

Ghiotto F, Fais F, Bruno S (2010) BH3-only proteins: the death-puppeteer's wires. Cytometry A 77:11-21.

Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp Neurol 209:321-332.

Harrington AW, Kim JY, Yoon SO (2002) Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22:156-166.

Harrington AW, Leiner B, Blechschmitt C, Arevalo JC, Lee R, Morl K, Meyer M, Hempstead BL, Yoon SO, Giehl KM (2004) Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. Proc Natl Acad Sci U S A 101:6226-6230.

Jansen P, Giehl K, Nyengaard JR, Teng K, Lioubinski O, Sjoegaard SS, Breiderhoff T, Gotthardt M, Lin F, Eilers A, Petersen CM, Lewin GR, Hempstead BL, Willnow TE, Nykjaer A (2007) Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. Nat Neurosci 10:1449-1457.

Jiang M, Deng L, Chen G (2004) High Ca(2+)-phosphate transfection efficiency enables single neuron gene analysis. Gene Ther 11:1303-1311.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD (2006) Ligand-dependent cleavage of the P75 neurotrophin receptor is

necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219-232.

Kim T, Hempstead BL (2009) NRH2 is a trafficking switch to regulate sortilin localization and permit proneurotrophin-induced cell death. EMBO J 28:1612-1623.

Koshimizu H, Hazama S, Hara T, Ogura A, 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, Hazama S, Suzuki S, Uegaki K, Nagappan G, Zaitsev E, Hirokawa T, Tatsu Y, Ogura A, Lu B, Kojima M (2009) Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. Mol Brain 2:27.

Krantic S, Mechawar N, Reix S, Quirion R (2005) Molecular basis of programmed cell death involved in neurodegeneration. Trends Neurosci 28:670-676.

Kroemer G, Petit P, Zamzami N, Vayssiere JL, Mignotte B (1995) The biochemistry of programmed cell death. FASEB J 9:1277-1287.

Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P (2008) Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods 44:205-221.

Kvam E, Nannenga BL, Wang MS, Jia Z, Sierks MR, Messer A (2009) Conformational targeting of fibrillar polyglutamine proteins in live cells escalates aggregation and cytotoxicity. PLoS One 4:e5727.

Lebrun-Julien F, Bertrand MJ, De Backer O, Stellwagen D, Morales CR, Di Polo A, Barker PA (2010) ProNGF induces TNFalpha-dependent death of retinal ganglion cells through a p75NTR non-cell-autonomous signaling pathway. Proc Natl Acad Sci U S A 107:3817-3822.

Ledgerwood EC, Morison IM (2009) Targeting the apoptosome for cancer therapy. Clin Cancer Res 15:420-424.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. Science 294:1945-1948.

Lessene G, Czabotar PE, Colman PM (2008) BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov 7:989-1000.

Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res 65:11-22.

Lim KC, Tyler CM, Lim ST, Giuliano R, Federoff HJ (2007) Proteolytic processing of proNGF is necessary for mature NGF regulated secretion from neurons. Biochem Biophys Res Commun 361:599-604.

Lin CY, Wu HY, Wang PL, Yuan CJ (2010) Mammalian Ste20-like protein kinase 3 induces a caspase-independent apoptotic pathway. Int J Biochem Cell Biol 42:98-105.

Lindholm D, Wootz H, Korhonen L (2006) ER stress and neurodegenerative diseases. Cell Death Differ 13:385-392.

Linggi MS, Burke TL, Williams BB, Harrington A, Kraemer R, Hempstead BL, Yoon SO, Carter BD (2005) Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. J Biol Chem 280:13801-13808.

Liu WW, Goodhouse J, Jeon NL, Enquist LW (2008) A Microfluidic Chamber for Analysis of Neuron-to-Cell Spread and Axonal Transport of an Alpha-Herpesvirus. PLoS One 3:e2382.

Lockshin RA, Zakeri Z (2001) Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol 2:545-550.

Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. Nat Rev Neurosci 6:603-614.

Luo L, O'Leary DD (2005) Axon retraction and degeneration in development and disease. Annu Rev Neurosci 28:127-156.

Mann DM (1991) Is the pattern of nerve cell loss in aging and Alzheimer's disease a real, or only an apparent, selectivity? Neurobiol Aging 12:340-343; discussion 352-345.

Mari M, Bujny MV, Zeuschner D, Geerts WJ, Griffith J, Petersen CM, Cullen PJ, Klumperman J, Geuze HJ (2008) SNX1 defines an early endosomal recycling exit for sortilin and mannose 6-phosphate receptors. Traffic 9:380-393.

Michalski B, Fahnestock M (2003) Pro-brain-derived neurotrophic factor is decreased in parietal cortex in Alzheimer's disease. Brain Res Mol Brain Res 111:148-154.

Millet LJ, Stewart ME, Nuzzo RG, Gillette MU (2010) Guiding neuron development with planar surface gradients of substrate cues deposited using microfluidic devices. Lab Chip 10:1525-1535.

Mok SA, Lund K, Campenot RB (2009) A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. Cell Res 19:546-560.

Moquin D, Chan FK (2010) The molecular regulation of programmed necrotic cell injury. Trends Biochem Sci 35:434-441.

Movassagh M, Foo RS (2008) Simplified apoptotic cascades. Heart Fail Rev 13:111-119.

Mufson EJ, Wuu J, Counts SE, Nykjaer A (2010) Preservation of cortical sortilin protein levels in MCI and Alzheimer's disease. Neurosci Lett 471:129-133.

Niewiadomska G, Mietelska-Porowska A, Mazurkiewicz M (2011) The cholinergic system, nerve growth factor and the cytoskeleton. Behav Brain Res 221:515-526.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843-848.

Pagadala PC, Dvorak LA, Neet KE (2006) Construction of a mutated pro-nerve growth factor resistant to degradation and suitable for biophysical and cellular utilization. Proc Natl Acad Sci U S A 103:17939-17943.

Park J, Koito H, Li J, Han A (2009) A multi-compartment CNS neuron-glia Co-culture microfluidic platform. J Vis Exp.

PENG S, WUU J, MUFSON EJ, FAHNESTOCK M (2004) Increased proNGF Levels in Subjects with Mild Cognitive Impairment and Mild Alzheimer Disease. Journal of Neuropathology & Experimental Neurology 63:641-649.

Perlson E, Jeong GB, Ross JL, Dixit R, Wallace KE, Kalb RG, Holzbaur EL (2009) A switch in retrograde signaling from survival to stress in rapid-onset neurodegeneration. J Neurosci 29:9903-9917.

Perlson E, Maday S, Fu MM, Moughamian AJ, Holzbaur EL (2010) Retrograde axonal transport: pathways to cell death? Trends Neurosci 33:335-344.

Pihl J, Sinclair J, Karlsson M, Orwar O (2005) Microfluidics for cell-based assays. Materialstoday 8:46-51.

Pollard TD, Earnshaw WC (2008) Programmed Cell Death. In: Cell Biology, 2nd Edition, Elsevier 833-850.

Raff MC, Whitmore AV, Finn JT (2002) Axonal self-destruction and neurodegeneration. Science 296:868-871.

Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 5:897-907.

Rivieccio MA, Brochier C, Willis DE, Walker BA, D'Annibale MA, McLaughlin K, Siddiq A, Kozikowski AP, Jaffrey SR, Twiss JL, Ratan RR, Langley B (2009) HDAC6 is a target for protection and regeneration following injury in the nervous system. Proc Natl Acad Sci U S A 106:19599-19604.

Rosch H, Schweigreiter R, Bonhoeffer T, Barde YA, Korte M (2005) The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus. Proc Natl Acad Sci U S A 102:7362-7367.

Samejima K, Earnshaw WC (2005) Trashing the genome: the role of nucleases during apoptosis. Nat Rev Mol Cell Biol 6:677-688.

Sasaki Y, Vohra BP, Lund FE, Milbrandt J (2009) Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but

not increased levels of neuronal nicotinamide adenine dinucleotide. J Neurosci 29:5525-5535.

Schor NF (2005) The p75 neurotrophin receptor in human development and disease. Prog Neurobiol 77:201-214.

Schweigreiter R (2006) The dual nature of neurotrophins. Bioessays 28:583-594.

Seidah NG, Benjannet S, Pareek S, Savaria D, Hamelin J, Goulet B, Laliberte J, Lazure C, Chretien M, Murphy RA (1996) Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. Biochem J 314 (Pt 3):951-960.

Sekhon BS, Kamboj S (2010) Microdluidics Technology for Drug Discovery and Development - An Overview. International Journal of PharmTech Research 2:804-809.

Serup Andersen O, Boisguerin P, Glerup S, Skeldal S, Volkmer R, Willnow TE, Nykjaer A, Andersen OM (2010) Identification of a linear epitope in sortilin that partakes in pro-neurotrophin binding. J Biol Chem 285:12210-12222.

Smith DE, Rapp PR, McKay HM, Roberts JA, Tuszynski MH (2004) Memory impairment in aged primates is associated with focal death of cortical neurons and atrophy of subcortical neurons. J Neurosci 24:4373-4381.

Song W, Volosin M, Cragnolini AB, Hempstead BL, Friedman WJ (2010) ProNGF induces PTEN via p75NTR to suppress Trk-mediated survival signaling in brain neurons. J Neurosci 30:15608-15615.

Szegezdi E, Macdonald DC, Ni Chonghaile T, Gupta S, Samali A (2009) Bcl-2 family on guard at the ER. Am J Physiol Cell Physiol 296:C941-953.

Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL (2005) A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat Methods 2:599-605.

Taylor AM, Dieterich DC, Ito HT, Kim SA, Schuman EM (2010) Microfluidic local perfusion chambers for the visualization and manipulation of synapses. Neuron 66:57-68.

Taylor AM, Rhee SW, Tu CH, Cribbs DH, Cotman CW, Jeon NL (2003) Microfluidic Multicompartment Device for Neuroscience Research. Langmuir 19:1551-1556.

Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9:231-241.

Teng KK, Felice S, Kim T, Hempstead BL (2010) Understanding Proneurotrophin Actions: Recent Advances and Challenges. Dev Neurobiol 70:350-359.

Troy CM, Friedman JE, Friedman WJ (2002) Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases. J Biol Chem 277:34295-34302.

Van Herreweghe F, Festjens N, Declercq W, Vandenabeele P (2010) Tumor necrosis factor-mediated cell death: to break or to burst, that's the question. Cell Mol Life Sci 67:1567-1579.

Vitzthum L, Chen X, Kintner DB, Huang Y, Chiu SY, Williams J, Sun D (2010) Study of Na+/H+ exchange-mediated pHi regulations in neuronal soma and neurites in compartmentalized microfluidic devices. Integr Biol (Camb) 2:58-64.

Volosin M, Song W, Almeida RD, Kaplan DR, Hempstead BL, Friedman WJ (2006) Interaction of survival and death signaling in basal forebrain neurons: roles of neurotrophins and proneurotrophins. J Neurosci 26:7756-7766.

Volosin M, Trotter C, Cragnolini A, Kenchappa RS, Light M, Hempstead BL, Carter BD, Friedman WJ (2008) Induction of proneurotrophins and activation of p75NTR-mediated apoptosis via neurotrophin receptor-interacting factor in hippocampal neurons after seizures. J Neurosci 28:9870-9879.

Walensky LD (2006) BCL-2 in the crosshairs: tipping the balance of life and death. Cell Death Differ 13:1339-1350.

Wang J, Ren L, Li L, Liu W, Zhou J, Yu W, Tong D, Chen S (2009) Microfluidics: a new cosset for neurobiology. Lab Chip 9:644-652.

Wang YJ, Valadares D, Sun Y, Wang X, Zhong JH, Liu XH, Majd S, Chen L, Gao CY, Chen S, Lim Y, Pollard A, Aguilar E, Gai WP, Yang M, Zhou XF (2010) Effects of proNGF on neuronal viability, neurite growth and amyloid-beta metabolism. Neurotox Res 17:257-267.

Weibel DB, Whitesides GM (2006) Applications of microfluidics in chemical biology. Curr Opin Chem Biol 10:584-591.

Whitesides GM (2006) The origins and the future of microfluidics. Nature 442:368-373.

Willnow TE, Petersen CM, Nykjaer A (2008) VPS10P-domain receptors - regulators of neuronal viability and function. Nat Rev Neurosci 9:899-909.

Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8:1069-1077.

Yang F, Je HS, Ji Y, Nagappan G, Hempstead B, Lu B (2009) Pro-BDNF-induced synaptic depression and retraction at developing neuromuscular synapses. J Cell Biol 185:727-741.

Yano H, Torkin R, Martin LA, Chao MV, Teng KK (2009) Proneurotrophin-3 is a neuronal apoptotic ligand: evidence for retrograde-directed cell killing. J Neurosci 29:14790-14802.

Zamzami N, Kroemer G (2003) Apoptosis: mitochondrial membrane permeabilization--the (w)hole story? Curr Biol 13:R71-73.

Zhai Q, Wang J, Kim A, Liu Q, Watts R, Hoopfer E, Mitchison T, Luo L, He Z (2003) Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron 39:217-225.