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NGF-induced pro-survival signalling

Molecular engineering of NGF to enhance pro-survival signalling

A thesis submitted to the National University of Ireland in fulfilment of
the requirement for the degree of

Doctor of Philosophy

by

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Thesis Supervisors:

Dr. Adrienne Gorman and Prof. Afshin Samali
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Acknowledgements

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Lastly a ginormous thanks to my Joey, thank you for your unwavering support in everything that I try. I couldn’t ask for a better partner.
List of abbreviations

NGF  Nerve growth factor
AD   Alzheimer’s disease
AIF  Apoptosis inducing factor
APAF-1 Apoptosis protease activating factor-1
ATCC American tissue culture collection
ATG  Autophagy-related gene
Aβ   β-amyloid
BBB  Blood brain barrier
BDNF Brain derived neurotrophin factor
BFCN The basal forebrain cholinergic neurons
BH   Bcl-2 homology
BIR  Baculovirus IAP repeat
BSA  Bovine serum albumin
CARD Caspase recruitment domain
c-FLIP Cellular FLIP
CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
ciAP Cellular inhibitor of apoptosis protein
CIPA Congenital insensitivity to pain with anhidrosis
CMA  Chaperone mediated autophagy
CNS  Central nervous system
CRD  Cystine rich domains
CTF C-terminal fragment of p75\textsuperscript{NTR}
DD  Death domain
DED  Death effector domain
DISC Death inducing signalling complex
DMEM Dulbecco’s modified Eagle medium
DR  Death receptor
Dbs  Disulfide bond forming proteins
ECACC European Collection of Cell Cultures
EDAR Ectodysplasin A receptor
EGTA Ethyleneglycoltetraacetic acid
ELISA Enzyme linked immunosorbent assay
EndoG Endonuclease G
ER  Estrogen receptor
FADD Fas-associated death domain containing protein
FBS  Fetal bovine serum
FDA Food and Drug Administration
FLICA FAM-DEVD-FMK reagent
Gab Grb associated binder
GRB Growth factor receptor bound
HEPES 2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid
HER2 Human epidermal growth factor receptor 2
Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum ()</td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>IBM</td>
<td>IAP-binding motif</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain of p75NTR</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin-like</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRAK1</td>
<td>Interleukin 1 receptor-associated kinase 1</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrates</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrates 1</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase ()</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>MMP7</td>
<td>Matrix metalloprotease 7</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution</td>
</tr>
<tr>
<td>NADP</td>
<td>p75NTR-associated Cell Death Executor</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis-inhibitory factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NRAGE</td>
<td>Neurotrophin receptor-interacting MAGE homolog</td>
</tr>
<tr>
<td>NRIF</td>
<td>Neurotrophin receptor interacting factor</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotrophin</td>
</tr>
<tr>
<td>p75NTR</td>
<td>75 kDa neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing tween</td>
</tr>
<tr>
<td>PC</td>
<td>Proconvertases</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>Phospholipase C-γ</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RIP</td>
<td>Ribosome inactivating protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
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<td>Son of sevenless</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming factor-β-activated kinase 1</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3′,5,5′-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF receptor 1</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-receptor associated death domain</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNFR-associated factor 2</td>
</tr>
<tr>
<td>TRAILR1</td>
<td>TNF-related apoptosis-inducing ligand receptor</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin-related kinase A</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked IAP</td>
</tr>
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Abstract

Nerve growth factor (NGF) is a potent pro-survival growth factor that has diverse roles in development and in the adult nervous system. It has two cognate receptors that stimulate distinct signalling pathways, tropomyosin-related kinase A (TrkA) and the 75 kDa neurotrophin receptor (p75NTR). NGF activation of TrkA induces proliferation and survival in neuronal cells, while activation of p75NTR causes either cell death or cell survival depending on the cell context. Alteration of NGF signalling has been implicated as a possible therapeutic in many human diseases such as Alzheimer’s disease, chronic pain and breast cancer. However, poor understanding of p75NTR receptor stimulation, limits the therapeutic potential of the NGF ligand. My research aim is to investigate a novel mechanism of NGF stimulated pro-survival signalling through TrkA, where at late stages of the apoptosis pathway it protected cells and increased their long-term survival. A further aim is to generate NGF variants to enhance NGF-mediated pro-survival signalling.

It is shown here that NGF activation of TrkA prevents cell death by targeting two stages of the apoptosis pathway, i.e., both before and after mitochondrial outer membrane permeabilisation (MOMP). MOMP is considered to be the point in which a cell commits to undergoing apoptosis. However, NGF treatment, at a time point where a significant amount of cells have lost their mitochondrial membrane potential, can induce an increase in the long term survival of the cells and a reduction in the activity and protein level of active caspases. The data shown here suggest that the loss in active caspase-3, often considered the main executioner of apoptosis, is mediated by lysosomal degradation, which is stimulated by NGF-mediated activation the ERK arm of TrkA signalling.

A further aim was to develop a protocol for the transient expression and purification of His tagged NGF and NGF variants. All NGF constructs contained a His tag for the purification of the protein. The protocol was developed with wild type, HA and FLAG-tagged NGF. Briefly, this method involved the generation of NGF cDNA constructs that were transiently transfected into HEK293T where the protein was
expressed and secreted into the media. The conditioned media was collected for purification by Nickel affinity chromatography. The binding affinities of these tagged NGFs as well as their biological activity through p75NTR and the TrkA receptor was analysed and compared to commercially available NGF. Using this protocol a range of NGF variants (8 variants) were also expressed. These variants were computationally designed by the FoldX computer algorithm to have altered affinity to its receptors with the aim of generating an NGF variant with enhanced pro-survival signalling.
1 Chapter 1. Introduction
1.1 Cell death

Cell death is a natural process that is necessary for multicellular organisms to remove unwanted or damaged cells. Many kinds of cell death occur through a number of mechanisms. These are classified according to their morphological features (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement nucleases or proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental) or immunological characteristics (immunogenic or non-immunogenic) (Kroemer et al., 2009). The cell death pathways that are relevant to this thesis are necrosis, necroptosis, autophagic cell death and apoptosis which can be defined into different categories according to morphological and biochemical characteristics (Fig. 1.1) (Hotchkiss et al., 2009; Kroemer et al., 2009; Klionsky et al., 2012).

Apoptosis and autophagic cell can occur under normal physiological conditions as part of the growth and renewal of cells. They are described as controlled cellular processes, as initiation causes cell death by a defined signalling cascade that terminates in their engulfment by neighbouring phagocytes, thereby removing the cell from the system without initiating pro-inflammatory immune responses (Savill and Fadok, 2000). Necrotic cell death mainly occurs when cells have been severely injured. It results in cell lysis and spillage of cellular contents into the extracellular space causing an inflammatory response (Lockshin and Zakeri, 2004). Necroptosis, a controlled form of necrosis, is challenging the original understanding of necrosis (Vandenabeele et al., 2010). Other forms of cell death have also been described as being necrotic-like and apoptotic-like (Lockshin and Zakeri, 2004) which are often a result of a disruption in cell death pathway (Leist and Jäättelä, 2001). The forms of cell death relevant to the work presented in this thesis will be discussed here (Fig. 1.1).
Chapter 1. Introduction

The main forms of cell death of importance to this thesis are, apoptosis, autophagic cell death, necrosis and necroptosis. The morphological features of apoptosis are cell shrinkage, membrane blebbing, nuclear condensation and externalisation of PS for immune cell detection. The main features of autophagy and autophagic cell death are the presence of double membranated vacuoles called autophagosomes and vacuolisation of the cytoplasm. A cell undergoing autophagy either recovers from the cell stress or continues to undergo autophagy and cell death ensues. The cell can then die by apoptosis, necrosis or by increased autophagy digestion of the cellular organelles. The main features of necrosis and necroptosis are cell and organelle swelling, increased translucency of the cytoplasm and activation of the inflammation response following cell lysis.
1.1.1 Necrosis and necroptosis

The morphological features of necrosis include swelling of organelles, translucent cytoplasm, dilation of the nuclear membrane, condensation of the nucleus into irregular well-defined spots and increased cell volume that disrupts the plasma cell membrane (Fig. 1.1) (Hotchkiss et al., 2009; Kroemer et al., 2009; Galluzzi et al., 2012). Necrosis was originally understood to be a passive cell death, which occurs when the cell is under particularly acute stress and as a result of culture conditions where there is no immune response to remove the dying cell. It has been described as the unregulated bursting of a cell causing inflammation and an immunogenic response (Kaczmarek et al., 2013). The idea that necrosis is an unregulated process has been challenged by numerous reports where necrosis requires the activation of a distinct signalling pathway. This process is called necroptosis. The most extensively studied pathway is the tumor necrosis factor (TNF) induced necroptosis that will be detailed in Section 1.1.3.

1.1.2 Autophagy and autophagic cell death death

Cells undergoing autophagy are characterised by the presence of double-membraned organelles called autophagosomes, which contain partially digested organelles such as mitochondria, and an increase vacuolisation of the cytoplasm (Fig. 1.1) (Hotchkiss et al., 2009; Kroemer et al., 2009; Galluzzi et al., 2012).

Autophagy is the bulk degradation process of the cell and is important for maintainance of cellular homeostasis. There are several forms of autophagy, macroautophagy, chaperone mediated autophagy (CMA), microautophagy, aggrephagy and chaperone-assisted selective autophagy, this will be discussed in more detail in Chapter 3. Macroautophagy has been linked to autophagic cell death. It is a process controlled by a family called autophagy-related genes (ATG) whose knockdown causes an inhibition of autophagosome formation. Autophagosomes are the defining feature in autophagy and are unusual as they possess a double membrane that must be actively formed by the ATG proteins. The double membrane forms around the cargo for degradation to form a vesicle called the
autophagosome. The autophagosome then fuses with lysosomes and generate the autolysosome, which completely digests the cargo in the autophagosome, generating energy and metabolites in the process (Levine and Klionsky, 2004).

When a cell is under stress, as is the case when a cell is experiencing nutrient deprivation, autophagy is induced to meet the energy requirements of the cell and thus acts as a pro-survival mechanism. This will be described in more detail in Section 1.2.1. However, if this stress becomes prolonged or particularly acute autophagy becomes self-destructive and the cell undergoes autophagic cell death (Fig. 1.1) (Maiuri et al., 2007; Kroemer and Levine, 2008). The term autophagic cell death is debated in the field. Two terms are used to define the cell death that has been described with autophagy morphology. The first term is autophagic cell death, in which a cell commits to death due to activation of the autophagic machinery and digestion of the cell contents. The second term is cell death with autophagy, which describes the condition where autophagy is induced and is contributing to the cell death. These terms have been generated to distinguish the role of autophagy as a true cell death mechanism or as a mechanism which contributes to apoptotic or necrotic cell death (Fig. 1.1) (Kroemer and Levine, 2008).

### 1.1.3 Apoptosis

The morphological features of apoptosis include detachment from neighbouring cells, plasma membrane blebbing, nuclear condensation and fragmentation of the cell into apoptotic bodies exposing phosphatidylserine (PS) that signals to macrophages for phagocytic engulfment (Fig. 1.1) (Hotchkiss et al., 2009; Kroemer et al., 2009; Galluzzi et al., 2012). There are two activation mechanisms of apoptosis, the intrinsic (mitochondrial pathway) and extrinsic (death receptor mediated) apoptosis pathways, which converge on the point of caspase activation (Fig. 1.2).
Figure 1.2. The extrinsic and intrinsic apoptosis pathways

Schematic of the extrinsic (on the left hand side) and intrinsic (on the right hand side) apoptosis pathways. TNF induced activation of the death receptor TNFR1 is used as an example of activation of the extrinsic apoptosis pathway. TNF binds to the TNFR1 death receptor trimer and stimulates the recruitment of the DISC complex of TRADD, FADD and caspase-8. Caspase-8 is then autocatalysed by induced proximity. The caspase-8 can then directly activate effector caspases or can amplify the apoptotic signal and activate the intrinsic apoptosis pathway by cleaving Bid to tBid to act with the BH3 only proteins Bax and Bak to form a pore in the mitochondria causing MOMP. Cytochrome c and other pro-apoptotic proteins are then released. The apoptosome is then formed which can activate the effector caspase-9 that in turn activates the effector caspases-3 and -7. These caspases then cleave their intracellular substrates and cause many of the morphological features of apoptosis. The extrinsic apoptosis machinery can be inhibited when the DISC complex is disrupted and recruits TRAF2 and cIAP1/2 to the death domain, this causes the ubiquitination of RIP and the activation of neuclear factor- κB (NF-κB) survival pathway and cFLIP expression which can inhibit the activation of caspase-8 in the DISC complex. Another inhibitor of the apoptosis is XIAP. It can bind and prevent the activity of caspase-9,-3 and -7. XIAP is inhibited on MOMP by the release of Smac/DIABLO which binds with high affinity to XIAP.
The extrinsic apoptosis pathway is activated by ligand-bound death receptors of the tumour necrosis factor receptor (TNFR) gene superfamily. Binding of the ligand causes a conformational change in the receptor trimer complex. This activates the death domains stimulating the formation of the death-inducing signalling complex (DISC) (Ashkenazi and Dixit, 1998). For example activation of TNF receptor 1 (TNFR1) by TNF causes a conformational change in the preformed receptor trimer that allows binding of TNF-receptor associated death domain (TRADD). The Fas-associated death domain (FADD) then binds to TRADD via its death domain. FADD then binds procasaspe-8. The trimerisation of the receptor allows multiple units of the FADD and procaspase-8 to bind in close proximity to one another that causes the autocatalysis of procaspase-8, by a process known as induced proximity, to its active form. The active caspase-8 dissociates from the receptor and activates other caspases either directly (Movassagh and Foo, 2008) or indirectly by cleaving the Bcl-2 pro-apoptotic protein Bid to its truncated form (tBid) enabling it to translocate to the mitochondria (Li et al., 1998) where it facilitates Bax and Bak oligomerisation leading to the formation of pores in the outer membrane of the mitochondria. This process is known as mitochondrial outer membrane permeabilisation (MOMP) (Tait and Green, 2010).

As mentioned previously programmed necrosis or necroptosis is best studied on treatment of cells with TNF. In the presence of a pan-caspase inhibitor TNF treatment of the cell causes necroptosis. This is initiated by the deubiquitination of ribosome inactivating protein 1 (RIP1) by Lys63-deubiquitylating enzyme cylindromatosis. RIP1 and RIP3 are then recruited to a supramolecular complex with TRADD, FADD and caspase-8 causing necrosis of the cell (Vandenabeele et al., 2010).

The intrinsic apoptosis pathway is initiated by a wide variety of stimuli inside the cell such as Endoplasmic reticulum stress, DNA damage, radiation and growth factor withdrawal (Hotchkiss et al., 2009). But all stress responses converge on MOMP. MOMP allows the release of pro-apoptotic proteins into the cytoplasm of the cell
such as cytochrome c, Smac/DIABLO, HtrA2/Omi, endonuclease G (EndoG) and apoptosis inducing factor (AIF) that can mediate apoptosis when the caspases are inhibited or potentiate the apoptotic signal (Martinou and Green, 2001; Zamzami and Kroemer, 2001). Upon release cytochrome c binds to and activates apoptosis protease activating factor-1 (APAF-1) which oligomerizes in presence of dATP to form a very large (approximately 700-1400 kDa) apoptosome complex. The apoptosome allows proximity induced auto activation of caspase-9, which in turn processes and activates the effector caspases-3 and -7 (Acehan et al., 2002; Cain et al., 2002). Activation of caspase-3 and -7 is responsible for many of the morphological changes during apoptosis such as membrane blebbing (Coleman et al., 2001), nuclear condensation (Enari et al., 1998) and PS externalisation (Martin et al., 1996; Naito et al., 1997; Suzuki et al., 2013). Release of Smac/DIABLO and HtrA2/Omi from the mitochondria also facilitates the execution of apoptosis by eliminating the anti-apoptotic effects of the inhibitor of apoptosis proteins (IAPs) (Du et al., 2000; Suzuki et al., 2001).

A family of proteins called the Bcl-2 proteins controls MOMP. Members of the Bcl-2 family are described as either pro-apoptotic or anti-apoptotic. They can be defined further based on their homology within four conserved regions termed Bcl-2 homology (BH) domains. They roughly correspond to α-helices dictating structure, function and localisation (Fig. 1.3) (Cory and Adams, 2002; Danial, 2007).
Figure 1.3. Classification of the Bcl-2 protein family members

A schematic of the Bcl-2 protein members separated into pro-apoptotic and anti-apoptotic sub-groups. The BH domains are highlighted. BH1 is blue, BH2 is beige, BH3 is red and BH4 is green. The transmembrane domain is grey. The anti-apoptotic Bcl-2 proteins contain 2 or more BH domains. The pro-apoptotic Bcl-2 proteins can be divided into two subgroups. The Bax family which contain more than one BH domain and the BH3 only contain BH3 domains.

The anti–apoptotic Bcl-2 family members inhibit the actions of the pro-apoptotic proteins. Thus the balance between pro- and anti-apoptotic Bcl-2 proteins determines cell fate (Cory and Adams, 2002). Apoptotic stimuli increase the transcription of pro-apoptotic Bcl-2 proteins shifting the balance leading to initiation of the pro-apoptotic Bcl-2 protein response. This includes the activation of the BH3 only proteins that are essential for initiating apoptosis. In the intrinsic apoptotic pathway the BH3 only proteins act as sensors of cellular stress and in response to a cytotoxic stimulus they are activated and up-regulated to inactivate the pro-survival members of the Bcl-2 family and thus allow the activation of Bax/Bax-like pro-apoptotic family members (Bouillet and Strasser, 2002). Bax and Bak oligomerisation and form pores in the outer mitochondrial membrane capable of allowing the release of mitochondrial pro-apoptotic factors such as cytochrome c.
to initiate the formation of the apoptosome and activate caspases. The caspases are a group of cysteine proteases that are responsible for the cleavage of cellular substrates leading to apoptosis and will be described in more detail in the following Section (Timmer and Salvesen, 2007).

1.1.4 Caspase structure and proteolytic activation

The two key characteristics of caspases are that they are cysteine-dependent, aspartate–specific proteases (Alnemri et al., 1996). Caspases use a cysteine side chain in their active site for highly specific substrate cleavage, at a XEXD amino acid sequence (Thornberry et al., 1997). A major specificity determinant of the caspases is the active site of the protein, which is polar and highly catalytically active. Caspases cleave after the C-terminal aspartic acid (XEXD), the three amino acids preceding the aspartic acid (XEXD) give the substrate specificity for a particular caspase (Table 1.1).

Caspases can be categorized based on the structure, function and substrate specificity (Table 1.1). Functionally there are two different families of caspases, namely the inflammatory caspases and the apoptotic caspases. Inflammatory caspases include (caspase-1, -4, -5, -11, -12 and -14). The apoptotic caspases can be further divided into initiator caspases or executioner caspases. The initiator caspase include (caspases-2, -8, -9 and -10) and the executioner caspases include (caspase-3, -6 and -7) (Thornberry et al., 1997). Caspases are synthesised as catalytically inactive zymogens, called the procaspases, which share structural similarities. The capases consist of a highly homologus catalytic domain consisting of a (~10 kDa) small subunit and (~20 kDa) large subunit. The initiator and inflammatory caspases contain an N-terminal prodomain that allows interaction of the caspases with adapter proteins. The domains include, the death effector domain (DED), the caspases recruitment domain (CARD) or the pyrin domain (PYD) (Table 1.1).
Table 1.1 Mammalian caspases categorised based on their structure, function and substrate specificity

<table>
<thead>
<tr>
<th>Function</th>
<th>Caspase</th>
<th>Pro-domain</th>
<th>Substrate sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory caspases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CARD</td>
<td></td>
<td>YVAD</td>
</tr>
<tr>
<td>4</td>
<td>CARD</td>
<td></td>
<td>LEVD</td>
</tr>
<tr>
<td>5</td>
<td>CARD</td>
<td></td>
<td>WEHD</td>
</tr>
<tr>
<td>11</td>
<td>CARD</td>
<td></td>
<td>VEHD</td>
</tr>
<tr>
<td>12</td>
<td>CARD</td>
<td></td>
<td>ATAD</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td></td>
<td>Not specified</td>
</tr>
<tr>
<td><strong>Apoptotic initiator caspases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CARD</td>
<td></td>
<td>VDVAD</td>
</tr>
<tr>
<td>8</td>
<td>DED</td>
<td></td>
<td>IETD</td>
</tr>
<tr>
<td>9</td>
<td>CARD</td>
<td></td>
<td>LEHD</td>
</tr>
<tr>
<td>10</td>
<td>DED</td>
<td></td>
<td>AEVD</td>
</tr>
<tr>
<td>15</td>
<td>PYD</td>
<td></td>
<td>IETD</td>
</tr>
<tr>
<td><strong>Apoptotic executioner caspases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td></td>
<td>DEVD</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td></td>
<td>VEID</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td></td>
<td>DEVD</td>
</tr>
</tbody>
</table>

In apoptosis the mechanism of caspase activation is tightly regulated. I will discuss this further in Section 1.2. The initiator caspases are responsible for starting the cascade of caspase activation. Once the initiator caspases are activated this leads to the activation of effector caspases. Effector caspases can initiate and activate each other and thus the caspase activation signal is amplified and grows exponentially (Budihardjo et al., 1999).

As explained earlier, the initiator caspases are often activated on binding to adapter molecules (such as the death receptors and the apoptosome). The executioner caspases lack adapter molecule-binding domains. Thus, they rely on the activation of the executioner caspases for cleavage and activation. The main executioner caspase, caspase-3, is synthesised as a 32 kDa protein which can be further subdivided into a 3 kDa pro domain, a 17 kDa (also referred to as p17) large subunit and a 12 kDa (p12) small subunit. Activation of caspase-3 involves two steps (Han et al., 1997; Nicholson, 1999). The initiator caspase cleaves pro-caspase-3 after Asp residue at the IETD site between the large and small subunit, forming the p20/p12
heterodimer. This partially active caspase-3 removes the 3 kDa pro-domain from the p20 subunit, to generate the p17, or active caspase-3 subunit (Han et al., 1997).

1.2 Cellular regulation of apoptosis

The apoptosis machinery has many internal controls to inhibit unregulated cell death (Lockshin and Zakeri, 2004). The mechanisms a cell utilises to evade apoptosis are most readily studied in cancer cells as they have induced a number of pro-survival mechanisms to achieve immortality (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

As explained earlier, MOMP, which is often believed to be the point in which a cell commits to apoptosis, is controlled by the Bcl-2 family proteins and the levels of pro and anti-apoptotic family members are altered in many cancer cells to prolong the life of the cell. Another example of inhibition of apoptosis is in altering the TNFR1 induced cell death. TRADD can bind RIP1, TNFR-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis protein 1 (cIAP). The cIAP then catalyses the addition of Lys63-linked polyubiquitin of RIP1 that provides a docking site for transforming factor-β-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3 which stimulate the canonical nuclear factor-κB (NF-κB) pro-survival pathway and an increase in cellular FLIP (c-FLIP) which can inhibit the TNF induced apoptosis (Fig. 1.2) (Vandenabeele et al., 2010). Other examples of inhibition during the apoptosis pathway are the IAPs direct control of the caspases by post-translational modification, autophagy survival signalling and growth factor survival signalling.

1.2.1 Inhibitor of apoptosis proteins (IAPs)

Apoptosis can be regulated downstream of MOMP by a group of proteins known as the IAPs. These proteins can bind active caspases and inhibit them (Roy et al., 1997; Takahashi et al., 1998; Deveraux and Reed, 1999; Yang and Li, 2000) or may send them to the proteasome for degradation (Huang et al., 2000; Yang et al., 2000). IAPs are a family of proteins characterised by the presence of a baculovirus IAP repeat (BIR) domains. There are 6 members of this family. They are X-linked IAP (XIAP),
cellular IAP 1 (cIAP1), cIAP2, IAP-like protein, neuronal apoptosis-inhibitory factor (NAIP), Survivin, and Bruce (Fig. 1.4). Not all IAPs, however, are involved in the regulation of caspases and to date only XIAP, cIAP1 and cIAP2 have been shown to have caspase inhibition abilities (Deveraux and Reed, 1999; Eckelman et al., 2006).

**Figure 1.4. The classification of the IAP family members**

A schematic of the human IAP proteins with their amino acid size. Each member of the IAP family must contain a baculovirus IAP repeat (BIR) domain. The IAP proteins can contain a really interesting new gene (RING) domain which has E3 ligase activity. cIAP1 and cIAP2 contain a caspase activation and recruitment domain (CARD) which is known to have binding affinity for death domains and other CARD domains.

The most potent caspase inhibitor is XIAP (Eckelman et al., 2006). XIAP has been demonstrated to block the caspase cascade at the initiation phase of caspase-9 (Deveraux et al., 1999), and the execution phase at caspases-3 and -7 (Deveraux et al., 1997). In-depth studies of the IAP family have shown that other human IAPs do not inhibit caspases with the same potency as XIAP. cIAP1 and cIAP2 are the closest homologues of XIAP however, their caspase inhibition is weak (Eckelman and Salvesen, 2006). Two separate domains mediate XIAP inhibition of effector caspases-3 and -7. Firstly, the BIR2 domain of XIAP binds to the effector caspases IAP-binding motif (IBM) that is exposed during caspase processing (Takahashi et al., 1998; Deveraux et al., 1999; Scott et al., 2005). The second domain required for complete caspase inhibition is a peptide strand preceding BIR2 of XIAP (Chai et al., 2001; Huang et al., 2001). This peptide strand, containing the critical residues Leu141 and Asp148, stretches across the catalytic binding cleft of the caspase,
preventing substrate access to the caspase active site (Huang et al., 2000; Chai et al., 2001; Scott et al., 2005).

In addition to their function as direct caspase inhibitors, IAPs have been suggested to mediate the proteasomal degradation of active caspases. This is due to the fact that a number of the IAPs contain Really Interesting New Gene (RING) domains, which are involved in targeting proteins for proteasomal degradation. Proteins containing RING domains have E3 ligase activity, meaning that they have the ability to recruit an E2 ubiquitin conjugating enzyme and ubiquitinate both itself (auto-ubiquitination) and other proteins to which it is bound (Deshaies and Joazeiro, 2009). XIAP, cIAP1 and cIAP2 contain a RING domain. However to date, little evidence of XIAP, cIAP1 and cIAP2 mediated poly-ubiquitination of caspases has been shown (Suzuki et al., 2001; Bader and Steller, 2009; Choi et al., 2009; Nakatani et al., 2013). One study showed that cIAP2 could function as an E3 ligase and catalyse the mono-ubiquitination of caspases-3 and -7 (Huang et al., 2000). This study, however, was carried out using in vitro ubiquitination assays, and verification of this result in vivo has not been published. It has been shown in Drosophila that the Drosophila Inhibitor of Apotosis 1 (DIAP) can block effector caspases by targeting them for poly-ubiquitylation and nonproteosomal inactivation. They found that on ubiquitin conjugation the catalytic activity of effector caspase drICE (Ditzel et al., 2008). More recently it has been shown that the IAPs can also promote the conjugation of ubiquitin-like protein NEDD8 in both drosophila and mammalian cells (Broemer et al., 2010). They showed that XIAP promotes autoneddylation as well as neddylation of caspase 7. They also show that cIAP-1 was able to target both itself and known other substrates for neddylation. However, the role of these modifications in vivo were not identified (Broemer et al., 2010)

It is unsurprising that the caspases are tightly regulated to prevent aberrant apoptosis. The regulation of the caspases can also be influenced by post translation modifications. The best example of inhibition of activation of caspase activity is in the matrix of the mitochondria where they are nitrosylatesd within their active site
and thus cannot be activated until MOMP (Mannick et al., 2001). The second example of post-translational modification of caspases is on caspase-9 where a phosphorylation in the active site renders it resistance to cleavage and activation (Cardone et al., 1998)

1.2.2 Growth factor signalling

Another mechanism induced by cancer cells to inhibit apoptosis is the disruption of growth factor signalling. The best studied mechanism of growth factor stimuli preventing apoptosis in cancer cells is in breast cancer cells. To highlight this fact most breast cancer cells are classified on the basis of growth factor receptor expression. They can be described as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) positive or negative. Cells which express none of these endocrine markers are called triple negative breast cancer cells (TNBC). The expression of these receptors determines treatment as inhibition causes an increase in the death of cancer cells, especially on combination treatment with chemotherapeutic reagents (Harvey et al., 1999; Schlotter et al., 2008).

The main endocrine derived treatments for breast cancer are anti-estrogens. The most common therapy is tamoxifen which blocks estrogen -induced proliferation of cancer cells. Tamoxifen was first used in the treatment of metastatic breast cancer, and led to a 30 % regression of cancers (Osborne, 1998,). Not all cancers express ER and are often targetted with an anti-HER2 blocking antibody which has proven to be effective in cancers that overexpress HER2 (Lewis Phillips et al., 2008; Schlotter et al., 2008). The TNBC are the most difficult to treat as they are resistant when used alone and in combination with other therapies. Other growth factors have been implicated in the pro-survival signalling of breast cancer cells, one such ligand is nerve growth factor (NGF).

NGF is a known to be a potent anti-apoptotic factor during development of the nervous system (Crowley et al., 1994; Smeyne et al., 1994) and in the adult brain (Berry et al., 2012). It has been proposed as a possible therapeutic target for breast
cancer cells as it was found to be expressed in breast cancer cells but not in normal breast epithelial cells (Descamps et al., 1998). In addition, the expression of NGF and its two receptors tropomyosin-receptor-kinase A (TrkA) as well as the 75 kDa neurotrophin receptor p75NTR correlated with poor patient outcome (Descamps et al., 2001). NGF and its receptors were also shown to increase proliferation (through TrkA) and protect the cells from C2 ceramide induced apoptosis (through p75NTR) (Descamps et al., 2001).

1.3 Commitment point in apoptosis signalling

Mitochondrial permeabilization or MOMP is often considered the point-of-no-return in the apoptosis pathway (Hotchkiss et al., 2009; Tait and Green, 2010). Once MOMP has occurred the classical apoptosis pathway can be activated, cytochrome c is released from the mitochondria and thus binds to APAF1 to form the apoptosome and activate caspases (Leist and Jäättelä, 2001; Hotchkiss et al., 2009). However, cell death still occurs upon inhibition or knock down of caspases and APAF1. This is called caspase-independent cell death and may result in necrosis or an apoptosis like cell death morphology that is mainly executed by AIF which is released by the mitochondria (Leist and Jäättelä, 2001; Cândé et al., 2002).

This would suggest that MOMP is the point of no return in the apoptosis pathway, as inhibition downstream of MOMP has no effect on the cell fate. Cells, however, have developed techniques to push back or delay their commitment point. This is particularly clear in cancer cells which have developed several mechanisms to evade apoptosis, including increased expression of the Bcl-2 pro-survival proteins, IAPs and enhanced endocrine signalling (Fernald and Kurokawa, 2013).

A report has also described cells as undergoing incomplete MOMP (iMOMP). This is the condition when not all mitochondria in the cell have undergone MOMP and intact mitochondria can still be seen (Tait et al., 2010). Under these conditions if the cell stressor is removed and caspases are inhibited the mitochondria can repopulate and increase the cells clonogenic survival. This effect was found to be dependent on Bcl-2 anti-apoptotic proteins as the effect was lost on ABT-373 treatment (Tait et al., 2010). This suggests that iMOMP by overexpression of the anti-apoptotic Bcl-2 proteins can
protect cells from caspase independent cell death on MOMP by further loss of MOMP (Tait and Green, 2010). Taken together these reports show that the commitment point to apoptosis is no longer as clear as once believed.

1.4 Neurotrophin Signalling

The neurotrophins are a small family of dimeric proteins comprising of NGF, brain derived neurotrophic factor (BDNF) and neurotrophin-3 or -4 (NT3/4). The neurotrophins bind to the pan p75NTR and their specific Trk receptor. Each neurotrophin binds specifically to its Trk receptor. NGF activates TrkA, BDNF and NT4 activates TrkB and NT3 activates TrkC (Huang and Reichardt, 2003).

As described earlier neurotrophins are expressed in breast cancer cells and contribute to their resistance to chemotherapeutic reagents but the neurotrophins key role in the development of the nervous system (Snider, 1994). Extensive apoptosis occurs in the developing nervous system as neurons are produced in excess during development and compete with each other, in acutely stressed environments, for limited amounts of the survival-promoting trophic factors secreted by target tissues (Deshmukh and Johnson, 1997). The pro-survival signalling of NGF are under investigation in this thesis and will be described in more detail here.

1.4.1 NGF

NGF is the founding member of the neurotrophin family (Levi-Montalcini, 1987). It is a 28 kDa protein consisting of two covalently linked 14 kDa monomers. Each monomer contains a cysteine knot essential for the protein’s structure: Cys58-108 and Cys63-110 form a loop through which the third disulfide bond, Cys15-80, passes (McDonald et al., 1991).

Members of the neurotrophin family are synthesised as precursor forms that can be cleaved intracellularly by Furin as well as other proconvertases (PC) (Seidah et al., 1996) and extracellularly by matrix metalloprotease 7 (MMP7) as well as plasmin (Lee et al., 2001) to generate mature secreted ligand. The NGF precursor forms a 35
kDa protein, which is N-linked glycosylated on residues -52 and -7 to form the principal 40 kDa species (Fig. 1.5) (Lessmann et al., 2003). The prodomains of the neurotrophins show sequence homology and it is known that the prodomain is necessary for protein expression, secretion and folding of the mature NGF (Suter et al., 1991; Rattenholl et al., 2001; Rattenholl et al., 2001; Lessmann et al., 2003; Kliemannel et al., 2004; Kliemannel et al., 2007). In addition to it’s function as a protein folding chaperone for the mature NGF, proNGF has pro-apoptotic functions that will be described in more detail in Section 1.6.4

![Figure 1.5. Structure of NGF and proNGF](image)

proNGF is a 241 amino acid protein that has two N-linked glycosylation sites highlighted in red and five proteolytic cleavage sites. The first site is the pre domain that is cleaved when the protein is transported from the Golgi. The second and third sites of cleavage are at aa site 193 and 162. The exact proconvertase that converts the protein are not known but the candidates are PACE and PC4/6. The fourth site of cleavage is 7 amino acids from the furin cleavage site. This site can be cleaved extracellularly by MMP7. The final cleavage site generates the mature NGF and can be targeted intracellularly by furin and extracellularly by plasmin. The molecular weights of the cleavage products are also displayed.

The crystal structure of NGF has been resolved (McDonald et al., 1991). NGF is a 120 amino acid protein consisted mainly of β-sheets. The NGF structure resolved was from Glycine 10 to alanine 116 with the remaining N-terminal and C-terminal not displaying electron density due to fluidity. The crystal structure of NGF, that was determined on co-crystallised with the TrkA, shows stark difference from the unbound NGF crystal structure. The N-terminal domain was resolved from residue serine 2, this region was unresolved on other structures but forms an important interface in the NGF/TrkA interaction. Thus, it has a well-defined electron density and with little flexibility in the NGF/TrkA crystal structure (Wiesmann et al., 1999;
Wehrman et al., 2007). Another difference was in the 10 amino acid loop from alanine 40 to arginine 50 of the NGF in the receptor complex with TrkA (Wehrman et al., 2007). This region has a secondary structure resembling a beta hairpin. This difference suggests that the N-terminal domain and the amino acid stretch from A40 to R50 are important for the NGF interaction with TrkA. When the crystal structure of unbound NGF was compared to that of NGF in complex with p75\textsuperscript{NTR} the most striking difference is that NGF binds to the receptor in the opposite orientation to TrkA (Fig. 1.6). The NGF was rotated 180° in Fig. 1.6 to illustrate that NGF bound to p75\textsuperscript{NTR} is similar to the unbound ligand (McDonald et al., 1991; He and Garcia, 2004; Wehrman et al., 2007).

![Figure 1.6. Changes in the conformation of NGF on binding to TrkA and p75\textsuperscript{NTR}](image)

The crystal structures were obtained using PDB codes 1BET (NGF), 21FG (NGF_TrkA) and 1SG1 (NGF_p75\textsuperscript{NTR}) respectively. NGF binding to p75\textsuperscript{NTR} is in the opposite orientation to NGF binding to TrkA. Thus, the NGF has been rotated 180° for ease of comparison. The NGF monomer structures were then isolated from the co crystal structure of NGF_TrkA as well as NGF_p75\textsuperscript{NTR} and compared to NGF crystallised alone. The N- and C-terminal domains of NGF are labelled and the first amino acid with a distinguishable electron density is also labelled. The differences in the N-terminal domain are highlighted in red and the difference in the A40–R50 loop it indicated with a red box.
1.5 NGF signalling through TrkA

TrkA expression in the nervous system has been linked to neuronal cell survival during development, growth cone guidance and synaptic plasticity. Its receptor structure and the activation of its pro-survival signalling will be described in more detail below.

1.5.1 TrkA receptor structure

The TrkA receptor extracellular domain consists of 3 leucine-rich repeats (LRR) flanked on either side by cysteine clusters and followed by two C2-type immunoglobulin-like (Ig-1 and Ig-2) domain, a single transmembrane domain as well as the cytoplasmic tyrosine kinase domain (Fig. 1.7) (Huang and Reichardt, 2003).

NGF binds to the Ig-2 domain proximal to the membrane of the TrkA receptor that is also called domain 5 (Wiesmann et al., 1999; Wehrman et al., 2007). This causes dimerization of the receptor and auto-phosphorylation of the conserved tyrosine residues of the Trk receptors. However, removal of the ligand binding site of TrkA by deletion of the Ig-1, Ig-2 domain or both domains increases spontaneous dimerization and activation of the receptor (Arevalo et al., 2000). There is also a novel single point mutation in a site between the LRR and the Ig-1 domain of TrkA, TrkA-P203A. This mutation decreased the ligand dissociation rate and thus increases NGF induced activity. This TrkA mutant was also found to be constitutively phosphorylated and capable of spontaneous dimerisation (Arevalo et al., 2001).

TrkA affinity for the NGF ligand is of the order of 10 nM. It is important, however, to note that when p75NTR is co-expressed with the TrkA receptor it increases the rate of association of NGF to TrkA 25-fold (Mahadeo et al., 1994). This was first thought to be association of TrkA and p75NTR to generate a high affinity binding site for the NGF in what is described as the ligand passing model (Wehrman et al., 2007). However, on further analysis the C-terminal cytosolic fragment (CTF) of p75NTR was found to be required to enhance the activity of TrkA (Wehrman et al., 2007; Matusica et al., 2013). This suggests that the conformation of TrkA is an important factor for the activation and signalling of the NGF.
The intracellular domain of TrkA contains a tyrosine kinase domain. There are 5 tyrosine residues that are critical for receptor function. These are Tyr490 in the juxtamembrane domain, Y670, 674 and 675 in the activation loop of the tyrosine kinase domain and Y785 in the C-terminal region. Binding of NGF to the receptor leads to the phosphorylation of these tyrosine residues and adapter protein docking. A schematic of the NGF homodimer binding to TrkA can be seen in (Fig. 1.7). (Bibel and Barde, 2000; Huang and Reichardt, 2003).

![Figure 1.7. Schematic of TrkA](image)

A schematic of the NGF homodimer binding to TrkA. NGF binds to TrkA at one site. Site I contains the interaction between NGF and domain 5 (the second Ig-like domain). The TrkA receptors ligand binding domain contains a LRR flanked by cysteine rich domains (CRD) and followed by two C2-type immunoglobulin-like (Ig-1 and Ig-2) domain. The intracellular domain contains the tyrosine kinase domain. It has key Tyr residues that get phosphorylated. These are Y490, Y670, Y674 and Y675, Y785.

### 1.5.2 NGF in complex with TrkA

The crystal structure of NGF in complex with the TrkA receptor has been determined (Wiesmann et al., 1999; Wehrman et al., 2007). The binding of NGF to TrkA activates three signalling pathways, namely the Ras/Raf/Mek/MAP, the
PI3K/AKT and the phospholipase C-γ (PLC-γ) pathway. This results in stimulating differentiation, neurite outgrowth, synaptic plasticity and anti-apoptotic effects.

On NGF stimulation Y490 phosphorylates, this allows the binding of Shc via its phosphotyrosine-binding domain and phosphorylates it. Phosphorylated Shc binds to the growth factor receptor bound 2 (Grb2)/ son of sevenless (SOS) complex and activates the Ras/Raf/Mek/MAPK pathway. Phosphorylation of FRS2 can also recruit the SHP-2/GRB2/SOS complex to activate the sequential phosphorylation of Ras, Raf, Mek, MAPK and ERK activation (Bibel and Barde, 2000; Huang and Reichardt, 2001; Huang and Reichardt, 2003). This complex activation allows for the sustained activation and phosphorylation of ERK that is seen on NGF stimulation (Santos et al., 2007) and is essential for NGF differentiation of neurons and PC12 cells (Greene and Tischler, 1976; Marshall, 1995). Activated PI3K and phosphatidylinositol-dependent protein kinase (PDK-1) activates the protein kinase Akt. Akt then phosphorylates several protein kinases important for cell survival. PI3K can be activated by a number of mechanisms. PI3K are activated through Ras-dependent and independent mechanisms (Yuan and Yankner, 2000; Vanhaesebroeck et al., 2001). Direct activation of PI3K by Ras is the major pathway by which NGF activates Akt through TrkA, but it is not the only mechanism (Vaillant et al., 1999; Bibel and Barde, 2000). phosphorylated Grb2 recruits the adapter protein Grb associated binder 1 (Gab1) and Gab2 and act as adapter proteins to recruit and activate PI3K and subsequent phosphorylation of AKT (Fig. 1.8) (Holgado-Madruga et al., 1997; Vaillant et al., 1999; Bibel and Barde, 2000). A second phosphorylation of Y785 on the TrkA receptor induces binding via SH2 domain and phosphorylates PLC-γ to activate the protein (Sofroniew et al., 2001). Activation of PLC-γ hydrolyses PtdIns(4,5)P₂ to generate inositol tris-phosphate (IP3) and diacylglycerol (DAG). IP3 promotes release of Ca²⁺ from internal stores that lead to the activation of Ca²⁺-regulated isoforms of protein kinase C and other Ca²⁺-calmodulin-regulated protein kinases. DAG stimulates DAG-regulated protein kinase C isoforms. It has been reported that one of these protein kinase C isoforms, PKCδ, is required for NGF-promoted neurite outgrowth and activation of ERK1/2 (Corbit et al., 1999).
Figure 1.8. NGF stimulated TrkA signalling

NGF causes the dimerization of the TrkA receptor. On dimerization the receptor autophosphorylates and recruits its downstream signalling adapters to activate three distinct pathways the PI3K/AKT, the Ras/Raf/MEK/ERK pathway and activation of PKC. Activation of the TrkA receptor results in pro-survival signalling.

1.6 NGF signalling through P75NTR

The p75NTR receptor is a member of the TNF family receptor superfamily. It is alternatively known as TNFRsf16, CD271 or NGFR. The role of P75NTR in the nervous system has been under continuous investigation since its discovery in 1973 (Herrup and Shooter, 1973). P75NTR is a 75 kDa single pass transmembrane protein and is a member of the TNF Receptor superfamily receptors (Nagata, 1997; Ashkenazi and Dixit, 1998; Locksley et al., 2001). There are eight members of the TNF receptor superfamily, TNFR1, FAS, death receptor 3 (DR3) TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1), TRAILR2, DR6, ectodysplasin A receptor (EDAR) and the p75 neurotrophin receptor (p75NTR) (Park, 2011). P75NTR displays structural similarity to DR6 and the EDAR. It contains three regions that are vital for its
function, namely the extracellular ligand binding domain, the transmembrane domain and the intracellular death domain (Underwood and Coulson, 2008).

1.6.1 \textit{p75}\textsuperscript{NTR} receptor structure

The extracellular region of \textit{p75}\textsuperscript{NTR} contains four highly conserved cystine rich domains (CRD). The crystal structure of the receptor reveals a large ligand-binding domain that would enable the binding and interaction of all members of the neurotrophin and proneurotrophin family (He and Garcia, 2004; Feng et al., 2010). The CRD repeats are followed by an α-secretase cleavage site. When this site is cleaved it yields a 25 kDa protein fragment called the C-terminal fragment (CTF). Cleavage at the γ-secretase site then releases this membrane bound receptor and yields a 20 kDa fragment called the intracellular domain (ICD), which is thought to have important functions in \textit{p75}\textsuperscript{NTR} signalling (Fig. 1.9)(Skeldal et al., 2011).

The transmembrane region of \textit{p75}\textsuperscript{NTR} contains a conserved Cys257, which is crucial for transducing NGF/p75\textsuperscript{NTR} signals. This will be described in more detail in Section 1.6.2 (Vilar et al., 2009).
A schematic of the NGF homodimer binding to p75<sup>NTR</sup>. NGF binds to p75<sup>NTR</sup> at two sites. Site I contains the interaction between NGF and the p75<sup>NTR</sup> at CRD2. Site II contains the interaction between NGF and the p75<sup>NTR</sup> at CRD3 and CRD4. P75<sup>NTR</sup> can be divided into several domains, a ligand-binding domain (comprised of 4 CRD), transmembrane domain (contains a conserved Cys257), juxtamembrane domain and death domain. P75<sup>NTR</sup> can undergo proteolytic cleavage in which generate different cleavage products of the receptor.

### 1.6.2 NGF in complex with p75NTR

Currently there is one crystal structure of NGF in complex with the p75<sup>NTR</sup> (He and Garcia, 2004) and one crystal structure with proNGF in complex with p75<sup>NTR</sup> (Feng et al., 2010) which allow for detailed information on the NGF/p75<sup>NTR</sup> interaction.

The crystal structure of p75<sup>NTR</sup> in complex with NGF shows an NGF homodimer binding to a monomer of p75<sup>NTR</sup>, this is called the asymmetric model of NGF binding, whereas the crystal structure of p75<sup>NTR</sup> in complex with proNGF shows the NGF homodimer binding to a p75<sup>NTR</sup> dimer, this is called the symmetrical model of NGF binding. The prodomain of NGF was not resolved in this structure, this was due to the fluidity of the prodomain and not extracellular cleavage of the protein as extensive analysis on the protein crystals formed showed that the correct molecular weight of proNGF was contained in the crystals (Feng et al., 2010).
Analysis of the binding sites of NGF to p75\textsuperscript{NTR} in these two structures shows that proNGF and mature NGF interact with p75\textsuperscript{NTR} at the same sites. Site 1 involves the CRD1 and CRD2 of the p75\textsuperscript{NTR} and contains extensive hydrogen bonds with the NGF at the site. Site 2 is formed between the junction of CR3 and CR4 that contain two salt bridges and two hydrogen bonds. However, in the asymmetric model of NGF binding the NGF displays structural differences between the two monomers, and this torsion of the NGF monomer is absent in the symmetrical model of NGF binding and prevents a second p75\textsuperscript{NTR} molecule from recognising the other face of the NGF (He and Garcia, 2004). This torsion of the NGF monomer and thus asymmetrical NGF binding may be an artefact of the crystal formation and packing. It, however, may differ from proNGF because proNGF has increased binding affinity for the receptor (Lee et al., 2001). In addition, the mature NGF is known to interact with the pro-domain and is essential for the folding of the NGF protein (Rattenholl et al., 2001; Rattenholl et al., 2001). The exact way NGF binds to the receptors is not yet known but more recent structural studies have produced further evidence for NGF binding to p75\textsuperscript{NTR} dimers. A conserved cysteine residue (Cys257) was found in the transmembrane region of P75\textsuperscript{NTR} that proved crucial for receptor function as mutation of the conserved Cys257 abolished the ability of the receptor to recruit downstream signalling partners (Vilar et al., 2009; Vilar et al., 2009). This has lead to two models of NGF binding and activation of downstream signalling (Fig. 1.10) (Vilar et al., 2009; Vilar et al., 2009).
Figure 1.10. Models of NGF binding to p75NTR

(A) NGF binds to a p75NTR monomer, which causes its dimerization and activation, the dimerization of the receptor could cause the activation of docking sites adapter molecules. The receptors can exist as both monomers and dimers in this model as they can dissociate from one another. (B) NGF binds to p75NTR as a preformed dimer. This causes a conformational change in the receptor around Cys257, which causes the death domains of the receptor to part and allows the recruitment of adapter proteins.

1.6.3 NGF stimulated p75NTR signalling

Activation of p75NTR signalling can activate either cell survival or cell death depending on cellular context, including expression of the receptors and of adapter proteins (Yoon et al., 1998; Casaccia-Bonnefil et al., 1999; Masoudi et al., 2009). p75NTR has been reported to activate cell survival signals by causing a conformational change in the TrkA receptor, increasing its on/off rate for NGF (Mahadeo et al., 1994), and by activating nuclear factor-κB (NF-κB) pro-survival pathway (Casaccia-Bonnefil et al., 1999). In contrast to this, NGF has also been shown to activate apoptosis (Roux and Barker, 2002). The signalling cascades activated by NGF stimulation of p75NTR will be summarised here.

It was first noted that NGF/p75NTR exerts a pro-survival function as its expression enhanced TrkA signalling. This activity is attributed to the ICD of p75NTR (Wehrman
et al., 2007; Matusica et al., 2013). \(p75^{NTR}\) is also known to signal alone in Schwann cells and stimulate the NF-κB pro-survival pathway through direct interaction of TRAF6 proximal to the death domain and interestingly, deletion of the death domain increased the association of TRAF6 for the \(p75^{NTR}\) in immunoprecipitation experiments (Khursigara et al., 1999). Further studies showed that activation of NF-κB required a complex formation of \(p75^{NTR}\) with TRAF6, the atypical protein kinase C-interacting protein p62 and the TrkA receptor (Wooten et al., 2001) and interleukin 1 receptor-associated kinase 1 (IRAK1) (Mamidipudi et al., 2002). More recently TRADD has been shown to be required for \(p75^{NTR}\)-mediated NF-κB activation where overexpression of a dominant negative TRADD mutant blocked \(p75^{NTR}\)-mediated anti-apoptotic signalling in breast cancer cells (El Yazidi-Belkoura et al., 2003). \(p75^{NTR}\) has also been shown to signal through phosphatidylinositol 3-kinase (PI3K) and activate the AKT kinase pathway as well as Ras to promote survival, through a yet unidentified pathway (Fig. 1.11) (Roux et al., 2001; Bui et al., 2002; Blochl et al., 2004).
NGF signalling through p75<sup>NTR</sup> is known to activate diverse signalling pathways depending on the cellular context. Binding of NADE, NRAGE and NRIF to the cytoplasmic domain on the receptor can activate JNK as well as phosphorylate Bad causing cell death. Binding of TRAF6, IRAK, p62 and TRADD have shown to contribute to NGF p75<sup>NTR</sup> NF-κB activity and cell survival. p75<sup>NTR</sup> has also shown to activate pro-survival signals through activation of the MAPK pathway.

Pro-death signalling through p75<sup>NTR</sup> is widely reported in the literature. The exact mechanism, however, used to execute cell death remains elusive. There have been some reports on adapter proteins that contribute to the cell death machinery. These include, neurotrophin receptor interacting factor (NRIF), p75<sup>NTR</sup>-associated Cell Death Executor (NADE), neurotrophin receptor-interacting MAGE homolog (NRAGE), and c-Jun N-terminal kinase (JNK) (Roux and Barker, 2002). NRIF was the first pro-apoptotic adapter protein discovered to interact with the intracellular domain of p75<sup>NTR</sup>. It was shown to be involved in developmental cell death in the mouse embryonic neural retina as NRIF, p75NTR and NGF knock-out mice gave
comparable reduction in cell death (Casademunt et al., 1999). Another adapter protein involved in death inducing machinery of p75NTR is NADE. NADE associates to the C-terminal domain of the receptor and overexpression of NADE caused caspase activation in HEK293, PC12 and PC12nnr5 cells overexpressing the p75NTR (Mukai et al., 2000). NRAGE binding was found to disrupt association with TrkA causing cell cycle arrest and a decrease in cell viability (Salehi et al., 2000; Jordan et al., 2001). The NGF induced death was found to involve an increase in ceramide production and phosphorylation of pro-apoptotic Bcl-2 protein Bad which was dependent on JNK (Bhakar et al., 2003). Taken together a model of NGF induced p75NTR pro-apoptotic signalling can be defined (Fig. 1.11).

1.6.4 proNGF stimulated p75NTR signalling

Recently the pro-form of the neurotrophins were suggested as the pro-apoptotic ligand for p75NTR. However the exact signalling cascade and adapter proteins activated upon proneurotrophin binding have yet to be fully elucidated. ProNGF was highlighted as a pro-apoptotic ligand for p75NTR (Lee et al., 2001; Beattie et al., 2002). Its levels are increased during Alzheimer’s disease (Fahnestock et al., 2001) and have been found to contribute to neuronal cell death during disease progression (Pedraza et al., 2005). proNGF was found to induce apoptosis of basal forebrain cholinergic neurons even on activation of the trophic TrkA receptor (Fahnestock et al., 2001; Lee et al., 2001; Volosin et al., 2008). The proNGF has a higher binding affinity than mature NGF and requires the co-receptor sortillin. Sortilin is a widely expressed protein essential for proNGF-induced apoptosis. It is ubiquitously expressed in the nervous system and most notably during embryogenesis, where it is expressed at high concentrations in areas where NGF is active (Nykjaer et al., 2004). The mechanism of proNGF induced cell death is thought to be similar to mature NGF induced apoptosis and it has been shown to activate JNK and caspases resulting in increased ceramide production and apoptosis (Harrington et al., 2004; Diarra et al., 2009).
1.7 Nerve growth factor in disease

1.7.1 NGF and Alzheimer’s disease

Alzheimer’s disease (AD) is a neurodegenerative disorder characterised by loss of neuronal plasticity in the hippocampus and the cortical areas of the brain. It is characterised by pathological deposits, consisting primarily of β-amyloid (Aβ) peptide Aβ_{1-42}. The accumulation of Aβ peptide is a consequence of increased production, differential processing and/or reduced removal of the peptide (Huang and Mucke, 2012). NGF and its receptors have been implicated in the progression of Alzheimer’s disease as p75NTR is increased in AD in the cortical and hippocampal neurons (Mufson and Kordower, 1992; Hu et al., 2002).

The Aβ peptide has been shown to induce cell death through the p75NTR both in vitro, in cells overexpressing the receptor (Yaar et al., 1997; Kuner et al., 1998) as well as in primary hippocampal cultures (Sotthibundhu et al., 2008), and in vivo in basal forebrain cholinergic neurons (Sotthibundhu et al., 2008). These studies show that both the soluble and insoluble form of the Aβ peptide bind to the p75NTR receptor with a high binding affinity, 4-7 nM and 12 nM respectively (Yaar et al., 1997; Kuner et al., 1998). Furthermore, increasing concentrations of NGF could displace the Aβ peptide suggesting that the peptide binds in the active site of the receptor. Aβ peptide induced cell death was lost in p75NTR knock out cells (Sotthibundhu et al., 2008). This is of particular importance because during Alzheimer’s disease the oligodendrocytes of the basal nuclear cortex, which express a high level of p75NTR are sensitive to neuronal loss (Woolf et al., 1989) and could implicate p75NTR in Alzheimer’s disease progression and identify NGF as a possible therapeutic.

Using SK-N-MC cells, a neuroblastoma cell line, Aβ toxicity through p75NTR was found to involve activation of the transcription factor NF-κB (Kuner et al., 1998) and it was later shown that this activation was dependent on Aβ activation of p38 and c-JNK. The activation of NF-κB was dependent on the death domain of p75NTR as a
truncated form of the receptor abrogated Aβ toxicity (p75NTR lacking residues 352-427) (Costantini et al., 2005). This is in line with reports that show that the CTF of the p75NTR receptor is generated upon Aβ treatment and is important for Aβ toxicity (Sotthibundhu et al., 2008).

The benefits of NGF treatment in AD have long been appreciated and although exogenous injections of NGF were successful in reversing age-dependent changes in the cholinergic forebrain neurons, as well as the performance of the aged animals in spatial memory tasks (Martínez-Serrano and Björklund, 1998). Unfortunately, the side effects of the NGF on the surrounding tissue were too great. These included weight loss from hypothalamic stimulation, pain from stimulation of the nociceptive neurons of the dorsal root ganglion and Schwann cell migration. Gene therapy options to localise the delivery of NGF were then considered (Martínez-Serrano and Björklund, 1998; Carter and Schuchman, 2001). Rat pre-clinical trials showed that ex vivo administration of NGF using primary autologous fibroblasts genetically modified to produce NGF were able to graft to the desired brain area and sustain the secretion of NGF for at least 18 months. The effect these grafts prevented cholinergic degeneration, restored cholinergic function and improved memory in aged primate brains (Smith et al., 1999; Conner et al., 2001).

Based on these studies Phase I and Phase II clinical trials have been conducted and NGF showed promise as a possible therapeutic for AD. In the first clinical trial stereotaxically injected primary fibroblast, which secrete NGF were placed into 8 patients. Cognition was assessed in 6 patients that safely completed NGF delivery. These patients showed a lower rate of cognitive decline and an increase in glucose uptake of the cholinergic neurons (Tuszynski et al., 2005).

NGF is unlikely to be successful as a sole treatment for AD due to the local effect of NGF in the cholinergic neurons. It could, however prove to be more potent then other pro-survival treatments as it also has an effect in the prevention of AB induced cell death at high concentration.
1.7.2 NGF and chronic pain

During development NGF/TrkA is important for the survival and maturation of the nociceptive neurons, a specialised subset of sensory neurons that respond to tissue damage (Kumar and Mahal, 2012). The importance of NGF and TrkA in the development and maturation of these neurons is highlighted in mouse models with a null mutation in TrkA or NGF that display insensitivity to pain (Crowley et al., 1994; Smeyne et al., 1994). Also patients with congenital insensitivity to pain with anhidrosis (CIPA) show decreased sensitivity to pain presented by inability to sweat. It was found that patients with CIPA have mutations in the TrkA gene that leave it unresponsive to NGF (Indo et al., 1996). NGF signalling through TrkA is required for the survival of the peripheral nociceptive neurons during development and thus these patients have no way of detecting pain, as they lack peripheral nociceptive neurons (Indo et al., 1996). NGF continues to show importance after development as it is expressed by the nociceptors during adult life (Kumar and Mahal, 2012). Administering NGF to the terminal cell bodies in-vivo alters central pain processing by up-regulating sensory neuropeptides, neuromodulators, receptors, and ion channels (e.g. CGRP, substance P, TRPV1, P2X3 and TTXs) (Pezet and McMahon, 2006). The effect of NGF treatment on pain is also further highlighted during AD studies where exogenous injections of NGF caused acute pain (Martínez-Serrano and Björklund, 1998; Carter and Schuchman, 2001).

NGF has been implicated as an important mediator in chronic pain and is induced in many chronic pain sufferers. This has lead to the development of humanized anti-NGF monoclonal antibodies as potential treatments for chronic pain (Kumar and Mahal, 2012). One such antibody is tanezumab developed by Pfizer, which has reached Phase II and III clinical trials. This antibody is being used with particular effectiveness in pain caused from Osteoarthritis of the Knee (Lane et al., 2010; Brown et al., 2012). The Food and Drug Administration (FDA) recently put the clinical trials on halt due the increase in the development of rapid joint destruction. This joint destruction may be attributed to destructive joint events undertaken by the patient due to insensitivity to pain (Kumar and Mahal, 2012). However, after an
investigation the FDA approved further testing of the drugs (U.S. Food and Drug Administration arthritis advisory committee, 2012)
1.8 Aims of this research

- The purpose of this work was to investigate the mechanism of NGF stimulated inhibition and degradation of caspases. In particular to determine what role the lysosomes had on the removal of cleaved caspase-3 from the cells.

- A method of recombinant NGF expression was also developed and optimised. The recombinant NGF developed had His tags to aid purification of the protein and FLAG or HA tags. The FLAG tagged and HA tagged was generated for co-immunoprecipitation experiments.

- The expression system developed was then utilised to express and purify variant NGF proteins designed by the FoldX computer algorithm. The NGF variant proteins are predicted to have altered binding and thus biological activity through TrkA or p75NTR. The variants were developed for the use as potent therapeutic agents. The NGF variants were expressed and their biological activity was compared with that of wild type NGF.
2 Chapter 2.

Materials and Methods
2.1 Materials

2.1.1 Suppliers

Alomone Labs, Jerusalem 91042, Israel

Abcam, Cambridge, CB4 0FL, UK

BD Biosciences-PharMingen, San Diego, CA 92121, USA

Calbiochem, Nottingham, NG9 2JR

Cell Signalling Technology (CST), Inc., Beverley, MA 01915, USA

Fisher Scientific Ireland, Dublin 15, Ireland

Invitrogen, Crofton Rd., Dun Laoghaire, Dublin, Ireland

Merck Millipore, Billerica, MA 01821, USA

New England Biolabs (NEB), Ipswich, MA01938-2723, UK

Pierce, Subsidiary of Thermo Scientific Ltd, Rockford, IL 61105 USA

Promega, Mulhuddart, Dublin 15, Ireland

R&D systems, Abingdon, OX14 3NB, UK

Santa Cruz Biotechnologies (SantaCruz), Inc., Santa Cruz, CA 95060, USA

Sigma-Aldrich Ireland, Dublin, Ireland

Vector Laboratories Inc, Burlington, CA 94010, USA

All chemicals were obtained from Sigma-Aldrich unless otherwise stated
2.2 Methods.

2.2.1 Cell culture techniques

A summary of the cell lines used is illustrated in Table 2.1. All cell lines were maintained in growth medium supplemented with heat inactivated fetal bovine serum (FBS) or horse serum (HS) and 50 U/ml penicillin and 50 U/ml streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂. When cells reached a confluence of 85-90% they were subcultured into a 75 cm² culture dish at the seeding density described below and summarised in Table 2.1.

Rat pheochromocytoma 12 cells (PC12) were obtained from the European Collection of Cell Cultures (ECACC) Cat. No. 88022401. They were maintained in Dulbecco’s modified Eagle medium (DMEM) that contains 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate. The media was supplemented with 10% HS, 5% FBS, 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded onto poly-L-lysine coated culture dishes at a density of 3 x 10⁴ cells/cm² for experiments or a seeding density of 2.5 x 10⁴ cells/cm² for routine culture (every 72 h).

Human embryonic kidney cells 293T cells (HEK293T) were obtained from the American tissue culture collection (ATCC). They were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded at a density of 2 x 10⁴ cells/cm², cells were subcultured every 60 h.

SK-N-MC cells were obtained from ECACC. They are a human neuroepithelioma cell line derived from a metastatic supra-orbital human brain tumor (Biedler et al., 1973; Seeger et al., 1977). They were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded at a density of 5.5 x 10⁴ cells/cm², cells were subcultured every 72 h.
Mec1 cells are a chronic leukemic human B-cell cell line that contain a deletion mutation in the p53 gene (Stacchini et al., 1999). HG3 cells are a chronic leukemic human B-cell cell line that are p53 positive (Rosén et al., 2012). They are suspension cell lines and a kind gift of Prof. Corrado Santocanale. Mec1 and HG3 cells were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded at a seeding density of 250,000 cells/ml for experiments or 500,000 cells/ml for routine culture, cells were subcultured every 72 h into an upright 75 cm² flask.

Chinese hamster ovary-K1 cells (CHO-K1) are a subclone derived from the parental CHO cell line. They were maintained in DMEM containing Ham’s F12 nutrient media supplemented with 10% FBS, 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded at a seeding density of $1 \times 10^4$ cells/cm², cells were subcultured every 48 h.

MDA-MB-231 cells are a triple negative breast cancer cell line obtained from the ECACC. They were maintained in DMEM supplemented with 10% FBS, 1% L-glutamine (L-glut), 50 U/ml penicillin and 50 U/ml streptomycin. Once cells reached confluence they were seeded at a seeding density of $3 \times 10^4$ cells/cm², cells were subcultured every 72 h.
Table 2.1. Summary of the cell line specific culture conditions

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Seeding density cells/cm²</th>
<th>Seeding density cells/ml</th>
<th>Growth media</th>
<th>Serum</th>
<th>Subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12</td>
<td>ECACC</td>
<td>3 x 10⁴</td>
<td>-</td>
<td>DMEM</td>
<td>10% HS, 5% FBS</td>
<td>72 h</td>
</tr>
<tr>
<td>HEK293T</td>
<td>ATCC</td>
<td>4 x 10⁴</td>
<td>-</td>
<td>DMEM</td>
<td>10% FBS</td>
<td>60 h</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>ECACC</td>
<td>10 x 10⁴</td>
<td>-</td>
<td>DMEM</td>
<td>10% FBS</td>
<td>72 h</td>
</tr>
<tr>
<td>Mec1</td>
<td>Gift</td>
<td>-</td>
<td>500,000</td>
<td>RPMI</td>
<td>10% FBS</td>
<td>72 h</td>
</tr>
<tr>
<td>HG3</td>
<td>Gift</td>
<td>-</td>
<td>500,000</td>
<td>RPMI</td>
<td>10% FBS</td>
<td>72 h</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Gift</td>
<td>1.3 x 10⁵</td>
<td>-</td>
<td>HamsF12</td>
<td>10% FBS</td>
<td>48 h</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>ECACC</td>
<td>3.9 x 10⁴</td>
<td>-</td>
<td>DMEM</td>
<td>10% FBS, 1% L-glut</td>
<td>72 h</td>
</tr>
</tbody>
</table>

2.2.2 Poly-L-lysine coating of tissue culture flasks

PC12 cells were seeded into poly-L-lysine (PLL)-coated cell culture flasks. The cell culture dishes were incubated with 10 μg/ml PLL solution for 2 h. The PLL was then removed and the tissue culture flasks were washed 3 times with sterile dH₂O.

2.2.3 Plasmids

ATG4B(C74A) cDNA was cloned from an expression plasmid encoding mStrawberry (Addgene) into the lentiviral vector pWPT (Addgene) in which the GFP was removed. To amplify the mStrawberry-ATG4B(C74A) sequence from the expression vector, the primers were designed with a mlul restriction site sequence in the forward primer 5’-CGCGTATGGTGAGCAAGGGCCGAGGAG-3’ and salI restriction site in the reverse primer 5’-GTCGACTCAGAGGGATAAGATTCAAGTC-3’. The PCR product was ligated into a TA-TOPO vector (Invitrogen). Both the TA-TOPO vector containing PCR product and the pWPT vector were digested with restriction enzymes mlul and salI. T4 DNA ligase (NEB) was used to ligate the mStrawberry-ATG4B(C74A) insert into the pWPT vector to generate pWPT-mStrawberry-ATG4B(C74A) lentiviral vector (this lentiviral construct was generated by Dr S.
Mouse GIPZ lentiviral shRNAmiR individual clones V2LMM_15767 and V3LMM_429629 (Open Biosystems) were used to evaluate the functional effects of silencing of Hsc70 and LAMP2a respectively. The lentiviral NFκB promoter reporter plasmid (NFκB(EIF_Puro in the pGreenFire1 plasmid backbone from Open Biosciences) was used to evaluate the NFκB activity in cells. The plasmid expressing human α-synuclein under the synapsin promoter was kind gift from Dr. Eilís Dowd, National University of Ireland, Galway, Ireland.

2.2.4 Transient transfection of PC12 cells

PC12 cells were seeded into PLL-coated dishes at a seeding density of 3 x 10^4 cells/cm^2 and allowed to adhere overnight. The PC12 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) using a DNA to lipid ratio of 1:2.5. Briefly, for a 6 well plate containing 2 ml of antibiotic free media, 2 μg of DNA was diluted in 200 μl serum free media and in a separate tube 5 μl of lipofectamine transfection reagent was incubated in 200 μl of serum free media. The tubes were vortexed and incubated at room temperature for 5 min. The transfection reagent was added to the DNA and incubated at room temperature for 20 min and added dropwise onto the cells. The cells were placed back into the incubator for 6 h, after which the media containing the transfection reagent was removed and replaced with fresh media. The typical transfection efficiency obtained was 60-65%.

2.2.5 Transient transfection of CHO-K1 cells

CHO-K1 cells were seeded at a seeding density of 1 x 10^4 cells/cm^2 and allowed to adhere overnight. The CHOK1 cells were transfected using Effectene transfection reagent (Qiagen) with a DNA to lipid ratio of 1:4. Briefly, for a 6 well plate containing 2 ml of antibiotic free media, 2 μg of DNA was diluted in 100 μl Effectene TE buffer and made up to 150 μl using Effectene EC buffer 4 μl of enhancer was then added. The diluted DNA was vortexed and left at room temperature for 5 min. 8 μl of Effectene transfection reagent was then added to the diluted DNA and was incubated at room temperature for 20 min before dropwise addition onto the cells. The cells were placed back into culture conditions for 5 h, after which the media
containing the transfection reagent was removed and replace with fresh media. A typical transfection efficiency of 85-90% was obtained.

2.2.6 Transient transfection of HEK293T cells

HEK293T cells were seeded at a seeding density of 5.8 x 10⁴ cells/cm² and allowed to adhere overnight. The HEK293T cells were transfected using JetPEI transfection reagent (Polyplus transfection reagents) with a DNA to lipid ration of 1:2. Briefly, for a 6 well plate containing 2 ml of antibiotic free media, 2 μg of DNA was diluted in 250 μl 150 mM NaCl buffer, in a separate tube 4 μl of JetPEI transfection reagent was incubated in 250 μl of 150 mM NaCl. The tubes were vortexed and incubated for at room temperature for 5 min. The transfection reagent was added to the DNA, vortexed, incubated at room temperature for 20 min and then added dropwise onto the cells. The cells were placed back into the incubator for 5 h, after which the media containing the transfection reagent was removed and replace with fresh media. The typical transfection efficiency obtained was 90-95%

2.2.7 Generation of stable cell lines by lentiviral infection

Lentivirus was made by transfection of expression vectors along with a second generation lentivirus packaging system (pMD2.G, psPAX2 and pRSC-Rev from Addgene) into HEK293T cells using JET PEI transfection reagent (Polyplus Transfection). Transduction efficiency of DN-Atg4 construct into PC12 cells was >90% and was monitored by fluorescence microscopy and quantified by flow cytometry analysis using mStrawberry fluorescent tag as a marker for transduced cells.

Stable PC12 cell lines containing LAMP2a and Hsc70 shRNA constructs were selected with 0.5 μg/ml puromycin (Sigma) for 1 week. Knockdown efficiency of endogenous target genes was >70% and was monitored by fluorescence microscopy using GFP as a marker for transduced cells as the LAMP2a and Hsc70 shRNA constructs co-expressed GFP.
MDA-MB-231 cells stably expressing the NF-κB promoter reporter were selected with 4 μg/ml puromycin (Sigma) for 1 week.

2.2.8 Preparation of protein extracts

Cells were seeded into 25 cm² culture dish using the seeding densities outlined in Table 2.1. Following experimental treatments the cells were detached from the cell culture dish by scraping the cells into the culture medium. The cell suspension was then collected and transferred into a 15 ml tube and centrifuged at 775 x g at 4 °C for 5 min. The supernatant was then discarded and the cell pellet was resuspended in 500 μl of 1 x PBS and transferred into a 1.5 ml tube and centrifuged at 20,000 x g at 4°C for 10 sec. The 1 x PBS was removed and the cells were lysed in whole cell lysis buffer (1 M HEPES pH7.5, 350 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM Ethyleneglycoltetraacetic acid (EGTA) and 1% Nonidet-P40) containing protease inhibitors and reducing agents (0.5 mM dithiothreitol (DTT), 0.1% phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 1 mM sodium orthovanadate (Na₃VO₄) and 5 mM sodium fluoride (NaF)). The cell pellet was lysed by resuspending it in 35 μl of whole cell lysis buffer, the cells were then allowed to swell on ice for 25 min and centrifuged at 20,000 x g at 4°C for 10 min to remove the genomic DNA and cell debris. The supernatant, which contain the cytosolic proteins, was then removed and transferred to a new 1.5 ml tube. The protein concentration was ascertained using the Bradford method and bovine serum albumin (BSA) as the standard. The cytosolic proteins were separated by their size to charge ratio using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). If samples were not used immediately they were stored a -20 °C for future analysis.

Protein samples were resuspended in 5 x Laemmli’s SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 1 mM PMSF and 0.05% bromophenol blue) until a concentration of 1X Laemmli’s SDS-PAGE sample buffer was reached. The proteins were then denatured for 5 min at 95 °C. 30 μg of protein was loaded onto the gel and resolved using a 8-15% SDS-PAGE gel (see Table 2.2 for
components). The percentage of the gel depended on the size of the protein of interest. A broad range (7-175 kDa) prestained colourplus protein ladder (NEB) was run alongside the protein samples. Gels were electrophoresed at 80 V for 30 min after which the voltage was increased to 110 V and run for another 1.5–2 h. The gels were electrophoresed in running buffer (25 mM Tris base pH 8.3, 2 M glycine and 3 mM SDS). The proteins were then visualised by Coomassie stain as described in Section 2.2.10 or by Western blotting as described in Section 2.2.9.

For some experiments cells were also lysed directly in the tissue culture well by 2X sample buffer (10% glycerol, 4% SDS, 0.1 M DTT, 0.1% bromophenol blue and 120 mM Tris pH 6.8). In brief after treatments to the cells the media was removed and the cells were washed with 1 x PBS. 60 μl of 2X sample buffer was then added directly onto the cells which were left to lyse for 5 min. The samples were then transferred to 1.5 ml tubes and vortexed. The proteins were denatured immediately for 5 min at 95 °C. Equal volumes of protein was loaded onto the gel and resolved using a 8-15% SDS-PAGE gel as described above.

### Table 2.2. Components of SDS-PAGE running gels and SDS-PAGE stacking gel

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<tr>
<th>Component</th>
<th>8% running gel (ml)</th>
<th>12% running gel (ml)</th>
<th>15% running gel (ml)</th>
<th>Stacking gel (ml)</th>
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<td><strong>5</strong></td>
<td><strong>5</strong></td>
<td><strong>2</strong></td>
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</table>
2.2.9 Western blotting

The proteins in the SDS-PAGE gels were then electrophoretically transferred onto nitrocellulose membrane (Whatman) for 90 min at 110 V in transfer buffer (25 mM Tris base pH 8.3, 2 M glycine and 20% methanol). Membranes were then blocked for 1 h in blocking buffer (5% non-fat dried milk in 1 x PBS containing 0.05% Tween 20 (1 x PBS-T) or 1% BSA in 1 x PBS-T). The membrane was then incubated with primary antibodies according to the conditions detailed in Table 2.3. After the primary antibody incubation the antibody was removed and any unbound or nonspecifically bound antibody was detached from the membrane by 3 x 15 min washes in 1 x PBS-T. This was followed by a 1-1.5 h incubation with the appropriate horseradish peroxidise (HRP)-conjugated IgG antibody diluted in 5% non-fat dried milk in 1 x PBS-T according to the conditions outlined in Table 2.3. After the secondary antibody incubation the unbound antibody was removed by 3 x 15 min wash in 1 x PBS-T. The membrane was then incubated with 1 ml Western Chemiluminescent HRP substrate (Pierce) for 5 min and the protein bands were visualised using X-Ray film (AGFA).

Antibodies were stripped from the membrane by incubating with 200 mM NaOH for 2 min followed quickly by one wash with ddH₂O and two 5 min washes with 1 x PBS-T. The membrane was then blocked using blocking buffer and ready for incubation with an alternative primary antibody.
<table>
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<th>1° antibody</th>
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<th>1° antibody dilution and incubation conditions</th>
<th>2° antibody</th>
<th>2° antibody dilution and incubation conditions</th>
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2.2.10 Coomassie staining of SDS-PAGE gels

To analyse the total protein of a sample an SDS-PAGE gel was stained with Coomassie Brilliant Blue stain. Once the gel was electrophoresed as described in Section 2.2.8 the gel was immersed in Coomassie stain (1% Coomassie, 50% methanol and 10% acetic acid) the gel was then incubated for 1 h at room temp with rocking. The Coomassie stain was then removed and the gel was washed with destain solution (50% methanol and 10% acetic acid). The gel was washed 3 x 20 min in destain solution and then placed in a new container and incubated in destain solution until the background signal was reduced and the protein bands were visible.

2.2.11 Annexin V staining of adherent cells

Cells were seeded into a 24 well plate at the seeding densities outlined in Table 2.1 and allowed to adhere overnight. The cells were treated as desired and harvested by trypsinisation. In brief, the media and cell suspension was and placed into a 1.5 ml tube, the cells were washed with 250 μl of Hank’s balanced salt solution and the wash was added to the cell suspension. Any remaining adherent cells were then removed using 250 μl of 1 x trypsin-EDTA. The tissue culture plate was then transferred to a 37 °C incubator until the cells had detached. The trypsin was the inactivated by addition of media and the cell suspension was added to a 1.5 ml tube. The cells were then allowed to recover membrane integrity by incubation under normal culture conditions for 15 min.

After the recovery time the cells were placed on ice and centrifuged a 1000 g at 4°C for 5 min. The supernatant was then removed and the cells were gently resuspended in 100 μl 1 x PBS, the cells were centrifuged as before and the supernatant was removed. The cells were gently resuspended in 50 μl calcium buffer (10 mM HEPES pH 7.5, 140 mM NaCl and 2.5 mM CaCl₂) containing 0.5 μl of recombinantly expressed and purified Annexin V conjugated to FITC. The cells were then incubated in the dark for 15 min after which 300 μl of calcium buffer was
added and the samples were analysed immediately by flow cytometry using the BD FACSCanto on channel FITC. All analysis was carried out using Cyflogic software.

2.2.12 Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) staining

Cells were seeded into a 24 well plate at the density outlined in Table 2.1 and allowed to adhere overnight. The cells were then treated as desired and harvested by trypsinisation as described in Section 2.2.11.

2 mM of TMRE was added and the cells were resuspended gently. The cells were then incubated in the dark for 30 min and measured by flow cytometry using the BD FACSCanto on channel PerCP and PE. All analysis was carried out using Cyflogic software.

2.2.13 Detection of DEVDase activity

Cells were seeded using the seeding densities described in Table 2.1. The activity of caspase-3-like enzymes (DEVDase activity) was determined fluorometrically as reported previously (Nicholson, 1999) with some modifications (Gorman et al., 1999). Briefly, cells were scraped and centrifuged at 300 × g at 4 °C for 5 min. Pellets were washed in ice-cold PBS, re-suspended in PBS and then flash-frozen in liquid nitrogen. 50 μM of DEVDase-substrate (DEVD-MCA) in reaction buffer (100 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid (HEPES) pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS), 5 mM DTT, 0.01% NP-40) was added to the lysates. The release of free AMC was monitored at 37 °C at 60 s intervals over a 30 min period using a Wallac Victor x3 microplate reader (excitation 355 nm, emission 460 nm). Fluorescence units were converted to nanomoles of AMC released per minute per mg of enzyme using a standard curve generated with free AMC and subsequently related to protein concentration.

2.2.14 Clonogenic assay

PC12 cells were treated as described with TG and NGF, following treatments, cells were reseeded into 78 cm² dishes at a density of 7.8 x 10² cells/cm² and allowed to
proliferate for 14 days. The proliferation time was chosen based on the doubling time of the cells. Colonies were fixed and stained with 0.25% methylene blue in 50% ethanol 50% dH2O for 30 min, then rinsed with dH2O. Colonies containing greater than 50 cells were counted

2.2.15 Isolation of Annexin V positive cells

Following treatment with 1.5 μM TG for 20 h PC12 cells were seeded into a 175 cm² flask at a seeding density of 10 x 10⁶ cells. The cells were allowed to adhere overnight and were treated with 1.5 μM TG for 20 h. The media containing the apoptotic cells was then collected and transferred into a 50 ml tube and any remaining adherent cells were then removed by addition of 10 ml of 1 x trypsin-EDTA. Once the cells had lifted the trypsin was neutralised and the cell suspension was transferred to the 50 ml tube and centrifuged for 10 min at 300 g. The cells exposing phosphatidylserine (PS) on the outer leaflet of the plasma membrane were labelled with magnetic beads conjugated to Annexin V using the Annexin V MicroBead Kit (Miltenyi Biotec Headquarters) according to the manufacturer’s instructions, and separated from the rest of the population by magnetic separation. The PS-exposing cells were eluted from the column by removing the magnetic field. The eluant was then divided in two and reseeded onto two 1.9 cm² dishes, one of which was then treated with 100 ng/ml NGF. Every second day fresh NGF was added to the culture medium. Cells were incubated on dishes for 7 days. The number of cell colonies and cells that contained neurites were counted, and expressed as a percentage of the number of TG-treated surviving cells.

2.2.16 Live cell imaging using FLICA and lysotraker

PC12 cells were seeded onto 8-well PLL-coated µ-slides (Ibidi) at a density of 3.5 x 10⁴/cm². The cells were treated with 1 μM TG for 20 h followed by treatment with 100 ng/ml NGF for 3 h. The 1X FAM-DEVD-FMK reagent (FLICA) (from the Image-iT™ LIVE Green Caspase Detection Kit) was then added for 1 h, along with 50 nM Lysotracker red DND99 for the final 0.5 h. 4 h after NGF treatment the cells were washed with DMEM. The nuclei were stained using 1 nM Hoechst 33258 for 5 min
after which the cells were washed twice with 1X apoptosis wash buffer (from the Image-iT ™ LIVE Green Caspase Detection Kit). The cells were maintained in DMEM and visualised immediately using a DeltaVision core system (Applied Precision) which controlled an interline charge-coupled device camera (coolsnap HQ2;Roper) mounted on an inverted microscope (1X-71;Olympus). Images were collected using a 100X oil objective at 0.2 µm z sections. All images were deconvolved and maximum intensity was projected using SoftWoRx software programme (Applied Precision).

2.2.17 Immunoprecipitation of cleaved-caspase 3

PC12 cells were seeded into PLL coated 75 cm² flasks at a seeding density of 3.5 x 10⁴/cm². Following experimental treatments cells were scraped from the culture flasks and centrifuged at 150 x g for 5 min at 4 °C. The cells were then washed in ice cold 1 x PBS and lysed using lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% NP-40) containing EDTA free protease inhibitor cocktail tablet (Invitrogen). The lysate was the centrifuged at 14,000 rpm for 10 min and the supernatant was incubated with 1.5 mg of pre-prepared Dynal beads (Invitrogen). The Dynal beads were prepared as per manufacturer’s guidelines and conjugated overnight with a 1/100 dilution of cleaved-caspase-3 antibody at 37 °C. The lysates were incubated with the conjugated beads for 12 h at 4 °C. The beads were collected by magnetic separation and the supernatant was removed. The beads were washed in this way a total of four times in lysis buffer. The beads were then resuspended in 40 µl of elution buffer (low pH buffer supplied with the kit) for 5 min at room temperature and collected by magnetic separation. The eluent was removed and subjected to Western blotting for cleaved caspase-3 and Hsc70

2.2.18 Neurite outgrowth assay

PC12 cells were seeded at a density 8 x 10³ cells/cm² onto PLL-coated plates. Sarstedt plates were used for optimal adherence of differentiated PC12 cells. The cells were allowed to adhere for 24 hours and then treated with commercially available human recombinant NGF 100 ng/ml, mouse NGF 100 ng/ml (Alomone
labs) or conditioned media from the HEK293T cells (Section 2.2.35). The PC12 cells were then returned to normal culture conditions and the cells were allowed to differentiate for 7 days. After the 7 days phase contrast images of the cells were taken and the cells were then stained with 1 g/L hematoxylin solution to aid the quantification of the neurite outgrowth. The media was removed and the cells were washed twice with 1 x PBS, the cells were then fixed in 100% methanol for 15 min at room temperature. The methanol was removed and the cells were washed twice with dH2O. The hematoxylin stain was then applied to the cells for 20 min at room temperature. The stain was removed from the cells and they were washed twice with dH2O and allowed to dry. The samples can be stored at 4 °C for up to 4 weeks.

2.2.19 Immunoprecipitation of tagged NGF

MDA-MB-231 cells were seeded into 175 cm² flasks at a density of 5 x 10⁴/cm². The cells were allowed to adhere overnight. The culture flasks were then treated with EV, HA_NGF or FLAG_NGF HEK293T conditioned media, expressed as described in Section 2.2.35. The MDA-MB-231 cells were treated with the NGF for 15 min after which the media was promptly removed and the cells washed with 10 ml ice-cold 1 x PBS. The 1 x PBS was removed and the cells were then harvested by scraping. The cells were centrifuged at 150 x g for 5 min at 4 °C and then washed in 1 ml ice-cold 1 x PBS. The cells were lysed at 4 °C for 20 min with rotating using NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 10% glycerol and 10 mM Tris pH 8) containing EDTA-free protease inhibitor cocktail tablet (Invitrogen). The lysate was then centrifuged at 14,000 rpm for 10 min and the supernatant was incubated with 1.5 mg of pre-prepared Dynal beads (Invitrogen). The Dynal beads were prepared as per manufacturer’s guidelines and conjugated overnight at 37 °C with 1 μg/ml of anti-HA or anti-FLAG antibodies. The lysates were incubated with the conjugated beads for 4 h at 4 °C. The beads were collected by magnetic separation, the supernatant was removed and the beads were washed 4 times in lysis buffer. The beads were then resuspended in 40 μl of elution buffer (low pH buffer supplied with the kit) for 5 min at room temperature and collected by magnetic separation. The eluant was removed and subjected to Western blotting (Section 2.2.8).
2.2.20 Nuclear morphology studies

Cells were seeded in a 24 well plate in 0.5 ml complete medium. The cells were allowed to adhere overnight and then treated with proNGF at 100 ng/ml, 250 ng/ml and 500 ng/ml. After the treatment time the medium was removed and the cells were harvested by trypsinisation as described in Section 2.2.11. The cells were then centrifuged at 1500 rpm for 5 min on soft acceleration the supernatant was removed and the pellet was washed in 1 x PBS. The cells were then fixed in 4% formaldehyde for 15 min. After fixation the cells were centrifuged as before and the pellet was washed in PBS. To mount the cells onto slides the cell pellet was resuspended in equal volumes of 1 x PBS and using DAPI in VECTASHIELD (Vector Laboratories Ltd). 5 μl of cell suspension was placed on the slide which was overlaid with a cover slip and the edges were sealed with varnish.

The DAPI stained nuclei were then visualised using the Olympus fluorescence microscope (excitation 360 nm, emission 460 nm). 200 cells were counted and the number of cells with condensed nuclei were quantified and expressed as a percentage of the total number of cells.

2.2.21 MTT assay

Cells were seeded into a 96 well plate at the seeding density described in Table 2.1 and allowed to adhere overnight. For the NGF protection assays in PC12 cells were pre-treated with 100 ng/ml NGF 2 h before treatment with 1.5 μM TG. 48 h after TG treatment the cell viability was determined by adding 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT) at 5 mg/ml in Hank’s balanced salt solution to each well. The cells were then returned to normal culture conditions for 3 h and the reaction was stopped by adding 100 μl of stop solution (20% SDS, 50% dimethylformamide in dH2O) to the wells. Rocking the plate at room temperature overnight redissolved the resulting formazan precipitate. The absorbance was then determined at 550 nm using the Victor x 3 plate reader.
Chapter 2. Materials and Methods

2.2.22 Luciferase assay

To study the effect NGF has on the activation of NF-κB in MDA-MB-231. The cells were stably transduced with an NF-κB promoter reporter construct. This construct contains an NF-κB promoter sequence upstream of the firefly luciferase and GFP genes as described in Section 2.2.7. Treatments that induce NF-κB activation and thus its promoter activity will induce the expression of luciferase and GFP and can be quantified.

MDA-MB-231 NF-κB reporter cells were seeded into a 96 well plate suitable for reading a luciferase assay. The cells were allowed to adhere overnight and treated as desired. The extent of luciferase expressed in the cells was then analysed using the ONE GLO luciferase assay system (Promega) according to manufacturer's guidelines. The luminescence was then read using the Victor x3 plate reader.

2.2.23 RNA extraction and RT-PCR

Poly(A)+ RNA extraction from cells was performed according to the protocol for the Total mammalian RNA extraction kit (Sigma). Reverse transcription was carried out with 2 μg total RNA and oligo(dT) (Invitrogen) using 20 U Superscript II Reverse Transcriptase (Invitrogen). cDNAs for genes of interest were amplified during 33 cycles with the following primers: XIAP 5’-primer (5’-GACAGGCCGTCGGACTC-3’); XIAP 3’-primer (5’-GTGCTCTGACCAGGCACGG); IAP1 5’-primer (5’-TGGCTACTTCAGTGGCTCCT-3’); IAP1 3’-primer (3’-GCAGGCCACTCTATCTCCAG-5’); IAP2 5’-primer (5’-GCCCTCTTAATTCTAGAGCAG-3’); IAP2 3’-primer (5’-ACATCTCAAGCCACCATCAC-3’); GAPDH 5’-primer (5’-ACCACAGTCCATGCCCAC-3’); and GAPDH 3’-primer (5’-TCCACCACCCTGTGCTG-3’). GAPDH was used as an endogenous control.

2.2.24 Transformation of plasmids into E. coli cells

Plasmid DNA was transformed into DH5α competent E. coli cells to amplify and purify plasmid DNA. 50 ng/ml of the plasmid DNA was added to 50 μl of DH5α competent E. coli cells. The plasmid DNA was mixed gently with the E coli cells and
left on ice for 20 min. The cells were then heat shocked at 42°C for 60 sec followed by incubation on ice for 2 min. 450 μl of Luria-Bertani (LB) broth (20% LB broth in dH₂O) was then added to the cells and incubated at 37 °C for 1 h with at shaking at 250 rpm. 100 μl of the cell suspension was then removed and spread onto LB agar (20% LB Agar in dH₂O) containing the appropriate selection antibiotic. The plates were then incubated at 37 °C overnight. A single colony was inoculated into 5 ml of LB broth containing selective antibiotic and incubated at 37 °C overnight with shaking at 250 rpm. The culture was centrifuged at 775 x g at 4 °C for 5 min. The supernatant was discarded into 1% Virkon solution and the plasmid was purified from the *E. coli* cells as outlined in the QIAfilter plasmid purification kit (Qiagen).

### 2.2.25 Site-directed mutagenesis using the megaprimer method

The NGF variants were generated by site directed mutagenesis using the megaprimer method, which requires a two-step PCR reaction (detailed in Fig. 2.1).

The first PCR reaction contained 1 μl of hβNGF cDNA (GeneCopoeia), 1 μM mutagenic primer, 1 μM reverse primer, 5 μM dNTPs, 1U of Phusion ‘Hotstart’ high fidelity DNA polymerase and 1X Phusion buffer. This reaction was placed in the thermocycler to generate the megaprimer. The thermocycler conditions were a 3 min hot-start at 95°C followed by 1 min denaturation at 95°C, 1 min annealing at 68°C, 1 min extension at 72°C and a final extension of 10 min at 72°C. 30 cycles were carried out. 5 μl of the PCR product was then separated by gel electrophoresis with 1% agarose in 1X Tris-acetate-EDTA (TAE) buffer, pH 8 at 70 V. If the PCR product travelled through the gel at its predicted size the PCR reaction was purified using the QIAquick PCR Purification Kit (Qiagen) standard protocol for the use in the second PCR reaction. The concentration of the megaprimer was then determined using the NanoDrop spectrophotometer ND-1000 (260 nm). The second PCR reaction contained 1 μM of megaprimer from the first PCR reaction, 1 μM of forward primer, 5 μM dNTPs, 1U of Phusion ‘Hotstart’ high fidelity DNA polymerase and 1 x Phusion buffer. This reaction was placed in the thermocycler to generate the full-length cDNA sequence. The same thermocycler conditions described above
were used. The PCR products were separated by gel electrophoresis with 1% agarose in 1X TAE buffer at 70 V. The full-length NGF PCR reactions were then purified using the QIAquick PCR Purification Kit (Qiagen) standard protocol. The PCR product was then subject to restriction end digestion for ligation into the pcDNA3.1 plasmid. The primers used can be seen in Table 2.4

![Diagram](A)  

![Diagram](B)

**Figure 2.1. Mutagenesis of NGF cDNA using the megaprimer method**

The megaprimer method requires a two-step PCR reaction. (A) The first PCR reaction contains a forward mutagenic primer (mutant primer) and an outer primer containing the restriction enzyme site (NGF rev primer). This reaction generates the megaprimer which contained the desired mutation. (B) The second PCR reaction uses the product of the first PCR reaction, the megaprimer, and the forward outer primer containing the restriction enzyme site (NGF fwd primer). This reaction generates the full-length cDNA sequence containing the desired mutation.
Table 2.4 List of primers

The list of mutagenic primers used with the mutation shown in red. The list of tagged primers used with the tag sequence highlighted in grey.

<table>
<thead>
<tr>
<th>Mutagenic NGF primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>H4D</td>
<td>5’-TCATCATCCGATCCCATCTTCA-3’</td>
</tr>
<tr>
<td>H4E</td>
<td>5’-CATCATCGAAACCACATCTTCA-3’</td>
</tr>
<tr>
<td>I31R</td>
<td>5’-CCCCCGCAGACACGTAAGGGCAAGAGGGTGTG-3’</td>
</tr>
<tr>
<td>K32R</td>
<td>5’-CCGCCACACGACATCCGTGGCAAGGAGGTGTGG-3’</td>
</tr>
<tr>
<td>G33M</td>
<td>5’-GACATCAAGTAGAAGAGGT-3’</td>
</tr>
<tr>
<td>R69D</td>
<td>5’-GCAGGTGCGATGCATTTGA-3’</td>
</tr>
<tr>
<td>H84Q</td>
<td>5’-CCACGACTCAAACCTTTGTCA-3’</td>
</tr>
<tr>
<td>A98I</td>
<td>5’-GGATGGCAAGCAGGCATTGTCGCGTTGTTTATCCCGATA-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Restriction site primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>NGF (Fwd)</td>
<td>5’-GGATCCATGTCCATGTTGTCTAC-3’</td>
</tr>
<tr>
<td>NGFhis (Rev)</td>
<td>5’-AGCGGCGCTCAATGTTGTGATGGTGATGGTGTTGGGCTCTTCATCTTC-3’</td>
</tr>
<tr>
<td>FLAGNGF (Fwd)</td>
<td>5’-GATTACAAGGATGACGACGATAAGATCATCATCCCATCCCATCCCATCCCATCTTC-3’</td>
</tr>
<tr>
<td>FLAGNGF (Rev)</td>
<td>5’-CTTATCGTCGTCACTCTTGTAAATCCCGCTCTGTGTCCTGTGAATCTTGTTGAA-3’</td>
</tr>
<tr>
<td>HANGF (Fwd)</td>
<td>5’-TACCCATACGACGTCCCAGACTACGTTCATCATCCCATCCCACATCCCA-3’</td>
</tr>
<tr>
<td>HANGF (Rev)</td>
<td>5’-ACCGTAGTCTGGAGGACGTGGTGATGGGTACGCTTGTCTGCCATCC-3’</td>
</tr>
</tbody>
</table>

2.2.26 Fusion PCR

The tagged NGF constructs were generated by fusion PCR. The fusion PCR method requires three PCR reactions. The first PCR reaction contains the 5’ forward NGF primer and the 3’ reverse tagged primer. The second PCR reaction contains the 3’ reverse NGF primer and the 5’ forward tagged primer. These two PCR reaction
products are then combined in the third PCR reaction with the 5’ forward NGF primer and the 3’ reverse NGF primer to generate the full length NGF. The forward and the reverse tagged NGF primers are generated to have overlapping complement cDNA sequences and thus enable the insertion of the tag cDNA sequence. A schematic of the fusion PCR method can be seen in (Fig. 2.2).

The first PCR reaction contained 1 µl of hβNGF cDNA (GeneCopeia), 1 µM tagged forward primer, 1 µM NGF reverse primer, 5 µM dNTPs, 1U of Phusion ‘Hotstart’ high fidelity DNA polymerase and 1X Phusion buffer. The second PCR reaction contained 1 µM tagged reverse primer, 1 µM NGF forward primer, 5 µM dNTPs, 1U of Phusion ‘Hotstart’ high fidelity DNA polymerase and 1X Phusion buffer. These reactions were placed in the thermocycler to generate the two overlapping tagged constructs. The thermocycler conditions were a 3 min hot-start at 95 °C followed by 1 min denaturation at 95 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C and a final extension of 10 min at 72 °C. 30 cycles were carried out. 5 µl of the PCR product was then separated by gel electrophoresis with 1% agarose in 1 x TAE buffer at 70 V. If the PCR product travelled through the gel at its predicted size the PCR reaction purified using the QIAquick PCR Purification Kit (Qiagen) standard protocol.

The third PCR reaction contained purified PCR products of the first and second PCR reaction. 10 µl of the first PCR reaction and 10 µl of the second PCR reaction, 1 µM of NGF forward primer, 1 µM of NGF reverse primer, 5 µM dNTPs, 1U of Phusion ‘Hotstart’ high fidelity DNA polymerase and 1 x Phusion buffer was then added to a tube. This reaction was placed in the thermocycler to generate the full-length cDNA sequence. The same thermocycler conditions described above were used. The PCR products were separated by gel electrophoresis with 1% agarose in 1 x TAE buffer at 70 V. The full-length NGF PCR reactions were then purified using the QIAquick PCR Purification Kit (Qiagen) standard protocol. The PCR product was then subject to restriction end digestion for ligation into the pcDNA3.1 plasmid. The primer sequences can be seen in Table 2.4.
Figure 2.2. Generation of the tagged NGF constructs using fusion PCR

The tagged NGF constructs were generated by fusion PCR. The fusion PCR method requires three PCR reactions in. (A) The NGF cDNA containing the full WT_NGF sequence containing a HIS tag was generated. The first PCR reaction contains the forward NGF primer (NGF fwd primer) and the reverse tagged primer (tag rev primer). (B) The second PCR reaction contains the reverse NGF primer (NGF rev primer) and the forward tagged primer (tag fwd primer). (C) The PCR products from (A) and (B) were combined in the third PCR reaction with the NGF fwd primer and NGF rev to generate the full length NGF containing the desired tag.

2.2.27 Digestion of vector and insert

The full length NGF PCR products obtained using the method described in Section 2.2.27 and 2.2.28 were then subjected to restriction enzyme digestion. The forward primer contained the restriction site BamHI and the reverse primer contained the restriction site NotI. These restriction sites were incorporated into the full-length NGF PCR products to permit insertion of the full length NGF PCR product into the destination vector, pcDNA3.1+ (Invitrogen).
For the restriction enzyme digestion 100 ng of the full-length NGF PCR product (insert) or the pcDNA3.1+ (vector) was placed in an eppendorf tube along with 1 U of BamH1, 1 U of NotI, 1 x BSA and 1 x NEB Buffer 3. The reaction was then incubated at 37°C for 4 h. After the incubation 5 μl of the reaction products was visualised by gel electrophoresis with 1% agarose in 1 x TAE at 70 V.

2.2.28 Ligation of vector and insert

Following digestion of the vector and the insert with complementary restriction enzymes they were incubated together to ligate the insert into the vector. 50 ng of the restriction enzyme digested vector was placed into an eppendorf tube along with 100 ng of insert. To this 1 U of T4 DNA ligase was added with 1 x ligase buffer. The reaction was incubated for 6 h at 16 °C.

2.2.29 Transformation of ligated DNA into competent E-coli cells

The ligated products in Section 2.2.31 were transformed into DH5α competent E-coli cells as described in Section 2.2.26. The transformed cells were spread onto LB agar plates containing selective antibiotic and grown at 37 °C overnight. 4 single colonies were chosen and inoculated into 5 ml of LB broth containing selective antibiotic. The culture was incubated at 37 °C overnight with shaking at 250 rpm and then centrifuged at 775 x g at 4 °C for 5 min. The supernatant was discarded into 1% Virkon solution and the plasmid was purified from the E. coli cells as outlined in the QIAfilter plasmid purification kit (Qiagen). The purified plasmid was then tested for insert incorporation. A sample of the plasmid was subject to restriction enzyme digestion as described in Section 2.2.29, the products of this reaction were visualised by gel electrophoresis with 1% agarose in 1 x TAE at 70 V. Plasmids that contained the insert were retained and sent for sequencing to verify the sequence (LGC Genomics).

2.2.30 Recombinant NGF expression in E. coli cells

The preproNGF construct containing a C terminal His tag was ligated into the pET15b (WT_NGF pET15b) E. coli expression plasmid using the restriction sites
BamHI and NotI. The WT_NGF pET15b plasmid was transformed into competent *E. coli* cells, plated onto agar plates containing the appropriate selection antibiotic and grown overnight, the competent *E.coli* cell lines used are outlined in (Table 2.5). A single colony was then picked and incubated overnight in 2X TY broth (16% Bacto-tryptone, 10% Yeast extract, 5% NaCl in dH2O) containing ampicillin at 37°C with rotating at 200 rpm. The starter culture was then diluted in 1 L 2xTY media containing antibiotic. The 1 L culture was then returned to the incubator and expression of the recombinant NGF was induced using 1 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher) when the O.D. (600 nm) reached 0.6-0.8. The culture was placed back into the incubator at the desired temperature with rotating at 200 rpm for 6 h or overnight. The expression of the recombinant NGF was then determined. 5 ml of the culture was centrifuged at 775 x g at 4 °C for 5 min. The pellet was resuspended in Laemmli buffer to lyse the cells. After denaturation the crude lysate was then applied to an SDS-PAGE gel and the proteins were stained with Coomassie protein stain as outlined in Section 2.2.9 and 2.2.10

Table 2.5. List of chemically *E. coli* cells used

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Strain information</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)pLysS</td>
<td>Invitogen</td>
<td>Chemically competent cells that express T7 polymerase upon IPTG induction. The pLysS plasmid produces T7 lysozyme to reduce basal level expression of the gene of interest</td>
</tr>
<tr>
<td>Rosetta</td>
<td>Invitrogen</td>
<td>Chemically competent cells similar to BL21(DE3) which have an additional mutation in the lac permease gene, enabling adjustable levels of protein expression throughout cell culture. Cells also carry tRNAs for the codons AUA, AGG, AGA, CUA, CCC and GGA under chloramphenicol selection</td>
</tr>
<tr>
<td>BL21-CodonPlus-RIL</td>
<td>Invitrogen</td>
<td>Chemically competent cells similar to BL21(DE3) which also carry tRNAs for AGA, AGG, AUA and CUA</td>
</tr>
</tbody>
</table>
2.2.31 Autoinduction of NGF expression in *E. coli* cells

To increase the yield of recombinant proteins a method called autoinduction is used (Studier, 2005). The WT_NGF pET15b construct was transformed into BL21(DE3)pLysS competent *E. coli* cells and grown overnight on agar plates containing the appropriate selection antibiotic. A single colony was picked and incubated in 2xTY broth containing selection antibiotic at 37°C with rotating at 200 rpm overnight. The starter culture was then diluted in 20 ml 2xTY media containing ampicillin. The culture was returned to the incubator and diluted in 2 L of ZYP50-52 media as described in (Studier, 2005) for autoinduction when it reached an O.D (600 nm) between 0.90-1.00. The culture was then placed at the desired temperature overnight. The expression of the recombinant NGF was then determined. 5 ml of the culture was centrifuged at 775 x g at 4°C for 5 min. The remaining culture was centrifuged and the pellet was retained for lysis and insoluble protein extraction. The 5 ml culture pellet was resuspended in Laemmli buffer to lyse the cells. After denaturation the crude lysate was applied to an SDS-PAGE gel and the proteins were stained with Coomassie protein stain and were subject to Western blotting for NGF as outlined in Section 2.2.9 and 2.2.10.

2.2.32 Solubilisation of NGF from inclusion bodies of *E. coli* cells

The bacterial pellet was resuspended in 10 ml of ice-cold lysis buffer (20 mM sodium phosphate, 300 mM NaCl, 10% glycerol and 20 mM imidazole pH 7). The sample was then placed in ice and sonicated at 35% amplitude for 60 sec with 0.5 sec pulse this was followed by a 1 min rest on ice. This avoids overheating of the sample and denaturation of proteins. The sonication was repeated 4 times. The bacterial lysate was then centrifuged at 20,000 x g for 30 min and the supernatant which contains the soluble proteins was removed. The bacterial pellet was resuspended in solubilisation buffer (8 M Urea, 20 mM HEPES pH 7.4) and incubated at 37°C with shaking to solubilise the proteins in the inclusion bodies of the *E.coli* cells. The crude lysate from Section 2.2.30, the soluble protein fraction and the insoluble proteins form the inclusion bodies of the *E. coli* cells were then
loaded onto an SDS-PAGE gel and the induction of NGF was then accessed by visualising the proteins with Coomassie stain as outlined in Section 2.2.9 and 2.2.10.

2.2.33 Recombinant NGF expression in mammalian cells

Once the wild type and mutant NGF cDNAs were generated and the sequences verified the plasmids were transfected into HEK293T cells using JetPEI transfection reagent as outlined in Section 2.2.6. The cells were seeded into penicillin and streptomycin-free media at a density 10 x 10^6 cells into a T175 cell culture flask and allowed to adhere overnight. The cells were then transfected using JetPEI transfection reagent. 5 h after transfection the media was removed and replaced with serum free media. The cells were then left in culture for 48 h or 72 h and secreted NGF was harvested from the HEK293T-conditioned media. To harvest the NGF, the media was removed under sterile conditions and retained in a 50 ml tube. The cells are then washed with 7 ml serum free medium and retained. The media was then centrifuged at 775 g at 4 °C for 10 min and the supernatant was transferred into a fresh tube. The NGF-containing media was stored at -80 °C until purification. The cells were then harvested for Western blotting as described in Section 2.2.8 and 2.2.9.

2.2.34 Determination of NGF concentration using enzyme linked immunosorbent assay (ELISA)

For the detection and quantification of recombinant NGF an ELISA was developed. 100 μl of a 1/1000 dilution of NGF antibody (SantaCruz) was coated into each well of a 96 well plate. The plate was incubated at room temperature overnight. After the overnight incubation any unbound antibody was removed from the plate and the plate was washed 3 times with 300 μl 1 x PBST and blocked with 1% BSA in 1 x PBS. The plate was then washed in 1 x PBST and 100 μl of NGF-containing sample was applied to the plate. hβNGF (Alomone) was used to generate a standard curve, with concentrations ranging from 10 ng/ml to 0.375 ng/ml. The samples were incubated at room temperature for 2 h, after which any proteins that did not bind to the anti-NGF were washed from the wells using 1 x PBST. The bound NGF was
then detected using a biotin-tagged anti-NGF antibody (R&D systems). 100 µl of 50 ng/ml antibody was applied to the plate and incubated at room temperature for 2 h. The unbound antibody was removed and the plate was washed with 1 x PBST. 100 µl of a 1/400 dilution of Strep-HRP (R&D systems) was then applied to the plate and incubated in the dark at room temperature for 20 min. The plate was washed in 1 x PBST to remove excess HRP. 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was then applied to the plate and incubated in the dark for 20 min, the reaction was stopped by addition of 50 µl of 2 M H₂SO₄ and the absorbance was read at 450 and 550 immediately using the Victor x3 microplate reader.

2.2.35 NGF protein purification

The NGF-containing media was subjected to 0.2 U/ml furin (NEB) digestion for 6 h at 37 °C and then concentrated using Amicon ultra 15 filters with a molecular weight cut off of 10 kDa (Millipore). The media was centrifuged in the Amicon filters at 3000 rpm for 40 min to concentrate it to 1 ml and then diluted in wash buffer (50 mM NaH₂PO₄, 0.5 M NaCl and 20 mM imidazole pH 8) to change the buffer to one that is compatible with the affinity chromatography. The concentrated NGF was then applied to pre-prepared Ni-NAT agarose beads (QIAGEN). To prepare the Ni-NAT agarose beads they were resuspended to get an even cell suspension and 100 µl of slurry was removed and used for each protein purification. The beads were then centrifuged at 1500 rpm for 5 min, the supernatant collected and the beads were washed in 1 ml wash buffer. The beads were resuspended in 50 µl of wash buffer. The NGF was incubated with the beads overnight at 4 °C with rotating. The beads were then centrifuged at 1500 rpm for 5 min the supernatant was then removed and the beads were resuspended in 1 ml of wash buffer and centrifuged again. The beads were washed a total of 4 times. After the wash steps the beads were then resuspended in 250 µl of elution buffer (50 mM NaH₂PO₄, 0.5 M NaCl and 250 mM imidazole pH 8) and rotated for 5 min and then centrifuged. The supernatant was retained. The beads were eluted a total of 3 times. The NGF purification was then quantified by Western blotting and ELISA for NGF.
2.2.36 NGF binding assay

After purification of the recombinant NGF and protein determination by ELISA the binding activity of the NGF was determined. 100 μg/ml of soluble fc linked TrkA and p75NTR receptors (R&D systems product code 175-TK-050 and 367-NR-050/CF, respectively) was coated onto a MaxiSorp 96 well plate (Nunc) covered and then incubated at room temperature overnight. The unbound receptor was removed and the plate was washed 3 times with 300 μl 1 x PBST. The plate was then blocked for 1 h with 300 μl 1% BSA in 1 x PBST. After blocking the buffer was removed was washed 3 times in 1 x PBST. 100 μl of the protein sample was loaded onto the plate. A concentration range of 0.625-15 ng/ml was used. The sample was incubated with the receptors for 2 h. Next any unbound protein was removed and the plate was washed in 1 x PBST. The concentration of NGF bound to the receptor was then quantified using a biotin-tagged anti-NGF antibody (R&D systems). 100 μl of 50 ng/ml biotin-tagged anti-NGF antibody was applied to each well. The plate was incubated at room temperature for 2 h. Next 100 μl of Strep-HRP diluted 1 in 400 (R&D systems) was applied to the plate and incubated in the dark at room temperature for 20 min. The HRP was removed and the plate was washed in 1 x PBST. 100 μl of TMB was then applied to the plate and incubated in the dark for 20 min, the reaction was stopped by addition of 50 μl of 2 M H₂SO₄ and the absorbance read at 450 and 550 immediately using the Victor x3 microplate reader.

2.2.37 Statistical analysis

Statistical analysis was carried out using SPSS software. Values are expressed as means ± SEM of at least 3 separate experiments. All data were analysed using repeated-measures ANOVA followed by LSD multiple comparisons post hoc test unless otherwise stated. Differences were considered statistically significant at p<0.0.
Chapter 3. Results

3 Nerve growth factor-mediated inhibition of apoptosis post-caspase activation is due to removal of active caspase-3 in a lysosome-dependent manner

Mnich K†1,3, Carleton LA†1, Kavanagh ET1, Doyle KM2, Samali A1, Gorman AM*1

Affiliations: 1Apoptosis Research Centre, School of Natural Sciences, National University of Ireland, Galway, Ireland; 2School of Medicine, National University of Ireland, Galway, Ireland; 3Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

†These authors have contributed equally to this work
3.1 Contribution of the Authors

Figure 1. Kinetics of apoptosis-related changes in TG-treated PC12 cells
(a) Carleton LA
(b) Carleton LA
(c) Carleton LA

Figure 2. NGF induces loss of active caspase-3
(a) Kavanagh ET
(b) Kavanagh ET
(c) Carleton LA
(d) Mnich K
(e) Kavanagh ET
(f) Carleton LA

Figure 3. NGF-induced removal of p17 involves TrkA-dependent activation of ERK1/2 and de novo protein synthesis
(a) Mnich K
(b) Mnich K
(c) Mnich K
(d) Mnich K
(e) Mnich K
(f) Mnich K
(g) Mnich K

Figure 4. IAPs do not target cleaved caspsase-3 for ubiquitin-proteasome-mediated degradation in NGF treated cells.
(a) Mnich K
(b) Carleton LA
(c) Mnich K
(d) Mnich K
(e) Carleton LA
(f) Mnich K
(g) Mnich K

Figure 5. NGF targets cleaved caspase-3 for lysosomal degradation
(a) Carleton LA
(b) Carleton LA

Figure 6. NGF-induced removal of active caspase-3 does not involve macroautophagy
(a) Mnich K
(b) MnichK

**Figure 7.** Chaperone-mediated autophagy is not involved in NGF-induced removal of p17

(a) Carleton LA  
(b) Carleton LA  
(c) Carleton LA

**Figure 8.** Schematic showing pre-and post-mitochondrial inhibition of apoptosis by NGF

(a) Carleton LA

**Supplementary Fig. 1.** NGF and EGF induce persistent and transient ERK phosphorylation respectively

(a) Mnich K

**Supplementary Fig. 2.** Lack of effect of lactacystin on p17 removal

(a) Kavanagh ET

**Supplementary Fig. 3.** Effect of zVAD-fmk on p17 removal

(a) Mnich K

**Supplementary Fig. 4.** Schematic showing the amino acid sequences of mouse, rat and human pro-caspase

(a) Carleton LA

**Supplementary Fig. 5.** Knockdown of Hsc70 or Lamp2a impairs CMA

(a) Carleton LA  
(b) Carleton LA  
(c) Carleton LA
3.2 Introduction

Apoptosis is a key process in the elimination of unwanted or damaged cells in multicellular organisms. A detailed understanding of the apoptotic pathway commitment point is crucial for the development of new therapeutic strategies to combat undesirable apoptosis that occurs in certain degenerative diseases and in acute injury such as ischemia (Galluzzi et al., 2009). Central to the apoptotic process is the activation of the caspase family of proteases that play a significant role in cellular disassembly (Samali et al., 1999; Taylor et al., 2008). In healthy cells caspases are maintained as inactive zymogens. One of the main means by which caspases are activated is through mitochondrial outer membrane permeabilization (MOMP)(Tait and Green, 2010). Upon MOMP cytochrome c translocates into the cytosol to initiate formation of the apoptosome complex that leads to proximity-induced auto-activation of initiator caspase-9 (Zou et al., 1999). Once activated caspase-9 cleaves and activates executioner caspases such as caspase-3 and -7 (Slee et al., 1999). Caspase activation is commonly regarded as the commitment point or ‘point of no return’ in the apoptosis process, such that once it occurs the cell is irrevocably destined to die.

Regulation of apoptosis occurs mainly upstream of mitochondrial changes, through the altered expression and/or post-translational modification of pro- and anti-apoptotic Bcl-2 family members (Cory and Adams, 2002; Kutuk and Letai, 2008). However, there also exist mechanisms for the regulation of caspases downstream of mitochondria. It is thought that caspases may be regulated either by inhibitors (e.g., XIAP) or by their rapid degradation. The E3 ubiquitin ligase XIAP can directly interact with active caspases-9, -3 and -7 and inhibit their enzymatic activity, although it remains controversial whether active caspases can be targeted for proteasomal degradation by IAPs (Eckelman et al., 2006; Pop and Salvesen, 2009; Galban and Duckett, 2010).

Nerve growth factor (NGF) is a potent pro-survival factor for sub-populations of neuronal cells, both during development and in post-mitotic neurons (Huang and
Reichardt, 2001). Withdrawal of this trophic factor from NGF-dependent neurons results in apoptosis (Deshmukh and Johnson, 1997). The pro-survival effects of NGF are mediated by the receptor tyrosine kinase TrkA. We have previously shown that NGF activation of phosphatidylinositol 3-kinase (PI3K)/Akt signalling can protect cells upstream of MOMP through regulation of pro-apoptotic Bcl-2 family members (Szegezdi et al., 2008).

Here, we explored whether NGF could also interfere with the apoptotic pathway downstream of MOMP, i.e., post-caspase activation, using the NGF-responsive model cell line PC12. We show for the first time that administration of NGF post-caspase activation can protect cells via ERK-dependent removal of active caspases by targeting them to lysosomes. This provides a novel mechanism by which NGF can protect cells from apoptosis.
3.3 Results

3.3.1 NGF promotes long-term survival of PC12 cells downstream of caspase-3 activation

In order to establish a suitable time for studying NGF treatment post-caspase activation, we initially investigated the kinetics of Thapsigargin (TG)-induced apoptosis in PC12 cells. TG is an endoplasmic reticulum (ER)-stress inducing reagent. Loss of ΔΨ_m was observed 18 h after TG treatment as judged by reduction in TMRE staining (Fig. 3.1A). Activation of caspases-9, -3 and -7 was detectable by 16-18 h, at which time the processed fragments were visible (Fig. 3.1B). This was temporally associated with PARP cleavage (Fig. 3.1B). Annexin V labelling, another indicator of caspase activation, was observed after 20 h of TG treatment (Fig. 3.1C).
PC12 cells were treated with 1 µM TG for the indicated times. **(A)** Loss of mitochondrial transmembrane potential (ΔΨm) was analysed using TMRE stain. Cells were harvested by trypsinisation and treated with 100 nM TMRE for 30 min in the dark. Fluorescence of the cells was measured at 582 nm by flow cytometry. Cells with low TMRE fluorescence were expressed as a percentage of the total cells. *p<0.01 using Bonferroni post hoc test. **(B)** TG-induced activation of caspases was analysed by Western blotting using antibodies to caspase-9, caspase-7, caspase-3 and PARP. Actin was used as a loading control. **(C)** Externalization of PS was analysed using Annexin V-FITC labelling. Cells were harvested by trypsinisation, allowed to recover for 15 min and then resuspended in calcium buffer containing Annexin V-FITC. The fluorescence of the cells was measured at 495 nm by flow cytometry. *p<0.005 using LSD post hoc test.

**Figure 3.1 Kinetics of apoptosis-related changes in TG-treated PC12 cells.**
To determine the effect of NGF addition at times pre- and post-caspase activation PC12 cells were treated with TG for 24 h and with 100 ng/ml NGF 2 h before thapsigargin (TG), or 18 or 23.5 h after TG and DEVDase activity was monitored. Pre-treatment with NGF provided robust inhibition of DEVDase activity (Fig. 3.2A). Unexpectedly, addition of NGF at 18 h after TG also led to a robust reduction in DEVDase activity (Fig. 3.2A). This effect was not observed with NGF addition 23.5 h after TG. Furthermore, addition of NGF at 18 h post-TG treatment caused reduction in the levels of the p17 subunit of active caspase-3 (Fig. 3.2B), indicating that the reduced DEVDase activity was due to removal of active caspases. A time course of TG treatment showed that NGF addition post-caspase activation caused a rapid loss in the active subunits of both executioner caspases-3 and -7 and initiator caspase-9, which was accompanied by a reduction in PARP cleavage (Fig. 3.2C).

In order to determine if this effect of NGF is specific to TG-induced apoptosis, cells were treated with other apoptosis inducers, i.e., etoposide (topoisomerase II inhibitor causing DNA damage), staurosporine (STS; general protein kinase C inhibitor) and tunicamycin (inhibitor of N-linked glycosylation inducing ER stress). Addition of NGF post-caspase activation resulted in reduction of p17 levels in all cases (Fig. 3.2D), demonstrating that the effect of NGF on active caspases is not limited to TG or to ER stress-induced apoptosis.

Next we performed clonogenic assays in order to examine the effect of NGF treatment post-caspase activation on the long-term survival of cells. Cells were treated with TG for 24 h with or without NGF treatment at 2 h before, 18 or 23.5 h after TG treatment. The cells were then re-seeded and the number of single cells that grew into colonies of cells after 14 days in culture was quantified. Treatment with TG alone resulted in a 55% decrease in clonogenic survival compared with untreated cells (Fig. 3.2E). Pre-treatment with NGF conferred complete protection against TG and these cells exhibited the same clonogenic survival as untreated cells. Surprisingly, addition of NGF 18 h after TG treatment also completely restored clonogenic survival, while treatment with NGF 23.5 h after TG treatment did not
provide any protection (Fig. 3.2E). The details on how the clonogenic assay was preformed are outlined in Section 2.2.14.

These data suggest that NGF can rescue cells post-caspase activation. However, an alternative interpretation is that the cells which display long-term survival had not yet initiated the death programme, and did not include those with active caspases. In order to isolate cells with active caspases we used an Annexin V Microbead kit. As phosphatidylserine (PS) exposure is dependent on caspase activity, the isolated population should include only cells containing active caspases. (Martin et al., 1996) Following isolation the PS-exposing cells, cells were divided into two samples that were reseeded for a clonogenic assay, one of which was treated with NGF every 2 days and the other untreated. Prolonged treatment of PC12 cells with NGF causes them to differentiate and extend neurites. (Greene and Tischler, 1976) Therefore, to assess long term survival of the cells, the number of surviving colonies plus cells with neurites was counted after 7 days. The details on how the clonogenic assay was preformed are outlined in Section 2.2.15. The NGF-treated cells exhibited a 1.9-fold increase in survival compared with those not treated with NGF (Fig. 3.2F). These data support the hypothesis that NGF promotes long term survival of cells post-caspase activation.
Figure 3.2. NGF induces loss of active caspase-3, -7 and -9.

(A) PC12 cells were exposed to 1.5 μM TG in the presence or absence of NGF which was added either 2 h before TG, or 18 or 23.5 h after TG. Cells were harvested at 24 h after TG treatment and DEVDase activity was measured. The times shown on the graph indicate the total duration of TG or NGF treatment. *p<0.05 using Bonferroni post hoc test. (B) PC12 cells were exposed to 1.5 μM TG and NGF was added at 18 h. Cells were harvested at 24 h after TG treatment and whole cell lysates were analysed by Western blotting using anti-caspase-3. Actin was used as a loading control. (C) PC12 cells were treated with 1.5 μM TG for 20 h, followed by treatment with NGF for indicated periods of time. Whole cell lysates...
were analysed by Western blotting using antibodies against cleaved caspase-3, caspase-9, caspase-7 and PARP. Actin was used as a loading control. The data are representative of three separate experiments. 

(D) PC12 cells were treated with 40 μM etoposide, 500 nM staurosporine (STS) or 1.5 μM tunicamycin (Tm) for 18 h, followed by treatment with NGF. Cells were harvested 24 h after addition of cytotoxins. Western blotting was performed using an antibody against cleaved caspase-3. Actin was shown as a loading control. Results are representative of three separate experiments. 

(E) PC12 cells were treated with 1.5 μM TG for 24 h. NGF was added 2 h before TG treatment or at 18 or 23.5 h after TG treatment. Cells were then replated and allowed to proliferate into colonies for 14 days. Colonies containing greater than 50 cells were counted. Clonogenic survival was expressed as a % of untreated cells ± SEM from three separate experiments. *p<0.05 using Bonferroni post hoc test. 

(F) PC12 cells were treated with 1 μM TG for 20 h. Cells with externalised PS were labelled by Annexin V MicroBeads and isolated in a magnetic field from non-apoptotic cell population. The isolated population was divided into two, and one half was treated with NGF every second day and the other was left untreated. Colonies containing greater than 10 cells or cells containing neurites were counted to determine the number of surviving cells. The data shown are average of three separate experiments ± range. Values are normalised to the TG-treated surviving cells.

### 3.3.2 Role of TrkA/ERK signalling

To investigate the signalling pathway that is involved in the removal of active caspases as determined by the removal of the p17 subunit of active caspase-3, we used several pharmacological inhibitors. Initially, we used an inhibitor of the TrkA receptor, K252a (Angeles et al., 1998), which revealed that this effect is mediated through the TrkA receptor (Fig. 3.3A).

TrkA can activate PI3-kinase/Akt, MEK/ERK1/2 and phospholipase C signalling pathways (Yao and Cooper, 1995; Xue et al., 2000). To determine which of these pathways are involved in NGF-induced removal of active caspase-3, TG-treated PC12 cells were incubated with LY294002 (PI 3-kinase inhibitor), U73122 (phospholipase C inhibitor) or UO126 (MEK1/2 inhibitor), before NGF addition. Neither PI3-kinase inhibition nor phospholipase C inhibition could block NGF-induced removal of p17 (Fig. 3.3B and C). In contrast, ERK1/2 inhibition partially restored the level of p17 (Fig. 3.3D). Together these data indicate an involvement of ERK1/2 signalling in regulation of active caspase-3 levels by NGF, and a lack of involvement of PI3-K.
The duration of ERK activation is known to regulate various cell fate decisions. For example, sustained phosphorylation by NGF induces PC12 cell differentiation (Greene and Tischler, 1976), while transient ERK phosphorylation by epidermal growth factor (EGF) stimulates proliferation of PC12 cells (Huff et al., 1981). In order to determine whether transient ERK activation could promote removal of active caspase-3 the effect of EGF on active caspases was compared with that of NGF. NGF induced a sustained phosphorylation of ERK1/2 while that induced by EGF was transient (Supplementary Fig. 3.1). In contrast to NGF, transient ERK phosphorylation by EGF did not stimulate the removal of p17 (Fig. 3.3E), suggesting that sustained ERK activation may be required for the removal of active caspase-3. ERK signalling is known to lead to activation of gene transcription (Murphy et al., 2004). It was found that pre-treatment with either actinomycin D or cycloheximide inhibited NGF-induced removal of active caspase-3 (Fig. 3.3F, G), indicating the role of gene transcription and protein translation.
Chapter 3. Results

Figure 3.3. NGF-induced removal of p17 involves TrkA-dependent activation of ERK1/2 and de novo protein synthesis.

PC12 cells were treated with 1.5 μM TG for 19 h, followed by incubation of cells with (A) 100 nM K252a, (B) 40 μM LY294002, (C) 2 μg/ml U73122 or (D) 10 μM U0126 for 1 h before an addition of NGF. Cells were harvested at 20-26 h as indicated and the levels of p17 were analysed by Western blotting using antibody against cleaved caspase-3. Actin was used as a loading control. Images are representative of three separate experiments. (E) PC12 cells were treated with 1.5 μM TG for 20 h followed by treatment with NGF or 150 ng/ml EGF. The cells were harvested at 20-26 h as indicated and the changes in p17 subunit levels was measured by Western blotting for cleaved caspase-3. Actin was used as a loading control. Cells were treated as above either with or without 10 μg/ml actinomycin D (F) or 10 μg/ml cycloheximide (G) for 1 h prior to addition of NGF. Cells were harvested and Western blotting performed as described above. Results shown are all representative of three separate experiments.
Supplementary Figure 3.1. NGF and EGF induce persistent and transient ERK phosphorylation respectively

PC12 cells were treated with 100 ng/ml NGF or 150 ng/ml EGF as indicated and ERK phosphorylation was monitored by Western blotting. The data are representative of two individual experiments.

3.3.3 Active caspase-3 is not targeted for proteasome-dependent degradation

The E3 ligases of the IAP family have previously been implicated in ubiquitination and proteasomal degradation of caspase-3 (Suzuki et al., 2001; Schile et al., 2008; Choi et al., 2009) although this remains controversial (Pop and Salvesen, 2009; Galban and Duckett, 2010). NGF treatment induced an increase in XIAP, cIAP1 and cIAP2 mRNA levels (Fig. 3.4A). However, this induction did not correlate with increased XIAP or cIAP1/2 protein levels (Fig. 3.4B). To further test the role of the IAPs the SMAC mimetic BV6 was used. BV6 can bind to BIR2 and BIR3 domains of XIAP, inhibiting its ability to bind and antagonize caspases (Varfolomeev et al., 2007). BV6 can also bind to cIAP1 and cIAP2 leading to their auto-ubiquitination and proteasomal degradation (Varfolomeev et al., 2007). This can be seen in Fig. 3.4C which confirms that BV6 was active in these cells. Although BV6 slightly increased the levels of the p17 caspase-3 fragment due to TG treatment, it did not block the ability of NGF to reduce p17 levels (Fig. 3.4D), suggesting that IAPs do not mediate NGF-induced removal of active caspase-3.

We next determined whether the ubiquitin-proteasome degradation machinery is involved (Chen et al., 2003). Treatment of PC12 cells with proteasome inhibitors, MG132 or lactacystin, caused an increase in the cellular level of ubiquitinated
proteins confirming proteasome inhibition by these inhibitors (Fig. 3.4E, and Supplementary Fig. 3.2). However, neither proteasome inhibitors were able to abrogate NGF-stimulated removal of p17 (Fig. 3.4F and Supplementary Fig 3.2). In addition, Ub-EI, an inhibitor of ubiquitin-activating enzyme E1,(Yang et al., 2007) was also found to have no effect on NGF-induced removal of p17 (Fig. 3.4G). Together, these data indicate a lack of involvement of the ubiquitin-proteasome degradation machinery in NGF-induced degradation of active caspase-3.
Fig. 3.4. IAPs do not target cleaved caspase-3 for ubiquitin-proteasome-mediated degradation in NGF treated cells.

PC12 cells were treated with NGF for indicated times and changes in the levels of IAPs were analysed. **(A)** Semi-quantitative RT-PCR. GAPDH was used as a house-keeping control. **(B)** Western blotting. Actin was used as a loading control. **(C)** PC12 cells were treated with 5 μM BV6 for indicated times and protein levels of IAP1/2 were analysed using Western blotting. **(D)** PC12 cells were treated with 1.5 μM TG for 14 h followed by addition of 5 μM BV6. At 20 h NGF was added. The cells were harvested for Western blot analysis at 22-26 h as indicated. The p17 subunit levels were analysed using the cleaved caspase-3 antibody. Actin was used as a loading control. Results shown are representative of three distinct experiments. **(E)** PC12 cells were treated with 10 or 20 μM MG132 for indicated times. The cells were then harvested and the total levels of ubiquitinated proteins were analysed using an anti ubiquitin antibody. **(F and G)** PC12 cells were treated with 1.5 μM TG for 19 h followed by incubation with 20 μM MG132 (F) or 10 μM Ub-EI (G) for 1 h before addition of NGF. Cells were harvested at 20-26 h as indicated. The p17 subunit levels were analysed using the cleaved caspase-3 antibody. Actin was used as a loading control. Results shown are representative of three distinct experiments.
Supplementary Fig. 3.2. Lack of effect of lactacystin on cleaved caspase-3 removal

PC12 cells were treated with 1.5 μM for 19.5 h followed by 10 μM lactacystin for 30 min before NGF addition. The total level of ubiquitinated proteins on cleaved caspase-3 levels were then analysed by Western blotting. Data is representative of three individual experiments.

3.3.4 NGF induces lysosomal localization and degradation of p17

Lysozomes are membrane-bound organelles containing acid hydrolases that mediate the degradation of cellular components, including organelles, protein aggregates and specific proteins (Pryor and Luzio, 2009). To examine the role of lysosomes in NGF-induced removal of caspase-3, PC12 cells were treated with chloroquine (a lysosomotropic agent that accumulates within lysosomes and neutralises their acidic interior, thus inhibiting lysosomal enzymes)(Ohkuma and Poole, 1978; Seglen et al., 1979). Chloroquine caused p17 accumulation in cells treated with NGF (Fig. 3.5A). Moreover, chloroquine treatment alone did not induce the cleavage of pro-caspase-3 (Fig. 3.5A). These data suggest a role for lysosomes NGF-stimulated degradation of p17. Next, the lysosomal localization of active caspase-3 was assessed using LysoTracker red to visualize lysosomes in combination with FAM-DEVD-FMK (FLICA; a fluorogenic peptide that binds irreversibly to active caspases-3 and -7 and can thus be used to visualize their localisation). In all treatment conditions, cells displayed punctate red fluorescence consistent with distribution of LysoTracker to lysosomes (Fig. 3.5B). FLICA staining was observed in cells treated with 1 μM TG indicating active caspase-3/7 (Fig. 3.5B). Similarly, TG-
treated cells that were exposed to NGF for 4 h exhibited FLICA staining. However, in contrast to TG treated cells, there was co-localization of FLICA with lysosomes in cells treated with TG and NGF (Fig. 3.5B). Notably, this co-staining was only observed in cells that were treated with both NGF and TG. Taken together, these data suggest that NGF-induced removal of p17 is dependent on lysosomal degradation of active caspases.

![Figure 3.5. NGF targets cleaved caspase-3 for lysosomal degradation](image)

**Figure. 3.5. NGF targets cleaved caspase-3 for lysosomal degradation**

**(A)** PC12 cells were treated with 1.5 μM TG for 19 h followed by treatment with 50 μM chloroquine for 1 h before addition of NGF. Cells were harvested at 20-26 h as indicated. The p17 subunit levels were analysed by Western blot using the cleaved caspase-3 antibody. Actin was used as a loading control. Results shown are representative of three distinct experiments. **(B)** PC12 cells were seeded onto 8 well PLL coated µ-slides (Ibidi) and allowed to adhere overnight. The cells were treated with 1 μM TG for 20 h and then with NGF for a further 3 h. The cells were then stained in normal culture conditions with 1X FAM-DEVD-FMK reagent (green) for 30 min and then with 50 nM Lysotracker red DND99 (red) for a further 30 min. Hoechst 33342 (blue) was used to visualise the nuclei. The cells were maintained in DMEM and visualised immediately using a DeltaVision core system (Applied Precision). Arrows indicate co-staining with Lysotracker and FLICA. Images are representative of three separate experiments.
3.3.5 Macroautophagy does not contribute to the removal of p17

Lysosomes are the terminal degradative compartment of several pathways including autophagic pathways such as macroautophagy (a non-selective form of autophagy whereby cytosolic particles, including protein aggregates, directed for degradation are sequestered in autophagosomes, by which they gain access to lysosomes) (Levine and Kroemer, 2008; Pryor and Luzio, 2009). To assess the contribution of macroautophagy to NGF-induced degradation of p17, we established a stable clone of PC12s with impaired autophagic degradation system due to overexpression of functionally inactive Atg4 (DN-Atg4) (Fujita et al., 2008). Atg4 is a cysteine protease that activates Atg8, which is a key protein in autophagosome formation (Fujita et al., 2008). Neo PC12 cells and DN-Atg4 PC12 cells were treated with chloroquine to compare basal autophagic flux. DN-Atg4 PC12 cells had decreased LC3-II compared with the Neo PC12 cells indicating decreased autophagosome formation in the DN-Atg4 PC12 cells (Fig. 3.6A). Treatment of DN-Atg4 PC12 cells with TG resulted in activation of pro-caspase-3 but to a lesser extent than in parental cells or Neo PC12 cells (Fig. 3.6B). Nevertheless, NGF treatment induced the removal of cleaved caspase-3 (Fig. 3.6B), suggesting that macroautophagy does not play a role in this process.
Figure. 3.6. NGF-induced removal of active caspase-3 does not involve macroautophagy.

PC12 cells were stably transduced with a lentiviral vector encoding functionally inactive Atg4 (DN-Atg4). (A) PC12 cells were treated with 50 μM chloroquine and the lipidation state of LC3 protein was analysed by Western blotting with anti-LC3 antibody. Actin was used as a loading control. (B) Parental PC12 cells, PC12 cells transduced with empty vector (Neo), and those transduced with DN-Atg4 were treated with 1.5 μM TG for 20 h followed by NGF cells. The cells were harvested at 20-26 h as indicated and the levels of the p17 subunit analysed by Western blotting using the cleaved-caspase-3 antibody. Actin was used as a loading control. Images are representative of three independent experiments.

3.3.6 The catalytic activity of caspase-3 is not required for NGF-stimulated removal of p17

It has previously been reported that the catalytic activity of active caspase-3 is required for its rapid turnover in Hela cells and that stabilisation of the active complex with caspase inhibitor abrogated the turnover of active caspase-3 (Tawa et al., 2004). We next investigated whether the catalytic site of active caspase-3 is involved in NGF-stimulated removal of p17, using the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk), to
stabilize the active enzyme complex (Tawa et al., 2004). Inhibition of TG-induced DEVDase activity by zVAD-fmk was initially confirmed (Supplementary Fig. 3.3A). However, treatment of PC12 cells with zVAD-fmk for 1 h prior to NGF addition was unable to prevent NGF-dependent removal of p17 (Supplementary Fig. 3.3B), suggesting that the catalytic activity of caspase-3 is not required for NGF-stimulated removal of p17.

Supplementary Figure 3.3. Effect of zVAD-fmk on p17 removal

(A) DEVDase activity after 24 h treatment with 1.5 μM TG and zVAD-fmk treatment (B) PC12 cells were treated with 1.5 μM and 1 h before NGF addition the cells were treated with 25 μM or 50 μM zVAD-fmk Cleaved caspase-3 levels were then analysed by Western blotting. Data is representative of three individual experiments.

3.3.7 Chaperone-mediated autophagy does not contribute to the degradation of p17

Chaperone-mediated autophagy (CMA) is a form of selective autophagy by which client proteins are targeted to lysosomes for degradation without the requirement for autophagosome formation (Majeski and Dice, 2004). A unique feature of CMA is that client proteins contain a sequence biochemically related to KFERQ (Majeski and Dice, 2004). Examination of the primary sequence of caspase-3 did not reveal any sequences that exactly match the requirements for a CMA client, but it did reveal a KLFIIQ sequence within the p17 subunit (residues 156-161), which lacks only a required acidic residue (Supplementary Fig. 3.4). Since this sequence is next to the active site of the enzyme (Supplementary Fig. 3.4) we hypothesized that the
cleavage site of caspase substrates (DEVD) could provide the acidic residues that are missing from this potential CMA-targeting target sequence. Therefore, we next examined whether CMA played a role in the degradation of p17. We used shRNA lentiviral constructs to stably knockdown essential proteins in the CMA machinery, heat shock cognate protein 70 (Hsc70) and lysosome-associated membrane protein type 2A (LAMP2a) (Supplementary Fig. 3.5A, B) (Kaushik et al., 2011). Impairment of CMA by knockdown of Hsc70 and LAMP2A was confirmed by monitoring the accumulation of α-synuclein, a known CMA substrate (Supplementary Fig. 3.5C) (Cuervo et al., 2004). In contrast, Hsc70 or LAMP2A knockdown did not have an effect on NGF-induced removal of p17, with comparable levels of cleaved caspase-3 reduction in Neo, LAMP2a KD and Hsc70 KD PC12 cells (Fig. 3.7A, B). Furthermore, immunoprecipitation of p17 did not reveal any interaction with Hsc70 in cells treated with NGF (Fig. 3.7C). Together these data suggest that CMA is not involved in NGF-induced degradation of active caspase-3.

Supplementary Figure. 3.4. Schematic showing the amino acid sequence of mouse, rat and human pro-caspase-3

The cleaved caspase-3 subunit is indicated above the amino acid sequence by the white bar. The cleavage sites to generate the p17 subunit are indicated by the arrowheads. The sequence in bold and highlighted in grey is the potential CMA-targeting sequence and the sequence in bold and underlined is the active site cleaved caspase-3.
Supplementary Figure 3.5. Knockdown of Hsc70 or LAMP2a impairs CMA

PC12 cells were stably transduced with Hsc70 shRNA (A), LAMP2a shRNA (B) or empty vector (Neo). Western blot confirmed knock down. (C) Accumulation of endogenous α-synuclein was observed following knockdown. Positive control: transient transfection of α-synuclein.

Figure 3.7. Chaperone-mediated autophagy is not involved in NGF-induced removal of p17

(A, B) PC12 cells stably transduced with shRNA to Hsc70 (A) or LAMP2a (B) were treated with 1 μM TG for 20 h followed by addition of NGF for further 2, 4 and 6 h. The p17 subunit levels were analysed by Western blotting using the cleaved caspase-3 antibody. Actin was
used as a loading control. Results shown are representative of three distinct experiments. (C) PC12 cells were treated with 1 μM TG for 19 h followed by treatment with 25 μM chloroquine (CQ) for 1 h before addition of NGF. The cells were then harvested and the lysate incubated with cleaved caspase-3 antibody conjugated to Dynal Beads overnight. The beads were then applied to a magnet and washed. The protein was then eluted from the column and the lysate and the immunoprecipitated proteins were analysed by Western blotting for p17 (using the cleaved caspase-3 antibody) and Hsc70. The data are representative of three separate experiments.
3.4 Discussion:

It is commonly accepted that the commitment point for apoptosis is caspase activation. In the intrinsic pathway, this occurs downstream of MOMP. In this chapter we describe the ability of the NGF Ras/MAPK pathway, to protect PC12 cells downstream of MOMP and post-caspase activation via a mechanism that involves the removal of active caspases and requires functional lysosomes.

We have previously reported that NGF protects PC12 from TG induced apoptosis. We then tested at what time point could NGF no longer protect the cells. To this end we determined the kinetics of cell death on TG treatment. It was determined that there was a significant activation of the apoptosis pathway 18 h post TG treatment. This was illustrated by a significant increase in cells with low mitochondrial membrane potential, an increase in protein levels of cleaved caspase-3, -7, -9 and detectable levels of caspase substrate processing. Treatment with NGF at this time caused a decrease in caspase activity, as determined by DEVDase assay. NGF treatment also caused a time dependent decrease in the protein levels of the caspase-3, -7 and -9 and increased the long-term survival of the cell as determined by clonogenic assay. This loss in caspase-3 on NGF treatment was found to be apoptotic stimulus independent. Interestingly NGF treatment also caused a reduction in the levels of the caspase substrate, PARP.

All data were obtained using whole populations of cells so one possible explanation for the decrease in cleaved caspases and PARP observed is that NGF treatment protected the portion of cells that had not yet lost their mitochondrial membrane potential (Szegezdi et al., 2008). Therefore, the loss of cleaved caspases and PARP is a result of reduced production of active caspases and degradation of the caspases present because of their half-life (the half life of cleaved caspase-3 and -7 is 8 h and 11 h respectively)(Walsh et al., 2011). This explanation was ruled out based on two pieces of evidence. The first piece of evidence is that disruption of NGF-induced protection upstream of MOMP, by inhibiting the PI3K/Akt pathway (Szegezdi et al., 2008), had no effect on cleaved caspase-3 levels. We show here NGF-stimulated
Ras/MAPK activation caused the loss of cleaved caspase-3. The effect also appeared to be due to the sustained phosphorylation of ERK as EGF, which activates transient activation of ERK, was not able to reduce the levels of cleaved caspases-3. The second piece of evidence to show that NGF can inhibit apoptosis downstream of MOMP is that PS-exposing cells isolated after treatment with TG, survived when treated with NGF. Exposure of PS is a marker for cells which have active caspases (Martin et al., 1996), when this subpopulation were treated with NGF they displayed greater long term survival than those which did not receive NGF. Together these show that NGF can interfere with apoptosis events downstream of MOMP via ERK signalling.

The gene expression profile of PC12 cells is dramatically altered in response to ERK stimuli (Pellegrino and Stork, 2006). NGF treatment mediates sustained ERK phosphorylation to promote PC12 differentiation (Greene and Tischler, 1976). In contrast EGF treatment mediates transient ERK phosphorylation and leads to PC12 cell proliferation (Lazarovici et al., 1997). Our results suggest that it is sustained ERK signalling that stimulates the loss of caspase-3. Activated ERK phosphorylates protein substrates of the cytosol and the nucleus and thus indirectly controls protein transcription (Chen et al., 1992; Lenormand et al., 1993). As inhibition of protein transcription and translation using Actinomycin D and cycloheximide, prevented NGF-mediated reduction of cleaved caspase-3, it suggests that ERK activation of de novo protein synthesis is required for the NGF-induced effect. It has also been shown that sustained ERK signalling, by overexpression of the B-Raf oncogene, protects cells from apoptosis downstream of MOMP by an unidentified pathway (Erhardt et al., 1999). It would be interesting to see if the loss of cleaved caspases is a phenomenon attributed to sustained ERK activation. A good model to test this hypothesis is the mutant PC12 cell line described in which is not capable of sustained ERK activation (Yaka et al., 1998) or the generation of a stable cell line capable of sustaining ERK activation without growth factor activation, as described in (Balmanno and Cook, 1999).
A number of reports demonstrate that caspases are activated in various physiological non-apoptotic processes such as long-term depression in hippocampal neurons (Li et al., 2010), sperm maturation (Arama et al., 2003), axon pruning during development (Cusack et al., 2013) and during the differentiation of certain cell types such as erythroid cells (Boehm et al., 2013) and neural stem cells (Fernando et al., 2005). The mechanisms that restrict these active caspases from causing complete apoptosis during these physiological conditions are poorly understood. The work described here is relevant to these studies as it describes a mechanism of restricting caspase activity thus allowing the cells to survive although the caspases were activated.

One of the most widely studied mechanisms of caspase inhibition are the IAPs such as XIAP which can bind to and prevent activation of caspase-9 and can bind to and inhibit the proteolytic cleavage activity of caspase-3 and -7 (Pop and Salvesen, 2009). Some of the reports demonstrating, physiological non-apoptotic, activation of caspases have been shown require XIAP (Cusack et al., 2013). In this study we investigated the role of the IAPs using two different approaches, analyses of the expression of IAP proteins on treatment with NGF and inhibition of IAPs. NGF treatment caused an increase in the mRNA of XIAP, IAP1 and IAP2 but had no effect on IAP protein levels. BV6 is an IAP inhibitor, it is a comprised of two SMAC-derived peptides connected together by a linker (Varfolomeev et al., 2007). Use of a dimeric SMAC derived peptide is reported to have high binding affinity for XIAP and releases the executioner caspases from their inhibition (Gao et al., 2007). BV6 has also been shown to cause the autoubiquitination and proteosomal degradation of IAP1 and IAP2 (Varfolomeev et al., 2007). Treatment of PC12 cells with BV6, still caused decrease in the levels of cleaved caspase-3 on NGF treatment. However, It is important to note that on treatment with NGF and BV6 there was an increase in the levels of cleaved caspases in the cell, visualised by an increase in cleaved caspase-3, in comparison to cells treated with NGF alone. This could be due to the increase in cleaved caspase-3 that is seen on co-treatment of cells with BV6 and TG (Fig. 3.4D). In addition to their function as direct caspase inhibitors, IAPs have also been
suggested to mediate the proteasomal degradation of active caspases. This is due to the RING domains, which are involved in targeting proteins for proteasomal degradation (Suzuki et al., 2001; Schile et al., 2008; Choi et al., 2009; Deshaies and Joazeiro, 2009). To test whether the IAPs were causing proteasomal degradation of cleaved caspase-3 we inhibited the E1 activating enzyme in the ubiquitin proteasomal pathway with Ub-E1, and the proteasome using MG132 and lactacystine. Treatments with the proteasomal inhibitors caused an accumulation in the levels of ubiquitinated proteins, but did not prevent the NGF-stimulated removal of cleaved caspase-3. This suggests that NGF stimulates an alternative mechanism of degradation of active caspases.

Treatment of cells with the lysosomal inhibitor, chloroquine, blocked NGF-induced removal of active caspase-3, causing its accumulation and on NGF treatment the active caspase-3 was also co-localised to lysosome, suggesting an involvement of lysosomes in the removal of cleaved caspase-3 from the cells. The co-localisation data was obtained using a fluorescent inhibitor of caspases (FLICA). FLICA is a caspase activity probe, it consists of a fluoromethyl ketone (FMK) moiety, with a caspase-3 and 7 recognition sequence (DEVD) attached to a carboxyfluorescein (FAM) reporter (FAM-DEVD-FMK). FLICA reagent is thought to interact with the caspase active site via the DEVD recognition sequence, and then covalently attaches to the caspase through the FMK moiety (Ekert et al., 1999). Therefore it is not a probe for cleaved caspase-3 but rather a reporter for caspase-3 and -7 activity. It is also important to note that FLICA is acting as a covalent inhibitor of the caspases as it has been found that inhibition of the catalytic activity of caspase-3 can stabilise the active caspase-3 subunit preventing its rapid turnover in Hela cells through interaction with its active site (Tawa et al., 2001). To determine if the catalytic activity of cleaved caspase-3 had a role in reducing the levels of cleaved caspase-3, the cells were treated with zVAD-fmk. However, zVAD-fmk did not stabilise cleaved caspase-3. This suggests that not only is the cleaved caspase-3 decreasing independently of its catalytic activity on NGF treatment, but also, that even though FLICA is acting as an inhibitor of apoptosis it is not affecting the NGF-induced
disappearance of cleaved caspase-3 and thus can be used to visualise active caspases-3 in the cell.

Lysosomes are known to be involved in degradation of proteins and protein aggregates via various types of autophagy, namely macroautophagy, CMA and microautophagy (Wirawan et al., 2012). Although lysosomal inhibition by chloroquine blocked NGF-induced removal of active caspase-3, macroautophagy did not appear to play a role in this effect as disruption of autophagosome formation, by overexpression of a DN-ATG4 (Fujita et al., 2008), had no effect on NGF-induced removal of cleaved caspase-3. This is in contrast to a recent report concerning degradation of initiator caspase-8 via macroautophagy (Hou et al., 2010). This paper used Bax-/ Hct116 cells that are TRAIL-resistant despite detection of a significant level of cleaved caspase-8. They investigated the localisation of active caspase-8 during TRAIL-mediated autophagy and after inhibition of autophagy. Their results suggest that the TRAIL-mediated autophagic response continuously sequestered the caspase-8 large subunit and its cleaved fragments in autophagosomes for elimination in lysosomes as treatment with the cathepsin inhibitors, E64D and pepstatin A, which significantly block lysosomal degradation, maintained the expression levels of cleaved caspase-8. However, in our study disruption of autophagosome formation had no effect on the disappearance of cleaved caspase-3 it excludes macroautophagy and forms of selective autophagy such as aggrephagy and chaperone-assisted selective autophagy as the mechanism of cleaved caspase-3 removal, as these require intact autophagosomes, which subsequently fuse with lysosomes to degrade their protein load (Arndt et al., 2010; Lamark and Johansen, 2012).

In contrast to macroautophagy, CMA directly delivers client proteins to the lysosome lumen, and thus there is no requirement for intact autophagosomes. This process is mediated by Hsc70 and LAMP2a (Kaushik and Cuervo, 2012). CMA is a targeted degradation of client proteins. The protein that is distant to be degraded is first recognised by Hsc70 through a KFERQ-like sequence (Dice, 1990). The protein
is then transported to the lysosomes and degraded. Caspase-3 was seen to have a KFERQ-like sequence, which was found within the active site of protein. The criteria to define a KFERQ-like sequence are quite lax, it is however, essential to have a Q/N followed or preceded by, a basic amino acid (K/R), an acidic amino acid (D/E) and a hydrophobic amino acid (F/L/I/V). The sequence KLFIIQ, was suggested to be a putative KFERQ-like sequence in active caspase-3. This sequence is missing the required acidic domain, but it was hypothesized that the caspase-3 substrate recognition sequence (DEVD) would provide the acidic amino acid required. This would also explain the data obtained using the caspase inhibitor zVAD. Inhibition of the caspase-3 with zVAD still caused the levels of cleaved caspsase-3 to decrease on NGF stimulation, in contrast to published data that suggests that the catalytic activity of caspase-3 is required for its degradation (Tawa et al., 2004). zVAD works as a caspase substrate which binds covalently to the active site of the caspase. It therefore maintains the caspase-substrate interaction and the acidic amino acid that would be required for Hsc70 recognition, thus it would be degraded. However, knockdown of the key proteins in CMA, Lamp2a and Hsc70, had no effect on NGF-stimulated degradation of p17. The knockdown was sufficient to decrease basal CMA as determined by an accumulation of α-synuclein (a protein known to be degraded by CMA).

It has been reported that TrkA can stimulate autophagy (Swanson and Watts, 1995; Hansen et al., 2007; Commissso et al., 2013). NGF was found to induce cell death in a glioblastoma cell line, which overexpresses the TrkA. This cell death was characterised as autophagic cell death that was dependent on ERK signalling. It displayed several characteristics of autophagy such as LC3 cleavage, autophagosome formation and large vacuole formation (Hansen et al., 2007). On further analysis of TrkA overexpression induced cell death in medulloblastoma Daoy cells overexpressing TrkA, siRNA knockdown of key molecules in the autophagic machinery (beclin-1, Atg5, LC3 and Atg9) failed to block NGF-induced vacuole formation and cell death (Li et al., 2010). Instead, the authors observed that hyperstimulation of macropinocytosis. Macropinocytosis is a form of bulk fluid
endocytosis to enable pathogen entry into the cell (Swanson and Watts, 1995) but more recently has been shown to be an adaptation of Ras transformed cancer cells (Commissio et al., 2013). The authors illustrate that stimulation with NGF in the TrkA overexpressing Daoy cells increases the uptake of fluorescent probes from the supernatant. However, this increase in cytosolic fluorescence was not differentiated from endocytosis caused by NGF stimulation of TrkA and there may be another mechanism at work, such as microautophagy that is also characterised by vacuole formation (Mijaljica et al., 2011).

Previous results from our lab show that the degradation of cleaved caspase-3 could be due to proteolytic cleavage. A previous PhD student, Edel Kavanagh, showed that NGF treated PC12 lysate mixed with TG treated PC12 lysate caused a decrease in the DEVDase activity in the lysate compared to untreated cells, however there was no degradation of the cleaved caspase-3. This experiment was then repeated without the addition of protease inhibitors it was found that the NGF treated lysate was able to inhibit the DEVDase activity and cause the degradation of caspase-3 compared to untreated cells. This implies the proteases (possible those in the lysosome) have a role in the degradation of the caspases. Miroautophagy is a process that non-specifically engulfs proteins into the lysosome for degradation and could be possible mechanism of caspase-3 degradation in this study. A more extensive study on the localisation of the cleaved caspase products on treatment with NGF would need to be carried out to show that microautophagy has a role in the degradation of cleaved caspase-3. As mentioned earlier the TrkA receptor can be internalised by pincher-mediated Macropinocytosis (Shao et al., 2002; Valdez et al., 2005). On Macropinocytosis of the receptor complex a large phase-bright vacuole is formed with a diameter >0.2 μM, this is called the macropinosome. The macropiosome matures, this is characterised by accumulation of the small GTPase Rab7 and lysosomal glycoprotein A. The macropinosomes then fuse with the lysosomes to complete degradation of the internalised protein (Kerr et al., 2006). This process could present a novel mechanism of caspase-3 degradation where by caspases are taken up by the macropinosomes where they are retained until they
are terminally degraded by the lysosomes.

As over-active Raf-MEK-ERK pathways are often seen in cancer cells (Roberts and Der, 2007; Little et al., 2013) and this work has implications on cancer cell survival however it still remains controversial whether cells can survive following MOMP even if caspases are inhibited. It is generally accepted that cells with damaged mitochondria die in a caspase-independent manner if caspase activity is inhibited (Leist and Jaattela, 2001; Tait and Green, 2008; Galluzzi et al., 2009). However, in sympathetic neurons caspase inhibition (by synthetic caspase inhibitors or XIAP) is reported to prevent cell death or significantly extend the commitment to death in cells exhibiting MOMP (Martinou et al., 1999; Deshmukh et al., 2000; Potts et al., 2003). It was also recently reported that not all mitochondria undergo MOMP at once, an apoptotic state called incomplete MOMP (iMOMP), and that the persistence of intact mitochondria confers long-term resistance to caspase-independent cell death (Tait et al., 2010). NGF protection is not only due to post-MOMP effects and can protect cells from apoptosis, before MOMP, through regulation of PI3K-dependent of BH3-only proteins (Szegezdi et al., 2008). Thus it could be hypothesised, NGF-stimulated removal of active caspases acts as a means of caspase inhibition to allow surviving mitochondria to repopulate the cell and promote long-term survival (Tait et al., 2010)

Given its importance as a key trophic factor during neuronal development for certain neuronal subpopulations and in promoting survival of long-lived post-mitotic neurons, it is perhaps not surprising that NGF stimulates a dual block in the apoptosis pathway that includes careful regulation of caspase activity to prevent inadvertent cell death (Fig. 4.8).
NGF can inhibit apoptosis upstream of MOMP through PI3K-dependent regulation of Bim expression and downstream of caspase activation through ERK-dependent removal of active caspases to the lysosomes.

The finding that NGF/ERK signalling, can protect downstream of MOMP decreasing caspase activation and protein level could have relevance to the development of therapeutics where there is a need to rescue cells that have already initiated death pathways, such as in stroke and spinal cord injury (Galluzzi et al., 2009). The activity of NGF may act to delay the commitment point of apoptosis and thus increase the long-term survival of damaged cells. In fact, NGF has previously been suggested as a possible neuroprotective therapy for Alzheimer’s disease (Tuszynski et al., 2005; Weissmiller and Wu, 2012).
Chapter 4. Results

4 Expression, purification and bioactivity of recombinant NGF and tagged NGF

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4.1 Contribution of authors

Figure 4.1. Schematic and sequencing results of NGF constructs
(A) Carleton LA
(B) Carleton LA and McKeown N

Figure 4.2. The recombinant NGF is expressed and secreted in mammalian HEK293T cells
(A) Carleton LA
(B) Carleton LA
(C) Carleton LA
(D) Carleton LA

Figure 4.3. Biological activity of the tagged NGF through TrkA
(A) Carleton LA
(B) Carleton LA

Figure 4.4. Biological activity of the tagged NGF through TrkA
(A) Carleton LA

Figure 4.5. Immunoprecipitation of FLAG tagged and HA tagged NGF
(A) Carleton LA
(B) Carleton LA

Figure 4.6. Purification of the tagged NGF
(A) Carleton LA
(B) Carleton LA
(C) Carleton LA
(D) Carleton LA

Figure 4.7. Optimisation of the NGF binding assay to p75NTR and TrkA
(A) Carleton LA and Healy E
(B) Carleton LA and Healy E

Figure 4.8. Binding of the tagged NGF to the p75NTR and TrkA
(A) Carleton LA
(B) Carleton LA

Figure 4.9. Schematic of the NGF expression and purification
(A) Carleton LA

Figure 4.10. Schematic of the cysteine bonds within the NGF monomer
(A) Carleton LA

Supplementary Fig. 4.1. Optimisation of the expression of WT NGF
(A) Carleton LA
(B) Carleton LA
Supplementary Fig. 4.2. Immunoprecipitation of FLAG tagged NGF

(A) Carleton LA

Supplementary Fig. 4.3. Optimisation of the NGF Furin digestion

(A) Carleton LA
(B) Carleton LA

Supplementary Fig. 4.4. Coomassie stained gel of the WT_NGF, HA_NGF and FLAG_NGF expression

(A) Carleton LA
(B) Carleton LA
4.2 Introduction

NGF is the founding member of a small family of dimeric secreted growth factors called neurotrophins which have diverse roles in development and in the adult nervous system (Bibel and Barde, 2000). It has been suggested as a possible therapeutic for Alzheimer’s disease (AD) (Lad et al., 2003; Tuszynski et al., 2005; Wahlberg et al., 2012). AD is characterised by a loss in cognitive functions and memory. The basal forebrain cholinergic neurons (BFCN) are particularly sensitive to neuron cell loss in AD, and neuronal loss in this area correlates with cognitive decline (Granholm et al., 2000). The loss of neurons from this area is thought to be due to a decrease in the protein levels of NGF and its receptors (Scott et al., 1995). NGF is currently being used in Phase I and Phase II clinical trial and has shown promising results in decreasing the rate of cognitive decline in AD patients (Tuszynski et al., 2005; Wahlberg et al., 2012).

NGF is synthesised as a 35 kDa pro protein (Lessmann et al., 2003) which is cleaved intracellularly by furin and other proprotein convertases such as PACE-4 and PC2 (Seidah et al., 1996; Pagadala et al., 2006) and extracellularly by matrix metalloprotease 7 (MMP7) and plasmin (Lee et al., 2001) to generate mature secreted ligand comprising two 14 kDa monomers. Both the mature NGF and the proNGF have been recombinantly expressed in eukaryotic mammalian cells (Seidah et al., 1996; Lee et al., 2001; Colangelo et al., 2005), Sf19 insect cells (Fahnestock et al., 2004; Pagadala et al., 2006) and prokaryotic E. coli cells (Rattenholl et al., 2001; Clewes et al., 2008). Much of the knowledge of NGF processing is mammalian cells, insect cells and in prokaryotic E. coli cells

The prodomain of NGF is known to increase protein expression, secretion and folding of the mature form (Suter et al., 1991; Rattenholl et al., 2001; Rattenholl et al., 2001; Lessmann et al., 2003; Kliemannel et al., 2004; Kliemannel et al., 2007) and has functions that are independent of the mature form when the processing is altered by oxidative modifications (Kichev et al., 2009).
In this chapter we describe the development of a protocol for the expression of wild type NGF (WT_NGF) and N-terminally HA and FLAG tagged NGF (HA_NGF and FLAG_NGF). The NGF proteins produced were also engineered to contain a C-terminal His tag to ease purification (Fig. 4.1). The NGF was produced for the use in biological assays, with results that would be therapeutically relevant as recombinant NGF expression is used in the treatment of AD.

The use of recombinant fusion proteins, can facilitate the purification and detection of recombinant proteins The many different proteins, domains, or peptides can be fused with the target protein, calmodulin-binding peptide, cellulose-binding domain, DsbA, c-myc tag, glutathione S-transferase, FLAG tag, HA tag, His tag, maltose-binding protein and Strep tag to name a few (Terpe, 2003). The structure of the N and C-terminal of NGF is quite flexible, so incorporation of tags in these domains should not interfere with the activity of the ligand (McDonald et al., 1991). The His tag was incorporated in the C-terminal domain of NGF for protein purification and two different tags, HA and FLAG, were incorporated into the N-terminal domain of the protein. The FLAG tag is a polypeptide protein tag made up of 8 amino acids DYKDDDDK. It is more hydrophilic than other epitope tags and thus, decreases the probability of denaturing or inactivation to the fused protein. It is also an attractive tag as it is removable due to its trypsinogen motif DDDDK (Terpe, 2003). FLAG peptides are used for protein purification (Einhauer and Jungbauer, 2001) and can also be used to isolate protein complexes (Terpe, 2003). The HA tag is made up of 9 amino acids YPYDVPDYA. It can be used in fusion production because it has the ability to detect, isolate and purify proteins. HA tag and FLAG tag are widely used in many areas especially in immuno-blotting and immuno-fluorescence.

Here we describe, in detail, a technique for the transient expression and purification WT_NGF, HA_NGF and FLAG_NGF in mammalian cells. The WT_NGF, HA_NGF and FLAG_NGF were produced at high concentrations and easily purified with the use of His-trap affinity chromatography and exhibited biological activity.
through the TrkA receptor and binding affinity for TrkA and p75\textsuperscript{NTR}. This method was generated for the transient expression of NGF variants that will be discussed in Chapter 5. The HA\_NGF and FLAG\_NGF were generated as a biochemical tool that could be readily used by the scientific community, specifically for the immunoprecipitation of TrkA and p75\textsuperscript{NTR}. However, further studies will be required to confirm that these constructs are suitable for immunoprecipitation.
4.3 Results

4.3.1 Generation of tagged NGF construct

To optimise the expression of recombinant NGF the WT_NGF construct, depicted in Fig. 4.1A, was ligated into a mammalian expression plasmid pcDNA3.1 and an *Escherichia coli* (*E. coli*) expression plasmid pET15b using the restriction enzymes BamHI and NotI. The prodomain of the NGF was retained in the *E. coli* WT_NGF construct, as it is essential to increase the yield of correctly folded mature NGF (Rattenholl et al., 2001; Rattenholl et al., 2001). The prodomain was also included in the mammalian WT_NGF constructs to ensure the correct folding, post-translational modifications and secretion (Lessmann et al., 2003; Kliemannel et al., 2004; Kliemannel et al., 2007) of the mature form of the NGF from the mammalian cells. Both constructs contained a C-terminal His tag to aid protein purification.

Similarly, the tagged NGF constructs, HA tagged NGF (HA_NGF) and FLAG tagged NGF (FLAG_NGF), were ligated into pcDNA3.1 using the BamHI and NotI restriction enzymes (Fig. 4.1A). The HA or FLAG tag was inserted into the NGF cDNA sequence downstream of the furin cleavage site by fusion PCR. Both the HA_NGF and FLAG_NGF constructs contained a C-terminal His tag.

The constructs were generated and the plasmids were then verified by DNA sequencing. The multiple sequence alignment of the amino acid sequence obtained from the sequencing can be seen in Fig. 4.1B.
Figure 4.1. Schematic and sequencing results of NGF constructs

(A) Schematic of NGF proteins expressed by pcDNA3.1 vector. The cleavage sites are indicated arrowheads. The pre domain is removed during transportation through the Golgi and the prodomain is removed by furin intracellularly before secretion into the media. (B) The amino acid sequence obtained from DNA sequencing of the NGF constructs. The poly-His tag is highlighted in grey, the HA tag is highlighted in yellow and the FLAG tag is highlighted in blue.

4.3.2 Optimisation of an expression system for recombinant NGF

The preferred expression system for protein production is *E. coli* because of its high yield, fast growth, simple fermentation and extensive characterisation (Terpe, 2006). NGF has a complex secondary protein structure and must be refolded from the inclusion bodies of *E. coli* cells (Rattenholl et al., 2001; Rattenholl et al., 2001).
Thus the expression system used to for recombinant NGF production was optimised. *E. coli* or mammalian systems were tested.

The WT_NGF pET15b plasmid was transformed into three different *E. coli* strains, BL21(DE3)pLysS, BL21-CodonPlus-RIL and Rosetta. Bacterial expression of the WT_NGF construct was tested under a number of different conditions indicated in Table 1. After induction of protein expression with Isopropyl β-D-1-thiogalactopyranoside (IPTG) or autoinduction using the method described in (Studier, 2005) and outlined in Section 2.2.31, 5 ml of the growth culture was lysed in 2X sample buffer. The crude bacterial protein lysate, containing both the insoluble and soluble proteins, was analysed for induction of the WT_NGF protein by Coomassie Brilliant Blue staining an SDS-PAGE gel. The result of these experiments are summarised in Table 4.1. Autoinduction of WT_NGF at 25 °C using BL21(DE3)pLysS cells yielded the highest levels of proNGF. The bacterial protein pellet was processed further into soluble and insoluble proteins as described in Section 2.2.34. Samples of the crude lysate, soluble proteins and insoluble proteins from BL21(DE3)pLysS cells, not induced, induced with 1 μM IPTG and auto-induced were loaded onto an SDS-PAGE gel and proteins were visualised by Coomassie Brilliant Blue staining (Supplementary Fig. 1A). The 25 °C autoinduction method of bacterial expression gave the largest induction of NGF protein as seen by an induction of protein at 35 kDa, the size of proNGF, in the insoluble protein fraction in comparison to the uninduced control (Supplementary Fig. 4.1A).
### Table 4.1. Summary of induction methods tested

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Induction method</th>
<th>Induction temp</th>
<th>Induction time</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)pLysS</td>
<td>1 μM IPTG</td>
<td>37 °C</td>
<td>8 h Overnight</td>
<td>Low expression and no visible induction on Coomassie staining of an SDS-PAGE gel</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>8 h Overnight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21-CodonPlus-RIL</td>
<td>1 μM IPTG</td>
<td>37 °C</td>
<td>8 h Overnight</td>
<td>Low expression and no visible induction on Coomassie staining of an SDS-PAGE gel</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>8 h Overnight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosetta</td>
<td>1 μM IPTG</td>
<td>37 °C</td>
<td>8 h Overnight</td>
<td>Low expression and no visible induction on Coomassie staining of an SDS-PAGE gel</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>8 h Overnight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>Auto induction</td>
<td>37 °C</td>
<td>16 h</td>
<td>At 37 °C there was low expression and no visible induction on Coomassie staining of an SDS-PAGE gel. At 25 °C there was induction of a band corresponding to the molecular weight of proNGF, on Coomassie staining of an SDS-PAGE gel</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>16 h</td>
<td></td>
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</tbody>
</table>
Supplementary Fig. 4.1. Optimisation of the expression of WT_NGF in BL21(DE3)pLysS E. coli cells

(A) The WT_NGF pET-15b construct was transformed into BL21(DE3)pLysS chemically competent E. coli cells. Using ampicillin as the selection antibiotic a starter culture was grown and diluted into 2 L of 2X TY broth. The culture was grown until it reached an OD 0.6 (at an absorbance of 600 nm). The bacteria were then induced using 1 μM IPTG or using autoinduction media at 25 °C overnight. A 5 ml fraction of the cells was lysed in 50 μl 2X sample buffer and the remaining culture was separated into soluble and insoluble fractions. The induction of the proNGF was analysed by loading 25 μl of sample and staining a 12 % SDS-PAGE gel with Coomassie. (B) Western blotting using anti-NGF was used to compare the expression of proNGF obtained using BL21(DE3)pLysS E. coli cells autoinduced at 25 °C overnight with the expression of proNGF and mature NGF obtained from transient transfection of HEK293T cells.

The WT_NGF pcDNA3.1 plasmid was transfected into HEK293T using JetPEI transfection reagent. The cells were lysed and the media collected 48 h post transfection. The concentration of WT_NGF expressed in the mammalian cells was compared to the bacterial expression system using Western blotting for NGF.
WT_NGF levels in the crude bacterial lysate and the insoluble protein fraction of 25 °C auto-induced BL21(DE3)pLysS cells were compared with that in the mammalian cell lysate and media (Supplementary Fig. 4.1B). The lysate of the mammalian cells contains a faint band for proNGF and mature NGF is secreted into the media. In the BL21(DE3)pLysS E. coli cells. Western blotting revealed a faint band corresponding to the size of proNGF. The signal for mature NGF is stronger in the media of the HEK293T cells (Supplementary Fig. 4.1B) in comparison to the signal for proNGF in the BL21(DE3)pLysS cells. Based on these results the expression system used to generate recombinant mature NGF was mammalian HEK293T cells.

4.3.3 Optimisation of recombinant NGF expression in mammalian cells

In order to establish optimal expression of the WT_NGF in mammalian cells the WT_NGF construct in pcDNA3.1 plasmid was transfected into HEK293T cells using JetPEI transfection reagent and into CHOK1 cells using Effectene transfection reagent. Effectene transfection reagent was used as it gives high transfection efficiency. HEK293T cells were used as proNGF has been successfully expressed in these cells (Lee et al., 2001) and CHOK1 cells were chosen because the CHO cell lines are a secretory cell line and are often used for high titre protein yields in biotechnology (Wurm, 2004).

At 5 h after transfection of the cells the media was changed to either serum free or serum containing media and harvested 24 h and 48 h after transfection. The concentration of NGF secreted into the media was then quantified using an NGF enzyme linked immunosorbent assay (ELISA). The HEK293T cells secreted higher concentrations of NGF than the CHOK1 cells. The cells produced similar concentrations of NGF in serum free and serum containing conditions, thus serum free conditions were used for subsequent experiments (Fig. 4.2A). This eliminated the extra contaminants contained within the serum in the NGF-containing media. The HEK293T cells were then transiently transfected as before and samples were harvested 24 h, 48 h and 72 h after transfection. NGF secretion into the media was similar 48 h and 72 h after transfection. 48 h post transfection there was a strong
expression of proNGF that decreased at 72 h (Fig. 4.2B). ProNGF is known to be glycosylated and it has been shown to be necessary for the efficient secretion of mature NGF from mammalian cells (Lessmann et al., 2003). The proNGF expressed in HEK293T cells was found to be N-linked glycosylated as determined by PNGase digestion (Fig. 4.2C). Next, the tagged NGF constructs were transfected into HEK293T cells and their secretion was analysed 72 h after transfection. The WT_NGF, HA_NGF and FLAG_NGF were secreted into the media as determined by Western blotting for NGF, His, HA and FLAG (Fig. 4.2D). The HA_NGF had a higher concentration of proNGF secreted in the media. The HA_NGF was secreted into the media as a doublet and can be seen as a doublet in both the HA and NGF Western blots (Fig. 4.2D). The lower band is not evident on the His blot, suggesting that the lower band that is obtained may be NGF without the His Tag. Further optimisation of the expression of NGF increased the typical protein yield from 5 ng/ml (Fig. 4.1) to 250 ng/ml. Conditions tested were seeding density and DNA:lipid ratio of the transfection reagent. The conditions used were transfecting 500,000 cells/ml with 30 μg of DNA. The media was changed 5 h post transfection and collect after 48 h for use in biological activity assays.
Figure 4.2. The recombinant NGF is expressed and secreted in mammalian HEK293T cells

(A) The WT_NGF construct was transfected into HEK293T cells using JetPEI transfection reagent and into CHOK1 cells using Effectene transfection reagent. 5 h after transfection the media was changed to complete or serum free media and samples were harvested after 24 and 48 h. The concentration of NGF was quantified using an NGF ELISA n=1. (B) The WT_NGF construct was transfected into HEK293T, 24, 48 and 72 h after transfection the cells and media were harvested and the concentration of NGF was determined by Western blotting for NGF. (C) 72 h after transfection with WT_NGF construct the media and lysate were harvested and incubated with 1 U of PNGaseF at 37 °C for 4 h. The molecular weight shift of NGF on PNGaseF digestion was analysed by Western blotting for NGF (D) HEK293T cells were transfected with WT_NGF, HA_NGF and FLAG_NGF constructs. The secretion of the tagged NGF constructs into the medium at 48 h was analysed by Western blotting for NGF, His, HA and FLAG. Data are representative of 3 independent experiments unless otherwise stated.
4.3.4 Biological activity of tagged NGF

It has been described previously that NGF (obtained from the mouse submaxillary gland) can protect PC12 cells from thapsigargin (TG)-induced apoptosis via TrkA mediated activation of AKT and reduction in the induction of BimEL (Szegezdi et al., 2008). To test the protection of tagged human β-NGF (hβNGF), PC12 cells were pretreated with hβNGF or conditioned media containing EV, WT_NGF, HA_NGF and FLAG_NGF, diluted to 100 ng/ml. The tagged NGF protected PC12 cells from TG-induced apoptosis to levels similar to that of commercial hβNGF (p<0.05) (Fig. 4.3A). This protection was inhibited on co-treatment with K252a, a TrkA inhibitor (Fig. 4.3A). Long-term treatment of PC12 cells with NGF causes them to differentiate and extend neurites, an effect mediated by sustained ERK phosphorylation (Greene and Tischler, 1976). PC12 cells were treated with hβNGF or conditioned media containing EV, WT_NGF, HA_NGF and FLAG_NGF, diluted to 100 ng/ml. The conditioned media containing the tagged NGF induced neurite outgrowth in PC12 cells to a level similar to that of WT NGF and commercially available hβNGF (Fig. 4.3B). The conditioned media was also applied to PC12 cells for 15 min or 2 h, and the NGF stimulated phosphorylation of ERK and AKT analysed by Western blotting. The WT_NGF and tagged NGF stimulated phosphorylation of ERK and AKT similar to that of commercial NGF and the cells treated with EV containing conditioned media caused no increase in phosphorylation (Fig. 4.4).
Figure 4.3. Biological activity of the tagged NGF through TrkA

The biological activity of the tagged NGF constructs was then analysed. (A) PC12 cells were seeded at a seeding density of 200,000 cells/ml. 24 h after seeding the media was removed and replaced with conditioned media containing 100 ng/ml tagged NGF or media containing 100 ng/ml hβNGF, with or without the addition of 100 ng/ml K252a. 2 h after NGF addition the cells were treated with 1.5 μM TG. The cell viability was analysed 48 h after TG addition, by MTT assay. The results were expressed as a percentage of the untreated cells. The data are representative of 3 independent experiments +/- the Std Dev *p<0.05 **p<0.01. (B) PC12 cells were treated with 100 ng/ml hβNGF, or conditioned media containing EV, WT_NGF, HA_NGF and FLAG_NGF. The cells were allowed to differentiate for 7 days and phase contrast images showing neurite outgrowth were taken. The data are representative of 3 independent experiments.
Figure 4.4. Biological activity of the tagged NGF through TrkA

24 h after seeding the PC12 media was removed and replaced with conditioned media containing 100 ng/ml tagged NGF for indicated times. The NGF-stimulated phosphorylation of ERK or AKT was analysed by Western blotting using an antibody specific to phosphorylated ERK or AKT. Total ERK and AKT was used as a loading control. The data are representative of 3 independent experiments.

4.3.5 Immunoprecipitation of NGF

The tagged NGF constructs were generated for use in biological studies, specifically for the immunoprecipitation of NGF with its receptors p75NTR and TrkA. The ability of the tagged NGF to co-immunoprecipitate with the TrkA and p75NTR receptor was thus analysed. MDA-MB-231 cells, a human cell line which are known to express both TrkA and p75NTR (Descamps et al., 2001), were seeded at a seeding density of 5 x 10⁴ cells/cm². The cells were left to adhere overnight after which the media was removed and replaced with 10 ml of EV, HA_NGF or FLAG_NGF conditioned media from HEK293T cells at a concentration of 250 ng/ml. The cells were treated with the tagged NGF or EV control for 15 min. After the incubation time the media was removed and cells were washed with PBS to remove unbound NGF. The cells were then lysed (PreIP) and NGF was immunoprecipitated using HA or FLAG conjugated magnetic Dynabeads for 4 h after the immunoprecipitation was complete the lysate was removed (PostIP) and the beads were then washed and the proteins bound were eluted (IP). The lysates were then subjected to Western blotting for NGF, p75NTR. The HA_NGF and FLAG_NGF proteins were precipitated successfully as seen by a decrease in the concentration of NGF after the 4 h immunoprecipitation
reaction (PostIP). However, there was no co-immunoprecipitation of p75<sup>NTR</sup> (Fig. 4.5A and 5B). However, on long exposures there was a faint band for p75<sup>NTR</sup> in FLAG IP lane (Supplementary Fig.4. 2). The band is indicated with a star.

Figure 4.5. Immunoprecipitation of FLAG tagged and HA tagged NGF

MDA-MB-231 cells were treated with EV, HA_NGF or FLAG_NGF-containing conditioned media for 15 min. The media was then removed and the cells were washed with 1X PBS. The cells were then scraped and lysed in NP40 lysis buffer. The lysates were then incubated with (A) FLAG antibody or (B) HA antibody conjugated to Dynabeads. The lysates were incubated with the beads for 4 h, washed 4 times in lysis buffer and then eluted in 40 μl elution buffer supplied with the kit. The IP samples were then subjected to Western blot for NGF and for p75<sup>NTR</sup>.
Supplementary Figure 4.2. Immunoprecipitation of FLAG tagged NGF

MDA-MB-231 cells were treated with EV and FLAG_NGF conditioned media for 15 min. The media was then removed and the cells were washed with 1X PBS. The cells were then scraped and lysed in NP40 lysis buffer. The lysates were then incubated with FLAG antibody to Dynabeads. The lysates were incubated with the beads for 4 h, washed 4 times in lysis buffer and then eluted in 40 μl elution buffer supplied with the kit. The IP samples were then subjected to Western blot for p75<sup>NTR</sup> and exposed for 40 min.

4.3.6 Purification of recombinant NGF

For binding studies the tagged NGFs were purified. To permit the purification of the recombinant NGF from the media all constructs were generated to have a C-terminal His tag (Fig. 4.1A). After harvesting the conditioned media the WT_NGF was concentrated using Amicon ultra 15 centrifugal filters. The concentrated WT_NGF was then incubated with 0.25 U/ml of furin for 6 h at 37°C. The incubation time used was 6 h and concentration of furin used (0.25 U/ml) was optimised as seen in (Supplementary Fig. 4.3A). The furin digestion ensured that the proNGF remaining in the protein sample was digested to the mature form. The buffer containing the WT_NGF was then exchanged to wash buffer containing a low concentration of imidazole (20 mM) to allow the NGF to bind to the NiNAT nickel agarose beads. The NGF was incubated with the nickel agarose beads overnight. The beads were then washed and NGF was eluted from the beads using elution buffer that contains a high concentration of imidazole (250 mM). Samples were taken at each step of the purification and subject to Western blotting for NGF (Fig. 4.6A) and His (Fig. 4.6B). During the wash steps the NGF remained on the column and was only present after elution. This can be seen by the immunoblotting for NGF.
(Fig. 4.6A) and His (Fig. 4.6B). However, the His antibody did not give as strong a signal as the NGF antibody.

Using this protocol the EV, WT_NGF, HA_NGF and FLAG_NGF were purified and the amount of NGF in the three-elution steps was determined by Western blotting for NGF (Fig. 4.6C) and ELISA (Fig. 4.6D). There was no detectable levels of NGF in the purified EV. However, after purification the yield of WT_NGF was 950 ng/ml +/- 10 ng/ml, of HA_NGF was 437 ng/ml +/- 48.9 ng/ml and of FLAG_NGF was 426 ng/ml +/- 49.8%. The HA_NGF eluant contained a higher molecular weight band on the Western blot corresponding to a partial cleavage of proNGF protein as this 25 kDa band appears in the media of the HEK293T cells when incubated with a furin Inhibitor (Supplementary Fig. 4.3B). The total protein in the lysates, the media and in the three-elution steps of the EV, WT_NGF and, HA_NGF and FLAG_NGF were visualised by Coomassie staining. The concentration of contaminant proteins decreased during the purification procedure. However, the concentration of NGF was too low to be visualised by Coomassie staining (Supplementary Fig. 4.4A and 4.4B).
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Figure 4.6. Purification of the tagged NGF

The conditioned media from HEK293T cells transfected with EV, WT_NGF, FLAG_NGF and HA_NGF were incubated with 0.25 U/ml furin enzyme for 6 h at 37°C. The media was concentrated and buffer exchanged before application to the His trap affinity chromatography beads. After overnight incubation the beads were washed 5 times with 1 ml of buffer containing 20 mM imidazole and then eluted with 3 times with 250 μl buffer containing 250 mM imidazole. Samples were taken after each step and the efficiency of the NGF purification was analysed by Western blotting for using an antibody for (A) NGF and (B) His. The data are representative of 3 independent experiments. The label C, indicates the concentrated NGF, FD, indicates the NGF after furin digestion and UB indicates the NGF that is not bind to the column. (C) The WT_NGF, HA_NGF and FLAG_NGF constructs were then concentrated and purified in the same way. 10 μl of each elution step was loaded onto a gel and the NGF eluted form the beads was analysed by Western blotting. (D) Elution 1 and elution 2 of WT_NGF, HA_NGF and Elution 2 and 3 of FLAG_NGF were combined and the concentration of the NGF was determined by ELISA for NGF. The data are average of three independent experiments +/- the Std Dev.
Supplementary Fig. 4.3. Optimisation of furin digestion of NGF

HEK293T cells were transfected with WT_NGF plasmid using JetPEI transfection reagent. (A) 5 h after transfection the media was changed to serum free media. 48 h post transfection the media was harvested and was incubated with increasing concentration of furin enzyme at 37 °C for 6 h from 1 U/5 ml. The molecular weight of NGF was then determined by Western blotting for NGF. (B) 5 h after transfection the media was changed to serum free media +/- 1 μM furin inhibitor II. 48 h post transfection the media was collected. 5 ml of media was then incubated with 0.25 U/ml of furin enzyme at 37 °C for 6 h and the processing of NGF to the mature form was determined by Western blotting for NGF.
Supplementary Fig. 4.4. Coomassie stained gel of the WT_NGF, HA_NGF and FLAG_NGF expression.

(A) HEK293T cells were transfected with EV, WT_NGF, HA_NGF and FLAG_NGF constructs using JetPEI transfection reagent. 5 h after transfection the media on each transfection was changed to serum free media. 48 h post transfection the media was collected and the cells were lysed. The amount of NGF expressed in the cells was then analysed by Coomassie Brilliant blue staining an SDS-PAGE gel. (B) The conditioned media from the HEK293T cells was then purified by His trap affinity chromatography. The NGF was eluted from the column with three 250 μl steps containing 250 mM imidazole. These elutions were loaded onto an SDS-PAGE gel and the contaminants in the elution were visualised by staining all proteins with Coomassie Brilliant blue.

4.3.7 Binding activity of tagged NGF to TrkA and p75NTR

The binding affinities of the tagged NGF constructs for the p75NTR and TrkA receptors were then analysed. The binding assay developed involved the immobilisation of soluble TrkA or p75NTR receptors to MaxiSorp 96-well plate. The plates were coated with 10 ng of soluble receptor overnight, the unbound receptor was then removed and the plate was washed with 3 times with 1X PBST. The plate
was blocked and washed with 1X PBST before incubation with the indicated concentrations of commercially available hβNGF with or without pre-incubation with a 1/100 dilution of neutralising antibody (R and D systems Cat No. MAB256), which binds to NGF and disrupting the receptor-ligand interaction. The NGF was incubated with the immobilised TrkA or p75NTR for 2 h, the unbound NGF was removed and the plate was washed. The amount of NGF bound to the receptor was quantified using the NGF ELISA (Fig. 4.7A and 7B). The binding of commercial hβNGF to TrkA or p75NTR increased in a dose-dependent manner. The absorbance at 15 ng/ml NGF was set to 100% and all other concentrations were compared to this. Addition of a neutralising antibody decreased the percent binding of NGF to both TrkA and p75NTR. The percent NGF bound to the plate when no receptor was immobilised onto the well was also determined, this represents the non-specific binding of NGF to the MaxiSorp.

Purified EV, WT_NGF, HA_NGF and FLAG_NGF were applied to the developed assay and the percent binding of these NGFs to TrkA and p75NTR was compared with commercial hβNGF. The EV gave no signal. The percent binding of WT_NGF, HA_NGF and FLAG_NGF to TrkA was similar to that of the hβNGF (Fig. 4.8A). The percent binding of WT_NGF to p75NTR was similar to that of the hβNGF, however both HA and FLAG-tagged NGF bound with higher affinity to the p75NTR receptor but this was found not to be statistically significant (Fig. 4.8B)
Figure 4.7. Optimisation of NGF binding assays to p75<sup>NTR</sup> and TrkA

10 ng of soluble (A) TrkA and (B) p75<sup>NTR</sup> were immobilised onto each well of a 96 well plate overnight at room temperature. The commercial NGF was then incubated with the receptor for 2 h with or without pre-incubation with NGF neutralising antibody. The amount of NGF bound to the receptor was then quantified using an NGF ELISA. The graphs (A) TrkA and (B) p75<sup>NTR</sup> show the percent binding of the hβNGF with the reading at 15 ng/ml set to 100%. The data are the mean of two independent experiments.
10 ng of soluble (A) TrkA and (B) p75<sup>NTR</sup> were immobilised onto each well of a 96 well plate overnight at room temperature the purified WT_NGF, HA_NGF and FLAG_NGF were then incubated with the receptor using the indicated concentrations. The nonspecific binding of the NGF to the plate was then determined by incubating the WT_NGF, HA_NGF and FLAG_NGF on the plate without the receptors. The amount of NGF bound to the receptor was then quantified using an NGF ELISA. The graph shows the percent binding values of the hβNGF, EV, WT_NGF, HA_NGF and FLAG_NGF binding to (A) TrkA and (B) p75<sup>NTR</sup> with 15 ng/ml of hβNGF set to 100% bound. The values presented are the average of 3 independent experiments +/- the Std Dev, with the non-specific binding subtracted.
4.4 Discussion

In this chapter we describe a method for the expression and purification of WT_NGF, FLAG_NGF and HA_NGF that display biological activity similar to that of commercially available hβNGF. The tagged NGF proteins bind to the TrkA and p75NTR and can be used for immunoprecipitation experiments. The method developed for the expression of NGF and the tagged constructs is summarised in Fig. 4.9.

Figure 4.9. Schematic of the NGF expression and purification

A schematic summarising the NGF expression and purification method developed. In brief, the EV, WT_NGF, HA_NGF and FLAG_NGF constructs were transfected into HEK293T cells using JetPEI transfection reagent. 5 h post-transfection the media was removed and replaced with serum free media. The cells were placed into normal culture conditions for 72 h after which the media is removed and NGF levels quantified and diluted for the use in biological activity assays. For further biochemical studies the NGF is subject to furin digestion at 37 °C for 6 h. The media is then concentrated using Amicon Ultra 15 centrifugal filters. Once the media is concentrated to 1 ml the media is diluted in wash buffer and buffer exchanged. The NGF is then incubated with NiNAT nickel agarose beads at 4 °C overnight with rotating. The beads are then centrifuged at 300 rcf for 5 min and the supernatant is removed. The beads are washed 5 times in this way. The NGF is then eluted from the beads and can be quantified using an NGF ELISA.
NGF expression was first tested in E. coli cells, as they are the preferred method of recombinant expression due to the high titres that can be obtained (Terpe, 2006). The active mature NGF ligand has a complex secondary structure consisting of two monomers each containing two beta sheets held together by a 3 disulfide bridges called a cysteine knot, that is essential for the proteins structure: Cys58-108 and Cys63-110 form a loop through which the third disulfide bond, Cys15-80, passes through (Fig. 4.10) (McDonald et al., 1991). When disulfide bond rich proteins are expressed in E. coli cells they usually build up as insoluble deposits in the inclusion bodies of the bacterial cells, which can be toxic and hinder growth and protein production (Studier, 2005). However, proNGF has been successfully expressed and refolded from the inclusion bodies of BL21(DE3) E. coli cells (Rattenholl et al., 2001; Rattenholl et al., 2001). On expression under similar conditions that were outlined in Rattenholl et al 2001a, induction with 3 nM IPTG at 37 oC for 4 h, we found that the yield of the proNGF was very low with no visible induction on Coomassie Brilliant Blue stained SDS-PAGE gels. Lowering the induction temperature and increasing the length of protein expression usually reduces the toxicity of the insoluble protein to the E. coli cells and therefore increases the yield of protein obtained. However, this failed to have an effect on WT_NGF expression. A number of different mechanisms were used to increase the yield of the protein obtained which will be detailed below.

**Figure 4.10. Schematic indicating the cysteine bridges within NGF monomer**

*E. coli* cells maintain a reduced environment that hinders the formation of disulphide bridge. The glutathione- and thioredoxin-dependent reduction systems are responsible for maintaining the reduced environment of the *E. coli* cells (Carmel-Harel and Storz, 2000). A strain called Origami has been generated that
contains a mutation in the thioredoxin (trxB) and glutaredoxin (gor) genes, making it a more favourable environment for disulphide bridge formation. The disadvantage of using these cells is that the exact formation of these bridges i.e., in NGF, Cys58-108, Cys63-110 and Cys15-80, is not directed by chaperones and thus could generate soluble but inactive mutant forms of the protein (Messens and Collet, 2006). Our collaborators in the centre for genomic research (CRG) Barcelona tested the expression of the WT_NGF construct in Origami(DE3) competent cells and saw no increase the yield of soluble or insoluble protein obtained. 3 additional bacterial strains were used to enhance the protein expression in E. coli cells, BL21(DE3)-pLysS, BL21-CodonPlus-RIL, Rosetta. The difference between these cell lines is outlined in Section 2.2.32. These different E. coli stains were chosen because the additional transfer RNAs in the stain could increase the level of protein expression as it increases the amino acids transcribed by the bacterial cell. Using these bacterial cells caused no increase in the expression of NGF as visualised by Coomassie Brilliant Blue stained SDS-PAGE gels.

The induction of the target protein by specific nutrient media by a method called auto-induction has also been successful in increasing protein yields (Studier, 2005). Using the auto-induction technique at 25 °C yielded the highest induction of WT_NGF protein, as visualised Coomassie Brilliant Blue stained SDS-PAGE gels. Auto-induction is a technique developed for high-level protein expression of proteins under the control of the T7 promoter, without the need to monitor cell growth. It has demonstrated 2-fold higher protein yield and twice the cell density compared to IPTG induction (Grabski A, 2005; Studier, 2005; Wang et al., 2007). The media was developed based on the observation that IPTG inducible proteins were expressed non-specifically due to low levels of lactose and that glucose prevented lactose induction. Regulation of the glucose and lactose levels in the growth media can sustain the ‘non-specific’ lactose induced expression, thus there is no requirement for IPTG addition (Studier, 2005).
However, there are additional mechanisms to increase the solubility and yield of recombinant proteins rich in disulphide bridges in *E. coli* cells that were not tested here. Such as overexpression of *E. coli* chaperone proteins in BL21(DE3) cells. Such chaperones are the disulfide bond metabolizing proteins (Dsb) such as DsbA, a thiol oxidant or DsbC, a disulphide isomerase. There are also chaperone-competent cells offered by the company Takara. These BL21(DE3) *E. coli* cells are transformed with 5 plasmids containing chaperone proteins, similar to that of the mammalian heat shock protein family, that aid the protein folding and intracellular bonds of the proteins ternary structure (Beckwith, 2007).

Mammalian expression of NGF has previously been used for the expression of recombinant NGF (Lee et al., 2001; Colangelo et al., 2005; Pagadala et al., 2006). Mammalian cells are the dominant system for the production of recombinant proteins for the use in clinical applications. This is because of their capacity for proper protein folding, assembly and post-translational modifications (Wurm, 2004). The main disadvantages of mammalian expression is the low yield of proteins produced and the cost, however this is becoming less problematic with the improvements of cell line productivity

Using a method adapted from (Colangelo et al., 2005) where they describe the expression of a mature human NGF in Hela cells and from (Pagadala et al., 2006) where they express a triple mutant proNGF. The mutations were within the prodomain of NGF at amino acid K46, K47, K77, R78, K119 and R120 and inhibited the native intracellular proteolytic cleavage to the mature form which was observed on expression of recombinant human proNGF in (Lee et al., 2001). Using a combination these expression system as a guideline we developed a method of expressing WT_NGF. Using this technique yielded active mature NGF, which was secreted into the media of the HEK293T cells. The cells were transfected with proNGF, as the prodomain is known to increase protein expression, secretion and folding of the mature form (Suter et al., 1991; Rattenholl et al., 2001; Rattenholl et al., 2001; Lessmann et al., 2003; Kliemannel et al., 2004; Kliemannel et al., 2007).
We found here that on transfection of proNGF constructs into HEK293T cells the proNGF was expressed in the lysate and the mature NGF was secreted into the media. Concentration of the media using the Amicon Ultra centrifugal filters increased the detection of the partially processed precursor forms of the NGF, as furin protease digestion removed the higher molecular weight proteins.

To determine which expression system would be used for future experiments the concentration of proNGF produced in the E. coli cells was then compared to the concentration of mature NGF secreted into the media of HEK293T cells. The mammalian expression system produced higher concentrations of mature NGF than the E. coli cells as determined by Western blotting for NGF. Another attractive benefit of using the mammalian expression system is that NGF is secreted in its mature folded form and thus does not require the refolding step necessary when using bacterial/prokaryotic expression systems. This eliminates the problem of lot variation in the protein productions. It was also reported that only 35% of the total protein isolated from the inclusion bodies of E. coli cells is refolded into its active form reducing the concentration of bioactive NGF further (Rattenholl et al., 2001). Thus, for all further studies we chose to express the NGF in mammalian cells as it was produced in its active native form and underwent all the post-translation modifications required for correct folding and secretion of the protein.

CHO cells are the most frequently used cell line for the production of recombinant proteins in pharmaceutical companies (Wurm, 2004). To achieve the optimal expression and secretion of WT_ NGF and tagged NGF proteins CHOK1 secretion of WT_NGF was compared to that which was secreted from HEK293T cells. This was tested in both serum free and serum containing conditions as most large scale cultures are undertaken in serum free conditions due to its cost and it eliminates contaminant proteins in the media (Wurm, 2004). HEK293T cells secreted higher amounts of mature NGF than CHOK1 cells and protein production was unaltered in serum free conditions. Thus HEK293T cells were used for all further expression experiments. Serum free conditions yielded concentration similar to the of serum
containing, serum free conditions were chosen for all experiments as it decreased the concentration of protein contaminants in the conditioned media. However, it is important to note that the cell viability decreased after 72 h in serum free conditions, so longer expression times or scale-up productions would require serum supplements. The secretion of WT_NGF was optimised from 5 ng/ml to 270 ng/ml +/- 11 ng/ml, by increasing the seeding density to 0.5 x 10^6 cells/ml in a 175 cm^2 tissue culture flask and by harvesting the recombinant protein 72 h post transfection. This method yielded concentrations below what has previously been published in Colangelo et al., 2005 where in similar culture conditions before scaling up they obtained a 433 ng/ml +/- 36 ng/ml. This is likely due to the transient transfection of the WT_NGF plasmid into HEK293T cells. In Colangelo et al.,2005, the Hela cells were stably transfected with the NGF plasmid and the clone that secreted the highest concentration of NGF was chosen.

On recombinant expression of the WT_NGF there was an increase in the secretion of the proNGF protein and intermediate processed forms. This has also been reported in Lee et al., 2001. These proprotein intermediates were completely converted to the desired mature form on furin digestion. However, on expression of the tagged NGF proteins there was a marked increase in the secretion of the proproteins, particularly on HA_NGF expression. This is likely due to the incorporation of the highly aromatic, hydrophobic, HA tag (amino acid sequence YPYDVPDYA) just upstream of the furin cleavage site. The incorporation of this peptide into the sequence decreased the activity of the protease, as it was still present after incubation with recombinant furin enzyme. However, incorporating the charged, hydrophilic, FLAG tag (amino acid sequence DYKDDDDK) did not disrupt the processing to mature NGF. The likely reason for this is the hydrophilic nature of the FLAG tag, the HA and FLAG tag were incorporated into the N-terminal domain of the NGF which is known to have little to no protein secondary structure and thus the amino acids in this region are exposed to the surrounding solute. This is an unfavourable environment for the hydrophobic HA tag and likely causes a change in the conformation of the N-terminal domain of mature NGF to bury the
tag in the protein structure, thus concealing the furin cleavage site. To combat this problem another protease could be used to remove the prodomain, plasmin, which has been reported to cleave proNGF extracellularly (Lee et al., 2001). However, further structural analysis of the HA_NGF would be required to ensure it maintains its native structure.

Detection of recombinant WT_NGF, HA_NGF and FLAG_NGF with the NGF antibody also generated a doublet band at the predicted size for NGF 14 kDa. This is particularly evident in the HA_NGF protein sample. This double band was present on detection with HA and NGF but not present on detection with the His antibody, suggesting that mature NGF has been expressed, without the His tag, and not a higher molecular weight form of NGF as it is also present after furin digestion. Surprisingly the doublet is present after elution from the His affinity chromatography column. This could be due to the fact that the active NGF is a dimer and consists of two NGF monomers. It is likely that an NGF dimer could contain two different monomers, one that is His tagged and one that is not, thus this would allow the purification of NGF monomer not containing the His tag from the conditioned media of the HEK293T cells.

The WT_NGF and tagged constructs were generated for use in biological assays, the activity of the NGF in comparison to commercially available NGF was analysed. For all biological assays the cells were treated with conditioned media of the HEK293T cells containing the serum requirements for the cell. PC12 cells are a model organism for NGF activity as they express both the TrkA and p75NTR receptor, they have well characterised activities in response to NGF such as neurite outgrowth (Greene and Tischler, 1976), protection from ER stress-induced apoptosis (Szegezdi et al., 2008) and activation of Ras/MAPK and PI3K/AKT signalling (Bibel and Barde, 2000; Huang and Reichardt, 2003). Stimulation with WT_NGF, HA_NGF and FLAG_NGF induced TrkA signalling indistinguishable to that of commercially available hβNGF. The conditioned media containing WT_NGF, HA_NGF and FLAG_NGF was applied to PC12 cells and protected them from TG-induced
apoptosis, caused extensive neurite outgrowth and robust phosphorylation of ERK and AKT. The NGF variants were then purified. The WT_NGF, HA_NGF and FLAG_NGF were all designed to contained a C-terminal His tag, after digestion of the NGF with furin, the NGF was apple to the His trap affinity chromatography. After purification the concentration and purity of the NGF increased. This pure NGF protein sample was then suitable for the use in binding studies.

The binding of purified WT_NGF and tagged NGF to p75\textsuperscript{NTR} showed that the tagged construct, particularly the HA-NGF, bound with higher affinity than the WT_NGF and commercial hβNGF. This increase in binding affinity, was not evident in the biological activity assays used which were focused on NGF/TrkA induced signalling. The increase in binding affinity could be due to the increase levels of proNGF in the protein sample. The proNGF ligand is known to bind to p75\textsuperscript{NTR}, with a $K_d$ of $\sim 0.2$ nM (Lee et al., 2001; Beattie et al., 2002) when it’s co-receptor sortilin is expressed (Nykjaer et al., 2004). The NGF ligand is known to bind with to p75\textsuperscript{NTR}, with a $K_d$ of $\sim 1$ nM (Mahadeo et al., 1994; Esposito et al., 2001; Lee et al., 2001). The development of a biological assay for NGF/p75\textsuperscript{NTR} and proNGF/p75\textsuperscript{NTR} would ascertain if the altered binding affinity at p75\textsuperscript{NTR} was due to the increased expression of proNGF or because of incorporation of the tag. The development of a p75\textsuperscript{NTR} assay will be described in more detail in Chapter 5.

The tagged NGF was developed for co-IP experiments of NGF with TrkA or p75\textsuperscript{NTR}. To develop our understanding of how NGF interacts with the p75\textsuperscript{NTR} and determine the pro-survival and pro-death complexes initiated on NGF treatment. The co-IP was tested in MDA-MB-231 cells are they are known to express both Trk and p75\textsuperscript{NTR}. On treatment of MDA-MB-231 cells with HA_NGF and FLAG_NGF, the HA and FLAG conjugated beads were able to pull down the NGF. This suggests that the tagged NGF was capable of binding to and interacting with the cells and was not washed off. The IP reaction was robust as there was depletion in the concentration present in the cell lysate after the IP reaction. However there was no co-IP with p75\textsuperscript{NTR} and only a faint band was detected on long exposures. To have the optimal
conditions for NGF and p75_{NTR} co-IP an alternative cell line should be used for future experiments, HEK293T cells that overexpress p75_{NTR} (a kind gift of Prof Bruce Carter). IP of p75_{NTR} has been described as having an optimal time of 10 min so a shortening the incubation time with NGF may also result in co-IP of p75_{NTR}.

In summary, we have developed a mechanism for the expression of biologically active mature NGF in mammalian cells, adapted from the expression system for proNGF (Lee et al., 2001; Colangelo et al., 2005; Pagadala et al., 2006) and NGF in mammalian cells. We have also generated tagged NGF constructs, suitable for IP reactions, which show biological activity and binding affinity similar to that of the WT_NGF and commercially available hβNGF. We have furthermore developed an NGF construct that can be adapted to E coli expression. Although we were unable to express large concentrations of NGF using E coli cells other groups have had success, and we have developed an NGF construct that allows expression of mature NGF with a N-terminal HA or FLAG tag while still maintaining the C-terminal inexpensive and efficient mechanism of protein purification, His trap affinity chromatography. It is important to have the prodomain of NGF included (which is included in the construct designed in this thesis) as this helps the refolding of the mature protein.
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5 Generation of NGF variants predicted to have altered receptor binding

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(a) Carleton LA
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5.2 Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that is characterised by a loss in cognitive functions and memory. The number of AD patients was estimated at 36 million in 2010, due to the aging population this number is predicted to triple by 2050. This is set to put a huge financial burden on health care systems worldwide with the estimated cost for treatment in 2010 of $604 billion (Huang and Mucke, 2012). AD currently has no effective treatment so the need for a therapeutic strategy to delay the progression and cognitive decline of AD is required.

AD pathology is characterized by the loss of neurons and the presence of extracellular deposits of fibrillar amyloid-β (Aβ) peptides (amyloid plaques). One of the most consistent areas of neuronal loss and dysfunction in AD occurs in the basal forebrain cholinergic neurons (BFCN). Nerve growth factor (NGF) and its receptors tropomyosin kinase A (TrkA) and 75 kDa neurotrophin receptor (p75NTR) have been implicated in sensitising this area to neuronal cell loss in AD. It has been shown by the BFCN have lower NGF, TrkA and p75NTR (Scott et al., 1995). The reduction in NGF is thought to be due to disruption in the retrograde transport of NGF and thus to sensitise this area to neuronal cell loss (Lad et al., 2003; Salehi et al., 2004) as mature NGF supports survival of these neurons (Williams et al., 1986; Fischer et al., 1987; Kromer, 1987). However, more recently the disruption in the processing of NGF has been proposed to contribute to the cell death in AD, as the predominant from of NGF in AD brains is proNGF (Fahnestock et al., 2001; Kichev et al., 2009). Isolation of proNGF from AD brains induced apoptosis through p75NTR suggesting that it is factor contributing to neuronal cell loss (Pedraza et al., 2005).

Not only is proNGF involved in the pathology of AD but also the expression levels of its receptor p75NTR. P75NTR is involved in cell death in during the nervous system as two knock out mice models leads to increases in the number of BFCN (Naumann et al., 2002). It has been shown to induce cell death in neurons when TrkA is not expressed on the cell (Casaccia-Bonnefil et al., 1996; Frade et al., 1996). Another
P75\textsuperscript{NTR} mediated mechanism of neuronal cell loss in AD is P75\textsuperscript{NTR} induced cell death on binding of soluble Aβ (Rabizadeh et al., 1994). The soluble Aβ\textsubscript{1-42} peptide has been shown to induce cell death through the p75\textsuperscript{NTR} both \textit{in-vitro}, in cells overexpressing the receptor (Yaar et al., 1997; Kuner and Hertel, 1998) and \textit{in-vivo} in basal forebrain cholinergic neurons (Sotthibundhu et al., 2008).

In spite of the disruption in NGF pro-survival signalling, NGF has been suggested as a potential therapeutic for neuronal loss in AD. It has been shown to stimulate cholinergic function, improve memory and prevents cholinergic degeneration in animal models of injury and aging (Fischer et al., 1987; Tuszynski et al., 1990; Capsoni et al., 2002; Tuszynski, 2007). However, the side effects of NGF treatment such as pain, due to stimulation of the nociceptors, require targeted treatment of NGF by stereotaxic surgery, there are currently phase I and phase II clinical trials underway that allow the targeted treatment of BFCN with NGF (Tuszynski et al., 2005; Wahlberg et al., 2012).

Whether NGF or its precursor form elicits a pro-survival or pro-apoptotic effect depends on the relative level of receptors (Yoon et al., 1998; Masoudi et al., 2009). In AD brains the TrkA levels decreasing and pro-apoptotic proNGF and soluble Aβ are increasing, limiting the therapeutic value of NGF. In this study we generated rationally designed NGF variants, by the FoldX computer algorithm, which were predicted to have altered receptor binding to TrkA and p75\textsuperscript{NTR}. The algorithm was used to generate a NGF variant that acts as an agonist at TrkA, increasing its pro-survival signalling, and blocks p75\textsuperscript{NTR} receptor apoptotic signalling and thus generating an NGF variant with enhanced pro-survival signalling and therapeutic potential.

Many mutations of NGF have been generated reviewed in Bradshaw et al., 1994. these were generated to determine the ligand receptor interaction between NGF/TrkA and NGF/p75NTR, a summary of these mutations can be seen in Appendix II. These structure function studies of NGF and other members of the neurotrophin family were quite extensive and allowed the generation of a pan-neurotrophin (Ilag
et al., 1995). The pan-neurotrophin was genetically engineered neurotrophic factor combinig the active domains of the NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3) into an NT-3 backbone which displayed multiple specificities both in vitro and in vivo (Ilag et al., 1995).

High-resolution crystal structures are now available for NGF in complex with its receptors (Wiesmann et al., 1999; He and Garcia, 2004; Wehrman et al., 2007). The FoldX computer algorithm is designed to calculate the energy of unfolding (ΔG) of a protein complex. It has proven to be a useful tool in predicting amino acid mutations, which would alter ligand receptor binding affinities (van der Sloot et al., 2006). The FoldX computer algorithm calculates the free energy of unfolding for a protein complex (ΔG). The free energy of unfolding of a mutated protein complex is then determined. The differences in free energy of unfolding are determined (ΔΔG). Mutations that resulted in a − ΔΔG indicate mutations that saw a increase in the free energy of unfolding which theoretically indicates a increase in the binding affinity of NGF to its receptor and in contrast + ΔΔG indicates a mutation that will decrease the binding affinity of NGF for its receptor(Guerois et al., 2002). This mutational analysis was carried out on the NGF ligand in complex with TrkA and in complex with p75NTR to generate mutants with altered binding affinity for their receptors.
5.3 Results

5.3.1 Generation of NGF variant constructs

The FoldX computer algorithm is a powerful tool to determine the free energy of unfolding ($\Delta G$) of protein-protein interactions, i.e., it gives a value ($\Delta G$) to the amount of energy it takes to disrupt intramolecular interactions in a protein complex. The crystal structures of NGF in complex with the TrkA (Fig. 5.1A protein data bank (PDB) code 2IFG) (Wehrman et al., 2007) and p75$\text{NTR}$ (Fig. 5.1B PDB code 1SG1) (He and Garcia, 2004) are available and were used to generate $\Delta G$ of the NGF/TrkA and NGF/p75$\text{NTR}$ protein complex. The $\Delta G$ of wild type NGF interaction with TrkA and p75$\text{NTR}$ ($\Delta G_{\text{int}}$) was determined using the method described in (Guerois et al., 2002; Schymkowitz et al., 2005). Single point mutations were then made in the protein-protein interaction sites between NGF and its receptor and the free energy of unfolding for this NGF variant protein-protein interaction was generated. The difference between the free energies of unfolding was then determined ($\Delta \Delta G_{\text{int}}$). If the $\Delta \Delta G_{\text{int}}$ is positive the NGF variant protein-protein complex had a lower $\Delta G_{\text{int}}$ and took less energy to unfold then the WT protein interaction. If the $\Delta \Delta G_{\text{int}}$ is negative the NGF variant protein-protein complex had a higher $\Delta G_{\text{int}}$ and thus took more energy to unfold then the wild type protein interaction. When the $\Delta \Delta G_{\text{int}}$ is negative, the prediction of the algorithm is that this variant NGF has stronger affinity to the receptor than wild type NGF. The stability of the protein complex was also determined in this way ($\Delta \Delta G_{\text{stab}}$) (Guerois et al., 2002). The $\Delta \Delta G_{\text{int}}$ and the $\Delta \Delta G_{\text{stab}}$ for the FoldX variants generated by the computer algorithm are listed in Table 5.1 and 5.2. The $\Delta \Delta G_{\text{int}}$ and the $\Delta \Delta G_{\text{stab}}$ for the 8 NGF that were expressed are shown in Fig. 5.2 A. These were chosen based on their $\Delta \Delta G_{\text{int}}$ and $\Delta \Delta G_{\text{stab}}$ values and the position of the single point mutations in the receptor complex.
Figure 5.1. Schematic of the NGF in complex with TrkA and p75\textsuperscript{NTR}.

Schematic of NGF receptor complexes used to determine the FoldX free energy of unfolding (A) TrkA (light blue). The TrkA receptor consists of a leucine rich repeat (LRR) between two cysteine rich domain (CRD) that is followed by two large Immunoglobulin like domains (Ig-like domains). The Ig-like domain that is proximal to the membrane interacts with the NGF dimer. This domain is also called domain 5. (B) p75\textsuperscript{NTR} (light green). P75\textsuperscript{NTR} consists of 4 CRD labelled consecutively from the amino terminal of the receptor. The NGF interacts with the receptor at two sites. Site I is the interaction of CRD2 with the NGF and site II is where the C-terminal and N-terminal domain of the NGF interacts with CRD3 and CRD4. The NGF (dark blue) is in opposite orientation in the complexes.

Table 5.1 NGF variants designed by the FoldX algorithm predicted to have altered receptor binding to TrkA.

The NGF variants highlighted in grey indicate the variants whose cDNA sequences have been generated and are shown in Appendix I (A).

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Table 5.2 NGF variants designed by the FoldX algorithm predicted to have altered receptor binding to p75<sub>NTR</sub>.

The NGF variants highlighted in grey indicate the variants whose cDNA sequences have been generated and are shown in Appendix I (B).

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<th>ΔΔG&lt;sub&gt;stab&lt;/sub&gt;</th>
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Figure 5.2. The FoldX change in free energy values of the 8 NGF variants that were chosen for protein expression and their location in the receptor complex.

(A) The ΔΔG interaction and stability calculation for the NGF variants to be expressed. A Schematic of NGF (dark blue) in complex with (B) TrkA receptor (light blue) (C) p75NTR receptor (light green). The 4 NGF variants predicted to have altered binding to the receptor are labelled and highlighted in red. (D) A Schematic of proNGF (dark blue) in complex with p75NTR. The prodomain of NGF was not resolved because of its flexibility. The NGF variants predicted to have altered binding to the receptor are labelled and highlighted in red. When the amino acid side chains are in close proximity to the receptor the receptor changes to the colour of the side chain (red). The surface of TrkA and p75NTR electron density is shown to simplify the diagram.
Of the variants chosen H4D, H4E, G33M and H84Q are predicted to have higher affinity for TrkA (Fig. 5.2A). H4D, H4E and H84Q are predicted to increase intramolecular hydrogen bonds. The G33M variant is predicted to improve packing between the ligand and receptor. The position of these mutants in the protein-protein complex is highlighted in Fig. 5.2B. The variants I31R, K32R and A98I are predicted to have higher affinity for the p75\textsuperscript{NTR} (Fig. 5.2A). The I31R variant is predicted to increase hydrogen bonding to P70 or D75 of the receptor, K32R variant is predicted to improve hydrogen bonding with D75 of the receptor while the A98I NGF variant is predicted to increase packing of the ligand receptor complex. R69D is predicted to have lower affinity to the receptor through the loss of a hydrogen bond with D134 of the receptor. The position of these mutants in the protein-protein complex is highlighted in (Fig. 5.2C).

There are two current hypotheses on how NGF binds to the p75\textsuperscript{NTR}. One is derived from the structure determined by He and Garcia where they show that the NGF dimer binds to a monomer of the p75\textsuperscript{NTR} receptor, this is known as the 2:1 stoichiometry. Using this structure the NGF dimer is suspected to bind to a monomer of the receptor causing its dimerization (He and Garcia, 2004). The second hypotheses for NGF binding, is supported by the crystal structure of NT3 in complex with p75\textsuperscript{NTR} (Gong et al., 2008) and the crystal structure of proNGF in complex with p75\textsuperscript{NTR}, where the electron density of the prodomain was not resolved due to its flexibility (Feng et al., 2010). The second hypothesis is that NGF binds to p75\textsuperscript{NTR} as a preformed dimer, this is known as the 2:2 stoichiometry. In this model the binding of NGF causes a conformational change around a conserved cysteine residue (Cys257) in the transmembrane domain of the receptor and thus allows the recruitment of downstream adapter molecules (Vilar et al., 2009). The position of the NGF mutants in complex with the p75\textsuperscript{NTR} monomer and dimer is shown in Fig. 2C and D (PDB code 3IJ2) (Feng et al., 2010). The NGF variants that were chosen interacted with the receptor on the same sites in both the 2:1 and the 2:2 stoichiometry. The $\Delta \Delta G_{\text{int}}$ and the $\Delta \Delta G_{\text{stab}}$ of the 8 NGF variants are given in Fig.
5.2A. The $\Delta\Delta G_{\text{int}}$ and the $\Delta\Delta G_{\text{stab}}$ of additional NGF variants designed by FoldX are listed in Table 1 and 2.

5.3.2 Expression of the NGF variants

The NGF variants described in Fig. 5.2A were generated from wild type NGF (WT_NGF) cDNA that contained the prepro domain of the NGF protein and a C-terminal poly His tag as described in Chapter 4. The NGF variants were generated using site-directed mutagenesis and ligated into the pcDNA3.1 plasmid using BamHI and NotI restriction enzymes. DNA sequencing confirmed incorporation of the single point mutation and the presence of the His tag. The multiple sequence alignment of the amino acid sequence of NGF and the 8 NGF variants selected for protein expression are shown in Appendix 1A. The cDNA constructs of the NGF variants highlighted in Table 1 and 2 were generated and there multiple sequence alignments are shown in Appendix 1B.

The empty vector (EV) WT_NGF or the NGF variant constructs (henceforth called by variant name) were transfected into HEK293T cells using JetPEI transfection reagent, 48 h post transfection the cells and the media were harvested. The expression of NGF in the lysate of the cell and secreted into the media of the HEK293T was determined using Western blotting for NGF (Fig. 5.3A) and by NGF ELISA (Fig. 5.3B). On transfection with the EV control there is no detectable level of NGF in the HEK293T cells. The NGF variants are expressed in the lysate of the HEK293T cells in the precursor form and secreted into the media. The WT_NGF, H4E, I31R, K32R, H84Q and A98I NGF variants are secreted into the media at high concentrations after 48 h, with concentration range between 100 to 250 ng/ml (Fig. 5.3B). The H4D and R69D variants are not expressed well in the cell lysate of the HEK293T cells and are secreted into the media in low concentrations (Fig. 5.3A) of 12 ng/ml and 24 ng/ml respectively (Fig. 5.3B). 48 h post transfection the G33M NGF variant is expressed in high concentrations in the lysate of the HEK293T cells (Fig. 3A) and is secreted into the media at a concentration of 42 ng/ml (Fig. 5.3B). 72 h post transfection the G33M variant was secreted into the media at higher
concentrations as seen by Western blotting the HEK293T conditioned media for NGF (Supplementary Fig. 5.1). For all further experiments the HEK293T cells conditioned media was collected 48 h after transfection to reduce the secretion of NGF without the His tag into the media (as seen in Chapter 4) and (Supplementary Fig. 5.1).

Figure 5.3. The recombinant NGF variants are expressed and secreted into the media by mammalian HEK293T cells

The NGF variant constructs were transfected into HEK293T cells using JetPEI transfection reagent. 48 h after transfection the media was collected and the cells were lysed. NGF expression in the HEK293T cells and the secretion of the NGF into the media was analysed by (A) Western blotting using an anti-NGF antibody. The data is representative of 3 repeats. (B) The concentrations of NGF secreted into the media were quantified using an NGF ELISA. Commercial available hβNGF from Alomone Labs was used as standard for NGF concentration. The data is the average of three individual experiments +/- StdDev.
Supplementary Figure 5.1. Expression of secreted NGF variants 72 h post transfection

The NGF variant constructs were transfected into HEK293T cells using JetPEI transfection reagent. 72 h after transfection the media was collected and the cells were lysed. NGF expression in the HEK293T cells and the secretion of the NGF into the media was analysed by. (A) Western blotting using an anti NGF antibody. The data is representative of 3 repeats

5.3.3 Biological activity of the NGF variants through TrkA

In order to access the biological activity of the NGF variants their activity was compared to WT_NGF. The HEK293T conditioned media containing EV, WT_NGF or the NGF variants was supplemented with the serum requirements of PC12 cells. Using the NGF ELISA data, the NGF was then diluted to 50 ng/ml NGF in complete media. For the variants that gave a low yield of NGF secretion, H4D and R69D, the conditioned media was supplemented with the serum requirements of PC12 cells and left undiluted. Thus the PC12 cells were treated with 12 ng/ml, and 24 ng/ml of H4D and R69D NGF variant respectively (Fig. 5.3B). The activity of the variants was then compared to a standard curve for hβNGF activity. The NGF variant was then applied to different TrkA activity assays in PC12 cells and the activity of the NGF variants in comparison to the WT_NGF was determined. The activity was not compared to the commercially available NGF as the activity of WT has been proven to be similar to commercially available NGF (Chapter 4).

To ensure that NGF variants were not causing adverse effects on the cells 50 ng/ml of EV, WT_NGF and NGF variants were applied to PC12 cells for 48 h. Phase contrast
images of the cells were then taken and the cell morphology was analysed (Fig. 5.4A). Treatment with EV and I31R had no effect on the cells. WT, H4D, H4E, K32R, G33M, H84Q and A98I caused the cells to flatten and differentiate, with visibly fewer neurites present in cells treated with G33M. PC12 cells treated with R69D showed visible signs of cell stress such as cell shrinkage. The morphological changes described are indicated with arrows in Fig. 4A. Prolonged treatment of PC12 cells with NGF causes them to differentiate and extend neurites (Greene and Tischler, 1976). 50 ng/ml of WT_NGF and NGF variant was applied to PC12 cells and their effect on neurite outgrowth was analysed. The PC12 cells were allowed to grow and differentiate in the NGF variant containing media for 7 days. Phase contrast images of the cells were taken (Fig. 5.4B) and the percentage of cells extending neurites was quantified (Fig. 5.4C).

The results show that there was no neurite outgrowth in cells treated with EV, I31R or R69D NGF variant. Of the 300 cells counted the number of cells extending neurites in the WT, H4D, H4E, K32R, H84Q and A98I was above 90% of the total number of cells counted. The number of cells extending neurites with G33M treatment was 81.1 ± 0.5 % (Fig. 5.4C).
(B)
Figure 5.4. The NGF variant induction of neurite outgrowth

The NGF containing HEK293T conditioned media was diluted to 50 ng/ml (A) The NGF was applied to PC12 cells and light microscopy images were taken 48 h after NGF addition, morphological features are indicated with arrows. The black arrow heads indicate the normal morphology of PC12 cells, the white arrow shows flattened cells, black filled arrows indicate neurite outgrowth and red arrows show cell shrinkage (B) The NGF was then applied to PC12 and 7 days later light microscopy of the neurite outgrowth were taken. Data is representative of 3 independent experiments (C) The neurite outgrowth was then quantified, 100 cells were counted and the number of cells extending neurites was quantified. Data is the average of 3 independent experiments +/- StdDev.

We have previously shown that pre-treatment with NGF can protect PC12 cells from TG induced apoptosis. This was found to be due to NGF stimulation of PI3K/AKT signalling through TrkA (Fig. 5.5A) (Szegezdi et al., 2008). Here we show that this protection from TG induced apoptosis was dose dependent (Fig. 5.5B) and thus a standard curve for NGF activity can be used to compare to the NGF variants. The PC12 cells were incubated with 100 ng/ml EV, WT_NGF and NGF variant without (Fig. 5.6A) or with (Fig. 5.6B) the addition of a TrkA inhibitor K252a. 2 h after NGF addition the cells were treated with 1.5 μM TG. The viability of the PC12 cells was analysed by MTT assay and the % viability of the cells was determined by comparing the cells to EV treated cells.
Figure 5.5. Standard curve for NGF-mediated protection of PC12 cells against TG-induced apoptosis

PC12 cells were treated with the indicated dose of NGF 2 h before treatment with 1.5 μM TG. 48 h after TG treatment (A) Light microscopy images of the cells treated with 100 ng/ml NGF were taken to show protection from TG induced apoptosis (B) The cell viability was analysed 48 h after TG addition by MTT assay. The untreated cells were set to a viability of 100% and the results are expressed as a percentage of their viability. The data are representative of 3 independent experiments +/- the Std Dev *p<0.05 **p<0.01.
Figure 5.6. NGF variant protection from TG-induced apoptosis

The NGF containing HEK293T conditioned media was diluted to 50 ng/ml NGF where possible, H4D and R69D, were treated with 12 ng/ml, and 24 ng/ml respectively (A) The media was applied to PC12 cells 2 h before addition of 1.5 μM TG. 48 h post TG treatment the cell viability was analysed by MTT. (B) The media was applied to PC12 cells with the addition of K252a 2 h before addition of 1.5 μM TG. The cell viability was analysed 48 h after TG addition by MTT assay. The viability of the cells treated with EV containing conditioned media was set to 100% and the remaining results were expressed as a percentage of their viability. The data are representative of 3 independent experiments +/- the Std Dev *p<0.05 **p<0.01.

The results show that the EV, I31R and R69D variant were unable to protect the PC12 cells from TG induced apoptosis. The H4D, H4E, K32R, G33M, H84Q and A98I variants were able to protect the PC12 cells to the same extent as WT_NGF (Fig.
5.6A) and this protective effect was lost on inhibition of the TrkA receptor (Fig. 6B). It was also seen that treatment with WT_NGF, H4D, H84Q and A98I without the addition of TG gave an increase in cell viability and NGF variant R69D gave a decrease in cell viability in comparison to EV treated cells, however this was not found to be statistically significant.

Application of NGF onto PC12 cells is known to cause phosphorylation of AKT and prolonged phosphorylation of ERK (Huang and Reichardt, 2001). The ability of the NGF variants to phosphorylate the downstream adapter molecules of TrkA was analysed by application of the 50 ng/ml EV, WT_NGF or NGF variant to PC12 cells for the indicated times (Fig. 5.7). NGF variants induced phosphorylation of ERK and AKT as analysed by Western blotting, using the total proteins as a loading control (Fig. 5.7).

**Figure 5.7. NGF variant activation of AKT and Erk**

The NGF containing HEK293T conditioned media was diluted to 50 ng/ml NGF where possible, H4D and R69D, were treated with 12 ng/ml, and 24 ng/ml respectively (A) phosphorylated ERK and total ERK and (B) phosphorylated AKT and total AKT were analysed by western blotting. The data is representative of 2 repeats.
The results show that EV and R69D treated cells do not induce phosphorylation of ERK or AKT, however it is important to note here that 24 ng/ml of R69D variant was used not 50 ng/ml. The I31R variant does not induce phosphorylation of ERK and has delayed phosphorylation of AKT. The H4E variant induces robust phosphorylation of ERK after 15 min however there is no phosphorylation of AKT at this same time point. The NGF variants K32R, G33M, H84Q and A98I induced phosphorylation of ERK and AKT to a similar level to WT_NGF. Treatment with H4D decreased phosphorylation, in comparison to WT_NGF, this may be due to its concentration of 12 ng/ml.

In summary the NGF variants H4D, H4E, K32R, G33M, H84Q and A98I showed activity through the TrkA receptor. Interestingly the H4D, which was used at a concentration 12 ng/ml, exhibited activity similar to that of WT_NGF in all assays except in the phosphorylation of ERK where there was a reduction in the level of phosphorylation at 2 h in comparison to WT_NGF treated cells. Using the standard curve of hβNGF protection the activity of H4D is most clearly seen. Cells treated with 12 ng/ml of hβNGF gave a viability of 12 % when treated with TG but the H4D variant at a concentration of 12 ng/ml gave a viability of 31 %. The NGF variants I31R and R69D showed no activity through TrkA as they exhibited no neurite outgrowth, no protection from TG induced apoptosis and little to no phosphorylation of ERK and AKT.

5.3.4 Binding activity of the NGF variants to TrkA and p75NTR

The EV, WT_NGF and the NGF variants were purified to determine their binding affinity for TrkA and p75NTR. The variants were purified as previously described in Chapter 4. The concentration of NGF was then analysed by Western blotting for NGF (Fig 5.8A). NGF was eluted at high concentrations in elution 1 and elution 2 for all variants excluding H4D, G33M and R69D. This was also shown using the NGF ELISA (Fig. 5.8B). The NGF was then diluted to 15 ng/ml and serial dilutions were made of the NGF to apply it to the NGF binding assay.
Figure 5.8. Purification of the NGF variants

The NGF variants were purified by affinity chromatography. The concentration of NGF eluted from the His trap affinity chromatography column was determined by (A) western blot using an anti NGF antibody and by (B) an NGF ELISA. Commercially available hβNGF from Alomone Labs was used as the NGF standard. The data is the average of 3 independent repeats +/- StdDev.

The WT_NGF and NGF variants were applied to 10 μg of immobilised TrkA or p75NTR on a 96 well plate. After 2 h incubation with the receptor the unbound NGF was removed and the concentration of NGF bound to the receptor was determined using the NGF ELISA. The absorbance which 15 ng/ml of WT_NGF bound to the receptor was set to 100%. The binding curves for the NGF variants were generated by expressing the amount of NGF detected on the plate as a percentage of the...
amount of WT_NGF that was detected. The binding curves for the NGF variants binding to TrkA are in Fig. 5.9A and B. The variants that were predicted to have altered receptor affinity for the TrkA are shown in Fig. 5.9A and the variants predicted to have altered receptor affinity to p75NTR are in Fig. 5.9B. The binding curves for the NGF variants binding to p75NTR are found in Fig. 1.10A and 5.10B. The variants that were predicted to have altered receptor binding to the TrkA receptor are shown in Fig. 5.10A and the variants that were predicted to have altered receptor affinity to p75NTR are shown in Fig. 5.10B.

The NGF variant A98I and K32R have a higher binding affinity to the TrkA receptor then the WT, with A98I only showing a modest increase and K32R 40 % +12 %. The H4D and H4E they exhibited an increase in binding in comparison to the WT_NGF of 11 % and 19 %, respectively. This increase in binding was only seen at the lower concentrations of NGF and was lost at concentrations above 5 ng/ml of NGF. The H84Q and G33M variant gave a 50 % decrease in binding in comparison to the WT_NGF. The NGF variants I31R and R69D, which exhibited no biological activity at TrkA also showed no affinity for the TrkA receptor.

H4E and R69D exhibited no affinity for the p75NTR receptor. The I31R variant displayed decreased affinity for p75NTR relative to WT_NGF. NGF variants H4D and I31R had similar binding affinity to WT_NGF. The A98I variant had a 50 % increase in binding to p75NTR in comparison to WT_NGF. The K32R variant had the largest increase in binding affinity for p75NTR showing a 100 % increase in the amount of NGF bound to the receptor compared to WT_NGF. The data is summarised in Table 5.2.
Figure 5.9 Binding of the NGF variants to TrkA

The soluble TrkA receptor was immobilised to NUNC MaxiSorp plates overnight. The unbound receptor was removed and the plate was blocked with 1% BSA in PBS. The purified NGF variants were then incubated with the immobilised receptor for 2 h using a concentration range of 0.375 ng/ml to 15 ng/ml. The unbound NGF was removed and the amount of NGF bound on the TrkA was determined using the NGF ELISA. (A) The absorbance obtained for the WT, H4D, H4E, A98I and H84Q variants (B) the absorbance obtained for the WT, I31R, K32R, G33M, and R69D NGF variants. The data are an average of 3 independent experiments +/- SdtDev.
Figure 5.10. Binding of the NGF constructs to the p75<sub>NTR</sub>

The soluble p75<sub>NTR</sub> receptor was immobilised to NUNC MaxiSorp plates overnight. The unbound receptor was removed and the plate was blocked with 1% BSA in PBS. The purified NGF variants were then incubated with the immobilised receptor for 2 h using a concentration range of 10 ng/ml to 0.375 ng/ml. The unbound NGF was removed and the concentration of NGF bound on the p75<sub>NTR</sub> was determined using the NGF ELISA. (A) The absorbance obtained for the WT, H4D, H4E, A98I and H84Q variant (B) the absorbance obtained for the WT, I31R, K32R, G33M, and R69D NGF variant. The data are an average of 3 independent experiments +/- SdDev.
Table 5.3. Summary of NGF variant binding data

The variants are displayed. The dash (−) denotes a variant that had unchanged binding to the indicated receptor. zero (0) denotes a variant that had no binding to the receptor the up arrow denotes a variant that had increased binding relative to WT to the receptor (↑) and the down arrow denotes a variant that had decreased binding to the receptor (↓).

<table>
<thead>
<tr>
<th>NGF variant</th>
<th>TrkA</th>
<th>P75NTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4D</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>H4E</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>I31R</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>K32R</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>G33M</td>
<td>↓</td>
<td>–</td>
</tr>
<tr>
<td>R69D</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H84Q</td>
<td>↓</td>
<td>–</td>
</tr>
<tr>
<td>A98I</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

5.3.5 Biological activity of the NGF variants through p75NTR

To test the biological activity of the NGF variants through p75NTR an assay needed to be developed. P75NTR is known to activate pro-survival signalling through nuclear factor-κB (NF-κB) or pro-apoptotic signalling through the adapter proteins NADE, NRAGE and NRIF. Activation of the contrasting pathways depends on the expression levels of TrkA (Yoon et al., 1998). A number of different assays were tested to show NGF stimulated pro-survival or pro-apoptotic activity through p75NTR. The assays tested are summarised in Table 5.3. An assay representing these results will be described in detail below.
Table. 5.4. Summary of conditions tested for the development of a \( p75^{NTR} \) assay

<table>
<thead>
<tr>
<th>Assay background</th>
<th>Treatment</th>
<th>Cell line</th>
<th>Assay type</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>proNGF/NGF can induce apoptosis through ( p75^{NTR} )</td>
<td>proNGF</td>
<td>PC12</td>
<td>Nuclear Morphology - Dapi staining</td>
<td>No significant cells death observed</td>
</tr>
<tr>
<td></td>
<td>proNGF</td>
<td>RN22 schwannoma</td>
<td>Nuclear Morphology - Dapi staining</td>
<td>No significant cells death observed</td>
</tr>
<tr>
<td></td>
<td>proNGF</td>
<td>RN22 schwannoma</td>
<td>Cell Viability - MTT assay</td>
<td>No significant loss in cell viability observed</td>
</tr>
<tr>
<td></td>
<td>proNGF</td>
<td>RN22 schwannoma</td>
<td>DEVDase activity</td>
<td>No significant increase in DEVDase observed</td>
</tr>
<tr>
<td>NGF</td>
<td>HEK293 <em>p75^{NTR}</em></td>
<td>Western blotting for pJNK</td>
<td>No significant increase in pJNK</td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>SK-N-MC</td>
<td>Western blotting for cleaved caspase-3</td>
<td>No increase in the cleavage of caspase-3 observed</td>
<td></td>
</tr>
</tbody>
</table>

| NGF induced pro-survival signalling to NF-\( \kappa B \) | NGF | HEK293 _p75^{NTR}_ | Western blotting for cleaved caspase-3 | No cleavage of caspase-3 observed |
| | NGF | HEK293 _p75^{NTR}_ | Western blotting for phospho \( \kappa B \) | No phosphorylation of \( \kappa B \) observed |
| | NGF | MDA-MB-231 | Western blotting for phospho \( \kappa B \) | No phosphorylation of \( \kappa B \) observed |
| | NGF | MDA-MB-231 | Western blotting for Total \( \kappa B \) | No degradation of \( \kappa B \) observed |
| | NGF | MDA-MB-231 _N\kappa B_ reporter | N\kappa B promoter activity with time and dose for mNGF | No luciferase activity observed |
| | | | N\kappa B promoter activity with time and dose for hNGF | No luciferase activity observed |
| | | | N\kappa B promoter activity with inhibition of NGF using Ro 08-2750 | Decrease in the basal luciferase activity observed |
| | | | N\kappa B promoter activity with inhibition of \( p75^{NTR} \) signalling using pep5 | Decrease in the basal luciferase activity observed |
NGF induced apoptosis through p75\textsuperscript{NTR} was tested first. Based on reports treatment of SK-N-MC cells with NGF causes cell death by apoptosis as assayed by DNA ladder (Kuner and Hertel, 1998) and tunel staining (Niederhauser et al., 2000). SK-N-MC cells were treated with NGF using the conditions described in (Niederhauser et al., 2000), briefly the cells were treated with 100 or 250 ng/ml NGF, 48 h after seeding. Before treatment the media was changed to either serum free or complete media. The ability of NGF to induce apoptosis was analysed 48 h and 72 h after treatment by Western blotting for cleaved caspases-3 (Supplementary Fig. 5.2A). NGF did not cause cleavage of caspase-3.

The next approach to develop an assay to test the NGF variants was to show that, increasing concentrations of the NGF or NGF variant can displace proNGF from p75\textsuperscript{NTR} and thus protect cells from uncleavable proNGF induced apoptosis. ProNGF, has been shown to induce cell death both in-vivo and in-vitro due to higher binding affinity to p75\textsuperscript{NTR} (Fahnestock et al., 2001; Lee et al., 2001; Beattie et al., 2002; Fahnestock et al., 2004; Harrington et al., 2004; Nykjaer et al., 2004; Massa et al., 2006; Masoudi et al., 2009; Capsoni et al., 2011). To optimise this assay PC12 and RN22 schwannoma cells were treated with proNGF at a concentration range of 50-200 ng/ml, in either serum free or serum containing conditions. The amount of apoptosis induced was analysed 24, 48 and 72 h after treatment by DAPI staining and DEVDase assay. Data obtained using the RN22 schwannoma cells has previously been shown to induce robust cell death on treatment with proNGF (Pagadala et al., 2006). However, proNGF caused no cell death in the cells lines as determined by a DEVDase assay (Supplementary Fig. 5.2B) and a morphological assay (DAPI) staining (Supplementary Fig. 5.2C). Another approach used was to transfect p75\textsuperscript{NTR} plasmid (a kind gift of Prof. Moses V Chao) into HEK293T cells using JetPEI transfection, this caused the HEK293T cells to die upon transfection, in an NGF independent manner, as described in (Bamji et al., 1998; Bhakar et al., 2003). The cell death obtained made it was unsuitable for data collection.
Supplementary Figure 5.2. Development of a p75NTR assay. NGF/proNGF induced pro-apoptotic signals

(A) SK-N-MC cells were treated with indicated concentrations of NGF in serum free or serum containing conditions. The NGF induced apoptosis was analysed by Western blotting for cleaved caspase-3. The data is representative of 3 repeats. (B) RN22 schwannoma cells were treated with indicated concentrations of proNGF, in serum containing conditions. The induction of apoptosis was analysed 24, 48 and 72 h after treatment by DEVDase assay. (C) RN22 schwannoma cells were treated with 500 ng/ml proNGF, in serum free or serum containing conditions 72 h after treatment the cells were fixed in 4% formaldehyde and apoptosis was analysed by staining with DAPI and counting the number of condensed nuclei as a percentage of the total number of cells counted. The data is the average of 3 independent experiments +/-SEM.
The second approach was to test systems where NGF has been reported to activate NF-κB pro-survival signalling. HEK293 cells that were stably transfected with the p75NTR receptor (HEK293_p75) (a kind gift of Prof Bruce Carter) were used to test the activity of NGF through the p75NTR receptor (Kenchappa et al., 2010). The inhibitors of NF-κB (IκB) proteins sequester NF-κB in the cytosol of the cell, thus preventing its activity. On stimulation of the NF-κB pathway the IκBs are phosphorylated causing their degradation. NF-κB can then translocate into the nucleus. NGF induced NF-κB activity was analysed using Western blotting for phosphorylated IκBα. NGF did not induce phosphorylation of IκBα (Supplementary Fig. 5.3A). 25 ng/ml tumor necrosis factor (TNF), a known activator of NF-κB, was used as a positive control and induced phosphorylation of IκBα 10 min after treatment (Supplementary Fig. 5.3B). MDA-MB-231, a breast cancer cell line, is reported to activate the NF-κB pathway on NGF treatment (Descamps et al., 2001). MDA-MB-231 cells were treated with NGF and the effect of NGF treatment on IκBα was analysed (Supplementary Fig. 5.3B). There was modest increase in the phosphorylation of IκBα. To this end the MDA-MB-231 cells were transfected with a lentiviral NF-κB promoter with a luciferase reporter (MDA-MB-231_NF-κB promoter cell). Treatment of cells with a dose of NGF (0 -150 ng/ml) had no effect on NF-κB activity in MDA-MB-231_NF-κB promoter cell line (Supplementary Fig. 5.3C). The MDA-MB-231_NF-κB promoter cells were then treated with inhibitors of p75NTR signalling and the effect of NF-κB activity was analysed. Treatment of cells with Ro 08-2750, a compound that can bind to NGF and disrupts its affinity for p75NTR (Niederhauser et al., 2000) and with Tat-pep5, a cell penetrating peptide that can bind to the death domain of p75NTR and inhibit it’s activity (Yamashita and Tohyama, 2003), decreased the basal NF-κB activity of the cells (Supplementary Fig. 5.3D). This suggests that NGF and p75NTR have a role in the basal activation of NF-κB in these cells. However, this would not be a suitable system to test the NGF induced activity.
Supplementary Figure 5.3. Development of a p75NTR assay. NGF induced NF-κB activity

(A) HEK293_p75NTR cells or (B) MDA-MB-231 cells were treated with 100 ng/ml NGF for indicated times. The NGF induced activation of NF-κB was then analysed by Western blotting for phosphorylated IκB α. 25 ng/ml TNF was used as a positive control. Data is representative of 3 individual experiments (experiment preformed by Hillis J). (C) MDA-MB-231_NF-κB promoter cells were treated with NGF for 18 h or (D) NGF inhibitors for 18 h (experiment preformed by Carleton L and Hillis J). The activation of NF-κB was measured by luciferase activation. The fold luciferase change was caluculated by setting the untreated cells as 1. The data is the average of 3 individual experiments +/- StdDev.
In the search for a biological assay for p75NTR, it was found that Ro 08-2570, an inhibitor of p75NTR activity, caused cell death in two chronic lymphocytic leukemia (CLL) cell lines, HG3 and Mec1 cells (assay preformed as part of a drug screen by Natoni A. in Prof. Corrado Santocanale lab). These results were then verified by treating the Mec1 cells with 2.5 and 5 μM Ro 08-2750 for 48 and 72 h. Ro 08-2750 decreased the viability of the cells in a dose and time dependent manner as determined by MTT assay (Supplementary Fig. 5.3A). The effect of PEP5 on the on the viability of Mec1 (Supplementary Fig. 5.3B) and HG3 (Supplementary Fig. 5.2C) cells was analysed. Tat-pep5 treatment decreased the viability of the cells in a dose dependent manner as determined by MTT assay. Cellular lysates from the CLL were then taken and the expression of NGF and p75NTR was analysed using Western blotting. The Mec1 and HG3 cells expressed both NGF (visualised as the precursor form) and p75NTR (Supplementary Fig. 5.3D). The predominant band for p75NTR was 55 kDa, not the expected 75 kDa. This could be due to the glycosylation status of the receptor on the cells, as extensive O-linked glycosylation increases the molecular weight of the protein from 55 - 75 kDa. As p75NTR is known to undergo α- and γ-secretase cleavage crucial to its function (Skeldal et al., 2011), the p75NTR present in the CLL cells was analysed further. The cells were treated with or without Ro 08-2750 for 24 h, lysates were then compared to PC12 cell lysate as a positive control. Ro 08-2750 treatment had no effect on Mec1 or HG3, p75NTR levels or processing. The predominant band for p75NTR is 55 kDa and there is a faint band visible at 75 kDa in the HG3 cells. The HG3 cells also have a band visible at 25 kDa that represents the size of the membrane bound C-terminal fragment of p75NTR (Supplementary Fig. 5.3E). The cells Mec1 cells were then treated with Ro 08-2750 for 48 h and the levels of cleaved caspase-3 was analysed by Western blotting (Supplementary Fig. 5.3F). Ro 08-2750 caused an increase in the processing of cleaved caspase-3 suggesting that the decrease in cell viability seen was due to an increase in apoptosis. These data suggest that the CLL cells may have a dependence on the NGF/p75NTR pathway for there survival. These cells could be used to analyse p75NTR receptor antagonists.
**Supplementary Figure 5.4. Preliminary characterisation of Mec1 and HG3 response to NGF and p75<sup>NTR</sup> inhibitors**

(A) Mec1 cells were treated with indicated concentrations of Ro 08-2750 48 and 72 h post treatment the cells viability was analysed by MTT assay. (B) Mec1 cells and (C) HG3 cells were treated with Tat-pep5. 48 h post treatment the cells viability was analysed by MTT assay. The percentage viability was calculated by setting the untreated cells to 100 % and comparing the cells to the untreated samples. The data is the average of 3 independent experiments +/- StdDev (n=2) (D) HG3 and Mec1 lysates were analysed for NGF and p75<sup>NTR</sup> expression by Western blotting. The changes in (E) Mec1 and HG3, p75<sup>NTR</sup> cleavage, and (F) Mec1, caspase-3 cleavage, after 48 h Ro 08-2750 treatment was analysed by Western blotting. The data is representative of 3 independent experiments.
5.3.6 Results Summary

The results obtained in this chapter and the overall conclusions will be described in Table 5.4.
Table 5.5 Summary of NGF variant data

<table>
<thead>
<tr>
<th>NGF variant</th>
<th>Predicted binding to TrkA</th>
<th>Predicted binding to p75NTR</th>
<th>Experimentally determined binding to TrkA</th>
<th>Experimentally determined binding to p75NTR</th>
<th>TrkA mediated protection</th>
<th>TrkA mediated neurite outgrowth</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4D</td>
<td>↑</td>
<td>no change</td>
<td>↑</td>
<td>no change</td>
<td>↑</td>
<td>no change</td>
<td>Increased binding and activity through TrkA while maintaining binding to p75NTR</td>
</tr>
<tr>
<td>H4E</td>
<td>↑</td>
<td>no change</td>
<td>↑</td>
<td>↓</td>
<td>no change</td>
<td>no change</td>
<td>Increased binding to TrkA but no change in activity</td>
</tr>
<tr>
<td>G33M</td>
<td>↑</td>
<td>no change</td>
<td>↓</td>
<td>no change</td>
<td>no change</td>
<td>↓</td>
<td>Increased binding to TrkA but no change in activity through ERK.</td>
</tr>
<tr>
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<td>↓</td>
<td>no change</td>
<td>no change</td>
<td>no change</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>↑</td>
<td>↑</td>
<td>no change</td>
<td>no change</td>
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</tr>
<tr>
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<td>no binding</td>
<td>no protection</td>
<td>no outgrowth</td>
<td>No binding to TrkA or p75NTR or activity through TrkA</td>
</tr>
<tr>
<td>A98I</td>
<td>no change</td>
<td>↑</td>
<td>↑</td>
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<td>no change</td>
<td>no change</td>
<td>Increased binding to p75NTR but no activity through TrkA</td>
</tr>
</tbody>
</table>
5.4 Discussion

This chapter focused on the generation of NGF variants and examination of the biological activity of these variants. Mammalian expression yielded active mature NGF variants suitable for biological activity assays. Biological and binding affinity testing of the NGF variants demonstrated that they had activity that was different from that of WT_NGF. Specifically the H4D variant showed increased activity at TrkA. The I31R variant showed no biological or binding activity at TrkA but retained affinity for p75 NTR, the A98I and K32R variants were biologically active at TrkA and displayed a marked increase in p75 NTR binding. A discussion of the results for each variant will be described in detail below.

The NGF variants were designed using the FoldX design algorithm. This algorithm has previously been used to successfully design variants of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) with death receptor specificity (van der Sloot et al., 2006). Many of the amino acids essential for the NGF-TrkA and NGF-p75 NTR structure/function relationship have been determined by extensive mutation of the NGF ligand, reviewed in Bradshaw et al., 1994. Several of the variants designed by FoldX are in sites that have been previously reported to be important for receptor interaction. This exhibits the accuracy in the design algorithm. The published data relevant to the 8 NGF variants will be described with each variant below.

The NGF variants that were predicted to have altered binding at the TrkA receptor are H4D, H4E, H84Q and G33M. The TrkA immunoglobulin like domain proximal to the membrane (domain 5) has been shown to be necessary for NGF binding (Fig. 5.1)(Urfer et al., 1995). This is also shown in the crystal structure of NGF in complex with the TrkA receptor (Wiesmann et al., 1999; Wehrman et al., 2007). The NGF ligand binds at two locations in domain 5 of TrkA. One interaction face is in the amino terminal of the NGF ligand and the second is on the central β sheet of the protein (Fig. 5.1) (Wiesmann et al., 1999; Wehrman et al., 2007). The FoldX
programme predicted that mutation in these key areas on NGF would alter the \( \Delta G_{\text{inter}} \) with the receptor.

H4D and H4E are in the amino terminal of NGF that interacts with the solvent exposed face of TrkA-domain 5. This site has previously been reported as having an important role in receptor binding (Ibáñez et al., 1993). The importance of the N-terminal domain in the interaction with TrkA can clearly been seen on comparison of the crystal structures of NGF. On co-crystallization with TrkA the amino terminal of NGF is well defined but on co-crystallization with p75\textsuperscript{NTR} this domain was not resolved because of it’s fluidity, suggesting it had little interaction with the receptor (Fig. 1.11). The mutation H4E showed no altered protein expression in comparison to WT_NGF and exhibited activity at the TrkA receptor similar to that of WT_NGF. H4E induce neuronal differentiation, phosphorylation of ERK and delayed phosphorylation of AKT and could protect PC12 cells from TG-induced apoptosis to a level similar to that of WT_NGF. In contrast to the H4E variant, H4D showed a marked decrease in NGF expression. The protein concentration values obtained were between 10-20 ng/ml. However, the H4D variant showed biological activity similar to that of 50 ng/ml WT_NGF when treated at these low concentrations. In the protection assay the activity of H4D matches that of the activity of WT_NGF, however there was a concentration difference of 4.1 fold between the WT_NGF and the H4D. This suggests that it may have enhanced activity at the TrkA receptor. The H4D and H4E bound to the immobilised TrkA receptor with affinity lower then that of the WT_NGF. However, at lower concentrations the H4 NGF variants had a higher binding affinity compared to WT_NGF. In contrast, the binding of H4D to the immobilised p75\textsuperscript{NTR} was similar to that of WT_NGF and there was a 50% decrease in the binding of H4E to the p75\textsuperscript{NTR}.

The variant H84Q was predicted by FoldX to have increased TrkA affinity. H84 has been shown to interact with TrkA-domain 5 (Ibáñez et al., 1993; Wiesmann et al., 1999). H84Q was active through TrkA. Treatment with 50 ng/ml of H84Q induced neuronal differentiation, phosphorylation of ERK and AKT and could protect PC12 cells
from TG-induced apoptosis to a level similar to that of WT_NGF. However, it showed a 43% decrease in binding to TrkA receptor and no difference in binding to $p75^{NTR}$, compared to WT_NGF. It is possible that the substitution of H with Q affects the binding of the NGF variant for the TrkA but has no effect on overall activation of the receptor. There is a high sequence identity between the various Trk receptors and between the neurotrophins at this interaction face (Wiesmann et al., 1999). Thus, it has been speculated that all neurotrophin-trk receptor complexes activate their preferred Trk receptor by a similar mechanism involving domain 5 of their Trk receptor and the central $\beta$ sheet of the neurotrophin (Wiesmann et al., 1999). The H84Q variant may have lost some specificity for the TrkA receptor but retained its activity. Binding assays with the other Trk receptors would verify this.

G33M was predicted by FoldX to have increased TrkA affinity. G33 is in loop 1 of NGF and was not shown to have extensive binding affinity with the TrkA receptor (Wiesmann et al., 1999). However substitution of G with M in this area retains the amine group and increases the hydrophobicity of the side chain. This was predicted to improve the packing between the NGF variant and the TrkA receptor. Treatment with 50 ng/ml of G33M induced neuronal differentiation of PC12 cells, phosphorylation of AKT and ERK. and protected PC12 cells from TG induced apoptosis. It showed a 55% decrease in binding to TrkA receptor but no difference to in $p75^{NTR}$ binding compared to WT_NGF. The G33M variant had no difference in TrkA activity in comparison to the WT_NGF. G33 is conserved within all neurotrophins (Bradshaw et al., 1994) and is solvent exposed (Wiesmann et al., 1999; Wehrman et al., 2007). This would suggest an important role for G33 in the neurotrophin family. The G33M variant showed reduced expression 48 h post transfection in comparison to WT_NGF, suggesting that this hydrophobic substitution at G33 had some effect on protein expression but no effect on the activity of the protein.

The NGF variants that were predicted to have altered binding at $p75^{NTR}$ are I31R, K32R, R69D and A98I. $P75^{NTR}$ binds to the homodimeric interface of NGF. The NGF interacts with the receptor at 2 sites. Site I it the interaction of NGF with cysteine rich domain 2 (CRD2), the mutations I31R, K32R and A98I are located in this
interaction site. Site II contains the interaction of NGF with CRD3 and CRD4, the NGF mutation R69D located in this interaction site (Fig. 5.2C and 5.2D).

I31R variant was predicted to have an increase in binding affinity to p75NTR. I31 has been shown to interact directly with p75NTR at interaction site I (He and Garcia, 2004) and with TrkA (Ibáñez et al., 1993). Thus it is predicted that alteration of this amino acid will have an effect on both TrkA and p75NTR. I31R showed no TrkA biological activity, as it did not induce neuronal differentiation, phosphorylation of ERK and AKT or protect PC12 cells from TG induced apoptosis. I31R showed no affinity for immobilised TrkA receptor and retained binding to p75NTR. Other variants of I31 have been generated, I31A, I31M and I31V (Bradshaw et al., 1994). Variants I31A and I31M demonstrated 30% TrkA binding, whereas I31V demonstrated 130% TrkA binding relative to wtNGF (Ibáñez et al., 1992). These data demonstrate the importance I31 in TrkA receptor interaction and the effect of amino acid substitution on TrkA activity. Based on our data, the amino acid substitution I31R has generated a NGF variant with specific binding and activity at p75NTR. However, a p75NTR biological activity assay would be required to confirm this conclusion.

K32R is predicted to have an increased binding affinity to p75NTR. K32R was active through TrkA as treatment induced neuronal differentiation, phosphorylation of ERK and AKT and protected PC12 cells from TG induced apoptosis, to levels similar to WT_NGF. The variant K32R exhibited strong increase in p75NTR binding, it was twice as effective at binding to p75NTR then WT_NGF. Previously published data suggested that K32 was important to the NGF/p75NTR interaction as the variant K32A exhibited strongly decreased p75NTR binding . A double variant of K32A, K34A or K32A, K35A and a triple variant of K32A, K34A, K35A exhibited no p75NTR binding and retained wtNGF TrkA binding (Ibáñez et al., 1992; Rydén et al., 1997). However, the substitution of a lysine with arginine, which like lysine, possesses a hydrophobic and positively charged side chain. Unlike previous studies the amino acid substitution was not expected to have a negative effect on the affinity of NGF for p75NTR. The K32R variant is predicted to improve hydrogen bonding with A75 of the receptor, in contrast to the K32A NGF variant which would lack this hydrogen bond
thus disrupting receptor binding. A biological activity assay for p75\textsuperscript{NTR} would demonstrate if this increase in binding affinity correlates with an increase in the biological activity.

The R69D variant was predicted to have decreased affinity for p75\textsuperscript{NTR}. The TrkA biological assays are consistent with this observation. R69D demonstrated no TrkA biological activity, as it did not induce neuronal differentiation, phosphorylation of ERK or AKT and did not protect PC12 cells from TG-induced apoptosis. The R69D variant also displayed no binding to immobilised TrkA or p75\textsuperscript{NTR}. The variant was predicted to decrease hydrogen bonding with the receptor, however R69 is also involved in intramolecular hydrogen bonding with R16 in the NGF homodimer (Bradshaw et al., 1994). It is likely that this mutation caused structural changes within the NGF rendering it inactive. NGF ELISA and the Western blotting results indicate a possible disruption in the structural change in the R69D variant. Detection of the R69D variant by Western blotting indicated that the variant was expressed at mildly reduced concentrations compared with the WT\_NGF. However, detection of R69D by NGF ELISA showed that R69D was secreted at low concentrations of ~ 24 ng/ml. ELISAs are developed for the detection of proteins in their native form and cannot detect denatured protein. The inconsistency between the Western blot and the ELISA might indicate a change in the structure of the R69D variant, making it functionally inactive.

A98I is predicted to have increased affinity for p75\textsuperscript{NTR}. A98 has not shown to be involved in the NGF/TrkA interaction (Wiesmann et al., 1999). A98I maintained activity at TrkA as it induced neuronal differentiation, phosphorylation of ERK and AKT and could protect PC12 cells from TG-induced apoptosis. A98I shows a 10 % increase in binding to TrkA and a 50 % increase in p75\textsuperscript{NTR} binding. A biological activity assay for p75\textsuperscript{NTR} would demonstrate if this increase in binding affinity has any effect on biological activity. A98I is binding with higher affinity to p75\textsuperscript{NTR} however, without a biological assay it is not known if it is acting as an agonist or antagonist for p75\textsuperscript{NTR} signalling.
To summarise, the NGF variant H4D shows promising results as a possible agonist of TrkA without disruption in p75\textsuperscript{NTR} binding affinity, and thus activity. The I31R, K32R and A98I variants show promising binding activities at the p75\textsuperscript{NTR}, however there activity through the p75\textsuperscript{NTR} pathway needs to be characterised further. The increase in binding affinity, seen for K32R and A98I, might not confer an increase in biological activity. The NGF variants might bind with high affinity to p75\textsuperscript{NTR} but be biologically inactive, and acting as an antagonist not agonist of p75\textsuperscript{NTR}.

The FoldX programme uses already determined crystal structures to design the NGF variants, thus the mutations designed are dependent on the crystal structures. However, in the crystal structure for NGF in complex with TrkA, the TrkA sequence resolved ends at domain 5 and possible interaction sites with loop 2 (L2) and loop 4 (L4) of NGF are not defined (Wiesmann et al., 1999; Wehrman et al., 2007). L2 and L4 have sequence diversity among the neurotrophin family and generation of chimeric NGF and brain derived neurotrophic factor (BDNF) variants demonstrated that L2 and L4 were important for NGF function (Ibáñez et al., 1991). On structural analysis of the NGF ligand in comparison to NGF in complex with TrkA, and p75\textsuperscript{NTR} a difference in L2 of the NGF was observed. The 10 aa loop from A40 to R50 of NGF was determined to be a beta hairpin, in the complex with TrkA. This region had no secondary structure in the crystal structure determined for the NGF ligand or for NGF in the co-crystallization with p75\textsuperscript{NTR}. This further suggests that this region plays an important role in the interaction and specificity of NGF for TrkA (Fig. 1.11) (McDonald et al., 1991; He and Garcia, 2004; Wehrman et al., 2007). The FoldX variant S47D is contained within this loop and is predicted to have increased binding to p75\textsuperscript{NTR}, however it would be interesting to see what effect this mutant had on TrkA activity.

The main limitation of the work presented is the lack of an assay to test for p75\textsuperscript{NTR} activity. The NGF variants are tested for binding to p75\textsuperscript{NTR}. This does not allow us to completely determine the role the NGF variants have at the receptor as it does not
determine if they are acting as agonist, enhancing the NGF/p75NTR, or antagonist, disrupting and blocking the WT_NGF or proNGF activity.

Two approaches were used to develop a p75NTR assay and have been summarised in Table 3. The first approach was to stimulate NGF/p75NTR activity using SK-N-MC cells that express p75NTR and have little or no TrkA (Biedler et al., 1973). However, using similar conditions to published data (Kuner and Hertel, 1998; Niederhauser et al., 2000), failed to give detectable levels of cleaved caspase-3 by Western blotting. PC12 cells and RN22 schwannoma cells were then treated with proNGF to induce cell death in PC12 cells and RN22 schwannoma cells as reported in the following studies (Fahnestock et al., 2001; Harrington et al., 2004; Nykjaer et al., 2004; Masoudi et al., 2009). The conditions used for proNGF induced apoptosis were those used in (Pagadala et al., 2006). However, treatment with proNGF had no effect on cell death as determined by DEVDase assay and on analysis of nuclear morphology.

The rational of this assay was if an NGF variant, with higher binding affinity for p75NTR then proNGF, would displace proNGF from the receptor and favour cell survival. This assay could be verified in-vitro, by altering the binding assay developed. The plate could be coated with p75NTR and proNGF could incubate the receptor with one concentration of in the presence of increasing amounts of variant, to determine if the proNGF could be displaced from the receptor. The concentration of NGF and the concentration of proNGF would then be determined. This assay would determine if an NGF variant would be able to compete for binding to the p75NTR and displace pro-apoptotic ligands from the receptor and hence, favour pro-survival signalling. A version of this assay could also be used to analyse the ability of the NGF variant to disrupt the affinity between the Aβ peptide and p75NTR. The soluble and insoluble forms of the Aβ peptide are reported to bind to p75NTR with a high binding affinity 4-7 nM and 12 nM respectively. Increasing concentrations of mature NGF disrupted the binding of the Aβ peptide for p75NTR (Yaar et al., 1997; Kuner et al., 1998). The NGF variants shown to have higher
affinity for p75NTR but unaltered TrkA activity, K32R and A98I, may be able to displace Aβ peptide or proNGF from the p75NTR and act as a potent pro-survival signal for the cells.

The second approach taken in the development of a p75NTR assay, was to activate NGF induced NF-κB signalling. HEK293_p75 were used to test the activity of NGF through the p75NTR receptor. However, NGF treatment did not induce the activation of NF-κB, as determined by phosphorylated IkBα. MDA-MB-231, a breast cancer cell line has been reported to activate the NF-κB pathway on NGF treatment (Descamps et al., 2001). MDA-MB-231 cells were treated with NGF and the effect NGF had on IkBα was analysed. There was modest, and often inconsistent increase in the phosphorylation in IkBα. The MDA-MB-231_NF-κB promoter cells were then used and they showed high basal levels of NF-κB activity, which was decreased after treatment with NGF inhibitors Ro 08-2750 and Pep5. These cells could be used as an assay to test p75NTR receptor antagonism.

Another possible assay developed for p75NTR receptor antagonism is in the HG3 and Mec1 CLL cells. These cells displayed a dependence on the NGF/p75NTR pathway for their survival as inhibition of the p75NTR receptor pathway decreases the viability of the cells. Further analysis of this pathway might reveal what role NGF has in the survival of these cells and could then be used for assay development.

Other possible assays that could be tried are changing the cellular context of the cell, changing the levels of receptors and adapter proteins, to enhance the p75NTR-induced activity. Previous studies have shown p75NTR mediated NF-κB activation on co-expression of adapter proteins such as the atypical protein kinase C-interacting protein p62 (Wooten et al., 2001) or interleukin 1 receptor-associated kinase 1 (IRAK1) (Mamidipudi et al., 2002). In these studies, over-expression of adapter proteins increased NGF-induced NF-κB activity (Wooten et al., 2001; Mamidipudi et al., 2002). This could be used to determine the effect the NGF variants have on p75NTR activity. Another alternative assay is to show that NGF variants can induce
NF-κB activity in stressed cell conditions or that NGF variant treatment increases the TNF induced activation of NF-κB (Bhakar et al., 1999).

In spite of the fact that the biological activity of the NGF variants could only be determined through the TrkA receptor, the NGF variants H4D, I31R, K32R and A98I show promise. The I31R variant is interesting as it displays no biological activity at the TrkA receptor but still retains binding to p75\textsuperscript{NTR}, suggesting that it is an NGF variant that may have specific activity. Further analysis on the biological activity of the I31R variant at p75\textsuperscript{NTR} would verify this. The H4D variant shows enhanced TrkA activity and no change in p75\textsuperscript{NTR} affinity. The expression of the H4D variant was greatly decreased, however, the expression of the H4E variant showed no difference to WT_NGF. The D and E are similar amino acids both are negatively charged amino acids containing an amine group. The difference between the amino acids is that glutamic acid contains a longer carbon chain. As the difference between the amino acids mutations are so minimal, the difference in cell expression was not expected. For further studies with this variant different cell lines would be tested, such as CHO and Hela cells, to express this variant. The increased TrkA biological activity and unaltered affinity for p75\textsuperscript{NTR} that H4D variant displays suggests that it would be a good variant for combination mutations with one of the variants, K32R and A98I, that showed a marked increase in p75\textsuperscript{NTR} affinity and no altered TrkA biological activity. Theoretically, such a combination variant would act as a potent pro-survival factor at the TrkA receptor and could act as an antagonist at p75\textsuperscript{NTR} decreasing the proNGF and Aβ induced cell death. Determining the K32R and A98I variants activity at p75\textsuperscript{NTR} is necessary for further analysis on the NGF variants.
Chapter 6. Conclusions and future perspectives
6.1 Conclusions and future perspectives

This thesis extends the role of NGF as a potent pro-survival factor that can protect cells from apoptotic stimuli and increase the long-term survival of the cells. It is an effective pro-survival factor as it can protect cells both upstream and downstream of MOMP by two distinct signalling pathways, and thus causes a dual block in the apoptosis pathway. NGF stimulation of PI3K/Akt signalling increases the defence at the mitochondria by altering the levels of the ‘guardians of the mitochondria’, the Bcl-2 proteins, and by stimulation of ERK signalling, which decreases pro-apoptotic caspases from the cell that would otherwise cause irreversible damage. The data presented here supports the theory that the combined effect of these pathways, results in the long-term survival of cells undergoing apoptosis via the intrinsic apoptosis pathway.

In general terms these studies show a potent block in apoptosis, upstream and downstream of MOMP by NGF. It is likely that NGF’s dual block in apoptosis has developed to sustain the survival of neuronal cells during development, particularly the sympathetic neurons which are dependent on NGF for their survival from approximately embryonic day 16 to 1 week postnatally (Coughlin and Collins, 1985). Extensive apoptosis occurs in the developing nervous system as neurons are produced in excess during development and compete with each other, in acutely stressed environments, for limited amounts of the survival-promoting trophic factors secreted by target tissues (Deshmukh and Johnson, 1997). This is not the first report to show NGF as a potent pro-survival factor that is able to protect cells in an advanced stage of apoptosis. Deshmukh et al., 1996 reported that NGF protected sympathetic neurons at a time point indistinguishable from caspase inhibition with boc-aspartyl-(OMe)-fluoromethyl-ketone (BAF). Caspase inhibition alone is rarely considered sufficient to increase the long-term survival of the cell. On caspase inhibition the cells have usually suffered extensive damage to the mitochondria and are said to die in a caspase-independent cell death, from the release of apoptosis-inducing factor and endonuclease G release from mitochondria (Galluzzi et al., 2012). During neuronal apoptosis there is a characteristic decrease in protein synthesis.
however, NGF treatment prevented the loss decrease in protein translation. This is in contrast to BAF treatment where although the cells did not undergo apoptosis they still showed a decrease in protein translation. The authors suggested that NGF was not acting as just a caspase inhibitor and was having additional activity to BAF treatment. A separate study then showed that NGF can extend the commitment point of apoptosis in post-mitotic neurons, beyond cytochrome c release until the point when mitochondrial transmembrane potential is lost (Deshmukh et al., 2000). Results in this thesis show that cells treated with NGF after caspase activation, do not undergo caspase-mediated apoptosis and retain the ability to survive and proliferate.

Therapeutically, these studies are of most relevance to neurodegenerative diseases, such as AD lack of effective therapies, current therapies ameliorate symptoms, but they do not prevent disease progression (Lad et al., 2003). Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that is characterised by a loss in cognitive functions and memory. The number of AD patients was estimated at 36 million in 2010, due to the aging population this number is predicted to triple by 2050 (Huang and Mucke, 2012). This is set to put a huge financial burden on health care systems worldwide with the estimated cost for treatment in 2010 of $604 billion (Huang and Mucke, 2012). NGF has been extensively studied in various models of disease, particularly in AD due to its ability to prevent the loss of cholinergic neurons in this disease (Fischer et al., 1987; Tuszynski et al., 1990; Capsoni et al., 2002; Tuszynski, 2007). Clinical trials with NGF-based therapies in AD have produced modest but promising results. Using targeted NGF treatment, by stereotaxic surgery, and continuous NGF treatment by implantation of NGF-expressing fibroblasts improved side effects associated with NGF treatment, such as pain, weight loss and antisera generation against NGF. Currently there are Phase I and Phase II clinical trials underway that allow the targeted treatment of BFCN with NGF (Tuszynski et al., 2005; Mandel, 2010; Wahlberg et al., 2012).

NGF treatment in AD has its limitations. In AD, basal forebrain cholinergic neurons have lower TrkA and NGF expression levels (Scott et al., 1995). NGF/TrkA signalling supports survival of these neurons and thus it is thought to be the cause of
neuronal cell loss in this area (Williams et al., 1986; Fischer et al., 1987; Kromer, 1987). More recently, proNGF is thought to contribute to the cell death in AD (Fahnestock et al., 2001; Kichev et al., 2009). ProNGF was found to be the predominant form of NGF in AD brains, isolation of which was found to bind to p75NTR and induced apoptosis through p75NTR (Pedraza et al., 2005). The Aβ1-42 peptide has been shown to induce cell death through the p75NTR both in vitro, in cells overexpressing the receptor (Yaar et al., 1997; Kuner et al., 1998) and in primary hippocampal cultures (Sotthibundhu et al., 2008), and in vivo in basal forebrain cholinergic neurons (Sotthibundhu et al., 2008).

In spite of the disruption in NGF pro-survival signalling in AD brains, it still shows therapeutic effects on treatment (Tuszynski et al., 2005; Mandel, 2010; Wahlberg et al., 2012). This shows that NGF is a potent pro-survival factor capable of increasing neuronal cell survival and cognitive function in the patients. However, it also indicates a disconnection between AD treatment and what is reported in the literature and exhibits the limitations of NGF in the treatment of AD i.e., the pro-survival TrkA receptors are decreasing and the pro-apoptotic proNGF and Aβ1-42 peptide are increasing.

To this end we molecularly engineered NGF to enhance its pro-survival potential through TrkA and thus its therapeutic potential. We generated a method for the transient expression and purification of recombinant NGF variants in mammalian cells. As the work presented here shows that the NGF is secreted into the media and exhibits biological activity when conditioned media is used. This is directly relevant to the therapeutically produced NGF as it is secreted from fibroblast cells (Mandel, 2010; Tuszynski et al., 2005; Wahlberg et al., 2012). The NGF variants were designed by the FoldX computer algorithm to have altered binding affinities for TrkA and p75NTR. Variants were chosen based on their predicted increased binding to TrkA and p75NTR and their location in the receptor-protein complex. 8 variants were generated H4D, H4E, I31R, K32R, G33M, R69D, H84Q and A98I and there pro-survival activity was analysed. Of these H4D, K32R and A98I show promise as a
possible variant NGF capable of enhancing pro-survival signalling in AD. H4D can protect PC12 cells from TG induced apoptosis similar to WT_NGF at a five-fold lower concentration. K32R and A98I show pro-survival activities similar to that of WT_NGF but also show increase in the binding affinity for p75<sup>NTR</sup>, which could prevent the unfavourable effects of p75<sup>NTR</sup> in AD.

As mentioned earlier the soluble Aβ peptide and proNGF have been shown to induce apoptosis through p75<sup>NTR</sup>. It would be of interest to analyse the ability of the NGF variants to competitively bind to p75<sup>NTR</sup> preventing Aβ peptide and proNGF binding, thus preventing p75<sup>NTR</sup>-induced apoptosis in AD. However, as mature NGF has also been implicated in neuronal cell death during the development of the nervous system it would be important to test the NGF variants’ abilities to protect neuronal cells from apoptosis and their ability to induce apoptosis in the absence of TrkA or in cells with low expression of the receptor, as is seen in AD.

NGF has suboptimal pharmacological properties, such as low stability in serum (half life of a few minutes), negligible oral bioavailability (Saltzman et al., 1999), minimal blood brain barrier (BBB) penetration and restricted diffusion within the central nervous system (CNS) (Poduslo and Curran, 1996). Small–molecule modulation of the neurotrophins and their receptors is a strategy used in neurotrophin research to overcome these problems (Massa et al., 2006; Longo and Massa, 2013). Some such compounds are TrkA activators asterriquinone (1H5) and monoindoyl-quinone (5E5) which were shown to promote PC12 cell survival at micromolar concentrations (Lin et al., 2007). Two compounds, LM11A-31 and LM11A-24 were also found to be capable of inhibiting the death of cultured neurons at picomolar concentrations (Massa et al., 2006). These compounds inhibited the binding of pro-NGF to the extracellular domain of p75<sup>NTR</sup> and diminished pro-NGF-induced apoptosis of oligodendrocytes (Massa et al., 2006). Treatment with these compounds after a spinal cord contusion injury, led to decreased binding of proNGF to p75<sup>NTR</sup> (Tep et al., 2013). In a model of Alzheimer’s disease in which amyloid-β oligomers are added to cultured hippocampal neurons or slices, LM11A-31 and LM11A-24
inhibited amyloid-β-induced deleterious signalling (Yang et al., 2008). These compounds were found in an in-silico screen for compounds that would interact with loop 1 of NGF, thus disrupting (site I) in the p75NTR/NGF interaction.
7 Capter 7. Bibliography


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Appendix I

8 Appendix I

The cDNA of the 8 NGF variants were produced by the megaprimer method and ligated into pcDNA3.1 vector using restriction sites BamHI and NotI. The plasmids were sent for sequencing to confirm the cDNA mutation was generated. Multiple sequence alignment of NGF amino acid sequences.
Appendix I

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Appendix IB. Additional NGF variants designed by FoldX

The NGF variants were prioritised based on the free energy of unfolding determined by the FoldX computer algorithm. The algorithm described other single point mutations that were predicted to have altered receptor binding. Multiple sequence alignment of additional cDNA sequences of NGF variants generated.
9 Appendix II

NGF mutations relevant to this study are described here and displayed in a Table. Appendix IIA displays the single point mutations and Appendix IIB displays the combination mutants generated. All information is presented as a percentage or fold change compared to wild type NGF. No assays for p75\textsuperscript{NTR} were preformed and thus are not described in the tables.

List of Abbreviations used

NOG – neurite outgrowth of PC12 cells

phopo-Trk – T409 phosphorylation of the TrkA receptor
<table>
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<th>Amino acid</th>
<th>Amino acid location</th>
<th>Mutation</th>
<th>Effect TrkA binding</th>
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<th>Effect p75 binding</th>
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(Ibáñez et al., 1992)
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(Ibáñez et al., 1990)
(Capsoni et al., 2011)
(Guo et al., 1996)
(Ibáñez et al., 1990)
### Additional information

#### Effect p75 binding

- **Loss in affinity to A875-cells**
- **In NGF secretion into media of COS cells**
- **Undetectable secretion of NGF into media of COS cells**

#### Effect TrkA binding

- **73% decreased biological activity**
- **35% loss in biological activity**
- **35% decrease in biological activity**
- **90% loss in activity**
- **Reduced NOG 35%**

#### Effect TrkA binding

- **Reducing binding affinity 10 fold**
- **99% decrease in binding affinity to PC12 cells**

### Combination mutants

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<th>Effect p75 binding</th>
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<td>R100G/R100K</td>
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<td>T26A/T27A/T29A</td>
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</table>

**Publication**

- (Woo and Neet, 1996)
- (Ibáñez et al., 1992)
- (Ibáñez et al., 1990)
- (Ibáñez et al., 1992)