Creating recombinant fusion protein conjugates for targeting SNARE protease into neuroendocrine cells

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Creating recombinant fusion protein conjugates for targeting SNARE protease into neuroendocrine cells

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Declaration

I hereby certify that this material, which I now submit for assessment in the programme of study leading to the award of Master of Science is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 4th July 2016

Dedication

I would like to dedicate this work to

Shivji….

Acknowledgement

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List of Abbreviations

Abbreviation	**Explanation**
/A	Serotype A
/AΔH_{CC}	LC.H_N.HC_N/A.ΔH_{CC}
/AΔH_{CC}N	LC.H_N.HC_N/A.ΔH_{CC}.βNGF (see in page xv)
BoNT	Botulinum toxin

βNGF	β form of Nerve Growth Factor
BoNT/A, B, C1, D, E, F and G	Botulinum neurotoxin serotype A, B, C1, D, E, F and G
BOTOX®	Botulinum toxin A-haemagglutinin complex
BSA	Bovine serum albumin
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
C-terminal	Carboxyl-terminal
DAPI	4', 6'-diamino-2-phenylindole
DC	Di-chain
DMEM	Dulbecco's modified Eagle's medium
DRG	Dorsal root ganglion
DTT	Dithiothreitol
D/W	Molecular biology grade deionized water

ECL,	Enhanced chemiluminescence
EDTA	Ethylenediamintetracetic acid
HBS	Hanks buffered salt solution
H_C	Binding domain
HEK 293	Human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_N	Translocation domain
TRPV1	Transient receptor potential vanilloid subfamily, member 1
ICC	Immunocytochemistry
ICNT	International Center for Neurotherapeutics
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani

LC	Light chain
LCH_N	Light chain and translocation domain
$LC.H_N.H_{CN}/A\Delta H_{CC}$	Comprised of the light chain, translocation domain and N-terminal heavy chain of BoNT/A, but lacking the binding domain
$LC.H_N.HC_N/A.\Delta H_{CC}.\beta NGF$	Comprised of the light chain, translocation domain and N-terminal heavy chain of BoNTA with the binding domain replaced by βNGF
N	Sample size
NGF	Homo dimer of β form of Nerve Growth Factor
NSAIDs	Nonsteroidal anti-inflammatory drug
O.D.	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween -20
PCR	Polymerase chain reaction

PEI	Polyethylenimine
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
PVDF	Polyvinyllidene Fluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNAP-25	Synaptosomal-associated protein of Mr = 25 K
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SP	Substance P
SV2	Synaptic vesicle protein 2

TBS	Tris-buffered saline
TBST	TBS with Tween-20
TM	Transmembrane
Vamp	Vesicle-associated membrane protein
WB	Western blot

Abstract

Title: Creating recombinant fusion protein conjugates for targeting SNARE protease into neuroendocrine cells

Author: Nilesh Baliram Patil

Chronic pain poses major healthcare and economic burdens. The aim of this study was to develop a strategy for pain management by generating a biotherapeutic that requires targeting of soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE) within the hyper-active sensory nerves. This entailed conjugating nerve growth factor (NGF) and the SNARE-cleaving protease and internalization domains of botulinum neurotoxin A (BoNT/A). The methodology involved genetic fusion and use of a protein stapling technology with a view to replacing the BoNT/A C-terminal neuronal binding subdomain (H_{CC}) with βNGF which is a specific ligand for preferential sensory neuronal targeting. A first generation of BoNT/AΔH_{CC} fused to βNGF was expressed in *E. coli* and purified. An alternative strategy was also adopted with a view to stapling a recombinant BoNT/AΔH_{CC}-SNAP-25 protein (without the neuronal binding domain) with Vamp2.βNGF produced in *E. coli*, and a synthetic syntaxin-1 peptide via SNARE complex formation. Treatment of PC-12 cells with BoNT/AΔH_{CC} fused to βNGF did not give any cleavage of intracellular SNAP-25. Failure to redirect BoNT/A protease into PC-12 cells was attributed to inactivity of the βNGF producing *E. coli*. After a number of attempts, the precursor peptide (Pre-Pro signal peptide) of NGF was identified as an essential element in making a biologically-active βNGF. Encouragingly, after inclusion of this leader peptide sequence into a Vamp2.βNGF construct, it was expressed as an active protein, although with low yield. The latter was addressed by creating a construct encoding a Pre-Pro signal peptide followed by βNGF.Vamp2 in the hope of increasing the yield. Expression of the

resultant protein was not attempted. In summary, expression of functionally-active βNGF.Vamp2 was achieved. Further optimization is needed before its conjugation to the BoNT core-therapeutic domains, for redirecting SNARE-cleaving protease into hyper-active sensory nerves.

1 Introduction

Preamble

A European consensus report by Pfizer (Chronic pain management 2010) revealed that the total costs to the Irish GDP arising from chronic pain were estimated at €5.34bn per year. The same report asserted that one in three people in Ireland who have chronic pain are struggling with their condition and fear that their illness and absences from work may mean loss of their jobs. Effective means to manage chronic pain are urgently needed to both improve the quality of life of sufferers and reduce health care costs (Katz *et al.* 2010, Lippe *et al.* 2010). This is borne out by a survey on chronic pain within 16 European countries conducted by Breivik *et al.* 2006, which stated that chronic pain occurred in 19% of 46394 respondents, two thirds of which were moderately affected and one third were severe cases, having a negative impact on their daily activities, social and working lives. Another study carried out by Rafferty *et al.* 2011 in the Republic of Ireland reported that chronic pain was prevalent within 35.5% of the 1204 respondents. Their study reported that 12% of respondents were unemployed or were working on reduced working hours due to chronic pain. Blyth *et al.* 2003 highlighted the effects of chronic pain on the ability to work and the consequent implications for the economy. This report included factors such as, the cost related to the loss of productivity due to time off work, reduced work effectiveness and the cost in loss of skills in cases where people reduced their working hours or stopped working altogether.

The International association for the study of pain (IASP) published on their website a meta-analysis on prevalence of chronic pain based on 13 worldwide studies. The data reported in these studies indicate a higher prevalence of chronic pain suffers among females and significant use of health care resources by chronic pain sufferers. The prevalence figures show that the costs associated with severe chronic pain are significant

for health systems in the countries surveyed (International association for study of pain 2003).

1.1 Neurobiology of pain

IASP defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Bonica 1979; National institute of Health 2014). The word pain is derived from the Greek word meaning ‘penalty’.

1.1.1 Nociceptors

Receptors on the peripheral ending of different nerves respond to a variety of stimuli. Their shape, location and field of reception indicate that they are designed for response to their stimuli. The pain receptors (nociceptors) have primitively-organized nerve endings and a weed-like appearance. The strength of the stimulus is a critical factor in the production of pain. When a certain threshold of intensity has been surpassed, any stimulus can be interpreted as painful to nociceptors. This threshold stimulus is called the noxious stimulus and it causes tissue damage (Hall 2002; Julius and Basbaum 2001).

1.1.2 Afferent fibers

An afferent nerve fiber is the nerve fiber (axon) of an afferent neuron (sensory neuron). First order afferent nerve fibers are classified according to their size and conduction rates (Fig.1). A-fibers are the largest in diameter and most rapid in conduction rates. B-fibers are intermediate in size and have a slow conduction rate. C-fibers are smallest in size and slowest conduction rate.

There are two types of primary afferent nociceptors, Aδ and C fibers. Aδ is one of four types of A-fibers (Aα, Aβ, Aγ, Aδ). Aδs are the most rapid pain conducting fibers compared to C conducting fibers. Pain signals conducted by Aδ fibers are described as sharp, shooting and intense, with a velocity of ~6-25 m/s. Aδ fibers are lightly myelinated, medium diameter size (1-5 μm) and have free nerve endings that transduce high threshold thermal, mechanical and chemical noxious stimuli. Aδ fibers make up 20% of pain afferents and mediate pain from superficial areas such as skin. C fibers are the slowest pain conducting. The pain conducted by C fibers is described as steady slow (<2 m/s) and constant. C fibers are unmyelinated and their diameters are small in size. C fibers account for 80 % afferent pain fibers and arise from non-localized polymodal nociceptors *i.e.* nociceptors that respond to wide range of noxious stimuli. Cell bodies of C fibers are located in dorsal root (spinal cord) or trigeminal ganglia (brain) of spinal cord and their axons terminate in the dorsal horn Hall James 2002; Julius and Basbaum 2001).

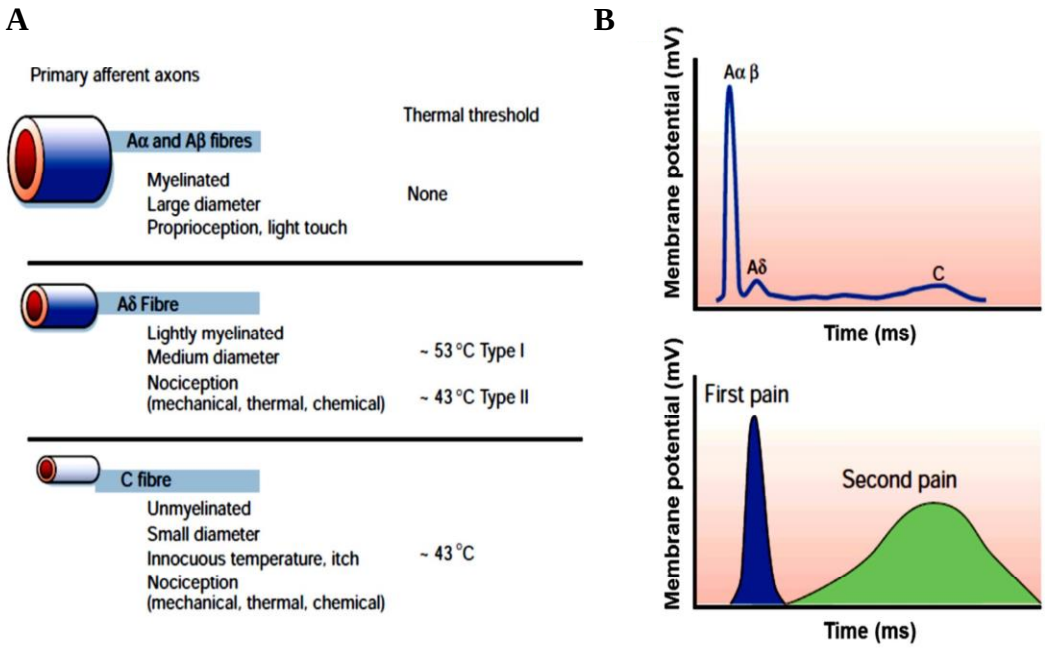

Fig.1 Classification of nociceptors. *A) Peripheral nerves consist of myelinated afferent fibers Aδ (medium-diameter), Aα, β (large-diameter) and unmyelinated afferent C fibers (small diameter). B) Schematic of peripheral nerve action potential. Aδ and C fibers differ in their conduction velocity (6-25 and ~1 m/s, respectively) and represent their first and second responses to painful stimuli. Image taken from Julius and Basbaum, 2001.*

1.1.3 The spinal cord and transmission of signal

The role of the spinal cord in the transmission mechanism of pain is important because information processing occurs in the dorsal horn grey matter. The spinal cord consists of two symmetrical halves, the dorsal median sulcus and the ventral median fissure separated with a small central canal in the center. The canal is surrounded by grey matter composed of nerve cell bodies, dendrites and synaptic connections. The outer region of the spinal cord contains white matter comprising of ascending and descending nerve fibers. The grey matter forms an H butterfly shape and the dorsal horn forms an arrangement of six layers (numbered in Fig. 2) as per their Rexed classification system (identified in the early 1950s by Bror Rexed to label portions of the grey columns of the spinal cord). Acute nociceptive pain, stimulates the release of pain mediators from primary afferent terminals that project to laminae I, IV and V in the spinal cord dorsal horn. Having been relayed to the lateral spinothalmic tract, pain signals from Aδ fibers travel to the ventrobasal to posterior nuclei or to the thalmic relay nuclei and after that to the post central somatosensory area of the cerebral cortex. Similarly, signals sent by the C fibers having reached the medial nucleus of the thalamus, continue to the prefrontal somatosensory region of the cerebral cortex (Rudolf *et al* 2007).

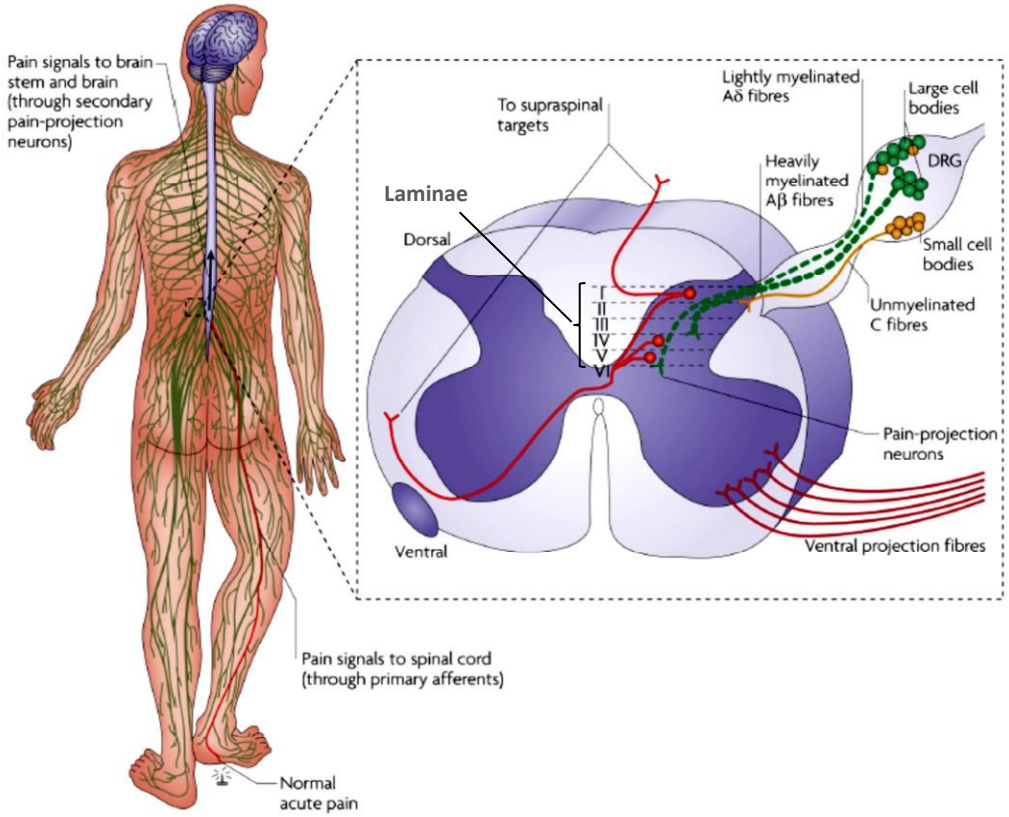

Fig. 2 Spinal cord and transmission of pain signals. *Pain receptors transmit pain signals to the dorsal horn of spinal cord. Primary afferent terminals that project to dorsal horn laminae I, IV and V, release pain mediators upon acute pain stimulation. Aβ, Aδ and C fibers also project to laminae II–VI. Dorsal root ganglia (DRG) propagate pain signal to dorsal spinal cord, brain stem and eventually to the brain where pain is perceived. Image taken from Milligan and Watkins, 2009.*

1.1.4 Classification of pain

Pain is an unpleasant subjective sensory experience induced by noxious stimuli, inflammation or damage to the nervous system which can be classified according to its type (Fig. 3). **Nociceptive pain** is the most widely experienced short-acting sensation to a noxious stimulus. It acts as an essential alarm system to alert the nociceptors *i.e.* sensory receptors in the peripheral nervous system, which are activated by painful stimuli. These pain signals are propagated from the periphery through the spinal cord, brain stem, and thalamus to the cerebral cortex, where the sensation is perceived. Nociceptive pain is an

important and essential alarm system that alerts the individual of fore coming danger to prevent the body from damage. Those that have inefficient nociceptive control systems, including patients with **congenital analgesia** (resulting from a nerve growth factor tyrosine kinase A mutation that leads to a loss of high-threshold sensory neurons), have reduced life expectancies due to their inability to protect themselves from harmful noxious stimuli (Miranda et al.,2002).

Acute, **inflammatory pain** is produced by tissue damage resulting in the release of peripheral inflammatory mediators to the affected inflamed area. The purpose of this short-term response is to encourage healing. Following inflammation, the sensory nervous system adapts by lowering the nociceptor activation threshold, so that normally non-painful stimuli produce pain **(allodynia)** and responses to noxious stimuli are enhanced **(hyperalgesia).** Once the site of injury heals, this pain usually subsides; however, in some cases it may become chronic.

Where there are lesions to the peripheral or central nervous systems, **neuropathic** pain results because of alterations in plasticity and the lowering of nociceptive thresholds, which in turn produce allodynia, hyperalgesia and **secondary hyperalgesia** (transfer of sensitivity to the non-injured area). (Woolf, 2004; Miranda *et al.* 2002)**.**

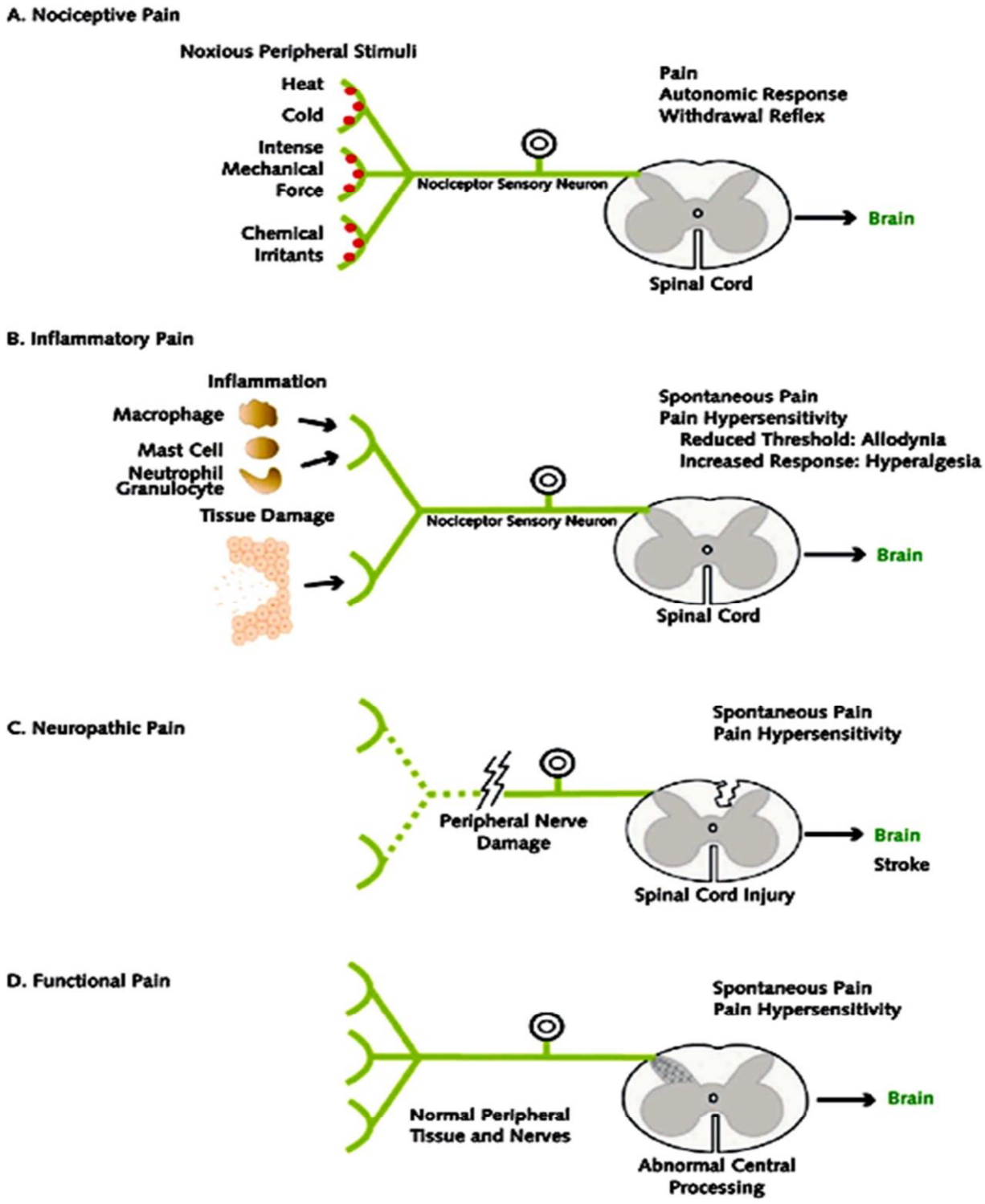

Fig. 3 Classification of pain. *Pain can be divided according to its types (nociceptive, inflammatory, and neuropathic). Nociceptive pain is produced in response to noxious stimuli, while inflammatory pain arises from tissue damage. Neuropathic pain is caused by lesion to the peripheral or central nervous systems. Image taken from Woolf, 2004.*

1.2 Chronic pain

Long-term inflammatory, neuropathic and functional pain is described as chronic pain which can arise in a range of conditions as for example arthritis, multiple sclerosis, chronic shoulder and back pain, chronic migraine and myofascial pain.

1.2.1 Peripheral sensitization and central sensitization

The presence of repeated stimuli in chronic pain situations reduces the threshold of nociceptive afferents and enhances the responsiveness of the peripheral terminals of nociceptors, resulting in what is termed as **peripheral sensitization.** This peripheral sensitization, resulting in normally innocuous insults being perceived as noxious, is induced by the action of neuropeptides and inflammatory mediators released by either Ca^{+2} regulated exocytosis such as substance P, CGRP, bradykinin, nerve growth factor, serotonin or neuropeptide Y or by non-exocytosis mechanisms (e.g. nitric oxide, prostaglandins or H^{+}) (Dray, 1995). Released neuropeptides and inflammatory mediators amplify the conduction of nociceptive impulses and, thereby, transfer to the cell bodies in the dorsal root or trigeminal ganglia, producing **central sensitization** (Latremoliere and Woolf, 2009; Woolf, 2011). Both sensitizations contribute enormously to inappropriate hyper-responsiveness to signaling substances.

1.2.2 Elevated levels of βNGF in chronic pain

NGF was discovered more than half a century ago as a protein involved in a variety of processes including cell signaling, cell differentiation, cell survival, and cell death within the peripheral nervous system (Wiesmann *et al.* 1999; McKelvey *et al.* 2013). There is growing evidence that NGF contributes to a variety of chronic states. McKelvey *et al.*

(2013) asserts that NGF plays an important role in mediating chronic pain, based on their findings that levels of NGF are abnormally elevated in a number of health conditions including rheumatoid arthritis, spondyloarthritis (Aloe *et al.* 1992; Halliday *et al.* 1998; Barthel *et al.* 2009), neurogenic overactive bladder, interstitial cystitis (Lowe *et al.* 1997; Oddiah *et al.* 1998; Jacobs *et al.* 2010; Liu *et al.* 2010) and cancer-induced pain (Manthy *et al.* 2010; Ye *et al.* 2011). In regard to the latter, Bradshaw *et al.* (2015) suggest that NGF and Pro-NGF (a precursor form of NGF) regulate breast cancer cell survival and enhance cell invasion, respectively. They suggested that prostate cancer cells over-express ProNGF whereby they induce neuritogenesis which increases cancer progression. The mechanism for this process remains unclear. Genetic mutations in the NGF and TrkA genes result in rare and severe condition of congenital insensitivity (loss of pain perception) (Indo *et al.* 1996; Einarsdottir *et al.* 2004; Carvalho *et al.* 2011; McKelvey *et al.* 2013). Genetic mutations in NGF or its receptor TrkA can cause the inability in humans to perceive pain (Rotthier *et al.* 2012 and McKelvey *et al.* 2013). These conditions come under the broader category of hereditary sensory autonomic neuropathies (HSANs) (Rotthier *et al.* 2012). Five types of HSAN's have been suggested (McKelvey *et al.* 2013; Rotthier *et al.* 2012), where in each condition the affected person loses the ability to perceive pain. HSAN IV and HSAN V are caused by mutations in TrkA and NGF, respectively (McKelvey *et al.* 2013).

1.2.3 Mechanisms of NGF in pain mediation

A subset of sensory neurons express TrkA (Fang *et al.* 2005; Franklin *et al.* 2009) and TRPV1 (a non-selective ligand-gated cation channel). In nociceptive neurons, transmission of the pain signal results when an external or internal source such as thermal, mechanical or chemical (acids and lipids) stimulate the influx of Ca^{2+} via the opening of a channel e.g. TRPV1 (Moran *et al.* 2004; Ramsey *et al.* 2006). Lewin *et al.* (2014) provided a detailed description of how NGF regulates pain in nociceptive neurons via the action on mast cells (Fig 4). Following injury or inflammation, innate immune response mechanisms involving the action of mast cells, interleukins (IL -1, IL3), cytokines *etc*. are activated in the affected area. While the mechanism is unclear, it has been hypothesized (Lewin *et al.* 2014) that these cells cause an increase in NGF expression. NGF decreases the threshold for action potential generation through (1) producing an internalized NGF-TrkA complex that activates phospholipase C in sensory neurons. Breakdown of phosphotidyl inositol phosphates (PiPs) by the latter causes a decrease in threshold at which the transient receptor potential vanilloid type I (TRPV1) (which binds PiPs) channel opens (also known as TRPV1 sensitization) (Chuang *et al.* 2001), and (2) NGF induces an upregulation of TRPV1 expression and its trafficking to the plasma membrane (Stein *et al.* 2006 and Ji *et al.* 2002; McKelvey *et al.* 2013). Kawamoto *et al.* (2002) suggests that NGF plays a crucial role in sensitizing the adjacent nociceptive neurons of the PNS. They stated that NGF facilitates the release of both other pain mediators such as histamines and prostaglandin and causes release of NGF from mast cells, leading to the formation of a positive feed-back loop in the transmission of pain signal. Hefti *et al.* (2006) and Mantyh *et al.* (2011) stated that NGF additionally plays an important part in sensitizing nociceptive neurons in the CNS via the upregulation of genes encoding substance-P, sodium ion channels such as Nav1.8, brain-derived neurotrophic factor; this central role of NGF signaling in pain is not

relevant to this study described here and will not be discussed further. Rather I will focus on the peripheral role of NGF signaling in pain.

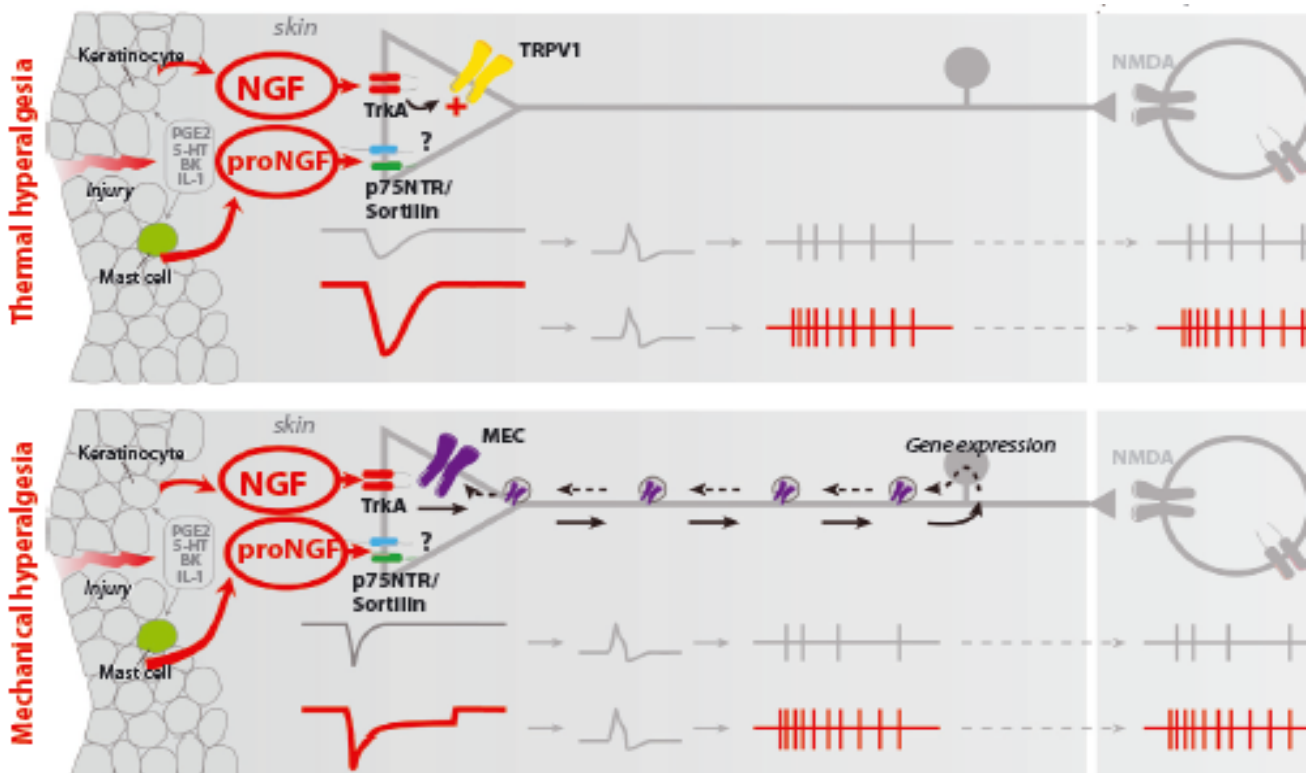

Fig. 4 Mechanisms through which NGF facilitates pain transmission. *An increase in NGF expression follows injury or inflammation. The factor binds to TrkA and activates phospholipase C (PLC) which leads to TRPV1 sensitization. NGF also increases expression and trafficking to the plasma membrane of TRPV1 and other nociceptive channels such as mechanosensitive channels (MEC), which decrease the threshold for action potential generation in nociceptive neurons. NGF also induces the release of pain mediators such as histamine, prostaglandins and NGF itself from mast cells which result in a positive-feedback loop that sensitizes nociceptive neurons (McKelvey et al. 2013). Image taken from Lewin et al. 2014.*

1.2.4 Structure of NGF, TrkA and NGF-TrkA complex

To understand the molecular mechanisms of NGF in mediating pain, it is important to understand the structure of NGF, Tropomyosin-related kinase A (TrkA) and the NGF-TrkA complex, described in Fig 5, 6 and 7.

1.2.4.1 Structure of NGF

The following description of the NGF follows that given by Weismann *et al.* (2001). NGF, as found in the submandibular salivary glands of mice, is a complex 7S structure, which consists of two copies of α-NGF, two copies of γ-NGF and a homo-dimer of βNGF. The stoichiometric arrangement is described as *a*2*b*2*g*2, around a two-fold symmetry axis relating the two βNGF molecules (Fig. 5A). To stabilize the *a*2*b*2*g*2 complex, conditions are created to allow two zinc ions to bind at the interfaces between the βNGF and the γ-NGF.

The α-NGF and γ-NGF belong to kallikrein family of serine proteases. The homo-dimer βNGF is the biologically-active form. The molecular weight of the βNGF monomer (Fig. 5B) is 13 kDa. The two βNGF monomers have three di-sulphide bridges linking them.

Each β-NGF monomer is expressed in an immature form namely, Pre-Pro peptide consisting of an 18 amino acid signal peptide (Pre), as well as a 103 residue Pro peptide. The γ-NGF is a specific protease which cleaves the Pre-Pro signal to its mature form, the βNGF monomer. The *α*-NGF is inactive and has been described as a ‘locked zymogen’. The importance of the Pre signal and the Pro peptide in the βNGF monomer became

evident in the course of this study for reasons which will be discussed in detail in Chapter 3. The homo-dimer βNGF is referred to as NGF in the following pages of this report.

Neurons respond to NGF via two cell surface receptors, namely TrkA and p75 (Luberg *et al.* 2015). In this study, p75 was not examined for a number reasons including the literature caveat that it causes cell apoptosis (Rabizadeh & Bredesen 2003). On the other hand, evidence from the literature intimated that TrkA receptor was responsible for cell survival and development (Huang & Reichardt 2003). Therefore, the experiments employed in this study were designed to exploit the reported benefits of TrkA.

The signaling of NGF in nociceptor neurons is mediated through two cell surface receptors, namely TrkA and p75 (Luberg *et al.* 2015). NGF binds to TrkA with high affinity (K_d ~10^{-11} M) by comparison with p75 (K_d ~10^{-9} M). In this study, p75 was not examined for a number reasons including the literature caveat that it causes cell apoptosis (Rabizadeh & Bredesen 2003). Instead, the experiments focused on TrkA for the following reasons: (1) TrkA signaling mediates neurotropic effects such as cell survival, differentiation and cell development. (2) NGF/TrkA signaling mediates the nociceptive functions of sensory neurons *i.e.* NGF/TrkA signaling pathway leads to the generation of action potentials for neuronal signaling as described in section 1.2.3

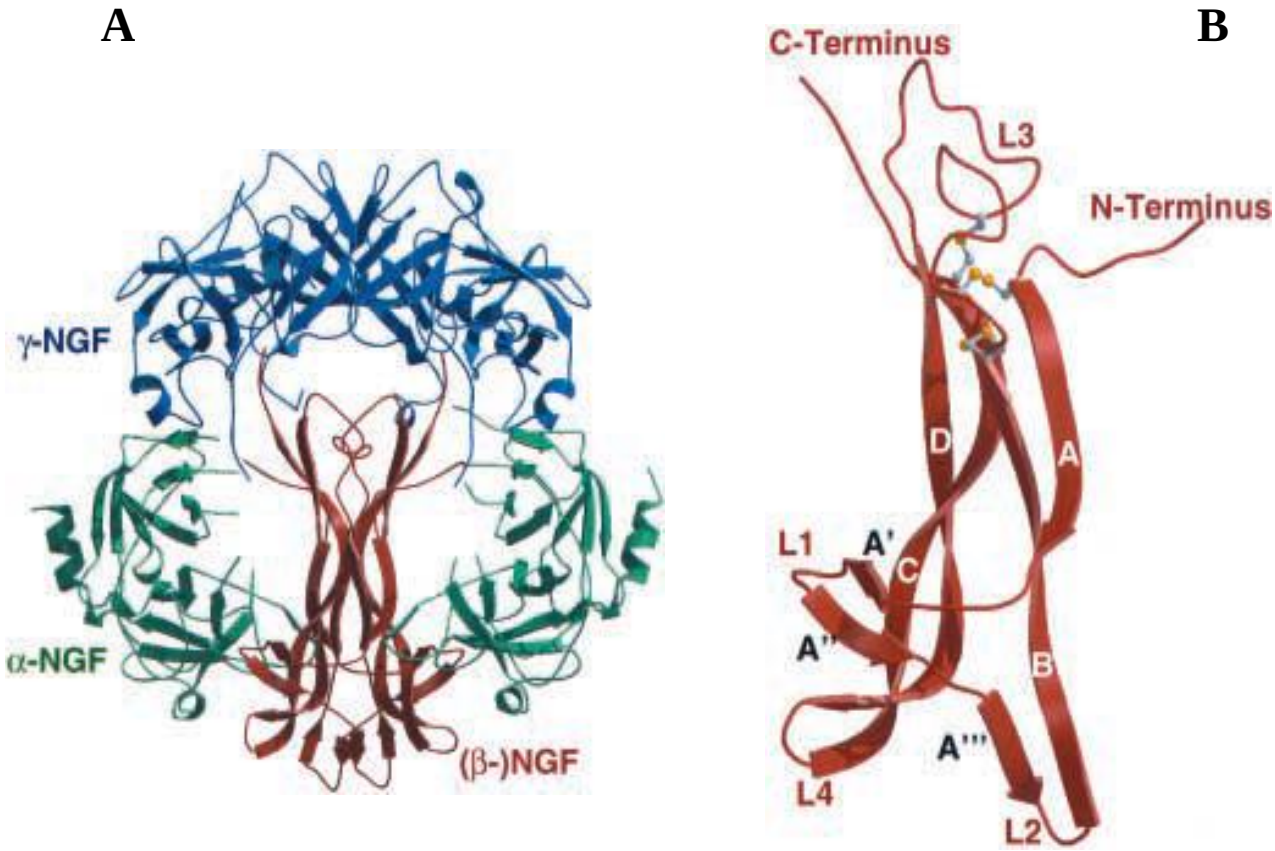

Fig. 5 Structure of the 7S NGF complex (PDB access code I SGF) and the NGF monomer (Protein Data Bank code 1BFT: *(A) 7S NGF complex with the homo-dimer of βNGF, depicted in red at the center, two copies of α-NGF shown in green, and two copies of γ-NGF in blue. (B) The secondary structure elements of NGF monomer are labelled and depicted according to McDonald et al. (1991). The termini as well as the loop regions, L1-L4, are labelled in red, and the cysteine residues forming the cysteine-knot motif near the top of the molecule are shown in gray and yellow in ball-and-stick rendering. Images taken from Weismann et al 2001.*

1.2.4.2 Structure of TrkA

Three forms of Tropomyosin receptor kinase (Trk) are expressed on cell surface of host of cells, TrkA, TrkB and TrkC; NGF binds to TrkA with a binding affinity of 10^{-11}KD, whereas different growth factors bind to the others (Luberg *et al.* 2015).

Trk receptors are glycoproteins that possess an extracellular ligand-binding domain, a hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain. Extracellularly, Trk receptors contain an arrangement of three leucine-rich 24-residue (LRR1-3) motifs which are bordered by two cysteine clusters (C1 and C2), followed by two immunoglobulin-like domains (Ig1 and Ig2). These are followed by a single transmembrane and a cytoplasmic domain that consists of a tyrosine kinase and several tyrosine-containing motifs. Phosphorylation of the cytoplasmic tyrosine in the TrkA receptor, regulates tyrosine kinase activity and provides phosphorylation-dependent recruitment sites for adaptor molecules and enzymes that mediate initiation of intracellular signaling cascades (Huang & Reichardt 2003; Marlin *et al.* 2015, Rabizadeh & Bredesen 2003) (Fig. 6).

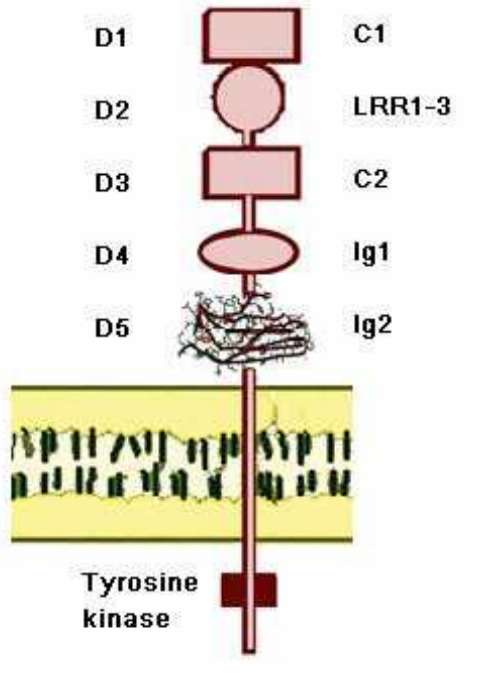

Fig. 6 Schematic presentation of the TrkA receptor. *The three leucine-rich motifs (LRR1-3) are flanked by two cysteine clusters (C1 and C2) and after these the two Ig domains. The*

tyrosine kinase domain is located at the intracellular domain. Image modified from Allen and Dawbarn 2006.

Resolution of the crystal structure of the NGF-TrkA complex showed that NGF engages with the second immunoglobulin-like domain, known as domain 5 (d5)(Fig. 6) of TrkA through two distinct patches. The first patch involves the four β-sheets present in the center of NGF molecule together with the first loop. The second patch is formed by the N-terminus of NGF, which is important in binding to TrkA (Wiesmann *et al.* 1999; Butte 2001).

The transduction of signal between NGF-TrkA is crucial in the development and growth in various host cells of peripheral nervous system (Huang & Reichardt 2003), including sensory neurons mediating pain (Marmigère 2006), postganglionic sympathetic neurons (Smeyne *et al.* 1994) and basal forebrain cholinergic neurons (Fagan *et al.* 1997). NGF secreted by the target cell interacts with TrkA on the axonal tip. The interaction of NGF to the extracellular domain Ig-C_2 domain of TrkA leads to conformational changes inducing TrkA receptor dimerization, followed by rapid transphosphorylation of five tyrosine residues on the cytoplasmic tail by the neighboring TrkA receptors. These phosphorylated sites on TrkA receptors serve as docking sites for adaptor for proteins containing phosphotyrosine-binding (PTB) or Sarcoma tyrosine-protein kinase (Src) homology 2 (SH2) domains such as Shc (Huang & Reichardt 2003). These cytoplasmic adaptor proteins bind to phosphorylation sites on the cytoplasmic tail where they are phosphorylated leading to activation of intracellular signaling pathways, Ras-mitogen activated protein kinase (MAPK) cascade pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt kinase pathway and phospholipase C γ -1 (PLC-γ1) (Huang & Reichardt 2003; Sanse *et al. 2011;* Marlin *et al. 2015).*

The signals arising NGF/TrkA complex are transported from the tip of axons to cell soma and then into dendrites by retrograde transport (An axonal transport mechanism responsible for moving molecules destined for degradation by lysomes from the axon back to the cell body) by the mechanism of internalization and signaling endosome formation.

TrkA undergoes internalization following engagement to NGF and this internalization at the tip of axons seems to be essential for retrograde survival signaling. Receptor-mediated endocytosis mechanisms can be broadly classified as being either clathrin-dependent or clathrin-independent, and recent findings suggest that TrkA may be internalized by both mechanisms. Controversy exists regarding the precise mechanism of TrkA internalization (Harrington and Ginty 2013; Doherty and McMahon 2009).

In clathrin-dependent mechanism for the endocytosis of TRKA, NGF causes a redistribution of the clathrin heavy chain to the plasma membrane and promotes the formation of protein complexes that contain TRKA, clathrin and adaptor protein 2 (AP2) (Doherty and McMahon 2009). Clathrin-independent mechanism (macropinocytosis) underlying TRKA endocytosis involves the formation of plasma membrane protrusions that eventually fuse together and engulf large volumes of membrane and extracellular fluid. Pincher, an NGF-upregulated GTPase, is involved in this mechanism of TRKA internalization (Harrington and Ginty 2013;Shao *et al.* 2002). The signaling events of both clathrin-mediated and pincher-mediated endocytosis for the internalization of TRKA and initiation of the NGF retrograde signal are poorly understood.

As mentioned earlier in this section, three major effector pathways for TRKA are the PI3K, ERK and PLCγ pathways. Of these, the PI3K and PLCγ pathways have the most important roles in receptor internalization. PI3K is activated by TRKA via the adaptor protein GAB1 (GRB2-associated-binding protein 1) (Harrington and Ginty 2013; Kaplan *et al.* 2000*).*

PI3K itself participates in internalization and the phosophoinositide products of its enzymatic activity bind and regulate several proteins implicated in endocytosis, including dynamin Krag *et al.* 2010, RAS-related protein RAB5 (Harrington and Ginty 2013; Christoforidis *et al.* 1999*)*, AP2 (Harrington and Ginty 2013; Abe *et al.* 2008*)* and synaptotagmin (Harrington and Ginty 2013; Radhakrishnan *et al*. 2009). Dynamin, a GTPase involved in pinching off the endocytic vesicle from the plasma membrane, is a required component for NGF–TRKA internalization and subsequent retrograde transport (Harrington and Ginty 2013; Zhang *et al.* 2000*)*. The pleckstrin homology (PH) domain of dynamin is required for its function in clathrin-mediated endocytosis. This activity requires the binding of phosphoinostides to the PH domain (Harrington and Ginty 2013; Bethoney *et al.* 2009*)* . Phosphoinostides themselves participate in vesicle coat formation and vesicle targeting through recruitment of AP2. Thus, PI3K activity is probably required at multiple stages in TRK-mediated endocytosis (Harrington and Ginty 2013).

Phosphorylation of TRKA at Tyr785 following NGF binding leads to recruitment and activation of PLCγ (Harrington and Ginty 2013; Vetter *et al* 1991). PLCγ is a multifunctional enzyme, acting as both a lipase and as an interacting partner for additional receptor tyrosine effectors through its SRC homology domains (Harrington and Ginty 2013; Bunney and Katan *et al.* 2011). The downstream effects of PLCγ can be categorized as those mediated by the second messengers of its lipase activity (that is, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3)) and those mediated by direct interactions. A recent study found that PLCγ activity is required for ligand-mediated TRKA internalization (Bodmer *et al.* 2011; Harrington and Ginty 2013;) possibly through the control of dynamin. Indeed, the release of Ca2+ from internal stores into the cytoplasm that is caused by PLCγ-mediated formation of IP3 activates the phosphatase calcineurin, which in turn catalyses dephosphorylation of key dynamin residues that are required for receptor

internalization (Harrington and Ginty 2013). Thus, events that are essential for different steps of TRK internalization are controlled by the early effectors PI3K and PLCγ Harrington and Ginty 2013).

Literature evidence suggests that the NGF-TrkA complex is proposed to be sorted into short lived recycling endosomes or long-lived signaling endosomes. The long-lived signaling endosomes are proposed to promote the cell survival and differentiation signals, during their journey towards the somatodendritic area via the retrograde transport mechanism (Harrington *et al.* 2011; Harrington *et al.* 2013). The fate of endocytosed NGF-TrkA is complex and it is unclear whether it is sorted into recycling (short lived) or long-lived signaling endosomes (Marlin *et al*. 2015). This ability of NGF-TrkA complex to be sorted into endosomes may enable the LC of BoNT/A to be targeted to sensory neurons (see later). Exploitation of this is the key objective of this study

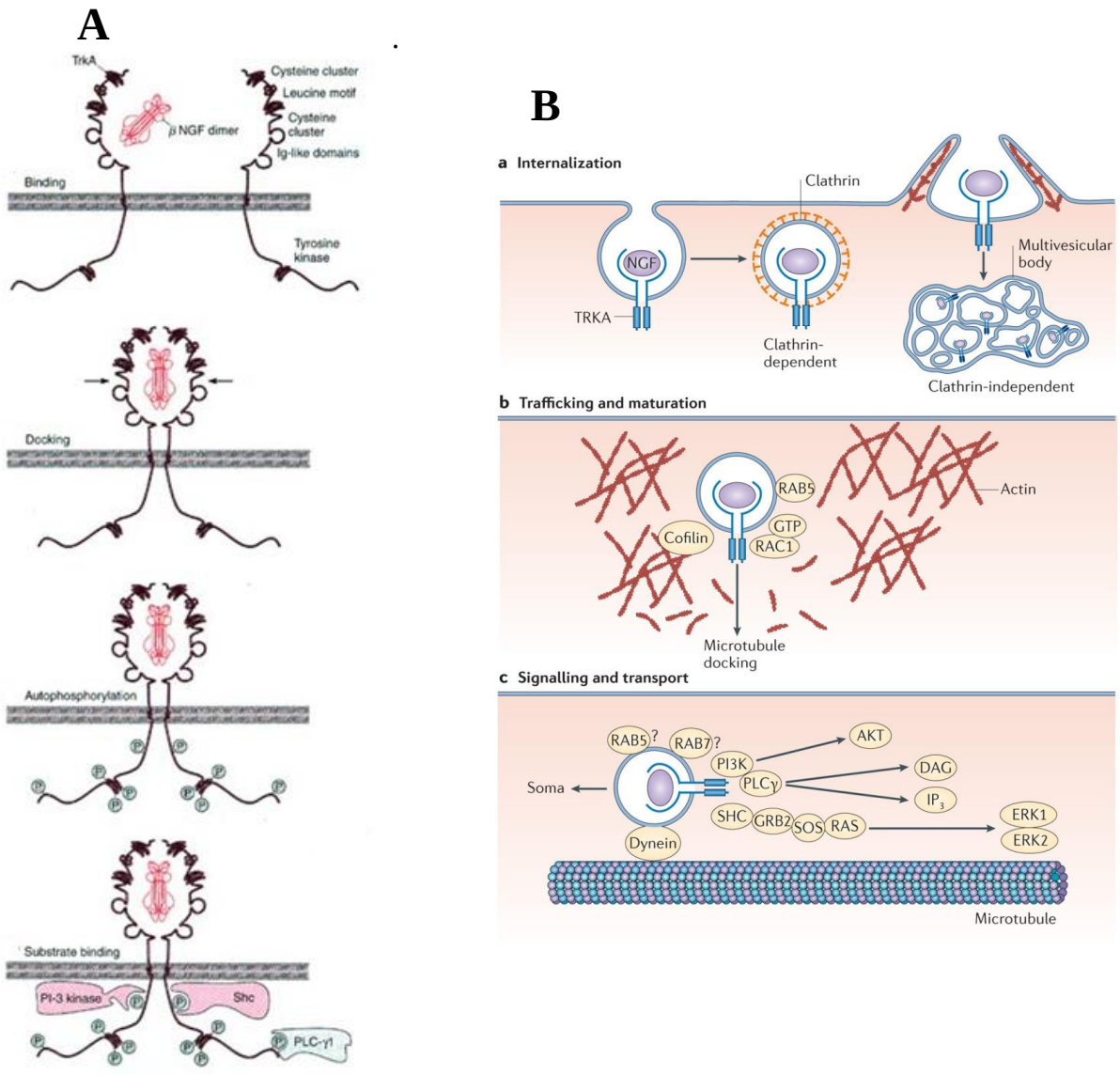

Fig. 7 NGF - TrkA complex *A) Sequence of events when NGF binds to TrkA. The biologically active forms of βNGF are dimers of identical 13 kDa peptide chains. The βNGF dimer binds to the TrkA protein. The binding induces two TrkA receptors to dimerize. This NGF-TrkA complex leads to transphosphorylation of five tyrosine residues on the cytoplasmic tail of the adjacent TrkA receptor. The cytoplasmic adaptor proteins such as Shc) bind to specific phosphorylation sites on the cytoplasmic tail where these substrates are phosphorylated. The bound adaptor proteins provide docking sites for downstream signaling proteins PI3 Kinase and PLC- γ1. Image adapted from Sanes et al. 2011. (B) Mechanisms of neurotrophin internalization, signalling and retrograde transport. (a) It has been proposed that internalization of tropomyosin receptor kinase A (TRKA) following binding to nerve growth factor (NGF) occurs through clathrin-dependent and clathrin-independent mechanisms. Although clathrin-mediated endocytosis results in the formation of early endosomes, internalization via macropinocytosis leads to the generation of multivesicular bodies. (b) Newly internalized TRKA endosomes must overcome an F-actin barrier before docking with microtubules for long distance transport. This is achieved by activation of RAS-related C3 botulinum toxin substrate 1 (RAC1), which leads to actin depolymerization through recruitment of cofilin to the signalling endosome. (c) TRKA endosomes that are competent for transport are linked to the retrograde motor protein dynein and move towards the neuron's soma. Signalling from the phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and phospholipase Cγ (PLCγ) signalling pathways persists during endosome transport. The specific RAB composition of these endosomes remains controversial. DAG, diacylglycerol; GRB2, growth factor receptor-bound protein 2; IP_3, inositol 1,4,5-triphosphate; SOS, son of sevenless. Imahe take from Harrington and Ginty 2013.*

1.3 Chronic pain management

The prevalence of chronic pain in modern societies has been discussed in the preamble to this chapter and the cost, in terms of quality of life and the drain on the exchequer, indicated. The need for governments to address the relevant issues through adequate and fit-for-purpose health services has been noted. In general, the treatments currently applied focus on pharmacological methods on the one hand and, on the other, education programs to inform healthy lifestyle decisions.

1.3.1 Current pharmacological treatment and limitations

The most commonly prescribed drugs for chronic pain in 15 European countries reported by Breivik *et al.* 2006 were NSAIDs (Nonsteroidal anti-inflammatory drug) (44%), weak opioid analgesics e.g. codeine (23%), paracetamol (18%) and strong opioid analgesic e.g. morphine (5%). Out of the 124 from UK and 129 from Ireland, 12% and 13 % used strong opioids. Kalso *et al.* 2003 and the pain society (2004) provided comprehensive guidance on the use for opioids to non-cancer-related pain.

Combating the side effects of medication is a matter of urgent concern. Much of the literature supports the view that, despite significant advances in the understanding of the pathophysiology of chronic pain (Gold *et al.* 2010) its management continues to challenge physicians and fails to serve patients. Breivik *et al.* 2006 reported that NSAIDs have adverse effects on the cardiovascular, and the gastrointestinal system where an overdose of paracetamol, for example, has the risk of hepatotoxicity. Their study highlighted that all analgesics have side effects including stomach upset, constipation, diarrhea, dizziness, or headache. Stannard and Johnson, 2003 reported that over one-third of respondents were concerned about the dangers of becoming addicted to pain medications and the remaining

two third expressed concern about other side effects. On the other hand, 64 % of respondents to one study of chronic pain patients using prescribed pain medications reported that they were inadequate to control their pain (Breivik *et al.* 2006). They also reported that 74% of GPs in the UK cited side effects of drugs as a major barrier to pain control.

Whelton *et al.* 2000 and Benyamin *et al.* 2008 give further examples of the need to address the side effects and risks of analgesic drugs in clinical practice. It is accepted that, as stated by Manthy *et al.* 2011, there are very few treatments that efficiently regulate chronic pain without unwanted side effects. The work reviewed in this report sought to maximise the wanted and minimise the unwanted side effects of pain killers. Currently, international best practise guidelines recommend a multimodal combination of pharmacologic and non-pharmacological modalities as the most effective strategy to manage the disability associated with chronic pain (Kimura *et al.* 2010). The approach used in this study focuses on the pharmacological ones. There are unmet needs which require the advancement of drug discovery research dealing with chronic pain for development of new class of drugs with long-lasting effect and which do not contribute towards side effects like drug dependence.

This study describes a new approach that seeks to exploit the ability of BoNT/A in the management of pain. Therefore, it is important to understand its structure and its role in blocking the exocytosis mechanism. This is illustrated in the next section.

1.3.2 Botulinum toxin type A as an emerging therapeutic

Since approval of Botox® in 1989 in the United States, Allergan has confirmed its safety as a therapeutic for the treatment of strabismus, benign essential blepharospasm and

conditions of the VIIth nerve. As demonstrated by fluorescent immuno-microscopy in a study of rodent nerve-muscle preparations paralysed with BoNT/A, its ability to accumulate the mediator Calcitonin gene-related peptide (CGRP) in the pre-synapse presented the first clues to its potential benefits in pain (Lande *et al.* 1989). Further evidence of pain relief was provided by successful treatment of dystonia patients with intra-muscular injections of BOTOX® (Brin *et al.* 1987). In the treatment of migraine, the injection of BOTOX® caused a reduction in the severity and the frequency of migraine episodes (Binder *et al.* 1998). Its success has encouraged a new interest in BoNT as a potential anti-nociceptive. A study of 30 migraine suffers revealed that BOTOX® treatment of any severity of migraine was well tolerated and that it relieved in a significant manner the frequency of migraine attacks at day 90, and the frequency of severe attacks at day 60 and 90 (Barrientos and Chana, 2003). In a study to discover its success in treatment of chronic tension type headaches, local injections of BOTOX® resulted in less headaches and precranial muscle tenderness (Relja and Telarovic, 2004). In a study on 29 patients with focal painful neuropathies and mechanical allodynia, BOTOX® resulted in a direct pain-killing effect on muscle tone of these subjects (Ranoux *et al.* 2008). Their effect on refractory neck pain was investigated on 47 subjects, showing a significant decrease in the mean pain intensity (Miller *et al.* 2009). More recently (Diener *et al.* 2010), in a large trial with 1384 chronic migraine suffers treated with onabotulinumtoxinA (BOTOX®), the results showed after one dose the occurrence of headache days was significantly reduced and this benefit lasted for six months. Following such encouraging results from these clinical studies, Allergan Inc. was granted approval by the FDA and MHRA for BOTOX® as a treatment for chronic migraineuers (headaches for 15 days or more per month) in the United States and UK, respectively (Aoki and Francis, 2011). BoNT have been exploited as muscular relaxant and in treatment of chronic pain. Because this study exploits the

ability of BoNT/A in the management of pain, it is important to understand the structure and the mechanisms of BoNT.

1.3.3 Botulinum neurotoxin

Botulinum neurotoxin (BoNT) is produced from *Clostridum botulinum*, a rod shaped gram-positive bacterium which grows in anaerobic conditions (Karalewitz *et al.* 2012). BoNT causes flaccid paralysis or botulism. *Clostridium botulinum* was cultured and isolated successfully in 1944 by Edward Schantz. Its success in the blocking of neuro-muscular transmission was reported by Burgen in 1949. In the last decade, BoNT/A research has gained considerable pace because of its impact on public health, potential utilization as a bio-terrorist molecule, and clinical applications in medical industry (Poulain *et al.* 2008). Seven categories of BoNTs from A-G, all of which are toxic to non-human primates have been identified, described and reviewed (Poulain *et al.* 2008). Type A toxin is the most broadly studied and applied for therapeutic treatments (Davletov *et al.*, 2005; Foran *et al.*, 2003; Jankovic, 2004).

1.3.3.1 Structure

BoNT/A consists of three independent functional domains, namely, (1) a heavy chain C-terminal domain which binds to the acceptors (H_C), described below. (2) a translocation domain (H_N). (3) a light chain which is a zinc-dependent catalytic endopeptidase (LC) (Fig. 8). The 150k single-chain BoNT/A becomes activated upon proteolysis by endogenous bacterial proteases (or by trypsin when proteolysis is carried out *in vitro*). Proteolysis leads to di-chains of $H_N.H_{CN}.H_{CC}$ and LC, linked via a disulphide bond and non-covalent interactions. The molecular weight of each of the three BoNT/A domains is ~50 k. The C-terminal acceptor-binding (H_C) domain is sub-divided further into two structural domains:

C terminal domain (H_{CC}) and N terminal domain (H_{CN}). Both play an important role in binding to gangliosides and high affinity protein synaptic vesicle protein (Chai *et al.* 2006; Jin *et al.* 2006). The N terminus of the H_C (H_{CN}) appears to form jelly-roll folds similar to legume seed lecithin-like structures (L-type lecitins) and the C-terminus of H_C (H_{CC}) appears to form a β-trefoil fold similar to ricin–type lectins (R-type) (Karalewitz *et al.* 2012; Varki *et al.* 2009; Thomas *et al.* 2009). H_{CN} consists of a series of anti-parallel strands, which are connected by short loops, to form two β-sheets (Karalewitz *et al.* 2012). The function of H_{CN} was reported to facilitate neurotoxin entry in co-ordination with H_{CC}. However, its independent function is unclear (Rummel *et al.* 2011). The H_{CC} is known as the receptor binding domain, which binds to gangliosides and protein acceptor contributing to neuronal tropism of BoNT/A (Karalewitz *et al.* 2012). An engineered variant of BoNT/AΔH_{CC} lacking the H_{CC} domain of BoNT/A created in ICNT displayed a greatly decreased ability to enter neurons isolated from the dorsal root ganglia (unpublished data). This has been exploited in this study as a control in the experiments described in Chapter 3.

It is reported that H_N consists of long helices which appears as a tunnel via which the LC passes into the neuronal cytosol (Fischer *et al.* 2007, Fischer *et al.* 2009). The LC is a zinc dependent metalloprotease, which cleaves SNARE proteins. LC is joined to H_N by a disulphide bond and non-covalent interactions by a loop from H_N, referred as the belt, around the LC region (Karalewitz *et al.* 2012). The literature provides evidence on recombinant variants of LH_N, described later in this chapter. Because this report focuses on BoNT/A and its entry into motor neurons leading to the blockage of neurotransmission signal, a description of the exocytosis mechanism follows.

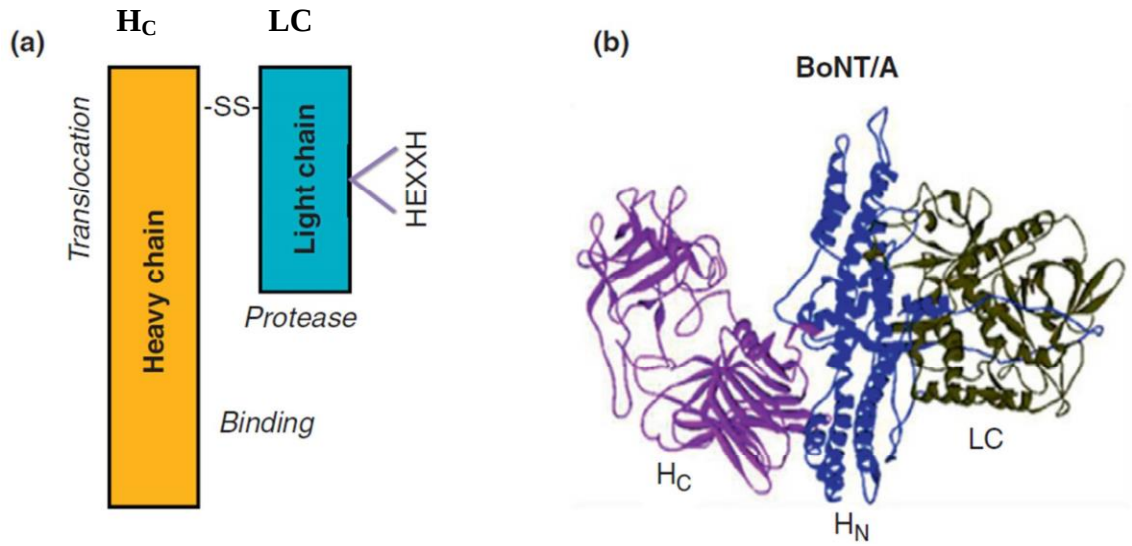

Fig. 8 BoNT/A *(a) Schematic of BoNT/A. (b) Structure of BoNT/A. Proteases cleave BoNT into their active forms which consists of a Mr ~100 k heavy chain (H_C) and a Mr ~50 k light chain (LC) linked by a disulphide and non-covalent bonds. The H_C region is important for binding to gangliosides and protein acceptor, the H_N for translocation and the LC for protease activity. Image taken from Dolly and O'Connell (2012).*

1.3.3.2 Exocytosis mechanism

The fusion of an intracellular trafficking vesicle with the plasma membrane is described as exocytosis. It plays a crucial role for growth and differentiation because it is the only mechanism whereby a cell can add additional membrane to the plasmalemma (a semi-permeable membrane enclosing the cytoplasm of a cell). Exocytosis regulates delivery of secretory products such as neurotransmitters, transporters, enzymes, or channels. At the molecular level, exocytosis involves specialized protein families such as the SNAREs, the Rab/ypt proteins, and the Sec1/Munc18-like (SM) protein family. Because of the relevance of SNAREs to this study, their role in exocytosis is described below (Jahn *et al.* 2004*)*.

SNAREs represent membrane-anchored proteins and are crucial for intracellular fusion events (Jahn *et al.* 2003; Jahn *et al.* 1999; Rizo *et al.* 2002; Fasshauer *et al*. 2003).

SNAREs referred to in this study include 1) the syntaxin-1 and 2) SNAP-25, localized on the plasma membrane, and 3) the synaptobrevin (also known as Vamp), concentrated on the membrane of synaptic and neurosecretory vesicles.

SNARE motifs show a tendency to homo-oligomerize into helical bundles (Fig 9). Literature suggests that during neuronal exocytosis, a synaptosomal associated protein with molecular weight 25 kDa (SNAP-25) is commonly bound on the plasma membrane due to various palmitoyl chains that include cysteine amino acids. Syntaxin-1 and Vamp, on the other hand, are bound on plasma and synaptic vesicle membranes, respectively, via their C-terminal domains (Foran *et al.* 2003; Humeau *et al.* 2000; Yamasaki *et al.* 1994). From the combination of syntaxin-1, Vamp and SNAP-25, a tight SNARE complex emerges, represented by elongated bundles of four α helices each of which is contributed by one of the participating SNARE motifs (two from SNAP-25), with the transmembrane domains being located at one end of the bundle (Hanson *et al. 1997;Sutton et al.* 1998). The core of the bundle consists of the helices, connected by 16 layers of hydrophobic amino acid side chains. The assembled SNARE complex is stable and resistant to treatment with the detergents sodium dodecyl sulfate (Hayashi *et al.* 1995; Fasshauer *et al.* 2002), a property exploited for bi-chemical coupling of recombinants (Darios *et al.* 2010) described in Chapter 3. Formation of the SNARE complex is triggered by Ca^{2+} at micro-molar concentrations (Rizzo *et al.* 2002). In addition to the three SNARE proteins, a large number of accessory proteins exist that enhance the SNARE-driven exocytosis process (Barclay *et al.* 2005).

A Ca^{2+} sensor namely, synaptotagmin interacts with SNAP-25 in early synaptic vesicle docking within the presynaptic membrane. The C-terminal cytoplasmic region of synaptotagmin binds to Ca^{2+} and its N-terminal domain is present within the lumen of pre-

synaptic vesicle (Littleton *et al.* 2001). Upon binding to Ca^{2+}, synaptotagmin facilitates the Ca^{2+}-evoked synaptic vesicle fusion with the presynaptic membrane (Humeau *et al.* 2000). Truncation caused by cleavage of the SNARE proteins by BoNTs prevents the formation of SNARE complex (Fig.9) and inhibits exocytosis.

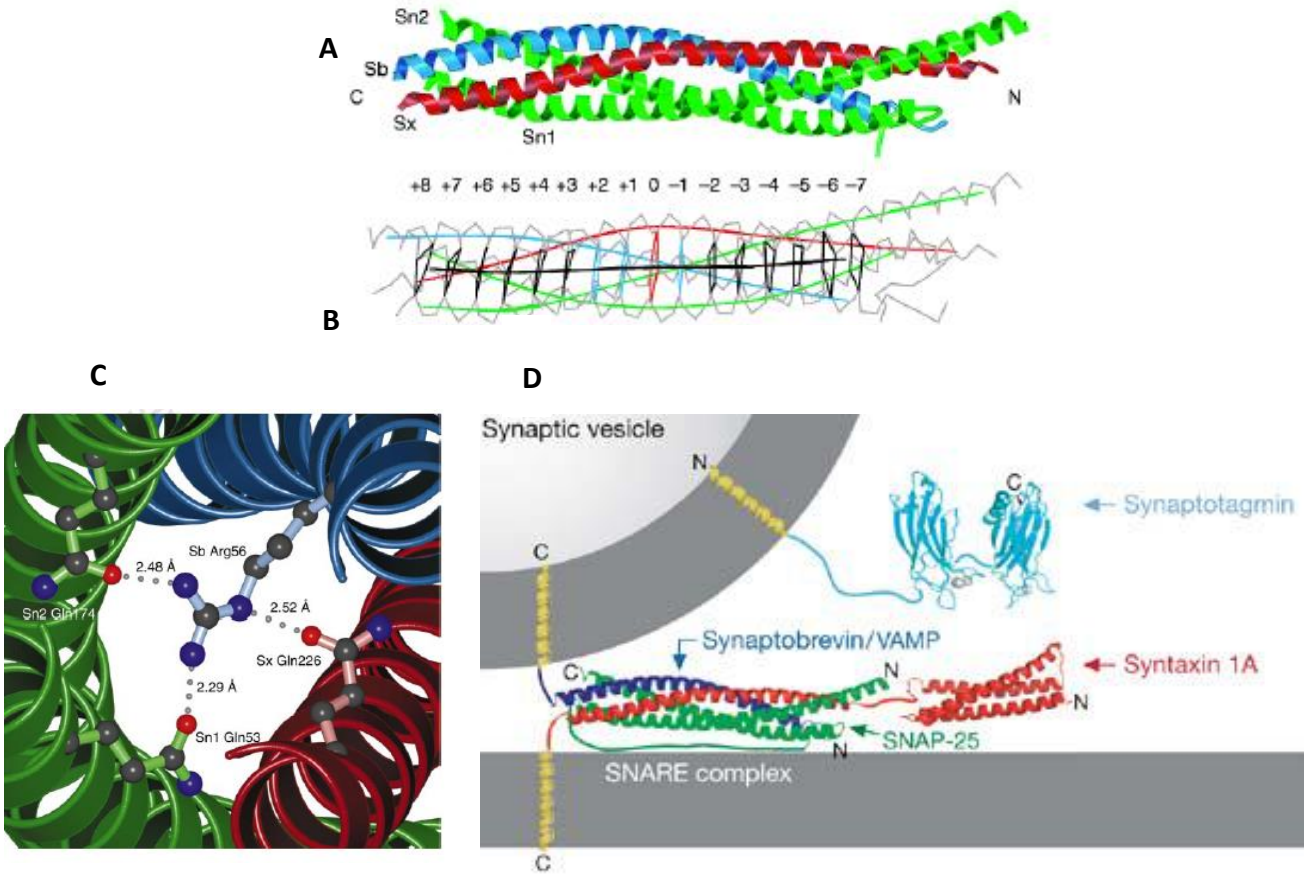

Fig.9 SNARE complex and synaptotagmin driven exocytosis. *(A-C) represents topology and organization of the synaptic fusion complex. (A) Showing backbone ribbon structure of neuronal SNARE core complex. Synaptobrevin 2 (Sb) is shown in blue; Syntaxin 1 (Sx) is in red; and SNAP-25 (Sn1 and Sn2) is in green. (B) Organization of the SNARE core complex shows 15 hydrophobic layers and 1 ionic "0" layer. Cα traces (grey), local helical axes (blue, red and green for synaptobrevin-II, syntaxin-1A and SNAP-25B, respectively), the super-helical axis (black), and layers refer to the Cα carbons that are closest to the center of the four helix bundle (0, red;-1, +1 and +2, blue; all others black; layer numbering is centered at the ionic layer) are shown for one of the three complexes in the asymmetric unit. (C) The structure of the central ionic "0" layer of the*

synaptic fusion complex. Side chains involved in the layer are shown as balls and sticks; backbone is shown as a ribbon. The total buried surface area for the sidechain atoms in this layer is 742A° (Picture is adapted from Sutton 1998) (D) The core of SNARE complex consists of α-tetra helices; two from SNAP-25 and one each from Vamp and syntaxin-1; synaptotagmin (Ca^{2+} sensor) that maintains SNARE zipping and vesicle fusion. Synaptotagmin consists of an N-terminal transmembrane region and two C-terminal domains namely, C2A and C2B, which bind to Ca^{+2} (Image is adapted from Carr and Munson, 2007).

1.3.3.3 Blockage of the exocytosis mechanism

There are four steps (Fig. 10A) by which BoNTs causes blockage of the exocytosis mechanism, namely, 1) the binding domain (H_C) attaches to the ecto-acceptors on cholinergic nerve terminals, 2) acceptor-mediated endocytosis and 3) translocation of a zinc-dependent catalytic endopeptidase LC 4) cleavage and inactivation of protein(s) essential for neurotransmitter release (Dolly and Lawrence 2014).

Specifically in relation to BoNT/A mechanisms, on entry into the neuronal cytoplasm, the zinc dependent LC/A selectively cleaves 9 amino acids from the C-terminal of SNAP-25. As already mentioned cleavage of SNAP-25 blocks the fusion of small clear synaptic vesicles (SCSVs) and large dense-core vesicles (LDCVs) to the plasma membrane and thereby blocks the exocytosis of neurotransmitters (Black and Dolly 1986; Dolly *et al.* 1984; Dolly *et al.* 1994; Humeau *et al.* 2000; Schiavo *et al.* 2000; Simpson 1979, Simpson *et al.* 1981).

Step 1: binding to the ecto-acceptors

BoNT/A recognizes cholinergic neurons by its $H_{CC,}$ ganglioside and synaptic vesicle protein binding domain (Baldwin *et al.* 2007, Emsley *et al.* 2000, Shapiro *et al.* 1997, Rummel *et al.* 2004, Stenmark *et al.* 2008, Chai *et al.* 2006, Jin *et al.* 2006). Yowler *et al.*

2004 suggest that H_{CC} of BoNT/A binds to gangliosides and glycosphingolipids predominantly found in the plasma membrane of cholinergic motor neurons. There is evidence (Emsley *et al.* 2000, Shapiro *et al.* 1997, Rummel *et al.* 2004, Stenmark *et al.* 2008) that a conserved ganglioside binding region, consisting of amino acid residues starting with Histidine and ending with Tyrosine 'HSXWY' exists within the H_C of BoNT/A. This conserved region is also known as the ganglioside binding pocket (GBP). Amino acids H and W are in direct contact with the ganglioside. On neuronal stimulation, the C-terminal end of H_{CC}, beside the GBP, facilitates the interaction of H_C with a second receptor namely, synaptic vesicle protein (Chai *et al.* 2006; Jin *et al.* 2006).

Step 2: acceptor-mediated endocytosis

Literature evidence suggests that BoNT/A binds with high affinity to synaptic vesicle protein 2 (SV2). After binding to the surface, BoNT/A is internalized by SV2 endosome recycling. SV2 is reported as an integral membrane glycoprotein with three isoforms namely, SV2A, SV2B, and SV2C. SV2A is predominantly found throughout the nervous system. Whilst SV2C is reported in neurons of some brain areas, very little is known about SV2B in the literature (Bajjalieh *et al.* 1994; Bajjalieh *et al.* 1993; Janz *et al.* 1998; Janz and Sudhof, 1999).

Step 3: translocation of a zinc-dependent catalytic endopeptidase LC

The low pH environment of endosomes (due to the proton pumps present in the endosomes), leads to conformational changes of BoNT/A during which the H_N forms a membrane pore through which the LC passes and escapes from the endosome into the cytosol (Koriazova *et al.* 2003) (Fig. 10B).

Step 4: cleavage and inactivation of protein(s) essential for neurotransmitter release.

After entry into the neuronal cytosol, LC specifically cleaves either vesicle or plasma membrane SNAREs. The LC of different serotypes of BoNTs has varied SNARE preferences. The literature review highlighted that SNAP-25 is cleaved by LC of BoNT/A, C1 and E. Syntaxin IA/IB is reported to be also cleaved by LC of BoNT/C1 (Wang *et al.* 2011) and Vamp isoforms 1, 2 and 3 are cleaved by LC of BoNT/B, /D, /F and /G (Foran *et al.* 2003; Humeau *et al.* 2000; Yamasaki *et al.* 1994). The resultant effect of LC cleavage of SNARE proteins leads to blocking of the exocytosis mechanism, which stalls the neuro-muscular communication causing flaccid paralysis (Poulain *et al.* 2008, Chen *et al.* 2011, Schiavo *et al.* 1992). It must be noted that only BoNT/A experiments were carried out in this research, in which the cleavage of SNAP-25 by BoNT/A was exploited. The results of these experiments are presented and discussed in Chapter 3 and 4, respectively.

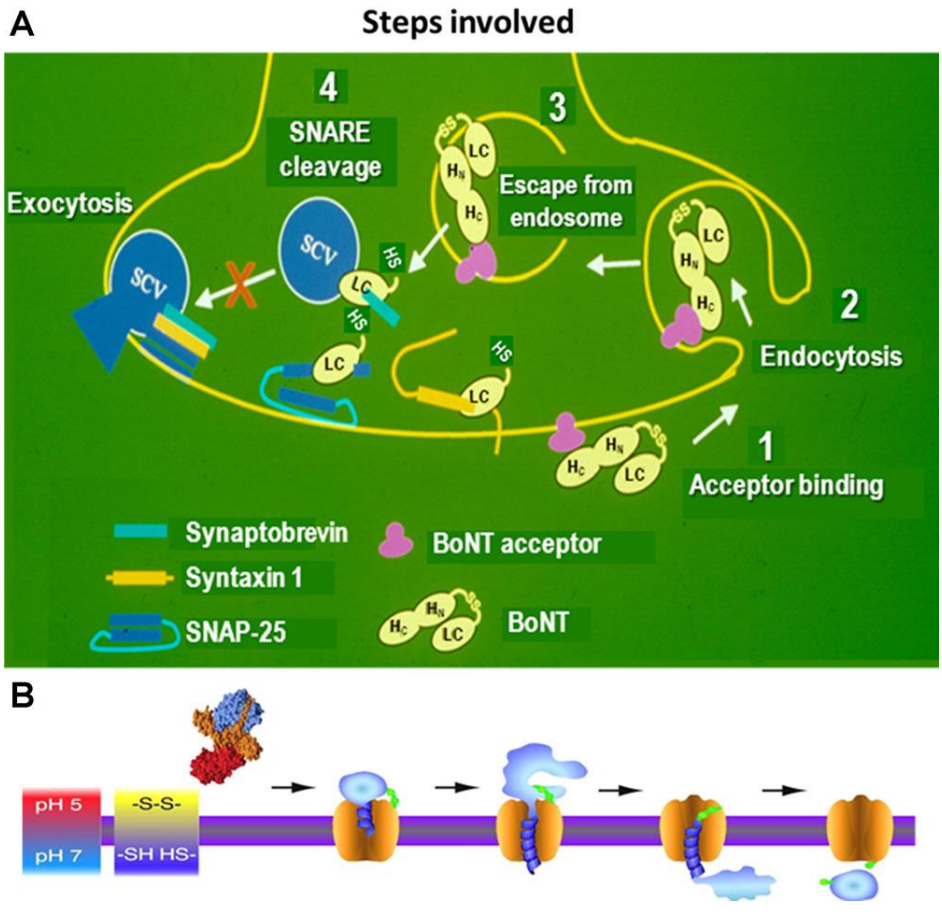

Fig 10. BoNT/A block exocytosis at neuro-muscular junction (NMJ). *A) BoNT/A blocks the release of transmitters via a multi-phasic mechanism culminating in the cleavage/inactivation of intracellular SNAP-25. Involving step 1 - acceptor binding, step 2 - endocytosis, step 3 - escape pf LC from the endosome and step 4 SNARE*

cleavage B) Suggested mechanism of translocation of the light chain, adapted from Fischer et al. 2007. Image taken from Dolly and Lawrence 2014.

1.3.3.4 Applications of re-engineered BoNT/A

The modular arrangement of BoNT and the independent functions of its three domains have encouraged several researchers to create variant BoNTs, using recombinant DNA technology (a common feature among all seven serotypes of BoNT) (Chen 2012; Masuyer *et al.* 2014). Natural variants of BoNT/A like subtype BoNT/A2 composed of LC of BoNT/A1 and HC of BoNT/A3 (Arndt *et al.* 2006) are formed due to natural genetic recombination process which, furthermore, encouraged researchers to exploit protein engineering technology. Literature (Chen 2012, Masuyer *et al.* 2014) suggests that with the help of genetic engineering technology, researchers had particularly identified various therapeutic applications of BoNT/A. They explored the following functional characteristics of BoNT/A to enhance its:

i. Neurotoxin activity
ii. Potential as a neurotoxin transporter
iii. LCH_N fragment
    a. Ability of LCH_N to act as targeted secretion inhibitor
    b. Ability of LCH_N to assemble into a functional molecule via protein stapling technology

1.3.3.4.1 Neurotoxin activity

Neurotoxin activity of BoNT/A relates to its ability to enter neuronal cytosol, cleave SNAP-25 and block the exocytosis of neurotransmitter. Literature describes that the intracellular speed of translocation of BoNT is dependent on the translocation domain (H_N), while its potency and toxicity are directly related to LC (Wang *et al.* 2008). The

literature argues that both, BoNT/A and BoNT/E cleave SNAP-25, but the latter by comparison to the former was faster and more potent in blocking the neurotransmission but for a shorter duration of time (Wang *et al.* 2008; Foran *et al.* 2003). While acknowledging the advantages of BoNT/E, the longer duration of BoNT/A neurotoxin activity determined the decision to exploit in this study, its use in the cleavage of SNAP-25.

Researchers focused on using protein engineering to improve the stability of LC/A after translocation step, for long-lasting effect in cleaving SNAP-25. Wang *et al.* 2008 demonstrated that the C-terminal end dileucine plays an important role in persistent SNAP-25 cleavage, and mutations of these residues shorten the direction of SNAP-25 cleavage.

Tsai *et al.* 2010 showed that, by comparison to LC/A, the ubiquitin-proteasome facilitates the degradation of LC/E via the ring finger protein TRAF2; they suggested that LC/A was not targeted by the ubiquitination degradation pathway which make them an ideal tool for therapeutic application.

1.3.3.4.2 Botulinum neurotoxin as a transporter

The H_C (binding domain) specifically binds to neuronal targets and allows the entry of BoNT/A into the neuronal cytosol. BoNT/A H_C domain as a protein transporter was reported by Drachman *et al.* 2010, who exploited the conjugating property of biotin and streptavidin. Drachman *et al.* 2010 illustrated a method in which H_C of BoNT/A linked to streptavidin, allows targeting of biotinylated viral vectors into neuronal cells. They reported that this design had the potential for treating muscular neuropathies including, spinal muscular atrophy and amyotrophic lateral sclerosis, as the biotinylated viral vectors carried therapeutic genes inside the motor nerve cells.

1.3.3.4.3 The LCH_N fragment

Shone *et al.* (1985) demonstrated that controlled proteolytic digestion of BoNT/A by trypsin results in the di-chain of LC/A and H_N linked by di-sulphide bond, named LCH_N/A fragment, lacking the binding domain (H_C). By replacing the H_C with alternative ligands, the LCH_N/A fragment has been targeted to various neuronal and non-neuronal cells, blocking secretory functions of that targeted cell via the cleavage of SNAP-25 (Foster, Hambelton, & Clifford, 2006; Foster 2009).

1.3.3.4.3.1 Ability of LCH_N/A to act as targeted secretion inhibitors

In the last 15 years, researchers have explored chemical conjugation as a method to couple LCH_N/A fragment to various ligands, to develop targeted secretion inhibitors. Chaddock *et al.* 2000a were the first to describe a method for chemically conjugating LCH_N/A with NGF. They reported the retargeting of LC/A to neuro-endocrine cells like PC-12 cells, which led to inhibition of noradrenaline release due to the cleavage of SNAP-25. Chaddock *et al.* 2000b described the chemical conjugation of LCH_N/A to wheat germ agglutinin (WGA), and reported the delivery of the endopeptidase into several neuronal and non-neuronal cell types. They reported that LCH_N/A was responsible for the inhibition of neurotransmitter secretion in neuronal cells, and attenuation of insulin secretion from pancreatic cells.

Darios *et al.* 2010 described that issues such as poor yield and high cost in preparing large quantities of coupled product, led them to the discovery of protein stapling technology. This technology, illustrated in fig 22, has the potential to surpass the obstacles arising from recombinantly-engineering BoNT/A such as large size, its dependence on additional structural units for translocation into the neuronal cytosol, and its variable production yields. Of particular interest to this study was the reported ability of this technology to

address issues of poor yield and inactive protein. How this was exploited in this study is described in detail in Chapters, 3 and 4.

1.3.3.4.3.2 Ability of LCH_N to assemble as a functional molecule via protein technology

The literature describes the term 'protein stapling' to indicate fusion of two proteins into a single polypeptide chain by recombinant means or chemically cross-linked. This study refers to the term 'protein stapling technology' with regards to a new linking method described by Darios *et al 2010*. This method is based on two distinct polypeptide linkers which self-assemble and stabilize a given protein conformation in the presence of a specific peptide brace (staple) (Ferrari *et al. 2012).*

As described in section 1.3.3.2, during neuronal exocytosis, synaptic vesicles bind to SNARE to form a tight tetra helical complex. Protein stapling technology exploits the self-assembling property of three helical SNAREs such as SNAP-25, Vamp and Syntaxin - 1 to form a tight tetra-helical protein complex. Of the four helices in the complex, SNAP-25 contributes two domains, syntaxin-1 contributes one domain and Vamp contributes one domain.

Darios *et al.* 2010 was the first to exploit this tight interacting property of helical SNAREs to combine H_C of BoNT/A and LCH_N/A fragments. They recombinantly created individual components of both HC/A fused Vamp and LCH_N/A fused SNAP-25 and combined them in presence of syntaxin-1 to form a fully-functional molecule, which showed cleavage of intra-neuronal SNAP-25. This activity was found to act similarly to native BoNT/A. Arsenault *et al.* 2013 used this concept and described and designed novel chimeric BoNT/A variants where the LC/A was linked to a selection of ligands to target neuroendocrine tumor cells. They suggested that in this approach new ligands can

substitute the botulinum receptor-binding domain (H_C) and allow targeting of LC/A to distinct neurons and cells of neuroendocrine origin. Of particular interest to this study was a design in which individual helical SNARE fused proteins like SNAP-25 (22-206) fused LCH_N/A and Vamp2 (25-84) fused to epidermal growth factor (EGF) were created. In the presence of syntaxin-1 (1-45), a stapled protein complex of EGF fused LCH_N/A could be formed. In this way, the endopeptidase of BoNT/A was delivered into the neuroblastoma SH-SY5Y cells, which blocked neuroendocrine secretion due to cleavage of SNAP-25. The relevance of protein stapling technology to this study is discussed in detail later in Chapters 3 and 4.

1.4 Aim of this study

The original aim was to develop a strategy to generate a new generation of bio-therapeutics to alleviate pain. This was to involve the targeting of SNAP-25, in the hyperactive sensory nerves, with a conjugate of NGF and LC protease of BoNT/A. As time progressed, the research began to converge on the creation of biologically-active βNGF.Vamp2 using protein stapling technology. It was decided to leave the conjugation of βNGF.Vamp2 with SNAP-25.LC.H_N as future work.

This study involved gaining competence in a number of related areas as stepping stones towards the objectives stated above. The process required familiarization with the relevant literature and laboratory skills like cell culture and maintenance of cells, vector creation using genetic engineering, protein expression, purification and testing of recombinant proteins on model cells

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2 Materials and Methods

2.1.1 Antibodies

Item	$1^O/2^O$ Ab	Catalogue number	Supplier	Species raised in	WB dilution	IHC dilution
SNAP-25 (binds to amino-terminal of the C-terminal peptide).	1^O	SMI81	Sternberger Monoclonals Inc.	Mouse	1:1000	1:1000
NGF (binds to mouse 2.5S NGF Accession P01139)	1^O	AN240	Alomone Labs Ltd.	Rabbit	1:200	N/A
LC/A (binds to N-terminal of LC/A)	1^O	Evorequest	Allergan Inc.	Rabbit	1:1000	N/A
Vamp 1, 2, 3 (binds to the rat recombinant cellubrevin 1 – 81 at the cytoplasmic part).	1^O	104102	Synaptic Systems	Rabbit	1:5000	1:500
p44/42 MAPK or Erk1/2 (binds with	1^O	9102	Cell Signaling Systems	Rabbit	1:1000	1:250

specificity and sensitivity to p44/42 MAPK (Erk1/2)						
Phospho-p44/42 MAPK or Erk1/2 (binds specifically with high activity to Phospho-p44/42 MAPK (Erk1/2) residues Thr202/Tyr204)	1^{O}	9101	Cell Signaling Systems	Rabbit	1:1000	1:100
TrkA (binding to rat TrkA extracellular domain)	1^{O}	N/A	Provided by *Louis Reichardt* (*University of California*, San Francisco)	Rabbit	1:1000	1:5000
Anti-mouse Alexa-488	2^{O}	A21121	Bio-Sciences Ireland Ltd	Goat	N/A	1:500
Anti-rabbit Alexa-568	2^{O}	A11011	Bio-Sciences Ireland Ltd.	Goat	N/A	1:500
Anti-rabbit-HRP	2^{O}	711-035-152	Jackson Immuno research Europe Ltd.	Donkey	N/A	1:5000

Table 1 Primary and secondary antibodies.

2.1.2 Purchased reagents

Name	**Supplier**
pET32b expression vector	Novagen
E. coli Origami B	Novagen
PC-12 cells	American Type Cell Culture Collection (ATCC)
SH-SY5Y cells	Dr. Hans-Georg König Laboratory (Royal College of Surgeons, Dublin).
Restriction enzymes	New England Biolabs
TALON® superflow resin	Clontech
UNO S1 and Q1 columns	Invitrogen
Bradford protein assay kit	Invitrogen
Precision plus™ all blue protein standards	Invitrogen
AccuPrime™ Pfx DNA polymerase	Invitrogen

Precast gels and reagents for SDS-PAGE	Invitrogen
PD-10 desalting columns	GE Healthcare
Substrates for ECL detection of horseradish peroxidase	Millipore
Enhanced chemiluminescence (ECL) reagents	Amersham (Millipore)
Immobilon™ PVDF membrane (0.45µm)	Merck Millipore
Bicinchoninic acid (BCA) protein assay kit	Pierce
Precast Bis-Tris gels	Invitrogen
Trypsin	Sigma
Trypsin inhibitor	Sigma
6'-Diamidino-2-phenylindole (DAPI)	Sigma
Isolectin B4-FITC	Sigma
Coomassie brilliant blue G 250	Sigma
Benzonase nuclease	Sigma

Leibowitz's L15 medium	Sigma
Dulbecco's modified Eagle's medium (DMEM)	Sigma
Roswell Park Memorial Institute 1640 (RPMI– 16) culture medium	Sigma
1:1 mixture of DMEM and Ham's F12 (DME F12)	Sigma
Fetal bovine serum	Sigma
Horse serum	Sigma
Poly-L-lysine	Sigma
Laminin	Sigma
Antibiotics (100 units/ml penicillin, and 100 µg/ml streptomycin)	Sigma
GlutMax I	Sigma
Mouse nerve growth factor (NGF-7S)	Sigma
Sodium acetate (3M), pH5.5	Sigma
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma

Propan-2-ol	Sigma
Collagenase I, Dispase II and DNase I	Roche Inc.
Sterile cell culture plates (including 6, 12 and 24 well plates etc.)	Sarstedt
Sterile flat bottom flasks (including T-25, T-75, T-125)	Sarstedt
Sterile pipettes (including 1ml, 5 ml, 10ml and 25 ml)	Sarstedt
Rat tail derived collagen	Thermo Fisher Scientific
Trypan blue solution 0.4%	Thermo Fisher Scientific
Miris transIT-LTI	Mirus Bio LLC
Ni-NTA super-flow resin	Qiagen
Thrombin	Merck Chemicals
Restriction endonucleases	New England Biolabs
1X DNA loading buffer	New England Biolabs
1 Kbp DNA ladder	New England Biolabs

Table 2 Reagents used for protein expression, purification and cell culture

2.1.3 Prepared reagents

Name	**Recipe**
4X Sample buffer (100ml)	0.8% (8g) SDS;4 m1 0.25 M Tris pH8.8 (10 ml of 1M);4 ml Glycerol; 1 ml 0.1 %(w/v) bromophenol blue; β-mercapthenol 5-10% (v/v) or 200mM 1,4-dithiothreitol (DTT)
PMSF	1M (174.19 g) soluble to 2-propanol (half-life 100µM PMSF at 25°C is, 110 min at pH 7/55 mins at pH 7.5/ 35 mins at pH 8)
Paraformaldehyde	4% paraformaldehyde, 1 N NaOH until solubilized, add 10% 10X PBS.
1000X trace metals mixture	50mM $FeCl_3$ (1.3g/100ml), 20mM $CaCl_2.2H_2O$ (0.222g/100 ml), 10 mM MnS04. H_2O (0.198 g/100ml), 10mM $ZnSO_4$ 7 H_2O (0.288 g/100ml), $CoSO_4$.$7H_2O$ (0.048 g/100ml), Cu SO_4. $5H_2O$ (0.034g/100ml), $NiCl_2$. $6H_2O$ (0.048g/100ml)
20X SDS-PAGE running buffer (NuPAGE MOPS) – 1L	50 mM MOPS (209.2 g), 50 mM tris Base (121.2 g), 0.2% SDS (10 g), 1 mM EDTA (6.0 g) pH 7.7
1X SDS-PAGE running buffer (NuPAGE MES) – 1L	50 mM MES (9.769 g), 50 mM tris base (6.069 g), 0.1% SDS (1 g), 1 mM EDTA (0.3 g) pH 7.3

Western blot 10X transfer buffer stock - 1 L	30.3g tris base, 144.1 g glycine
Western blot 1X transfer buffer	100 ml of 10X stock, 500ml H_2O, 200ml methanol
10X tris acetate EDTA buffer (agarose gel electrophoresis)	48.5g Tris, 11.4 ml acetic acid and 20 ml of 0.5m EDTA at pH 8 (18.6g in 100 ml of distilled water)
Phosphate buffer saline (10X) stock – 1 L	80 g NaCl, 2 g KCl, 14.4g Na_2HPO_4, 2.4 g KH_2PO_4
Phosphate buffer saline Tween -20 (10X) stock – 1 L	80 g NaCl, 2 g KCl, 14.4g Na_2HPO_4, 2.4 g KH_2PO_4, 1g Tween-20
Sterile1X phosphate buffer saline – 1 L	137 mM NaCl, 10 mM phosphate, 2.7 mM KCl. 100 ml 10 X Stock. 20 min, 121°C, liquid cycle.

Table 3 Reagents prepared in the lab

2.2 Methods

2.2.1 Molecular biology

Standard methods were used in this study as described in Gallagher (2011) and Sambrook *et al.* (1989).

2.2.1.1 Isolation of plasmid DNA

Luria Bertani (LB) medium with appropriate antibiotics were inoculated with a single colony of *E.coli* and grown by shaking at 220 revolutions per min (rpm) overnight at 37° C.

Cells were centrifuged at 1000 x g. Plasmid DNA was isolated by using Qiagen® plasmid mini kit. This involves alkaline lysis of cells, followed by binding of plasmid DNA to an anion exchange resin under appropriate low salt and pH conditions, as per manufacturer's protocol. The DNA pellet was washed two times with 70% ethanol and dissolved in 30 µl sterile deionized water (D/W).

2.2.1.2 DNA manipulation

2.2.1.2.1 Restriction digestion

Restriction digestion of plasmid DNA (~5 µg) was completed using appropriate buffer and 20 units of enzyme per µg DNA. The samples were incubated, as instructed in the protocol provided by New England Biolabs.

2.2.1.2.2 Agarose gel electrophoresis

Agarose gel was prepared (0.7%-1%) by melting agarose in 1X Tris acetate EDTA pH 8 (TAE); 1 in 1000 (dilution factor) of ethidium bromide was added to make up final concentration of 250 ng/ml. An appropriate amount of loading buffer was added to DNA sample. A 1 Kbp DNA marker and an appropriate amount of samples were loaded into each well. Electrophoresis was performed in 1X TAE with 100 V for the time required to obtain satisfactory separation, usually 40 mins. DNA was visualized under UV light on trans-illuminator and digitally photographed using G-box imaging instrument and GeneSys analysis software.

2.2.1.2.3 Agarose gel purification

Using a clean scalpel, the approximate size DNA fragment was excised from the gel. The slice was weighed in a 1.5 ml eppendorf tube. Purification proceeded using the Qiagen® gel extraction kit, in accordance with manufacturer's instruction.

2.2.1.2.4 Polymerase chain reaction (PCR)

PCR was performed in a 0.2 ml tubes (Sarstedt) in Verity® – in a 96 well thermal cycler (Applied Biosystems). The PCR mix was prepared containing the following:

10X buffer for KOD hot start DNA polymerase, 1.5 mM of 25 $MgSO_4$, 0.2 mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 0.3 μM of each primer, 0.02 U of KOD hot start polymerase; 10 ng of plasmid DNA and PCR grade water to make up final volume 50 μl.

The following method was used on the thermal cycler:

Reaction volume: 50ul

Cover temperature: 105°C

Stage 1 temperature 95.0°C; time (M:S): 2:00

Stage 2 - for 20 cycles

Step 1 temperature: 95.0°C; time (M:S): 0:25

Step 2 temperature: 66.7 (varies as per primer melting temperature); time(MM:SS): 0:55

Step 3 temperature: 72.0; time (M:S): 1:00

Stage 3

Step 1 temperature: 70.0; time (M:S): 10:

Step 2 temperature: 4.0; time: infinity

The success of amplification was assessed using agarose gel electrophoresis.

2.2.1.2.5 Primer design

Where possible, primers were selected using the following parameters:

- Melting temperature between 57°C and 63°C
- (G+C) content between 30-80%
- Length between 18-22 bp
- Less than 2°C difference in melting temperature between the two primers.

Wherever necessary, the parameters were relaxed to the following:

- Length between 18-27 bp
- Melting temperature 55°C and 65 °C

Blast searches were performed to test the specificity of primer pair.

2.2.1.2.6 Direct PCR purification

PCR products (50 µl) were purified using the Qiagen® PCR purification kit in accordance with the manufacturers protocol.

2.2.1.2.7 DNA quantification

DNA was quantified by applying Beer-Lambert Law (A = ECL) relating absorbance to DNA concentration (C). Absorbance (A) readings was measured at 260 nm (L). The extinction co-efficient (E) used for double stranded DNA was 50. Absorbance readings were determined by NanoDrop® spectrophotometer (NanoDrop technologies).

2.2.1.2.8 Ligation

The ligation reaction contained 10X buffer (1 µl), 3X insert, 100 ng vector DNA, T_4 DNA ligase (1µl) and molecular grade deionized water (D/W) to make up the final volume of 10 µl. The reaction was mixed and incubated overnight at room temperature (~22°C).

2.2.1.2.9 Transformation

Rubidium chloride competent origami B strain of *E.coli* cells were thawed for 2 mins on ice. ligation product (5 µl) was inserted into an Eppendorf tube with a 50 µl aliquot of bacterial suspension, followed by 1 min on ice. Immediately after heat shock (42°C for 90 sec), 950 µl of LB-medium was added, followed by incubation with shaking at 200 rpm for 1 hour. After incubation, 100 µl was plated on an agar plate containing ampicillin (100 µg/ml), kanamycin (50 µg/ml), and tetracycline (15 µg/ml) and incubated at 37°C overnight.

2.2.1.2.10 Plasmid sequencing

Plasmid sequencing was performed by MWG Eurofins Germany

2.2.1.2.11 Computational analysis

Blast: web based BLAST analysis was performed on Ensemble (Ensemble, 2016)

Alignments of DNA sequence – DNA sequence were aligned using the program ClustalW_2 (Larkin *et al.* 2007*)*

Expassy translate – DNA sequences were translated to protein sequence using Expassy translate tool (Expassy translate, 2016)

Expassy compute – the theoretical molecular weight (Mr) of proteins were computed by entering protein sequence into Expassy compute (Expassy compute, 2016)

2.2.2 Protein expression

Origami B cells carrying the desired plasmid were picked and selected in 4 ml LB broth containing ampicillin (100 µg/ml), tetracycline (15 µg/ml) and kanamycin (50 µg/ml). Cells were grown overnight at 37°C at 200 rpm. 1:1000 dilution of each starter culture was inoculated into LB broth containing ampicillin (100 µg/ml), tetracycline (15 µg/ml) and kanamycin (50 µg/ml) and grown until an Abs 600nm 0.6-0.7 at Abs 600 nm was reached. Following this step, temperature was reduced to 22°C and 0.4 mM IPTG was added to the culture and grown at 200 rpm for 16 hours.

2.2.3 Cell lysis

Cultures were centrifuged at 12,000 x g for 30 mins at 4°C. Supernatants were discarded and cell pellets allowed to air dry. The cell pellets were re-suspended thoroughly in lysis buffer (150 mM NaCl, 150 mM HEPES, 1 mM PMSF, 2mg/ml lysosome) and placed on a roller at 4°C for 45 min. This solution was freezed/thawed-lysed in a -80°C freezer for 1 hour and thawed in a 20°C water bath. The solution was then centrifuged at 47,900 x g in an ultracentrifuge for 45 min at 4°C. An aliquot of the clarified supernatant was collected for analysis on SDS-PAGE and the remainder was purified by IMAC.

2.2.4 Purification of recombinant proteins

All recombinant proteins used in this study contained a hexa -histidine (his_6) tag for affinity purification using immobilized metal chelate affinity chromatography (IMAC).

IMAC separates proteins or peptides, based on co-ordinate covalent interaction between immobilized metals ions such as nickel (Ni^{+2}) or cobalt (Cu^{+2}) and specific amino acids (such as histidine). Recombinant proteins are genetically modified to include protein tag such as glutathione-S-transferase (GST), hexa-histidine (his_6) etc. The stationary phase consists of nickel or cobalt ions immobilized by co-ordinate covalent interaction with chelator. The hexa-histidine tags binds to immobilized nickel or cobalt ions, thereby, separating the non-selective proteins. The bound histidine tagged protein can be eluted by addition of imidazole, which displaces the histidine tagged protein.

Ni-NTA resin (2 ml) was added to a commercial 20 ml column and washed with equilibration buffer (20 mM HEPES, 150 mM NaCl, pH 8.0). The clarified supernatant was mixed with equilibration buffer at 1:1 ratio and incubated with the resin for 1 hour at 4°C on a roller. Following binding, the mixture was placed back into the column and the flow through collected and stored on ice. The resin bound recombinant proteins were washed three times with wash buffer (equilibration buffer + 50 mM imidazole). Bound proteins were eluted at least three times with one bed volume (2ml) of elution buffer per fraction (20 mM HEPES, 150 mM NaCl, 500 mM imidazole). The eluted fractions were buffer exchanged into PBS using a PD-10 column, following the protocol provided by GE Healthcare. Concentrations of the eluates were determined by Bradford assay, as described in Section 2.2.4.4. Samples from each of the IMAC purification steps were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and protein was stained as described in Section 2.2.4.1 and 2.2.4.2, respectively. Purified and buffered exchanged fractions were stored at -20°C.

2.2.4.1 SDS-PAGE

This was used to separate proteins on the basis of size with SDS detergent being added to remove any secondary or tertiary structures. Proteins were loaded onto manually prepared acrylamide gels and run with 1 x electrophoresis buffer at 120 volts (stacking) and 170 volts (resolving gel) until protein were separated according to their sizes, corresponding to the pre-stained protein marker.

For Western blotting, protein gels were electrophoretically transferred to a Immobilon™ PVDF membrane for immunoblot assay as described in Section 2.2.4.3.

2.2.4.2 Comassie blue staining

Gels were prepared as per 2.2.4.1 and placed in heated 0.25% Coomassie brilliant blue G 250 in 10% acetic and 45% methanol for 2-4 hours with gentle rocking to distribute the dye evenly over the gel. Gels were washed once with distilled H_2O and placed in e-stain using 30% methanol/10% acetic acid until protein bands became distinct.

2.2.4.3 Western blotting

A piece of PVDF membrane with a rated pore size of 0.45 μm (Millipore) was soaked for about 30 second in methanol followed by rinsing with deionized H_2O, before soaking in transfer buffer. The sponge and filter paper were also soaked in transfer buffer for 15 mins and assembled as "sandwich" as follows: sponge, filter paper, PVDF membrane, protein gel, filter paper and sponge. This sandwich cassette was tightly sealed with the membrane side facing towards the positive electrode and gently placed in a transfer tank (TE62, Hoefer Inc.) filled with transferring buffer as above and transferred for 2-3 hours at 45 volts at 4°C. The membrane was then blocked for 1 hour at room temperature in 5% bovine

serum albumin in tris-buffer saline tween-20 (0.1% Tween 20 in TBS). The membrane was incubated with primary antibody diluted in blocking buffer for 1 hour at room temperature or at 4°C overnight. After washing 3 x 10 min with 0.1 % Tween-20 in TBS, membranes were incubated for 1 hour at room temperature with horseradish peroxidase conjugated anti-species secondary antibodies diluted in blocking buffer. The membranes were washed as described above; proteins were detected by ECL reagent which contains a luminol substrate of horseradish peroxidase and can be converted to a light releasing substance. Images were recorded using G BOX Chemi-16 gel documentation system and intensities quantified with Image J software.

2.2.4.4 Protein concentration determination by BCA assay kit

The BCA protein assay combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid (Thermo Fischer 2016). The first step is the chelation of copper by protein in an alkaline solution to form a blue colored complex. In this step, known as the biuret reaction, peptides containing three or more amino acid residues form a light blue colored chelate complex with cupric ions in an alkaline environment (pH 8.0). Proteins will react to produce a light blue to violet complex that absorbs light at 540 nm. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. In the second step of the color development reaction, BCA, a highly sensitive and selective colorimetric detection reagent reacts with the cuprous cation (Cu^{+}) that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations (Thermo Fisher 2015).

Briefly, 25 µl of sample from each fraction or BSA standard were applied to a 96 microplate; 200 µl mixture of reagent A and B (50:1) supplied in the kit were added to each well. The plate was mixed thoroughly and incubated for 30 minutes at 37°C before absorbance at 562 nm was read. Concentrations were calculated from the linear range of the standard curve.

2.2.4.5 Statistical analysis

Data were calculated and graphs generated by GraphPad Prism 4.0; each point represents the means ± S.E.M as indicated in figure legends of section 3.4 (Millar 2001).

2.2.5 HEK 293 cell culture and maintenance

LentiX-HEK 293 was used because of the ease of handling, their robust growth rate, low cost medium requirements and high capacity for recombinant protein expression. HEK293T contains the SV40 large antigen that amplifies transfected plasmid and expression of desired gene products. HEK 293T cells were grown at 37°C , 5% CO_2 in sterile T-25 or T-75 flat bottom flasks in complete Dulbecco's modified Eagle's medium (DMEM). Full DMEM consisted of 10% fetal bovine serum (FBS), 1% penicillin (100units/ml)/streptomycin (100 µg/ml), 1% Glutamax (0.4mM-L-glutamine).

Cells were split every two-three days. During the passaging process, old medium was discarded. Cells were washed with sterile PBS (two times); 0.5% of trypsin was added and kept at 37°C for up to 2 mins. Fresh full DMEM was added to cells and transferred into sterile 50 ml conical tubes and centrifuged for 1 min at 300 x g. The supernatant medium was discarded and replaced with fresh full DMEM. Cells were subjected to gentle and

continuous aspiration by using sterile 1ml pipette, to disrupt any lumps formed due to centrifugation.

2.2.5.1 Transfection of HEK293 cells.

For ease of transfection, cells were grown in 154 cm^2 collagen coated petri-dishes. Freshly-passaged HEK 293T cells grown up to ~90% confluency were used for transfection. Prior to transfection, the medium from the plates to be transfected was replaced with full DMEM with lowered serum concentration (consisting of 2% of FBS instead of 10% FBS). Vector DNA (50µg) to be transfected was added to 5ml serum-free medium followed by addition of 75µl Polyethylenimine (PEI) stock (1 mg/ml) to make the DNA: PEI ratio 1:1.5. The solution was left at room temperature for 10 mins to allow formation of DNA-PEI. After 10 mins, the DNA-PEI complex was added to the petri-dishes with brief rotation to allow mixing. After 8-12 hours the conditioned medium with PEI was discarded and replaced by fresh full DMEM with (2% serum) low serum. 48 hours later, the conditioned medium was collected for purification.

2.2.6 PC-12 cell culture and maintenance

PC-12 cells were provided by ATCC (CRL 1721). The cells were maintained using T-25 and T-75 cm flasks in complete RPMI-16 medium at 37 °C under a humidified 5% CO_2 atmosphere. Complete RMPI-16 consist of 10% horse serum, 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged every three days; stocks were stored in liquid nitrogen in a medium consisting of 10% DMSO and 90% complete RPMI-16 media (Greene and Tischler 1976; Levi *et al.* 1985).

2.2.6.1 ERK phosphorylation on PC-12 cells

Cells ($1x10^5$ per well) were plated on poly-L-lysine coated 12 well plates. Cells were maintained for 24 hours in complete RPM-16 medium. Cells were serum starved for 2 hours prior to their treatment with control (7S NGF) or test samples for ERK phosphorylation.

2.2.6.2 SNAP-25 on PC-12 cells

Sterile 12-or 24-well plates were coated with collagen, and kept in the sterile environment of laminar air flow until they appear dry (this step was considered necessary to avoid minimum presence of acetic acid present in collagen). Cells ($1x10^5$ per well) were plated on collagen coated well plates. Addition of 7S NGF (200 ng/ml) to PC-12 cells maintained in complete RPMI-16 medium, led to differentiation and neurite outgrowth of PC-12 cells. PC-12 cells were supplemented with 7S NGF for 7 days in complete RPMI -16 medium. Every two days, cells were replenished with fresh complete RPMI-16 medium. Cells were serum starved for 24 hours prior to their treatment with control or test samples for SNAP-25 cleavage.

2.2.7 SH-SY5Y cell culture and maintenance

SH-SY5Y neuroblastoma cells were provided by Dr. Hans-Georg König (RCSI, Dublin). SH-SY5Y cells were maintained using T-25 and T-75 cm flasks in complete 1:1 mixture of DMEM and Ham's F12 (DME F12) at 37 °C under a humidified 5% CO_2 atmosphere. Complete DME F12 medium consists of 10% fetal calf serum, 1% L-glutamine 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged every three days, and stocks stored in liquid nitrogen in a medium consisting 10% DMSO and 90% complete DME F12.

2.2.7.1 ERK phosphorylation on SH-SY5Y cells

SH-SY5Y cells ($1x10^5$ per well) were cultured on sterile 12 or 24 well plates and maintained for 24 hours in complete DME F12 medium. Cells were serum starved for 2 hours prior to their treatment with control (7S NGF) or test samples for ERK phosphorylation.

Following treatment of SH-SY5Y cells with test samples as described in section 3.4, the wells were washed once with PBS before addition of 100 µl of loading buffer (56 mM sodium dodecyl sulfate, 0.05 M Tris-HCl, pH 6.8, 1.6 mM Ultrapure EDTA, 6.25% glycerol, 0.0001% bromophenol blue). Samples were boiled for 1 min at 95°C and then run on 12% SDS–PAGE gels. Following separation, proteins were transferred onto Immobilin-P polyvinylidene difluoride membranes, and then incubated for 30 min in blotting solution (5% BSA, Tris, buffered saline, (0.1%) Tween -20). The Rabbit Phospho p42/44 ERK and Rabbit p42/44 ERK primary antibodies were added at 1:1000 dilutions to the blotting solution and incubated for 18 h. Membranes were washed three times in Tris, buffered saline, Tween -20 for 10 min and then incubated for 30 min in the blotting solution containing secondary peroxidase-conjugated goat anti-rabbit antibodies, respectively. Membranes were washed three times in Tris, buffered saline, Tween -20 (0.1%) for 10 min Immunoreactive proteins were detected by ECL reagent which contains a luminol substrate of horseradish peroxidase and can be converted to a light releasing substance. Images were recorded using G BOX Chemi-16 gel documentation system and intensities quantified with Image J software. (Arsenault et al. 2010).

2.2.8 Cytochemical staining and microscopic recording of images

Cover slips were surface sterilized with 96 % ethanol under the laminar air hood, and placed in sterile 12-or 24-well plates. Cover slips were coated with rat tail derived collagen. Cover slips coated plates were kept in the sterile environment of laminar air flow until they appeared dry.

2.2.8.1 PC-12 cells

PC-12 cells ($1x10^5$) were cultured as described in Section 2.2.6. In order to detect SNAP-25 by cytochemical staining, neurite outgrowth of PC-12 cells was considered necessary and achieved by supplementing complete RPMI -16 medium with 7S NGF (500 ng/ml) for seven days. Cells were replenished with fresh complete RPMI-16 medium containing 7S NGF after every two days. To detect TrkA receptors by cytochemical staining, PC-12 cells were cultured in full RPMI-16 medium for three days.

Coverslips were washed three times with Dulbecco's phosphate buffer saline (lacking $Mg^{2+}$and Ca^{2+}), then fixed for 20 minutes with 3.7% paraformaldehyde at room temperature in PBS. The cells were then washed with PBS three times, followed by permeabilization for 5 minutes with 0.2% Triton X-100 in PBS before blocking with 1% goat serum in PBS for 1 hour. Primary antibodies were applied in the same solution and left overnight at $4^{o}C$; after extensive washing, fluorescently-conjugated secondary antibodies were added for 1 hour at room temperature. Counter-staining of nuclei was carried out with DAPI (1 μg/ml in water) added before the final wash.

Immuno-fluorescent pictures were taken with an inverted confocal (Zeiss) or an Olympus IX71 microscope equipped with a CCD camera. Images were analyzed using Zeiss

confocal software and Image-Pro Plus 5.1, respectively. The omission of primary antibody from the fluorescence staining gave the background for secondary antibody; the signal intensity above this was taken as positive reactivity.

2.2.8.2 SH-SY5Y cells

SH-SY5Y cells ($1x10^5$) were cultured as described in Section 2.2.7.SH-SY5Y cells were cultured in complete DM F12 medium. Every two days, cells were replenished with fresh complete DM F12 medium. Immuno-cytochemical staining steps of SH-SY5Y cells were same as described for PC-12 cells in Section 2.2.8.1.

2.2.9 Hemocytometer counting

A glass hemocytometer and coverslip were cleaned with alcohol before use. Coverslip was affixed to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicated proper adhesion. Cells were mixed well prior to aspirating 20 µl into an Eppendorf tube. Trypan blue (0.4%) 20 µl mixed with the 20 µl of cells (1:1 ratio of cells: trypan blue). In some cases, 1:2 ratio of cells: trypan blue was achieved by aspirating 20 µl trypan blue treated cells into another Eppendorf with 20 µl of trypan blue. Trypan blue-treated cell suspensions (20 µl) were applied very gently to both chambers underneath the coverslip of the hemocytometer, allowing the cell suspension to be drawn out by capillary action. Using a microscope, the grid lines of the hemocytometer were focused with a 10X objective. Using a hand tally counter, live unstained cells that do not take up trypan blue stain were counted in one set of 16 squares. A counting system was used, whereby cells were only counted when they were set within a square and were on the right-hand or bottom boundary line. Similarly, 4 sets of 16 corners were counted.

Formulae to determine number of cells per millimeter = counted number of cells X (dilution factor) X (10^4) / 4 (squares)

3 Molecular engineering of proteins for targeting SNARE-cleaving protease into neuroendocrine cells

This following section describes recombinant fusion proteins LC.H_N.H_{CN}/A.βNGF (abbreviated as /AΔH_{CC}N), Vamp2.EGF and Vamp2.βNGF expressed in *E.coli* cells. This work was performed in conjunction with Dr. Matthew King.

3.1 Construct for BoNT/A-based chimera /AΔH_{CC}N

In order to determine if the BoNT/A endopeptidase could be delivered into neuroendocrine cells via a non-native binding receptor (tropomyosin receptor kinase A - TrkA), Dr Matthew King designed chimera LC.H_N.H_{CN}/A.βNGF (/AΔH_{CC}N). It consists of the LC.H_N.H_{CN}/A domain from BoNT/A fused to a cDNA encoding the βform of human NGF

(Ullrich *et al.* 1983). Structural elements of BoNT/A, chimera /AΔH_{CC} and chimera /AΔH_{CC}N are shown in Fig. 11.

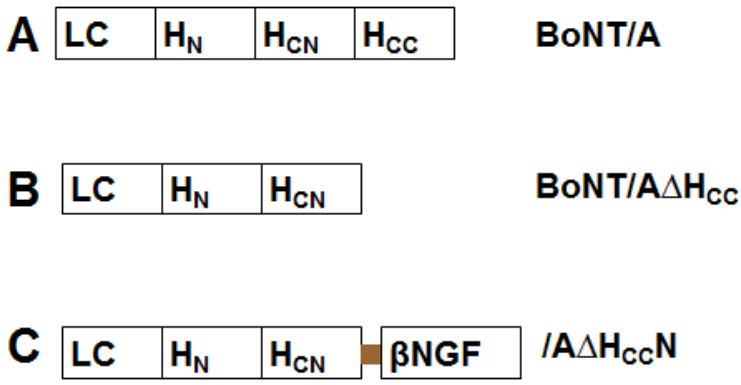


Fig. 11 Schematic representation showing structural domains of BoNT/A, chimera /AΔH_{CC} and chimera /AΔH_{CC}N. *(A) BoNT/A consists of light chain (LC), translocation (H_N), N- terminal (H_{CN}) and C-terminal (H_{CC}) binding domains. (B) /AΔH_{CC} comprises BoNT/A without the binding sub-domain H_{CC}. (C) Chimera /AΔH_{CC}N is comprised of gene fragments LC, H_N and H_{CN} from BoNT/A fused to the βNGF gene. The nucleotide sequence encoding a linker (GGGGSGGGGS) is indicated with (■).*

3.1.1 Generated chimera /AΔH_{CC}N: cloning and expression

The binding domain (H_{CC}) of BoNT/A is reported to bind synaptic vesicle protein (SV2) and gangliosides of motor neurons. The approach used in this study involved replacing the binding domain (H_{CC}) with βNGF which was hoped would bind and target the LC/A to sensory neurons, rather than motor neurons. This study focused on creating a conjugate of βNGF with BoNT/AΔH_{CC} (/AΔH_{CC}) using recombinant DNA technology to seek a cost-effective method for large scale production of such proteins.

In this study Dr Matthew King created pET32b.chimera/AΔH_{CC} and I carried out the expression and IMAC purification of the encoded protein. Dr Matthew King conjugated chimera /AΔH_{CC} with preceding sequences encoding thioredoxin (Trx), an S-tag and $his_{(6)}$ tag of the vector pET32b (Appendix 1). The resultant DNA encoded the Trx.$his_{(6)}$.S-tag.

LC.H_N.H_{CN}.βNGF protein, abbreviated as Trx./AΔH_{CC}. The details of this protein will be elaborated later in this section. Chimera Trx./AΔH_{CC}.N was present within SacI and XhoI sites of vector pET32b (Fig. 12A).

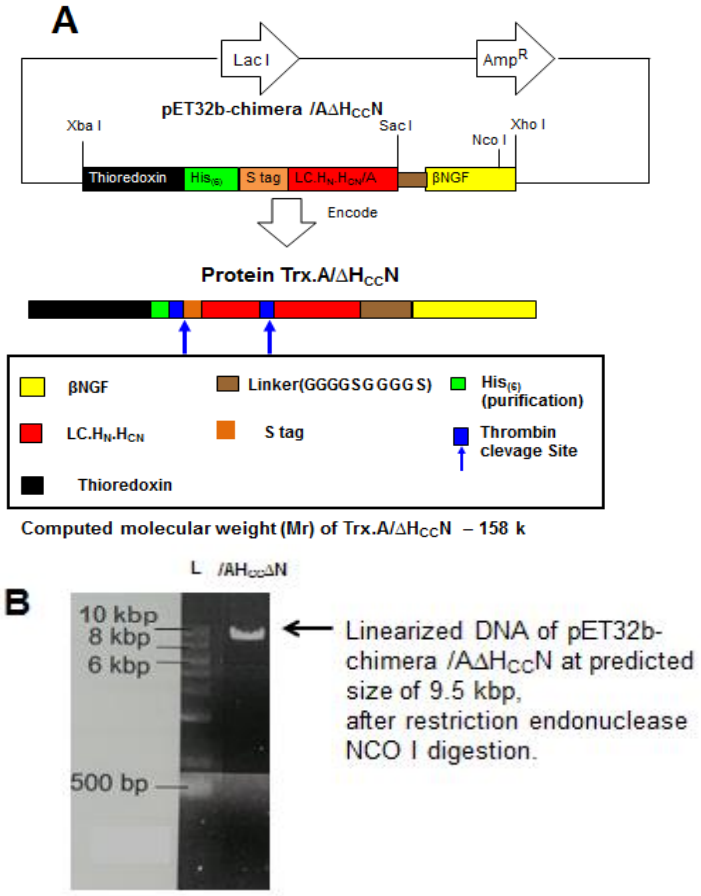


Fig. 12 Design and cloning of chimeric construct pET32b_/AΔH_{CC}N. *(A)pET32b-Chimera /AΔH_{CC}N encodes a single chain protein (abbreviated Trx. /AΔH_{CC}N) consisting of thioredoxin (Trx)tag (black), S tag known as solubility tag (orange), His $_{(6)}$ tag (green), LC.H_N.H_{CN}/A(red) fused to βNGF (yellow) by a flexible linker (brown). The molecular weight (Mw) of Trx. /AΔH_{CC}N is 158 k, predicted by its nucleotide sequence using an Expasy compute tool. (B) Image depicts ethidium bromide stained agarose gel consisting of a DNA Ladder (L) and linearized DNA of pET32b-Chimera /AΔHCCN after a single cut with NCO I restriction endonuclease for 1 hour at 37°C. Due to the presence of one NCO I restriction endonuclease site within the vector pET32b /AΔHCCN, cleavage at this locus gives a band at size 9.5 kbp indicating linearized DNA.*

Chimeric construct pET32b/AΔH_{CC}N was transformed into the origami B strain of *E.coli*, using the heat shock technique (see section 2.2.1.2.9), and the cells were cultured in Luria-Bertani medium (see section 2.2.2). This strain of *E.coli* was used as it is K-12 derivative that has mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which enhance the formation in the cytoplasm of the six disulphide bonds of the

βNGF homodimer. Trx is also commonly employed as a fusion tag to avoid incorporation into inclusion bodies, by taking advantage of its intrinsic oxido-reductase activity responsible for the reduction of disulfide bonds through thio-disulfide exchange (Prinz *et al.*1997; Aslund *et al.* 1999 and LaVallie *et al.* 2000). Thus, it was envisioned that the thioredoxin tag might facilitate oxidative refolding of Trx./AΔH_{CC}N in the periplasmic environment of *E.coli*. S-tag is a 15 amino acid oligopeptide (Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser) derived from pancreatic ribonuclease A. The abundance of its charged and polar residues makes it an extremely soluble structured peptide with a net charge near neutral pH that could facilitate expression of soluble Trx. /AΔH_{CC}N (Raines *et al. 2000*). In this study expression of chimera /AΔH_{CC}N was not tried without the Trx and S-tag. The $his_{(6)}$ tag consists of six consecutive histidine residues that coordinate, via the histidine imidazole rings, transition metal ions such as Ni^{+2} or Co^{+2} immobilized on beads or a resin for purification using immobilised metal chelate affinity chromatography (IMAC). Carboxymethylasparte agarose resin (Talon®, from ClonTech) has a high binding capacity, and was used for purification of Trx./AΔH_{CC}N from crude cell lysates. The advantages of using $his_{(6)}$ tag include: (a) its small size and charge minimises interference with Trx./AΔH_{CC}N structure and function (b) Trx./AΔH_{CC}N can be eluted under mild conditions by imidazole competition (Costa *et al.* 2014, Terpe, 2003;Kimple and Sondek, 2004; Li, 2010). Two thrombin cleavage sites, indicated by blue arrows (Fig. 13A), were inserted to allow removal of the $his_{(6)}$ tag and to convert the single polypeptide into disulphide-bridged dichains (Fig.13A).

Three antibiotics namely ampicillin (100 µg mL^{-1}), tetracycline (12.5 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) were used for selection. The origami B strain of *E.coli* consists of a gene for tetracycline resistance in its chromosome and is, therefore, able to degrade tetracycline present in the Luria-Bertani medium. The presence of kanamycin maintains a

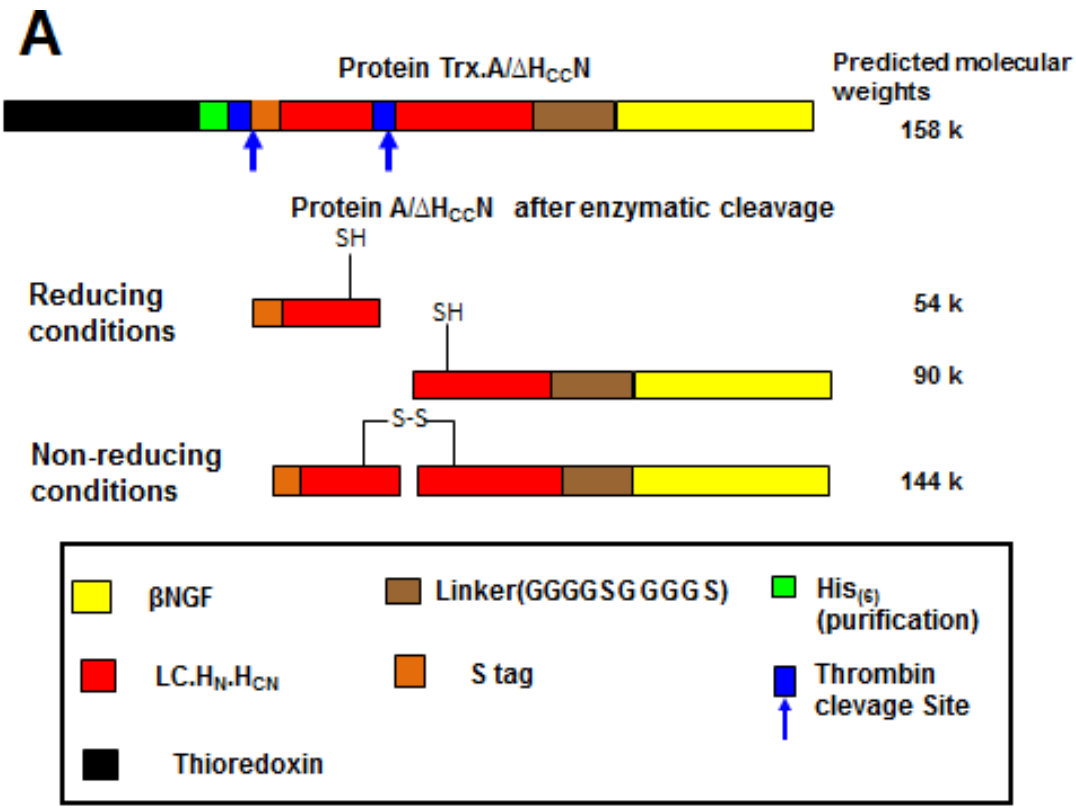
transgene trx B in origami B cells which, in turn, may facilitate formation of six disulphide bonds in βNGF homodimer within the cytoplasm (Prinz *et al.* 1997 and LaVallie *et al.* 1993). The gene for ampicillin resistance is present on the pET32b vector; therefore, ampicillin facilitates selection of cells transformed with chimeric construct pET32b /AΔH_{CC}N.

To trigger transcription from the lac operon (Lac I) and the expression of the protein Trx./AΔH_{CC}N, 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium used for the cultivation of the *E.coli* cells. The lac operon is required for the transport and metabolism of lactose, as a carbon source when glucose is not available. Lac I is a functional unit of *E.coli* genomic DNA consisting of three structural genes lac Z, lac Y and lac A, a promoter, a terminator and an operator. The gene product of lac Z is β-galactosidase (an enzyme that cleaves lactose to glucose and galactose). Lac Y encodes lactose permease, a protein that facilitates transport of lactose into the cell. Lac A encodes an enzyme galactoside O acetyltransferase that transfers an acetyl group from acetyl-CoA to β-galactosides. IPTG is a molecular mimic of allolactose, a lactose metabolite that does not get hydrolysed by β-galactosidase, but does trigger transcription from the lac operon. IPTG binds to the lac repressor (a region of DNA which inhibits transcription of lac Z, lac Y and lac A genes) and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing transcription of genes lac Z, lac Y and lac A. The sulphur atom of IPTG forms a chemical bond, which is non-hydrolysable by the cell and, thereby, prevents IPTG degradation. IPTG uptake at low concentrations involves lactose permease but at higher levels it enters cells independent of lactose permease (Malan *et al.*1984; Hansen *et al.*1998 and Marbach and Bettenbroch 2012).

3.1.2 IMAC purification followed by thrombin nicking yielded protein Trx/AΔH_{CC}N

IMAC yielded 1.2 mg of protein per litre of culture. Bands registered at molecular weight (Mr) 158 (Fig. 13A) by Coomassie staining on 10% SDS-PAGE suggest the presence of Trx/AΔH_{CC}N but as a minor fraction (Fig. 13B).

Incubation with thrombin achieved nicking of the chimera /AΔH_{CC}N, as demonstrated by the loss of the 144 k band and the appearance of bands at Mr ~90 k for H_N.H_{CN}.βNGF and molecular weight ~54 k for LC/A fused to S tag upon SDS-PAGE in the presence of 2-mercaptoethanol. A band appeared at Mr ~144 k, in the absence of reducing agent indicating that the inter-chain di-sulphide bond had been formed (Fig. 13C).



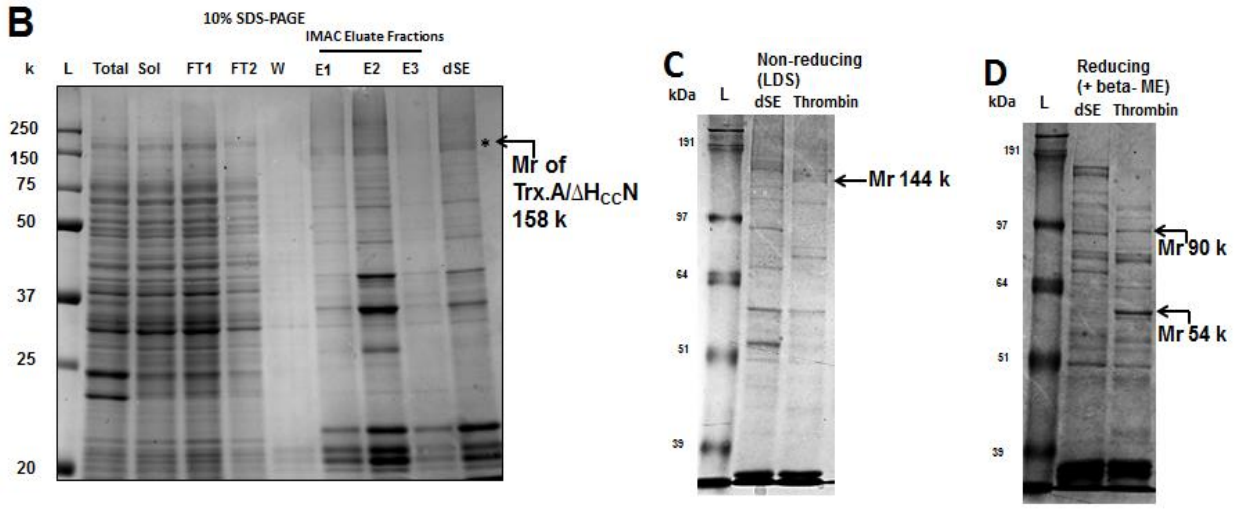


Fig.13 Expressed chimera A/H_{CC}ΔNGF. *(A) Depicts the predicted modular structure and molecular weight (Mr) of protein Trx./AΔH_{CC}N determined by using Expasy compute tool, which uses a coded algorithm to predict the iso-electric focusing point and molecular weight of proteins based on their amino acid sequences. Treatment of protein Trx. /AΔH_{CC}N with thrombin (200 µg of protein and 2 µg of enzyme in 0.2 ml 50 mM HEPES/50 mM NaCl, pH 7.4 for 1 hour at 37°C for 40 minutes) resulted in nicking of thioredoxin tag and formation of /AΔH_{CC}N dichain (ICNT unpublished data). The latter consists of two moieties namely, LC/A fused S tag and H_N.H_{CN}/A.βNGF. The dichain was inter-linked under non-reducing conditions due to presence of interchain disulphide bond formation. In the presence of reducing agent such as 2-mercaptoethanol, reduction of sulphur atoms involved in the disulphide bond formation led to separation of inter-linked dichains. The Mr of LC/A fused S tag and H_N.H_{CN}/A.βNGF were 54 k and 90 k, respectively. (B) Coomassie stained 10% SDS PAGE gel of Total lysate (total), soluble bacteria lysate (Sol) and various fractions obtained during IMAC purification of Trx./AΔH_{CC}N; FT1-FT2 - flow through (non-binding), W – wash, E1-E4 – eluate fractions, dSE – E1-E4 pooled and desalted by gel filtration. All samples excluding wash (w) and one imidazole eluate (E3) indicate the presence of a band at 158 Mr (labelled *), which is the predicted size of Trx. /AΔH_{CC}N. (C) Coomassie stained protein gel of Trx. /AΔH_{CC}N under non-reducing conditions after thrombin treatment Bands were detected at the molecular weight 144 k. (D) Coomassie stained protein gel of Trx. /AΔH_{CC}N under reducing conditions after thrombin treatment. Bands were detected at the molecular weights 90 k and 54 k*

corresponding to those predicted in Fig. 13A. A number of other bands visible in (C) and (D) may be due to contaminants from E.coli or degraded recombinant protein.

3.1.3 Characterization of generated protein /AΔH_{CC}N in neuroendocrine cells

The reason for designing chimera /AΔH_{CC}N was to ascertain whether its encoded protein LC.H_N.H_{CN}/A.βNGF can enter neuroendocrine cells via receptor TrkA, by determining SNAP-25 cleavage due to delivered LC/A protease activity. SH-SY5Y cells and PC-12 cells are derived from human neuroblastoma and rat adrenal medulla, respectively. They are accepted as models to evaluate the potency of BoNT/therapeutics due to several advantages like: ease of culture, generated in large quantities and sensitivity to both NGF and BoNT/A.

3.1.3.1 PC-12 cells and SH-SY5Y cells contain TrkA and SNAP-25 and, thus, could be used to assess receptor binding and uptake of chimera /AΔH_{CC}N

The presence of TrkA in SH-SY5Y cells and PC-12 cells was demonstrated by immuno-fluorescent microscopy and counter staining with DAPI (Fig. 14/A,B and 15/A,B).

Before assessing whether chimera /AΔH_{CC}N can enter neuroendocrine cells (such as PC-12 cells and SH-SY5Y cells) and if its LC/A protease cleaves SNAP 25, it was important to demonstrate the presence of SNAP-25. Using a specific antibody against SNAP-25 and counter staining using DAPI, both cells showed labelling of SNAP-25 in cell bodies and neuron like process (Fig. 14/C,D and 15/C,D).

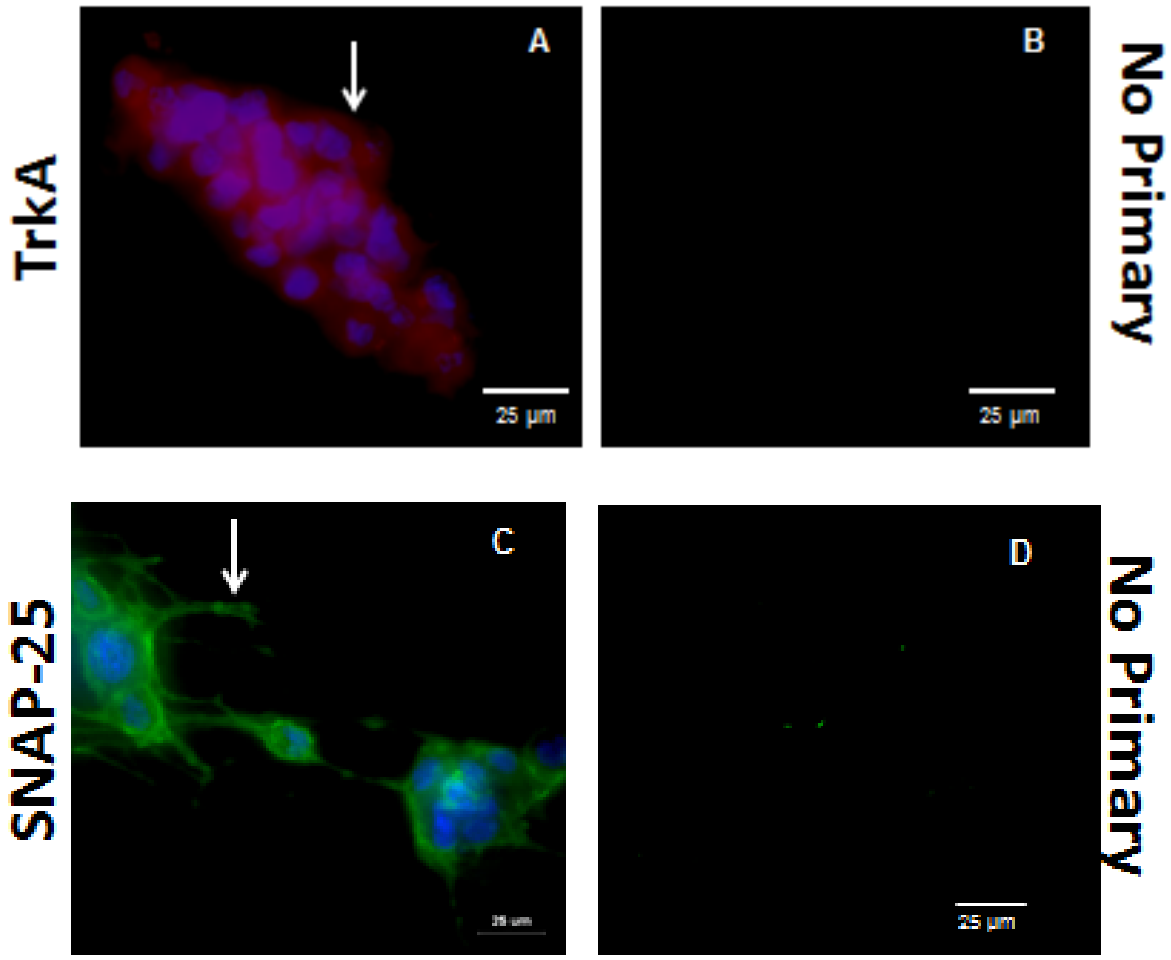


Fig. 14 Visualisation of TrkA receptor and SNAP-25 on PC-12 cells. *PC-12 were cultured in vitro on coverslips without 7S NGF as described in Chapter 2. Cells were fixed and permeabilised. (A) The PC-12 cells were stained with rabbit anti-TrkA (1:5000) overnight at 4°C, washed with phosphate buffer saline followed by goat anti rabbit Alexa Fluor 568 (1:1500). For (B) the PC-12 cells were exposed only to goat anti-rabbit Alexa Fluor 568 (1:1500). In (C) and (D), PC-12 cells were differentiated with 500 ng per ml of 7S NGF in order to visualize SNAP-25 along the differentiated neurites, as described in Chapter 2. Seven days post culture, cells were fixed and permeabilised. The cells were stained with mouse anti-SNAP-25 (1:5000), washed with phosphate buffer saline followed by goat anti-mouse Alexa Fluor 488 (1:1500). In the case of (D), the PC-12 cells were treated identically except that mouse anti-SNAP-25 (1:5000) was omitted. Samples (A) and (C) were counter-stained by DAPI. All the samples were mounted onto slides and viewed in an inverted microscope under fluorescent mode.*

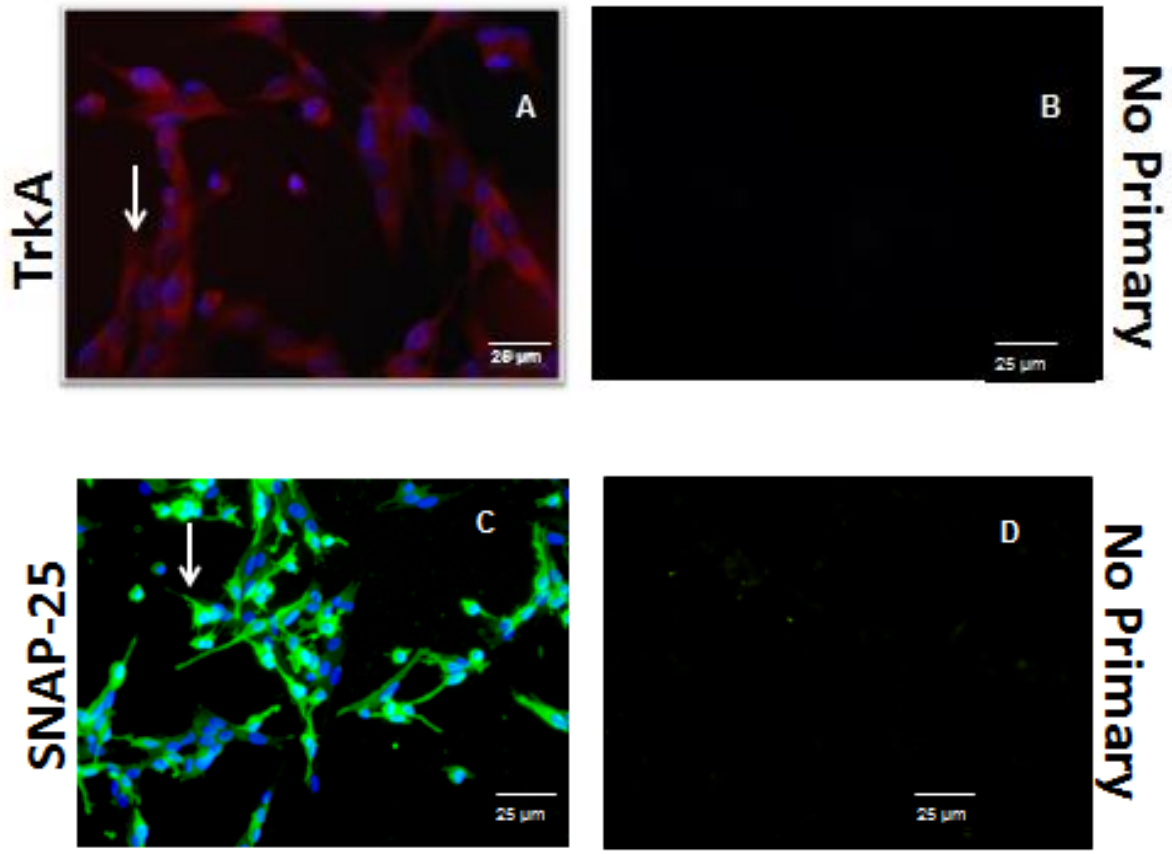


Fig. 15 Microscopic demonstration of the presence of TrkA receptors and SNAP-25 in SH-SY5Y cells. *SH-SY5Y cells were grown on cover slips as described in Chapter 2. (A) The SH-SY5Y cells were stained with rabbit anti-TrkA (1:5000) overnight at 4°C, washed with phosphate buffer saline followed by goat anti rabbit Alexa Fluor 568 (1:1500). For (B) the SH-SY5Y cells were exposed only to goat anti-rabbit Alexa Fluor 568 (1:1500). In (C) and (D), the SH-SY5Y cells were stained with mouse anti-SNAP-25 (1:5000), washed with phosphate buffer saline followed by goat anti-mouse Alexa Fluor 488 (1:1500). In the case of (D), the SH-SY5Y cells were treated identically except that mouse anti-SNAP-25 (1:5000) was omitted. Samples (A) and (C) were counter-stained by DAPI. All the samples were mounted onto slides and viewed in an inverted microscope under fluorescent mode.*

3.1.3.2 Protein /AΔH_{CC}N proved biologically inactive, as measured by antibodies to phospho-MAPK 42/44

NGF preferentially binds to receptor TrkA (Huang and Reichardt 2003) which consists of an extracellular, an intracellular domain, a transmembrane, an intracellular juxta-membrane domains followed by tyrosine kinase domain and C-terminal tail (Weismann and de Vos 2001). NGF stimulates TrkA receptor auto-phosphorylation at C-terminal tail at tyrosine 490 which causes dimerization of the receptor via a cascade of signalling proteins Ras, C-Raf and MEK, activated TrkA leads to phosphorylation of MAPK at sites Thr202 and Tyr204 (Segal, 2003; Cowley *et al.*1994)(Fig. 16A).

In order to discover if the chimeric /AΔH_{CC}N protein entered PC-12 cells, escaped the acidic compartments and cleaved SNAP-25, PC-12 cells were grown in presence of 7S NGF for seven days as described in Section 2.2.6.2 of Chapter 2 (Fig. 16B). LC/A domain of BoNT/A (control) is expected at Mr ~ 50 k. Because of the contribution of the S-tag, the LC/A of chimeric /AΔH_{CC}N protein is predicted at ~ 54 k (Fig. 12A); bands detected by antibody to LC/A at ~54 k indicated its presence. After exposure to 30 nM and 10 nM, /AΔH_{CC}N protein separated from the translocation domain. Western blotting with specific mouse anti – SNAP -25 antibody confirmed the presence of intact and cleaved SNAP-25 in PC-12 cells. Treatment of PC-12 cells with Trx./AΔH_{CC}N at concentrations of 30 nM and 10 nM, but not at 3 nM, showed faint bands of cleaved SNAP-25 detected by specific anti-SNAP-25 antibody, indicating that it is low in activity compared to control BoNT/A protease activity. The latter, in turn, could be due to 1) presence of S tag at the N-terminus of protein /AΔH_{CC}N, suggested to reduce toxicity of its LC/A (Chen and Barbieri 2011) and 2) due to impurities in the protein /AΔH_{CC}N. An additional explanation for the faint

bands could be due to non-specific binding and membrane uptake of /AΔH_{CC}N protein by PC-12 cells.

To confirm whether chimeric /AΔH_{CC}N protein interacts with cell surface TrkA receptors, PC-12 cells were incubated with /AΔH_{CC}N or control 7S NGF. Western blotting with rabbit anti-phospho MAPK 42/44 antibody showed that the specific control (7S NGF) led to phosphorylation of mitogen activate protein kinase (Mr = 42 k and 44 k), presumably at amino acid sites Thr202 and Tyr204 (Fig. 16C). However, chimeric /AΔH_{CC}N protein did not show a detectable increase in phosphorylation of MAPK relative to that in non-treated control cells, indicating failure of receptor TrkA activation at cell surface of PC-12 cells. Thus, replacing the binding domain of BoNT/A (H_{CC}) with βNGF (Fig. 11C) led to expression of chimeric protein LC.H_N.H_{CN}/A.βNGF, which proved to be biologically inactive. Reasons for this will be discussed in the General Discussion (Chapter 4).

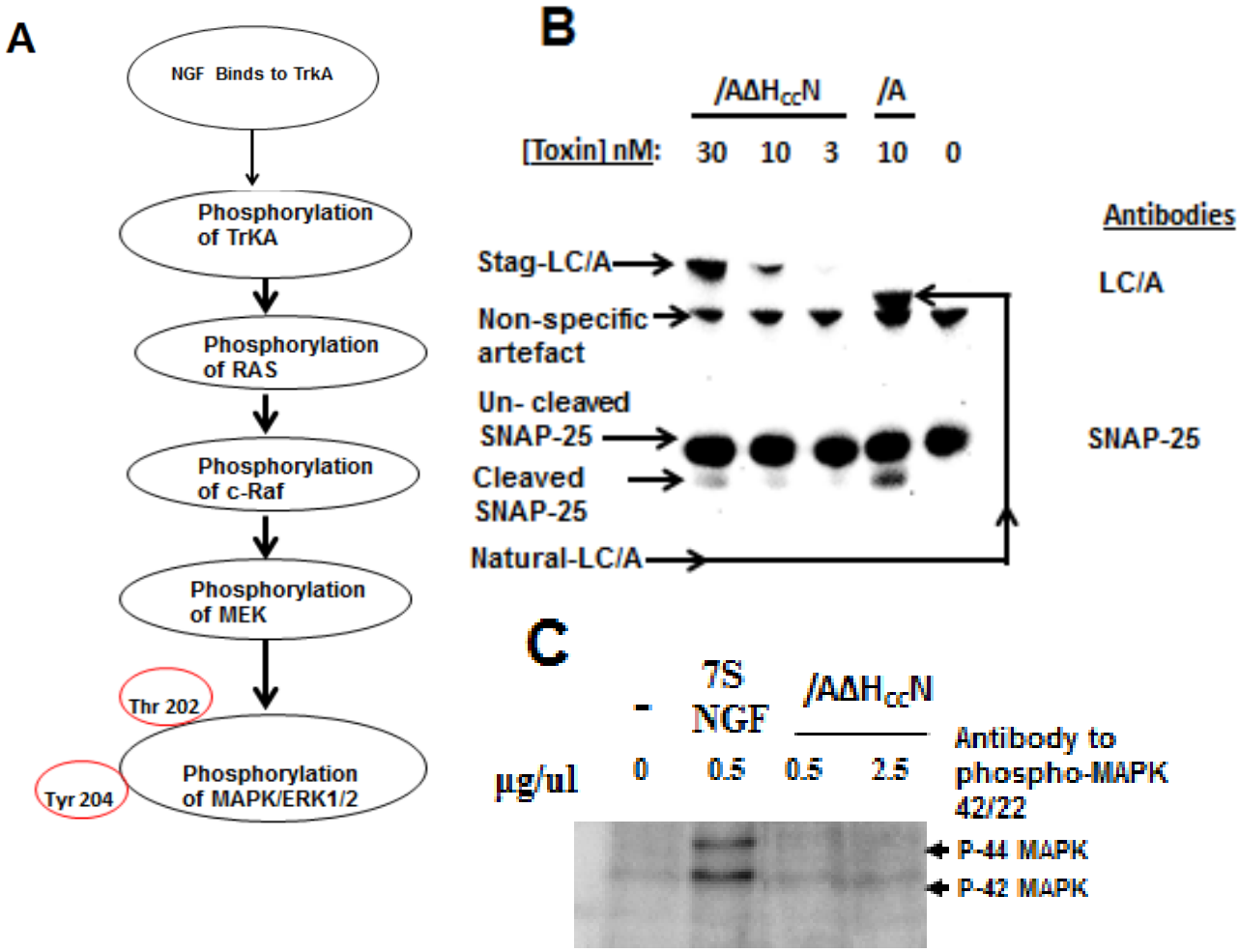


Fig. 16 Chimera /AΔH_{CC}N proved to be biological inactive on PC-12 cells. *(A) On binding to TrkA, NGF activates an intracellular cascade of signaling proteins, including Ras, c-Raf and MEK. This eventually leads to phosphorylation of MAPK 1/2 proteins at sites (circled red) Thr202/Tyr204. (B) Serum-starved PC-12 cells were treated with chimeric /AΔH_{CC}N protein at different concentrations (30, 10 and 3 nM) and, for comparison, 10 nM of BoNT/A were treated with PC-12 cells. Western blotting by specific anti-SNAP-25 antibody detected bands at Mr 25 k and Mr ~23 k indicating the presence of intact SNAP-25 and cleaved SNAP-25, respectively. As detailed in chapter 4, the higher bands detected by specific antibody to LC/A antibody at ~ Mr 54 k and Mr ~50 k indicate the presence of LC/A fused S tag and LC/A of BoNT/A, respectively. The lower set of bands in all lanes show non-specific proteins detected by antibody to LC/A in PC-12 cells. (C) 7S NGF induced robust phosphorylation of ERK 1/2 (MAPK) compared to negative control (-). LC.H_N.H_{CN}./A.βNGF (represented as /AΔH_{CC}N) failed to induce ERK 1/2 /MAPK phosphorylation.*

3.2 Protein stapling technology

Targeting the protease of BoNT/A to neuroendocrine cells by direct recombinant fusion of proteins, has several disadvantages such as, the unidirectional nature of protein translation (N–C), misfolding of protein, proteolytic degradation and low yields (Ferrari *et al* 2012; Arsenault *et al* 2013).

During neuronal exocytosis, synaptic vesicles bind to SNAREs to form a tight tetra-helical complex. Arsenault *et al.* (2013) suggested exploiting the established selective and high-affinity interaction of the α-helices from neuronal SNAREs to achieve biochemical coupling; this strategy has been termed protein stapling (Fig. 17). Protein stapling technology exploits the self-assembling property of three helical SNAREs such as SNAP-25, synaptobrevin (also termed Vamp) and syntaxin-1 to form such tetra helical complexes (Fig. 17). Of the four helices in the tetra helical complex, SNAP-25 contributes two, syntaxin-1 contributes one and the Vamp contributes the final component (Sutton *et al* 1998). The four α-helices spontaneously interact when mixed to form a tetra-helical tight interaction (Darrios *et al.* 2010). The assembled neuronal SNARE complex is stable at elevated temperatures and exhibits resistance to: chaotropic agents, strong detergents and proteases detailed in section 1.3.3.2 of this study (Sutton 1998, Sudhof and Rothman 2009).

Using this approach, Arsenault *et al.* (2013) described the delivery of type A botulinum protease into neuroendocrine cells, using EGF. Arsenault *et al.* (2013) created individual components Vamp2 (25-84) fused EGF and SNAP-25 (22-206) fused LC.H_N/A in *E.coli* via DNA recombination. They were stapled together into a functional unit by addition of a synthetic peptide corresponding in sequence to residues (1-45) of syntaxin-1.

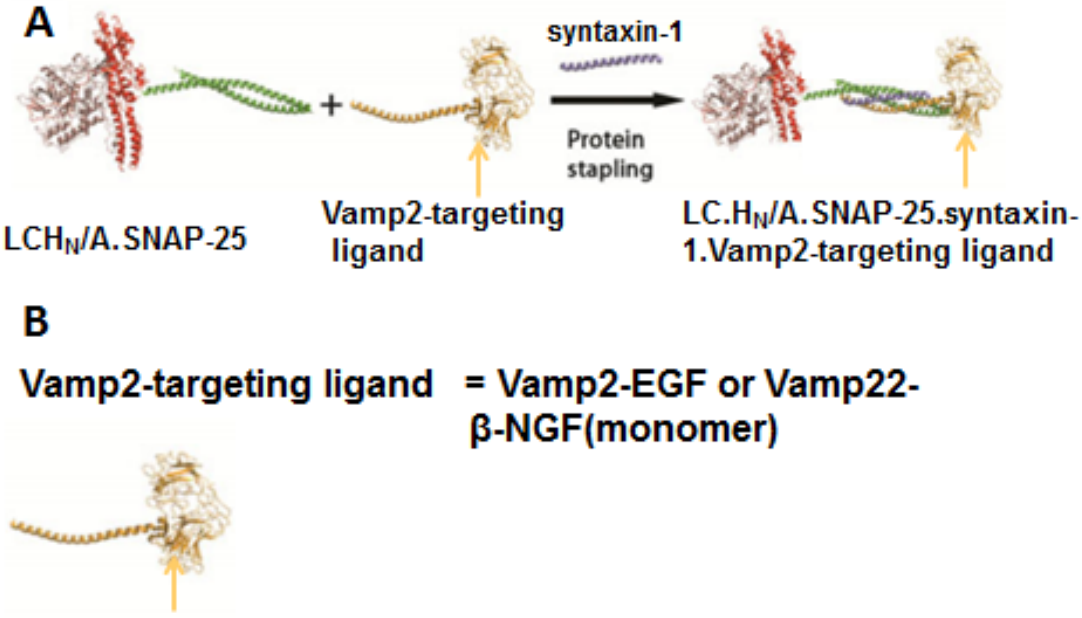


Fig. 17 Schematic representation of protein stapling. *(A) Stapling reaction using LCH$_N$/A-SNAP-25 (the protease domain, brown; the translocation domain, red, and a SNAP-25 (22-206) linker, green), the Vamp2 (25-84) linked to targeting receptor-binding ligand (yellow) and the syntaxin-1 peptide (1-45) (blue). Addition of the syntaxin-1 peptide (1-45) results in formation of an LC.H$_N$/A.SNAP-25.syntaxin-1.Vamp2-targeting ligand. (B) shows targeting of the receptor-binding ligand EGF and βNGF monomer (yellow arrow) linked to Vamp2. Image adapted from Arsenault et al. 2013.*

In view of the lack of success in the generating biological-active /AΔH$_{CC}$N fusion protein, it was decided to explore the potential of this stapling technology, as described by Arsenault *et al.* (2013) and in Chapter 1. In this section, the successful creation Vamp2 fused to EGF in *E.coli* is described, which involved 1) the creation of chimeric construct encoding Vamp2 (25-84) fused to EGF and 2) generation of a chimeric construct encoding Vamp2 (25-84) fused to NGF. The study described in Arsenault *et al* (2013) used Vamp2 fused EGF as the ligand to retarget the SNARE cleaving protease into sensory neurons whereas my study used NGF as the ligand to re-target the SNARE cleaving protease into

neuroendocrine cells. The difference between chimeric construct Trx.Vamp2.EGF and Trx.Vamp2.βNGF involved the replacement of gene encoding EGF with that encoding βNGF.

3.2.1 Generation of a chimeric construct encoding Vamp2 (25-84) fused to EGF (Vamp2.EGF) in *E.coli*

Dr Matthew King performed the cloning of Chimeric construct Trx.Vamp2.EGF and I expressed it in *E.coli* cells and purified it using IMAC.

Chimeric construct Trx.Vamp2.EGF was transformed into the origami B strain of *E.coli*, using the heat shock technique; the cells were cultured in Luria –Bertani medium. DNA of chimeric construct Trx.Vamp2.EGF (Fig. 18A) encodes Trx (see above), $his_{(6)}$ tag, a thrombin cleavage site to allow removal of the $his_{(6)}$ tag, Vamp2 (amino acid 25-84) for SNARE stapling, 10 amino acid (GGGGSGGGGS) flexible linker and EGF. Ampicillin (100 μg mL^{-1}), tetracycline (12.5 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) were used as before. To trigger transcription of the lac operon (Lac I), expression of chimera Trx.Vamp2.EGF was induced by addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the *E.coli* culture. During IMAC purification the total bacterial lysate was mixed with Talon® resin at 4°C for 1 hour before separating unbound proteins from the resin on gravity flow columns. After washing with wash buffer (150 mM NaCl, 20 mM HEPES and 10 mM imidazole), $his_{(6)}$ tagged proteins were eluted by 500 mM imidazole; eluted proteins were separated from imidazole salt and buffer exchanged into storage buffer (20 mM HEPES, 145 mM NaCl pH 7.4) by gel filtration columns. Expression of Trx.Vamp2.EGF in the origami strain of E.coli followed by IMAC purification yielded 1.5 mg of total protein per litre, of which Trx.Vamp2.EGF formed ~80% of total protein (Fig. 18/B,C). Thrombin treatment of IMAC purified

Trx.Vamp2.EGF protein for 1 hour at 20°C nicked Trx.his$_{(6)}$ tag. Incubation of thrombin treated Trx.Vamp2.EGF protein with Talon® resin, followed by collection of unbound flow through using gravity flow columns, separated Vamp2.EGF and Trx.his$_{(6)}$ tag (Fig. 19A).Western blotting with specific antibody to Vamp 1, 2, 3 showed thrombin cleavage separated Vamp2.EGF from the thioredoxin tag (Fig. 19B).

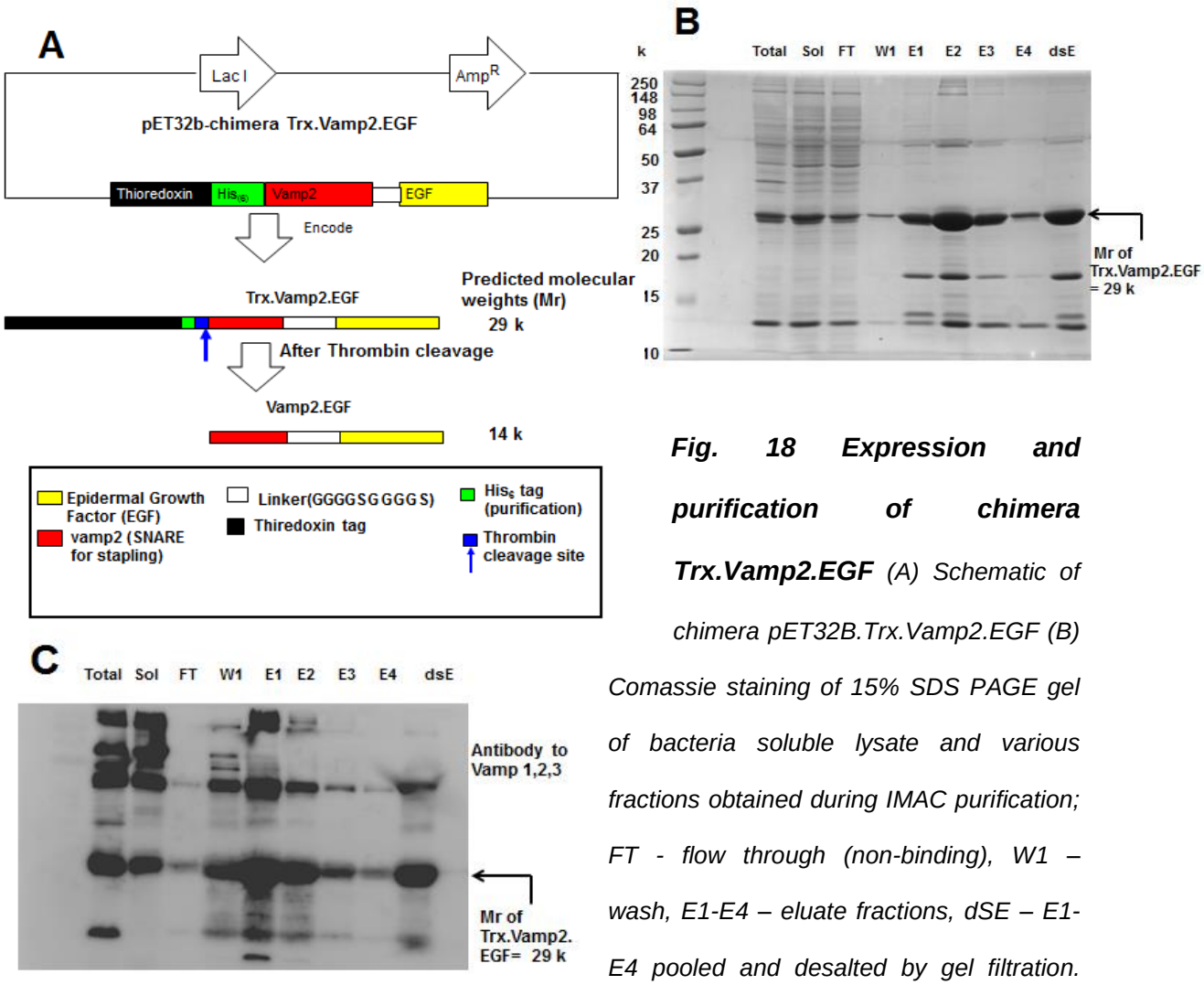


Fig. 18 Expression and purification of chimera Trx.Vamp2.EGF *(A) Schematic of chimera pET32B.Trx.Vamp2.EGF (B) Comassie staining of 15% SDS PAGE gel of bacteria soluble lysate and various fractions obtained during IMAC purification; FT - flow through (non-binding), W1 – wash, E1-E4 – eluate fractions, dSE – E1-E4 pooled and desalted by gel filtration. Prominent bands were displayed at ~ Mr 29 k, which is the predicted molecular weight of Trx.Vamp2.EGF indicating its presence. (C) Western blot using antibodies against Vamp 1, 2, 3 of samples as described in B. Total lysate (Total) was mixed with Talon® resin at 4°C for 1 hour before separating unbound proteins (FT) from the resin on gravity flow columns. After washing with wash buffer (150 mM NaCl, 20 mM HEPES and 10 mM imidazole pH 7.4), his$_{(6)}$ tagged proteins were*

eluted with 500 mM imidazole; eluted proteins were separated from imidazole salt and buffer exchanged (dSE) into storage buffer (20 mM HEPES, 145 mM NaCl pH 7.4) by gel filtration columns. Strong band at Mr 29 k confirms the presence of Trx.Vamp2.EGF.

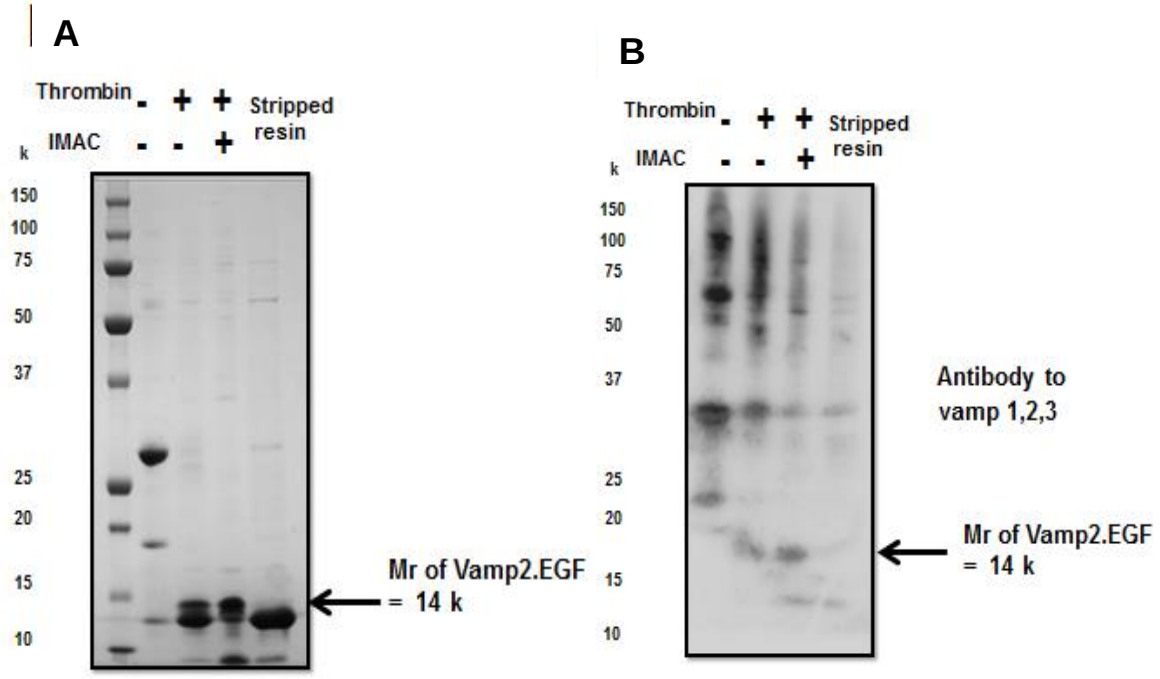


Fig. 19 Thrombin nicking of chimera Trx.Vamp2.EGF *(A) Coomassie staining of thrombin-treated Trx.Vamp2.EGF followed by IMAC purification on 15 % SDS-PAGE gel. At ~Mr 14 k, strong bands occur indicating the presence of Vamp2.EGF; slightly below, another band at Mr ~15 k, which is expected size of thioredoxin protein was detected indicating its cleavage following thrombin treatment of Trx.Vamp2.EGF. Occurrence of thioredoxin band (upper band) below the predicted Vamp2.EGF suggests that most of Vamp2.EGF protein was purified using IMAC purification. (B) Depicts the Western blot of Trx.Vamp2.EGF after thrombin treatment followed by IMAC purification. A band detected by antibody to Vamp 1, 2, 3 at Mr of 14 k, confirming the presence Vamp2.EGF upon removal of thioredoxin protein after thrombin treatment. Stripped resin in Fig. 19 (A), (B) represents the recombinant protein solubilized from the Talon® resin by SDS-PAGE.*

3.2.2 The creation of a chimeric construct encoding Vamp fused to NGF (Vamp2.βNGF) in *E.coli*

Using the construct Trx. pET32b.Vamp2.βNGF created by Dr Matthew King (unpublished data generated as part of his work at ICNT during the course of this study), the work of Arsenault *et al.* (2013) was adapted as described in Section 3.2 to generate a fusion protein of Vamp2.βNGF in *E.coli*.

Chimeric construct Trx.Vamp2.βNGF (Fig. 20) was transformed into the origami B strain of *E.coli,* using the heat shock technique; the cells were cultured in Luria –Bertani medium. Expression of chimeric protein Trx.Vamp2.βNGF in the origami strain of *E.coli* cells was followed by IMAC purification as described previously for other fusion proteins. Coomassie staining of SDS-PAGE (Fig. 21A) and Western blot (Fig. 21B) using antibody to Vamp 1, 2, 3 showed the majority of Trx.Vamp2.βNGF was insoluble. This suggested its presence in inclusion bodies due to the intracellular accumulation of aggregated Trx.Vamp2.βNGF protein. Attempts were made to separate inclusion bodies and isolate the Trx.Vamp2.βNGF from inclusion bodies as described by Rattenhol *et al.* 2001; however, during the dialysis step the protein aggregated.

Lessmann *et al.* 2003 suggests that the Pre-Pro domain plays an important role in proper folding and intracellular sorting of mature βNGF. Lack of precursor Pro domain in the designed chimera Trx.Vamp2.βNGF and absence of components necessary for post translational processing of ProNGF into soluble mature βNGF homodimer in *E.coli* may have led to the formation of protein aggregates and incorporation into inclusion bodies.

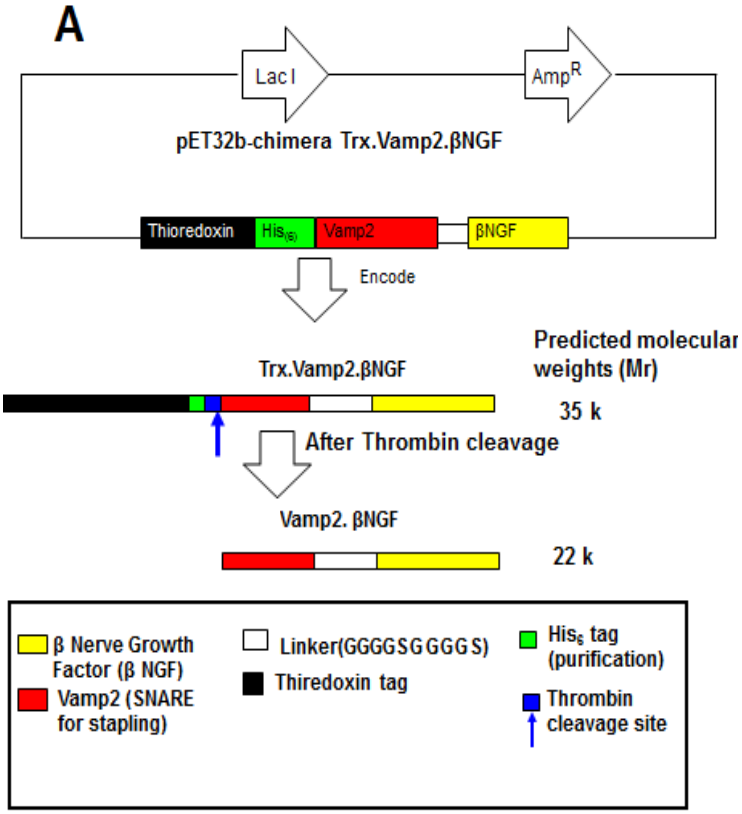


Fig. 20 Generation of chimera pET32B.Trx.Vamp2.βNGF *Schematic of chimera pET32B.Trx.Vamp2.βNGF*

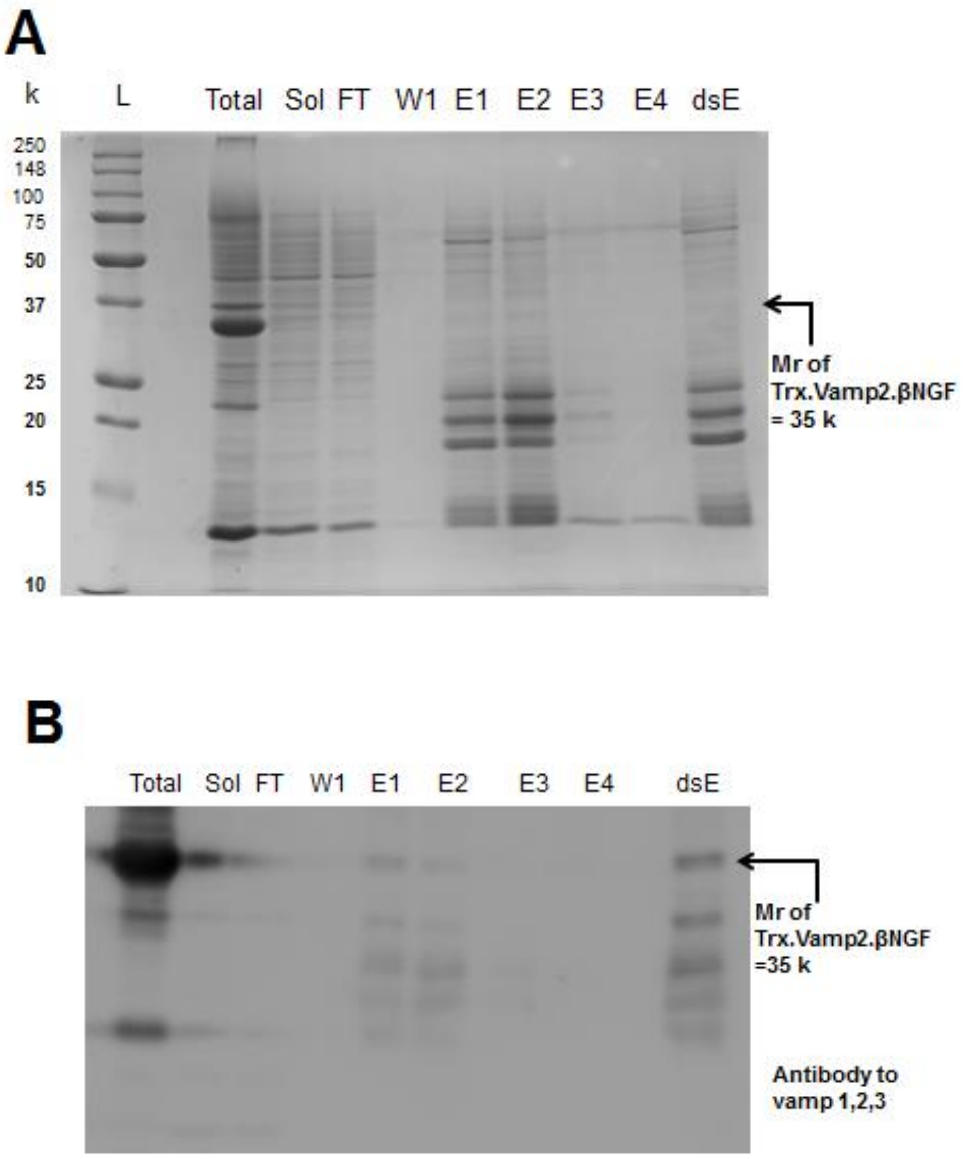


Fig 21 Expression and purification of Vamp2.βNGF in the inclusion bodies of E.coli.

(A) Coomassie staining of a 15% SDS PAGE gel of various fractions obtained during IMAC purification of Trx.Vamp2.βNGF protein as described in Fig. 18B. A strong band at the predicted molecular weight of approximately 35 Mr in (Total) indicates the presence of Trx.Vamp2.βNGF in the inclusion bodies. (B) Western blot on IMAC purified samples of Trx.Vamp2.βNGF on 10% SDS-PAGE. Although anti Vamp 1,2,3 immunoreactivity was clearly evident in lysed bacteria, only a minor amount was found in the soluble fraction.

3.3 Molecular engineering of proteins in mammalian cells for the targeted delivery of SNARE-cleaving protease into neuroendocrine cells.

To overcome the lack of success with expressing soluble βNGF fused to Vamp2 in *E.coli* cells, it was decided to explore a mammalian expression system. The study described in Colangelo *et al.*(2005) showed that active NGF could be expressed with good yield in mammalian cells. In the rough endoplasmic reticulum, naturally occurring NGF is synthesized as Pre-Pro precursor proteins. The precursor Pre-Pro signal of NGF plays an important role in post-translation modification and processing of biological-active homodimer, βNGF. The Pre (signal) peptides are removed by proteolytic processing in the lumen of the endoplasmic reticulum to yield stable Pro.NGF. Biophysical analysis reported by Lessmann *et al.* (2004) suggests that the Pro domain serves an important role in proper folding and intracellular sorting of mature βNGF. ProNGF is glycosylated at amino acid residues 61 and 121. Trimming of its N-linked oligosaccharide regulates the transport of Pro-NGF from endoplasmic reticulum to the trans-Golgi network, via clathrin-coated transport vesicles (Sutter *et al.* 1991). The Pro.βNGF is rapidly cleaved intracellularly at a four residue site (Arg-X, Lys/Arg-Arg) by furin (a protease of the prohormone convertase family) (Seidah *et al.* 1996). Furin (ubiquitously present in mammalian cells such as HEK 293T cells) converts Pro.βNGF to a mature βNGF form (Li *et al.* 2010).

It was decided that this could be applied with some adaptations, together with the protein stapling technology to generate a soluble and biologically-active Vamp2.βNGF. The stages involved were: 1) the transfection and characterization of a chimeric construct pCDNA3.1ProβNGF encoding ProβNGF in HEK 293T cells and 2) generation of chimeric

constructs pCDNA3.1.Pro.Vamp2.βNGF and pCDNA3.1.Pro.βNGF.Vamp2 3) and characterization of these chimeras in human neuroblastoma SH-SY5Y cells to ascertain biological activity. As stated earlier, HEK 293T cell line is a derivative of human embryonic kidney 293T cells, which contains the Simian Vacuolating Virus 40 TAg (SV40 T-antigen). SV40 T antigen is a hexameric protein that is a dominant-acting oncoprotein derived from the polyomavirus SV40 that allows for episomal replication of plasmid pCDNA3.1 containing the SV40 origin of replication. The function of T-antigen is controlled by phosphorylation, which attenuates the binding to the SV40 origin. Protein-protein interactions between T-antigen and DNA polymerase-alpha directly stimulate the SV40 origin of replication. This allows amplification of transfected pCDNA3.1 and extended temporal expression of the transgene.

In this case Pro.βNGF HEK 293 T cells were used as they were straightforward to grow and to transfect. Advantageously, these cells can be grown in low serum concentrations (2% fetal bovine serum), which facilitates efficient transfection using PEI. A more detailed description of transfection of HEK 293T cells can be found in Chapter 2.

pCDNA3.1 is a plasmid widely used for high level stable or transient expression of transgenes in mammalian cells. It consists of an early cytomegalovirus promoter (CMV) for high level expression in HEK 293T cells. Presence of a neomycin-resistance gene enables selection of stable cell lines. The Simian Vacuolating Virus 40 Tag, a hexameric oncoprotein facilitates episomal replication in HEK 293T via the large SV40 larger T antigen, as described above.

3.3.1 The Pro signal of NGF facilitates soluble expression of biologically-active NGF, determined by neurite outgrowth in PC-12 cells

The objective of this experiment was to determine if NGF expressed in HEK 293T was biologically active.

NGF binds to the high-affinity tyrosine kinase receptor, TrkA, leading to its phosphorylation and the subsequent activation of PI3K/Akt/GSK3β pathways. This, in turn, facilitates the cytoskeletal rearrangements necessary for neurite outgrowth (Patapoutian *et al.* 2001; Cantley *et al*. 2002; Zhou *et al.* 2004 and Hur *et al.* 2010; Sierra-Fonseca *et al* 2014). The Chimeric construct pCDNA3.1pro.βNGF (Fig. 22A) containing the Pre-Pro.βNGF gene (Ullrich *et al.* 1983) was provided by Dr Anna Maria Colangelo (Università degli Studi di Milano-Bicocca).

Due to the absence of a purification tag in the chimeric construct pCDNA3.1.Pre-Pro.βNGF, affinity purification was not possible. Instead, it was decided to collect the conditioned medium transfected with the chimeric construct pCDNA3.1.Pre-Pro.βNGF. This conditioned medium was tested on PC-12 cells and neurite outgrowth of PC-12 cells was detected by bright field microscopy (Fig. 22C), suggesting the presence of soluble and biologically active NGF in the conditioned medium. This may have resulted from the presence of: (a) Pro signal in the design of pCDNA3.1Pro.βNGF (Lessmann *et al.* 2003; Danielsen 1990; Naim and Naim 1996; Rothman and Orci 1992; Presley et al 1997) (b) use of HEK 293T cells which contain the components necessary for post translational processing of Pro.NGF into soluble mature βNGF homodimer, as described above in Section 3.3 (Aricescu *et al.* 2006; Mancia *et al.* 2004). The presence of endogenous furin in HEK 293T cells was also considered as a (possible) contributing cause (Wang *et al.* 2004; Seidah *et al.* 1996). For comparison, the control medium from non-transfected cells was

tested on PC-12 cells; no neurite outgrowth of PC-12 cells was detected by bright field microscopy (Fig. 22B).

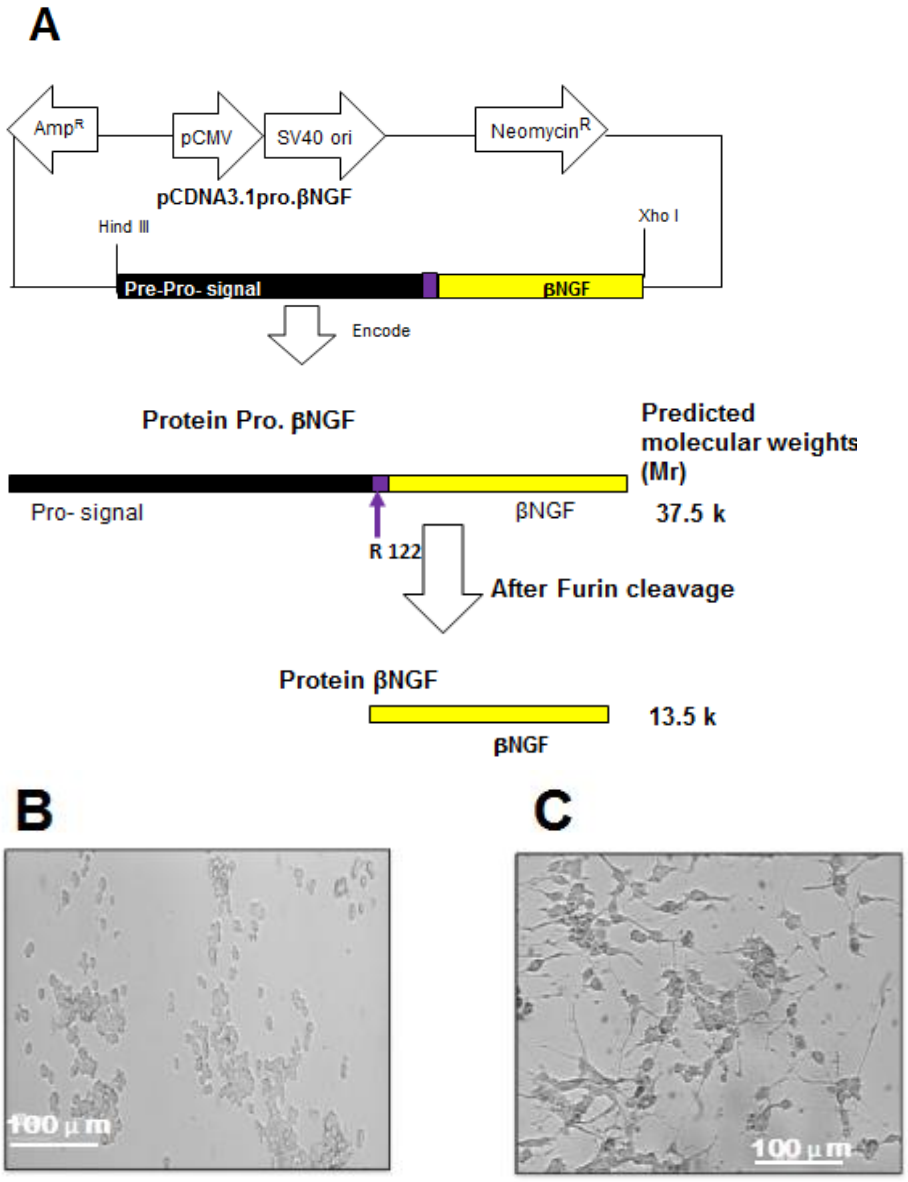
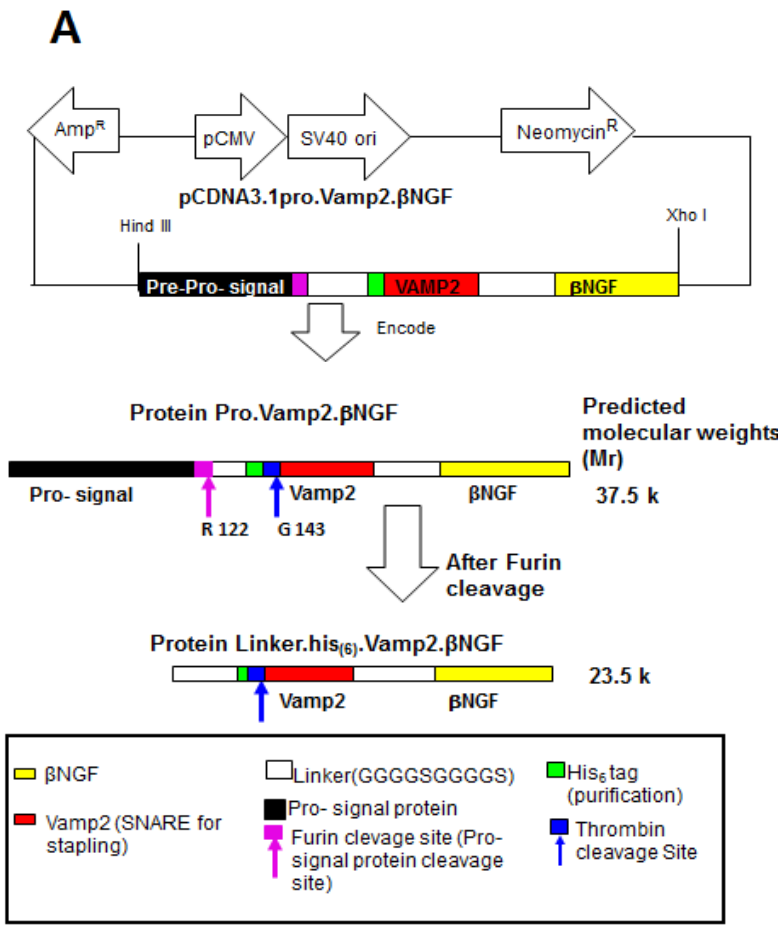


Fig. 22 Conditioned medium from pCDNA3.1Pro.βNGF transfected HEK 293T cells, but not from non-transfected cells, resulted in the growth of neurites from PC-12 cells. *(A) Schematic of chimera pCDNA3.1.Pro.βNGF. Representative micrographs, (B) PC-12 cells exposed for three days to conditioned medium from non-transfected HEK 293T cells. (C) HEK 293T transfected with pcDNA3.1.Pro.βNGF. Scale bar = 100 µm.*

3.3.2 Generated chimera pCDNA3.1.Pro.Vamp2.βNGF and pCDNA3.1.Pro.βNGF.Vamp2: cloning and transfection

To surmount the insolubility issue encountered previously for Trx.Vamp2.βNGF, I designed the chimeric construct pCDNA3.1.Pro.Vamp2.βNGF (Fig. 23A). The gene sequence encoding protein Pro.Vamp2.βNGF was synthesized and supplied by MWG Eurofins. It was inserted into the mammalian expression vector pCDNA3.1 within restriction endonuclease sites Hind III to Xho I (Fig. 23B). It is notable that this design included a $his_{(6)}$ tag to facilitate rapid affinity purification. This was inserted between the furin cleavage site and Vamp2 sequence so that mature Vamp2.βNGF could be captured. Sequencing of the resulting chimera (Appendix 2) confirmed the correct orientation of DNA encoding protein Pro.Vamp2.βNGF, beginning with methionine of Pre-Pro signal of NGF, within the translational frame of mammalian expression vector pCDNA3.1 (Fig. 23C).



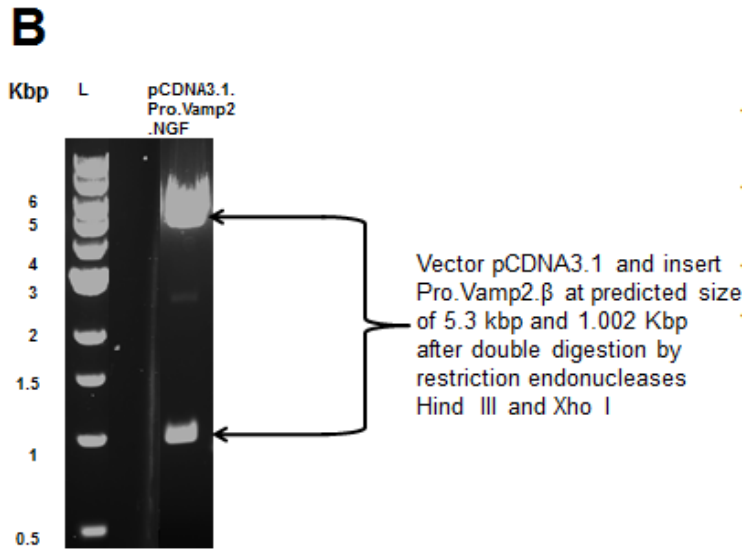


Fig. 23 Design and cloning pCDNA3.1.Pro.Vamp2.βNGF and pCDNA3.1.Pro.βNGF.Vamp2 *(A) Chimeric construct pCDNA3.1.Pro.Vamp2.βNGF. The DNA encodes Pre-Pro signal peptide (black box), a furin cleavage site R 122 (pink box), which separates Pre-pro signal, 10 amino acid (GGGGSGGGGS) flexible linker (white box), $his_{(6)}$ tag for IMAC purification (green box), Vamp2 for SNARE stapling (red box), a second 10 amino acid (GGGGSGGGGS) flexible linker (white box), human βNGF gene (yellow box). A thrombin cleavage site G 143 allows removal of the $his_{(6)}$ tag following IMAC purification. (B) Agarose gel image stained with ethidium bromide. Lanes (L) represent 10 kbp DNA ladder and vector pCDNA3.1. Pro.Vamp2.βNGF after double digestion with restriction endonucleases Hind III and Xho I for 1 hour at 37°C. Two bands visualized indicate the presence of excised vector pCDNA3.1 and insert Pro.Vamp2.βNGF at size 5.3 kbp and 1.003 kbp, respectively.*

HEK 293T cells were transfected with pCDNA3.1.Pro.Vamp2.βNGF by using PEI. As determined using the BCA method (see section 2.2.4.4), IMAC purification yielded 9 μg of total protein from 100 ml of pooled conditioned medium post transfection. Upon SDS-PAGE and Western blotting with antibodies against anti-Vamp 1, 2, 3, a band detected at molecular weight Mr ~35.5k corresponds to the expected migration of protein Pro.Vamp2.βNGF (Fig. 24 A,B). Western blotting using specific antibodies to phospho MAPK 42/44 and total MAPK 42/44 indicated that IMAC purified Pro.Vamp2.βNGF partially activated TrkA receptor (described in detail later in Fig. 25).

To optimize the biological activity, it was decided to modify the design of Pro.Vamp2.βNGF by interchanging the βNGF and Vamp2 gene and introduce it within the mammalian expression vector, pCDNA3.1 (DNA Sequence of the chimera pCDNAR3.1.Pro.βNGF.Vamp2 is described in Appendix 3). The aim of creating chimera pCDNAR3.1.Pro.βNGF.Vamp2 (Fig. 24C) by interchanging βNGF and Vamp2 gene was to ascertain, if the biological activity could be improved by including precedent Pre-Pro sequences adjacent to NGF, as it occurs in its natural form, as described in Ullrich *et al.* (1983). The gene encoding Pro.βNGF.Vamp2 was inserted into the mammalian expression vector pCDNA3.1 within restriction endonuclease sites Hind III to Apa I (Fig. 24D). Chimera pCDNA3.1.Pro.βNGF.Vamp2 was transiently transfected onto the HEK 293T cells. Western blotting using specific antibodies to phospho MAPK 42/44 and total MAPK 42/44 showed that, pCDNA3.1.Pro.βNGF.Vamp2, transfected conditioned medium from HEK293T cell culture activated TrkA mediated Ras, C-raf, MEK pathway (Fig. 25). Unfortunately, due to time constrains, scaling up of HEK 293T cells followed by IMAC purification could not be achieved.

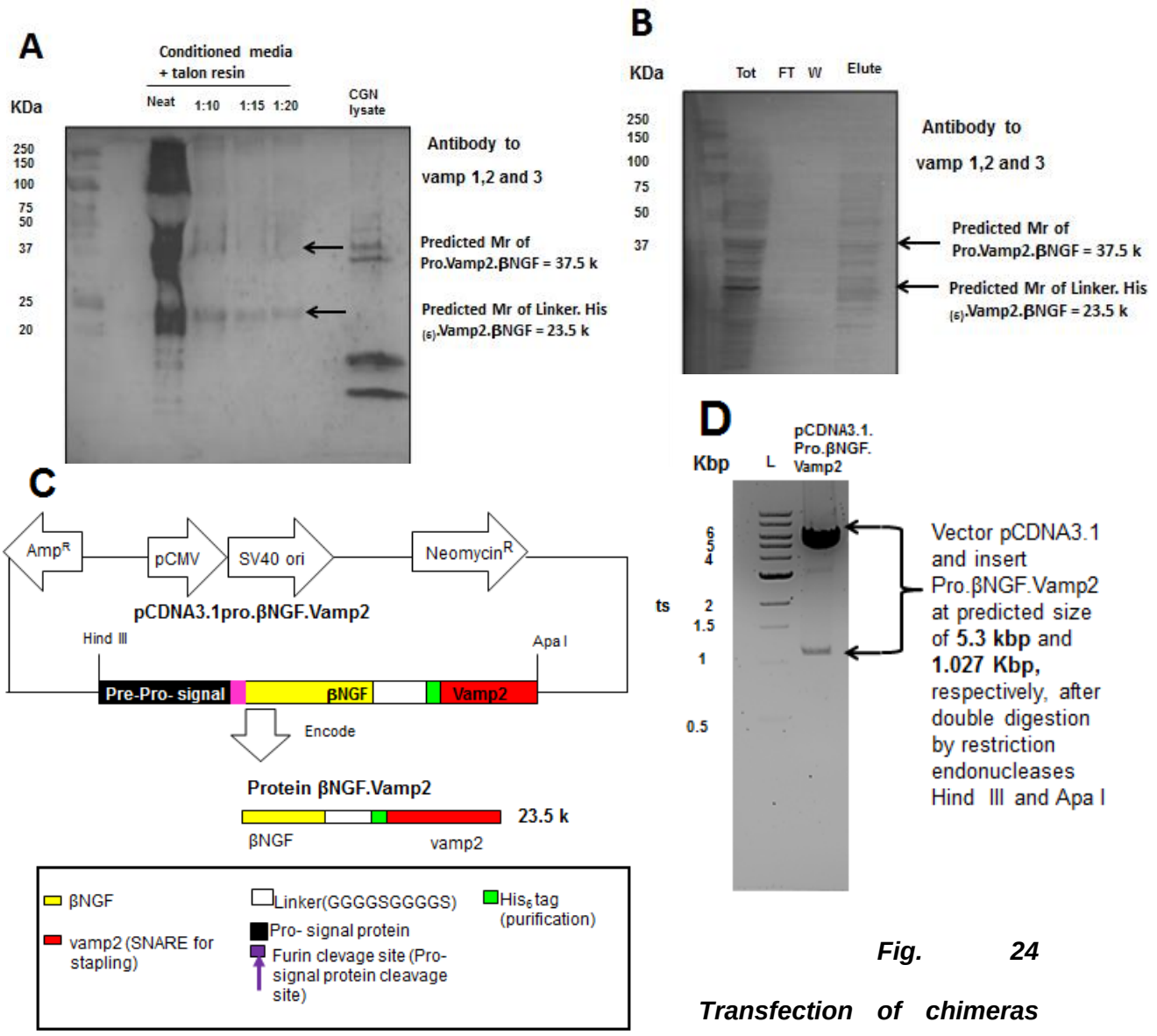


Fig. 24 Transfection of chimeras pCDNA3.1.Pro.Vamp2.βNGF and pCDNA3.1.Pro.βNGF.Vamp2 *(A) Western blotting of Talon® resin that has been incubated with conditioned medium ranging from neat to diluted in gradations 1:10, 1:15 and 1:20. Bands detected by specific antibody to Vamp 1,2,3 at Mr ~23.5 k and Mr~37.5 k indicate the presence of Linker.Vamp2.βNGF and Pro.Vamp2.βNGF, respectively. Bands determined at ~Mr 23.5 k maybe due to presence of endogenous furin activity present within HEK 293T cells. HEK 293T cells contain endogenous furin, which may have caused cleavage at the furin site to produce linker.Vamp2.βNGF. (B) Depicts Western blotting using antibodies against Vamp 1,2,3 following IMAC purification of conditioned medium from HEK293T cells transfected with pCDNA3.1.Pro.Vamp2. Conditioned medium (Total) was mixed with Talon resin at 4°C for 1 hour before separating unbound proteins (FT) from the resin by gravity flow columns. After washing with wash buffer (150 mM NaCl, 20 mM HEPES and 10 mM imidazole), $his_{(6)}$ tagged proteins were eluted by 500 mM imidazole; eluted proteins were separated from imidazole. salt and buffer exchanged into storage buffer (20 mM HEPES, 145 mM NaCl pH 7.4) by gel filtration columns. The*

expected bands at Mr ~23.5 k and Mr ~37.5 k were detected by antibody to Vamp1,2,3 indicating presence of protein Linker.Vamp2.βNGF and Pro.Vamp2.βNGF. (C) Schematic of mammalian expression vector pCDNA3.1.Pro.βNGF.Vamp2. It encodes a single chain protein consisting of Pre-Pro signal peptide (black box), human βNGF gene (yellow box), 10 amino acid (GGGGSGGGGS) flexible linker (white box), six residues of histidine for IMAC purification (green box) and Vamp2 for SNARE stapling (red box). The Pre-Pro signal peptide can be cleaved in the presence of furin (pink box) shown by arrow at amino acid position R122. (D) Ethidium bromide stained agarose gel loaded with DNA Ladder (L) and vector pCDNA3.1.Pro.βNGF.Vamp2 after double digestion with restriction endonucleases Hind III and Apa I for 3 hour at 25°C. The two bands indicate the presence of both double digested vector *pCDNA3.1 and insert Pro.βNGF.Vamp2 at sizes 5.3 kbp and 1.027 kbp, respectively.*

3.4 Proteins Vamp2.EGF and Pro.βNGF.Vamp2 induced phosphorylation of MAPK 42/44 in SH-SY5Y cells suggesting activation of their requisite growth factor receptors.

NGF binds to TrkA with high affinity (Kd = 10^{-11}M). Binding of NGF to TrkA stimulates dimerization of the latter, leading to phosphorylation of ERK 42/44 via activation of intracellular proteins such as Ras, c-Raf and MEK. Similarly EGF binds to epidermal growth factor receptor (EGFR); leading to its dimerization which, in turn, results in phosphorylation of ERK 42/44 via intracellular proteins such as c-Raf, PI3 and MEK.

SH-SY5Y cells have growth factors receptors such as TrkA and EGFR on their cell surfaces. The experiment discussed in this paragraph was performed as described in section 2.2.7.1. The effects of the growth factors listed in Fig.25 on serum-starved SH-SY5Y cells showed bands at ~ Mr 42 k and Mr ~44 k (Fig. 25A) detected by antibodies, total ERK 42/44 and phospho ERK 42/44, indicating the presence of total and phosphorylated MAPK, respectively. The bar chart (Fig. 25B) obtained after densitometric analysis of the bands detected using Image J shows the ratio of Phospho ERK to total ERK. The bar labelled (b) corresponds to protein 7S NGF, the positive control, known to activate Ras, C-raf /MEK pathway via ERK 42/44 phosphorylation was used for comparison purposes.

The ratio of phospho ERK 42/44 is a measure of the biological activity of the growth factors. Relative to 7S NGF (bar b), Vamp2.EGF (bar a) and Pro.βNGF.Vamp2 (bar g) gave approximately the same ratio indicating that both are biologically active. Similarly, relative to 7S NGF, Pro.Vamp2.βNGF (bars d and f) has approximately half the ratio suggesting that they were partially biologically active. This could be due to unavailability of free NGF N-termini to activate the TrkA receptors. The activity indicated in the proteins

corresponding to bars (f) and (g) arises from combination of the activity of the serum and the native biological activity of the proteins.

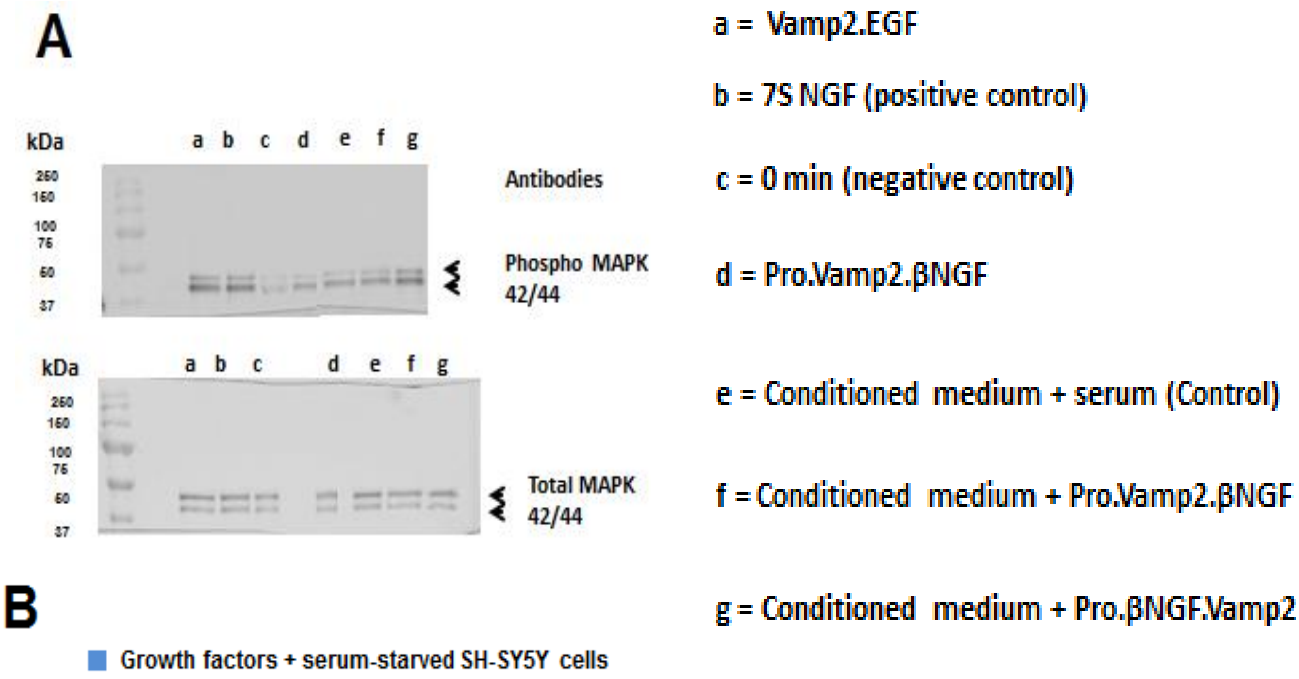


Fig. 25 Chimeras encoding proteins Vamp2.EGF, Pro.Vamp2.βNGF and Pro.βNGF.Vamp2 show biological activity on neuroendocrine cells *(A) Western blotting using specific antibodies to Total ERK 42/44 and phospho ERK 42/44 after exposure to growth factors as fusion proteins, as listed in the key. (B) Ratio of phospho ERK to total ERK detected by antibodies. The intensities of immune-signals were measured from digitized images using NIH Image J software. All data plotted in (B) are means ± S.E.M., n = 2.*

4 General discussion

In this chapter many of the themes described in chapter 1 are elaborated and discussed in the light of the findings of this study.

4.1 Effectiveness of pharmacological therapies for chronic pain

Given the clinical problem presented by chronic pain, considerable effort has been expended in finding methods for its alleviation. The therapies most prescribed for alleviation of pain are classified into three categories: 1) Non-steroidal anti-inflammatory drugs (NSAIDs), which are commonly known as aspirin and ibuprofen; 2) Opioids, such as morphine and pre-gabalin and 3) Surgery (Foster *et al.* 1999*)*. The pain relieving action of NSAIDs occurs by reducing the concentration of prostaglandins in the PNS. However, prostaglandins are only one of several mediators of pain (Dray A 1995); therefore, NSAIDSs are only effective in reducing mild pain. The uses of NSAIDs are associated with side effects such as initiation of ulceration in gastro-intestinal tract and gut which limit their use in chronic pain relief (Foster *et al*. 1999; Kalso *et al.* 2003; Foster *et al.* 1999).

Opioids act at the spinal cord level, inhibiting neurotransmission between primary nociceptive fibers (C fibers) and projection neurons; this is achieved by causing prolonged hyper-polarization at their synapses. Unfortunately, opioids have the following unwanted side effects (Foster *et al.* 1999*)*: 1) depression of respiratory system in the brain stem; 2) constipation by effects on smooth musculature of gastro-intestinal tract; 3) psychoactive effects including sedation and euphoria and 4) physical dependence leading to drug abuse (e.g. heroine). 5) Tolerance to opioids i.e. increasing dose of drug required to produce the same analgesic effect). Surgical lesions of pain pathways at various levels from peripheral nerves through dorsal root section have been used for severe chronic pain. However, these surgical operations are associated with significant risk to patients. Therefore, there is an

unmet need for development of new class of pharmaceuticals for pain treatments. The desired properties of new therapies should 1) have the ability to provide long-lasting pain relief 2) lack systemic side-effects that impair patient's quality of life (Foster *et al*. 1999).

4.2 Botulinum toxin as an emerging therapeutic

Local injection of BOTOX® (Allergan Inc., California) or its active component botulinum neurotoxin A (BoNT/A) has proved most successful in the treatment of human disorders such as dystonias, spasticity, over-active bladder, abnormalities of gastro-intestinal sphincters, hyper-hydrosis and sialorrhea (Dolly *et al.* 2012; Aoki *et al*. 2011). Common to all of these applications is that the BoNTs are taken up in the motor neuron terminal at the neuromuscular junction or parasympathetic axon terminal where the toxin acts to block the release of acetylcholine (Pantano *et al.* 2014; Pellett *et al.* 2015). Because of its remarkable effectiveness in alleviating numerous clinical conditions due to over-activity of nerves, research is being focused on other indications for this potent and specific inhibitor of exocytosis particularly in the area of pain (Mittal *et al*. 2016). The toxin has shown interesting analgesic activities on other neuronal pathways, including blockade of the release of pain mediators (glutamate, substance P, CRGP) from peripheral terminals, dorsal root ganglia (DRG), and spinal cord neurons (Lucioni *et al*. 2008; Meng J *et al.* 2007; Oh *et al*. 2015) decreasing local inflammation around nerve terminals (Cui *et al.* 2004; Kharatmal *et al.* 2015; Shin *et al.* 2012) inhibiting discharge of muscle spindles (Fillippi *et al*. 1993) and decreasing sympathetic transmission (Rand *et al. 1965).* Although the underlying mechanism is not as clearly understood as that for neuromuscular and other cholinergic conditions, it has led to the utilization of the toxin for various pain-related conditions such as myofascial pain syndrome and migraine (Moore *et al. 2007;* Masuyer *et al.* 2014).

Building on its success, described above, in the treatment with migraine, where it acts on sensory neurons, attempts have been made to apply it more widely. One such application involved replacing the binding domain of BoNT/A(H_{CC}) with βNGF which was hoped would bind and re-target the LC/A to sensory neurons, rather than motor neurons. This was the main focus of my research, in the attempt to create a conjugate of NGF with BoNT/AΔH_{CC} (abbreviated as /AΔH_{CC}).

4.3 Potential applications of re-engineered BoNT/A arising from this study

The potential applications of re-engineered BoNT/A arising from this study are discussed under three sub-headings.

4.3.1 Discussion of BoNT based chimera /AΔH_{CC} (first generation of recombinant proteins).

This study involved direct fusion of NGF to BoNT using recombinant DNA technology to seek a cost-effective method for large scale production of such proteins. For this purpose, chimeric construct encoding NGF fused to /AΔH_{CC} was created and expressed in *E.coli* (/AΔH_{CC} consists of the light chain, translocation domain and N-terminal heavy chain of BoNT/A, without its binding domain). The resultant protein was purified using IMAC and its biological activity tested on PC-12 cells. Western blotting using antibodies to phospho ERK 42/44 yielded indirect evidence that receptor uptake of /AΔH_{CC}N did not take place through the NGF high-affinity receptor, TrkA. Western blotting using specific antibodies to SNAP-25 indicated that at the higher concentrations tested (30 nM and 10 nM), /AΔH_{CC}N protein showed cleavage of SNAP-25, but none at 3 nM. This could be due to non-specific binding and membrane uptake of /AΔH_{CC}N protein by PC-12 cells. This avenue of research was not pursued further.

βNGF homodimer is the biologically-active form of mature NGF. It binds to receptor TrkA with high affinity i.e. Kd =10 $^{-11}$M. Protein Data Bank image of NGF-TrkA complex (Weismann *et al.* 2000) showed that the two N termini of the NGF homodimer are arranged parallel to each other. The N-termini, particularly residues 1-12, play an active role in the stimulation of the extracellular domain of TrkA receptor leading to its dimerization (Ullrich *et al.* 1983). This βNGF binding to TrkA, in turn, causes phosphorylation of threonine 202 and tyrosine 204 of MAPK via activation of Ras, C-raf, and MEK pathway. Herein, Western blotting using a specific antibody to phospho MAPK 42/44 showed that /AΔH_{CC}N did not lead to TrkA stimulated phosphorylation of MAPK proteins in PC-12 cells, indicating that it was biologically inactive.

Regarding the design of the chimeric construct /AΔH_{CC}N, the N-terminal of βNGF is not free but fused to a 10 amino acid flexible linker which conjugates it to domain H_N.H_{CN}.LC of BoNT/A; this could have occluded the critical N-terminal residues in such a way as to render them unavailable to stimulate the TrkA receptors. The first 8 N-terminal residues of LC/A not only contains a signal for localization to the plasma membrane but also contributes an important structural element of the protein (Aoki *et al.* 2003). Therefore it was considered preferable to have LC/A at the N-terminus rather than βNGF within the chimeric construct /AΔH_{CC}N. Because the uni-directional nature of protein translation (N-C terminus) was considered a major obstacle, it was concluded that a better method of producing active /AΔH_{CC} was required. As a possible development for future studies, it is suggested that attaching fluorophore tags to /AΔH_{CC}N, could serve as tracers to indicate the intracellular path of the /AΔH_{CC}N in PC-12 cells.

4.3.2 Discussion of recombinant proteins, created using stapling technology (Second generation recombinant proteins).

The discussion of recombinant proteins, created using stapling technology (second generation recombinant proteins) is sub-divided into three parts.

4.3.2.1 LCH_N/A fragment

Modular arrangement of BoNT and the independent functions of its three domains have encouraged several researchers to create variant BoNTs using recombinant DNA technology (Wang *et al.* 2008; Wang *et al.* 2011; Dolly *et al.* 2011; Dolly *et al.* 2014; Masuyer *et al.* 2014). Shone et al (1985) suggested that controlled proteolytic digestion of BoNT/A by trypsin results in the di-chain of LC/A and H_N linked by di-sulphide bond named, the LCH_N /A fragment. LCH_N /A lacks the binding domain (H_{CC}), which makes it non-toxic as it is unable to bind to neuronal receptors. Replacing the binding domain (H_{CC}), with alternative ligands, the resultant fusion has been targeted to enter neuronal and non-neuronal cells. The LCH_N /A fragment maintains protease activity and blocks secretion from the targeted cells via the cleavage of SNAP-25 (Alexander *et al.* 2002; Foster *et al.* 2006; Foster 2009). Under low pH conditions, the LCH_N /A fragment forms pores through the endosomal membrane, although the pore-formation mechanism for translocation remains elusive. (Foster *et al*. 2006).

4.3.2.2 The limitations of chemical coupling technology

Chaddock *et al* (2000) have described the chemical conjugation of $LC.H_N$/A to NGF and showed that this molecule was potent (IC_{50} for NGF-LCH_N/A= 1.71 ±0.39 nM , BoNT/A = 4.0 ± 1.27 nM, and LH_N/A = 688 nM) in inhibiting neurotransmitter release from PC-12 cells. Chemical conjugation of $LC.H_N$/A to NGF has several disadvantages such as: 1) both components are sensitive to over-derivatization by their respective linkers; this has been

reported to reduce catalytic activity (Chaddock *et al.* 2000); 2) the formation of non-specific aggregated proteins; 3) the need for several purification steps such as size exclusion chromatography, cation exchange chromatography to separate conjugated product and 4) poor conjugation efficiency i.e.~ 250 µg of conjugated product from 2 mg of NGF and ~1 mg of LC.H_N/A (Foster *et al.* 2015*).*

4.3.2.3 The use of protein stapling technology

As an alternative to chemical conjugation, Arsenault *et al.* (2013) suggested exploiting the established selective and high-affinity interaction of the α-helices from neuronal SNAREs to achieve biochemical coupling; this strategy has been termed protein stapling (Fig. 22). Four α-helices derived from three SNARE proteins such as SNAP-25 (22-206) Vamp2 (25-84) and syntaxin-1 (1-40) spontaneously interact when mixed to form a tetra-helical tight interaction (Darrios *et al.* 2010). The assembled neuronal SNARE complex is extraordinarily stable, exhibiting resistance to chaotropic agents, strong detergents, proteases, and elevated temperatures as detailed in Section 1.3.3.2 of Chapter 1 of this study (Sutton 1998, Sudhof and Rothman 2009). Arsenault *et al.* (2013) created individual components Vamp2 (25-84) fused EGF and SNAP-25 (22-206) fused LC.H_N/A in *E.coli* via DNA recombination. They were stapled together into a functional unit by addition of a synthetic peptide corresponding in sequence to residues (1-45) of syntaxin-1.

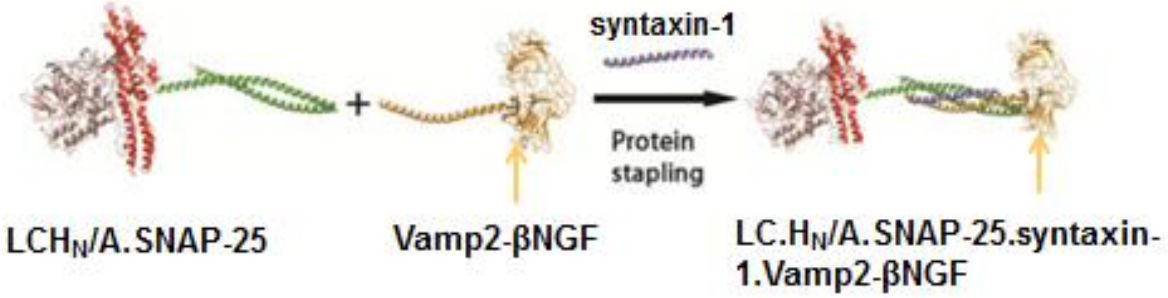


Fig. 26 Schematic representation of LC.H_N/A.SNAP-25.syntaxin-1.Vamp2- βNGF.

Stapling reaction using LCH$_N$/A-SNAP-25 (the protease domain, brown; the translocation domain,

red, and a SNAP-25 (22-206) linker, green), the Vamp2 (25-84) linked to βNGF (yellow) and the syntaxin-1 peptide (1-45) (blue). Addition of the syntaxin-1 peptide (1-45) results in formation of an LC.H_N/A.SNAP-25.syntaxin-1.Vamp2- βNGF (Image adapted from Arsenault et al. 2013).

Herein, I successfully replicated the creation of Vamp2 fused to EGF in *E.coli*, and then attempted to replace the EGF with βNGF, and apply the protein stapling method. Unfortunately, the Trx.Vamp2.βNGF resulting from expression of the chimeric construct pET32b.Trx.Vamp2.βNGF appeared in the insoluble fraction of the lysates. It is proposed that the insolubility could be due to a lack of Pre-Pro signal protein in the design of the chimeric construct pET32b.Trx.Vamp2.βNGF resulting in the accumulation of incorrectly folded protein in the inclusion bodies. This explanation in relation to the insolubility of Trx.Vamp2.βNGF concurs with arguments given by Rattenhol *et al.* (2001) that Pro sequence of NGF promotes folding of the mature βNGF. Despite being very hydrophobic, naturally- occurring NGF is soluble. Lessmann *et al.* (2003) reported that soluble expression of biologically-active NGF is facilitated by the presence of its Pre-Pro signal protein. Because of the lack of success in expression of soluble βNGF fused to Vamp2 in *E.coli*, it became clear that exploration of this modification system was necessary.

A paper by Colangelo *et al.* (2005) reported that transfection of the construct encoding Pre-Pro.βNGF expressed soluble NGF in mammalian cells. I was able to achieve the expression of NGF using pCDNA3.1.Pre-Pro.βNGF kindly provided by Prof.Anna Maria Colangelo (Università degli Studi di Milano-Bicocca). This successful production of soluble biologically-active NGF encouraged me, to design two new constructs 1) pCNDA3.1Pro.Vamp2.βNGF and 2) pCNDA3.1Pro.βNGF.Vamp2, to transfect these into HEK 293T cells and to generate chimeric proteins Pro.Vamp2.βNGF and Pro.βNGF.Vamp2, respectively. As already stated in section 3.3.2 the IMAC purification of Pro.Vamp2.βNGF yielded 9 µg of total protein from 100 ml of pooled conditioned

medium. As already pointed out in section 3.3.2 scaling up of Pro.βNGF.Vamp2 in HEK 293T cell culture followed by IMAC purification was not carried out. Notably, testing their biological activity on SH-SY5Y cells showed both Pro.Vamp2.βNGF and Pro.βNGF.Vamp2 were found to be active, detected by specific antibodies to total ERK and phospho ERK. Thus, the Pre-Pro signal can facilitate expression of soluble fusion protein containing a functional NGF moiety fused to a SNARE sequence that can be exploited for biochemical protein conjugation. The result may also be explained by the fact that HEK 293 cells contain the necessary components for soluble expression of proteins Pro.Vamp2.βNGF and Pro.βNGF.Vamp2. Chen *et al.* (2011) reported that the N terminal residues (1 -12) of βNGF played an important role in activating TrkA receptor. In this regard, it should be noted that cleavage of Pre-Pro signal from Pro.βNGF. Vamp2 by endogenous furin in HEK 293 cells would produce βNGF.Vamp2 with the N-terminal of βNGF.Vamp2 free to stimulate TrkA receptors and was identified as the core finding in this study.

4.3.3 Future work

The strategy for direct fusion of βNGF to LC.$H_{N.}H_{CN}$/A by genetic recombination was unsuccessful as demonstrated by the fact that LC.$H_{N.}H_{CN}$/A.βNGF generated was biologically inactive. Although protein stapling technology proved promising, the stapling of βNGF.Vamp2 to LC.$H_{N.}$SNAP-25 was not performed due to time constraints. If more time had been available, I would have proceeded to optimize the yield of βNGF.Vamp2, leading to the following future options: 1) to scale up the amount of conditioned medium with Pro.βNGF.Vamp2 by selecting stable HEK 293 cells transfected with pCDNA3.1.Pro.βNGF.Vamp2 (Lonza 2016); followed by protein purification via IMAC and protein analysis by SDS-PAGE and Western blotting (as described in Chapter 2), 2) to

generate βNGF.Vamp2 by nicking Pro.βNGF.Vamp2 using commercial furin, 3) to staple βNGF.Vamp2 to LC.$H_{N.}$SNAP-25 by addition of syntaxin-1 fused to fluorophore like FITC as described in Ferrari *et al (*2012*).* Currently, both LC.$H_{N.}$SNAP-25 and syntaxin-1.fused to fluorophore like FITC have been synthesized and are available in ICNT and 4) to test the biological activity of stapled product on PC-12 cells using assays such as *i.e.* ERK phosphorylation and SNAP-25 cleavage, as described in Chapter 2. Syntaxin-1.fused to FITC would be advantageous as it could act as a tracer to indicate the specific intracellular path of the stapled product in PC-12 cells.

Appendix 1 – Sequenced DNA of pET32b Trx./AΔH_{CC}N

```
     TGGCGAATGG   GACGCGCCCT   GTAGCGGCGC   ATTAAGCGCG   GCGGGTGTGG   TGGTTACGCG
60
     CAGCGTGACC   GCTACACTTG   CCAGCGCCCT   AGCGCCCGCT   CCTTTCGCTT   TCTTCCCTTC
120
     CTTTCTCGCC   ACGTTCGCCG   GCTTTCCCCG   TCAAGCTCTA   AATCGGGGGC   TCCCTTTAGG
180
     GTTCCGATTT   AGTGCTTTAC   GGCACCTCGA   CCCCAAAAAA   CTTGATTAGG   GTGATGGTTC
240
     ACGTAGTGGG   CCATCGCCCT   GATAGACGGT   TTTTCGCCCT   TTGACGTTGG   AGTCCACGTT
300
     CTTTAATAGT   GGACTCTTGT   TCCAAACTGG   AACAACACTC   AACCCTATCT   CGGTCTATTC
360
     TTTTGATTTA   TAAGGGATTT   TGCCGATTTC   GGCCTATTGG   TTAAAAAATG   AGCTGATTTA
420
     ACAAAAATTT   AACGCGAATT   TTAACAAAAT   ATTAACGTTT   ACAATTTCAG   GTGGCACTTT
480
     TCGGGGAAAT   GTGCGCGGAA   CCCCTATTTG   TTTATTTTTC   TAAATACATT   CAAATATGTA
540
     TCCGCTCATG   AGACAATAAC   CCTGATAAAT   GCTTCAATAA   TATTGAAAAA   GGAAGAGTAT
600
     GAGTATTCAA   CATTTCCGTG   TCGCCCTTAT   TCCCTTTTTT   GCGGCATTTT   GCCTTCCTGT
660
     TTTTGCTCAC   CCAGAAACGC   TGGTGAAAGT   AAAAGATGCT   GAAGATCAGT   TGGGTGCACG
720
     AGTGGGTTAC   ATCGAACTGG   ATCTCAACAG   CGGTAAGATC   CTTGAGAGTT   TTCGCCCCGA
780
     AGAACGTTTT   CCAATGATGA   GCACTTTTAA   AGTTCTGCTA   TGTGGCGCGG   TATTATCCCG
840
     TATTGACGCC   GGGCAAGAGC   AACTCGGTCG   CCGCATACAC   TATTCTCAGA   ATGACTTGGT
900
     TGAGTACTCA   CCAGTCACAG   AAAAGCATCT   TACGGATGGC   ATGACAGTAA   GAGAATTATG
960
     CAGTGCTGCC   ATAACCATGA   GTGATAACAC   TGCGGCCAAC   TTACTTCTGA   CAACGATCGG
1020
     AGGACCGAAG   GAGCTAACCG   CTTTTTTGCA   CAACATGGGG   GATCATGTAA   CTCGCCTTGA
1080
     TCGTTGGGAA   CCGGAGCTGA   ATGAAGCCAT   ACCAAACGAC   GAGCGTGACA   CCACGATGCC
1140
     TGCAGCAATG   GCAACAACGT   TGCGCAAACT   ATTAACTGGC   GAACTACTTA   CTCTAGCTTC
1200
     CCGGCAACAA   TTAATAGACT   GGATGGAGGC   GGATAAAGTT   GCAGGACCAC   TTCTGCGCTC
1260
```

GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCCGGTGAGC GTGGGTCTCG
1320
CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG TTATCTACAC
1380
GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC
1440
ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT
1500
AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA ATCTCATGAC
1560
CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA
1620
AGGATCTTCT TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAAA CAAAAAAACC
1680
ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT
1740
AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG
1800
CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA TCCTGTTACC
1860
AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT
1920
ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGCACACAGC CCAGCTTGGA
1980
GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT
2040
TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG
2100
CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG GGTTTCGCCA
2160
CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA
2220
CGCCAGCAAC GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGTT
2280
CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA
2340
TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA
2400
GCGCCTGATG CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC GCATATATGG
2460
TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA GCCAGTATAC ACTCCGCTAT
2520
CGCTACGTGA CTGGGTCATG GCTGCGCCCC GACACCCGCC AACACCCGCT GACGCGCCCT
2580

GACGGGCTTG TCTGCTCCCG GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT
2640
GCATGTGTCA GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGGCAGCTG CGGTAAAGCT
2700
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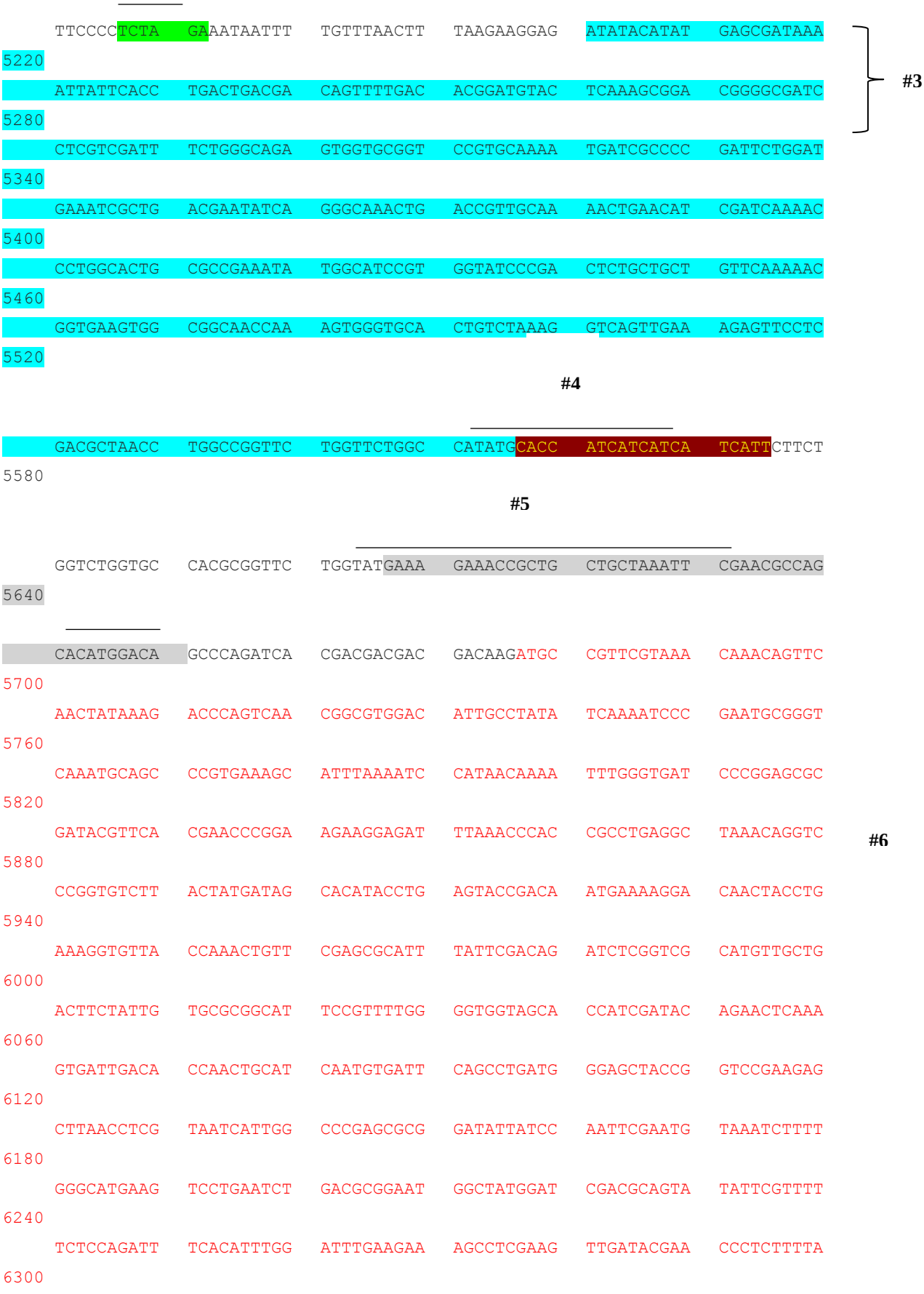
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Sequenced DNA of pET32b Trx./AΔH_{CC}N provided by Dr Matthew King, ICNT unpublished data

#1 – T7 promoter

#2 -Restriction enzyme Xho I site

#3 –Thioredoxin tag

#4 –$His_{(6)}$ tag

#5 –S-tag

#6 – LC.$H_{N.}H_{CN}$/AΔH_{CC}

#7 - Restriction enzyme XhoI site

#8 – human βNGF (Ullrich *et al.* 1983)

#9 - Restriction enzyme XhoI site

Appendix 2 – Sequenced DNA of pCDNA3.1 Pro.VAMP2.βNGF

Appendix 2

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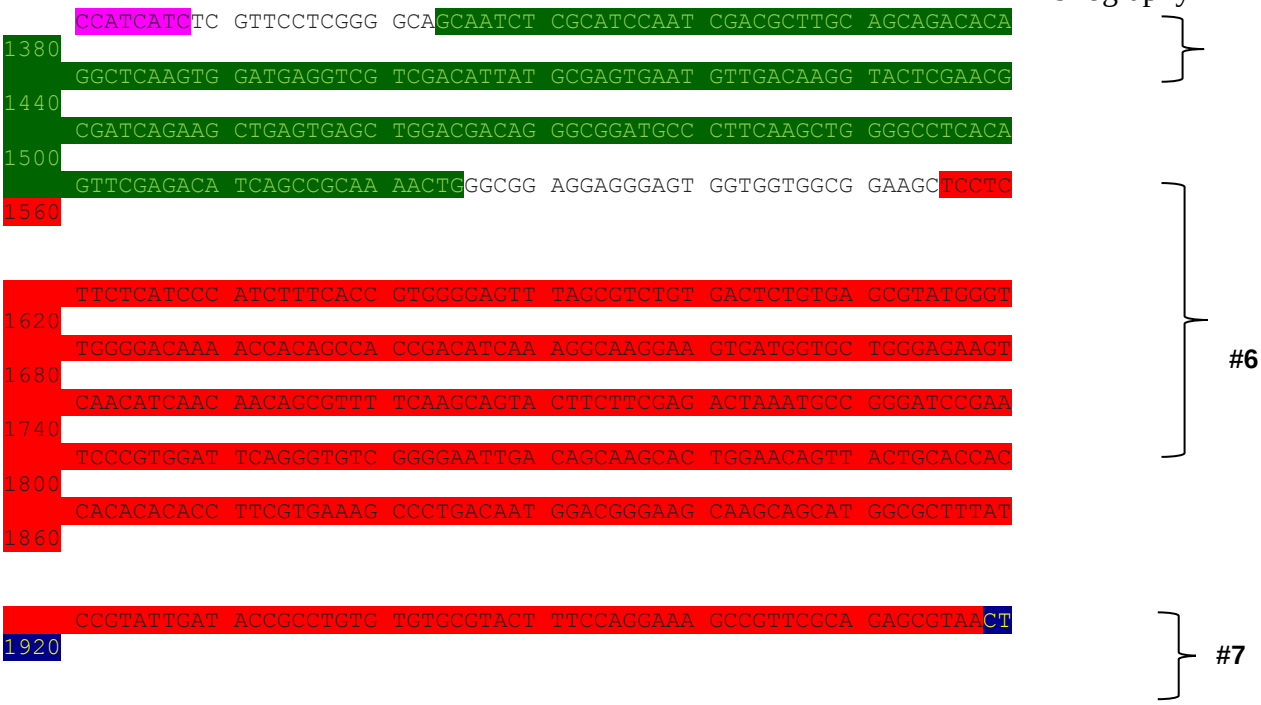


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     GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG
5880
     TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG
5940
     AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC
6000
     CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT
6060
     CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT
6120
     CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG
6180
     CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC
6240
     AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA
6300
     TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG
6360

TC
```

Sequenced DNA of pCDNA3.1 Pro.VAMP2.βNGF (MWG, Germany)

#1 – T7 promoter

#2 – Restriction enzyme Hind III site

#3 – Human derived Pre-Pro signal peptide (Ullrich *et al.* 1983)

#4 – $His_{(6)}$ tag

#5 – Rat derived Vamp2 (25-84) (Arsenault *et al.* 2013)

#6 – Human βNGF (Ullrich *et al.* 1983)

#7 - Restriction enzyme XhoI site

Appendix 3 - Sequenced DNA of pCDNA3.1 Pro.βNGF. VAMP2.

```
GACGGATCGG GAGATCTCCC GATCCCCTAT GGTGCACTCT CAGTACAATC TGCTCTGATG        60
     CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
120
     CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
180
     TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
240
     GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
300
     TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
360
     CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
420
     ATTGACGTCA ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
480
     ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
540
     ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
600
     TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
660
     ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
720
     AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
780
     GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
840

                                    #1
                                    ____________________
     CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
900

                  #2
                  _____
     GTTTAAACTT AAGCTTATGT CCATGTTGTT CTACACTCTG ATCACAGCTT TTCTGATCGG
960
     CATACAGGCG GAACCACACT CAGAGAGCAA TGTCCCTGCA GGACACACCA TCCCCCAAGT
1020
     CCACTGGACT AAACTTCAGC ATTCCCTTGA CACTGCCCTT CGCAGAGCCC GCAGCGCCCC
1080
     GGCAGCGGCG ATAGCTGCAC GCGTGGCGGG GCAGACCCGC AACATTACTG TGGACCCCAG
1140
     GCTGTTTAAA AAGCGGCGAC TCCGTTCACC CCGTGTGCTG TTTAGCACCC AGCCTCCCCG
1200
     TGAAGCTGCA GACACTCAGG ATCTGGACTT CGAGGTCGGT GGTGCTGCCC CCTTCAACAG
1260
     GACTCACAGG AGCAAGCGGT CATCATCCCA TCCCATCTTC CACAGGGGCG AATTCTCGGT
1320
     GTGTGACAGT GTCAGCGTGT GGGTTGGGGA TAAGACCACC GCCACAGACA TCAAGGGCAA
1380
     GGAGGTGATG GTGTTGGGAG AGGTGAACAT TAACAACAGT GTATTCAAAC AGTACTTTTT
1440
     TGAGACCAAG TGCCGGGACC CAAATCCCGT TGACAGCGGG TGCCGGGGCA TTGACTCAAA
1500
     GCACTGGAAC TCATATTGTA CCACGACTCA CACCTTTGTC AAGGCGCTGA CCATGGATGG
1560
     CAAGCAGGCT GCCTGGCGGT TTATCCGGAT AGATACGGCC TGTGTGTGTG TGCTCAGCAG
1620

                                                                  #4
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#3

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      GAAGGCTGTG AGAAGAGCCC TCGAGGGCGG CGGCGGCTCT GGTGGAGGAG GGTCCCACCA
1680

      TCACCACCAT CATCTCGTTC CTCGGGGCAG CAATCTCGCA TCCAATCGAC GCTTGCAGCA
1740
      GACACAGGCT CAAGTGGATG AGGTCGTCGA CATTATGCGA GTGAATGTTG ACAAGGTACT
1800
      CGAACGCGAT CAGAAGCTGA GTGAGCTGGA CGACAGGGCG GATGCCCTTC AAGCTGGGGC
1860
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#5

#6

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      CTCACAGTTC GAGACATCAG CCGCAAAACT GTAACCCGGG GTTTAAACCC GCTGATCAGC
1920
      CTCGACTGTG CCTTCTAGTT GCCAGCCATC TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT
1980
      GACCCTGGAA GGTGCCACTC CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA
2040
      TTGTCTGAGT AGGTGTCATT CTATTCTGGG GGGTGGGGTG GGGCAGGACA GCAAGGGGGA
2100
      GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG GGCTCTATGG CTTCTGAGGC
2160
      GGAAAGAACC AGCTGGGGCT CTAGGGGGTA TCCCCACGCG CCCTGTAGCG GCGCATTAAG
2220
      CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA CTTGCCAGCG CCCTAGCGCC
2280
      CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC GCCGGCTTTC CCCGTCAAGC
2340
      TCTAAATCGG GGGCTCCCTT TAGGGTTCCG ATTTAGTGCT TTACGGCACC TCGACCCCAA
2400
      AAAACTTGAT TAGGGTGATG GTTCACGTAG TGGGCCATCG CCCTGATAGA CGGTTTTTCG
2460
      CCCTTTGACG TTGGAGTCCA CGTTCTTTAA TAGTGGACTC TTGTTCCAAA CTGGAACAAC
2520
      ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG ATTTTGCCGA TTTCGGCCTA
2580
      TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG AATTAATTCT GTGGAATGTG
2640
      TGTCAGTTAG GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG
2700
      CATCTCAATT AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT
2760
      ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC
2820
      CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT
2880
      ATTTATGCAG AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC
2940
      TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTCCCGGGAG CTTGTATATC CATTTTCGGA
3000
      TCTGATCAAG AGACAGGATG AGGATCGTTT CGCATGATTG AACAAGATGG ATTGCACGCA
3060
      GGTTCTCCGG CCGCTTGGGT GGAGAGGCTA TTCGGCTATG ACTGGGCACA ACAGACAATC
3120
      GGCTGCTCTG ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG GGCGCCCGGT TCTTTTTGTC
3180
      AAGACCGACC TGTCCGGTGC CCTGAATGAA CTGCAGGACG AGGCAGCGCG GCTATCGTGG
3240
      CTGGCCACGA CGGGCGTTCC TTGCGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAGG
3300
      GACTGGCTGC TATTGGGCGA AGTGCCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCCT
3360
      GCCGAGAAAG TATCCATCAT GGCTGATGCA ATGCGGCGGC TGCATACGCT TGATCCGGCT
3420
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ACCTGCCCAT TCGACCACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATGGAA
3480
GCCGGTCTTG TCGATCAGGA TGATCTGGAC GAAGAGCATC AGGGGCTCGC GCCAGCCGAA
3540
CTGTTCGCCA GGCTCAAGGC GCGCATGCCC GACGGCGAGG ATCTCGTCGT GACCCATGGC
3600
GATGCCTGCT TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCTGGATT CATCGACTGT
3660
GGCCGGCTGG GTGTGGCGGA CCGCTATCAG GACATAGCGT TGGCTACCCG TGATATTGCT
3720
GAAGAGCTTG GCGGCGAATG GGCTGACCGC TTCCTCGTGC TTTACGGTAT CGCCGCTCCC
3780
GATTCGCAGC GCATCGCCTT CTATCGCCTT CTTGACGAGT TCTTCTGAGC GGGACTCTGG
3840
GGTTCGAAAT GACCGACCAA GCGACGCCCA ACCTGCCATC ACGAGATTTC GATTCCACCG
3900
CCGCCTTCTA TGAAAGGTTG GGCTTCGGAA TCGTTTTCCG GGACGCCGGC TGGATGATCC
3960
TCCAGCGCGG GGATCTCATG CTGGAGTTCT TCGCCCACCC CAACTTGTTT ATTGCAGCTT
4020
ATAATGGTTA CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC
4080
TGCATTCTAG TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGTATACCGT
4140
CGACCTCTAG CTAGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT
4200
ATCCGCTCAC AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG
4260
CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
4320
GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC
4380
GTATTGGGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC
4440
GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA
4500
ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
4560
CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT
4620
CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA
4680
GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC
4740
TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT
4800
AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
4860
CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG
4920
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4980
TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGAACAGT ATTTGGTATC TGCGCTCTGC
5040
TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG
5100
CTGGTAGCGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG
5160
AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG
5220
GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT
5280
GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT
5340
TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCTGAC
5400
TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA
5460

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     TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG
5520
     GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT
5580
     GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTTGCCA
5640
     TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT
5700
     CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG GTTAGCTCCT
5760
     TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG
5820
     CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG
5880
     AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG
5940
     CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA
6000
     AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT
6060
     AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT
6120
     GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT
6180
     GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA
6240
     TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT
6300
     TTCCCCGAAA AGTGCCACCT GACGTC
6326
```

Sequenced DNA of pCDNA3.1 Pro.VAMP2.βNGF (MWG, Germany)

#1 – T7 promoter

#2 – Restriction enzyme Hind III site

#3 – Human derived Pre-Pro βNGF (Ullrich *et al.* 1983)

#4 – $His_{(6)}$ tag

#5 – Rat derived Vamp2 (25-84) (Arsenault *et al.* 2013)

#6 – Restriction enzyme Apa I site

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