Medicus Sapentiae

Thesis Obsequium

Regulation of p75NTR Trafficking by Neurotrophins in the NSC-34 Motor Neuron Cell Line

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“The product of mental labor - science - always stands far below its value, because the labor-time necessary to reproduce it has no relation at all to the labor-time required for its original production”

Karl Marx (1818-1883)

“The most heated defenders of a science, who cannot endure the slightest sneer at it, are commonly those who have not made very much progress in it and are secretly aware of this defect”

Georg C. Lichtenberg (1742-1799)

CHAPTER 1: NEUROTROPHINS, NEUROTROPHIN RECEPTORS AND SIGNALING MECHANISMS VIA ENDOSOMAL TRANSPORT 1

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Abstract

Neurotrophins are a family of growth factors necessary for the development and maintenance of the nervous system. They produce their effects through receptor mediated signaling mechanisms that are highly regulated by sophisticated intracellular transport networks. The impairment of intracellular trafficking of neurotrophins in motor neurons has been identified as one possible factor in the development of motor neuron diseases, but remains inadequately studied. Aided by advances in imaging technology and the development of more powerful and sensitive detection tools for *in-vitro* studies, the dynamics of intracellular transport of neurotrophins are beginning to be unraveled. However, a primary limiting factor in the study of neurotrophin-transport dynamics in motor neurons has been the lack of alternative and easily available in-vitro systems able to substitute the often difficult and costly primary motor neuron cultures.

The aim of this project was to develop a suitable motor neuron model using the NSC-34 cell line for the study of receptor mediated trafficking events through endosomal transport pathways. Successful evaluation and characterization of NSC-34 cells for motor neuron specific markers would result in the investigation of the p75 neurotrophin receptor ($p75^{NTR}$) trafficking pathways in the presence of exogenous neurotrophins, with a variety of confocal imaging techniques.

Chapter 3 describes the optimisation of NSC-34 cell culture conditions through media modification and the development of a suitable growth substrate matrix, which significantly improved cell adhesion, differentiation and the ability to culture the cells for extended time periods in serum free conditions. Quantitative measurements of cell proliferation, culture viability, cell-body size and neurite length are described to highlight the increased value of the cell line for long-term culture and experiments examining a broad range of issues relevant to motor neurons.

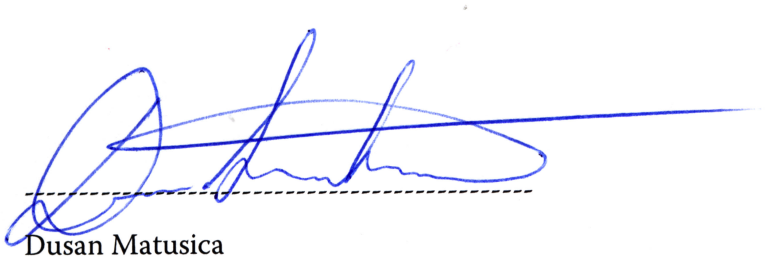
In Chapter 4, multiple experimental approaches were used to extensively screen the NSC-34 cell line for the presence of motor neuron-specific markers, neurotrophin receptors and proteins involved in regulation of endosomal transport. This characterization established the presence of a developing motor neuron-like neurotrophin receptor profile ($p75^{NTR}$, TrkB and TrkC), a genetic marker of developing motor neurons, cholinergic markers, proteins regulating transport within the endosomal pathway, and additional proteins previously shown to directly interact with neurotrophin receptors, including sortilin, and the lipid raft associated ganglioside GT1b. Furthermore, evidence is provided that NSC-34 cells undergo apoptosis in response to exogenous nerve growth factor (NGF) or neurotrophin-3 (NT-3), but not brain derived neurotrophic factor (BDNF) or neurotrophin-4 (NT-4). In addition characterization of mouse specific $p75^{NTR}$ antibodies is presented to establish their suitability for internalization studies without altering the binding of exogenous neurotrophins to the receptor.

Subsequent confocal microscopy examination focusing on $p75^{NTR}$ trafficking in Chapter 5 revealed that internalization and intracellular transport of this receptor is regulated by exogenous neurotrophins at the cell surface where ligand binding and internalization occur, and in endosomal compartments where the bulk of receptors and ligands are targeted to their specific destinations. Evidence is provided showing that $p75^{NTR}$ internalization is altered in the presence of NGF, NT-3, or NT-4, but not BDNF, and the receptor is diverted into non-clathrin mediated endosomal pathways in response to NGF but not BDNF. Immunofluorescence confocal microscopy suggests that $p75^{NTR}$ recycles to the plasma membrane in a Rab4 GTPase dependent manner in the absence of neurotrophins. Addition of neurotrophins diverted $p75^{NTR}$ from the recycling Rab4 positive pathway, into EEA-1 positive sorting endosomes in the presence of NGF or NT-3, or lysosomal degradation in the presence of BDNF or NT-4.

This study clearly demonstrates the suitability of the NSC-34 cell line as an alternate in-vitro system for the study of motor neuron biology, particularly the study of neurotrophin receptor trafficking. Taken together the results represented in this study suggest for the first time, that the fate of the $p75^{NTR}$ receptor depends on which neurotrophin is bound. These findings have important implications for understanding the dynamic mechanisms of action of $p75^{NTR}$ in normal neuronal function, and may also offer further insight into the potential role of neurotrophins in the treatment of neurodegenerative diseases.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge or belief it does not contain any material previously published or written by another person except where due reference is made in the text.



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Publications and manuscripts arising from this research

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MATUSICA, D., FENECH, M. P., ROGERS, M. L. & RUSH, R. A. (2007) Characterization and use of the NSC-34 cell line for study of neurotrophin receptor trafficking. ***Journal Of Neuroscience Research***. 86, (3):553-65

MATUSICA, D., ROGERS, M. L. & RUSH, R. A. (Submitted) NSC-34 cells: enhanced differentiation and adhesion increases value as a motor neuron cell line.

MATUSICA, D., ROGERS, M. L. & RUSH, R. A. (Submitted) NGF and NT-3, but not other neurotrophins, prevent trafficking of $p75^{NTR}$ to lysosomes in NSC-34 cells.

LIST OF ABBREVIATIONS

Ab	Antibody
Akt	Serine/threonine kinase / protein kinase B
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
EE	Early endosome
EEA-1	Early endosomal antigen 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Factor associated death domain
FAP-1	Fas associated phosphatase 1
HB9	Homeobox gene 9
IgG	Immunoglobulin
LE	Late endosome
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MC-192	Monoclonal antibody against rat p75 receptor
MEK	MAPK kinase / ERK kinase
MLR-2	Monoclonal antibody a
mRNA	messenger ribonucleic acid
MVB	Multi-vesicular body
NaCl	Sodium chloride
NADE	p75-associated cell death executor
NF-1	Neurofibromatosis-1
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
NRAGE	Neurotrophin receptor interacting melanoma associated antigen homolog
NRIF	Neurotrophin receptor interacting factor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
$p75^{NTR}$	p75 neurotrophin receptor
PC-12	Pheochromocytoma-12 cells
PI3K	Phosphatidyl inositol-3 kinase
PNS	Peripheral nervous system
Rab4	Rab 4 GTPase protein
Ras	Ras GTPase protein kinase
REX	Receptor external domain
RIP	Ribosome inactivating protein
RIP^{1}	Receptor interacting protein
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SC-1	Schwann cell factor 1

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Sorting endosome
TGN	Trans-Golgi network
TNF	Tumour necrosis factor
TRAD	TNF receptor associated death domain
Trk	Tropomyosin receptor kinase
Western Blot	Immunoblot

CHAPTER 1: Neurotrophins, Neurotrophin Receptors And Signaling Mechanisms Via Endosomal Transport

1.A General Introduction

Among the most morphologically complex cells, neurons reside at the pinnacle of cellular specialization (Kandel *et al.*, 2000). They are highly polarized cells with axonal and somatodendritic membrane surfaces that spatially separate signal-sending from signal-receiving membrane domains (Schmidt & Haucke, 2007). Neurons are characteristically involved in large-scale intracellular movement as a result of molecular synthesis, degradation, uptake and disposal (Almenar-Queralt & Goldstein, 2001). Their long (up to 1m) extended axons and elaborate dentritic trees establish and maintain the circuitry that detects, stores and transmits information that is essential to the function of all complex organisms.

1.A.1 The Neurotrophic Hypothesis

In the early 1900's it was demonstrated that neurons must contact their target organs in order to survive (Hamburger, 1958). Today, we know that throughout their life, all neurons depend on numerous target-released growth factors and other molecules to maintain their highly ordered functionality. In development, the survival and differentiation of neurons depends on competition for specific growth factors, which are available in limited amounts from target tissues (Hendry, 1992). Neurons that are able to obtain a sufficient amount of growth factors, survive and form synapses; the neurons that do not are removed by developmentally programmed cell death (Hendry *et al.*, 1974; Oppenheim, 1991). In the mature nervous system these factors play vital roles in the maintenance of activity dependent neuronal plasticity, and possibly regulate higher systemic brain function (Dechant, 2001).

Neurotrophins represent perhaps the most characterized, and intensely studied growth factors associated with neuronal development and functional maintenance (Thoenen & Sendtner, 2002; Lu *et al.*, 2005). Unlike conventional growth factors, neurotrophin signal transduction differs, in that the ligands activate their receptors at the synapse and require to be retrogradely transported to the cell body in order to elicit changes in gene expression (Howe & Mobley,

2005).

1.A.2 Diverse Roles Of Neurotrophins

At the cellular level, the biological effects of neurotrophins are highly diverse, cell type specific and often paradoxical. Although initially identified as growth factors, neurotrophins mediate a multitude of biological functions including survival, differentiation, cell cycle arrest, neurodegeneration, neuroprotection, apoptosis and modulation of synaptic plasticity (Chao, 2003). From a genetic point of view, the neurotrophin ligand-receptor system used to produce these diverse physiological phenomena appears simple. In higher vertebrates, neurotrophins are derived from four genes (Hallbook, 1999), with five genes encoding three unrelated receptors responsible for signaling effects (Bronfman & Fainzilber, 2004).

1.A.3 Current Progress In Neurotrophin Receptor Trafficking

The receptor-ligand relationships between neurotrophins and their receptors were mostly elucidated using various cell-lines overexpressing the receptors in recombinant form (Kaplan, 1998; Dechant, 2001). Although the mechanisms used to convey neurotrophin signals over great distances remain contentious (Howe & Mobley, 2005), the fact that binding of neurotrophins to their receptors initiates internalization of a receptor-ligand complex (Campenot & MacInnis, 2004), followed by a vesicular-based mechanism for transporting a biological signal to the cell body, has been substantiated by an increasing body of evidence (Grimes *et al.*, 1996; Grimes *et al.*, 1997; Bronfman *et al.*, 2003; Howe & Mobley, 2004). The experimental design in these studies focused primarily on the transport of nerve growth factor (NGF) and its cognate receptors (Bronfman *et al.*, 2003). The role of the PC-12 cell-line (Greene *et al.*, 1975), a rat adrenal pheochromocytoma cell line that responds to NGF and resembles sympathetic neurons, but also differs from neurons in many aspects, has been instrumental in the majority of these breakthrough discoveries (Kaplan & Stephens, 1994). This *in vitro* platform has provided valuable insights into these complex sub-cellular mechanisms (Grimes *et al.*, 1996; Grimes *et al.*, 1997), which have been

subsequently validated *in vivo* (Ye *et al.*, 2003; Howe & Mobley, 2004).

1.A.4 Neurotrophin Signaling Is Regulated By Intracellular Transport

A fundamental concept that has clearly emerged from decades of neurotrophin research is that the spatio-temporal regulation of neurotrophin signaling through receptor internalization and vesicular sorting is highly significant for the physiology of neurotrophins (Barker *et al.*, 2002; Ginty & Segal, 2002; Heerssen & Segal, 2002). These mechanisms are further complicated, as different cell types have the capacity to translate neurotrophin receptor activation into a myriad of cellular responses, clearly enforcing that it is paramount to examine these events on a cell-type specific basis (Bibel & Barde, 2000; Huang & Reichardt, 2001). At present there is no agreed way of specifically identifying the vesicular structures containing neurotrophin receptors and ligands, also referred to as cargo carriers, in relation to predicting the targeted destination of these vesicles, within the neuronal cytoplasm.

1.A.5 Significance Of Understanding Neurotrophin Receptor Trafficking In Motor Neurons

Historically motor neurons had a fundamental role in establishing the concept that neurotrophins have an important influence on the development of neurons (Thoenen *et al.*, 1993). Yet despite the importance of motor neurons in human health and disease, and in contrast to the importance of motor neurons in establishing the target dependent neurotrophic concept, ligand-receptor complex internalization and transport in these cells are relatively poorly understood when compared to the intensely studied sensory and sympathetic neurons (Sendtner *et al.*, 2000). Motor neuron diseases such as amyotropic lateral sclerosis (ALS) remain high priority neurodegenerative disorders due to their relentless progressive course and the invariably fatal prognosis (Bartlett *et al.*, 1998). The significance of neurotrophin receptor transport has recently come to attention, as it appears likely, that numerous neurodegenerative diseases could be caused by "indirect" effects on neurotrophin trafficking and signaling (Kruttgen *et al.*, 2003; Bronfman *et al.*, 2007).

1.A.6 Aims

Difficulties associated with obtaining, isolating and growing primary motor neuron cultures (Kuhn, 2003), the difficulty of transfecting primary motor neuron populations (Read *et al.*, 2005), and the lack of existing, well characterized motor neuron cell lines (Deinhardt & Schiavo, 2005), have to date been the primary obstacles in elucidating motor neuron specific knowledge regarding neurotrophin transport and signaling.

The aim of this thesis is to characterize and establish a motor neuron cell-line model to be used in the study of neurotrophin receptor transport and cytosolic localization. More specifically, attention will be focused primarily on one of the most enigmatic and controversial neurotrophin receptors, the p75 neurotrophin receptor ($p75^{NTR}$), and its sub-cellular localization, following neurotrophin dependent internalization. The following pages will cover the overview of current concepts detailing three converging areas including neurotrophin function, including detailed description of interactions between neurotrophins, their receptors; description of general and neurotrophin receptor-specific uptake pathways; and a brief but thorough introduction to motor neuron cell lines used in research.

1.B Neurotrophins

A large number of molecules have been identified based on the ability to support the survival of immature neurons *in vitro* (DiStefano, 1993). The majority of these represent the gene families of neurotrophins, neurocytokines and other related molecules (Mitsumoto & Tsuzaka, 1999b; a). Neurotrophic factors can be defined as polypeptides that are required for the survival of specific neuronal populations during development and at adult stages (Davies, 1994). A wide variety of factors are now considered to fit this definition, but it remains hard to apply this definition rigorously for two reasons. First, the physiological roles of many neurotrophic factors are still not clearly defined, and often they are inferred from pharmacological experiments *in vitro* or *in vivo.* Second, some molecules initially identified due to their survival-promoting activity may elicit other physiological effects that are at least as important (Henderson *et al.*, 1998), and neurotrophins are prototypical of such growth factors.

1.B.1 Nerve Growth Factor (NGF)

Neurotrophins are responsible for the regulation of neuronal survival and physiology in both the peripheral (PNS) and central (CNS) nervous system (Levi-Montalcini *et al.*, 1996; Bibel & Barde, 2000). The first neurotrophin to be discovered was nerve growth factor (NGF) (Levi-Montalcini & Hamburger, 1951). Subsequent systemic search of mammalian tissues revealed that male mouse salivary glands contained substantial amounts of NGF (Cohen, 1960). Ongoing work in the field established that NGF is released by the target tissues of sympathetic and sensory fibers, and is taken up by the fibers and retrogradely transported to the cell body (Hendry *et al.*, 1974).

Human NGF gene is located on the short arm of chromosome 1 and codes for a polypeptide of 307 amino acids which, when the signal peptide and pro region are cleaved gives rise to a mature protein of 118 amino acids that naturally exists as a non-covalently bound homodimer (Francke *et al.*, 1983). Although NGF is the only member of the neurotrophin family that has not been demonstrated to support motor neuron survival (Sendtner *et al.*, 1992; Hughes *et*

al., 1993), its represents by far the most studied and functionally defined molecule of all the growth factors in the nervous system.

1.B.2 Brain Derived Neurotrophic Factor (BDNF) And Neurotrophins 3 - 4 (NT-3-4)

Thirty years after the discovery of NGF, before another factor that demonstrated neuronal growth-promoting properties was purified from pig brain, and later called Brain Derived Neurotrophic Factor (BDNF) (Barde *et al.*, 1982). The eventual cloning of the gene some seven years later revealed a relatively close homology to NGF with 51 identical amino acids. This close similarity also suggested that BDNF occurred naturally as a homodimer. By using the contiguous regions between NGF and BDNF to design oligonucleotides for polymerase chain reaction (PCR) cloning, a third member of the family called neurotrophin 3 (NT-3) was cloned in 1990 (Ernfors *et al.*, 1990).

Just a year later, and after an exhaustive search the last of the neurotrophin family members, neurotrophin 4 (NT-4), was cloned and identified in *Xenopus* (Ibanez *et al.*, 1992). However the equivalent human cDNA proved to be sufficiently different from *Xenopus* NT-4, and was subsequently identified as a separate gene called neurotrophin 5 (NT-5) (Berkemeier *et al.*, 1991). Following further investigation it was later realized that NT-4 and NT-5 were orthologous genes, and therefore this neurotrophin has been often referred to as NT-4/5. A new member of the neurotrophin family was identified in 1994, named neurotrophin 6 (NT-6), but has so far been found only in fish (Hallbook, 1999).

1.B.3 Synthesis And Secretion Of Neurotrophins

All mammalian neurotrophins are synthesized as precursors called proneurotrophins which are approximately 30 ~ 35 kDa in size (Lee *et al.*, 2001b). Proneurotrophins are cleaved by furin and pro-convertases to form mature neurotrophins, all of which form non-covalently-linked homodimer with each monomer being approximately 12 ~ 13 kDa (Lee *et al.*, 2001b)(Figure 1.1). The X-ray crystal structures of mammalian neurotrophins have all been elucidated, and

because of their characteristic formation of a double loop formed by two disulfide bonds, penetrated by a third disulphide bond, they are classed as part of the Cysteine Knot Superfamily (Lu, 2003).

After synthesis in the endoplasmic reticulum (ER), proneurotrophins must be folded correctly, and subsequently sorted into the appropriate secretory pathway, regulated or constitutive, and then transported to the appropriate cellular compartment (Harrington *et al.*, 2004). In the majority of non-neuronal cells, neurotrophins are secreted through the constitutive pathway, where intracellular vesicle compartments fuse spontaneously with the plasma membrane to release them into the extracellular space (Lee *et al.*, 2001b). In most neuronal populations, neurotrophins are loaded into secretory granules and are subsequently transported to dendrites and spines to be released post-synaptically (Fahnestock *et al.*, 2004). However, neurotrophins are also loaded into vesicles and are targeted for anterograde transport to the axonal terminals where they undergo extracellular stimuli-dependent secretion (Harrington *et al.*, 2004). Ultimately there are three known fates of intracellular neurotrophins. Intracellular cleavage followed by secretion; secretion followed by extracellular cleavage; or excretion without extracellular cleavage (Lu *et al.*, 2005).

Traditionally, pro-neurotrophins have been considered as inactive precursors, with no significant biological function (Hempstead *et al.*, 1992). However the findings that in some tissues a substantial amount of proNGF and proBDNF escapes cleavage raised the possibility that these uncleaved forms of neurotrophins may have biological functions (Lee *et al.*, 2001b).

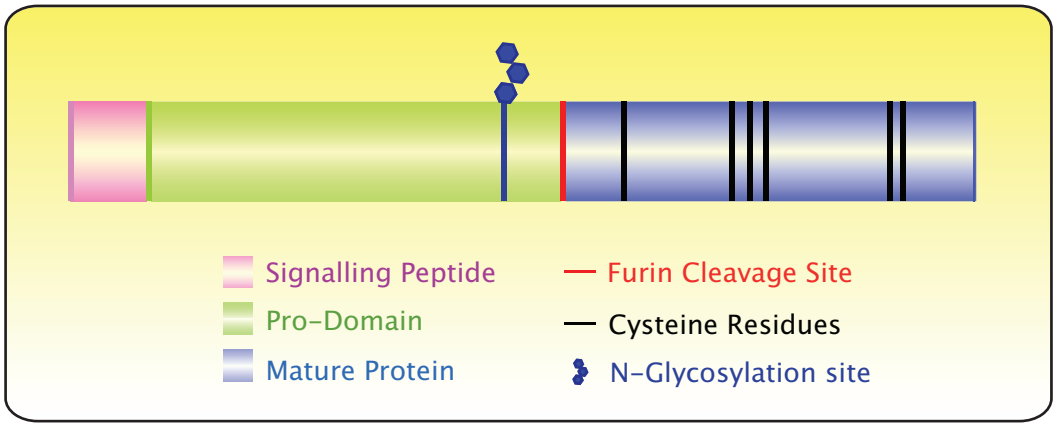


Figure 1.1 **The processed versus the unprocessed monomeric form of neurotrophins.** With the exception of NT4/5, neurotrophin sequences are highly conserved in mammals. They are synthesized as glycosylated pre-pro-proteins that undergo proteolytic cleavage. The pro-domain may be cleaved either intracellularly by furin or extracellularly by matrix metalloproteases (MMPs) and plasmin. The extracellular cleavage sites have not been specified yet, but do not coincide with the furin site. Both the processed and the unprocessed protomers form stable dimers in solution. The structural hallmark of neurotrophins is the cystine-knot motif, which comprises several conserved cysteine residues within the mature moiety. (adapted from *Schweigreiter, R. 2006*)

ProNGF and proBDNF are currently emerging as potent inducers of apoptosis (Harrington *et al.*, 2004; Teng *et al.*, 2005), but further investigations are needed as contradicting reports of proNGF mediated survival effects have also been observed (Fahnestock *et al.*, 2004; Buttigieg *et al.*, 2007).

1.C Neurotrophin Receptors

Neurotrophins bind two main types of cell surface receptors, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (Barbacid, 1994; Patapoutian & Reichardt, 2001), and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumour necrosis factor (TNF) receptor family (Barker, 1998). $P75^{NTR}$ was the first identified receptor for NGF (Chao *et al.*, 1986; Radeke *et al.*, 1987), and the first Trk receptor was originally discovered as a rearrangement of non-muscle tropomyosin, and a then unknown tyrosine kinase (Martin-Zanca *et al.*, 1986).

This tyrosine kinase was referred to as TrkA and subsequently identified as a receptor for NGF (Martin-Zanca *et al.*, 1989; Kaplan *et al.*, 1991a). The identification of TrkB and TrkC, based on their similarity to TrkA, followed thereafter (Klein *et al.*, 1989; Hempstead *et al.*, 1991; Lamballe *et al.*, 1991).

These receptors are often co-expressed on the same cell, and their function varies markedly as the signals generated by the two receptors are able to either augment or oppose each other (Segal, 2003). Hence, Trk's and $p75^{NTR}$ exist in a complex paradoxical relationship, in some instances each taking a role in either supporting or suppressing each other actions, as well as sharing and enhancing binding affinities for multiple neurotrophins. To add to the complexity of this two-receptor system, $p75^{NTR}$ also associates with a number of other receptors in a variety of functions not always associated with neurotrophin action.

1.C.1 Tyrosine Kinase Receptor Family (Trk's)

The trk proto-oncogene encodes three closely related tyrosine kinases, TrkA, TrkB and TrkC (Eide *et al.*, 1993; Barbacid, 1994), and numerous splice variants of each Trk (Barbacid, 1994). Trk receptors are prototypical receptor tyrosine kinases that possess a tripartite structure containing an extracellular domain composed of three leucine-rich motifs flanked by two cysteine clusters, two immunoglobulin-like C2 type domains (Ig-C2), a single transmembrane domain, and a cytoplasmic tail containing ten conserved tyrosines for the purpose of initiating signaling cascades (Barbacid, 1994; Kaplan & Miller, 1997)(Figure 1.2).

Binding of neurotrophins to Trk receptors occurs mainly through the Ig-C2 domains, with the domain closer to the transmembrane region playing a more prominent role (Perez *et al.*, 1995; Urfer *et al.*, 1998). The leucine-rich motifs and the cysteine clusters may also be involved (MacDonald & Meakin, 1996). In addition to ligand binding, the Ig-C2 domains can also stabilize the monomeric form of the Trk receptor to prevent spontaneous dimerization and activation in the absence of neurotrophins (Arevalo *et al.*, 2000). Neurotrophin binding to Trk receptors results in allosteric receptor dimerisation followed by kinase activation (Kaplan & Miller, 2000). Phosphorylation of several evolutionarily conserved tyrosine residues present in the cytoplasmic activation loop, including Y670, Y674, and Y675 lead to the recruitment of signaling proteins (Huang & Reichardt, 2003). Phosphorylation of the other residues promotes signaling by creating docking sites for a number of adaptor proteins that couple these receptors to intracellular signaling cascades (Lee *et al.*, 2001a; Huang & Reichardt, 2003), and to components of cytoplasmic motors for axonal transport (Yano *et al.*, 2001).

1.C.2 Alternative Splicing Of Trk Receptors

Splice variants of trk receptors represent another puzzling feature of neurotrophin signal transduction complexity. All Trk neurotrophin receptor genes are expressed in multiple naturally occurring protein isoforms that are

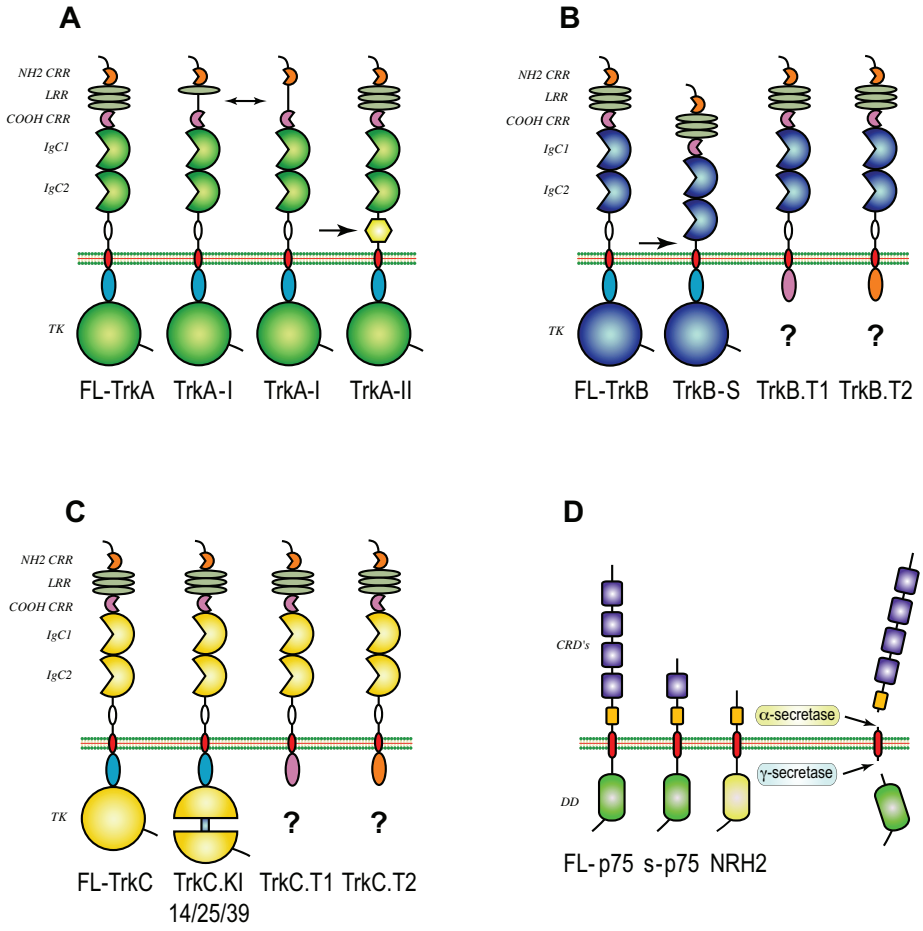


***Fig 1.2* Schematic representation of Trk and p75NTR structural isoforms.** Trk and p75NTR receptors occur in their full-length (FL) forms, but alternative splicing has also been reported for all neurotrophin receptors. **(A)** Two different isoforms of TrkA have been described and named TrkA-I and TrkA-II. Three TrkA-I isoforms (two are shown) have been isolated which carry a total or partial deletion of amino cysteine-rich region in the extracellular matrix. TrkA-II differs from TrkA-I in that it carries an extracellular six amino acid insertion next to the transmembrane domain. **(B)** Both TrkA and TrkB transcripts are alternatively spliced at a small exon in their extracellular domain creating a corresponding protein isoform termed TrkA/B-S. Alternative splicing on the intracellular domain of the TrkB transcript results in isoforms with truncated tyrosine kinase domains (TrkB-T1 and TrkB-T2). **(C)** Truncated tyrosine kinase domain isoforms Trk-T1 and Trk-T2 also exist for the TrkC receptor. In addition tyrosine kinase peptide insertion isorms for TrkC have been described (TrkC.KI-14, 25 or 39 equivalent to the number of amino acids forming the insertion). **(D)** A protein isoform has been described for p75NTR which lacks the cysteine-rich domains 2, 3 and 4 (s-p75NTR) due to alternative splicing of exon III of p75NTR-FL. p75NTR homologue NRH2 is also present in mammals. p75NTR also undergoes cleavage by metalloproteinases α-secretase and γ-secretase to produce an ectodomain piece and a transmembrane and cytoplasmic domain fragment with signaling capability.

Legend: ***NH2 CRD's****, amino cysteine-rich regions;* ***COOH CRD's****, carboxy cysteine-rich regions;* ***TK****, tyrosine kinase;* ***DD****, death domain;* ***LRR****, leucine-rich repeat;* ***IgC1****, Immunoglobulin C1 domain;* ***IgC2****, Immunoglobulin C2 domain.*

generated through alternative splicing of their primary gene transcripts. Splice variants of Trk receptors are expressed with insertions in the extracellular domain (TrkA and TrkB), insertions in the tyrosine kinase domain (TrkA and TrkC), or truncated forms lacking the kinase domains (TrkB and TrkC) (Barbacid, 1994; Dechant, 2001; Vega *et al.*, 2003)(Figure 1.2 A-C).

1.C.3 Extracellular Splice Variants

The splicing of a small exon in the extracellular domain of TrkA (Barker, 1993) (Clary & Reichardt, 1994) and TrkB (Strohmaier *et al.*, 1996) produces isoforms with altered ligand interactions compared with the full-length receptors (Dechant, 2001). These TrkB isoforms have reduced affinities for NT-3 and NT-4 (Dechant, 2001), and increased specificity for BDNF (Strohmaier *et al.*, 1996). The corresponding TrkA isoforms differ in their interaction with the non-preferred ligand NT-3 (Dechant, 2001). Alternate variants of TrkB with deletions of the leucine-rich domains completely lack ligand binding and signaling capabilities (Ninkina *et al.*, 1997).

1.C.4 Intracellular Splice Variants

Isoforms for TrkB (Klein *et al.*, 1990; Middlemas *et al.*, 1991) and TrkC (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993) with truncations or insertions in the catalytic tyrosine kinase domains have been described. Truncated TrkB receptors (T1 and T2) can function as dominant negative inhibitors of the TrkB kinase if both forms are expressed in *cis* on the same cell (Ninkina *et al.*, 1996; Eide *et al.*, 1996). If the same isoforms are expressed in *trans*, they function as scavenger receptors by sequestering BDNF and removing it from the extracellular space via internalization (Alderson *et al.*, 2000). This mechanism restricts the activation of the catalytic full-length TrkB temporally and spatially (Dechant, 2001). The full-length and truncated TrkB isoforms also differentially regulate dentritic growth of cortical neurons (Yacoubian & Lo, 2000). Evidence has also emerged suggesting that non-catalytic TrkB receptor isoforms may have intrinsic signaling capabilities (Baxter *et al.*, 1997; Esteban *et al.*, 2006), and a truncated form of TrkC promotes neuronal differentiation in neural crest cells, but only in the

presence of $p75^{NTR}$ (Hapner *et al.*, 1998). Further, the intricate differential expression of multiple Trk isoforms during development may play a crucial role in sculpting axonal morphology (Ichinose & Snider, 2000).

1.C.5 The p75 Neurotrophin Receptor

Initial efforts to identify the NGF receptors resulted in discovery of $p75^{NTR}$ by expression cloning of a trans-membrane protein capable of binding NGF (Johnson *et al.*, 1986). For many years $p75^{NTR}$ was believed to be a low affinity receptor for NGF, but studies have demonstrated that it binds to all of the mammalian neurotrophins (NGF, BDNF, NT-3 and NT-4/5) with a similar affinity (Chao & Hempstead, 1995). When initially discovered and cloned, $p75^{NTR}$ represented a novel type of receptor, but a large number of structurally related cytokine receptors have since been discovered, the best characterized of which is the tumour necrosis factor (TNF) receptor family (Chao, 2003). A prototypic member of the TNF receptor family, $p75^{NTR}$ contains four cysteine-rich extracellular domains, a single short serine- and theorine-rich trans-membrane domain, and a small and poorly conserved ~80 amino acid cytoplasmic domain (also termed the "death" domain) (Figure 1.2D)(Liepinsh *et al.*, 1997; Barker, 1998). Due to the presence of the "death domain", cell death and apoptosis attributed to $p75^{NTR}$ mediated signaling received considerable attention (Rabizadeh *et al.*, 1994). Cell death and apoptosis induced by $p75^{NTR}$ have been demonstrated in sensory neurons (Barrett & Bartlett, 1994), oligodendrocytes (Casaccia-Bonnefil *et al.*, 1996), cultured neonatal sympathetic neurons (Bamji *et al.*, 1998), motor neurons (Sedel *et al.*, 1999), and neuronal populations with reduced or non-existent Trk receptor activity (Davey & Davies, 1998).

After the discovery of Trk receptors as the signaling components of the neurotrophin receptor complex (Kaplan *et al.*, 1991a), reviewed in (Bothwell, 1995), $p75^{NTR}$ was relegated to the role of a binding protein modifying the binding and signaling capabilities of the Trk receptors. For many years, the $p75^{NTR}$ intracellular "death" domain was still believed to lack intrinsic ligand inducible enzymatic function. The continuous discovery of increasingly varied signaling capabilities of $p75^{NTR}$ were attributed to the recruitment of numerous cytosolic

interactors and adaptors that result in diverse and frequently opposing outcomes like survival (DeFreitas *et al.*, 2001), apoptosis (Rabizadeh *et al.*, 1994; Bamji *et al.*, 1998), axonal growth (Bentley & Lee, 2000), axonal collapse, and cell cycle arrest (Salehi *et al.*, 2000), reviewed in: (Dechant & Barde, 1997; Barker, 1998; Hempstead, 2002) (Figure 1.6). Most of these molecules also lack catalytic activity, suggesting that $p75^{NTR}$ signaling requires recruitment of multiple transducers through protein–protein interactions (Bai *et al.*, 2003; Roux & Barker, 2002; Tcherpakov *et al.*, 2002; Blochl & Blochl, 2007).

Recent investigations however, have revealed that the $p75^{NTR}$ intracellular domain contains two tyrosine kinase residues, Y337 and Y336, which undergo phosphorylation upon NGF binding (Blochl, 2004). Further, the tyrosine-phosphorylated $p75^{NTR}$ activates Ras through the same adaptor proteins as Trk receptors (Blochl *et al.*, 2004).

1.C.6 p75 Splice Variant And Homologue Receptors

The p75 gene is also expressed in multiple receptor isoforms. The alternative splice variant named short p75 (s-p75) (Figure 1.2D) contains an open reading frame that translates into a naturally occurring isoform of the receptor with a spliced exon III encoding the cysteine-rich domains 2, 3, and 4 which are essential for neurotrophin binding (Lee *et al.*, 1992; von Schack *et al.*, 2001). Even though the s-p75 variant does not bind any of the neurotrophins, it shares an identical intracellular domain, and it appears likely that it may bind some of the intracellular signaling proteins and interactors of the full-length $p75^{NTR}$ (Dechant, 2001). Because the relationship between ligand binding and activation of signaling is not clearly defined for $p75^{NTR}$, a ligand-independent function for s-p75 could be plausible.

A gene structurally related to $p75^{NTR}$ encodes for the neurotrophin receptor homologue (NRH) (Hutson & Bothwell, 2001). NRH is homologous to $p75^{NTR}$ with a strong sequence similarity in the transmembrane, juxtamembrane and death-domain regions (Figure 1.2D)(Kanning *et al.*, 2003). NRH1 and NRH2 have been identified (Hutson & Bothwell, 2001), however only NRH2 has been found

in the mammalian genome (Kanning *et al.*, 2003). NRH2 has been identified recently in rat and been alternatively termed PLAIDD (p75-like apoptosis inducing death domain protein) (Frankowski *et al.*, 2002) and also NRADD (neurotrophin receptor alike death domain protein) (Wang *et al.*, 2003).

1.C.7 Neurotrophin Receptors And Ligand Specificity

Neurotrophin ligand affinity and specificity is inherently complex due to the use of multi-receptor systems (Figure 1.3). In addition to Trks and $p75^{NTR}$ having their own signaling properties, $p75^{NTR}$ also has the ability to modulate and alter ligand specificity of Trk receptors (Hempstead, 2002). Although TrkA is able to bind NGF and NT-3, $p75^{NTR}$ co-expression with Trk A restricts NGF signaling via TrkA only (Mischel *et al.*, 2001). TrkB has the ability to bind BDNF, NT-3 and NT-4/5 (Bothwell, 1995). However in the presence of $p75^{NTR}$, only BDNF is able to elicit a functional response (Bibel *et al.*, 1999). In contrast, TrkC exhibits binding affinity for NT-3 only, but shows a decrease in its absolute specificity for NT-3 in the presence of $p75^{NTR}$ (Yuen & Mobley, 1999). In addition, recently investigated structure-function relationship studies of Trk receptors indicate, that when they are co-expressed with $p75^{NTR}$, extracellular Trk receptor domains are altered to offer additional docking sites for neurotrophin ligands (Zaccaro *et al.*, 2001). In 2001, utilizing a furin resistant form of proNGF, Hempstead and colleagues shattered this traditional view, by demonstrating that proNGF binds to $p75^{NTR}$ with an affinity 5 times higher than mature NGF, and is a potent inducer of $p75^{NTR}$ dependent apoptosis in sympathetic neurons, oligodendrocytes and in a vascular smooth muscle cell line (Barker, 2004; Nykjaer *et al.*, 2004). Furthermore, these studies also demonstrated that proNGF binds $p75^{NTR}$ and sortilin only, but not TrkA, and have therefore suggested that proNGF is an apoptotic ligand that is specific for $p75^{NTR}$ alone or in a partnership with sortilin.

1.C.8 Neurotrophin Receptor Interactors

In addition to its interactions with Trk receptors, $p75^{NTR}$ forms functional partnerships with other non-related membrane proteins (Figure 1.4). The sortilin receptor represents the most recent member and a conceptual twist in the neurotrophin receptor family Sortilin is a ~95-kDa type-I receptor first isolated from human brain by receptor associated protein (RAP) affinity chromatography (Petersen *et al.*, 1997), and recently shown to be identical with the neurotensin receptor-3 (Mazella *et al.*, 1998). It has strong homology to yeast vacuolar protein-sorting 10 protein (Vps-10p), with a single transmembrane domain, and a short cytoplasmic tail with a C terminus strongly homologous to that of the mannose 6-phosphate (M6P-R)/insulin-like growth factor-II (IGF-II) receptor (Petersen *et al.*, 1997). In mature sortilin, an N-terminal 44-residue propeptide has been cleaved off, and recent results show that furin-mediated propeptide cleavage conditions sortilin for ligand binding (Munck Petersen *et al.*, 1999). The Vps-10p family of receptors are involved in trafficking cargo from the trans-golgi network (TGN) to the endocytic and lysosomal compartments, and more than 90% of sortilin is found in intracellular compartments (Nielsen *et al.*, 2001). Sortilin is expressed in a wide variety of tissues but is most abundant in the central nervous system from development through to adulthood (Barker, 2004).

The intriguing twist in this tale has been added by the implication that neurotrophins use not two, but three distinct receptor classes to dictate and regulate opposing biological responses of survival and death. Sortilin binds pro-NGF molecules (Nykjaer *et al.*, 2004), and pro-BDNF (Teng *et al.*, 2005), in the presence of $p75^{NTR}$ (Figure 1.3C-D) with high affinity, subsequently leading to neuronal apoptosis.

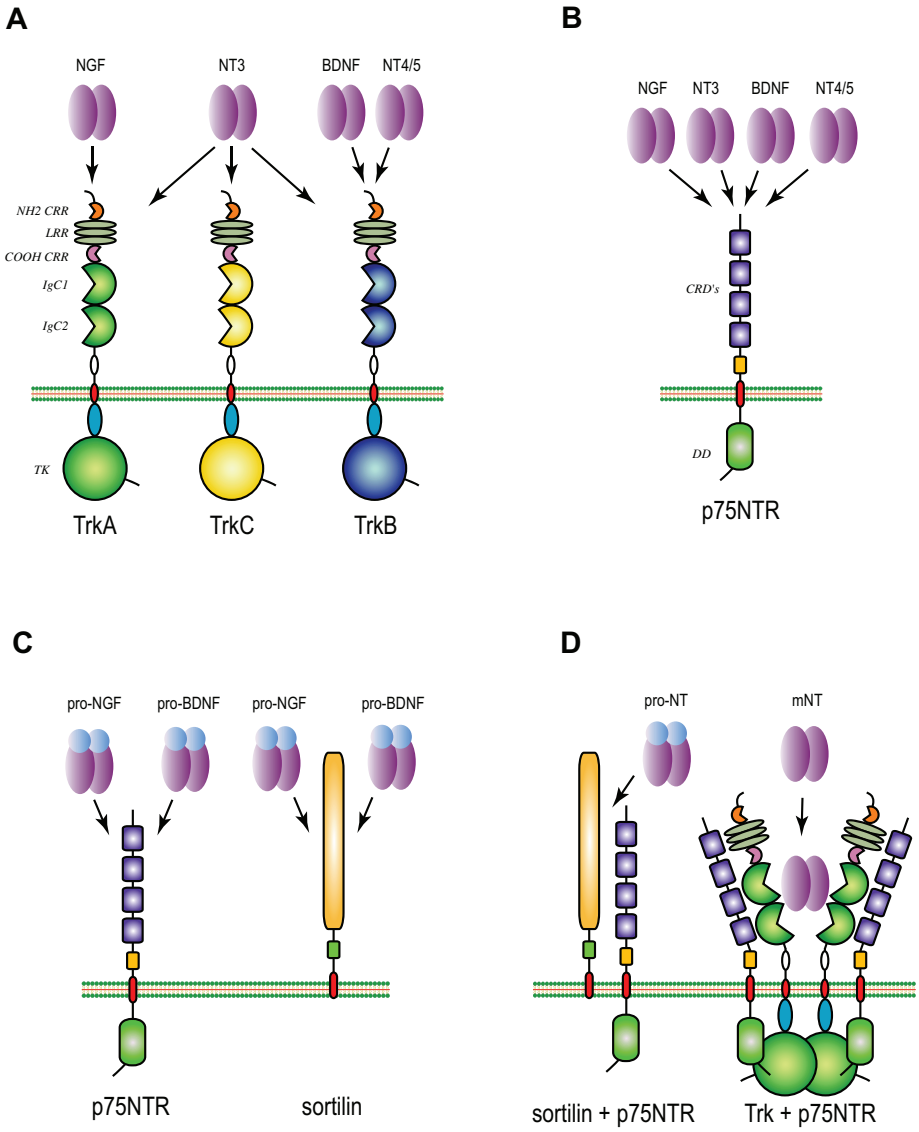


***Figure 1.3* Neurotrophin interactions with their receptors.** Trk A binds NGF and NT-3 (with a lower affinity), Trk B binds BDNF, NT-3 and NT-4. Trk C binds NT-3 only, with an affinity Kd of about 1-10 nM (**A**). p75NTR is able to bind all mammalian neurotrophins with similar affinity (**B**). Recent findings confirm that p75NTR is able to bind proNGF and proBDNF with a five times higher affinity than the mature neurotrophins, and add a third receptor to the family; sortilin. Sortilin serves as the neurotensin receptor, but the lack of processing of proNGF and proBDNF results in these ligands having a higher binding affinity for sortilin (**C**). Further, biochemical evidence supports the existence of complexes consisting of sortilin and p75NTR as a result of pro-neurotrophin binding, and Trk and p75NTR receptors in the presence of mature neurotrophins (**D**). The formation of Trk and p75NTR complexes has been correlated with higher ligand affinity and specificity.

*Legend: **NH2 CRD's**, amino cysteine-rich regions; **COOH CRD's**, carboxy cysteine-rich regions; **TK**, tyrosine kinase; **DD**, death domain; **LRR**, leucine-rich repeat; **IgC1**, Immunoglobulin C1 domain; **IgC2**, Immunoglobulin C2 domain.*

Although both $p75^{NTR}$ and sortilin are required for the transduction of proNGF and proBDNF induced apoptotic signaling, sortilin also binds proNGF (Nykjaer *et al.*, 2004) and proBDNF (Chen *et al.*, 2005b) in the absence of $p75^{NTR}$. The ability of sortilin to block the sorting of BDNF with a single substitution in its pro domain (BDNF-V66M) from TGN to secretory granules, suggests that a specific pro-domain region may be required for the efficient, regulated secretion of proBDNF from neurons (Chen *et al.*, 2005b). Therefore, sortilin functions not only as a co-receptor to $p75^{NTR}$ in apoptotic signaling, but it appears that it may be crucial in correctly sorting the intracellular movement of newly synthesized proNT's.

In addition to the multiple roles already attributed to $p75^{NTR}$, this versatile receptor has recently emerged as a key player in the regulation of neuronal growth (Yamashita *et al.*, 2002) Wang, K.C. et al. 2002). CNS-derived myelin-based growth inhibitors (MBGIs) including myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgP) are potent inhibitors of neurite outgrowth in many neuronal populations (Mukhopadhyay *et al.*, 1994; McGee & Strittmatter, 2003). $P75^{NTR}$ is also expressed during the time of neurite growth, and inhibition by MAG is blocked if neurons are exposed to neurotrophins before encountering the inhibitor (Cai *et al.*, 1999).

The Nogo receptor (NogoR), the ganglioside GT1b and Lingo-1 have been identified as components of the receptor complex responsible for attenuating responses to MBGIs (Figure 1.4A) (Yamashita *et al.*, 2002), Mi et al, 2004), and in vitro studies suggest that the co-expression of at least NogoR, $p75^{NTR}$ and Lingo-1 is required for MBGI signaling (Barker, 2004). Both Trk and $p75^{NTR}$ have been reported to interact with the ankyrin repeat-rich membrane-spanning protein substrate (ARMS) (Kong *et al.*, 2001).

1.C.9 Ligand Promiscuity Of $p75^{NTR}$

Apart from having important roles in the binding, internalization and trafficking of neurotrophins, recent reports show that $p75^{NTR}$ has an amazing ability to bind directly or indirectly to an astonishing array of proteins including lectins, pathogens and toxins (Figure 1.4B) (Butowt & Von Bartheld, 2003). Even more amazing is the fact that some of these molecules, such as tetanus neurotoxin (TeNT), have the ability to enter specific neurons and travel transneuronally among connected cell populations within the nervous system (Lalli *et al.*, 2003; Von Bartheld, 2004). Hence specific binding to $p75^{NTR}$ does not only play a role in the trafficking of neurotrophins, but seem to present an explicit transport entry system for foreign proteins.

The diversity of non-neurotrophin ligands reported to bind to $p75^{NTR}$ include the neuronal membrane-binding lectin wheat germ agglutinin (WGA) which competes partially with saturating doses of NGF, possibly because both molecules bind to $p75^{NTR}$ (Butowt & Von Bartheld, 2003). Studies have also revealed, that the glycoprotein responsible for neuronal penetration from certain rabies virus strains specifically binds the $p75^{NTR}$ neurotrophin receptor (Tuffereau *et al.*, 1998), as part of a multi-receptor complex (Von Bartheld, 2004). Rabies virus binds with high affinity to the first cystein- rich domain of mammalian $p75^{NTR}$ (Langevin *et al.*, 2002). This domain is retained in the partial p75-knock-out mouse (von Schack *et al.*, 2001), probably explaining unimpaired rabies virus infection in this model system (Jackson & Park, 1999).

Tetanus toxin and cholera toxin also appear to have a special relationship with $p75^{NTR}$. Tetanus toxin is transported in vesicles containing $p75^{NTR}$ bound NGF (Lalli & Schiavo, 2002). Tetanus toxin binds specifically to polysialogangliosides, GD1b, and GT1b, as well as other yet unknown cell surface proteins (Schiavo *et al.*, 2000). GT1b has already been established as a $p75^{NTR}$ interactor in Nogo signaling (Wang *et al.*, 2002). Cholera toxin subunit B binds to

the cell surface ganglioside GM1, as GM1 also associates with Trk receptors and may bind (and be trafficked) in a GM1-Trk-p75 complex (Butowt & Von Bartheld, 2003).

In Alzheimer's disease, $p75^{NTR}$ also appears to be involved in neuronal damage owing to β-amyloid's binding to $p75^{NTR}$ and activation of its death domain (Yaar *et al.*, 1997; Yaar *et al.*, 2002; Perini *et al.*, 2002). A series of recent studies provided an unexpected new twist to the role of $p75^{NTR}$ in Alzheimer's disease by showing that $p75^{NTR}$ directly interacts with β-amyloid and serves as a receptor for b-amyloid to promote cell death via NF-kB (nuclear factor kappa B) (Kuner *et al.*, 1998) and JNK (c-Jun N-terminal kinase (Perini *et al.*, 2002; Yaar *et al.*, 2002).

Prion peptides are responsible for the fatal neurological diseases named transmissible spongioform encephalopathies (TSEs) (Prusiner, 1987). The pathogenic form of the prion protein (PrP^{C}) has been demonstrated to bind, and be transported by $p75^{NTR}$ (Della-Bianca *et al.*, 2001). In addition, evidence supporting the role of $p75^{NTR}$ activation as a primary cellular damage mechanism through prion peptides has also emerged (Della-Bianca *et al.*, 2001).

The significance of alternate and often lethal ligands gaining entry into neurons remains a matter of speculation, but it may indicate the evolutionary importance of neuronal trafficking pathways established by neurotrophins.

However, the simplest evolutionary explanation is that these molecules hijack a less discriminatory receptor transport system to gain entry into cells. Direct proof for the precise role of the $p75^{NTR}$ mechanisms and activation by pathogens and toxins is still largely lacking. However, a sufficient body of data now supports further investigation, to unravel how $p75^{NTR}$ facilitates these harmful agents in accessing endosomal transport to gain access to, and ultimately destroy, neurons (Butowt & Von Bartheld, 2003).

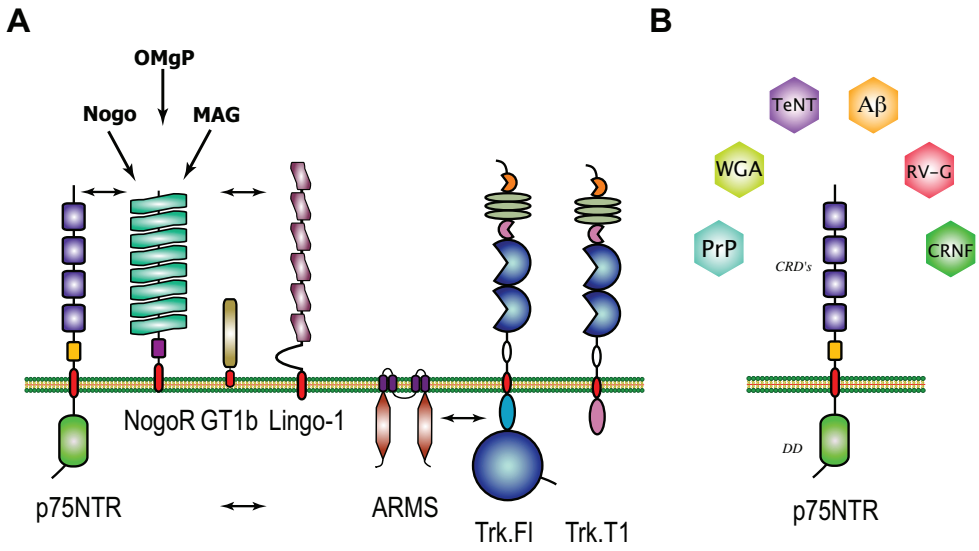


***Fig 1.4* Schematic representation of receptor interactors and p75NTR alternate ligands.** (**A**) Nogo receptor (NogoR), Gt1b and Lingo-1 are involved in inhibition of neurite outgrowth via CNS-derived myelin-based growth inhibitors (MBGIs), including myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgP). Ankyrin-rich membrane spanning protein (ARMS) interacts with p75NTR and Trk to distinguishing neurotrophin action from other growth factors. p75NTR may also interact with truncated forms of Trks.
(**B**) p75NTR also binds or associates with lectins, pathogens and toxins such as prion protein (PrP), wheat germ agglutinin (WGA) tetanus toxin (TeNT), amyloid peptide beta (Aβ), certain variants of rabies virus (RV-G), and cysteine-rich neurotrophic factor (CRNF).

1.D Neurotrophin Receptor Signaling

1.D.1 Tyrosine Kinase Mediated Signaling

The signaling pathways activated by neurotrophins through Trk receptors result in critical neuronal fates such as cell survival, differentiation, dentritic arborization, synapse formation, plasticity, axonal growth, and axonal guidance, and have been a topic of extensive reviews (Kaplan & Stephens, 1994; Huang & Reichardt, 2003; Arevalo & Wu, 2006; Schweigreiter, 2006), and only a brief overview is necessary for the purpose of this thesis.

Binding of neurotrophins to Trk receptors leads to dimerization and phosphorylation of the receptors, recruitment of various adaptors and enzymes, and activation of several signaling pathways (Summarized in Figure 1.5) (Bhattacharyya *et al.*, 1997; Senger & Campenot, 1997). Specific responses for neurotrophins are achieved through the unique combination of Trk receptor docking sites, recruitment of different cytoplasmic adaptors and enzymes, and regulated receptor trafficking (Whitmarsh & Davis, 2001). There are as many as ten tyrosine residues present in the Trk cytoplasmic domain (Stephens *et al.*, 1994). Several of these, including tyrosines Y490, Y670, Y674, Y675 and Y785 are phosphorylated in response to neurotrophins and serve as the major docking sites for binding of adaptor proteins and enzymes (Inagaki *et al.*, 1995; Zirrgiebel *et al.*, 1995).

The most well-defined signaling pathways activated by Trk receptors in response to neurotrophins are the Shc-Ras-MAPK, Rap-MAPK, PI3K-Akt, and PLCγ-PKC survival and differentiation pathways (Heumann, 1994; Huang & Reichardt, 2003), however new downstream effectors are continually being identified (Arevalo & Wu, 2006).

Transcriptional regulation of neuronal survival and differentiation occurs by four main cascades including transient or prolonged forms of extracellular signal-regulated kinases/mitogen activated protein kinases (ERK/MAPK) through

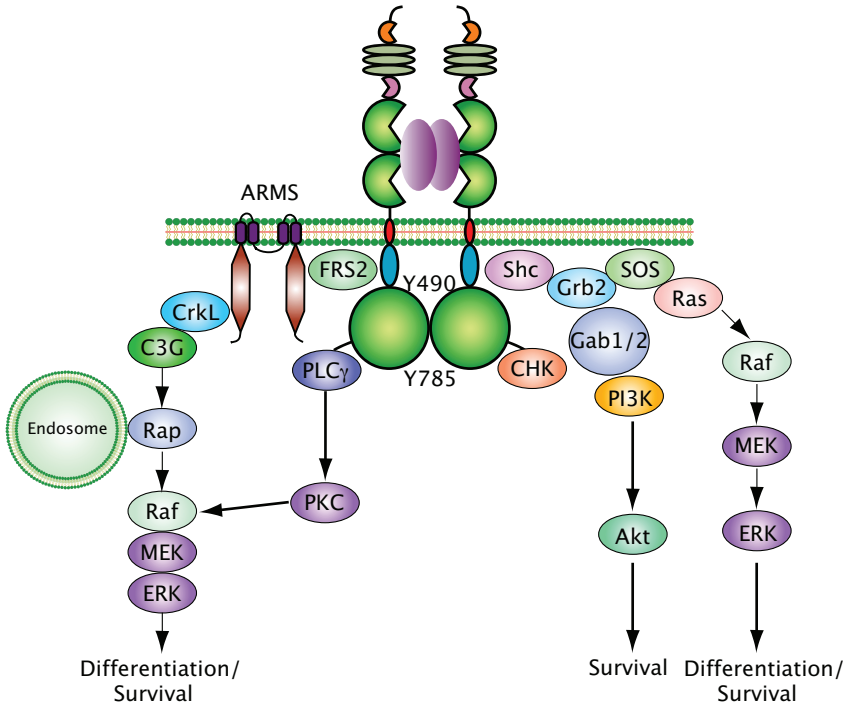


***Fig. 1.5* Trk receptor-mediated signaling pathways.** Neurotrophin binding to Trk receptors leads to their activation and to the recruitment of different proteins that associate with specific phosphotyrosine residues in the cytoplasmic domain of Trk receptors. Numerous tyrosine kinase interactions trigger the activation of signaling pathways, such as the Shc-Ras-MAPK, Rap-MAPK, PI3K-Akt, and PLCγ-PKC pathways, which result in survival, neurite outgrowth, gene expression, and synaptic plasticity. Some of these effectors are also associated with endosomal compartments for long range signaling.

the small GTPase Ras (Marshall, 1995). Transient activation engages via Shc-Grb2-GEF/SOS-Ras-B-Raf/Raf-1-MAPK protein activation (Kao *et al.*, 2001). Prolonged tyrosine kinase ERK/MAPK activation requires additional adaptors CrkII/CrkL, GEF-C3G and the GTPases Rap1/B-Raf (York *et al.*, 1998; Wu *et al.*, 2001a), but may also include interactions with fibroblast receptor substrate-2 adaptor (FRS2) (Kao *et al.*, 2001) and ankyrin repeat-rich membrane-spanning protein substrate (ARMS) (Kong *et al.*, 2001). In addition, sustained but not transient activation of ERK/MAPK pathways requires endosomal internalization of the Trk receptor (York *et al.*, 2000; Wu *et al.*, 2001a).

The third signaling cascade critical for the survival of both cell lines and neurons is the Phosphoinositide-3 kinase / serine/threonine protein kinase Akt (PI3K-Akt) pathway, which engages Shc in association with Grb2/Gab1 (Holgado-Madruga *et al.*, 1997; Atwal *et al.*, 2000; Encinas *et al.*, 1999). Moreover, Akt phosphorylation can also activate the NF-κB pro-survival response (Foehr *et al.*, 2000), or regulate axonal growth and branching through GSK-3β inactivation (Zhou *et al.*, 2004).

The fourth survival and differentiation response involves the activation of phospholipase C-gamma (PLCγ), which results in the generation of inositol tri-phosphate (IP3) and diacylglycerol (DAG), and triggers various protein kinase C (PKC) pathways (Vetter *et al.*, 1991; Corbit *et al.*, 1999).

PKC's in turn engage the already mentioned Raf-MEK-ERK1 survival and differentiation cascade (Corbit *et al.*, 1999). In addition, PLCγ signaling has been shown to also play an important role in BDNF mediated synaptic plasticity (Kang & Schuman, 1995). Investigations addressing the regulation of synaptic plasticity by neurotrophins have become a growing discipline, and have been recently reviewed in detail (Lu *et al.*, 2005).

Although the knowledge regarding Trk receptor signaling cascades is rapidly increasing, it needs to be highlighted that the majority of these pathways have been studied largely in the context of cell-line-derived NGF/TrkA signaling,

and subsequently extrapolated to the other Trk receptors. Further, many studies use transfection methods to insert Trk receptors of interest into cell lines. As a result of this technique, the receptor behavior may not represent the tangible outcomes associated with true physiological states. This means that many of the cell type specific details regarding Trk receptors and their isoforms remain to be addressed both *in vivo* and *in vitro*.

1.D.2 $p75^{NTR}$ Signaling

Unlike the well-defined trophic roles of the Trk receptors, $p75^{NTR}$ multi-functionality makes it extremely hard to formulate unified $p75^{NTR}$ signaling models. This high level of complexity is unavoidable considering variable effects ranging from trophism to programmed cell death, but further confusion stems from attempts to incorporate the fact that $p75^{NTR}$ functions are often integrated with other receptors and multiple ligands (Figure 1.4A-B), which mediate both synergistic and antagonistic interactions. As with Trk receptors, a summary of $p75^{NTR}$ signaling (Figure 1.6) is necessary to illustrate the complexity of signaling pathways.

As $p75^{NTR}$ was believed to be primarily involved in death signaling through protein interactors, its apoptotic pathways are the best-understood and described (Miller & Kaplan, 1998; 2001a; Roux & Barker, 2002; Blochl & Blochl, 2007). They can be triggered by neurotrophins, proneurotrophins and alternate $p75^{NTR}$ ligands (Schor, 2005). A core function of the p75 intracellular domain ($p75^{ICD}$) appears to be ceramide production (Brann *et al.*, 1999; Lievremont *et al.*, 1999), and ceramide is involved in almost all apoptotic mechanisms (Blochl & Blochl, 2007). Increased or high ceramide production upon $p75^{NTR}$ activation results in the upregulation of multiple apoptotic proteins (Roux & Barker, 2002).

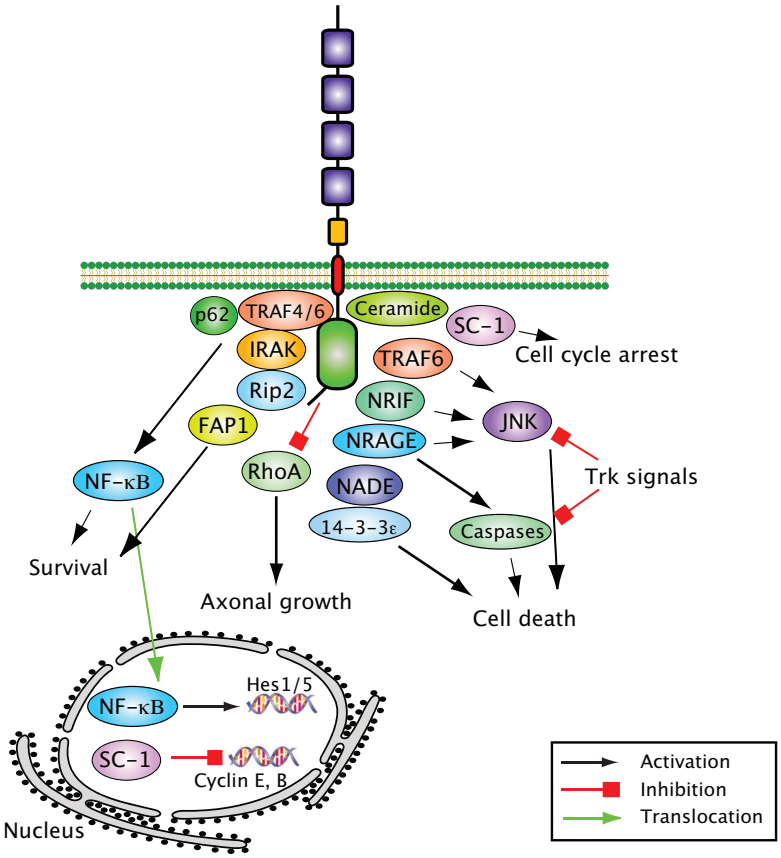


***Fig 1.6* p75NTR-mediated signaling pathways.** Binding of mature neurotrophins or pro-neurotrophins to p75NTR triggers the activation of multiple signaling pathways through different adaptors that result in diverse and at times opposite outcomes like survival, apoptosis, axonal growth, axonal collapse, and cell cycle arrest. Adapter proteins involved in p75NTR-mediated signaling include ceramide induced NRIF, NRAGE, TRAF6, NADE pathways converge on JNK and various caspases driving apoptosis. These effects can be blocked by Trk signals. SC-1 induces cell cycle arrest through nuclear regulation of cyclins E and B. Important survival signaling is transduced through TRAF4/6, p62, IRAK and RIP2 leading to NF-κB. p75NTR activation causes RhoA disassociation from the receptor leading to axonal growth. NF-κB translocation to the nucleus modulates dendritic growth via Hes1/5 expression.

The $p75^{NTR}$-associated cell death executor (NADE) (Mukai *et al.*, 2000), neurotrophin receptor interacting factor (NRIF) (Casademunt *et al.*, 1999), TNF receptor-associated factor (TRAF6) (Khursigara *et al.*, 1999), nuclear factor-κB (NF-κB), and neurotrophin receptor-interacting MAGE homologue (NRAGE) (Salehi *et al.*, 2000) have been identified as primary interactors associated with death signaling cascades. However, all apoptotic signaling pathways appear to converge on the activation of Jun kinase (JNK) (Blochl & Blochl, 2007), tumour suppressor protein p53 (Aloyz *et al.*, 1998), and several caspases (Kaplan & Miller, 2000; Roux & Barker, 2002). NRIF and NRAGE, in collaboration with the Schwann cell factor 1 (SC-1) are also involved in cell-cycle arrest, regulating levels of cyclins E and B upon NGF activation (Chittka *et al.*, 2004).

In contrast to high ceramide production resulting from continuous receptor activation, short pulse ceramide production activates PI3-K, which subsequently produces trophic effects (neurite extension and survival) through Akt or RhoA-Ras-Raf-Erk1/2 activation (Blochl & Blochl, 2007). However continued ceramide production in this pathway will negatively influence the Akt/Erk1/2 pathways, blocking trophic effects (Brann *et al.*, 2002). A pro-survival pathway associated with NGF binding to $p75^{NTR}$ utilizes NF-κB (Carter *et al.*, 1996), which influences gene transcription mediated through TRAF6, p62, interleukin-1 receptor-associated kinase (IRAK) and receptor-interacting protein-2 (RIP2) (Khursigara *et al.*, 1999).

In an alternate scenario NF-κB has the ability to translocate to the nucleus and trigger the expression of Hes1/5 to influence dentritic growth (Salama-Cohen *et al.*, 2005). Ligand binding can also initiate cleavage of $p75^{NTR}$, and the resulting $p75^{ICD}$ has also been reported in nuclear translocation (Frade, 2005), however the physiological relevance of this remains unclear. Like Trk receptors, $p75^{NTR}$ can also modulate synaptic transmission and plasticity in mature neurons (Rosch *et al.*, 2005; Zagrebelsky *et al.*, 2005).

1.D.3 Summary

Neurotrophins are a small family of growth factors including NGF, BDNF, NT-3 and NT-4, and exist in biologically active pro- and mature forms. They regulate critical and diverse aspects of developing and adult nervous system such as differentiation, survival, cell death, axonal elongation, myelination and synaptic plasticity. Neurotrophins cause these effects by binding to three distinct families of receptors, the tyrosine kinases TrkA, TrkB and TrkC, the TNF receptor family member $p75^{NTR}$, and the sortilin receptor. The signaling outcome of neurotrophins depends on several factors including cell type specific expression of their receptors (including numerous functional splice variants), and individual or compound interactions between the various receptors in multiple receptor ligand complexes. $P75^{NTR}$ plays a central role in attenuating survival signaling by increasing ligand specificity for Trk's, or partnering sortilin in apoptosis. Further, interactions with numerous other ligands and interactors determine $p75^{NTR}$ mediated cellular responses. An array of various cascades involved in neurotrophin signaling have been identified, providing opportunities to uncover the details of neurotrophin receptor functional biology, but the physiological relevance, and identity of compartments involved in Trk and $p75^{NTR}$ activity are emerging as key components in neurotrophin signal regulation and will be described in the next section. In addition, $p75^{NTR}$ internalization and processing is turning out to be increasingly fascinating, as it also appears to be exploited as an effective entry portal to many harmful molecules.

1.E Neuronal Membrane Dynamics

All neuronal processes depend on the fidelity of intracellular membrane transport. Lipids, proteins, receptor ligands and solute molecules are trafficked to distinct compartments within the cell through both the biosynthetic and endocytic pathways (Watson *et al.*, 2005). These pathways play a critical role in translating the external stimuli into biological responses through a high level of ordered compartmentalization into multiple intracellular structures possessing their own distinct identity and function (Ceresa & Schmid, 2000). Neurotrophins are some of the most crucial external stimuli producing signal transduction via ligand activated receptor internalization (Campenot & MacInnis, 2004). Over the last decade the role of neurotrophin receptor endocytosis has evolved from a mechanism merely responsible for the downregulation of signal transduction, to a critical component of neurotrophin signal distribution, delivery and spatio-temporal regulation (Heerssen & Segal, 2002). The following section will focus on the relationship between endocytosis and neurotrophin receptor trafficking.

1.E.1 General Overview Of Endocytosis.

Endocytosis occurs by multiple mechanisms that fall into two broad categories, 'phagocytosis' or "cell eating" (the uptake of large particles) and 'pinocytosis' or "cell drinking" (the uptake of fluid and solutes) (Rodman *et al.*, 1990). Phagocytosis is typically restricted to specialized mammalian cells, including macrophages, monocytes and neurophils, that function to clear large pathogens such as bacteria or yeast (Conner & Schmid, 2003), but macroautophagy is also constitutively active in healthy neurons and is vital to cell survival responsible for the turnover of organelles and long-lived proteins (Boland & Nixon, 2006). Pinocytosis is the primary mode of internalization for plasma membrane receptors and occurs in all cells by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae mediated endocytosis, and clathrin- and caveolae independent endocytosis (Perret *et al.*, 2005)

1.E.2 Macropinocytosis

Macropinocytosis, or fluid-phase uptake, can be measured by the intracellular accumulation of tracer molecules present in the medium (Cohen *et al.*, 2004). Macropinosomes (MPs) are large endocytic vesicles formed by signal based, actin-driven formation of membrane protrusions that fuse with the plasma membrane (Rodman *et al.*, 1990). They encapsulate large volumes of fluid and can be recycled back to the cell surface, or enter the degradative pathway and fuse with lysosomes (Lys) (D'Hondt *et al.*, 2000).

1.E.3 Clathrin Mediated Endocytosis

Clathrin mediated endocytosis (CME) is by far the most widely studied and best-understood mechanism of cellular uptake (Ceresa & Schmid, 2000). CME is often referred to as 'receptor mediated' endocytosis, but it is now becoming clear that this is a misnomer, as most pinocytic pathways have been demonstrated to involve specific receptor–ligand interactions (Cavalli *et al.*, 2001; Conner & Schmid, 2003). CME involves mechanisms where different receptors are internalized and trafficked through highly individualized endocytic mechanisms (Haucke, 2006).

CME involves the concentration of high-affinity transmembrane receptors and their bound ligands into 'coated pits' on the plasma membrane, which are formed by the sequential recruitment of accessory and recruitment proteins including AP-2, epsin, dynamin, actin and ARP2/3 (Conner & Schmid, 2003; Rappoport *et al.*, 2004), with the main assembly unit being the clathrin triskelions (Hirst & Robinson, 1998). Coated pits pinch off to form clathrin-coated vesicles (CCVs) that carry concentrated receptor–ligand complexes into the cell (Puthenveedu & von Zastrow, 2006). Following internalization the vesicles rapidly lose the clathrin coats and are sorted for fusion with early endosomes (EEs)/sorting endosomes (SEs), and/or multivesicular bodies (MVBs) (Mellman, 1996).

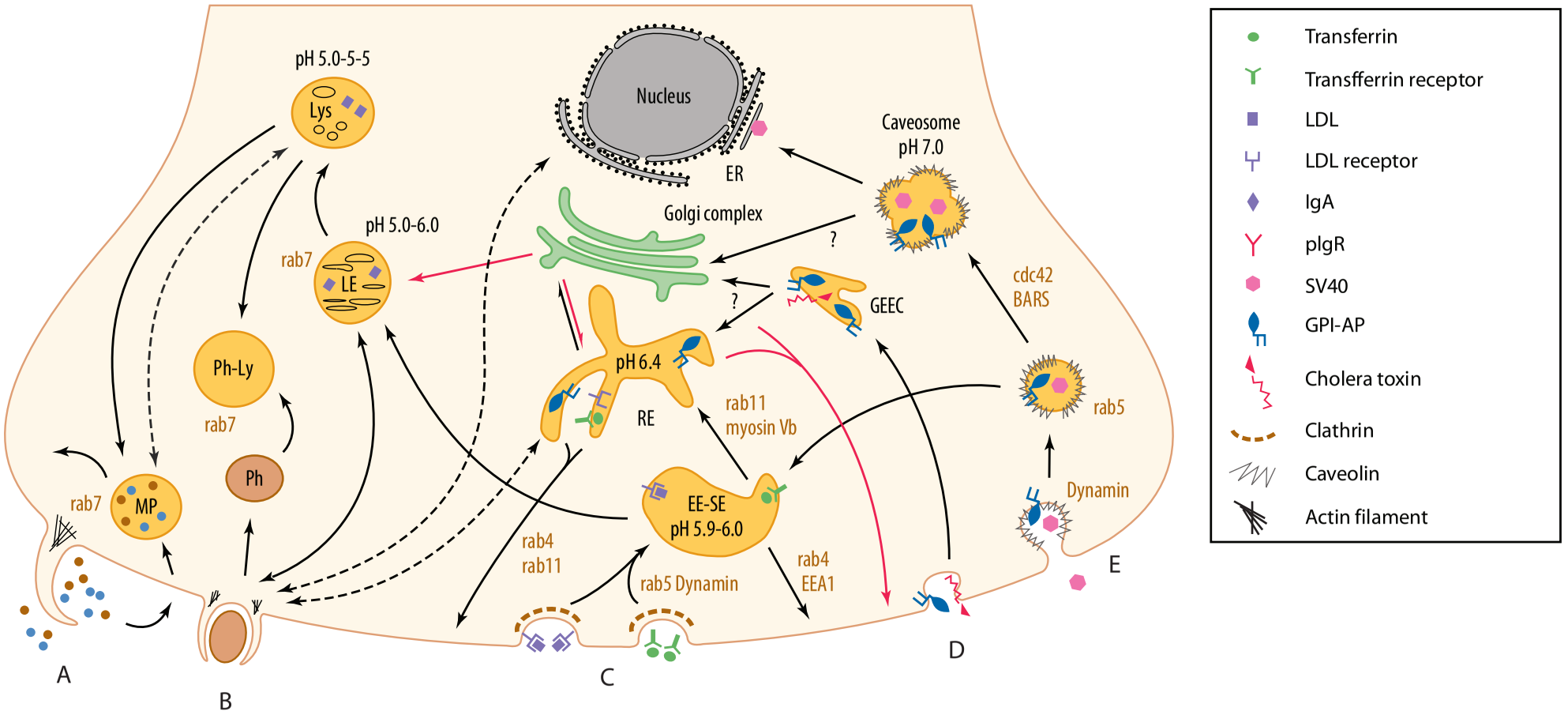

pH 5.0-5-5
Lys
Nucleus
ER
Caveosome
pH 7.0
Golgi complex
pH 5.0-6.0
rab7
LE
?
?
GEEC
cdc42
BARS
Ph-Ly
rab7
pH 6.4
RE
rab11
myosin Vb
rab5
Ph
rab7
MP
EE-SE
pH 5.9-6.0
Dynamin
rab4
rab11
rab5 Dynamin
rab4
EEA1
A
B
C
D
E
Transferrin
Transfferrin receptor
LDL
LDL receptor
IgA
pIgR
SV40
GPI-AP
Cholera toxin
Clathrin
Caveolin
Actin filament

Figure 1.7 **Biosynthetic and recycling routes and the endosomal network.** Various exo/endocytic routes and the endosomal network highlighting the intracellular itinerary of a typical ligand for each internalization route is depicted; some ligands such as cholera toxin (CTx) can use multiple routes (see text for details). Dashed two-headed arrows represent fusion between organelles. Biosynthetic traffic from the Golgi complex to the late endosome (LE), recycling endosome (RE) and the plasma membrane is shown by red arrows. Organellar pH, rab GTPases and other effectors necessary for organelle identity and sorting are also shown. (**A**) Macropinocytosis. Stimulation by growth factors or other signals causes the actin-driven formation of membrane protrusions that fuse with the plasma membrane to form large endocytic vesicles known as macropinosomes (MP) that encapsulate large volumes of the extracellular fluid. MPs can either fuse with lysosomes (Lys) or recycle back to the cell surface. (**B**) Phagocytosis. Signaling cascades trigger the rearrangement of the actin cytoskeleton and form membrane extensions that envelop the particle and engulf it. Membrane required for the formation of the phagocytic cup can be supplied by RE, LE and/or the ER. After internalization, the phagosome (Ph) fuses with lysosomes to form the phagolysosome (Ph-Ly) which requires hydrolytic enzymes to degrade its cargo. (**C**) Clathrin-mediated endocytosis. Receptors internalized into early endosomes/sorting endosomes (EE/SE) are segregated from their ligands due to the low pH (5.9) generated by vacuolar proton ATPases. Ligands and fluid are sorted into LE for subsequent degradation in lysosomes, while receptors (LDLR, TfR) are transferred to REs which lie deeper in the cell near the microtubule organizing center. REs are composed of tubules (60 nm diameter) and are characterized by a more neutral pH and a longer half-life than SEs. REs are also considered 'early' endosomes, as opposed to LEs, which are characterized by their commitment to a degradative route. Recycling from RE to the plasma membrane in non-polarized cells is under the control of rab4 and rab11. (**D**) Caveolae-mediated endocytosis. Glycosylphosphatidylinositol-anchored proteins (GPI-APs) and opportunistic ligands such as Siman virus 40 (SV40) and CTx can trigger the formation of caveolae, thus enhancing their uptake. These ligands are internalized into specialized vesicles known as caveosomes. From here, SV40 is transported to the endoplasmic reticulum (ER) while GPI-APs can traffic to the Golgi complex. SV40 can also be sorted into the EE/SE. (**E**) Clathrin- and caveolin-independent endocytosis. CTx and GPI-APs can be internalized via 40–50-nm raft-like structures into the GPI-anchored protein-enriched early endosomal compartment (GEEC), from where they are sorted to the Golgi complex (CTx) or to REs (GPI-APs). Mechanisms regulating these secondary endosomal sorting pathways are unclear. (adapted from: Perret et al., 2005)

EEs/SEs represent a dynamic array of tubules and vesicles distributed throughout the peripheral and perinuclear cytoplasm (Clague & Urbe, 2001).

Due to the slightly acidic pH (pH ~ 6.0 - 6.8) inside the EEs, many receptor-ligand complexes disassociate, and are subsequently sorted to establish the destination of the receptors and ligands (Von Bartheld, 2004). Receptors can be rapidly recycled back to the plasma membrane (Sheff *et al.*, 1999). The ligand may remain bound to the receptor and may be carried back to the surface membrane during recycling, or the ligand may dissociate from the receptor (Maxfield & McGraw, 2004). Thus, ligands and receptors can be targeted to different destinations. Different receptors may follow different fates, allowing for fine-tuned regulation of the internalization step and account for the sensitivity and specificity of the neuron's response to ligand exposure (Di Fiore & De Camilli, 2001). Free receptors selectively accumulate in early endosomal tubular extensions, which bud off to yield recycling endosomes (REs), which transport receptors, directly or indirectly to the plasma membrane (Maxfield & McGraw, 2004). Disassociated ligands collect in the vesicular compartments of EEs simply because of their high internal volume relative to the endosome tubular extensions. These vesicular structures subsequently pinch off, or are left behind following the budding of REs, and traverse to the perinuclear cytoplasm on microtubule tracks, and fuse with late endosomes (LEs), characterized by their commitment to lysosomal delivery (Piper & Luzio, 2001).

1.E.4 Caveolin Mediated Endocytosis

Caveolae are extremely abundant flask-shaped, sphingolipid-rich microdomain invaginations of the plasma membrane in which many diverse signaling molecules and membrane transporters are concentrated (Nichols, 2003). A dimeric cholesterol binding protein caveolin (van Meer & Sprong, 2004) confers the shape and structural organization of caveolae. In most cells, even after activation, caveolae are only slowly internalized (half-time, t 1/2, 20min) and the small vesicles (50–60 nm in diameter) carry little fluid-phase volume. However,

caveolae-mediated endocytosis seems to be highly regulated and driven by the cargo molecules themselves (Ikonen, 2001; Paratcha & Ibanez, 2002). The molecular basis for this link between cargo molecules, caveolae-localized receptors and triggered endocytosis remains to be elucidated.

1.E.5 Clathrin And Caveolin Independent Coated Pit Formation

Caveolae represent just one type of cholesterol-rich microdomain on the plasma membrane. Others, more generally referred to as 'rafts', are small structures, 40–50nm in diameter, which diffuse freely on the cell surface (Nichols & Lippincott-Schwartz, 2001). Their unique lipid composition provides a physical basis for specific sorting of membrane proteins and/or glycolipids based on their transmembrane regions. These small rafts can presumably be captured by, and internalized within, any endocytic vesicle (van Meer & Sprong, 2004).

1.E.6 Identification Of Endosomal Compartments

One of the distinguishing features of the endocytic pathway is the pH variation in different sub-compartments (Demaurex, 2002). Acidification increases progressively from plasma membrane to endocytic vesicles and early endosomes, through to multivesicular bodies, late endosomes, and ultimately lysosomes (Grabe & Oster, 2001). Conversely, the pH of the secretory pathway also becomes more acidic as the cargo travels from the ER and the Golgi network to towards the cell surface (Wu *et al.*, 2001b). Molecules entering cells through the endocytic pathway rapidly undergo a drop in pH from neutral (6.8), to pH 5.9 – 6.0 in the lumen of early endosomes / recycling vesicles (Watson *et al.*, 2005). Further reduction from pH 6.0 to 5.0 occurs through the transition from late endosomes to lysosomes (Watson *et al.*, 2005).

Regulation of the endocytic pathway pH is controlled by several factors, including vacuolar ATPase, chloride and sodium channels, NaK-ATPase, passive proton leaks and the buffering capacity of the organelle lumen (Grabe & Oster, 2001).

1.E.7 Dynamic Regulation Of Endocytosis

The dynamics of the endocytic network are regulated by small GTPases of the Rab family (Miaczynska & Zerial, 2002). Rab proteins are well recognized for their roles in vesicle fusion (Schimmoller *et al.*, 1998). However, recent evidence also supports their role in vesicle transport and motor protein recruitment (Jordens *et al.*, 2005). Although over 60 mammalian Rab proteins have been identified (Jordens *et al.*, 2005), only a small fraction of these have been implicated in neurotrophin receptor trafficking (Saxena *et al.*, 2005a; Deinhardt *et al.*, 2006). Rab5 regulates early endosomal sorting (Sonnichsen *et al.*, 2000); Rab4 and Rab11 regulate early and recycling transport (Cavalli *et al.*, 2001); and Rab7 is essential in transitions from EEs to LEs and through to lysosomes (Bucci *et al.*, 2000)(Figure 1.7). Perhaps not surprisingly Rab4, Rab5, Rab7 and Rab11 all represent GTPases that interact with cytoplasmic motor proteins (Jordens *et al.*, 2005).

1.E.8 Summary

Receptors activated at the plasma membrane can be internalized via several routes, each one with unique distinguishing membrane, adaptor and scaffolding protein composition. However all these internalization pathways converge on recycling, intracellular transport or degradation of cargo fates. The identity of the distinct endosomal compartments is dynamically regulated through vesicle acidification and specific regulatory protein association.

1.F Endocytosis And Signaling: An Evolving Partnership

The concept that the establishment and maintenance of target innervation in neurons depends on the coordinated effects of neurotrophins is supported by an array of data from morphological, biochemical and genetic studies, and the neurotrophic factor hypothesis represents one of the key tenets of neurobiology (Sofroniew *et al.*, 2001). It fails, however, to explain how neurotrophin signal transduction through multiple receptors is communicated across the distance separating the synapse and the cell body.

The retrograde transport of NGF was demonstrated decades ago, creating plausible theories and models for neurotrophin signaling, but receptor signaling was generally believed to occur on the plasma membrane only (Hendry, 1992). Observations that shortly after ligand activation, the majority of activated epidermal growth factor receptors (EGFR's), and their downstream signaling effectors were detected not on the plasma membrane, but on early endosomes (Di Guglielmo *et al.*, 1994), led to the deduction that signaling continues from these compartments after the internalization cascade (Baass *et al.*, 1995). Subsequent work with NGF in PC12 cells (Grimes *et al.*, 1996) was instrumental in the development of the "signaling endosome hypothesis".

It has become apparent in recent years that signaling processes depend not only on activation of a particular set of receptors and downstream secondary effectors, but also on the location and duration of the signal being transduced (Miaczynska *et al.*, 2004b). Furthermore, exciting new findings suggest that signaling machinery is able to achieve a high order of regulation by exploiting the compartmentalization and functional specialization of the endocytic pathway (Gonzalez-Gaitan & Stenmark, 2003). Although endosomal compartmentalization appears ideally suited for regulation of signaling events, alternative facets of long distance growth factor signaling events are also emerging (Howe & Mobley, 2005). The following section will describe the current views on endosomal neurotrophin signaling.

1.F.1 Receptor Internalization Mechanisms

The majority of the structural information regarding the internalization of neurotrophin receptors is derived from observations in PC12 cells and DRG neurons, and centers primarily on the interaction of NGF with the TrkA receptor (Grimes *et al.*, 1996; Grimes *et al.*, 1997; Beattie *et al.*, 2000; Howe *et al.*, 2001). So far, three distinct mechanisms of NGF induced TrkA internalization, have been reported (Figure 1.8A).

In PC12 cells NGF treatment results in the formation of dynamin-1, endophilin, β-adaptin and AP-2 dependent clathrin-coated pits situated close to the cell membrane (Grimes *et al.*, 1997; Beattie *et al.*, 2000; Howe *et al.*, 2001). In addition, NGF treatment increases the internalization of multiple membrane-bound and fluid phase markers of clathrin-mediated endocytosis (CME) (Beattie *et al.*, 2000). Ligand dependent $p75^{NTR}$ internalization also seems to depend heavily on CME (Bronfman *et al.*, 2003), but with slower internalization kinetics and different vesicular distribution to TrkA (Saxena *et al.*, 2004; Saxena *et al.*, 2005a; Saxena *et al.*, 2005b).

An alternate non-CME route for both Trks (Bilderback *et al.*, 1999; Suzuki *et al.*, 2004; Nagappan & Lu, 2005) and $p75^{NTR}$ (Higuchi *et al.*, 2003; Hibbert *et al.*, 2006; Gil *et al.*, 2007) involves internalization mediated by the structural protein caveolin-1 and lipid raft components, which is also dynamin-1 dependent (Nichols, 2003). Although Trks and $p75^{NTR}$ appear to have individual fates via caveolae, formation of a high-affinity binding complex between the two receptors relies on lipid raft localization (Ross *et al.*, 1998).

The third emerging route of internalization for both TrkA and TrkB but not $p75^{NTR}$ is a form of macropinocytosis mediated by a chaperone protein named pincher (Shao *et al.*, 2002; Valdez *et al.*, 2005). This process is independent of clathrin or caveolin assisted vesicle formation, and is initiated by plasma membrane ruffling (Shao *et al.*, 2002; Valdez *et al.*, 2005), possibly due to

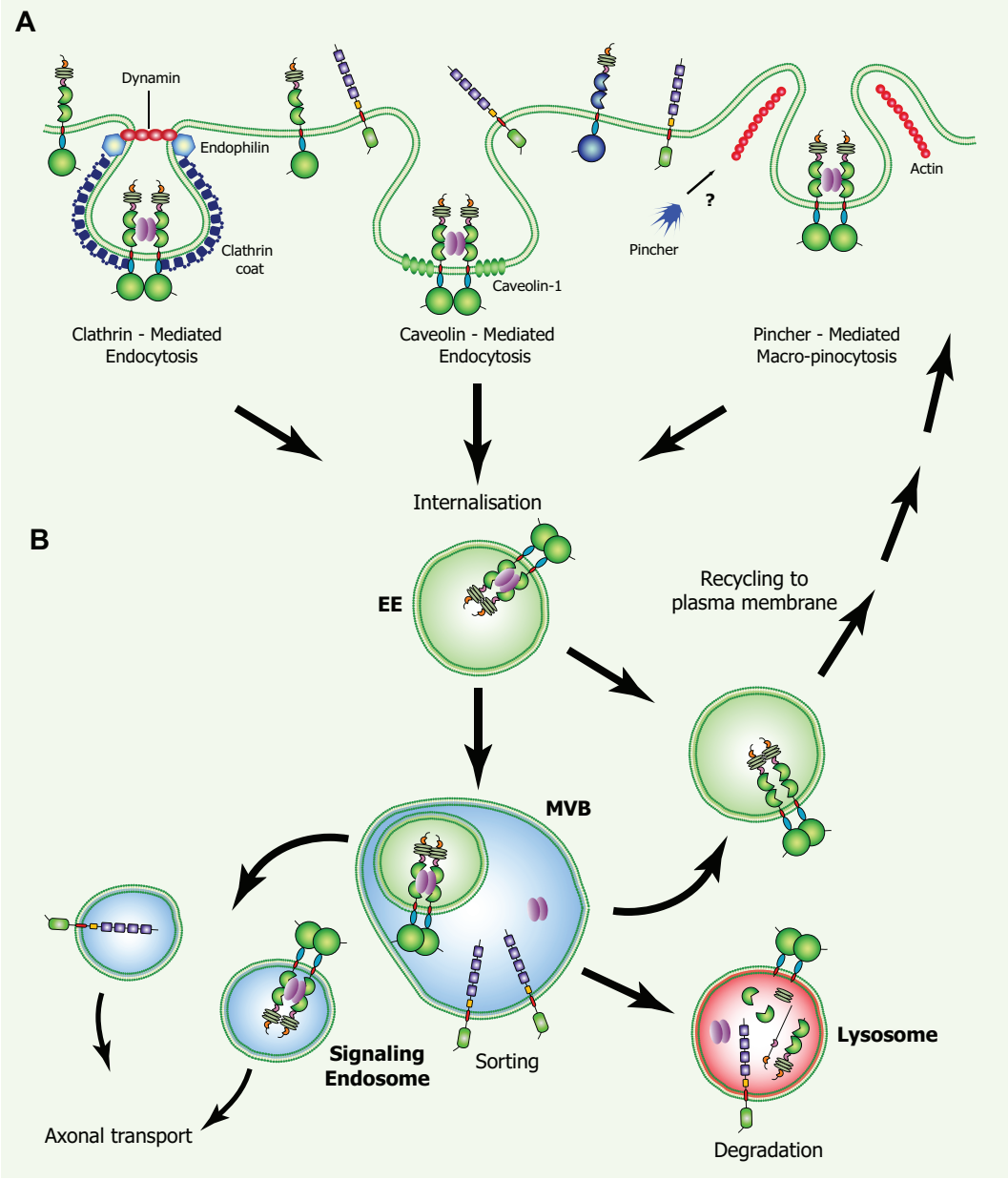


***Figure 1.8* Neurotrophin receptor internalisation.** (**A**) Neurotrophins and their receptors enter cells via clathrin dependent endocytosis and clathrin independent endocytic mechanisms, such as caveolin-mediated or Pincher-mediated endocytosis into small vesicles of poorly defined origin. These vesicles contain numerous markers of early endosomes, but differ in molecular composition depending on the receptor, ligand and cell population type. (**B**) Internalised receptors are sorted for different fates either from early endosomes (EEs) or multi-vesicular bodies (MVBs). They can be recycled back to the plasma membrane or undergo rapid degradation, possibly in proteasomes. Some are shuttled for retrograde axonal transport to the cell body where they are able to elicit their effect and be subsequently degraded in lysosomal compartments.

increased receptor aggregation (Nakamura *et al.*, 2002). Interestingly, pincher mediates internalization of activated and autophosphorylated TrkA and TrkB receptors independent of ligand activation (Valdez *et al.*, 2005). These observations are consistent with previously reported density-dependent Trk receptor activation (Hempstead *et al.*, 1992; Saragovi *et al.*, 1998), and Trk signaling in the absence of a ligand (MacInnis & Campenot, 2002; Heerssen *et al.*, 2004).

1.F.2 The Signaling Endosome Hypothesis

No consensus has been reached so far regarding the dominant mode of neurotrophin receptor internalization. What makes this issue so puzzling is that independent evidence for neurotrophin-signaling platforms has been provided for each of the three modes of internalization (Zweifel *et al.*, 2005). Internalized receptors can be sorted into various pathways (Figure 1.8B), which include recycling, retrograde transport or degradation (Dechant, 2001; Yano & Chao, 2004). Agreement has been reached, accepting that internalized neurotrophin-receptor pathways appear to converge on a population of poorly defined endocytic vesicles associated with various and specific signaling components (Howe & Mobley, 2004; Zweifel *et al.*, 2005; Bronfman *et al.*, 2007).

The "signaling endosome hypothesis" provides a mechanism for long distance communication between the synapse and the cell body (Howe & Mobley, 2004). It states that target derived NGF, bound to tyrosine phosphorylated TrkA at the pre-synaptic terminal, is internalized into membrane bound signaling organelles that are retrogradely transported along microtubules to the cell body. Moreover, it argues that delivery of tyrosine-phosphorylated TrkA to the soma activates effector-signaling molecules to evoke the changes in gene expression and metabolic activity required for survival and differentiation (Howe & Mobley, 2004) (Figure1.9).

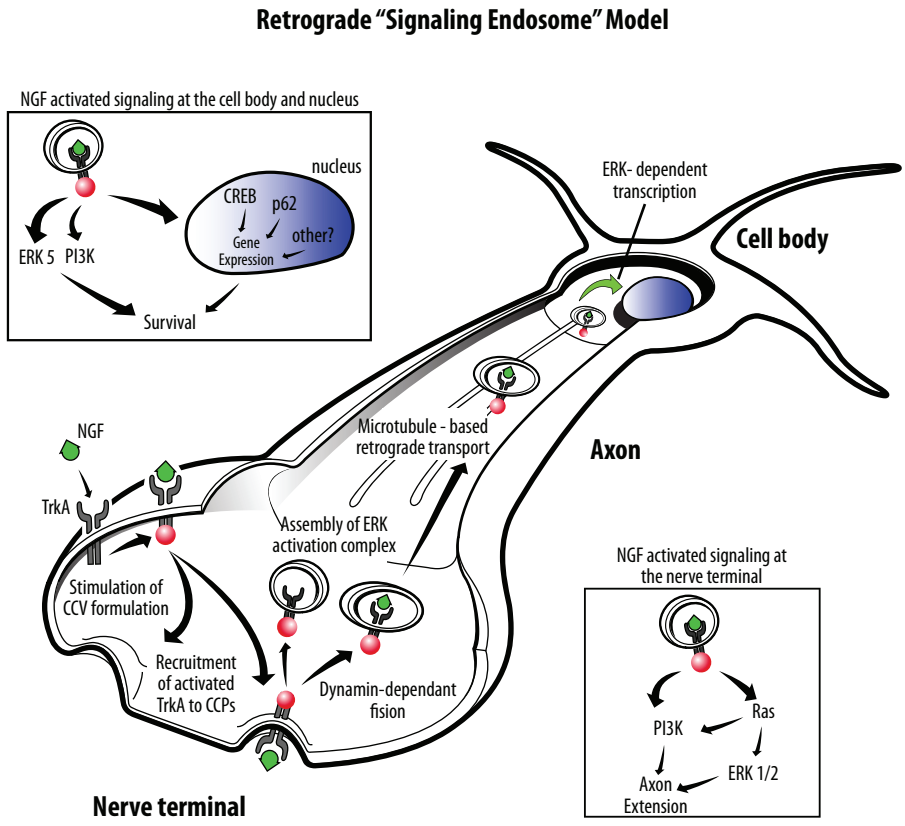


Figure 1.9 **The retrograde "signaling endosome" mechanism.** The main mode of retrograde neurotrophin signaling is through the retrograde transport of a membrane-enveloped ligand-receptor complex (clathrin coated pits, CCPs) with characteristics of early endosomes. Some of these endosomes become platforms for unique signaling effectors which are essential for activation of downstream signaling pathways and transport of retrograde neurotrophin signals. These "signaling endosomes" are retrogradely transported to the cell body with the assistance of dynein and microtubule dependent transport mechanisms. The vesicle-associated Trk receptors remains autophosphorylated (also possibly in the absence of the initially bound ligand) and capable of promoting unique sets of signaling cascades depending on whether their location is at the nerve terminal or cell body. Diverse signalling cascades (arrows in boxed diagrams) that are crucial for neurotrophin-dependent survival and axon growth are associated with nerve growth factor (NGF)-TrkA containing endosomes. These include MEK-(MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase and phosphatidylinositol 3-kinase (PI3K) signalling pathways. The mechanisms responsible for the nucleation of these complexes remain unresolved. Concepts adapted from (Barker et al. 2002)

Evidence supporting the signaling endosome hypothesis includes: accumulation of activated TrkA distal to a lesion site in sciatic nerves (Ehlers *et al.*, 1995; Bhattacharyya *et al.*, 1997), dynein motor based transport of NGF in sensory axons dependent on TrkA and PI3-K (Reynolds *et al.*, 1998), and the requirement of Trk internalization and kinase activity for retrogradely transmitted nuclear responses in compartmentalized sympathetic cultures (Riccio *et al.*, 1997; Watson *et al.*, 2001; Heerssen *et al.*, 2004). Moreover, NGF remains tightly associated with TrkA at an acidic pH generally responsible for receptor-ligand disassociation (Zapf-Colby & Olefsky, 1998), indicating these vesicles may have special properties different from regular endocytic carriers. Recent studies have begun to identify specific of the components of the signaling endosome (Howe *et al.*, 2001; Bronfman *et al.*, 2003; Valdez *et al.*, 2005). However, the identity of these vesicles remains elusive.

1.F.3 Signaling Endosome Components

The best characterized model for the study of signaling endosomes comes primarily from PC12 cell investigations of isolated NGF/TrkA (Grimes *et al.*, 1997; Howe *et al.*, 2001) or NGF/p75 complexes (Bronfman *et al.*, 2003). Trk activation induced signaling endosomes have been isolated from clathrin coated vesicles (CCV's), containing associated signaling effectors of the MAPK mediated neuronal survival pathway, and members of the Raf-MEK-Erk1/2 signaling pathway (Howe *et al.*, 2001). Remarkably, several of these components, including inactive Erk1/2, were present in CCV's in the absence of NGF (Howe *et al.*, 2001; Delcroix *et al.*, 2003; Valdez *et al.*, 2005).

Other constituents include members of the phospholipase C-γ (PLCγ) and phosphatidylinositol 3-kinase (PI3K) survival signaling pathways (Bartlett *et al.*, 1997; Reynolds *et al.*, 1998; Howe *et al.*, 2001; Watson *et al.*, 2001).

Direct evidence for the association of TrkA with the dynein light chain (Yano *et al.*, 2001), and microtubules (Delcroix *et al.*, 2003), suggests that retrograde transport of signaling endosomes requires dynein-dependent motor function and is necessary component of neurotrophin survival signaling (Figure 1.10).

The PC12 cell model has recently also provided some evidence to the existence of the $p75^{NTR}$ receptor in signaling endosomes, internalized via clathrin-coated pits (Bronfman *et al.*, 2003). These cellular interactors include the members of the MAGE family (Salehi *et al.*, 2000). Subcellular fractionation of PC12 cell early/recycling endosomes established that NGF stimulation increased the associated levels of the MAGE associated proteins Rab5, Necdin and NRAGE (Bronfman *et al.*, 2003). In addition it was demonstrated that $p75^{NTR}$ internalizes through the clathrin-mediated pathway to the recycling endosome but with kinetics distinct from NGF-TrkA containing vesicles (Bronfman *et al.*, 2003). Although further examination is required, experimental evidence so far supports the existence of spatially and temporally distinct vesicles that have the ability to propagate significantly different biological signals from neuronal terminals to cell bodies (Figure1.10).

1.F.4 Alternative Models Of Retrograde Signal Propagation

Despite evidence for the signaling endosome model, alternative models for growth factor propagation have been put forward in recent years. Once again the best evidence for these proposals comes from the study of NGF signal propagation (MacInnis & Campenot, 2002). However, none of these models should be considered mutually exclusive, and it makes good evolutionary and biological sense for a neuron to have multiple pathways for important events such as transmission of survival signals (Miller & Kaplan, 2001b).

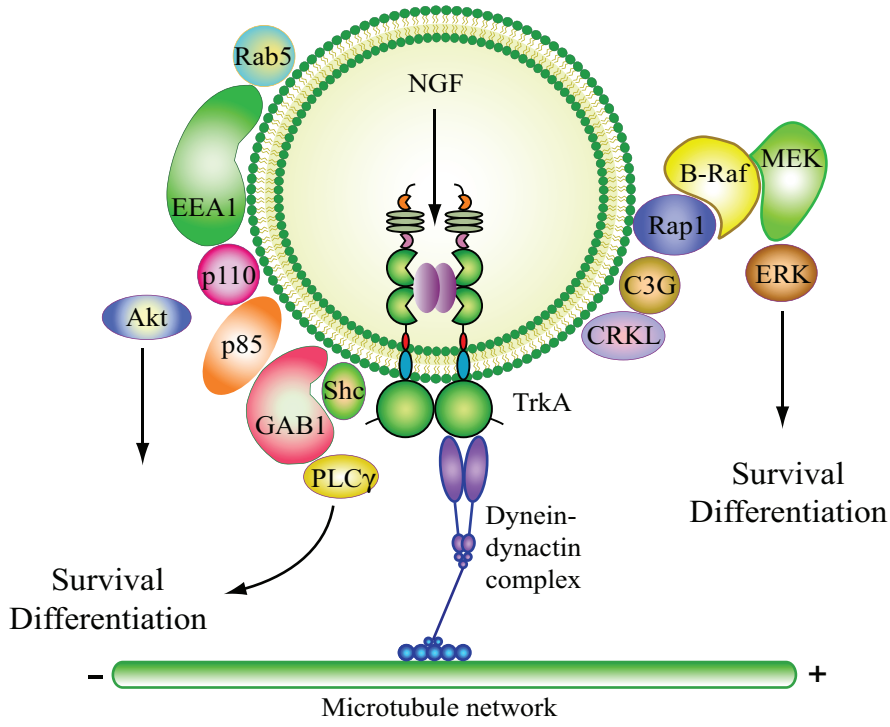


Figure 1.10 **The signalling endosome.** The main mode of retrograde neurotrophin signalling is through the retrograde transport of a membrane-enveloped ligand–receptor complex with characteristics of early endosomes. These signalling endosomes are platforms for unique signalling effectors such as the small G-proteins Rap1 and Rab5, which are essential for activation of downstream signalling pathways and transport of retrograde neurotrophin signals. Various signalling cascades (arrows) that are crucial for neurotrophin-dependent survival and axon growth are associated with nerve growth factor (NGF)–TrkA containing endosomes. These include constituents of the phospholipase C-γ (PLCγ), Raf (a serine/ threonine protein kinase)–MEK (MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase)–ERK and phosphatidylinositol 3-kinase (PI3K) signalling pathways. The mechanisms responsible for the nucleation of these complexes remain unresolved. Akt, v-akt murine thymoma viral oncogene homologue, also known as protein kinase B; ARMS, ankyrin-rich membrane-spanning protein; B-Raf, v-raf murine sarcoma viral oncogene homologue B1; CRKL, v-crk sarcoma virus CT10 oncogene homologue; C3G, Rap guanine nucleotide exchange factor (GEF) 1; EEA1, early endosome antigen 1; GAB1, GRB2 (growth factor receptor-bound protein 2)-associated binding protein 1; p85, regulatory subunit of phosphatidylinositol 3-kinase; p110, catalytic subunit of phosphatidylinositol 3-kinase; Shc, Src homology 2 domain-containing transforming protein C. Adapted from (Zweifel *et al*, 2005)

1.F.5 Unbounded Receptor Model

The unbounded receptor model is based on the modification of the signaling endosome model, but the receptors within the vesicles are not bound to a ligand (Ye *et al.*, 2003) (Figure 1.11A). The evidence for this concept comes from one of the more unusual properties of TrkA: its propensity to dimerize and auto-activate in the absence of NGF when its concentration in the plasma membrane reaches high enough levels (Hempstead *et al.*, 1992). Another unique property of TrkA, its sustained activity following NGF binding, might also support this model (Miller & Kaplan, 2001b).

In contrast to TrkA, other tyrosine kinases such as the EGF receptor are rapidly internalized and neutralized following EGF binding (Dikic, 2003). These properties suggest that TrkA is a receptor suited to be a retrograde signal carrier, even if NGF dissociates from the receptor during the process of, or after internalization (Miller & Kaplan, 2001b). However, the fact that these unique properties of TrkA have only been reported in PC12 cells, with over expressed TrkA receptors (Hempstead *et al.*, 1992), means that further experimental support of this model is needed, especially under conditions of native TrkA or other tyrosine kinase receptor expression.

1.F.6 Wave Propagation Model

The "wave propagation model" or "domino model" stipulates that a wave of activated TrkA on distal axons is propagated retrogradely along the plasma membrane of the cell, delivering the signal to its somatic destination independent of its ligand (Howe & Mobley, 2005). Support for this model of long distance retrograde signaling comes from the observed rapid kinetics of retrograde TrkA phosphorylation (Senger & Campenot, 1997). Other supporting evidence emerged from experiments using focal application of EGF to demonstrate a ligand-

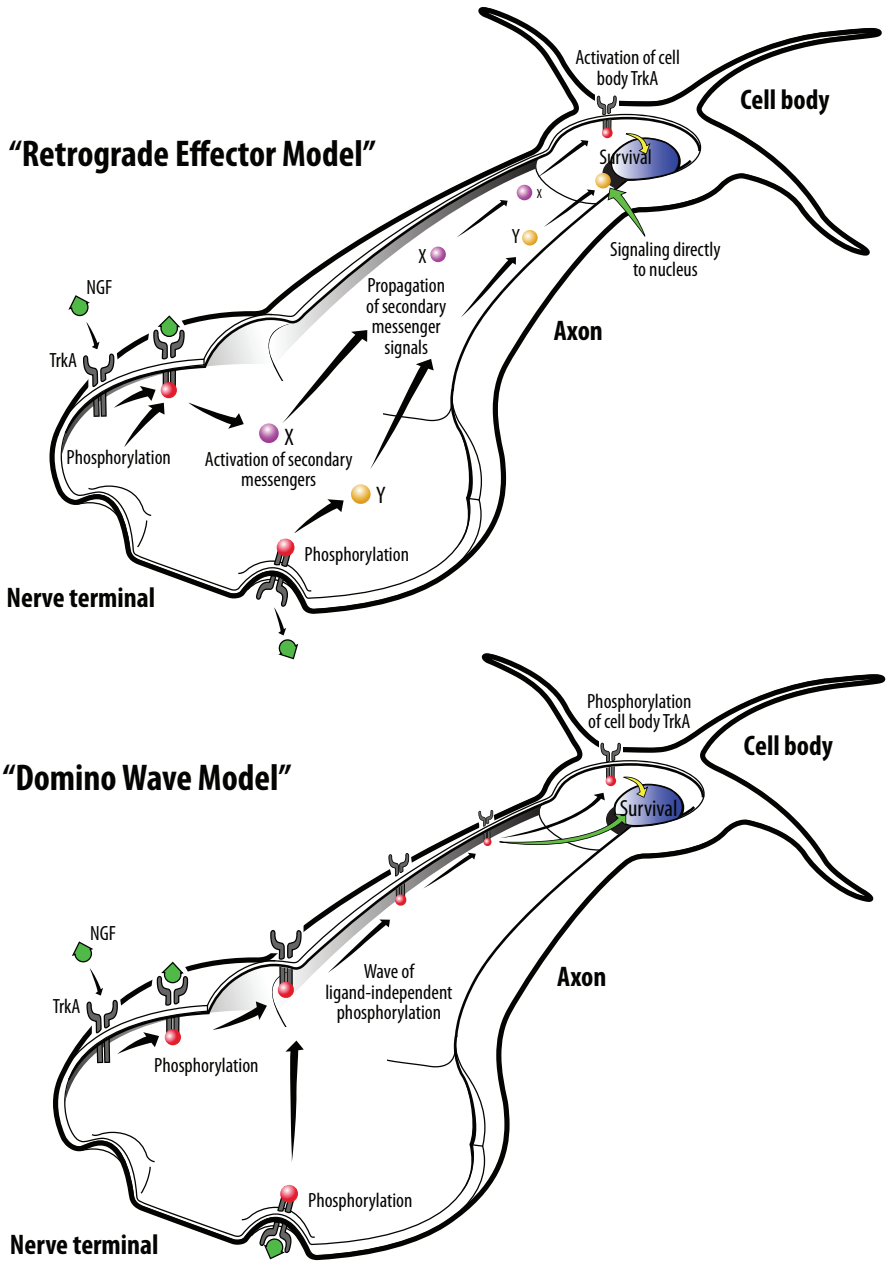


Figure 1.11 **Alternative models of retrograde neurotrophin signaling.** In the "retrograde effector model" local signals downstream from the Trk receptor ligand-dependent phosphorylation in the distal axons are propagated to the cell body where they support activation of other Trk receptors (**X**), or alternatively relay their signal directly to the nucleus to promote survival (**Y**). In the "domino wave model", neurotrophin binding to Trk receptors induces phosphorylation of the tyrosine kinase motifs which subsequently initiate a domino-like wave of ligand-independent Trk phosphorylation down the length of the axon. Upon reaching the cell body, this signaling cascade initiates phosphorylation of somatic Trk receptors that mediate cell survival. Alternatively, this wave of phosphorylation activates secondary messenger survival cascades directly. Concepts adapted from (Ginty et al, 2002).

independent wave of EGF receptor activation for several micrometers along the plasma membrane in transformed A431 cells (Verveer *et al.*, 2000).

As with the previous model, these findings suggest that the signaling endosome is not the only avenue available for the propagation of long distance signals, but further experimental evidence is required to determine whether lateral propagation of activated tyrosine kinases along the plasma membrane can truly account for long range (millimeters, centimeters or longer) signaling in neurons.

1.F.7 Signaling Effector Model

The possible existence of alternative retrograde signaling mechanisms not involving the transport of receptor ligand complexes came from experiments showing rapid NGF-induced retrograde tyrosine phosphorylation of TrkA at 1 minute, and other proteins at 15-30 minutes using [^{125}I] NGF beads, which are able to bind to TrkA but cannot internalize through its normal activation mechanism (Senger & Campenot, 1997). Further experiments demonstrating that this retrograde signal was not inhibited by tyrosine kinase inhibitor k252a at cell bodies, gave rise to the possibility that survival signaling was achieved without the TrkA-NGF complex, the retrograde transport of phosphorylated TrkA without NGF, or the serial propagation of plasma membrane phosphorylated TrkA (Campenot & MacInnis, 2004). This led to the signaling effector theory, which predicts that NGF binding to TrkA on the axonal membrane activates downstream survival proteins such as PI3-kinase, and that it's these downstream effectors that generate the retrograde signal (Miller & Kaplan, 2002). Interestingly, the essential PI 3-kinase-Atk survival-signaling pathway is activated both locally in axons and retrogradely in the soma by NGF (Miller & Kaplan, 2001b).

In contrast, the issue of spatial segregation has been highlighted, by NGF induced axonal-growth effector mitogen-activated protein (MAP) signaling (Watson *et al.*, 2001). A neurotrophin cocktail-induced activation at the distal

axon was able to activate MAP kinases ERK1 and ERK2 at the local axonal site (Watson *et al.*, 2001). However, an identical stimulus failed to activate ERK1 and ERK2 in the cell bodies, and instead caused the activation of another MAP kinase, ERK5 (Watson *et al.*, 2001). Not surprisingly, things get even more complicated from here. In the experiments using [^{125}I] NGF beads, MacInnis and Campenot demonstrated that NGF binding but not internalization, leads to PI 3-kinase-Atk pathway activation but not ERK1 and ERK2 (Miller & Kaplan, 2001b). This finding is similar to that reported when internalization of NGF is disrupted pharmacologically, or by blocking dynamin dependent endocytosis (Miller & Kaplan, 2001b).

These findings imply that secondary effector proteins are able to propagate a survival signal independent of receptor-ligand internalization, and that tyrosine kinase signaling substrates activated in this manner can differ, depending on the local or retrograde nature of this signal.

1.F.8 Summary

The study of retrograde signaling has leapt beyond the categorical principle that retrograde signals are carried to the cell body only in the form of receptor-ligand complex endosomes internalized at axon terminals. The described body of evidence divides NGF (and probably other growth factor) signaling into two very distinct mechanisms. One that requires the internalization of the ligand receptor complex which forms that basis of the signaling endosome hypothesis, and a number of possible mechanisms that are independent of ligand internalization.

Even with by far the most studied and experimentally supported model of signaling endosomes (Grimes *et al.*, 1996; Beattie *et al.*, 1996; Grimes *et al.*, 1997; Beattie *et al.*, 2000; Sandow *et al.*, 2000; Howe *et al.*, 2001; Delcroix *et al.*, 2003; Ye *et al.*, 2003), there is now enough evidence to support the existence of alternative models of retrograde growth factor signaling (Miller & Kaplan, 2002; Campenot & MacInnis, 2004). The functional significance of having multiple

retrograde signaling mechanisms is still being contested (Campenot & MacInnis, 2004; Von Bartheld, 2004; Howe & Mobley, 2005).

The concept of signaling endosomes is relevant and biologically important for a number of reasons. One obvious role for endocytosis in signaling is to provide temporal regulation (Miaczynska *et al.*, 2004b), as the duration of signaling is a crucial parameter in determining the biological output of a specific signaling cascade (Ginty & Segal, 2002). The duration of the signaling process also depends on the proportion of internalized receptors undergoing degradation, compared to those targeted for recycling back to the plasma membrane (Weible *et al.*, 2001).

All this evidence suggests the existence of numerous highly specific and regulated pathways in neurons designed to deliver signals crucial to the development and survival of the nervous system. The number of degenerative diseases that affect motor neurons suggests that the extremely long axons of these neurons may be particularly susceptible to defects in axonal transport (Hirokawa & Takemura, 2003). Given the evidence that neuronal retrograde transport is important for the survival and maintenance of neurons, it is not surprising that evidence is also emerging in support of failures in retrograde transport playing a crucial part in the degeneration of motor neurons (Hirokawa & Takemura, 2003).

1.G Motor Neuron Cell Line Clones in Research

1.G.1 Existing Motor Neuron Cell Lines

Clonal cell lines derived from specific neuronal populations with somatic cell fusion techniques have been widely used to identify and characterize properties specific to those neurons (Greene *et al.*, 1975; Heumann *et al.*, 1979; Hammond *et al.*, 1986; Crawford *et al.*, 1992; Lee *et al.*, 1990). A thorough database search of the literature, describing development and subsequent studies using immortalized cell lines with motor neuron-like characteristics reveals only four such clones in existence. Altogether, these amount to just over 60 peer reviewed publications, compared to well over 9600 published studies utilizing and

manipulating the PC12 cell, which has become the most commonly used neuronal cell line model system. Three of these hybrid cell lines have been produced through somatic fusion between the N18TG2 neuroblastoma and isolated embryonic spinal cord-enriched cells. These include, NSC-34 and NSC-19 (Cashman *et al.*, 1992), MN-1 (Salazar-Grueso *et al.*, 1991), and rat ventral spinal cord (VSC 4.1) motor neuron cells (Smith *et al.*, 1994). Other motor neuron-like cell lines, the human spinal cord cell lines (HSP1, HSP2 and HSP4) have also been reported in the literature (Li *et al.*, 2000). However, they were immortalized using a retroviral vector method.

1.G.2 Current Uses For Motor Neuron Cell Lines

Major motor neuron diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy remain high priority disorders due to their incessant progressive course and unfailingly fatal prognosis. Hence, it's no surprise that majority of studies using these motor neuron cell lines focus on areas of apoptotic cell death mechanisms and motor neuron disease patho-physiology (Durham *et al.*, 1993; Mosier *et al.*, 1995; Colom *et al.*, 1997; Turner *et al.*, 2003; Park *et al.*, 2004; Koh *et al.*, 2005; Wen *et al.*, 2006; Rizzardini *et al.*, 2006), as well as neuroprotection (Chapman & Chan, 1999; Mercer *et al.*, 2000; Keir *et al.*, 2001; Liu *et al.*, 2002; Rembach *et al.*, 2004; Das *et al.*, 2005; Zhai *et al.*, 2005; Weishaupt *et al.*, 2006).

Although the MN-1 and VSC 4.1 clones have been described as motoneuron-like, not a great deal of information has been forthcoming regarding more detailed physiological properties shared with motor neurons. Both of these clones express the cholinergic marker choline acetyltransferase (ChAT) (Salazar-Grueso *et al.*, 1991; Smith *et al.*, 1994), a property lacking in the parent neuroblastoma cell line (Amano *et al.*, 1974), and common with motor neurons. However the expression of ChAT in the spinal cord is not restricted solely to motor neurons (Phelps *et al.*, 1984), and should not be used as a definitive motor neuronal marker. Robust cell morphology including the extension of neurite-like processes has been partly used as a characteristic shared with the neuronal

phenotype, but offers little more in terms of further motor neuron-comparative biochemical analysis, such as acetyl choline (ACh) accumulation and release (Cashman *et al.*, 1992). Hence, in comparison with the NSC-34 cell line, relatively little is known about the biological similarities between MN-1 and VSC 4.1 cell lines and motor neurons.

Since its introduction, the NSC-34 cell line has become the best-characterized and most routinely used to investigate motor neuron biology and pathology, with relatively detailed biochemical characterization undertaken by the creators of the NSC clones (Hunter *et al.*, 1991; Cashman *et al.*, 1992; Durham *et al.*, 1993). To our knowledge, only the NSC-34 and NSC-19 clones originating from (Cashman *et al.*, 1992) and MN-1 cells (Salazar-Grueso *et al.*, 1991) have been briefly examined in relation to basic motor neuronal biology and behavior (Hunter *et al.*, 1991; Matsumoto *et al.*, 1995; Moscoso & Sanes, 1995; Usuki *et al.*, 2001), including studies describing the effects of growth factors (Trupp *et al.*, 1999; Usuki *et al.*, 2001; He *et al.*, 2002; Turner *et al.*, 2004).

1.G.3 NSC-34 Motor Neuronal Characteristics

Murine neuroblastoma × spinal cord (NSC) hybrid cell lines were developed as an inexhaustible resource available for the study of motor neuron biology (Cashman *et al.*, 1992). The availability of a motor neuron cell line could surmount many of the difficulties inherent in the use of motor neurons derived from primary tissues including low yield, limited purity of motor neuron preparations and poor survival rates (Fischbach & Dichter, 1974; Kato *et al.*, 1985; O'Brien & Fischbach, 1986). Of the 30 NSC hybrids produced only two, NSC-19 and NSC-34, expressed properties expected of motor neurons (Kato *et al.*, 1985). Only NSC-34 cells however, possessed further characteristics similar to those occurring in neuronal development (Cashman *et al.*, 1992).

All NSC hybrid cell lines were produced through somatic fusion between the N18TG2 aminopterin-sensitive neuroblastoma and motor neuron enriched embryonic day 12-14 spinal cord cells (Cashman *et al.*, 1992). NSC-34 express

many of the morphological and physiological properties of motor neurons including: extension of processes, synthesis and storage of ACh, support of action potentials, induction of myotube twitching, formation of contacts with cultured myotubes, and expression of neurofilament proteins (Cashman *et al.*, 1992). In addition NSC-34 cells are also able to induce ACh receptor (AChR) clustering upon co-culture with myotubes, suggesting that these cells might be able to model aspects of early neuromuscular synapse formation (Martinou *et al.*, 1991). It has also been shown that NSC-34 cells adhere specifically to the leucine-arginine-glutamate (LRE) motif of S-laminin, a neuromuscular synapse specific basal lamina glycoprotein (Hunter *et al.*, 1991).

Since the availability of the NSC-34 cell line a multitude of other neuronal characteristics have been reported (summarized in Table 1). Among these are findings reporting the expression of numerous receptors to motor neuron growth factors including: CNTF (Usuki *et al.*, 2001), responsiveness to NGF, BDNF, and NT-4 (Turner *et al.*, 2004), as well as tumour necrosis factor-alpha receptor (TNF-αR) (He *et al.*, 2002). Further studies have used NSC-34 cells in development of a glutamate-sensitive motor neuron cell line (Eggett *et al.*, 2000; Rembach *et al.*, 2004) and a novel NSC-34tTA^{40} cell line that permits tetracycline-regulated gene expression (Babetto *et al.*, 2005), as well as in detailed characterization of their membrane ganglioside profile (Matsumoto *et al.*, 1995).

1.G.4 Morphological Comparison Between NSC-34 Cells and Primary Motor Neuron Cultures

Spinal cord motor neurons have been studied in various forms of dissociated spinal cord preparations (Sobkowicz *et al.*, 1968; Peacock *et al.*, 1973; Berg & Fischbach, 1978; Phelps *et al.*, 1984; Lombard-Golly *et al.*, 1990) and purified motor neuron populations (O'Brien & Fischbach, 1986; Krieger & Kim, 1991; Citron *et al.*, 1997; Anderson *et al.*, 2004). Morphologically, motor neurons are typically identified as having a large soma harboring multiple dentritic arborizations and a long axon, usually identifiable by its relatively constant diameter (Figure 1.12A) (Deinhardt & Schiavo, 2005). However dissociated spinal cord cultures or pure isolated motor neuron cultures generally contain a range of large bipolar and multi-polar cell types with robust neurites (Figure 1.12 B and C) (Fischbach & Dichter, 1974; O'Brien & Fischbach, 1986). In the case of dissociated spinal cord cultures, these also contain other neuronal types and non-neuronal cells (Peacock *et al.*, 1973; Berg & Fischbach, 1978; Digby *et al.*, 1985).

An overwhelming majority of differentiated NSC-34 cells resemble cultures of spinal cord neurons and purified motor neurons (Figure 1.12C). These are typically large cells with multi-polar neurite projections, although a small percentage of the cell population exhibits a flat fibroblast-like morphology. However, these flat fibroblast-like cells also sometimes possess neurite like projections.

In summary, NSC-34 cell represent to date the best-characterized and most extensively studied immortal motor neuron-like cells. Their expanding characterization of many motor neuron properties, and close morphological resemblance to primary motor-neuronal cultures makes them a superior candidate for investigating various aspects of motor neuron biology. Nevertheless, due to the very different neural crest lineage of the NSC-34 cells, it would be of future interest to examine a number of other neuronal and non-neuronal characteristics compared to (1) neural crest-like cell lines that do not

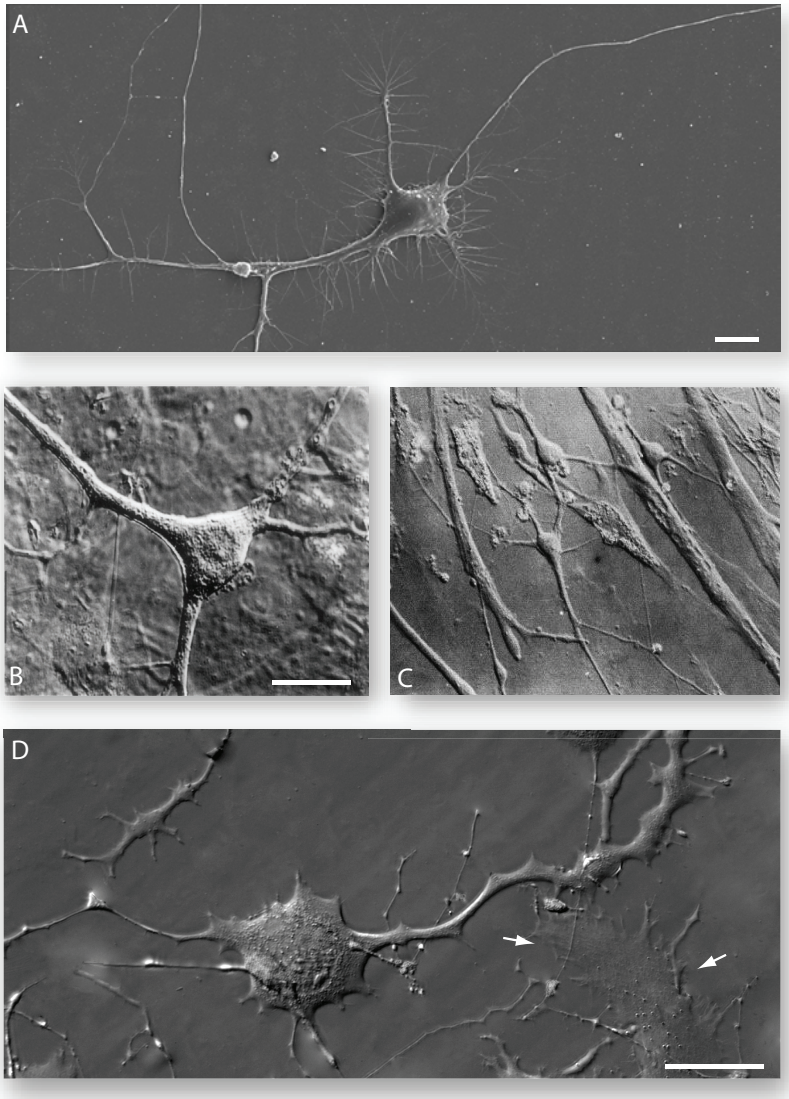


Figure 1.12 **Morphological comparison between cultured primary embryonic motor neurons and NSC-34 cells. (A)** Scanning electron micrograph of a cultured spinal cord motor neuron. Motor neurons typically have a large cell body, multiple dendritic arborizations and a single long axon with a relatively constant diameter, scale bar = 10 μm (figure adapted from : *Deinhardt and Schiavo, 2005*). Interference contrast micrographs of **(B)** dissociated embryonic spinal cord cell in culture; scale bar = 25 μm (adapted from fig.12a in: *Fischbach & Dichter, 1974*), and **(C)** 7-day old sorted embryonic motor neurons (taken from Fig.6 in: *O'Brian & Fischbach, 1986*). **(D)** Relief contrast micrograph of a differentiated NSC-34 cell cultured for 5 days. In a differentiated population there are neuronal type cells as well as cells resembling fibroblasts (arrows). When differentiated, the motor neuron-like cells posses a large soma and multible neuronal-like arborizations and projections, resembling motor neurons, scale bar = 25 μm.

Table 1 - Neuronal Properties and Markers of NSC-34 cell line

	N18TG2	NSC-34	NSC-34$_D$	Method	Reference
Cholinergic Markers					
ACh release	–	+	+	[^{14}C] Choline	(Cashman *et al.*, 1992)
ChAT	–	+	+	Fonnum Assay	(Cashman *et al.*, 1992)
nAChR	–	+	+	IHC	(Rembach *et al.*, 2004)
Growth Factor Receptors					
Trk A	ND	–	–	Western Blot	(Turner *et al.*, 2004)
Trk B	–	+	+	Western Blot	(Turner *et al.*, 2004)
p-75	–	+	+	Western Blot	(Turner *et al.*, 2004)
NRH2	–	+	+	Western Blot	(Turner *et al.*, 2004)
CNTF-r	–	+	+	IHF	(Usuki *et al.*, 2001)
Lif-R	–	ND	ND	N/A	(Usuki *et al.*, 2001)
Gp-130	–	+	ND	IHF	(Usuki *et al.*, 2001)
TNF-αR	ND	+	+	Viability Assay	(He *et al.*, 2002)
Cytoskeletal Proteins					
NF-68	–	+	+	IHC	(Cashman *et al.*, 1992)
NF-150	–	+	+	IHC	(Cashman *et al.*, 1992)
NF-200	–	+	+	IHC	(Cashman *et al.*, 1992)
MAP-Ia	–	+	+	IHC	(Cashman *et al.*, 1992)
MAP-2	–	–	–	IHC	(Cashman *et al.*, 1992)
GFAP	–	–	–	IHC	(Cashman *et al.*, 1992)
Vimentin	–	+	–	IHC	(Cashman *et al.*, 1992)
β-tubulin III	ND	+	+	Western Blot	(Rembach *et al.*, 2004)
Gangliosides					
GM-1	+	+	+	CT IHC	(Matsumoto *et al.*, 1995)
GM-2	+	+	+	IHC	(Matsumoto *et al.*, 1995)
GM-3	+	ND	ND	IHC	(Matsumoto *et al.*, 1995)
GD1a	+	+	+	IHC	(Matsumoto *et al.*, 1995)
GD1b	ND	ND	ND	Western Blot	(Matsumoto *et al.*, 1995)
Glutamate Receptor Proteins					
NMDAR1	–	–	+	IHC	(Eggett *et al.*, 2000)
NMDAR2a/b	–	–	+	IHC	(Eggett *et al.*, 2000)
GluR1	–	–	+	IHC	(Eggett *et al.*, 2000)
GluR2/3	–	–	+	IHC	(Eggett *et al.*, 2000)
GluR2	–	–	+	IHC	(Eggett *et al.*, 2000)
GluR4	–	–	+	IHC	(Eggett *et al.*, 2000)
KA2	–	–	+	IHC	(Eggett *et al.*, 2000)

Table 1 cont.- Neuronal Properties and Markers of NSC-34 cell line

	N18TG2	NSC-34	NSC-34$_D$	Method	Reference
Cell Adhesion Molecules (CAMs)					
L1	+	+	ND	mRNA/PCR	(Moscoso & Sanes, 1995)
Nr-CAM/Bravo	–	+	ND	mRNA/PCR	(Moscoso & Sanes, 1995)
Neurofascin/ABGP	+	+	ND	mRNA/PCR	(Moscoso & Sanes, 1995)
N-CAM	–	+	ND	mRNA/PCR	(Moscoso & Sanes, 1995)
Neuronal Nuclear Markers					
NeuN	ND	+	+	Western Blot	(Rembach *et al*., 2004)
Other Neuronal Properties					
Process extension	–	+	+	Phase Contrast	(Cashman *et al*., 1992)
Myotube twitch induction	–	+	+	dbcAMP add.	(Cashman *et al*., 1992)
Action potential generation	–	+	+	Electrical stim.	(Cashman *et al*., 1992)
AchR clusters on myotubes	–	+	ND	Co-culture	(Cashman *et al*., 1992)

Table legend: ND – Not Determined; N18TG2 – parent neuroblastoma, NSC-34$_D$ – Differentiated NSC-34 cells; IF – Immunofluorescence; IHC – Immunohistochemistry; CT – Cholera toxin; ES – Electrical Stimulation; PCM – Phase Contrast Microscopy.

differentiate in serum; (2) progenitor cell lines, some of which can partially differentiate in culture; and (3) mature neuronal cell lines or bipotential cell lines of murine origin.

1.H Concluding Remarks

Neurotrophins are essential regulators of neuronal development, which mediate their effects through multiple ligand-receptor complexes, and an array of downstream signaling effectors. Advances in understanding the nature and regulation of endocytic mechanisms have established this process as a suitable candidate for spatio-temporal regulation of complex signaling events. Subsequently, the emergence of structural details regarding multiple modes of ligand-dependent receptor internalization, and downstream signaling, have been instrumental in beginning to unravel the diverse and highly regulated mechanisms of neurotrophin signal transduction.

The use of PC12 cells and compartmentalized sympathetic neuron cultures have proven extremely valuable in these studies. However, neurotrophin actions can be highly cell type specific due to differential receptor expression and ligand availability. Neurotrophins are as vital to motor neurons as any other neuronal population, and motor neuron diseases have been recently linked with defects in neurotrophin signaling and transport. Unfortunately a reliable motor neuron model for the study of neurotrophin biology has not yet been established.

The NSC-34 cell line appears as the most suitable candidate for such a model. The increasing use of NSC-34 cells also means that they represent best-characterized motor neuron cell line available. Although they have been used extensively in studies of ALS associated apoptosis mechanisms, very little is known about their suitability for the study of neurotrophin biology, and receptor trafficking in particular. The following chapters describe the characterization and use of the NSC-34 cell line for the study of neurotrophin receptor trafficking.

Chapter 2: Materials And Methods

2.A Materials

2.A.1 Cell culture, media supplements and growth factors

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, F-12 Kaighn's modification medium, RPMI 1640 medium, fetal bovine serum (FBS), horse serum (heat inactivated), nonessential amino acids (NEAA), L-glutamine, penicillin-streptomycin-glutamine (PSG), HEPES buffer, N-2 Supplement and Trypsin-EDTA solution were obtained from Invitrogen Life Technologies (Mulgrave, Australia). Rat-tail collagen, poly-l-lysine (70,000MW), all trans-retinoic acid (RA), bovine serum albumin (BSA) and N-1 supplement were purchased from Sigma (Castle Hill, Australia). Purified mouse nerve growth factor (NGF) was a kind gift from Prof. Ian Hendry (ANU, Australia). Recombinant human BDNF, NT-3 and NT-4 were purchased from Chemicon (Boronia, Australia). Purified S-laminin was a gift from Prof. Salvatore Carbonetto (CRN, McGill University, Canada). Hybridoma fusion and cloning supplement (HFCS) was from Roche Diagnostics GmbH (Mannheim, Germany).

2.A.2 Tissue culture plastic and glassware

Tissue culture plastic-ware was purchased from Interpath Services (West Heidelberg, Australia) and Sardstedt (Adelaide, Australia). Glass coverslips, 12mm and 42mm in diameter and 0.17mm thickness were sourced from Stennick Scientific (Adelaide, Australia).

2.A.3 Fluorescent dyes and compounds

All Alexa-Fluor Succinmidyl ester dyes (488, 568, 594, 647), DAPI, Hoechst 33258, LysoSensor Yellow/Blue-Dextran (MW ~10,000), LysoSensor Green DND-189, Lysotracker Red DN-99, and transferrin 555 conjugates, were purchased from Invitrogen Life Technologies (Mulgrave, Australia). Fluorescein Isothiocyanate (FITC) isomer I, annexin-V-FITC apoptosis kit and propidium iodide (PI) were sourced from Sigma (Castle Hill, Australia). The C-fragment of tetanus toxin-FITC conjugate was provided by List Biological Labs (California,

USA).

2.A.4 Primary and secondary antibodies

Primary antibodies listed in (Table 2.1 -2.2) were obtained from various sources. MLR-1, MLR-2 and MLR-3 antibodies against mouse $p75^{NTR}$ (Rogers *et al*, 2006) were kindly supplied by Mary-Louise Rogers (Flinders University, Australia). Polyclonal antibodies 9560 and 9992 against the extracellular and cytoplasmic domains of $p75^{NTR}$ respectively, and phospo-TrkB (pTrkB-18664) rabbit polyclonal were kindly supplied by Prof. Moses Chao (NYU, New York, USA). Vesicular acetylcholine transporter (VAChT) antibody (V-007) was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). GT1b hybridoma clones were a kind gift from Prof. Ronald Schnaar (John Hopkins University, Baltimore, USA). The sortilin and neurofilament 200 (NF-200) polyclonal antibodies were purchased from Sapphire Biosciences (Redfern, Australia). Antibodies against clathrin-HC, AP-1 and AP-2 were provided by Antibody Technology Australia Pty. Ltd. (Adelaide, Australia). Monoclonal antibodies against EEA-1, Limp-II and Lamp-1 were produced by Dr. D.A. Brooks, Department of Chemical Pathology, Women's and Children's Hospital (Adelaide, Australia).

All secondary detection antibodies are listed in Table 3 and 4. Secondary detection antibodies conjugated with Cy-fluorophores, horseradish peroxidase (HRP) or alkaline phosphatase, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Secondary antibody Alexa-Fluor conjugates were obtained from Invitrogen Life Technologies (Mulgrave, Australia). Quantum dot 525 and 655 conjugated secondary [$F(ab)_2$ –fragments] antibodies were supplied by Chemicon (Boronia, Australia). All secondary detection antibodies purchased, were cross-absorbed against all other species to minimize non-specific binding in multiple-labeling experiments. All other antibodies were produced in our laboratory.

Table 2.1 - List of primary antibodies and working dilutions.

Name	Antigen	Host Species	Form	Dilution	Source
MC192	*rat p75^{NTR}*	Mouse	AP	FACS 1: 200	Flinders
				WB N/A	University
				IF 1: 1000	
MLR-1	*(rh) p75^{NTR}*	Mouse	AP	FACS 1: 200	Flinders
				WB N/A	University
				IF 1: 1000	
MLR-2	*(rh) p75^{NTR}*	Mouse	AP	FACS 1: 200	Flinders
				WB N/A	University
				IF 1: 1000	
MLR-3	*(rh) p75^{NTR}*	Mouse	AP	FACS 1: 200	Flinders
				WB N/A	University
				IF 1: 1000	
027RB-SW0	*(nr)TrkB*	Rabbit	WS	FACS 1:200	Flinders
(TrkB)				WB 1:500	University
				IF 1:1000	
TrkA	*(nr)TrkA*	Rabbit	WS	FACS 1:200	Chemicon
				WB 1:500	
				IF 1:1000	
Ab07-266	*(r)TrkC*	Goat	AP	FACS 1:200	Chemicon
(TrkC)				WB 1:500	
				IF 1:1000	
MabGT1b-1	*(rh)GT1b*	Mouse	AP	FACS 1:200	JHU
				WB 1:500	
				IF 1:1000	
MabGT1b-2a	*(rh)GT1b*	Mouse	AP	FACS 1:200	JHU
				WB 1:500	
				IF 1:1000	
AB312	*(nr)GQ1b*	Mouse	AP	FACS 1:200	Chemicon
(GQ1b)				WB 1:500	
				IF 1:1000	
H-V007	*(r)VAChT*	Goat	AP	FACS 1:200	Phoenix
(VAChT)				WB 1:500	Pharm.
				IF 1:1000	
Ab-31544	*(r)VAChT*	Sheep	AP	FACS 1:200	Abcam
(VAChT)				WB 1:500	
				IF 1:1000	

Table 2.2 - List of primary antibodies and working dilutions.

Name	Antigen	Host Species	Form	Dilution	Source
CB11R	*(nr) Clathrin*	Rabbit	WS	FACS N/A	Antibody
				WB 1:500	Technology
				IF 1:1000	Australia
CB21R	*(nr)AP1-*	Rabbit	WS	FACS N/A	Antibody
	Subunit			WB 1:500	Technology
				IF 1:1000	Australia
CBR31R	*(nr)AP1/2-*	Rabbit	WS	FACS N/A	Antibody
	Subunit			WB 1:500	Technology
				IF 1:1000	Australia
AB4680	*(nm) 200kDa*	Chicken	IgG	FACS N/A	Abcam
(NF-200)	*Neurofilament*			WB 1:500	
				IF 1:1000	
LAMP-1	*N/A*	Sheep	AP	FACS N/A	WCH
				WB 1:200	
				IF 1:1000	
LIMP-2	*N/A*	Mouse	AP	FACS N/A	WCH
				WB 1:200	
				IF 1:1000	
EEA-1	*N/A*	Goat	AP	FACS N/A	WCH
				WB 1:200	
				IF 1:1000	
EEA-1	*N/A*	Mouse	AP	FACS N/A	BD
				WB 1:500	Bioscences
				IF 1:1000	Pty. Ltd.
Ab-16640	*(sh)Sortilin*	Rabbit	AP	FACS 1:200	Abcam
(Sortilin)				WB 1:500	
				IF 1:1000	
anti-Rab-4	*(sh) Rab-4*	Rabbit	WS	FACS N/A	Chemicon
				WB 1:100	
				IF 1:100	
anti-ChAT	*(nm) ChAT*	Rabbit	WS	FACS	Chemicon
				WB	
				IF	
HB-9	*N/A*	Rabbit	WS	FACS N/A	Flinders
				WB 1:200	Tech.
				IF N/A	

Legend: AP, affinity purified; IgG, purified immunoglobulin fractions; WS, whole serum; FACS, fluorescence activated scanning; IF, Immunofluorescence assays; WB, Western blot assay; (r), recombinant; (rh), recombinant human; (sh), synthetic human; (nr), native rat.

Table 3 - List of fluorescent secondary antibodies and working dilutions.

Fluorescent Secondary antibody conjugates	Host Species	Form	Dilution
Cy-2 / Cy-3 AffiniPure Anti-Goat	Donkey	IgG (H+L)	FACS 1:250
			IF 1:400
Cy-2 / Cy-3 AffiniPure Anti-Mouse	Donkey	IgG (H+L)	FACS 1:250
			IF 1:400
Cy-2 / Cy-3 / Cy-5 AffiniPure Anti-Rabbit	Donkey	IgG (H+L)	FACS 1:250
			IF 1:400
Cy-2 / Cy-3 / Cy-5 AffiniPure Anti-Sheep	Donkey	IgG (H+L)	FACS 1:250
			IF 1:400
Alexa Fluor 488, 594, 647 Anti-Mouse	Goat	IgG (H+L)	FACS 1:200
			IF 1:1000
Alexa Fluor 488, 594, 647 Anti-Rabbit	Goat	IgG (H+L)	FACS 1:200
			IF 1:1000
Alexa Fluor 647 Anti-Chicken	Rabbit	IgG (H+L)	FACS 1:200
			IF 1:1000
Alexa Fluor 568 Anti-Goat	Donkey	IgG (H+L)	FACS 1:200
			IF 1:1000
Quantum Dot 525 Anti-Mouse	Goat	$F(ab)_2$	IF 1:1000
Quantum Dot 655 Anti-Rabbit	Goat	$F(ab)_2$	IF 1:1000

Legend: IgG (H+L), Immunoglobulin heavy and light chain; $F(ab)_2$, Fab immunoglobulin fragment; FACS, fluorescence activated cell scanning; IF, Immunofluorescence assays.

Table 4 - List of HRP and Alkaline Phosphatase conjugated secondary antibodies and working dilutions.

Secondary antibody conjugates	Host Species	Form	Dilution
Alkaline Phosphatase Anti-Goat,	Donkey	IgG (H+L)	ELISA 1:10000
Mouse, Rabbit and Sheep			WB 1:5000
Alkaline Phosphatase Anti-Goat,	Donkey	$F(ab)_2$	ELISA 1:10000
Mouse, Rabbit and Sheep			WB 1:5000
Horseradish Peroxidase conjugated Anti-	Donkey	IgG (H+L)	ELISA 1:10000
Goat, Mouse, Rabbit and Sheep			WB 1:5000
Horseradish Peroxidase conjugated Anti-	Donkey	$F(ab)_2$	ELISA 1:10000
Goat, Mouse, Rabbit and Sheep			WB 1:5000

Legend: IgG (H+L), Immunoglobulin heavy and light chain; $F(ab)_2$, Fab immunoglobulin fragment; ELISA, Enzyme-Linked ImmunoSorbent Assay; WB, Western blot assays.

2.A.5 Protein chromatography

Sephadex-G25-Fastflow gel and sepharose-6B gel, columns and chromatography reagents were acquired from GE Healthcare Bio-Sciences (Uppsala, Sweden). Protein-G Agarose Fastflow gel was purchased from Chemicon (Boronia, Australia).

2.A.6 Chemical reagents, kits and compounds

Caltag Fix & Perm Cell Permeabilization kit and NuPAGE 4-12% Bis-Tris gels were purchased from Invitrogen Life Technologies (Mulgrave, Australia). TMB Peroxidase EIA Substrate kit and SDS-PAGE pre-stained standards were supplied by Bio-Rad (Regents Park, Australia). ECL and ECF western Blot Kits were from GE Healthcare Bio-Sciences (Uppsala, Sweden). WST-1 ECS cell proliferation assay kits were provided by Chemicon (Boronia, Australia). All custom synthesis peptides and peptide conjugates for antibody production were manufactured by Auspep Pty. Ltd. (Parkville, Australia). (4)-nitrophenyl phosphate disodium salt hexahydrate, dimethyl sulfoxide (DMSO), Staurosporine, Picric Acid, Triton X-100 and other general chemicals were purchased from Sigma (Castle Hill, Australia).

2.A.7 Data analysis

Quantitative data analysis and graphs were compiled with GraphPad PRISM software (San Diego, USA). Unless otherwise stated, image processing and figure compilation was performed with NIH ImageJ and Adobe Photoshop CS software.

2.B Methods

2.B.1 Mammalian cell culture

Neuroblastoma x spinal cord cells (NSC-34) (kindly provided by Dr. Neil Cashman, University of Toronto), were maintained in Dulbecco's modified

Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin/glutamine solution (PSG) as previously described (Cashman *et al.*, 1992) Cells were subcultured every 3-4 days at a ratio of 1:5. To slow the proliferation and enhance the maturation of these cells, they were grown to 80% confluence and the maintenance medium (DMEM. 10% FBS, 1% PSG) was exchanged for differentiating medium comprising of 1:1 DMEM plus Ham's F12, 1% FBS, 1% PSG, and 1% modified Eagle's medium non-essential amino acids (NEAA). After 24-48 hours culture in the differentiating medium, there was a large amount of cell death. At this stage the medium was replaced with fresh differentiating medium, and the remaining populations of cells were allowed to proliferate for up to ~7 days, with fresh media change every 2 days. The differentiated cells (NSC-34_D) survived in the reduced serum medium and were subsequently serially passaged for up to 2 times without loss of viability (Eggett *et al.*, 2000).

MLR-1, MLR-2, MLR-3 (created by M-L. Rogers, Flinders University), X-63 and *MC-192* hybridoma cells were grown and maintained in RPMI 1640 with Glutamax, 10% FBS or TCS, 2 mM L-glutamine, 1% liquid mixture of sodium hypoxanthine and thymidine (HT**)** 1% (PSG).

GT1b-1 and *GT1b-2a* (kindly provided by R.L. Schnaar, Johns Hopkins School of Medicine) hybridoma clones were grown and maintained in DMEM containing high glucose, 10% FBS or TCS, 2 mM L-glutamine, 1% (100X) liquid mixture of sodium hypoxanthine and thymidine (HT**),** 1% (PSG), 200 U/I insulin, and 50 mg/l sodium pyruvate. All antibody producing hybridomas were cultured for 3 – 5 days, the conditioned media were then harvested, and cells were subcultured at a ratio of 1: 5 in fresh growth medium.

2.B.2 Adhesion matrix for cell culture

All cell cultures were grown successfully without added adhesion factors in plastic flasks or plates. Glass coverslips were coated with adhesion matrices dependent on the type of experiments performed. For short-term cultures,

0.1mg/ml poly-l-lysine solution in 0.15 M borate buffer (pH 8.3) was used to coat coverlips for 24 hours, which were then washed tree times with PBS, and cells were seeded immediately. For cultures longer than 48 hours, rat-tail collaged type-1 at 100 μg/ml in 0.02M acetic acid and 2 μg/ml laminin matrix stock solution were diluted twenty fold in 30% ethanol. Coverlips were coated and air-dried in the laminar flow hood.

2.B.3 Cell viability assays measuring effects of low serum media conditions

NSC-34 and NSC-34_D cells were seeded in 96 well microtiter plates at 1×10^4 per well in 100μl of DMEM containing one of the following: 1% FBS, 2%FBS or 3% FBS supplemented with 2 mM L-glutamine, 20mM HEPES, 1% NEAA, 1% PSG and incubated in time intervals of 24, 48, 72 and 96 hours, at 37°C and 5.1% CO_2. Cell viability was assessed as described in 2.B.6.

2.B.4 Cell viability assays measuring the effects of neurotrophins

NSC-34 and NSC-34_D cells were seeded in 96 well microtiter plates at 1×10^4 per well in 100μl of DMEM containing one of the following: 1% FBS, 2%FBS or 3% FBS supplemented with 2 mM L-glutamine, 20mM HEPES, 1% NEAA, 1% PSG and incubated for 18 hours at 37°C and 5.1% CO_2. Cells were then incubated in serum deficient DMEM for 4 hours prior to application of growth factors. Cells were treated with NGF, BDNF, NT-3, and NT-4 at 200, 100, 50, 20 and 10 ng/ml concentrations in 100μl fresh differentiating media for up to 48 hours.

2.B.5 Retinoic acid and BDNF treatment of cell cultures

NSC-34 and SH-SY5Y cells were cultured as described in 2.B.1, in T-75 flasks to about 40% confluence over the period of 2-3 days. 10 μM *all trans*-RA was then added in 10% FBS supplemented DMEM, with fresh media + *all trans*-RA change every two days. After 5 days in the presence of RA, cells were washed three times with DMEM and incubated with 50 ng/ml BDNF in DMEM (without serum) for different intervals. Cell lysates were prepared and analyzed on

Western blots as described in 2.B.11.

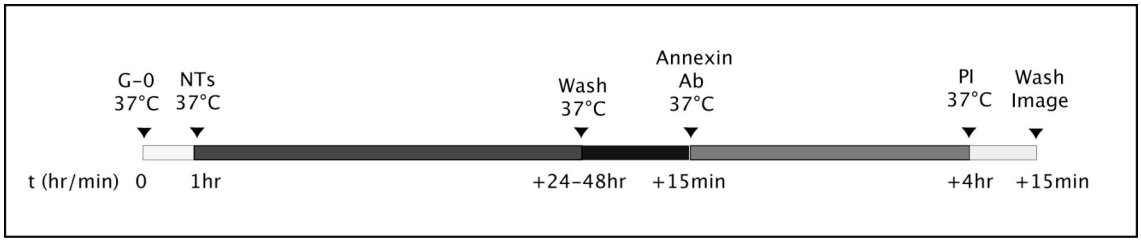
2.B.6 Acid phosphatase hydrolysis assay

Cell viability for all in-vitro assays was assessed by (4)-nitrophenyl phosphate hydrolysis. The assay is based on the hydrolysis of the p-nitrophenyl phosphate by intracellular acid phosphatases in viable cells to produce p-nitrophenol. Absorbance of p-nitrophenol at 405 nm is directly proportional to the cell number in the range of 10^3-10^5 cells. The assay can quantify as few as 1000 cells per well in 96-well microtiter plates, previously described by [Yang, 1996 #423]. Treated plates were gently washed with 100µl of PBS three times and then incubated with 100µl of 5 mM p- (4)-nitrophenyl phosphate, 0.1M sodium acetate, 0.1% (vol/vol) Triton X-100, pH 5.0 for 30 min ~ 1hr at 37°C and 5.1% atmospheric CO_2. Cell viability was expressed as a percentage of untreated cell cultures (100%).

2.B.7 Annexin-V-FITC apoptosis assay

Cells were grown in 24-well plates as described in 2.B.1 until 80% confluent. Apoptosis in control experiments was induced by the addition of 1µg/ml of staurosporine in 1.5% BSA supplemented culture media for 4 hours. Neurotrophin treated cells were incubated for up to 96 hours with NGF, BDNF, NT-3 or NT-4. Cells were washed with pre-warmed media and incubated with 3µg/ml annexin-V-FITC in media containing 10mM HEPES, 140mM NaCl and 2.5mM $CaCl_2$, for 4 hours at 37°C. Media solution containing PI (propidium iodide) at 2µg/ml and Hoechst 33258 at 1µg/ml were then added to cell cultures for 10 minutes. Cells were washed twice with pre-warmed media and observed under a fluorescence microscope. Cell counts were performed on individual images (6 images per treatment) as described in 2.B.14.

Figure 2.1 - Schematic representation of apoptosis assay timeline



2.B.8 Purification of antibodies

Conditioned media from all hybridoma clones was harvested and stored at -20°C until time of purification. This media was sterile filtered through a 0.2 μm filter and loaded onto a 0.15M PBS, pH 7.2 equilibrated protein-G column at a flow rate of 0.5 ml/min overnight at 4°C, using a Pharmacia P-1 peristaltic pump. The next day the column was washed with 200ml of 0.15M PBS, pH 7.2 at a flow rate of 2-3 ml/min, and antibodies were eluted with 0.1M glycine, pH 2.7, using a Pharmacia P-1 peristaltic pump connected to a single path UV-1 monitor. Collected fractions were neutralized with 2M Tris to a pH of 7.2. Purified antibodies were extensively dialyzed against 0.15M PBS at pH 7.2. All antibodies were stored in aliquots at - 80°C until used.

2.B.9 Conjugation of antibodies to fluorescent compounds

Antibodies were concentrated using a Nanosep 100K centrifugal device (Pall) to a final concentration > 2.0 mg/ml and conjugations with FITC, Alexa Fluor Succinimidyl esters were performed according to manufacturer's instructions. Conjugates that labeled the brightest positive cells, and which had the lowest background on negative cells, were used for the experiments. The conjugates were stored in PBS 7.2pH containing 2mM sodium azide at 4°C protected from light. For long term storage the conjugate can be aliquoted and freezed at -20°C.

2.B.10 Fluorescence Activated Cell Sorting (FACS) flow cytometry

All procedures were carried out at 4°C (or on ice) and in the dark. Single cell suspension in were prepared in 0.15M PBS, 2% FBS, 10 mM HEPES, at a concentration of 1×10^7 cells /ml. 100 μl of cell suspension were aliquoted into 5ml polystyrene (FACS) tubes. Primary antibodies including fluorescent conjugates were added at 10 -20 μg / ml to the cells and mixed gently, followed by incubation for 30 - 60 minutes. Cells were washed two times by adding 2ml 0.15M PBS, 0.2% BSA, 10 mM HEPES, 0.02% NaN_3 and centrifuged for 5 minutes at 1600rpm (300 x g).

Cells were re-suspended and appropriate secondary antibodies (when required) were added at 1:400 ~ 1:1000 followed by a further incubation for 30 – 60 minutes. Stained cell pellets were re-suspended in 0.15M PBS, 0.2% BSA, 10 mM HEPES 0.02% NaN_3 for flow cytometry. 20μl of PI solution was added 5 minutes prior to analysis to detect dead cells. Single-cell suspensions for each treatment were prepared and passed through a Becton-Dickinson FACScan flow cytometry. The pattern of viable cells was used to outline a gated region using CellQuest software (Version 3.2.1). Cells showing increased forward scatter fluorescence compared with control antigen stained cells, were judged to be positive.

2.B.11 FACS analysis of neurotrophin binding on antibody binding

The experimental procedure was carried out as described above with the addition of a 1hour pre-incubation step with NGF, BDNF, NT-3 or NT-4 at 100ng/ml to antibody treatment groups. Counting events of viable cells were gated at 20,000 and mean fluorescence measurements were plotted against antibody treated samples not treated with neurotrophins.

2.B.12 Cell lysate preparation and Western Blot analysis

Cells were lysed using chilled lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% (vol/vol) Triton-X100 with 1% (vol/vol) protease inhibitor cocktail for 10 min on ice. Lysates were solubilized in an equal volume of 2 × SDS sample buffer containing 4% (wt/vol) SDS, 2% (wt/vol) glycine, 0.015% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol, and 10% (vol/vol) β-mercaptoethanol in 100 mM Tris-HCl buffer pH 6.8. Cell lysates were electrophoresed through 12.5% Tris-glycine buffered SDS gels. Proteins from the gels were electroblotted onto nitrocellulose membrane at 100 V for 1 hr. The membranes were blocked in 4% (wt/vol) skim milk powder, 0.1% (vol/vol) Tween-20, and 0.02% (wt/vol) NaN_3 in Tris-buffered saline (TBS), pH 8.0, for 1 hr at room temperature and then incubated overnight with primary antibodies at 4°C. Neuron-specific antibodies in this study included tyrosine kinase B (TrkB), $p75^{NTR}$ rat specific antibody (Mc-192), $p75^{NTR}$ specific antibodies MLR-1, MLR-2 and MLR-3, choline acetyl transferase (ChAT), vesicular acetylcholine transporter (VAChT), gangliosides GT1b and GQ1b, brain derived neurotrophic factor (BDNF). Blots were then washed three times in Tris-buffered saline-Tween 20 (TBST), pH 8.0, for 10 min and incubated for 1 hr with respective horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:2,500) in TBS at room temperature and then washed three times in TBS for 10 min. Blots were developed with enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF).

2.B.13 $p75^{NTR}$ internalization and pharmacological inhibition of endocytosis

Cells were plated at 10,000 cells/coverslip on 30mm poly-D-lysine/Collagen/Laminin coated coverslips and cultured for 4-5 days (until differentiated) in 6-well plates with DMEM/F-12 differentiating medium containing no phenol red. After pre-incubation for 1 hr at 37°C with incubation buffer (DMEM/F-12, 25mM HEPES, 3 mg/ml BSA) to deplete endogenous transferrin and growth factors, cells were then incubated with transferrin-Alexa-555 (10μg/ml) and MLR-2-Alexa-488 antibody (3μg/ml) alone (vehicle) or in the

presence of NGF, BDNF, NT-3 or NT-4 (100ng/ml) for 45min at 6°C. Following Incubation cells were washed with ice-cold media, followed by pre-warmed incubation media for 0-60 minutes at 37°C in a temperature controlled sealed POC chamber.

Inhibition of receptor internalization in low temperature was assayed by incubation of cells at 4-6 °C for 45 min. Endocytosis inhibitors MDC and PAO were used after vehicle or neurotrophin incubation, by treating cells with 100 μM MDC for 1 h, or 10 μM PAO for 15 minutes in serum-free medium supplemented with 2% bovine serum albumin. Uptake of p75NTR via non clathrin-mediated endocytosis was measured by subtracting internalized staining in the presence of MDC not co-localized with transferrin, and 8 different cells were analyzed per time sequence in each experiment.

2.B.14 Immuno-endocytosis assays

Cells were cultured on poly-D-lysine/collagen/laminin coated coverslips in 24 well plates as above, but in DMEM/F-12 medium containing no phenol red. After pre-incubation for 1 hr at 37°C with incubation buffer (DMEM/F-12 HEPES, 1 mg/ml BSA) to deplete endogenous transferrin and growth factors, cells were treated with primary antibodies for 1 hr at 4°C in the presence of (100ng/ml NGF or BDNF). Cells were then washed with ice-cold incubation media three times and incubated with secondary antibodies and Lysosensor Blue/Green or Lysotracker Red DN-99 for a further 1 hour at 4°C. Cells were subsequently washed with ice-cold incubation media, followed with pre-warmed incubation buffer for 15–180 min at 37°C in the presence of (100ng/ml NGF or BDNF) followed by three washes in ice-cold PBS and fixation in 3% paraformaldehyde for 20 min. The fixation reaction was quenched in 0.1 mM glycine for 10 min. The schematic representation for individual experiment time lines (A: live and B: fixed), are shown in Figure 2.2. The presence of vesicles positive for individual and/or colocalised markers, were quantified by measuring the fluorescence intensity levels for each dye in individual channels. Results were presented as a percentage of fluorescently labeled intracellular structures within a single image plane. Intracellular structures were defined with the aid of direct

interference contrast (DIC) imaging. Intracellular regions were isolated by marking out the intracellular regions of interest (ROIs) from the extracellular ROIs, according to cellular morphology seen in DIC images. Background subtraction of 25% and threshold compensation of 30% (60 pixels) was applied to all merged images. A double-positive compartment was defined on the basis of the following criteria: the carrier was labeled in two different channels and the morphology of the carrier was very similar in the two channels. 8-12 individual cells were analyzed per time point in each experiment. Quantification of fluorescent data was subsequently performed using ImageJ software.

Figure 2.2 - Schematic representation of live and fixed immunoendocytosis assay time line.

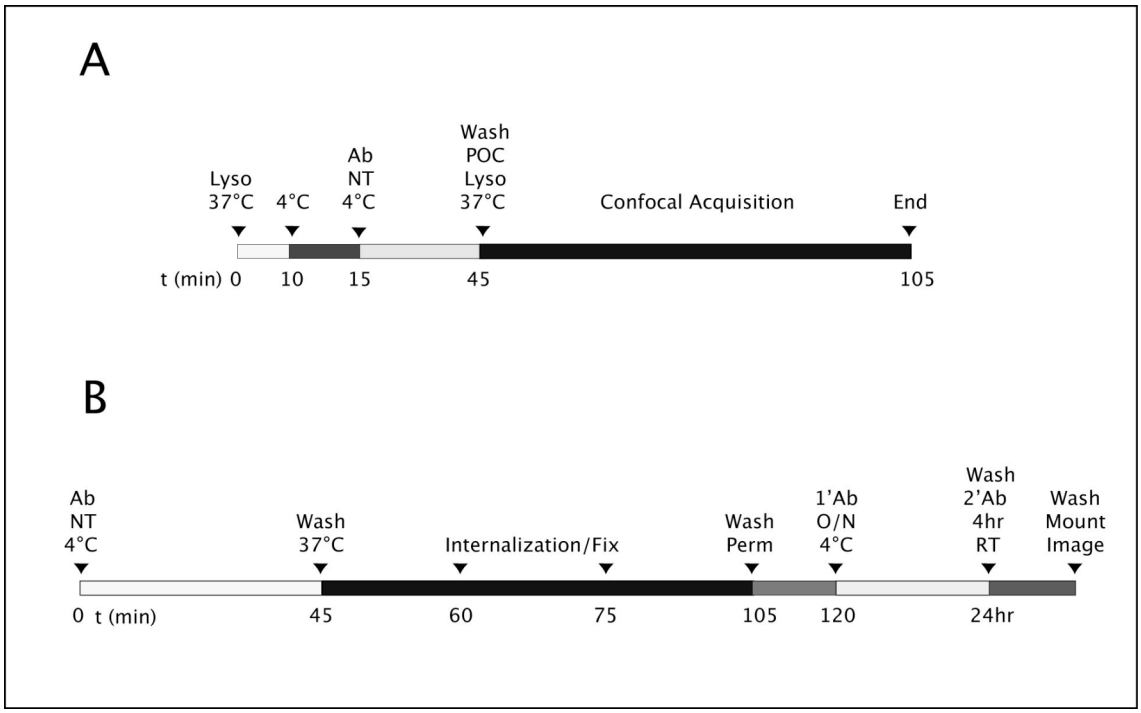


***Figure 2.2 legend: Ab**, antibody; **NT**, neurotrophins; **Perm**, Permeabilization step; **O/N**, overnight incubation; **RT**, room temperature incubation.*

2.B.15 Phase contrast microscopy

Phase contrast microscopy was performed using an Olympus IX71 inverted fluorescence microscope equipped with TH4-200 halogen lamp apparatus, U-RFL-T mercury lamp apparatus, F-View CCD camera and phase contrast / relief contrast (Hoffmans Contrast) objective lenses, including (culture dish, coverslip/slide correction rings. AnalySIS getIT image capture software was used for image collection, analysis and processing.

2.B.16 Confocal microscopy

Confocal imaging of initial characterization experiments using emission spectra detection at 488 nm and 568 nm, were performed using a Bio-Rad MRC 1024 scanning confocal microscope Olympus AX70 microscope, equipped with a Krypton/Argon laser. Analysis used BioRad LaserSharp software.

All other confocal imaging, including live cell imaging was accomplished using a Leica Microsystems TCS SP5 Tandem Scanner confocal microscope rigged with a Leica Tempcontrol 37-2 digital temperature regulator, Leica CTI-Controller 3700 digital CO_2 atmosphere regulator and a LaCon POC-Chamber System for "open" and "closed" applications. Leica LAS AF platform software was used for analysis and processing of images.

Live cell immunofluorescence experiments using NSC-34 cells were performed in a temperature controlled closed system chamber. Chamber temperature was regulated at 35°C. Experiments were done in media with N-2 neuronal cell supplement and 20mM HEPES. Laser power settings were adjusted according to the signal strength during individual experiments. Laser scanning speed varied between 200-400Hz and images were acquired at 1024x1024 pixel resolution. Images were acquired every 20s over a total of up to 60 frames for the first 20 min, and then every 60s for 50 frames for the remainder of the experiment. Multi-fluorescent live cell acquisition was performed with sequential scanning mode between scan lines, and averaging 2-4 lines. Pinhole size was set at

1 Airy unit for all confocal acquisition. Images were acquired in a single optical plane with a distance of 5-10µm between optical planes for all live and fixed experiments. Z-stacks were also taken and analyzed for fixed samples. PMT bandwidth detection settings for all fluorescent markers are summarized in Table 5 and 6. Dye separation was performed in experiments using transferrin-Alexa-555 in combination with Alexa-488 and Alexa-594 fluorescence.

Table 5 - PMT gate settings for cell membrane and nuclear marker emission.

Fluorophore Dye Selection (Primary and secondary antibody conjugates)	**Laser Source** Abs (nm)*	**PMT Collection** *E*m (nm)
DAPI / Hoechst 33258	405	**408-480**
Alexa 488 / Cy-2 / FITC	488	**500-540**
Alexa 555	514	**555-590**
Alexa 565 / Cy-3	561	**595-645**
Alexa 594	561	**605-650**
Alexa 647 / Cy-5	633	**660-710**

Table 6 - PMT gate settings for fluid-phase marker emission.

pH Sensitive Cellular Compartment Markers	**Laser Source** Abs (nm)*	**PMT Collection** *E*m (nm)
LysoSensor Green DND-189	443	**460-520**
Lysotracker Red DND-99	561	**575-610**

* Values represent laser excitation wavelength and not absorption maximum values obtained from datasheet of individual products.

Chapter 3: Optimization Of NSC-34 Culture And Differentiation

3.A Introduction

Studies utilizing embryonic spinal cord cultures, including motor neurons have been crucial in gaining knowledge of the development, but also adult physiology, injury, and disease of the nervous system (Kuhn, 2003). Numerous growth factor molecules, including among others the neurotrophins, have been identified and shown to promote the survival, differentiation as well as aid in the recovery of injured motor neurons in vivo (Thoenen *et al.*, 1993; Oppenheim, 1996; Henderson *et al.*, 1998; Sendtner *et al.*, 2000). It is from these discoveries that ideas emerged postulating the possible therapeutic role of neurotrophic factors in the treatment of various motor neuron diseases (Yan & Miller, 1993; Oppenheim, 1996). However, despite the importance of neurotrophins in motor neuron physiology and disease, a very limited number of studies have focused on the trafficking and intracellular sorting of these molecules in motor neurons.

Major reasons for this trend are the difficulties associated with obtaining, isolating and growing primary motor neuron cultures (Berg & Fischbach, 1978; Calof & Reichardt, 1984; Kuhn, 2003; Anderson *et al.*, 2004), the difficulty of transfecting primary motor neuron populations encoding foreign gene products (Yang *et al.*, 1997; Figlewicz *et al.*, 2000; Berry *et al.*, 2001; Read *et al.*, 2005), and the lack of existing, well characterized motor neuron cell lines (Cashman *et al.*, 1992; Smith *et al.*, 1994; Salazar-Grueso *et al.*, 1991). Available methods for purifying motor neurons to relative homogeneity from rodent spinal cords involve retrograde labeling and fluorescence-activated cell sorting (Eagleson & Bennett, 1983; Calof & Reichardt, 1984; O'Brien & Fischbach, 1986) and isolation and purification by panning against $p75^{NTR}$ antibodies (Camu & Henderson, 1992; Anderson *et al.*, 2004). Both methods are costly, time consuming, and provide only limited numbers of neurons, which rarely survive longer than seven days in culture (O'Brien & Fischbach, 1986; Camu & Henderson, 1992) with no viable cells at day-14 (O'Brien & Fischbach, 1986), although a more recent study has reported survival of some motor neuron populations up to 14 days (Anderson *et al.*, 2004).

Our interest is in the study of neurotrophin receptor trafficking within motor neurons with a view to better understand the mechanisms by which the neurotrophins elicit their various roles. For this purpose, any primary neuron population must also be further characterized for receptor expression, which is often difficult due to the lack of availability of sufficient cell numbers to perform biochemical analysis (Kaplan, 1998). Further complexity arises from the observations that neurons modulate their expression of neurotrophins, and their receptors during various stages of development (Yan *et al.*, 1993; Hughes *et al.*, 1993; Buck *et al.*, 2000). This means that results could vary depending on the age of the animal that the neurons were obtained from, or the duration of subculture of the isolated populations (Kaplan, 1998).

Many aspects of neurotrophin transport have been studied in primary or immortalized cell lines. Although each have their advantages and disadvantages, a relatively miniscule amount of knowledge has been extracted from motor neurons (Lalli & Schiavo, 2002), compared to sensory and sympathetic neuronal systems (Hendry *et al.*, 1974; Stoeckel *et al.*, 1975; Claude *et al.*, 1982; Johnson *et al.*, 1987; Ehlers *et al.*, 1995; Grimes *et al.*, 1996; Sandow *et al.*, 2000). The five primary neuronal populations used to study neurotrophin effects and behavior are made up of sympathetic, sensory, cortical, cerebellar, and hippocampal neurons (Kaplan, 1998). In contrast mainly two types of immortalized cell lines have been used for in-vitro investigations: the PC-12 pheochromocytoma cells and neuroblastoma cells (Kaplan, 1998).

The PC-12 cells have been to a great extent the major model system to study neurotrophin internalization and subsequent signaling events (Chandler *et al.*, 1984; Kaplan *et al.*, 1991b; Benedetti *et al.*, 1993; Kaplan & Stephens, 1994; Beattie *et al.*, 1996; Grimes *et al.*, 1996; Saxena *et al.*, 2004). The success of the PC-12 cell line as a model system lies in its extensive initial characterization (Greene & Tischler, 1976), followed by 30 years of extensive use. To date only four motoneuron-like cell lines have been generated (Salazar-Grueso *et al.*, 1991; Cashman *et al.*, 1992; Smith *et al.*, 1994 and Li, 2000 #512), and among these, the

NSC-34 clone has become the best characterized and also most frequently used to study motor neuron biology.

This chapter describes the characterization and optimization of the NSC-34 cell line growth conditions. This cell line has been previously characterized for motor neuron properties not expressed by the neuroblastoma parent cell line (summarized in Table 1.7.1), which indicate that NSC-34 cells share many properties with spinal motor neurons in primary culture (Fischbach & Dichter, 1974; Kato *et al.*, 1985; Peacock *et al.*, 1973). However one of the biggest drawbacks of the NSC-34 cells are extremely poor substrate adhesion compared to primary neuronal or non-neuronal cells, and the inability to grow the cells in defined media for longer than one week and one passage (Durham *et al.*, 1993). It is crucial that optimal culture conditions are well defined, in order to ensure that experimental observations result from specific inductive interactions rather than from a more general effect on the health of the cells (O'Brien & Fischbach, 1986), and to ensure the reproducibility of experimental procedures over time. The improvement of the substrate adhesion and defined media culture conditions for the NSC-34 cells is a necessary step required to use this cell line for experimental design and purpose requiring multiple treatments and manipulation.

3.B Culture, Maintenance and Differentiation of NSC-34 cells

3.B.1 Optimization of NSC-34 cell culture conditions

Some of the documented characteristics of NSC-34 cells, such as poor substrate adhesion (Durham *et al.*, 1993), present a considerable obstacle in being able to culture these cell for long periods of time required for their differentiation, and subsequently use them for intracellular trafficking experiments requiring multiple treatment steps. Conditions for the growth and differentiation of these cells have been previously described in the literature (Cashman *et al.*, 1992; Durham *et al.*, 1993), however these media environments were not suitable for growing NSC-34 cells on coverslips due to extremely poor adhesion.

Stock NSC-34 cell cultures were grown and maintained in standard 10% FBS supplemented medium (Figure 3.1A), for up to 20 passages. After 20 passages there was significant reduction in spontaneous cell differentiation. Cultures contained two morphologically distinct populations. Parent-like neuroblastic cells (N-type, arrowheads) with a rounded morphology and no neurite processes, and substrate adherent (S-type, arrows) with small neurite processes that undergo further differentiation until sub-cultured. Cell grown in differentiating media containing 1% FBS, were only grown for 1 passage. Although, NSC-34 cells differentiate more readily in low (1%) FBS supplemented media (Figure 3.1B), the sudden serum withdrawal results in massive loss of cells followed by surviving cells sprouting extensive neurite projections in a time span of 5-8 days. Cells differentiated in this manner also adhered poorly, and detached with a gentle shake of the culture vessel.

As FBS contains numerous adhesion factors NSC-34 cell were cultured in 2% and 3% FBS medium (Figure 3.1C-D) to investigate whether cell growth and adhesion would be improved under these conditions, while still producing high differentiation rates. The lack of ability to grow the NSC-34 cells in a defined or serum substituted medium in previous studies (Durham *et al.*, 1993), led these authors to omit quantitative measurements and analysis of cell proliferation and neurite extension. NSC-34 cells were successfully grown in serum-free defined media (DMEM/F-12) supplemented with commercially available N-1 (Contains 0.5 mg/ml insulin from bovine pancreas, 0.5 mg/ml human transferrin (partially iron-saturated), 0.5 µg/ml sodium selenite, 1.6 mg/ml putrescine, and 0.73 µg/ml progesterone) or N-2 (Contains 0.5 mg/ml insulin from bovine pancreas, 0.5 mg/ml human transferrin (partially iron-saturated), 0.52 µg/ml sodium selenite, 1.611 mg/ml putrescine, and 0.63 µg/ml progesterone) defined neuronal cell culture additives (Figure 3.1 E-F). Although both N-1 and N-2 media supplements supported NSC-34 cells over numerous passages, the N-2 supplement was significantly more efficient in inducing differentiation (Figure 3.1 E-F).

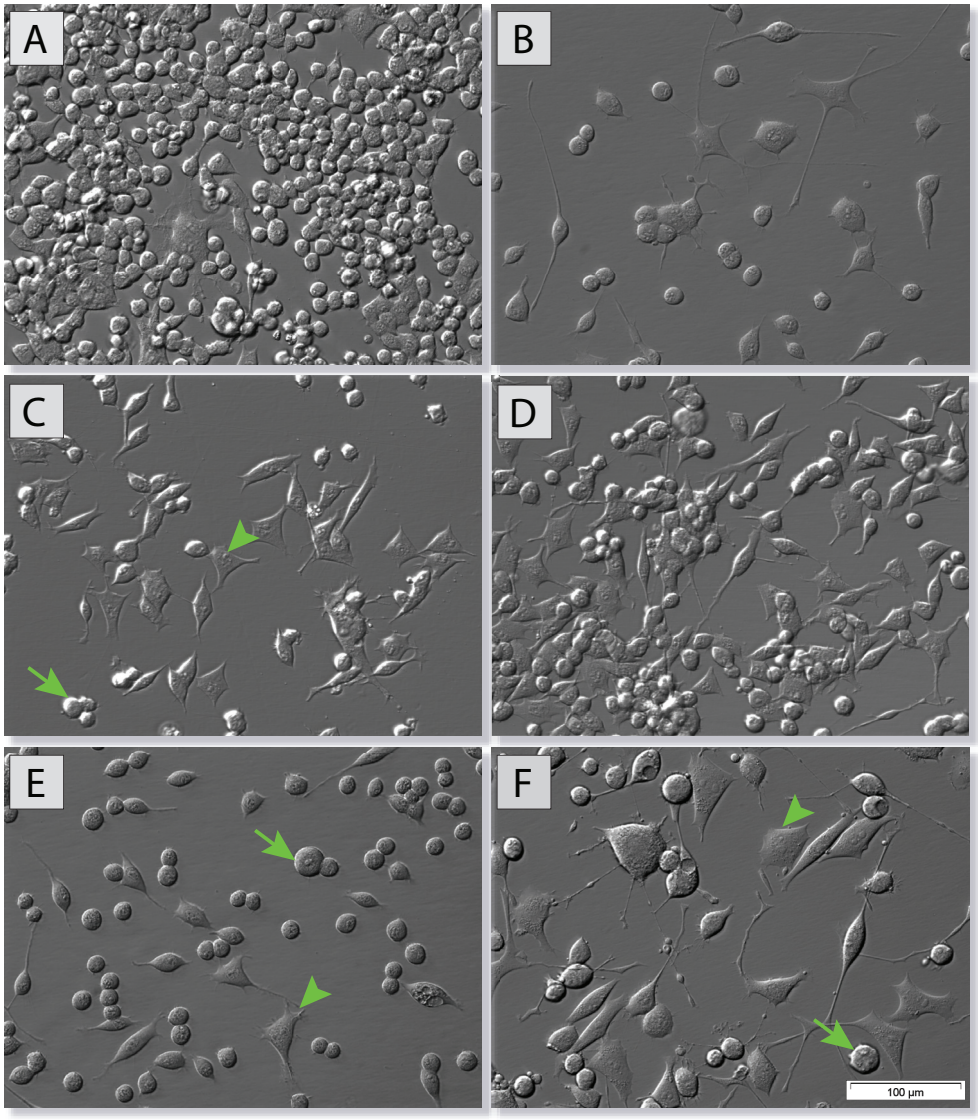


Figure 3.1 **Growth media associated morphological characteristics of NSC-34 cells.** **(A)** Cells cultured in 10% FBS growth medium, in **(B)** 1% FBS medium, **(C)** 2% FBS medium, and **(D)** in 3% FBS supplemented medium. NSC-34 cells do not survive in serum free media unless growth factors are added. **(E)** Cells cultured under serum free conditions with the addition of N-1 neuronal media supplement, and **(F)** N-2 neuronal media supplement. Relief contrast micrographs were taken 4 days after plating. A heterogeneous neuroblastic (arrows) and substrate adherent (arrowheads) population of cells can be observed under all growth conditions (Scale bar = 100µm).

3.B.2 Analysis of cell survival rates in serum-supplemented and defined media.

Quantification of cell survival in serum-supplemented media (10%, 1-3% FBS; Figure 3.2A), and defined media (no serum, 1%N-1 or 1%N-2; Figure 3.2B), shows that culture in low serum medium formulation results in large-scale cell death due to serum withdrawal and that defined media supplements N-1 and N-2 abolished cells death. Media containing 1% or 2% FBS resulted in a significant loss surviving cells in the first 48 hrs (*$p<0.005$, two-way ANOVA analysis), however cell numbers exhibited signs of increased proliferation by 96 hrs. Increasing serum concentration to 3% FBS almost completely reduced serum withdrawal associated cell death within the first 48 hrs observed in the 1-2% FBS colonies, however cell proliferation was reduced when compared to 10% FBS media cultures. Cells cultured in defined media containing N-1 or N-2 showed reduced rates of proliferation with the exception of N-2 media cultures, which proliferated more readily within the first 48 hours. Total withdrawal of FBS resulted in rapid cell death within 24 hours.

3.B.3 Effects of serum-supplemented and defined media on cell subtype

As described above, NSC-34 cells grown in standard 10% FBS exhibit two distinct morphological phenotypes. Culture in serum reduced and defined media compared to the standard growth media produced major shifts in morphological ratios of cell populations (Figure 3.3A-B). Significant increases in S-type cells in 1%, 2%, 3% FBS, N-1 and N-2 defined media were observed within 24 hours after subculture, and were subsequently quantitated at 48 hours (Figure 3.3B).

All serum reduced media and defined media initiated differentiation and the increased presence of S-type cells, in each case at a higher ratio than the N-type cells. In each treatment the S-type cell remained the dominant phenotype until cells were sub-cultured. However unlike cell grown in 10% FBS where upon sub-culture all the cells loose their projections and revert back to predominantly neuroblastic morphology, all the cells maintained in serum reduced, N-1 or N-2 defined media returned to a differentiating and substrate adherent phenotype

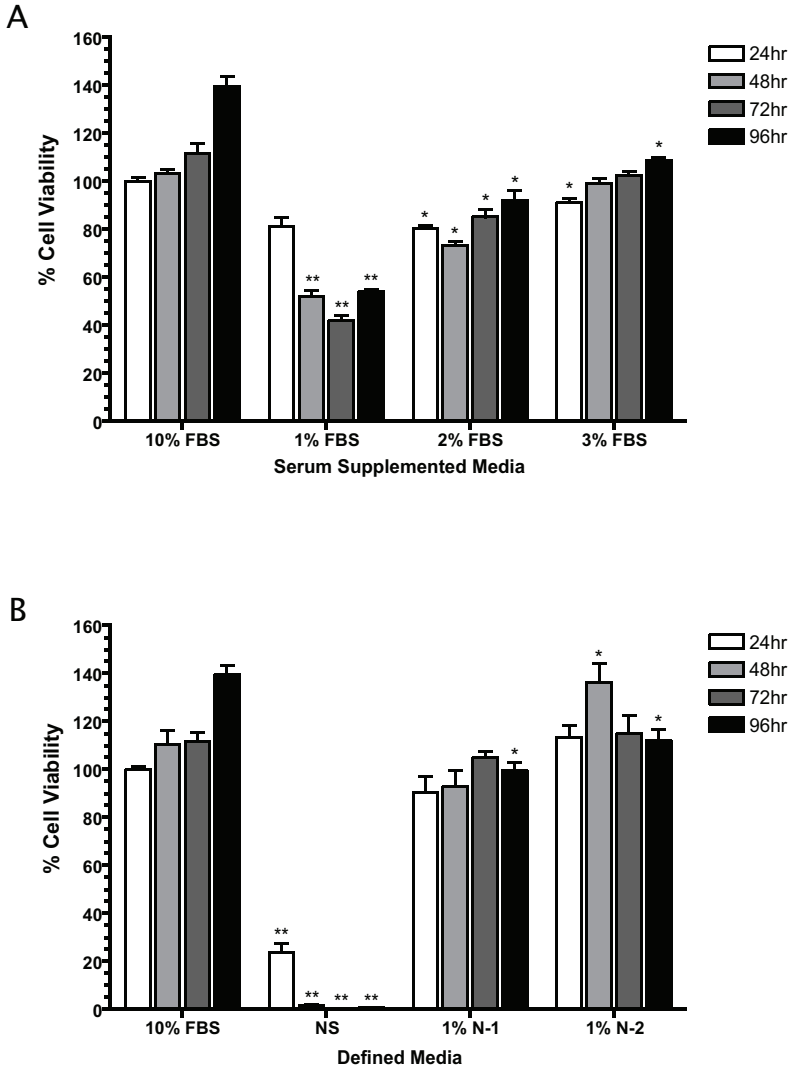


Figure 3.2 **Effects of serum supplemented and defined media on cell viability.** NSC-34 cell viability in FBS supplemented media **(A)**. Populations treated with normal (10% FBS) growth media versus low serum media conditions were compared in 24 hr intervals for up to 96 hrs. Culturing cells in 1% and 2% fetal calf serum supplemented media resulted in a significant loss of viable cells in the first 48 hrs of treatment, with signs of cell population recovery by 96 hrs post treatment. Culturing cells in 3% FBS abolished serum withdrawal dependent cell death. Cell cultures in serum-free defined media **(B).** Withdrawal of serum (NS) resulted in rapid loss of cell viability within 24 hr. Addition of 1% N-1 or N-2 supplement restored cell viability to levels comparable with cells grown in 10% FBS (n = 6, mean ± SEM; *$p<0.05$, ** $p<0.001$, two-way ANOVA analysis of difference to 10% FBS at each time interval).

within 24 hour after sub-culture at a 1:10 ratio into new vessels.

Although both N-1 and N-2 defined media maintained S-type morphology and neurite sprouting in the NSC-34 cells, the effects of the N-2 media superior in aspects of cell differentiation. The cell morphology of the N-1 media was very similar to the cells maintained in 2-3% FBS. This led us to discard the use of the N-1 media supplement in favor of the N-2 nutrient mixture for all subsequent experiments requiring defined media. A major problem with these cells being used in long-term studies testing morphological end-points was their characteristic to detach after 7-10 days, which limits their usefulness. Culture of NSC-34 cells in N-2 defined media can be maintained for up to 21 days with regular media change, eliminating difficulties of regular culture methods. Although cell proliferation is initially increased over the first 2 days of sub-culture in N-2 media (Figure 3.5A), this effect is not maintained with fresh media change and cell population decreases as neuroblastic cells are eliminated and terminally differentiated cells remain.

3.B.4 Effect of media on cell size and neurite extension in long term cultures

During the experiments described above, we noticed that the NSC-34 hybrids had different morphological characteristics relative to their soma size and neurite length and type of media used for culture. Measurements of population samples from each media treatment 96 hours after plating revealed that cells maintained in 1% FBS media had the overall smallest median cell soma size, although their median measurements neurite extensions were significantly longer than cells grown in 3% FBS or control (10% FBS) media (Figure 3.4A; n=12; *p>0.01, two-way ANOVA analysis).

Cells maintained in 3% FBS possessed a slightly larger cell body compared to the 1% FBS treatments, however their cell soma were significantly larger than control (10% FBS) cultures (Figure 3.4A; n=12; *p>0.01, two-way ANOVA analysis).

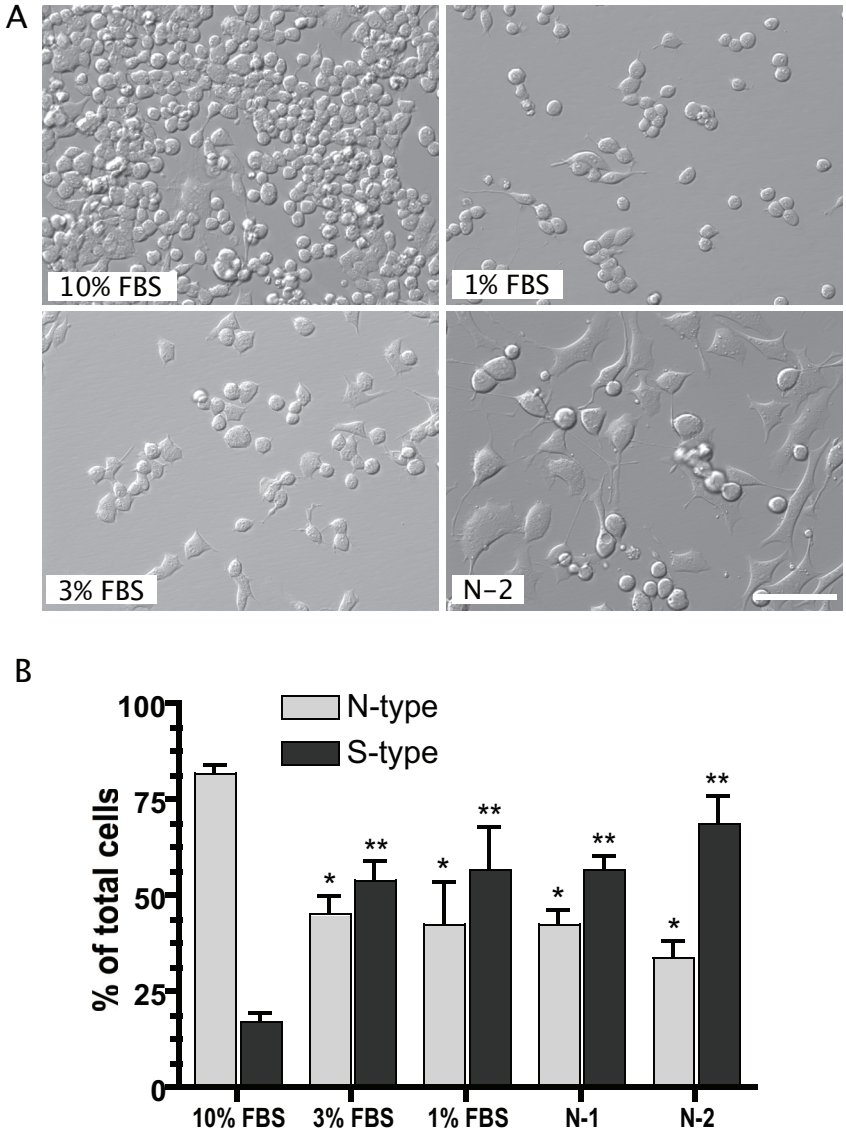


Figure 3.3 **Analysis of NSC-34 cell subtypes in serum supplemented and defined media.** Relief contrast micrographs of NSC-34 cells cultured in **(A)** 10%, 1% and 3% FBS control growth medium and serum free N-1 (micrograph not shown) or N-2 medium, for 48 hours. **(B)** Distribution of neuroblastic (N-type) versus substrate adherent (S-type) cells in total populations. Cells grown in normal growth medium (10% FBS) posses a predominantly neuroblastic appearance. Cells grown in serum reduced (1 or 3% FBS) or serum free defined N-1 or N-2 media have a characteristically higher populations of S-type than N-type cells. Quantitative analysis of cells subtypes expressed as a total of cell population (n=8; *$p>0.001$; **$p>0.0001$, two-way ANOVA analysis) scale bar = 200μm.

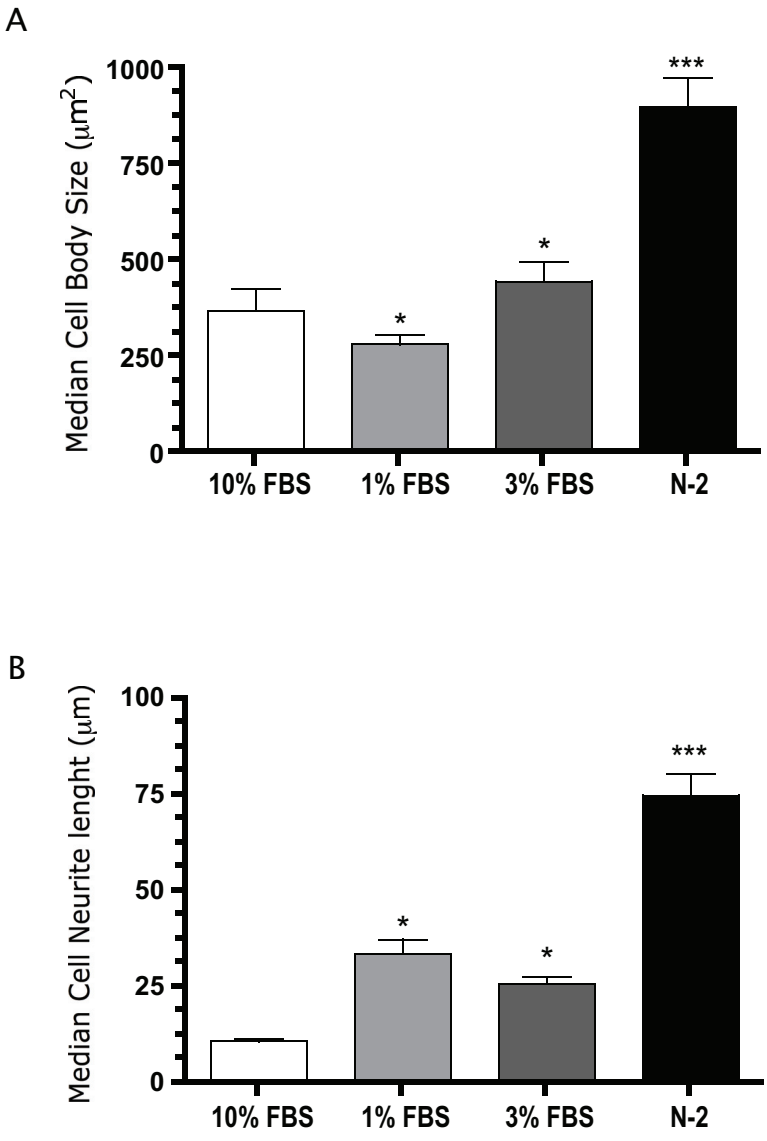


Figure 3.4. **Cell body size and neurite process measurements.** (**A**) Cell surface area measurements and **(B)** cell neurite projection length in cells after 96 hours in control (10% FBS), reduced serum (1% or 3% FBS) or serum free defined (N-2) media (n=12 sample micrographs for each treatment group). Reduced serum media mediate sprouting of longer projections, however 1%FBS media groups have reduced cell body size. N-2 defined nutrient mixture produces healthy cultures with the longest neurite projections and largest soma (*p>0.01; ***p>0.001; paired *t*-test).

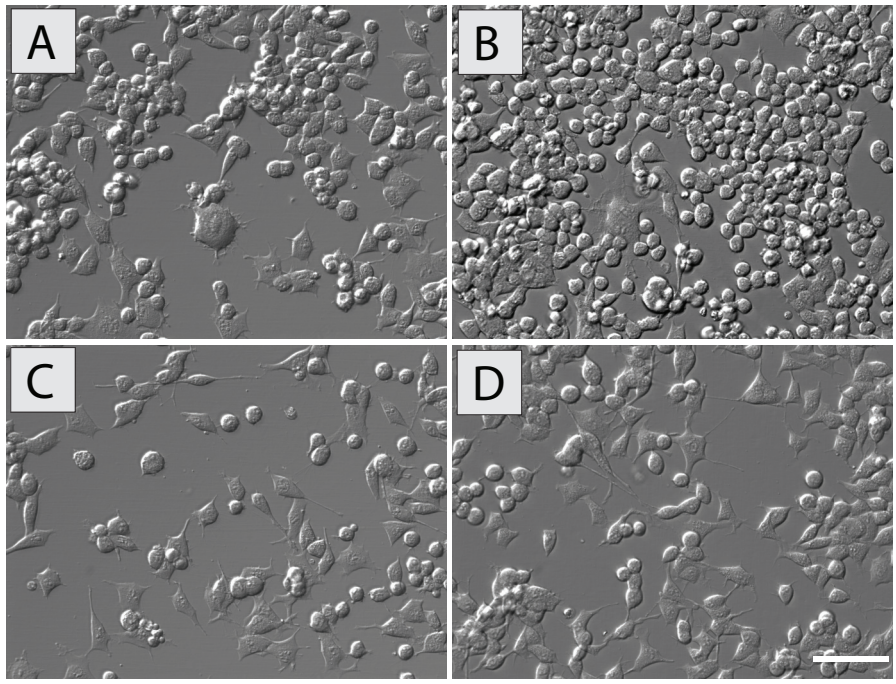
The median neurite length of the 3% FBS maintained cells was marginally shorter than the 1% FBS populations, but still significantly longer than the control cultures (Figure 3.4B; n=12; *p>0.01, two-way ANOVA analysis).

However the most profound differences were observed when cells were grown in the N-2 defined nutrient media. Cell some median size was twice as large when compared to the control cultures and low serum media treatments (Figure 3.4A; n=12; ***p>0.001, two-way ANOVA analysis). High degree of differentiation with neurite processes more than double the length of either the control cells or low serum treatments, were also observed in the N-2 cultures (Figure 3.4B; n=12; ***p>0.001, two-way ANOVA analysis). Although the neurite length in these cell increased over the first 4-6 days in culture with the N-2 media, and individual cells with neurites of up to 120 -150 μm in length were commonly observed, the number of total neurite projection sprouting from individual cells did not change compared to the control or low serum treatments.

3.B.5 Effects of long term RA treatment in NSC-34 cells

NSC-34 cell differentiate spontaneously upon serum reduction or sub-culture with serum free N-2 media. While some growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) have been reported to maintain attachment and differentiate NSC-34 cells, this effect only lasted one passage (Durham *et al.*, 1993).

Retinoic acid (RA), an analog of vitamin A is well documented to induce the arrest of cell growth in the G1 portion of the cell cycle (Kaplan *et al.*, 1993), and induce dramatic differentiation in neuroblastoma (NB) cells (Thiele *et al.*, 1985). Further, RA produces this effect in 10-15% FBS supplemented media formulations (Thiele *et al.*, 1985; Kaplan *et al.*, 1993; Edsjo *et al.*, 2003). More importantly, RA differentiated NBs possess many biochemical, ultrastructural and electrophysiological similarities to neurons (Abemayor & Sidell, 1989).



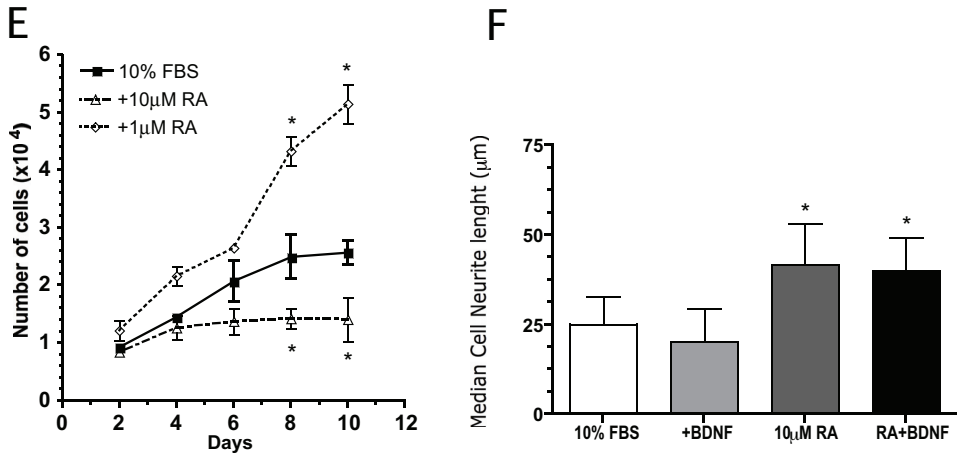


Figure 3.5 **Retinoic acid induced NSC-34 differentiation.** Phase contrast micrographs of NSC-34 cells grown in 10% FBS (**A**), with addition of 1 μM retinoic acid (RA) (**B**), 10 μM RA (**C**), or 10 μM RA with 100ng/ml BDNF (**D**) after 7 days in culture . Addition of 1 μM RA to 10% FBS media increases proliferation where as supplementation with 10μM RA significantly reduces cell growth (**F**). Quantification of cell neurite length as a marker of cell differentiation shows that 10μM RA treatment induces cell differentiation when compared to cells grown in 10% FBS alone, however the addition of BDNF does not induce increased differentiation (**F**) as reported in other neuroblastoma cell lines. (n = 6, mean ± SEM). (*$p<0.05$, two-way ANOVA difference to 10% FBS culture; Scale bar = 50μm).

RA also induces TrkB expression, which in turn mediates BDNF driven survival and differentiation of SH-SY5Y NB cells in serum free media (Kaplan *et al.*, 1993). Concentrations of 1-10μM RA are sufficient enough to induce this effect (Abemayor & Sidell, 1989).

NSC-34 cells were cultured in 10% FBS medium with the addition of 1μM RA, 1μM plus 100ng/ml BDNF, 10μM RA or 10μM RA with added 100ng/ml BDNF for up to 10 days (Figure 3.5A-D). Subculture in 1μM RA did not inhibit cell growth, nor did it result in an increased population of differentiated cells. Instead, we observed an increase in cell proliferation (Figure 3.5E), and a predominant N-type morphological population. Cells grown in 1μM RA supplemented media needed frequent media change would reach confluence and detach within 2-3 days after plating. Subculture in 1μM RA for 5-7 days followed by addition of 100ng/ml BDNF did not reduce cell proliferation, increase differentiation, or enhanced the survival of NSC-34 cells in a BDNF-dependent manner.

In contrast, a ten-fold increase of RA to 10 μM partially produced effects resembling observations previously described in other NB cells. NSC-34 cell growth was significantly reduced (Figure 3.5E), and cells differentiated more readily compared to control and 1μM RA treatments (Figure 3.5F). However, the addition of BDNF did not produce cell differentiation that was significantly different to cells grown in 10μM RA only (Figure 3.5F). NSC-34 cells grown in 10μM RA supplemented media remained adherent, and were suitable for further subculture.

3.B.6 Optimization of culture growth matrix for glass coverslips

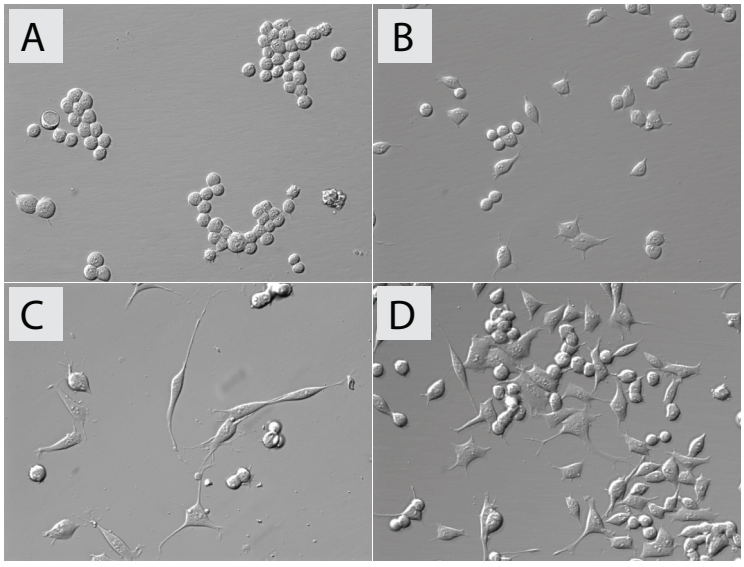
Sub-culturing NSC-34 cells on poly-D-lysine and / or rat-tail collagen coated glass coverslips, although necessary for adhesion of the cells, had negative effects on cell survival rates in low serum conditions. This problem was further complicated by the need to wash out the dead cells after the first 48 hrs of low serum treatment, prior to experimental treatments, which often resulted in detachment of the surviving cells. To overcome these difficulties we

experimented with various combinations of coating matrices. The cells grow extremely poorly on poly-D-lysine coated coverslips (Figure 3.6A), and very few cell extend processes under these conditions, regardless of the serum content of the media. Type 1 rat-tail collagen coated glass coverslips were better suited to cell growth with cells fairing substantially better in terms of adhesion and cell process extension, however cell detached after 4 to 5 days of sub-culture. A combination of poly-D-lysine and rat-tail collagen produced significantly better differentiation (Figure 3.6 B) irrespective of media type used, but also improved adhesion in long-term cultures.

Since the NSC-34 cells have been reported to adhere to laminin (Hunter *et al.*, 1991), we supplemented our poly-D-lysine or rat-tail collagen matrices with 2-5μg of laminin during the coating procedure. Cells grown on a combination matrix of poly-D-lysine, rat-tail collagen and laminin (PCL matrix) (Figure 3.3 C) sprouted processes more readily and did not detach even after 10 days in culture. Cells grown on PCL matrix with N-2 defined media (Figure 3.3 D), looked healthier and more robust with more cells sprouting projections within a 24-hour period after plating. Generally increasing FBS content of culture media in cultures plated on the PCL matrix from 1% to 3% significantly increased the survival rates as well as adhesion of these cells, making it possible to undertake experimental procedures more readily.

3.B.7 Long-term culture in serum free media

Under normal (10% FBS) or serum reduced (1% FBS) culture conditions the cells usually detached within 5-7 days or when they reached a confluent state. At this stage the cells required subculture, following which they reverted back to a neuroblastoma-like round morphology and subsequently differentiated 1-2 days after re-seeding. In Contrast, defined N-2 media maintained adherent and differentiated NSC-34 cells for periods of up to 21 days with regular media change every 2-3 days (Figure 3.7A-B). Cells maintained in N-2 media also differentiated



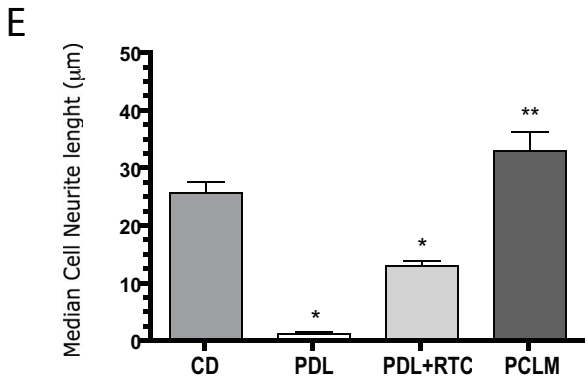


Figure 3.6 **Optimization of culture substrate matrix for glass coverslips.** Cells subcultured on 30mm glass coverslips 4 days after initial plating in 3% FBS media coated with **(A)** 0.1mg/ml poly-D-lysine (PDL); **(B)** poly-d-lysine and 0.1mg/ml rat tail collagen matrix (PDL+RTC). **(C)** poly-d-lysine, rat tail collagen and 2µg/ml laminin matrix (PCLM). **(D)** cells grown in culture dish (CD). Neuronal phenotype, including projection on glass coverslips is best achieved on a PCL substrate matrix. **(E)** Effects of different substrates on cell differentiation. Populations grown in plastic culture dishes (CD) versus cells grown on PDL only, PDL+RTC or PCL matrix 96 hrs (n = 4, mean ± SEM). (*$p<0.001$, **$p<0.05$ two-way ANOVA difference to CD growth conditions).

more rapidly following subculture, and provided highly differentiated populations within 3-4 days. In these cultures the signs of differentiation were prominent within 12 hours following subculture. Further, these cells remained attached to the substrate and were resistant to titration with a Pasteur pipette. Withdrawal of the N-2 nutrient mixture and reintroduction of 3% FBS supplemented media maintained these cells for 1-2 days, after which the cells detached and died. Reintroduction of NSC-34 cells to 10% FBS media following N-2 culture increased cell proliferation but decreased cell differentiation as seen in cells only grown in 10% FBS.

3.C Discussion

The choice of the NSC-34 clones over the other available hybrids (NSC-19, MN-1 and VSC-4.1) as an in-vitro model for motor neuron studies was based on many of its inherent morphological and physiological properties resembling primary motor neuron spinal cord cultures in neuronal development. Previously reported characteristics such as poor adhesion and limited ability to culture NSC-34 cells in defined media presented the fundamental obstacles in the ability to use these cells in experiments where adequate substrate adhesion and serum free culture are necessary.

Although existing growth protocols (Hunter *et al.*, 1991; Cashman *et al.*, 1992; Eggett *et al.*, 2000) for this cell line were adequate for studies evaluating neurotoxicity (Liu *et al.*, 2002; Rizzardini *et al.*, 2003; Weishaupt *et al.*, 2006; Wen *et al.*, 2006), the present study increases the future potential of NSC-34 cell line by establishing standardized growth conditions for highly differentiated and adherent cultures in serum supplemented or defined media types.

The effects of different media formulations on NSC-34 cells were analyzed by quantifying cell survival and proliferation, cell type morphology, cell body size and neurite outgrowth. NSC-34 cells maintained in 1% or 2% FBS have reduced survival rates when compared to cell grown in standard 10% FBS. Increasing the

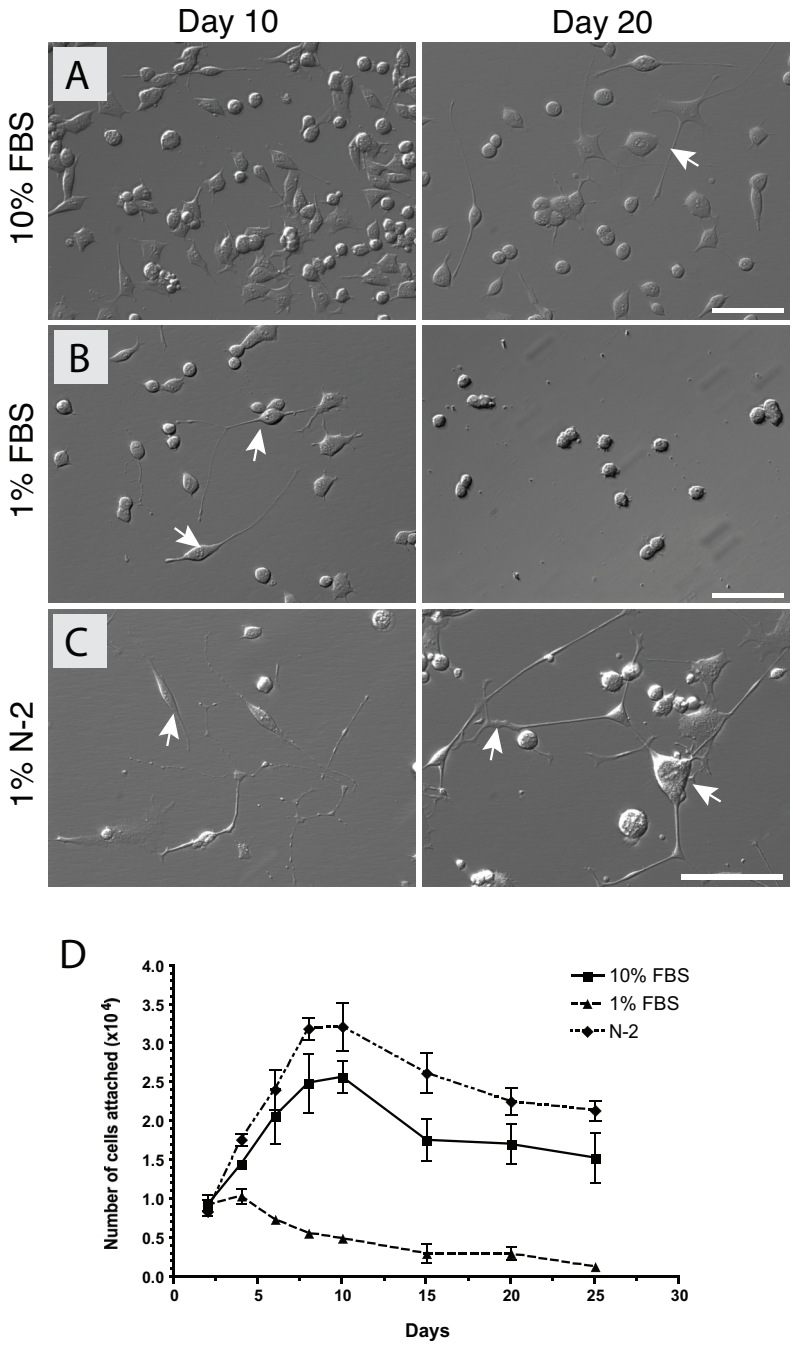


Figure 3.7 **Long term culture of differentiated NSC-34 cells.** Cells subcultured on 30mm glass coverslips coated with PCL matrix in 10% FBS **(A),** 1% FBS media **(B)** or 1% N-2 defined media **(C)** at 10 and 20 days with fresh media change every 48 hours. **(D)** Quantification of cell attached at given time intervals. Cells proliferate 7-10 days following subculture in 10% FBS or 1% N-2 media, but not 1% FBS media, which causes differentiation of cells that survive serum withdrawal for up to 10 days, after which the remaining cells also die . Both 10% FBS and N-2 media support cells for up to 21 days, however significant differences were observed in cell differentiation (arrows) and substrate adhesion (n = 4, mean ± SEM; Scale bar = 100μm).

FBS concentration in culture media to 3% abolished serum withdrawal associated cell death, and still produced a significant increase in cell differentiation. Switching cultures from standard growth conditions to serum free defined media supplemented with N-1 or N-2 also eliminated cell death as a result of serum withdrawal. Maintenance of NSC-34 cell in all serum reduced or serum free media formulations also produced significant increase in the morphological subtypes from N-type cells to differentiated S-type cells (Figure 3.7).

Reduction of serum in culture media is essential for profound differentiation of NSC-34 cells, and this is clearly supported by the observation that all reduced serum media initiated the growth of significantly longer neurites than observed in control cultures. The use of N-2 defined nutrient mixture produced the most notable improvements in terms of adhesion and the amount of neurite sprouting. Cells maintained in N-2 media also had the highest ratio of differentiating S-type cells, largest soma size and the longest neurites compared to cultures grown in FBS supplemented media.

Although NSC-34 cells differentiate spontaneously to varying degrees dependent on the media they're maintained in, some cell lines of neuroblastic origin such as the SH-SY5Y clone have been cultured in the presence of RA to reduce cell proliferation and induce drastic cell differentiation in 10-15% FBS containing media (Kaplan *et al.*, 1993; Encinas *et al.*, 2000; Edsjo *et al.*, 2003). Additional evidence suggests that RA induced neuronal differentiation of NBs occurs through modulation of TrkB neurotrophin receptor expression (Kaplan *et al.*, 1993; Encinas *et al.*, 2000). To establish whether this method may provide an alternative way of maintaining adherent and differentiated cultures, cells were treated with RA concentrations ranging from 1-10 μM. Long-term culture (7-10 days) of NSC-34 cells in 1μM retinoic acid resulted in increased cell proliferation. Subsequent addition of BDNF had no effect on proliferation of differentiation of these cells. In contrast, increased concentration of retinoic acid at 10 μM had the opposite effect on NSC-34 cells. Cell proliferation was significantly reduced, and cell differentiation increased compared to cells grown in standard 10% FBS media. However the addition of BDNF to RA cultures did not induce increased

differentiation measured by neurite length, when compared to the RA only groups. The observation that BDNF did not effect cell differentiation may reflect poor RA mediated upregulation of full-length TrkB receptors responsible for BDNF signaling.

Laminins are structural scaffolding glycoproteins of basement membranes that critically contribute to cell survival, differentiation, cell shape and movement, and maintenance of tissue phenotypes (Colognato & Yurchenco, 2000). Laminin is also necessary for the maintenance and differentiation of primary motor neuron cultures (Calof & Reichardt, 1985; Camu & Henderson, 1992). S-laminin, a homologue of laminin has been shown to bind NSC-34 cells through a motor neuron selective attachment site (Hunter *et al.*, 1989; Hunter *et al.*, 1991). Addition of 3μg/ml laminin to coated coverslips in a two-step process with poly-d-lysine and rat-tail collagen (PCL matrix) sufficiently maintained long-term, adherent cultures in both serum-supplemented and defined media. Although the presence of laminin enhanced the differentiation of NSC-34 cells, it was the increased cell adhesion that was most noticeable in all media types. In 10% FBS containing media the use of trypsin was required to disassociate the cells prior to subculture, where as cells cultured without the PCL matrix were easily detached by gentle pipetting. In addition, cultures maintained in media containing FBS never reached differentiation rates observed in serum-free cultures. This observation suggests that FBS may have inhibitory effects on the differentiation of NSC-34 cells. The combination of the PCL matrix and N-2 defined media provided the most suitable conditions for adherent and rapidly differentiating cultures on glass coverslips.

The combination of N-2 defined media and PCL matrix enabled prolonged culture of NSC-34 cell for up to 21 days. This represents an extension of 10 days compared to previously described methods for this cell line (Cashman *et al.*, 1992; Durham *et al.*, 1993). Although N-2 maintained cultures never reached confluence or a homogenous population of differentiated cells, they did not require subculture and always maintained a higher ratio of differentiated cells when compared to all other culture conditions.

In summary, this chapter describes an improved, standardized method for growing stably adherent and highly differentiated NSC-34 motor neuron-like cells for up to three weeks without the need for subculture in defined media. The only requirement for cell survival and differentiation within this time frame is regular media change. This critical step the in the characterization of NSC-34 cells provides an opportunity to diversify their usefulness in the field of general motor neuron-specific biology.

Chapter 4: Characterization Of NSC-34 Cells For The Use In Neurotrophin Receptor Trafficking Studies

4.A Introduction

Despite the fact that the NSC-34 cell line has been widely used to study various aspects of motor neuron biology, no studies to date have examined its potential for investigating neurotrophin biology. The preferred in-vitro cell lines for the study of neurotrophins have been the PC-12 cells and a handful of neuroblastoma cells (Greene & Tischler, 1976; Kaplan, 1998). The PC-12 cells in particular have been instrumental in the development of the first neurotrophin receptor specific monoclonal antibodies (Chandler *et al.*, 1984; Taniuchi & Johnson, 1985), molecular cloning of $p75^{NTR}$ (Chao *et al.*, 1986) and identification of tyrosine kinase receptors (Klein *et al.*, 1991; Kaplan *et al.*, 1991b) amongst others. The continual use and characterization of the PC-12 cell contributed greatly to investigations of neurotrophin specific receptor-ligand interactions (Venkatakrishnan *et al.*, 1990; Squinto *et al.*, 1991; Klein *et al.*, 1992; Wolf *et al.*, 1995), culminating in the development of the signaling endosome hypothesis (Beattie *et al.*, 1996; Grimes *et al.*, 1996; Howe *et al.*, 2001).

The wealth of knowledge gained from using PC-12 cells has greatly advanced our understanding of many critical aspects of neurotrophin interaction with their receptors. However, there is a critical aspect of neurotrophin biology that warrants the study of neurotrophins in different cell types. One of the most challenging aspects of neurotrophin biology is the well-documented observation that different neuronal populations co-express discreet combinations of $p75^{NTR}$ and Trk receptors (Bibel *et al.*, 1999; Chao, 2003; Ernfors, 2001; McAllister, 2001). As a consequence of cell type specific receptor expression, neurotrophins are able to elicit varied and contrasting effects such as survival and cell death (Bartlett *et al.*, 1998; Schweigreiter, 2006; Hennigan *et al.*, 2007).

The motor neuron specific expression of neurotrophin receptors (Chiu *et al.*, 1993; Escandon *et al.*, 1994; deLapeyriere & Henderson, 1997; Henderson *et al.*, 1998; Buck *et al.*, 2000) and the effect of neurotrophins on motor neuron populations (Yan *et al.*, 1993; Sendtner *et al.*, 1996; Sendtner *et al.*, 2000) are well documented, providing an information-rich framework for direct comparison

with the NSC-34 cell line. The previous chapter clearly established an improved cell culture protocol for maintaining differentiated and adherent cells within a time frame comparable to PC-12 cells (Greene & Tischler, 1976). The purpose of this chapter is to establish the whether NSC-34 cells share enough similarities with motor neurons to make them suitable for neurotrophin receptor trafficking studies.

The neurotrophin receptor profile of the NSC-34 cell line has not been clearly established. The presence of $p75^{NTR}$ and full-length TrkB receptors with biological activity has been briefly described (Turner *et al.*, 2004). However, the neurotrophin receptor profile expressed by motor neurons changes in the first postnatal days from $p75^{NTR}$ expressing cells, to $p75^{NTR}$ deficient cells (Escandon *et al.*, 1994). Since improved culture methods have made growing these cells routine for up to 3 weeks, it may be valuable to establish whether the receptor expression on the NSC-34 cells changes with age. One of the few studies to examine NSC-34 receptor expression demonstrated that changes in glutamate receptor expression occur over a period of 2 weeks (Eggett *et al.*, 2000). Establishing a physiological outcome as a result of exogenous neurotrophins and characterization of antibodies for the use in receptor trafficking will be the remaining focus of this chapter.

4.B Analysis of Cell Membrane and Cytosolic Markers

4.B.1 FACS and Immunofluorescence screening

FACS flow cytometry was used as a preliminary screening tool for all primary fluorescent antibody conjugates produced for the purpose of this study, as well as an initial detection method for receptors of interest outside of the neurotrophin family. A broad range of neuronal antibodies was screened on the NSC-34 cells. Many of the commercial, primarily polyclonal antibodies, lacked mouse antigen specificity and therefore only the positive results are discussed bellow.

NSC-34 cells express a number of standard markers for developing and

mature motor neurons. Neurotrophin receptors $p75^{NTR}$ (MLR-2, 20μg/ml), tyrosine kinase B (Trk-B, 10μl/ws), tyrosine kinase C (Trk-C Ab07-226, 20μg/ml), ciliary neurotrophic factor receptor-α (CNTFR-α) and glial derived neurotrophic factor (GDNF) receptor GFR-α were identified. The most recently included member of the neurotrophin family, the sortilin receptor is also expressed (Ab16640, 20μg/ml). Motor neuron cholinergic marker, vesicular acetylcholine transporter (VAChT V-007, 20μg/ml) and cell membrane lipid raft minor ganglioside subtypes GT1b (AbGT1b, 20μg/ml), which appeared to be expressed at higher levels (median cell counts) and GQ1b (MAb321, 20μg/ml) were also detected (Figure 4.1). The presence of GT1b and GQ1b provides evidence for expression of ganglioside family isoforms previously not examined due to the lack of availability of specific antibodies (Matsumoto *et al.*, 1995). Flow cytometry measurements represent fluorescent signal shift of viable gated populations (green phase) compared to X-63 FITC (red phase) conjugated control IgG as negative control for all experiments. Expression of cell surface proteins detected by FACS was confirmed with immunofluorescence of markers that returned positive results (Figure 4.2).

4.B.2 Western Blot analysis of neurotrophin receptors and endogenously expressed neurotrophins

The only study to investigate neurotrophin receptor expression of NSC-34 cells reported the presence of $p75^{NTR}$ and neurotrophin receptor homologue NRH2, as well as the full-length isoform of TrkB, which appeared to be physiologically active under normal growth conditions (Turner *et al.*, 2004). Western blot analysis of NSC-34 lysates from standard and reduced-serum culture conditions confirmed the presence of neurotrophin receptors detected by flow cytometry including sortilin and TrkC in addition to $p75^{NTR}$ and TrkB. However in contrast with the observations by (Turner *et al.*, 2004) the TrkB but also TrkC receptors detected in cell lysates appeared predominantly in the truncated isoforms at ~ 95kDa (Figure 4.3A), with less intense bands representing the full-length (~140kDa) isoforms of these receptors.

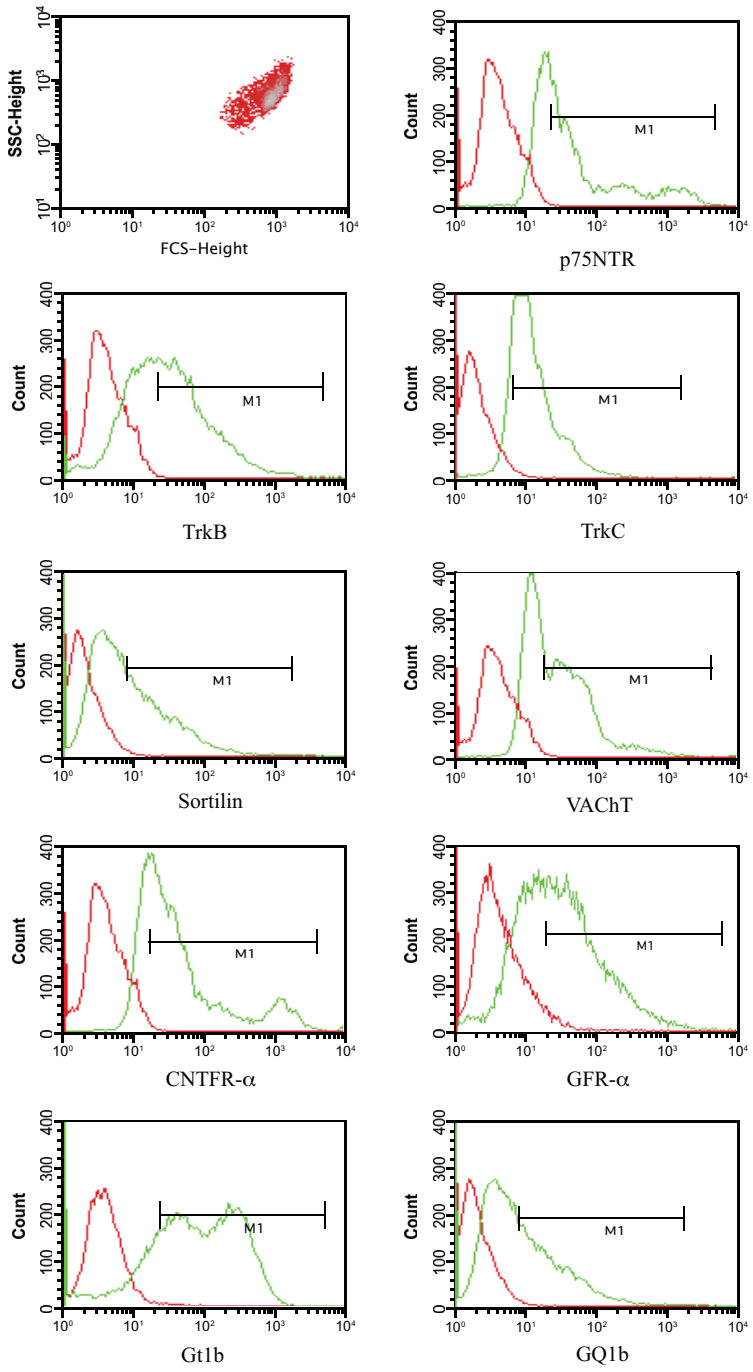


***Figure 4.1* FACS screening of NSC-34 cells.** Screening NSC-34 cells using fluorescence activated cell sorting (FACS) indicated the presence of the common neurotrophin receptor p75NTR, tyrosine kinase receptors B and C, and the sortilin receptor. Other growth factor receptors expressed include the CNTF receptor CNTFR-α and the GDNF receptor GFR-α. Cholinergic marker VAChT was detected with an antibody specific to the C-terminus of the transporter exposed on the extracellular membrane during ACh release. Presence of lipid raft components Gt1b and GQ1b (previously not described) was also confirmed. (Red lines represents control IgG X-63-FITC, green lines represent cell surface markers named bellow the x-axis). Cultures were grown in defined 1% N-2, 1% NEAA and 1% PSG medium for 5 days prior to experimental procedures.

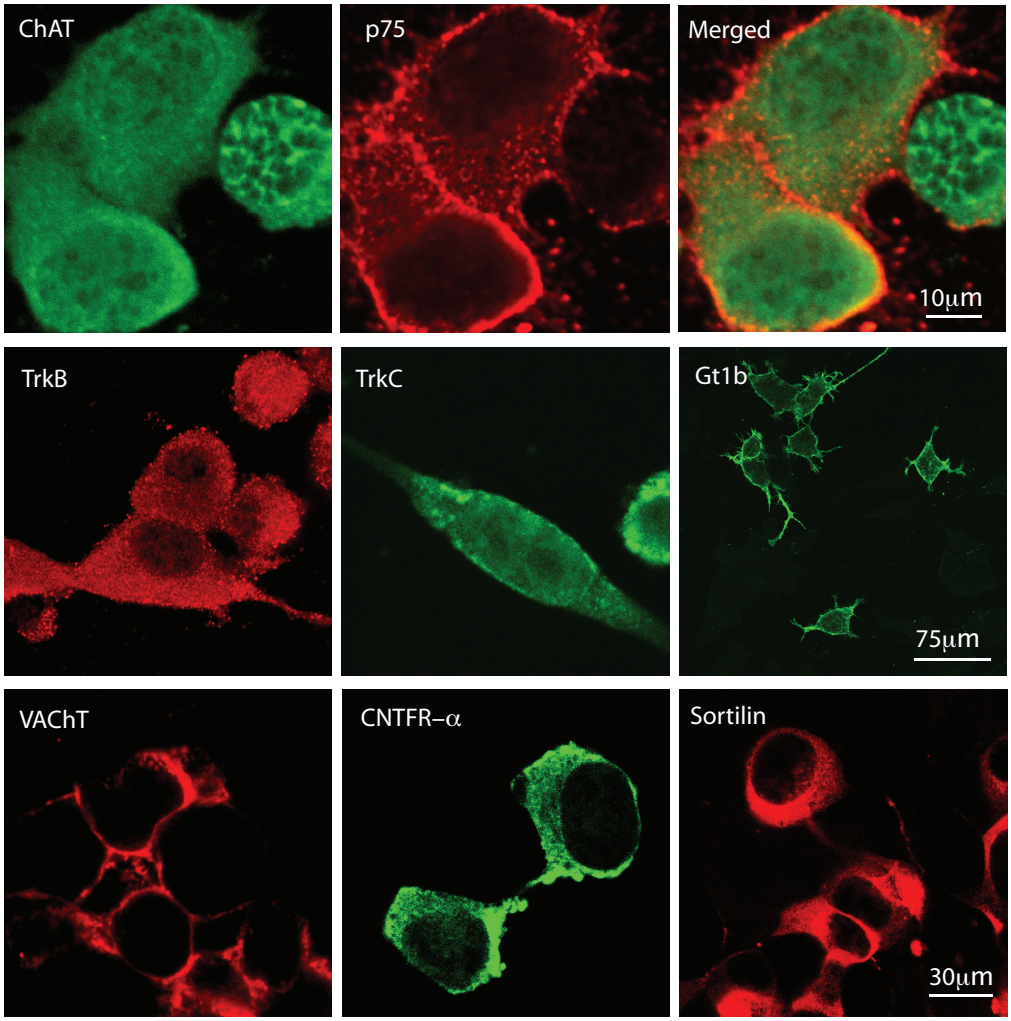


Figure 4.2 **Immunofluorescence of motor-neuronal markers expressed by NSC-34 cells.** NSC-34 cells express two standard markers for motor neurons, namely p75 neurotrophin receptor (p75NTR) which is preferentially expressed in the embryonic and neonatal spinal cord and the rate-limiting enzyme for the neurotransmitter acetylcholine, choline acetyltransferase (ChAT). Other motor neuron markers include neurotrophin tyrosine kinase receptors B and C (TrkB, TrkC). Another cholinergic marker, the vesicular acetylcholine transporter (VAChT), as well as ciliary neurotrophic factor receptor (CNTFR-α), ganglioside Gt1b and sortilin receptor expression was also detected with antibodies raised against the protein extracellular domains.

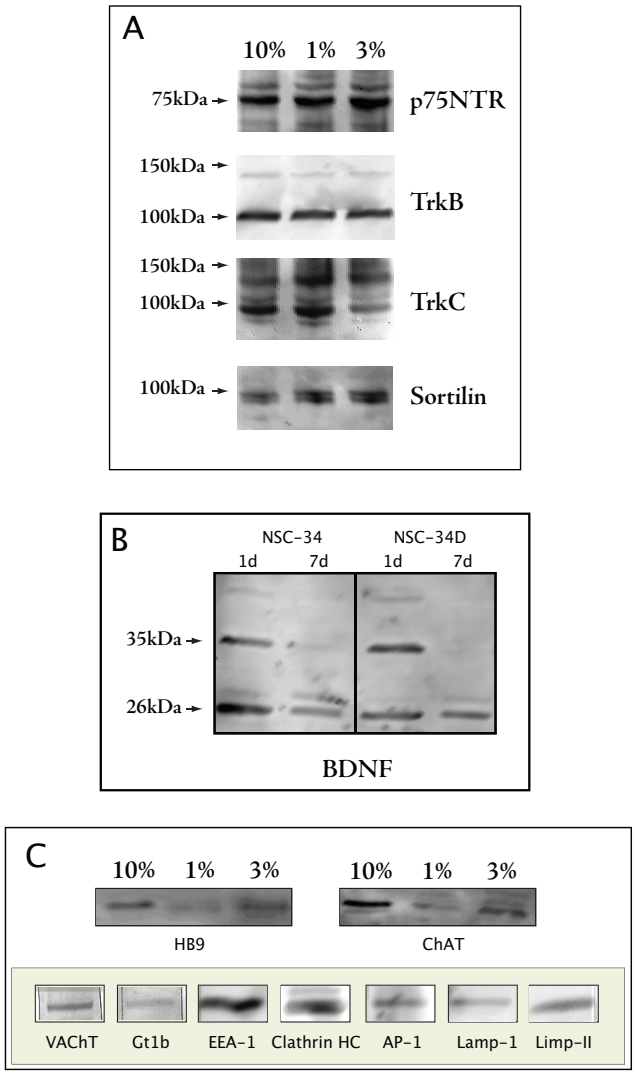


Figure: 4.3 **Western blot analysis of neurotrophin receptors and intracellular transport proteins.** (A) NSC-34 (10% FBS media) and NSC-34D (3% and 1% FBS media) cells express a neurotrophin receptor profile consistent with developing motor neurons, including the presence of p75NTR, TrkB, TrkC and sortilin, but no TrkA (data not shown). TrkB and TrkC receptors are present in both full length (140 kDa) and truncated (100 kDa) isoforms. The presence of BDNF (B), but no NGF, NT-3 or NT-4 (data not shown) was detected. BDNF was detected at 26 kDa indicating the homodimer and also at 35 kDa consistent with pre-proBDNF. Both isoforms of BDNF were detected in normal (NSC-34) and differentiated (NSC-34D) cells, but only the homodimer was detected in these cells after 7 days of culture. NSC-34 cells express motor neuron specific markers HB9 and ChAT in cells lysates from normal (10%) and low serum (1-3%) cultures. Other important motor neuron markers such as the cholinergic marker VAChT, and ganglioside Gt1b are expressed. (C) Critical markers of the endocytic pathway including EEA-1, clathrin heavy chain (clathrin-HC), adaptor protein-1 (AP-1), and the lysosomal markers Lamp-I and Limp-II were also shown to be present.

This is an interesting observation as the western blotting procedure was identical to the one described in (Turner *et al.*, 2004). In addition, preliminary data from mRNA detection in RT-PCR experiments using cDNA specific for truncated isoforms of TrkB (Klein *et al.*, 1990; Middlemas *et al.*, 1991) and TrkC (Tsoulfas *et al.*, 1993; Palko *et al.*, 1999), so far indicate the presence of TrkB.T1, an isoform lacking the kinase domain of the full-length receptor (data not shown). The expression of TrkA was not detected in any cell lysates or membrane preparations, providing additional support for NSC-34 motor neuron-like phenotype.

Spinal motor neurons express combinations of BDNF, NT-3 and NT-4 during development and in adulthood (Funakoshi *et al.*, 1993; Kobayashi *et al.*, 1996; Buck *et al.*, 2000) where these neurotrophins play important roles in synaptic differentiation and plasticity (Thoenen, 2000; McAllister, 2001). Taking into account the spinal cord origin of NSC-34 cells, cell lysates were also screened for the presence of endogenously expressed neurotrophins. The loading of 20μg cell lysate per lane was sufficient to detect the presence of endogenous BDNF in both freshly subcultured and differentiated NSC-34 cells in Western blots (Figure 4.3B). Two bands were detected that correlate with the known molecular weights (MW) for the BDNF homodimer and pre-proBDNF at 26 kDa and 35 kDa respectively. Interestingly, bands corresponding to the MW of pre-proBDNF were only detected in the early stages following subculture, and were absent in cells cultured for 7 days or longer (Figure 4.3B). NGF, NT-3 and NT-4 were not detected in any NSC-34 lysates or cell membrane preparations.

To assess whether NSC-34 cell released BDNF into the media, cells were cultured in both serum and serum-free media until the media in the culture flasks (Figure 4.3), was conditioned and cells started to die. The conditioned media was collected, filtered and subsequently used to culture native or RA primed SH-SY5Y neuroblastoma cells for up to 4 days. Culturing SH-SY5Y cells in RA has been demonstrated to produce cultures that are BDNF dependent for their survival (Kaplan *et al.*, 1993; Encinas *et al.*, 2000). The ability to culture BDNF responsive, but not normal SH-SY5Y cells for up to 96hr provides support for the

release of biologically active BDNF by NSC-34 cells. Although the NSC-34 cells share many characteristics with motor neurons including the above mentioned neurotrophin receptor profile and ChAT expression amongst others (Cashman *et al.*, 1992), to date no study has reported the expression of any motor neuron-specific genetic markers.

One possible criticism of using $p75^{NTR}$ expression and cholinergic characteristics as concrete evidence of motor neuron phenotype, is that motor neurons express $p75^{NTR}$ only during early development (Yan & Johnson, 1988; Wiese *et al.*, 1999) following injury (Bussmann & Sofroniew, 1999; Johnson *et al.*, 1999; Coulson *et al.*, 2000), or development of motor neuron disease (Chiu *et al.*, 1993; Copray *et al.*, 2003). Motor neurons are also not the only cells in the cortico-spinal tract to express cholinergic properties (Phelps *et al.*, 1984).

Since NSC-34 cells originated from a fusion with spinal-cord cells enriched neuronal population, evidence of motor neuron specific genetic markers should resolve any uncertainty regarding their motor-neuron phenotype. Antibodies against Islet-1 (Pfaff *et al.*, 1996) and Homeobox-9 gene (HB-9) (Arber *et al.*, 1999), as well as antibodies against established cholinergic markers ChAT and VChAT were used to screen NSC-34 lysates prepared from standard and reduced-serum cultures. Although the transcription factor Islet-1 was not detected in any NSC-34 lysate preparations, the presence of motor neuron specific markers HB9, in combination with $p75^{NTR}$, ChAT, VAChT and the lack of TrkA receptors provide further support for a motor-neuronal phenotype of NSC-34 cells (Figure 4.3C).

In addition to screening the cells for neurotrophin receptors, Western blots were also carried out to confirm antibody specific detection of endogenous proteins associated with endosomal compartments. Components of the clathrin mediated endocytosis pathways associated with neurotrophin signaling events such as clathrin heavy-chain (C-HC), adaptor protein AP-1, adaptor protein AP-2, early endosomal autoantigen-1 (EEA-1), Rab GTPase Rab-4, lysosomal markers Lamp-1 and Limp-II, and lipid raft component GT1b not previously reported in

the NSC-34 cells (Figure 4.3 C).

4.B.3 Effects of Culture Media on Neurotrophin Receptor Expression

Post-mitotic motor neurons undergo changes in neurotrophin receptor expression during their lifespan. In rats, $p75^{NTR}$ is expressed in on motor neurons from E15 until the second postnatal week (Yan & Johnson, 1988), and is subsequently re-expressed in 10-15% of motor neurons in aged animals (30 weeks) (Johnson *et al.*, 1999). The full-length TrkB and TrkC receptor expression in embryonic and postnatal motor neurons always precedes the expression of the truncated forms of these receptors (Escandon *et al.*, 1994).

Since changes of receptor expression after 7 days in culture have been reported in the NSC-34 cells for glutamate receptors (Eggett *et al.*, 2000), and in-vivo neurotrophin receptor expression changes during various stages of development we wanted to confirm that the neurotrophin receptors expressed on the NSC-34 cell were present after long-term culture. Due to the need to perform future experiments in serum free media, we also needed to certify that transitions from serum supplemented to defined media did not affect neurotrophin receptor profile of these cells.

Neurotrophin receptor expression profile in NSC-34 cells is maintained at 10 days after plating in both serum reduced (1% and 3% FBS) and serum free N-2 defined media (Figure 4.4). $P75^{NTR}$ was consistently detected at slightly lower molecular weight of 70kDa, but expression did not appear affected by change in media growth conditions. TrkB receptor was detected only in the truncated form at ~100kDa, corresponding to one or both known isoforms of this 95kDa receptor. Long term retinoic acid and BDNF treatments, alone and combined, failed to increase the presence of the 140kDa full-length isoform, reported in other neuroblastoma cell lines (Kaplan *et al.*, 1993; Encinas *et al.*, 2000).

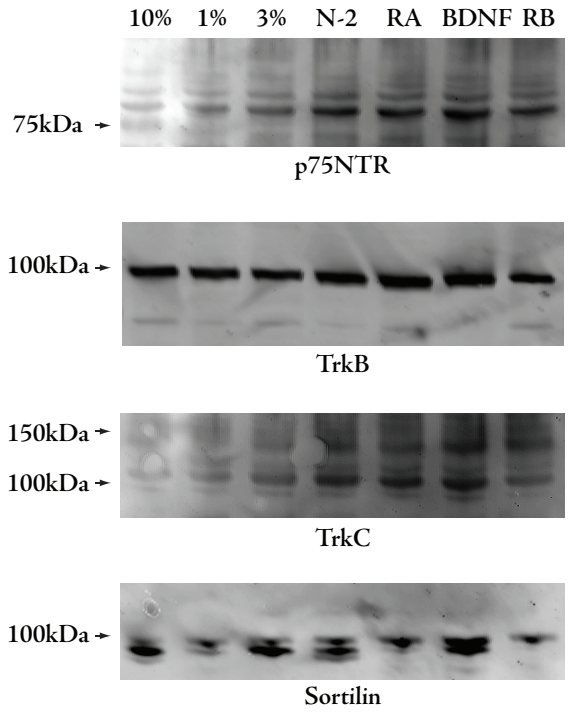


Figure 4.4. **Analysis of neurotrophin receptor expression in defined growth media.** NSC-34 express the neurotrophin receptors p75NTR, TrkB, TrkC and sortilin. This receptor expression profile is maintained at 7 days after plating in defined media (see legend bellow). p75NTR was detected at slightly higher molecular weight of aprox. ~ 78kDa. TrkB receptor was detected only in the truncated form at 100kDa. Retinoic acid and BDNF treatments alone and combined did not result in the increased presence of the 140kDa full length isoform as has been reported in other neuroblastoma cell lines. TrkC was present in both the truncated 100kDa and full length 140kDa isoforms. Sortilin expression seemed to decrease substantially in 1% FBS serum media , and media supplemented with retinoic acid. Interestingly the expression of sortilin in 1% FBS media supplemented with 100ng/ml BDNF showed stronger detection of the receptor compared to 1% FBS media alone.

Legend: **10%, 3%** and **1%** indicate percentage of FBS supplement in the growth media; (**N-2**) serum free media containing 1% N-2 supplement and 1% NEAA ; (**RA**) 1% FBS growth media supplemented with 10**μM** retinoic acid; (**BDNF**) 1% FBS media with added 100ng/ml BDNF; (**RB**) 1% FBS media with added 10**μM** retinoic acid and 100ng/ml BDNF.

In addition, TrkC appears to be present in both the truncated (95kDa) and full-length (140kDa) isoforms. The sortilin receptor expression appeared to decrease substantially in the low serum (1% FBS) media, and media supplemented with 10μM retinoic acid. Interestingly the expression of sortilin in 1% FBS media supplemented with 100ng/ml BDNF showed a stronger band of detection compared to 1% FBS media alone, however retinoic acid seemed to diminish the expression of sortilin in the presence of BDNF.

4.C Physiological responses of NSC-34 cells to neurotrophins

4.C.1 Effect of exogenous neurotrophins on NSC-34 cells

To determine whether neurotrophins provide any trophic support for NSC-34 cells consistent with the role of these growth factors in development (Klein, 1994; Henderson, 1996; Huang & Reichardt, 2001), cultures were exposed to individual neurotrophins for up to 4 days. Cell proliferation and differentiation rates between treated and untreated cultures were compared. Cell viability assays and neurite length measurements were used to investigate the effects of neurotrophins in undifferentiated (NSC-34) cultures and highly differentiated ($NSC\text{-}34_D$) cultures. Cells were exposed to NGF, BDNF, NT-3 or NT-4 in concentrations ranging from 10 – 200 ng/ml for 48 hours (Figure 4.5).

Treatment of normal and differentiated NSC-34 cells with individual neurotrophins failed to induce any significant differences in neurite extension. In undifferentiated cultures there was also no significant effect on cell proliferation when treated with NGF, NT-3 and NT-4. The only effect observed was a slight increase in cell viability at 10 ng/ml of BDNF treatment (Figure 4.5A).

In contrast, treatment of differentiated cultures with exogenous 200ng/ml NGF and NT-3 induced significant, dose-dependent cell death of 40-60% after 48 hours (NGF -$^{**}p<0.01$; NT-3 -$^{*}p<0.05$ difference compared to untreated controls). The rate of cell death did not reach 40% in any individual experiment (Figure 4.5C).

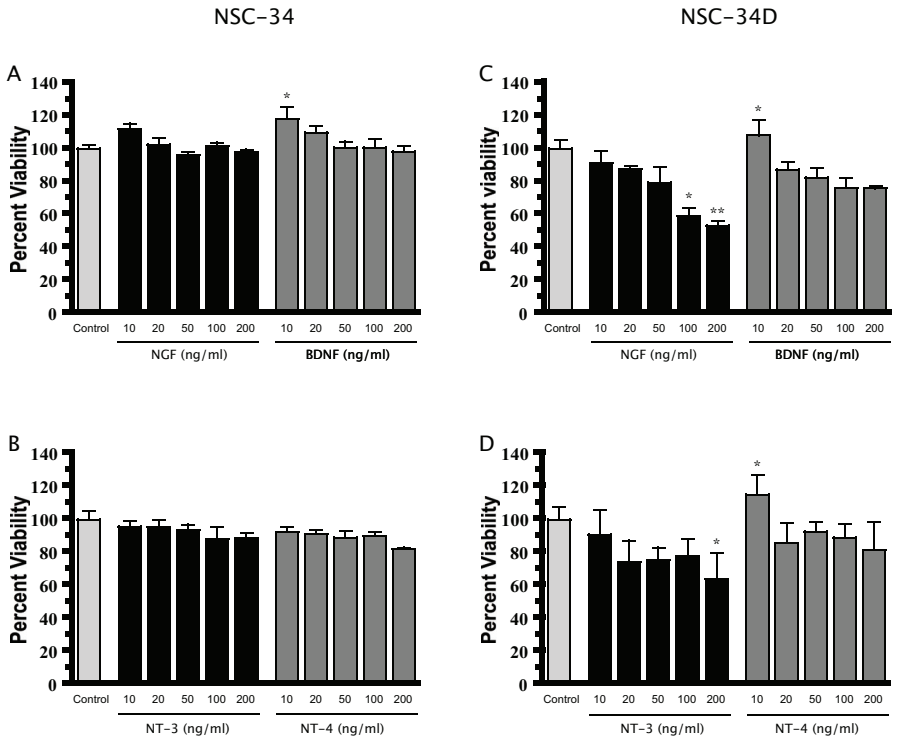


Figure 4.5 Effects of neurotrophins on NSC-34 and NSC-34D cell survival. NSC-34 and NSC-34D cells were treated with 10 – 200ng/ml concentrations NGF or BDNF **(A, C)**, NT-3 or NT-4 **(B, D)** in for 48 hr. Treatment of NSC-34 cells with neurotrophins under normal serum conditions (10% FBS) failed to produce any significant effects in these cells within 48 hours, with the exception of slight increase in viability at 10 ng/ml of BDNF treatment **(A)**. In NSC-34D cells high doses of NGF and NT-3 induced significant cell death, whereas BDNF and NT-4 stimulates marginal proliferation at small doses **(C, D)**. Cell viability under these conditions was assessed by acid phosphatase reduction (n = 5, mean ± SEM, *P < 0.05, **P < 0.01 vs. untreated cells).

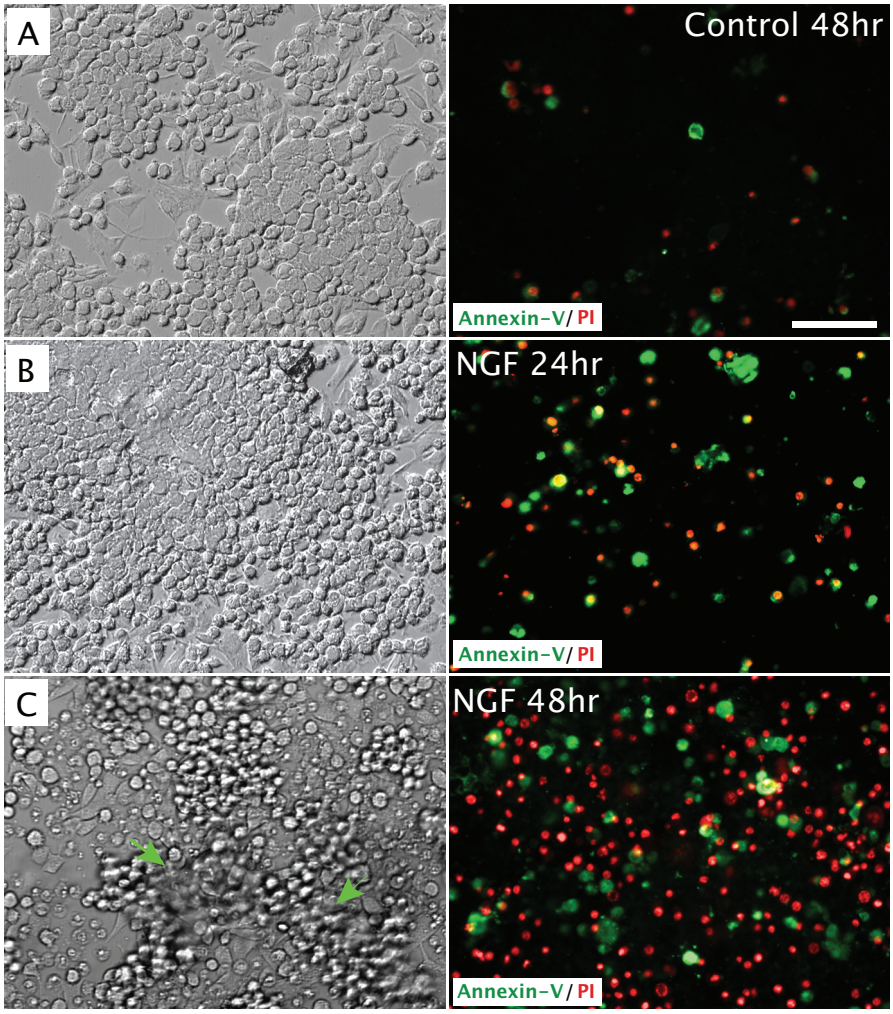
There was a small, but significant increase in proliferation with BDNF treatment (Figure 4.5C-D) and NT-4 (Figure 4.5D) treatments at 10ng/ml (*p<0.05 difference compared to untreated controls), but not at any higher concentrations tested.

4.C.2 Analysis of neurotrophin induced cell death

Since NGF and NT-3 treatment of NSC-34 cells results in cell death, further investigation into the type of cell death would contribute to understanding the possible mechanisms of neurotrophin induced cell death. We used annexin-V-FITC and propidium iodide as markers of early apoptosis and cell death respectively, on cells treated with 100ng/ml of NGF, NT-3 or BDNF. Nuclear DAPI stains were used as markers of total cell numbers in each experiment. Both NGF and NT-3 induced apoptosis in NSC-34 cell as indicated in (Figure 4.6) and (Figure 4.7) respectively. Only treatments at 24 and 48 hours were quantified. After 48 hours cells detached in significant numbers and produced significant irregularities in count analysis.

Apoptosis was evident within 24 hours in both NGF (25–30%) and NT-3 (20-25%) treated cells and the number of apoptotic cells in both treatments was significantly higher than in control groups (~5%) or cells treated with 100ng/ml of BDNF (~10%) (Figure 4.8; n=4; *p>0.01; **p>0.001, two-way ANOVA analysis). However, the rate of apoptosis was not as efficient as staurosporine (55-60%) treated positive controls (Figure 4.8; n=4; **p>0.001; *p>0.01).

At 24 hours, the ratio of annexin-V positive cells was slightly higher than PI stained cells. This trend was reversed 24 hours later. The rate of apoptosis at 48 hours was significantly higher compared to both control treatments and 24-hour time points in NGF and NT-3 cultures. However the ratio of PI stained cells at 48 hours was marginally higher that annexin-V positive cells, indicating cells in very late stages of apoptosis and cells already dead but not detached.



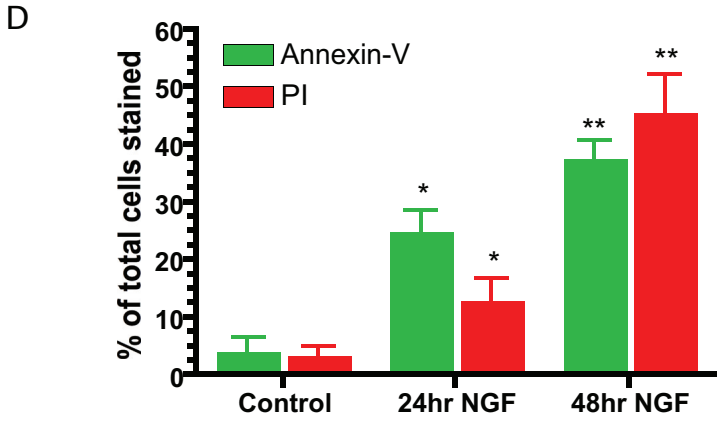


Figure 4.6 **Analysis of NGF induced cell death with anexin-V apoptotic stain.** Relief contrast and fluorescence micrographs of NSC-34 cells cultured in **(A)** 1% FBS control growth medium for 48 hours stained with annexin-V-FITC and PI. **(B)** Cells after 24 hour treatment with 100ng/ml NGF. Presence of both annnexin-V-FITC (green) and PI (red) stained indicating early and late apoptosis respectively. **(C)** An increase in both annexin-V (early apoptosis) and PI (late apoptosis/dead) staining was observed after 48 hours. **(D)** Quantitative analysis of apoptotic cells expressed as a total of cell population (n=4; *p>0.01; **p>0.001, two-way ANOVA). Number of apototic cells increased in a time dependent manner up to 48 hours. Data analysis after 48 hours was marred by poor substrate adhesion, with majority of apoptotic cells detaching (green arrows in C). Scale bar = 200μm.

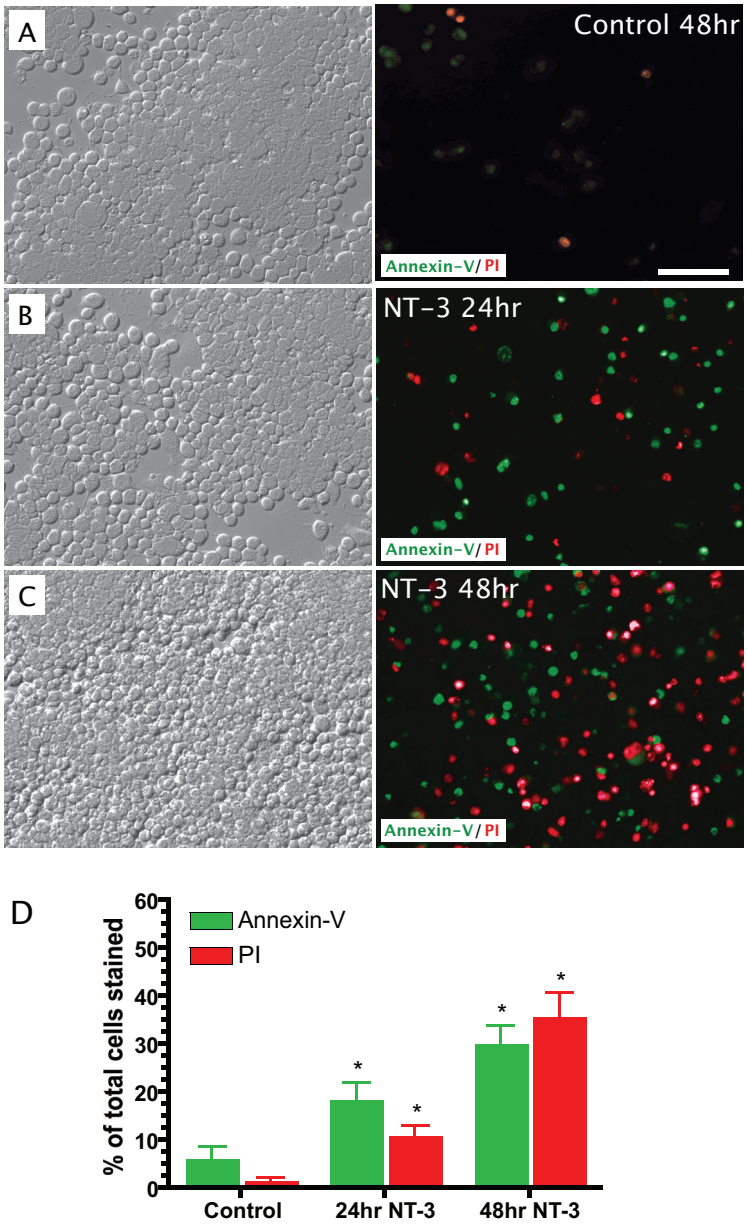
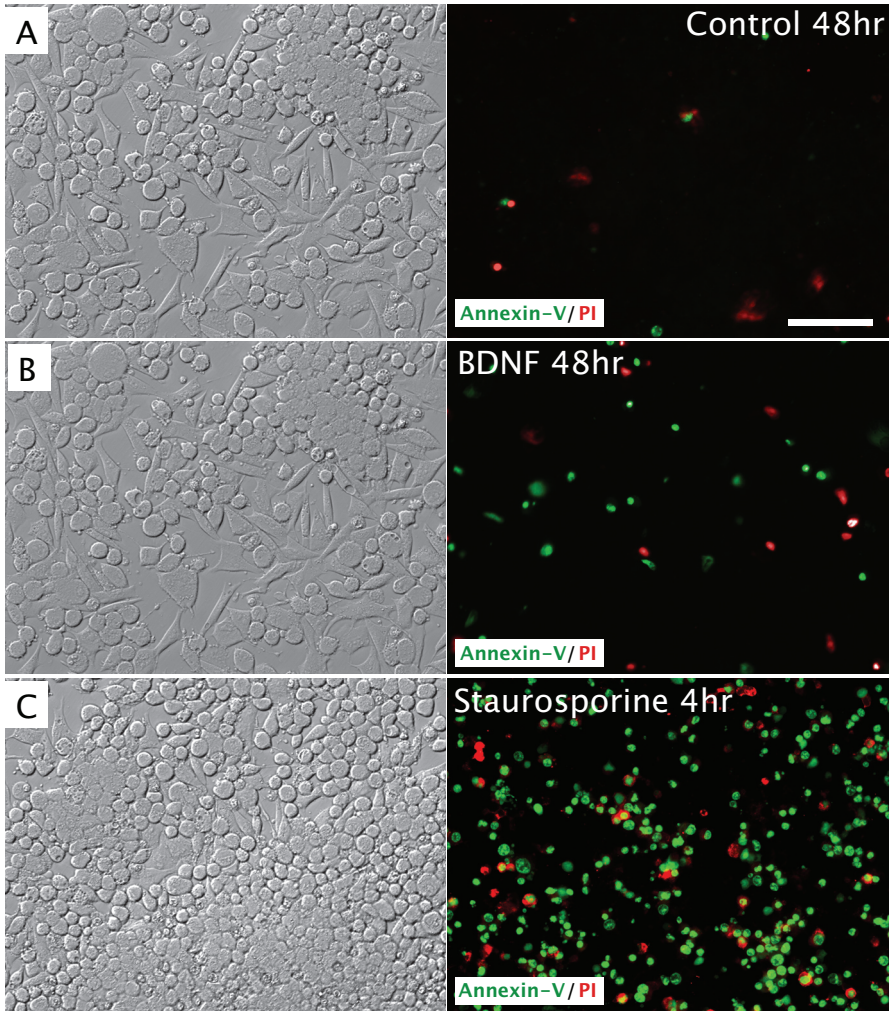


Figure 4.7. Analysis of NT-3 induced cell death with anexin-V apoptotic stain. Relief contrast and fluorescence micrographs of NSC-34 cells cultured in **(A)** 1% FBS control growth medium for 48 hours stained with annexin-V-FITC and PI. **(B)** Cells after 24 hour treatment with 100ng/ml NT-3. Similar to NGF, both annnexin-V-FITC (green) and PI (red) stained cells indicating early and late apoptosis were observed with NT-3 treatment, and both annexin-V (early apoptosis) and PI (late apoptosis/dead) staining increase was observed after 48 hours **(C)**. **(D)** Quantitative analysis of apoptotic cells expressed as a total of cell population (n=4; *p>0.01; two-way ANOVA). Number of apototic cells increased in a time dependent manner up to 48 hours. (Scale bar = 200μm).



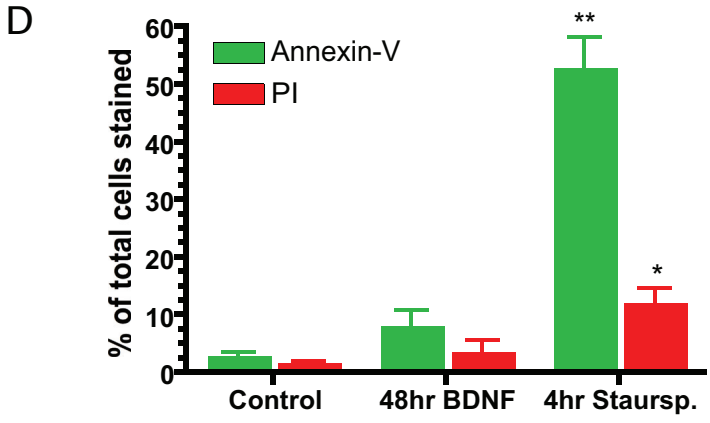


Figure 4.8 **Analysis of BDNF and Staurosporine induced cell death with anexin-V apoptotic stain.** Relief contrast and fluorescence micrographs of NSC-34 cells cultured in **(A)** 1% FBS control growth medium for 48 hours stained with annexin-V-FITC and PI. **(B)** Cells after 24 hour treatment with 100ng/ml BDNF. Similar to control treatments, both annnexin-V-FITC (green) and PI (red) staining is at background levels or marginally higher. Annexin-V staining was dominant in positive control cells treated with 10μM staurosporine for 4 hours, and PI staining observed after 48 hours was slightly but significantly higher **(C)**. **(D)** Quantitative analysis of apoptotic cells expressed as a total of cell population (n=4; **p>0.001; *p>0.01, paired t-test). (Scale bar = 200μm).

4.D. Neurotrophin receptor interactions with receptor specific antibodies

4.D.1 Effects of neurotrophins on binding of $p75^{NTR}$, TrkB, TrkC and Sortilin

Competition between antibodies and ligands targeting the same receptor may produce unusual observations when studying receptor internalization dynamics. In the case of $p75^{NTR}$, MC192 antibodies binding to $p75^{NTR}$ have been demonstrated to significantly increase binding of NGF to the receptor (Taniuchi & Johnson, 1985; Yan *et al.*, 1988), easily quantified by immunofluorescence (Bronfman *et al.*, 2003). In contrast, another antibody targeting the extracellular domain of $p75^{NTR}$ (REX) has been shown to completely inhibit receptor internalization (Weskamp & Reichardt, 1991).

Recently, TrkB antibodies have been generated that mimic the function of BDNF, including activation of downstream signaling molecules by the TrkB receptor (Qian *et al.*, 2006). Interestingly, these antibodies have been confirmed to recognize unique binding sites on TrkB, distinct from sites involved in BDNF docking (Qian *et al.*, 2006), highlighting the possible biological significance of ligand independent receptor-antibody interactions. Since none of the antibodies available against $p75^{NTR}$ recognize the mouse form of the receptor, we needed to characterize new $p75^{NTR}$ antibodies, which bind the mouse isoform of $p75^{NTR}$ (Rogers *et al.*, 2006). Although the primary focus of the next chapter is $p75^{NTR}$ trafficking observed via fluorescent antibody conjugates, we tested antibodies against all neurotrophin receptors expressed by the cell line.

To investigate whether neurotrophins interfered with the binding ability of $p75^{NTR}$, TrkB, TrkC or sortilin antibodies on NSC-34 cells, cultures were grown in N-2 defined media for 5 days. Cells were then treated in 1% BSA containing media with either the control antibody X-63 or antibodies to $p75^{NTR}$, (Figure 4.9A),

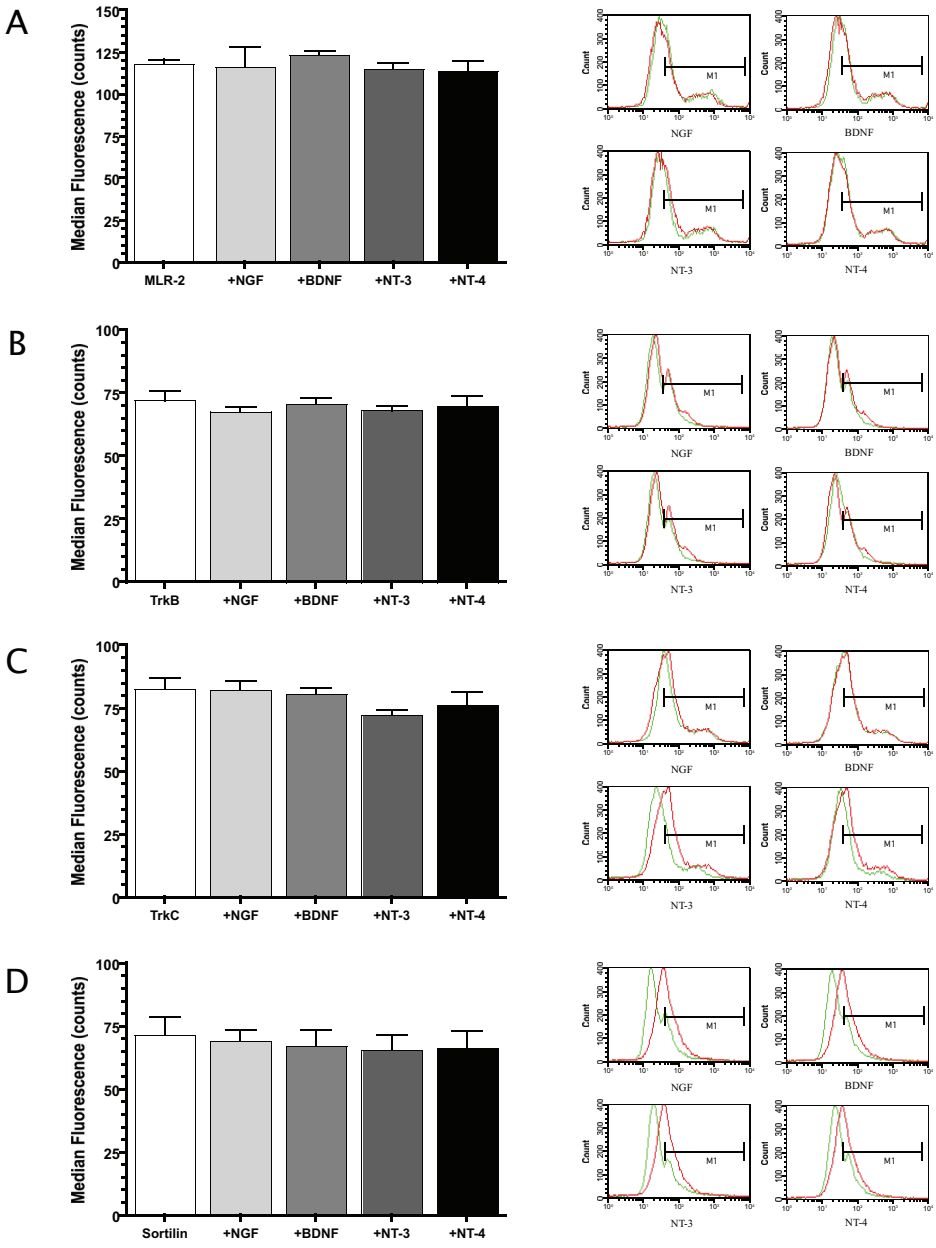


Figure 4.9 **Binding of p75NTR, TrkB, TrkC and Sortilin antibodies in the presence of neurotrophins.** The binding of antibodies alone (red line), or in the presence of 100ng/ml NGF, BDNF, NT-3 or NT-4 (green line) in NSC-34D cells was analysed by flow cytometry. **(A)**; MLR-2 FITC (10µg/ml), , **(B)**; anti-TrkB (20µl/Whole serum + anti-rabbit FITC secondary 1:500), **(C)**; anti-TrkC (20µl/Whole serum + anti-goat FITC secondary 1:500) and **(D)**; anti-Sortilin (20µl/Whole serum + anti-rabbit FITC secondary 1:500) (Median fluorescence measured by flow cytometry; n=4). X-63 is a control IgG. No significant statistical difference was observed with MLR-2 **(A)**, TrkB **(C)** or Gt1b **(D)** however, MLR-3 binding was significantly increased with the addition of 100 ng/ml NGF (n = 4, mean ± SEM, *P < 0.05, vs. NGF and BDNF untreated cells).

TrkB (Figure 4.9B), TrkC (Figure 4.9C) or sortilin (Figure 4.9D) alone, or either pre-incubated or co-incubated with 100ng/ml of NGF, BDNF, NT-3 or NT-4 for 1 hour on ice. Antibody binding was determined using median fluorescence of individual cell counts determined by flow cytometry (n = 4, mean ± SEM, *p< 0.05, NGF, BDNF, NT-3 or NT-4 vs. antibody only treated cells). Pre-incubation or co-incubation of cells with neurotrophins prior to the addition of receptor specific antibodies failed to produce any significant effects on fluorescence intensity, suggesting than neurotrophins do not actively compete for receptor binding sites with MLR-2, TrkB, TrkC or sortilin specific antibodies, at concentrations at or above commonly used for immunofluorescence experiments.

4.D.2 Effects of $p75^{NTR}$ antibodies on NGF induced apoptosis

It is well established that NGF (Sedel *et al.*, 1999) and proNGF (Domeniconi *et al.*, 2006) induce programmed cell death or apoptosis in motor neurons. We have demonstrated that exogenous NGF also induces apoptosis in NSC-34 cells in a dose dependent manner. To investigate whether binding of $p75^{NTR}$ antibodies MLR-2 and MLR-3 induced NGF-like activation of $p75^{NTR}$ in the absence of NGF, or had any effects on NGF mediated cell death, two experiments were carried out. First, antibodies were added to NSC-34 cultures in a dose-dependent manner, and incubated for 48 hours. Second, antibodies (10μg/ml) were co-incubated in the presence of 100 ng/ml NGF for the same time period. Cell survival of treated cultures was compared to controls (no NT) and NGF treated cells.

Incubation of MLR-2 or MLR-3 antibodies with NSC-34 cells ranging from 100 – 0.01μg/ml, failed to induce any changes in cell survival when compared to NGF treated cells (Figure 4.10A-B). Further, no significant difference was observed when compared to control cultures (no NGF). In experiments where MLR-2 and MLR-3 antibodies were co-incubated with NGF over 48 hours, analysis of cell survival revealed a small but significant inhibition of NGF mediated cell death (Figure 4.11C).

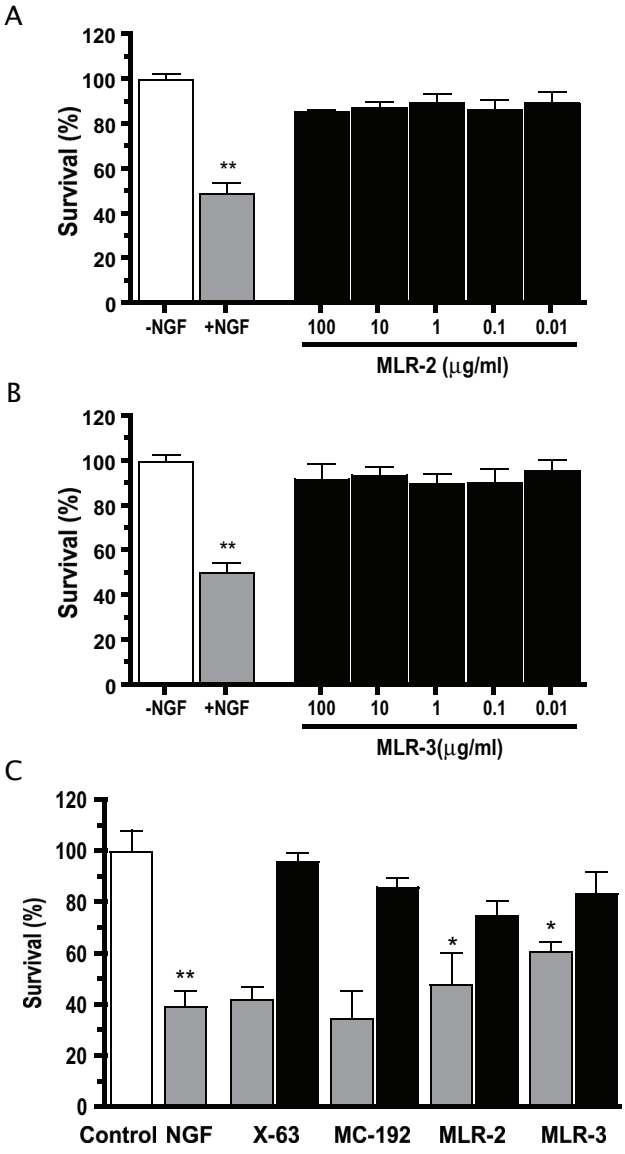


Figure 4.10 **Effects of p75NTR antibodies on NGF induced apoptosis.** NSC-34 cells were treated with 100ng/ml NGF, p75NTR antibodies MLR-2 (**A**), MLR-3 (**B**) or NGF in the presence of MLR antibodies (**C**). Treatment of NSC-34 cells with antibodies against p75NTR, MLR-2 & 3 in doses ranging from 0.01 - 100μg/ml does not induce apoptosis when compared to cells exposed to 100ng/ml NGF for 48 hours. Co-incubation with p75NTR antibodies MLR-2 & 3 (10 μg/m) and NGF (100ng/ml) for 48, did however produce a small but significant increase in cell survival when compared to NGF treatment controls. Co-incubation with MC-192 antibody against rat p75NTR and control antibody X-63 did not induce an increase in survival (n=6; **p>0.001; *p>0.05, two-way Anova - repeated measure with Bonferroni post-test).

Comparison to cells treated with NGF only, co-incubation with control antibody X-63, or co- incubation with rat specific $p75^{NTR}$ antibody MC-192, revealed that the presence of 10μg/ml of MLR-2 and MLR-3, increased cell survival of NGF treated cultures by 10-20%. This effect however, was not observed in cultures co-incubated with less than 10μg/ml of MLR-2 or MLR-3 (data not shown).

To gain an insight into the binding domains of $p75^{NTR}$ antibodies MLR-2 and MLR-3, mapping of the antibody binding epitopes was kindly determined by Christine Tuffereau at Laboratoire de Genetique des Virus, CNRS, France. Flow cytometry analysis suggests that binding of MLR-2 and MLR-3 appear to require at least cysteine rich domain (CRD) 3 and 4 (Figure AP.1A). In comparison, human anti-$p75^{NTR}$ ME20.4 requires only CRD 3. Western blot analysis of MLR-2 in non-reducing conditions shows that this antibody recognizes CRD 234, but not CRD 34 (Appendix Figure AP.1B), suggesting that CRD 2 plays an important role in MLR-2 binding site recognition. Both CRD 2 and CRD 3 contain important recognition sites for NGF binding (Yan & Chao, 1991; Baldwin & Shooter, 1995), and this may explain the partial inhibition of NGF induced apoptosis by MLR antibodies.

4.D.3 Time-lapse internalization of $p75^{NTR}$

To test whether mouse specific antibodies were suitable as an intracellular probe for NGF and BDNF dependent internalization, MLR-3 FITC was incubated on NSC-34 cells in the presence or absence of NGF and BDNF for up to 90 minutes at 37°C. Treatment with MLR-3 was able to induce internalization of p75NTR in the absence of neurotrophins (Figure 4.11A and D). However, the time or internalization was faster in the presence of 100ng/ml of NGF (Figure 4.11B, D) or BDNF (Figure 4.11C and D). The treatment with NGF appeared also to increase the total surface and intracellular staining with MLR-3 FITC, compared to BDNF treatment (Figure 4.11D), but this was not quantified.

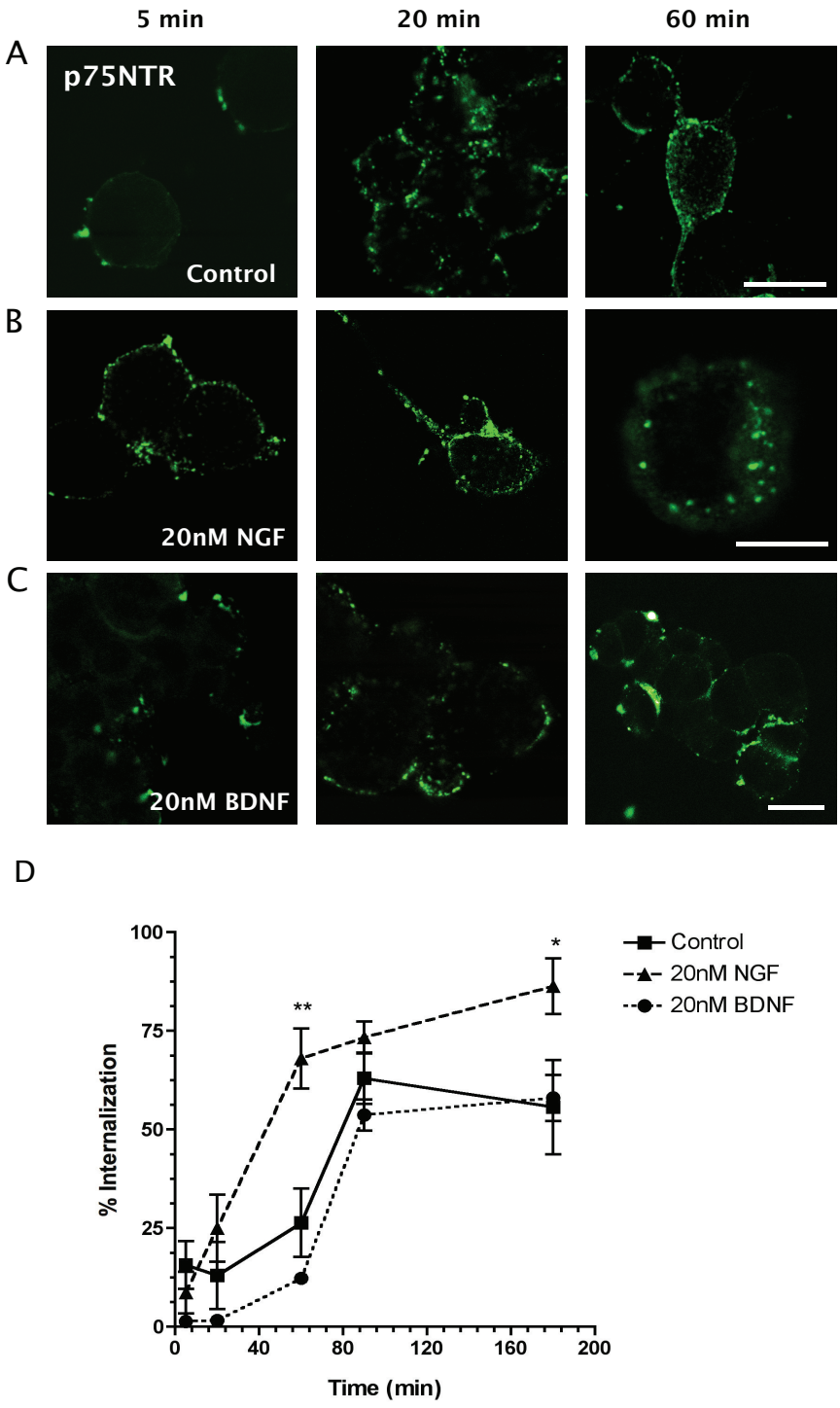


Figure 4.11 **Time-lapse ligand dependent and independent internalization of p75NTR.** Monoclonal antibody MLR-3 FITC was able to induce ligand independent internalization of p75NTR (A, D), however the time of internalization was faster with the addition of 100ng/ml of NGF (B, D) or BDNF (C, D). The addition of NGF appeared to increase total surface binding and rate of internalization of MLR-3 FITC, compared to BDNF treated cells (D), even though p75NTR has similar affinities for both ligands. The difference of p75NTR internalization rates in NGF compared to BDNF treated cells could possibly indicate a synergistic effect of the MLR-3 antibody with NGF on the p75NTR internalization. (Scale bar = 50 μm) Internalization was defined as any sub-cellular surface staining in a subset of randomly selected cells (n = 12; **p>0.001; *p>0.01, two-way ANOVA).

Further, the rate of p75NTR internalization in the presence of NGF was slightly faster compared to BDNF. The faster rate of internalization of p75NTR in the presence of NGF and MLR-3 compared to BDNF and MLR-3 could indicate a possible synergistic effect of the antibody interacting specifically with NGF but not BDNF to internalize the receptor-ligand complex. Internalization was defined as any sub-cellular surface staining in a subset of randomly selected cells (n = 12).

4.D.4 Time-lapse internalization of TrkB

To test whether rabbit polyclonal antibody to TrkB was suitable as an intracellular probe for NGF and BDNF dependent internalization, anti-TrkB was incubated on NSC-34 cells in the presence or absence of 100ng/ml NGF or BDNF for up to 90 minutes at 37°C. As with the $p75^{NTR}$ antibody, anti-TrkB was able to induce internalization of TrkB in the absence of neurotrophins (Figure 4.12A and D), however the time of internalization was increased with the addition of 100ng/ml of NGF (Figure 7B and D), but decreased with the addition of 100ng/ml BDNF (Figure 4.12C and D). The addition of BDNF did not appear to increase the binding and rate of internalization of anti-TrkB, compared to untreated cells (Figure 4.12D). One notable observation was the behavior of anti-TrkB under NGF treatment. Anti-TrkB internalization decreased when treated with NGF compared to untreated cells. Internalization was defined as any sub-cellular surface staining in a subset of randomly selected cells (n = 12).

4.D.5 Effects of k252a on neurotrophin receptor internalization

The binding of neurotrophins in the presence of $p75^{NTR}$ and trk receptors may result in the formation of high affinity binding sites, and subsequent internalization of the high affinity receptor complex (Wolf *et al.*, 1995; Bothwell, 1995; Dechant, 2001; Esposito *et al.*, 2001; Teng & Hempstead, 2004; Barker, 2007).

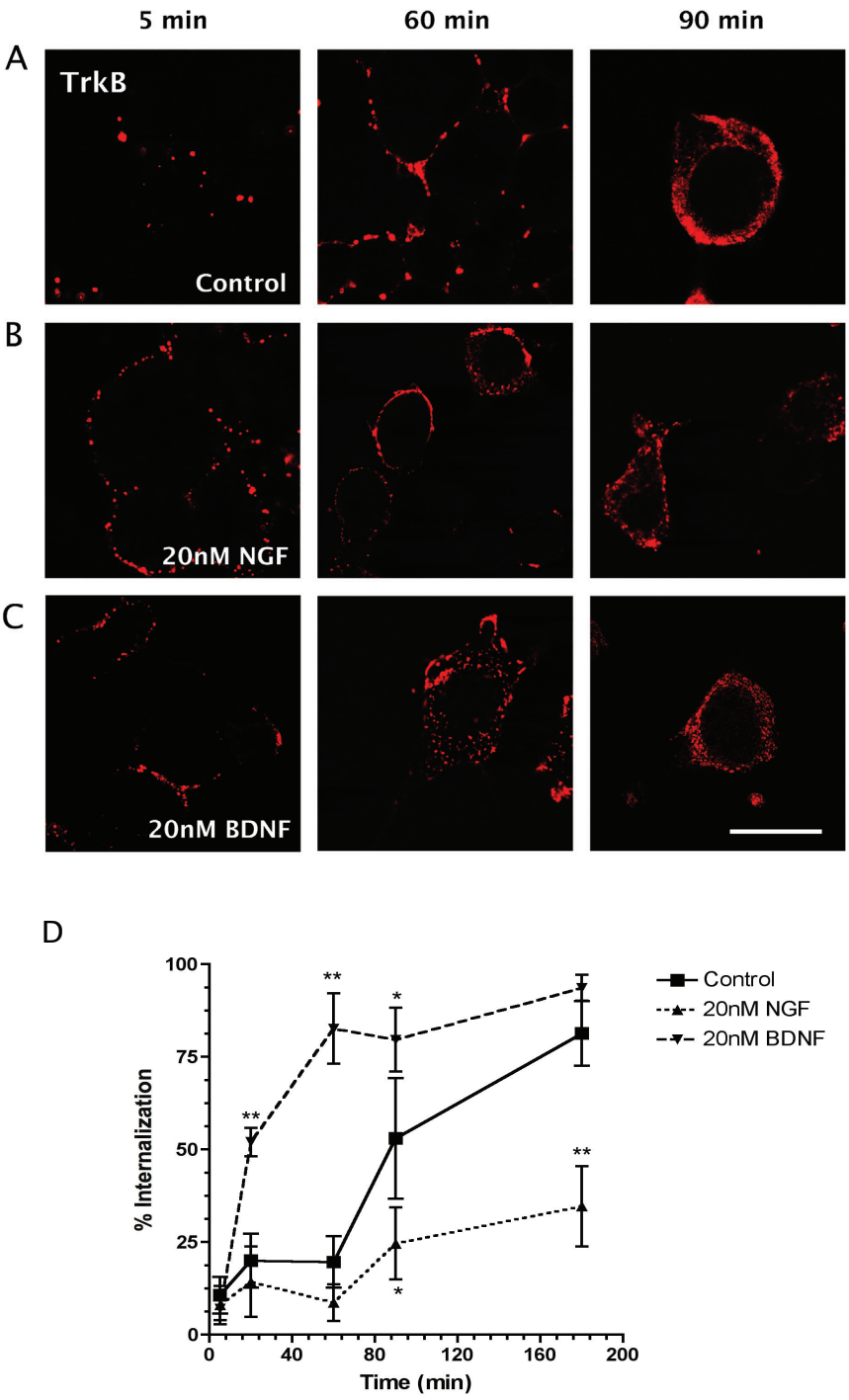


Figure 4.12 **Time-lapse ligand dependent and independent internalization of neurotrophin receptor TrkB.** Anti-TrkB sera was able to induce ligand independent internalization of TrkB in NSC-34 cells (A, D). The presence of 100ng/ml of NGF (B, D), and BDNF (C, D) resulted in altered internalization rates of the TrkB receptor. The presence of BDNF increased the speed of TrkB internalization, but did not appear to increase the cell surface binding, compared to untreated cells (D) (Scale bar = 50μm). One notable observation was the behavior of TrkB in the presence NGF. The speed TrkB internalization decreased when treated with NGF compared to untreated cells. Internalization was defined as any sub-cellular surface staining in a subset of randomly selected cells (n = 12; **p>0.001; *p>0.01, two-way ANOVA).

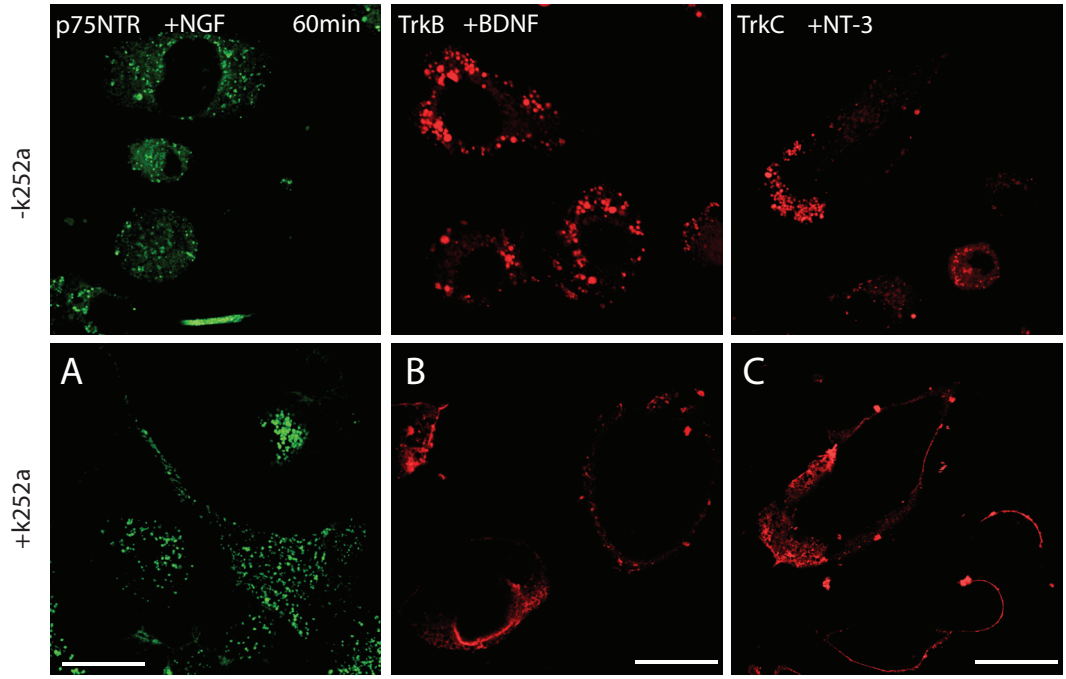
To investigate whether ligand dependent activation and internalization of Trk receptor could be inhibited without simultaneously affecting $p75^{NTR}$ internalization, we used the alkaloid-like compound K-252a. This CaM Kinase II inhibitor is widely used as a selective inhibitor of neurotrophin action on Trk receptors (Berg *et al.*, 1992; Kahle *et al.*, 1994; Auld *et al.*, 2001; Evangelopoulos *et al.*, 2004), and has also been demonstrated to inhibit Trk receptor internalization (Du *et al.*, 2003).

To asses the effects of K-252a on $p75^{NTR}$ and Trk receptor internalization, cultures were pre-incubated with 200nM K-252a for 60 minutes prior to the addition of 100ng/ml of NGF, NT-4 or NT-3 in the presence of receptor specific antibodies, and incubated for a further 60 minutes at 4°C (Figure 4.13 A-C).

Cells were then observed at 37°C in a POC chamber under a confocal microscope for up to 60 min. Fluorescence quantification revealed that treatment of NCS-34 cells with K-252a had no significant effect on $p75^{NTR}$ internalization when compared to control cultures (Figure 4.13C). In contrast, TrkB internalization in the presence of NT-4, and TrkC internalization with NT-3 was reduced by 60 – 65%. The remaining Trk receptor internalization observed in the presence of K-252a may indicate internalization of truncated forms of Trk receptors lacking tyrosine kinase domains.

4.E Discussion

Western blot and flow cytometry results confirmed that NSC-34 cells express a neurotrophin receptor profile typical of a developing motoneuron with $p75^{NTR}$ and tyrosine kinase receptors TrkB and TrkC present, but lacking the TrkA receptor (Yan *et al.*, 1993). NSC-34 cells also express the sortilin receptor, which was detected in a cell lysate preparation, but showed a relatively weak signal when detected by FACS analysis.



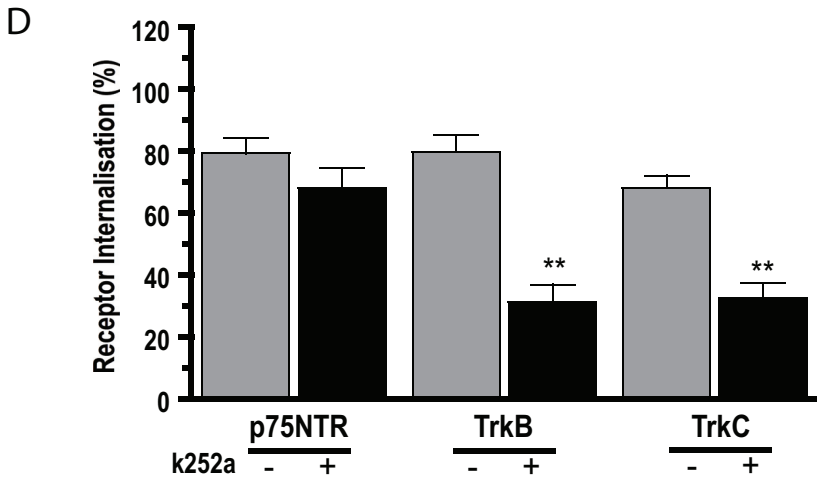


***Figure 4.13* Effects of k252a on neurotrophin receptor internalization.** NSC-34 cells were treated with 200nM k252a for 60 min and then with antibodies against p75NTR (**A**), TrkB (**B**) or TrkC (**C**) for 60 minutes in the presence of 100ng/ml NGF, BDNF and NT-3 respectively. (D) Pre-incubation with k252a had no significant effect on p75NTR internalization in the presence of NGF after 60 minutes. Pre-incubation with k252a significantly reduced both TrkB and TrkC internalization in the presence of receptor specific neurotrophins. (n=3; ***; **p>0.001; One-way Anova - repeated measure with Bonferroni post-test).

This observation seems to be consistent with reports that sortilin is primarily expressed in the cytoplasm (Petersen *et al.*, 1997), with significantly fewer receptors being expressed on the extracellular membrane (Nielsen *et al.*, 1999). Sortilin immunoreactivity has previously been reported in spinal cord lysates (Petersen *et al.*, 1997), and has recently been reported in cultured embryonic motor neurons, localized in the cell body and the neurites (Domeniconi *et al.*, 2006).

Analysis of molecular-weight bands on Western blots also support the presence of full length and truncated forms of the TrkB and TrkC receptors. Truncated isoforms of TrkB receptors that lack a tyrosine kinase domain (Middlemas *et al.*, 1991) predominate in the adult mammalian CNS (Cabelli *et al.*, 1996), yet their roles remain controversial. Co-expression of the full-length TrkB together with the truncated TrkB.T1 isoform results in decreased levels of full-length TrkB on the cell surface (Haapasalo *et al.*, 2002).

This appears to be the case in the NSC-34 cells as well. Although truncated forms of TrkB.T1 lack a signaling domain, they have been shown to recruit $p75^{NTR}$ via intra-membrane and extracellular interactions, and modulate dentritic growth through $p75^{NTR}$ signaling (Hartmann *et al.*, 2004).

TrkC is also commonly expressed in neuroblastomas (Brodeur *et al.*, 1997), is expressed in developing motor neurons (Tessarollo *et al.*, 1993; Yan *et al.*, 1993), is rapidly transported in embryonic motor neurons (Tessarollo *et al.*, 1993) and is able to rescue motor neurons after axotomy (Tessarollo *et al.*, 1993). The TrkC locus also encodes a number of kinase deficient isoforms defined as TrkCT1 (Tsoulfas *et al.*, 1993), TrkCNC2 (Menn *et al.*, 1998) and TrkCic158 (Valenzuela *et al.*, 1993). The function of full-length TrkC receptors and their interactions with NT-3 are well defined (Yuen & Mobley, 1999; Dechant, 2001), however the function of truncated TrkC isoforms has largely been attributed to the active inhibition of the kinase-active receptors (Huang & Reichardt, 2001). Despite the lack of initial evidence, to date a number of studies have suggested the potential for other functions mediated by kinase-deficient receptors,

including the activation of signaling pathways (Valenzuela *et al.*, 1993; Baxter *et al.*, 1997; Esteban *et al.*, 2006).

Sortilin has recently emerged as a co-receptor for $p75^{NTR}$ in the mechanism of pro-NGF (Nykjaer *et al.*, 2004) and pro-BDNF (Teng *et al.*, 2005) apoptotic signaling, however completely opposing reports are emerging (Fahnestock *et al.*, 2004) regarding the functional mechanisms and biological outcomes of this receptor-ligand complex. The observation that expression of neurotrophin receptors did not change over a 3-week period, with $p75^{NTR}$ readily detected at all time points, is consistent with the receptor profile of developing or injured motor neurons (Johnson *et al.*, 1999; Wiese *et al.*, 1999).

Other extracellular and intracellular markers of interest, and possibly connected with neurotrophin receptor trafficking were also identified. These included two members from the ganglioside b-series family; GT1b and GQ1b, which are direct analogues of GD1b. Gangliosides, are broadly distributed in the nervous system, and are sometimes associated with clinical symptoms that imply selective nerve damage (Allende & Proia, 2002; Gong *et al.*, 2002). They have been reported to act as functional nerve cell ligands involved in inhibition of nerve regeneration (Vyas *et al.*, 2002) but have also been implicated in tetanus toxin (Sinha *et al.*, 2000) and cholera toxin (Torgersen *et al.*, 2001) binding and transport. Members of the b-series family of gangliosides have been implicated as essential components of the tetanus toxin receptor complex (Kitamura *et al.*, 1999) and the tetanus toxin fragment TeNT has been reported to associate with $p75^{NTR}$ in lipid raft mediated endocytic transport (Lalli & Schiavo, 2002). A partial characterization of NSC-34 gangliosides has been previously described (Matsumoto *et al.*, 1995), however the presence of GT1b and GQ1b was not examined. Physiological markers of interest to intracellular trafficking, in particular markers of the regulated endocytic pathway such as clathrin, AP-1, EEA-1 and acidic organelle/lysosomal markers Lamp-1 and Limp-II, which are pivotal to endosomal compartment identification were detected in NSC-34 cells.

One of the more intriguing findings is the apparent presence of both pro and mature forms of BDNF in NSC-34 cell lysates. BDNF is well recognized as a neurotrophic factor for motor neurons. However, there is also sufficient evidence for BDNF synthesis within developing (Buck *et al.*, 2000) or injured (Kobayashi *et al.*, 1996; Qin *et al.*, 2006) rat motor neurons. Thus, the detection of BDNF protein within the NSC-34 cells is not surprising and consistent with a possible autocrine role for motor neurons (Lindsay, 1996). The observation that conditioned media from NSC-34 cultures was able to keep BDNF SH-SY5Y cells alive for up to 4 days secreted by NSC-34 cells, may be an additional factor contributing to the lack of BDNF responsiveness of the cell line.

None of the neurotrophins elicited visually detectable proliferation or differentiation of NSC-34 cultures, however addition of NGF and NT-3 produced a significant, and dose-dependent cell death in 48 hours of in differentiated NSC-34 cells. It is most likely that the serum in the non-differentiated cell cultures blocked or abolished the effects of NGF and NT-3. Further analysis of these mechanisms confirmed that NGF and NT-3 induced cell death through apoptotic mechanism, providing additional support for $p75^{NTR}$ associated cell death. Although NGF mediated cell death via $p75^{NTR}$ is consistent with observations in motor neurons (Curtis *et al.*, 1998; Sedel *et al.*, 1999), NT-3 mediated cell death has been seldom studied. However, NT-3 has been reported to induce cell death of adult BDNF-dependent cortico-spinal neurons via TrkC and $p75^{NTR}$ signaling (Giehl *et al.*, 2001). Whether production and secretion of BDNF by NSC-34 cells makes them BDNF dependent remains to be determined.

One of the paramount requirements for the study of receptor internalization in the presence of the native ligand is the ability of the antibody and the ligand to bind to the receptor simultaneously, and the ability of that receptor to carry out its function. To investigate the interactions of our antibodies against neurotrophin receptors with their respective ligands, we tested the ability of the antibodies to bind in the presence of neurotrophins. Pre-incubation with NGF, BDNF, NT-3 or NT-4 for 1 hour did not significantly affect the cell surface binding of $p75^{NTR}$, TrkB, TrkC or sortilin antibodies. This result

indicates that there is very little, or no hindrance to the specific antibodies used in binding to their receptors when neurotrophins occupy their respective binding sites.

Part of the characterization process of new antibodies recognizing mouse $p75^{NTR}$ included the assessment of detectable physiological effects. Commonly used MC192 antibodies enhance the binding of NGF to the receptor (Taniuchi & Johnson, 1985), and another antibody (REX) completely blocks NGF binding and $p75^{NTR}$ internalization (Weskamp & Reichardt, 1991). Mapping of $p75^{NTR}$ epitopes required for MLR antibody binding suggests that NGF and MLR antibodies bind to the same cysteine rich domains. Although, the antibodies themselves had no intrinsic biological activity on NSC-34 cells, co-incubation of high amounts of MLR-2 and MLR-3 (10μg/ml) with NGF produced a small but significant reversal of NGF induced cell death.

To test whether MLR-3 and polyclonal TrkB antibodies were suitable as a intracellular probes for the study of neurotrophin receptor internalization, the ability of each antibody to internalize in the presence as well as absence of NGF and BDNF was studied. MLR-3 induced ligand independent internalization of $p75^{NTR}$, however the time of internalization was altered with the addition of NGF or BDNF. The addition of NGF appeared to increase binding and rate of internalization of MLR-3-AF488, compared to BDNF, even though both ligands have similar affinity for $p75^{NTR}$. The slower rate of $p75^{NTR}$ internalization in the presence BDNF could indicate possible synergistic effect of MLR-3 antibody with NGF.

As with the $p75^{NTR}$ antibodies, polyclonal TrkB antibodies were able to induce ligand independent internalization of TrkB, however the time of internalization was increased with the addition of NGF, and decreased with the addition of BDNF. The addition of BDNF did not seem to increase the binding and rate of internalization of TrkB, compared to untreated cells. One notable observation was the behavior of TrkB under NGF treatment. TrkB internalization decreased when treated with NGF compared to untreated cells. The internalization rates observed in our experiments, for both $p75^{NTR}$ and TrkB, were

consistent with the internalization rates for these receptors reported in other cell lines (Grimes *et al.*, 1996; Saxena *et al.*, 2004; Iwasaki *et al.*, 1997). Inhibition of TrkB and TrkC but not $p75^{NTR}$ receptors with K-252a may also provide a useful mechanism for the study of $p75^{NTR}$ internalization with reduced association with Trk receptors.

The experiments reported in this chapter have expanded the characterization of the NSC-34 cell line to diversify its usefulness in not just the field of neurotrophin research, but also general motor neuron relevant biology. The neurotrophin receptor profile of the NSC-34 cell line offers broad scope to conduct investigations aimed at unraveling the complex receptor interactions that occur as a result of neurotrophin binding, that lead to multiple biological responses including survival, differentiation and programmed cell death. The presence of functional neurotrophin receptors should allow the exploitation of this cell line for studies of receptor trafficking, signaling and downstream responses including those initiated by the pro-neurotrophins through the sortilin and $p75^{NTR}$ receptor.

Chapter 5: Exogenous Neurotrophins Regulate The Sub-Cellular Fate Of $p75^{NTR}$

5.A Introduction

Internalization of the neurotrophin-receptor complex is fundamental in many aspects of neurotrophin function. In the last 10 years, a growing body of evidence has emerged in support of spatio-temporal regulation of neurotrophin signaling through vesicular complexes called signaling endosomes (Neet & Campenot, 2001; Barker *et al.*, 2002; Ginty & Segal, 2002; Heerssen & Segal, 2002; Howe & Mobley, 2005; Zweifel *et al.*, 2005). The majority of experimental evidence for the existence of signaling endosomes has been provided by the investigation of TrkA-NGF internalization, trafficking and signaling (Howe *et al.*, 2001; Ye *et al.*, 2003). Following ligand binding, TrkA can be internalized into clathrin-coated vesicles (CCV's) (Grimes *et al.*, 1996; Beattie *et al.*, 2000), caveolae (Bilderback *et al.*, 1999), or pincher-mediated macro-pinosomes (Shao *et al.*, 2002). After internalization receptors are sorted into recycling, retrograde transport or degradation pathways (Dechant, 2001; Yano & Chao, 2004). An established role of NGF signaling events involves a subset of still poorly defined vesicles called signaling endosomes, that associate with specific downstream signaling proteins and adaptors (Grimes *et al.*, 1996; Beattie *et al.*, 2000; Chao, 2003; Zweifel *et al.*, 2005).

In comparison to TrkA, $p75^{NTR}$ ligand induced internalization and trafficking remains a poorly understood and controversial topic. Distinct kinetics of neurotrophin binding between TrkA and $p75^{NTR}$ indicate that $p75^{NTR}$ is more important in surface binding than intracellular accumulation of NGF, BDNF and NT-3 (Saxena *et al.*, 2004). Internalization of $p75^{NTR}$ has been shown to be primarily clathrin-dependent in PC12 cells (Bronfman *et al.*, 2003; Saxena *et al.*, 2004), and lipid raft-dependent in sympathetic neurons (Hibbert *et al.*, 2006), and mouse cerebellar neurons (Higuchi *et al.*, 2003). The requirement of $p75^{NTR}$ for neurotrophin transport is well established (Johnson *et al.*, 1987; Curtis *et al.*, 1995), however direct evidence for retrogradely transported vesicles containing $p75^{NTR}$ and NGF has only recently been observed in motor neurons (Lalli & Schiavo, 2002). Subsequent studies indicate that $p75^{NTR}$ is involved in the formation of signaling endosomes (Bronfman *et al.*, 2003), and retrograde carriers

(Hibbert *et al.*, 2006) different to those containing Trks. Ligand stimulation in cell lines results in $p75^{NTR}$ being sorted to the recycling pathway (Bronfman *et al.*, 2003), but not in glial cells, where it's sorted to the lysosomal pathway (Kahle *et al.*, 1994). However, in motor neurons NGF bound $p75^{NTR}$ does not localize with lysosomal organelles, and is transported with a non-toxic tetanus fragment (Lalli & Schiavo, 2002).

Given the highly cell type specific effects of neurotrophins in the nervous system (Henderson *et al.*, 1998; Bartlett *et al.*, 1998), and the significance of neurotrophin events in motor neuron diseases (Bartlett *et al.*, 1998; Deinhardt & Schiavo, 2005; Bronfman *et al.*, 2007), very little is known about neurotrophin receptor trafficking in motor neurons. Unlike in PC12 cells or sympathetic neurons, NGF does not support survival of motor neurons, but induces apoptosis (Sedel *et al.*, 1999; Sendtner *et al.*, 2000). Motor neurons do not express the TrkA receptor (Escandon *et al.*, 1994), leaving $p75^{NTR}$ as the only NGF signaling receptor.

The previous 2 Chapters have established the suitability of the NSC-34 cell line for the study of neurotrophin receptor trafficking (Matusica *et al.*, 2008). This study focuses on the subcellular localization of $p75^{NTR}$ to establish neurotrophin dependent differences in $p75^{NTR}$ endosomal sorting and lysosomal localization.

5.B Internalization of $p75^{NTR}$ in the presence of neurotrophins

5.B.1 $p75^{NTR}$ internalization rates are altered in the presence of exogenous neurotrophins.

Ligand-dependent internalization of $p75^{NTR}$ has been studied and demonstrated in PC-12 cells (Bronfman *et al.*, 2003; Saxena *et al.*, 2004; Saxena *et al.*, 2005b). In these studies $p75^{NTR}$ internalization in the absence of NGF and BDNF was negligible when traced with anti-$p75^{NTR}$ (MC192) (Bronfman *et al.*, 2003), but clear differences were observed in $p75^{NTR}$ surface binding and internalization, depending on the type of neurotrophin bound (Saxena *et al.*,

2004), using Fab fragments of MC192. However, NSC-34 cells express a discreet combination of neurotrophin receptors in comparison to PC-12 cells, and may therefore possess distinct $p75^{NTR}$ internalization kinetics in response to neurotrophin stimulation.

Following a thorough characterization of the MLR clones, it was determined that the MLR-2 antibodies were more suitable for the use in receptor internalization studies. This decision was made based on results indicating that presence MLR-3 produced a significant decrease in NGF induced apoptosis in NSC-34 cells. Since the aim of this study was to investigate $p75^{NTR}$ trafficking, the ability of the antibodies to minimize the effect on the receptor function was considered to be important. Although MLR-2 produced a similar effect, the magnitude of apoptosis blocking observed was significantly smaller. The initial semi-quantitative $p75^{NTR}$ internalization study in chapter 4 used MLR-3 antibodies, however all experiments performed following the characterization of MLR antibodies were performed with MLR-2. The use of different MLR antibodies may explain the differences observed in $p75^{NTR}$ internalization rates in the initial results from chapter 4 and the results reported in this chapter.

Differentiated NSC-34 cells were incubated with MLR-2-AF488 (3µg/ml) in the presence or absence of 100ng/ml of NGF, BDNF, NT-3 or NT-4 for 45 min. at 6°C, and then observed for up to 60 minutes at 37°C (Figure. 5.1A-D) in a POC chamber. MLR-2 antibodies alone induced internalization of $p75^{NTR}$ in the absence of neurotrophins (control), with over 50% of total fluorescence observed in intracellular regions of cells within 10-15 minutes. The addition of exogenous neurotrophins resulted in slower internalization rates of $p75^{NTR}$, but significant differences were observed only in presence of NGF, NT-3 and NT-4 ($n = 6$; $^{*}p>0.05$; $^{**}p>0.01$, two-way ANOVA analysis).

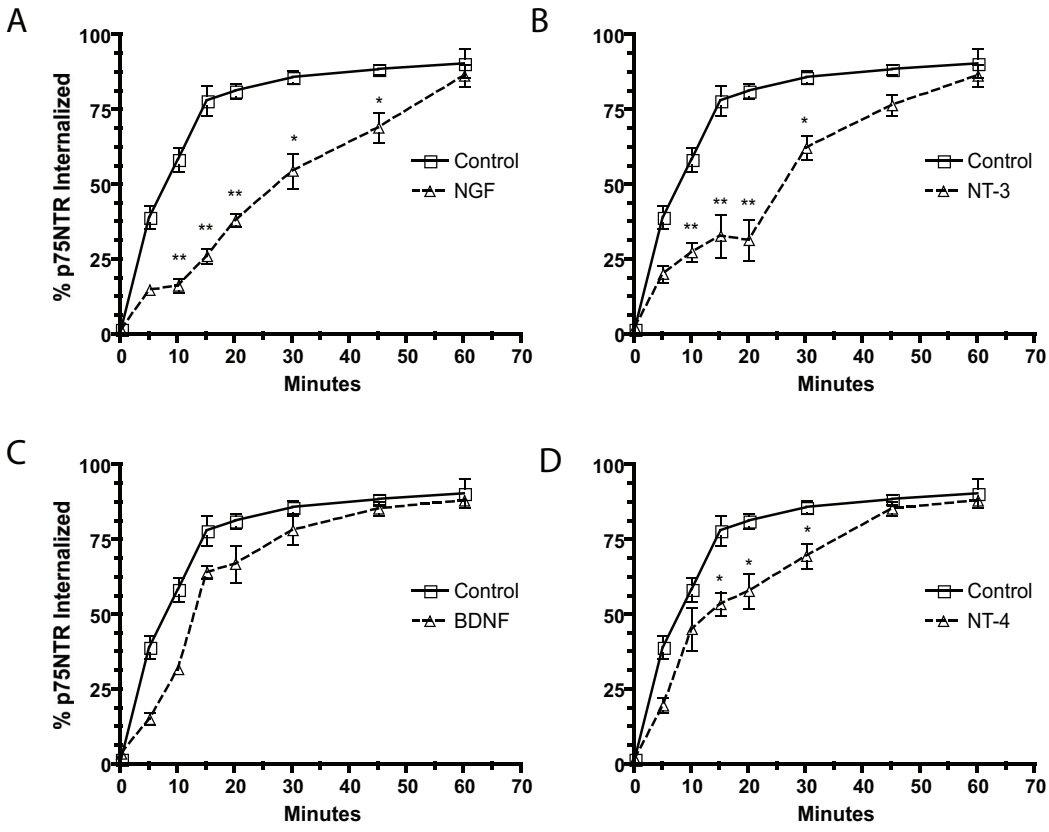


Figure 5.1 **Internalization kinetics of p75NTR following neurotrophin treatment.** Internalization of p75NTR (MLR-2-AF488) following treatment of NSC-34 cells with (A) NGF, (B) NT-3, (C) BDNF or (D) NT-4 over 60 minutes. Internalization kinetics of p75NTR were slower in all neurotrophin treatments when compared to antibody only treatments in the absence of neurotrophins. NGF and NT-3 mediated p75NTR internalization was marginally slower when compared to BDNF and NT-4 internalization rates (n=6; *p>0.05; **p>0.01, Two-way ANOVA analysis).

In the presence of NGF and NT-3, $p75^{NTR}$ internalization reached K_d50 in just over 30 minutes, where as NT-4 internalized slightly faster with K_d50 reached within 20 minutes. BDNF induced internalization rates were comparable to the control experiment in the absence of neurotrophins. At 60 minutes in all experiments, fluorescently labeled $p75^{NTR}$ was detected in intracellular compartments or bordering the intracellular membrane. Intracellular and extracellular membrane regions were selected according to DIC images of each individual cell.

5.B.2 $p75^{NTR}$ associates with GT1b on the cell membrane but is internalized via clathrin mediated endocytosis.

The polysialoganglioside GT1b has been reported to associate with $p75^{NTR}$ in two different aspects of neuronal physiology. Firstly, GT1b and $p75^{NTR}$ form part of a receptor complex in MAG-mediated inhibition of neurite outgrowth (Yamashita *et al.*, 2002). Secondly, the observation that $p75^{NTR}$ and tetanus toxin share the same endocytic transport vesicles in primary motor neurons (Lalli & Schiavo, 2002), combined with the fact that the ganglioside GT1b is essential for tetanus toxin binding and internalization (Staub *et al.*, 1986; Sinha *et al.*, 2000) suggests that $p75^{NTR}$ may also be part of GT1b containing receptor complex for tetanus toxin. To investigate whether $p75^{NTR}$ and GT1b are associated at the cell membrane, and share the same endocytic vesicle following internalization, the following experiment was carried out. Cells were incubated with MLR-2-AF488 and GT1b-AF568 antibody conjugates in the presence of neurotrophins for 1 hour on ice. Cells were then washed and incubated at 37°C for intervals up to 90 minutes, fixed and processed for microscopy.

Immunostaining of NSC-34 cells in the absence of neurotrophins, shows that approximately 50-55% of $p75^{NTR}$ colocalised with GT1b on the cell surface at the early stages of endocytosis (5min.) (Figure 5.2A).

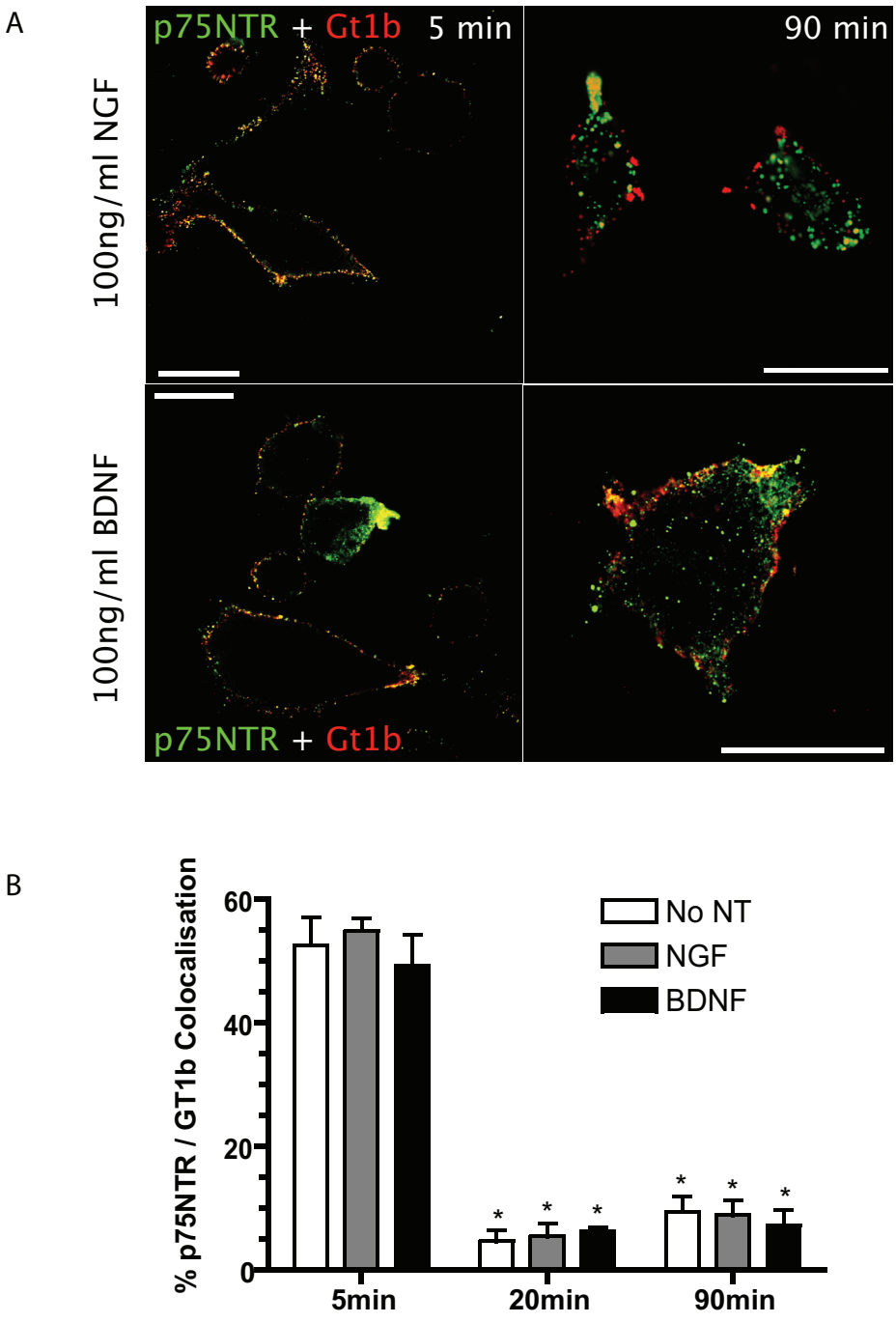


***Figure* 5.2 GT1b associates with p75NTR on the plasma membrane but is not internalized with the receptor.** Cell surface colocalization of p75NTR (MLR-2-AF488) and GT1b following treatment of NSC-34 cells with (**A**) NGF and BDNF at 5 minutes and 90 minutes respectively. (**B**) Quantitative analysis of p75NTR and GT1b colocalization. Colocalization between p75NTR and GT1b is substantial on the cell surface prior to large scale endocytosis, however following the internalization of p75NTR, GT1b remains located at the cell surface. No differences were observed between controls and NT treated cells. (All neurotrophins were tested NT-3/NT-4 not shown; n=4; *p>0.001; Two-way ANOVA analysis; scale bars = 25μm).

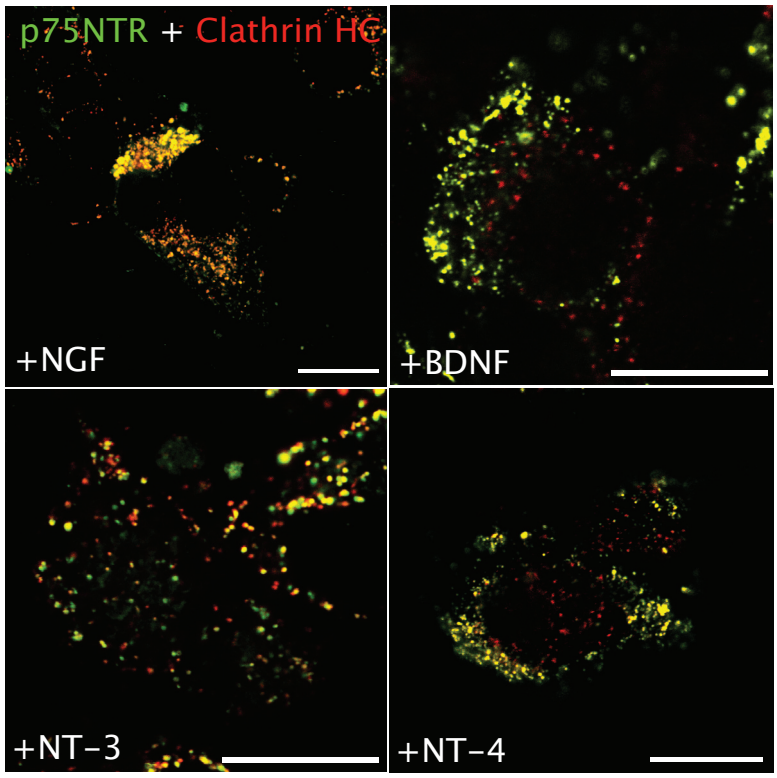
However the colocalisation of $p75^{NTR}$ and GT1b rapidly decreased following internalization of $p75^{NTR}$, and at the 20-minute time-point, very little intracellular colocalisation of the two markers was observed following internalization for up to 90 minutes (Figure 5.2A-B). Colocalisation rates were not affected by the addition of neurotrophins as no differences were observed when compared to controls (no NT).

5.B.3 $p75^{NTR}$ associates with clathrin HC and transferrin positive endocytic carriers.

The observation that GT1b associates with $p75^{NTR}$ at the plasma membrane but is not seen colocalised with the receptor following internalization suggests that GT1b may play a role in sorting of the $p75^{NTR}$ on the extracellular membrane prior to internalization. To confirm that $p75^{NTR}$ neurotrophin internalization through CME mediated pathway, cells were treated with neurotrophins and counterstained with anti-clathrin HC- AF595. Cells fixed at intervals correlating with timeframes for early endocytosis of $p75^{NTR}$ showed high degrees of colocalisation between p75NTR and clathrin HC (Figure 5.3a-A). Rates of colocalisation for p75NTR and clathrin HC were not influenced significantly by the presence of neurotrophins (figure 5.3a-B).

Transferrin is the best-characterized marker of the clathrin-mediated endocytic pathway involved in recycling to the plasma membrane. The presence of neurotrophin bound $p75^{NTR}$ in transferrin positive vesicles has been previously shown (Bronfman *et al.*, 2003). To determine whether the association of $p75^{NTR}$ with transferrin-positive endocytic vesicles is affected by the presence of neurotrophins, $p75^{NTR}$ internalization was monitored in the presence of NGF, BDNF NT-3 or NT-4 and transferrin-AF-555 for up to 60 min (Figure 5.3b-A). Analysis of colocalisation rates reveals an increased $p75^{NTR}$ trafficked via transferrin-associated pathways. NGF and NT-3 associated $p75^{NTR}$ shows a 10-20% decrease in transferrin colocalisation compared to control, BDNF or NT-4 experiments (Figure 5.3b-B).

A



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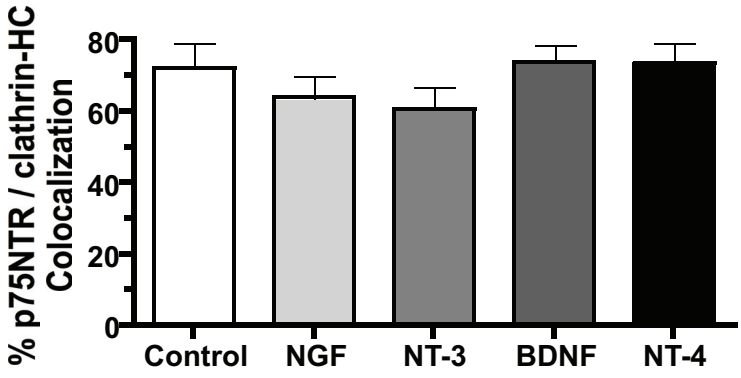


Figure 5.3a **p75NTR colocalization with clathrin-HC is not influenced by neurotrophin treatment.** (**A**) Colocalization of p75NTR (MLR-2-AF488) with clathrin-HC following treatment of NSC-34 cells with NGF, NT-3, BDNF or NT-4 at 30 minutes. (**B**) Quantification of p75NTR and clathrin-HC colocalization did not show significant differences between each of the neurotrophins and control treatments (n=6; Two-way ANOVA analysis).

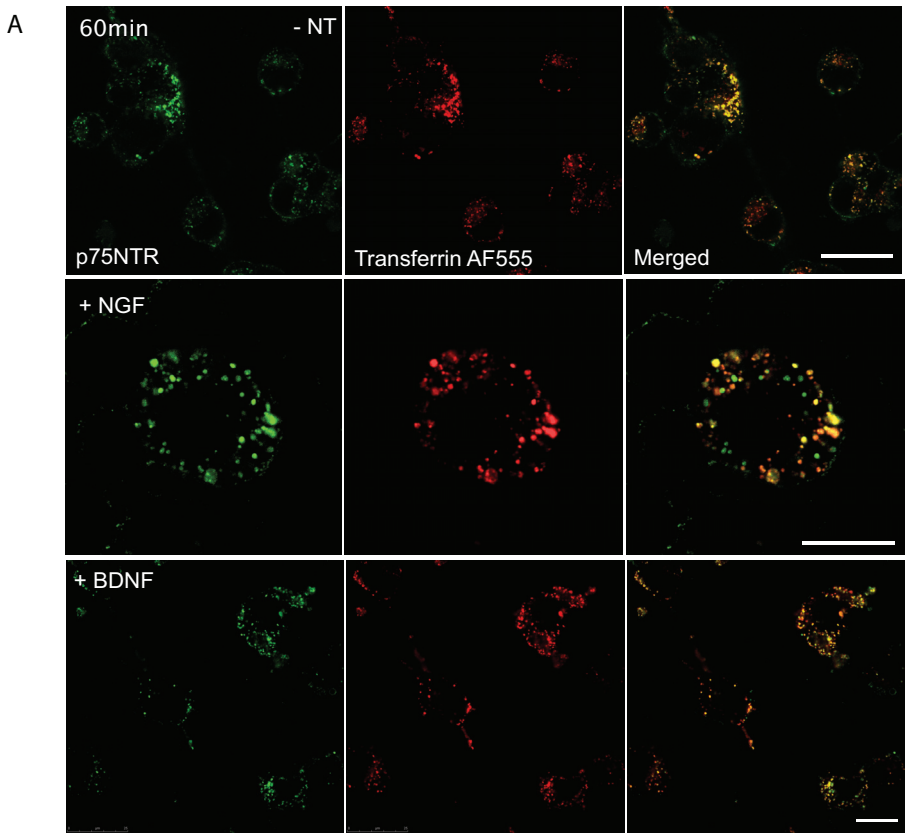


Figure 5.3b **The presence of NGF and NT-3 but not BDNF or NT-4 reduces p75NTR internalization into common endocytic carriers.** (**A**) Analysis of neurotrophin-mediated p75NTR internalization in the presence of transferrin-AF-555 indicates that the kinetics of transferrin internalization are faster than that of p75NTR when NSC-34 cells are treated with 100ng/ml of neurotrophins (NGF and BDNF shown) and observed through time-lapse confocal microscopy. (**B**) Analysis of p75NTR internalization in the presence of transferrin-AF-555 revealed a consistent decrease across all time points up to 60min of p75NTR/transferrin colocalisation in NGF and NT-3 treated cells and not BDNF or NT-4-mediated colocalisation of p75NTR in transferrin positive endocytic vesicles (n=3; *p>0.05; two-way ANOVA analysis against no NT control; Scale bar = 25μm).

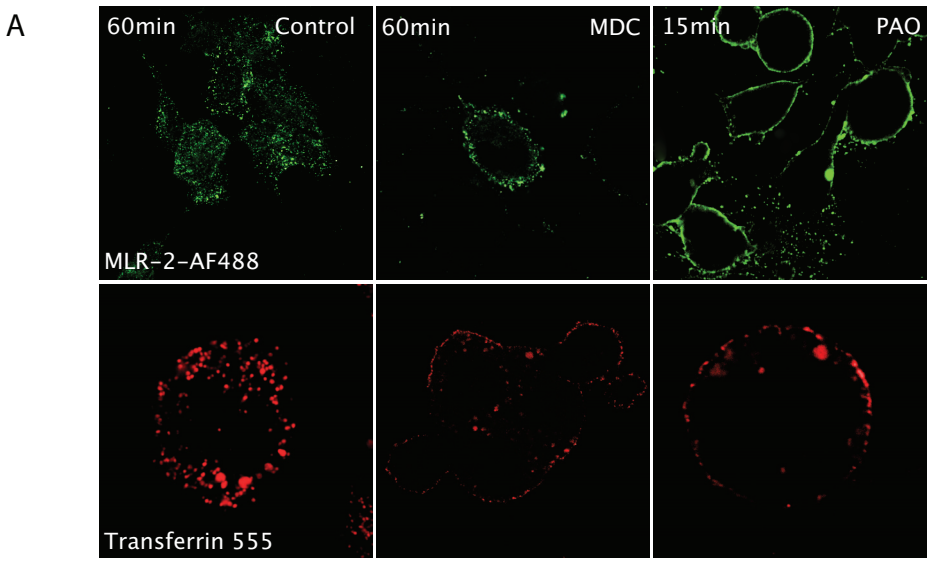
This decrease in colocalisation appears to be consistent over time and independent of total $p75^{NTR}$ internalization.

5.B.4 Inhibition of clathrin-mediated endocytosis prevents $p75^{NTR}$ internalization in the presence of BDNF and NT-4, but not NGF and NT-3.

Internalization of $p75^{NTR}$ has been shown to be primarily clathrin-dependent in PC-12 cells (Bronfman *et al.*, 2003; Saxena *et al.*, 2004), but also lipid raft-dependent in sympathetic neurons (Hibbert *et al.*, 2006), and mouse cerebellar neurons (Higuchi *et al.*, 2003).

To investigate whether $p75^{NTR}$ is internalized through different endocytic pathways depending on the neurotrophin present, NSC-34 cells were incubated with 100ng/ml of NGF, BDNF, NT-3 or NT-4 for 30 min at 4 °C, and then treated with either regular media, media containing 100mg/ml MDC for 1hr or 10mg/ml PAO for 15 minutes. The internalization of transferrin-AF-555 was used as a control for CME inhibition. $P75^{NTR}$ labeled with MLR-2-AF488 was localized inside cytosolic vesicles in the presence or absence of neurotrophins within 60 min (Figure 5.4A). MDC is a competitive inhibitor of transglutaminase, an enzyme essential for the formation of clathrin-coated vesicles. MDC was efficient in blocking 75-80%, of $p75^{NTR}$ internalization in control experiments and in cells treated with BDNF and NT-4, and a 10-15% fraction of $p75^{NTR}$ internalizing through non-CME pathways.

However cells inhibition of CME with MDC in NGF and NT-3 treated cells was not as effective, blocking only 50-65% of $p75^{NTR}$ internalization (Figure 5.4B). Non-specific inhibition of endocytosis with PAO blocked $p75^{NTR}$ internalization with an efficiency of 85-95% across all treatments, suggesting that NGF and NT-3 shifts the fraction of $p75^{NTR}$ being internalized through a non-CME pathway by more than 2 fold.



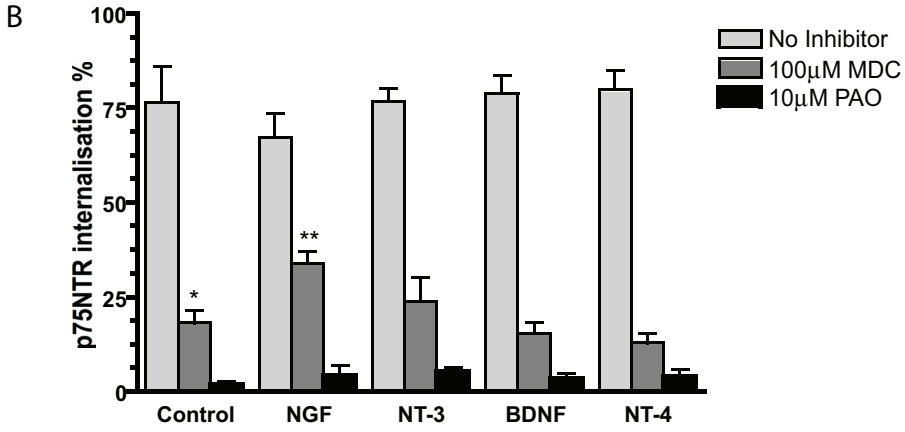


***Figure 5.4* Selective inhibition of p75NTR via clathrin-mediated endocytosis completely prevents BDNF and NT-4, but not NGF and NT-3 mediated p75NTR internalization.** Internalization of MLR-2-AF488 and transferrin AF-555 (A), under normal culture conditions (control), and in the presence of clathrin-mediated endocytosis inhibitors monodansycadaverine (MDC), or phenylarsine oxide (PAO). Quantification of fluorescence reveals that 15min (10 µg/ml) PAO incubation completely abolished endocytosis in all treatments. Some internalization was evident after 1hr of (100 µg/ml) MDC treatment, however the level of internal fluorescence was ~ 10% higher in cells treated with NGF and NT-3 (n=10; *p>0.05; two-way ANOVA analysis).

5.C $p75^{NTR}$ endosomal trafficking dynamics

5.C.1 NGF and NT-3, but not BDNF and NT-4 decrease lysosomal targeting of $p75^{NTR}$.

Unlike the Trk receptors, which have been reported to undergo lysosomal degradation (Jullien *et al.*, 2002; Saxena *et al.*, 2005b), NGF bound $p75^{NTR}$ has been shown to escape lysosomal targeting in PC12 cells (Saxena *et al.*, 2005b), primary motor neuron cultures (Lalli & Schiavo, 2002), and primary hippocampal cultures (Bronfman *et al.*, 2007). To investigate whether $p75^{NTR}$ lysosomal targeting, is influenced by the neurotrophin present, NSC-34 cells were treated with 100ng/ml of NGF, BDNF, NT-3 or NT-4. Colocalisation of $p75^{NTR}$ and the lysosomal marker Lysotracker DND-99 was quantified in a time dependent manner with all neurotrophins separately. In the absence of neurotrophins, 55% of $p75^{NTR}$ colocalised with lysotracker after 15 minutes, with the rate of $p75^{NTR}$/lysotracker colocalisation steadily increasing towards 75-80% at the one-hour mark (Figure 5.5.A-F). In contrast, in cells exposed to NGF and NT-3 $p75^{NTR}$ colocalisation with lysotracker was significantly reduced at 15 minutes and remained so after 60 minutes. Interestingly, the level of $p75^{NTR}$ and lysotracker colocalisation was marginally higher in NT-3 treated cells (20–30%) across the indicated time points when compared to NGF induced colocalisation rates of 5-20%. In comparison, the overlap observed between $p75^{NTR}$ and lysotracker in BDNF or NT-4 treated cells was much higher, with 30% and 50% of $p75^{NTR}$ respectively, targeted for degradation at 15 minutes. The levels of $p75^{NTR}$, colocalisation with lysotracker in the presence of BDNF or NT-4 then reached 70% at 60 minutes, marginally lower than control experiments performed in the absence of neurotrophins.

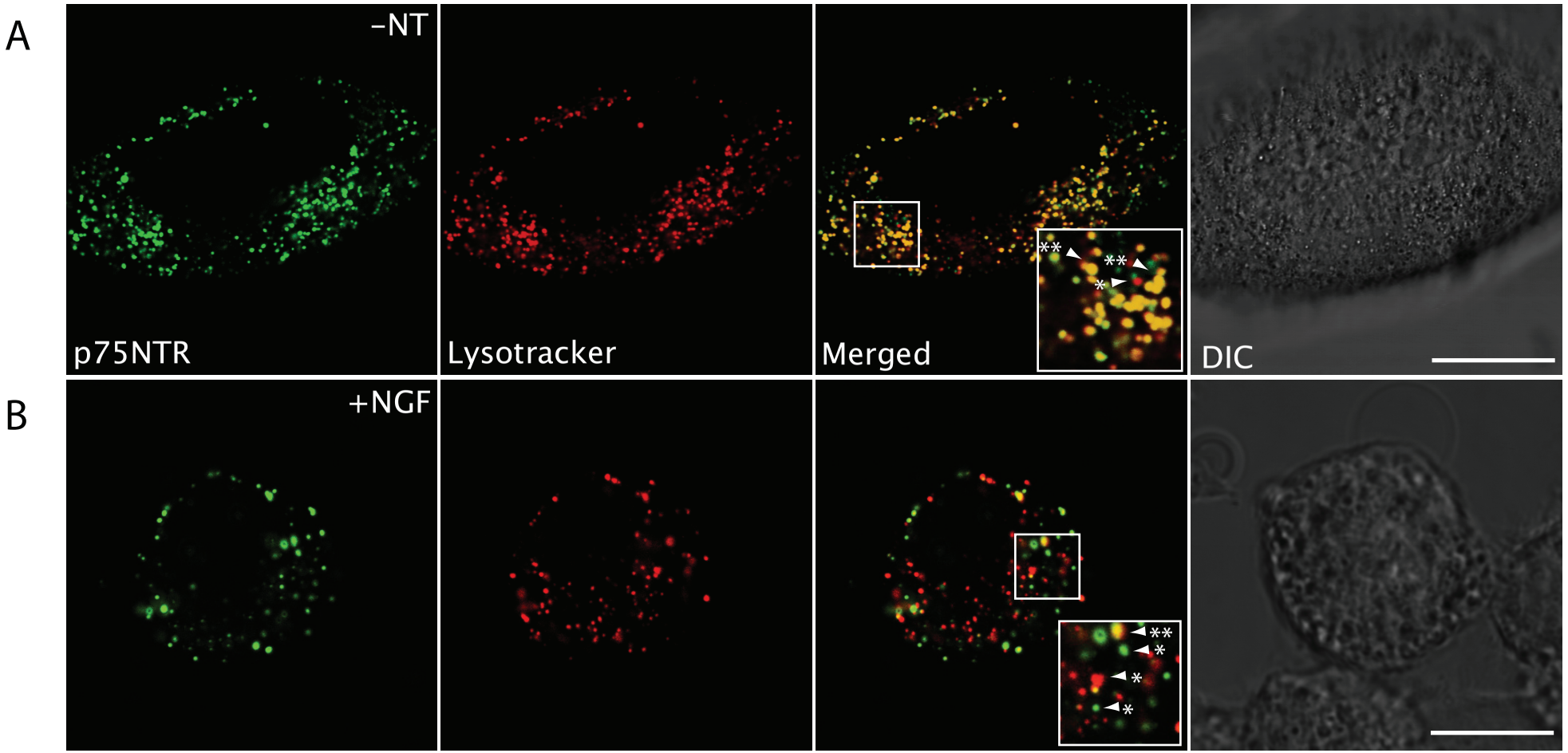


Figure 5.5a **Neurotrophins differentially regulate lysosomal fate of p75NTR.**

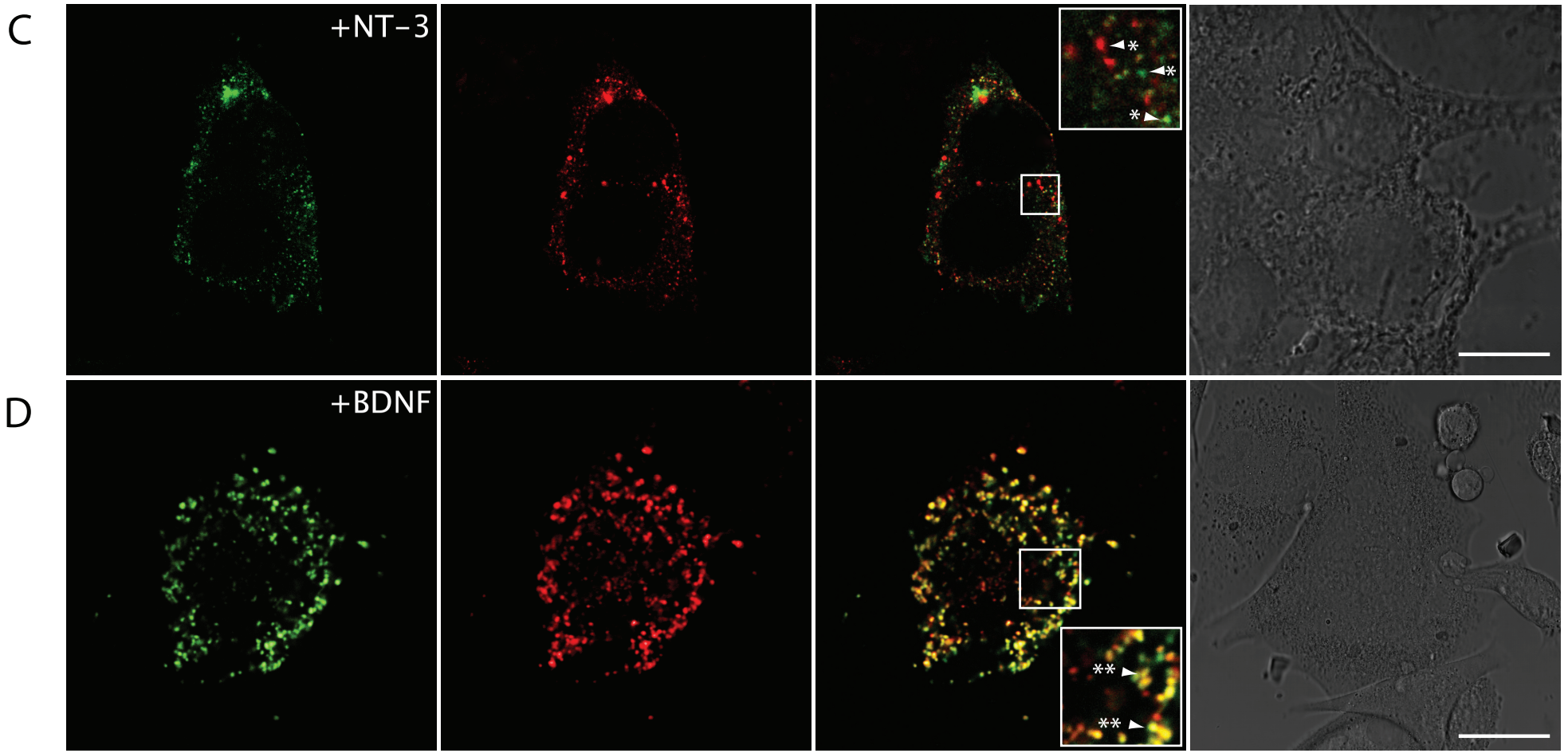


Figure 5.5*b* **Neurotrophins differentially regulate lysosomal fate of p75NTR.**

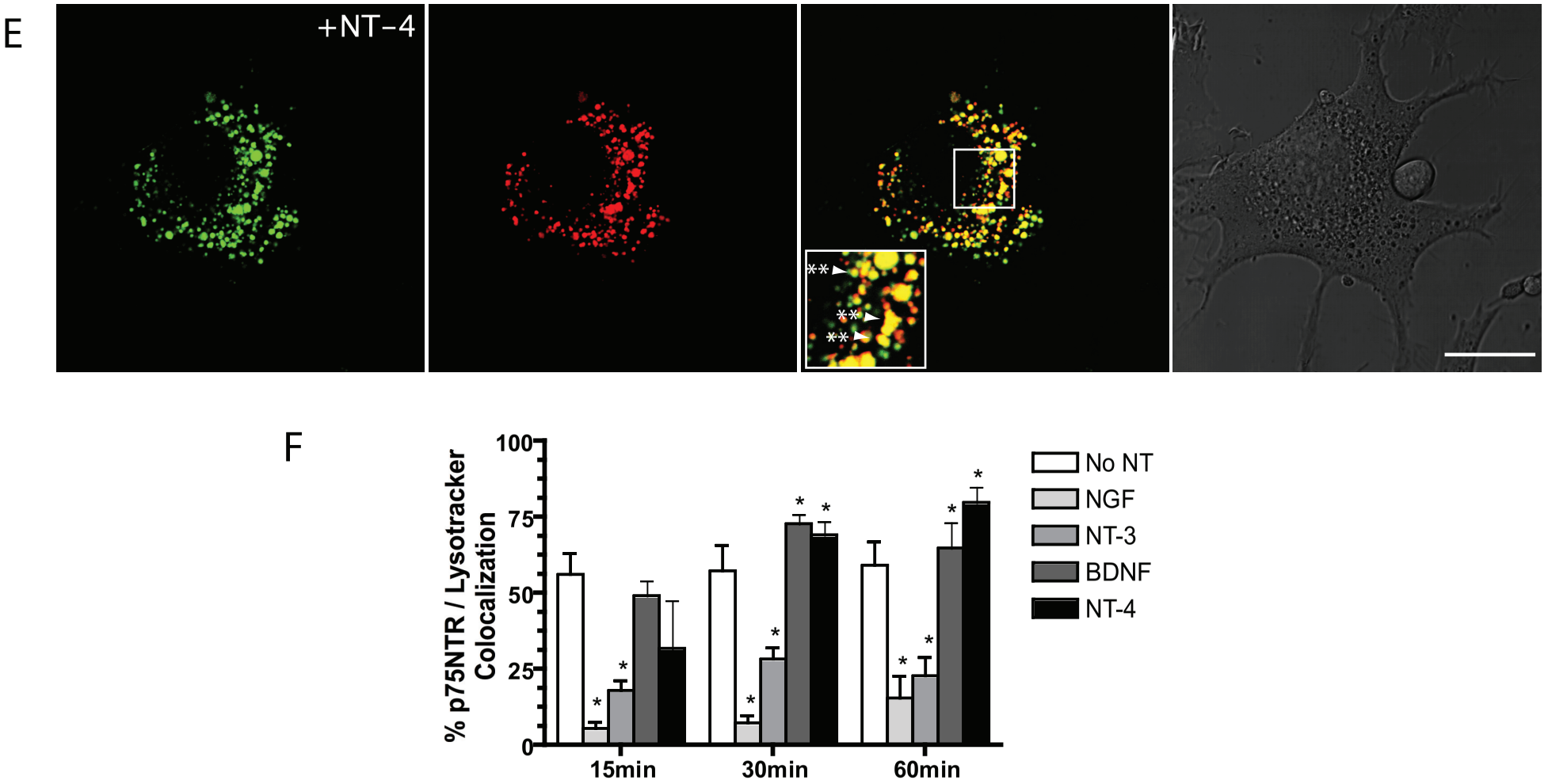


Figure 5.5c **Neurotrophins differentially regulate lysosomal fate of p75NTR.**

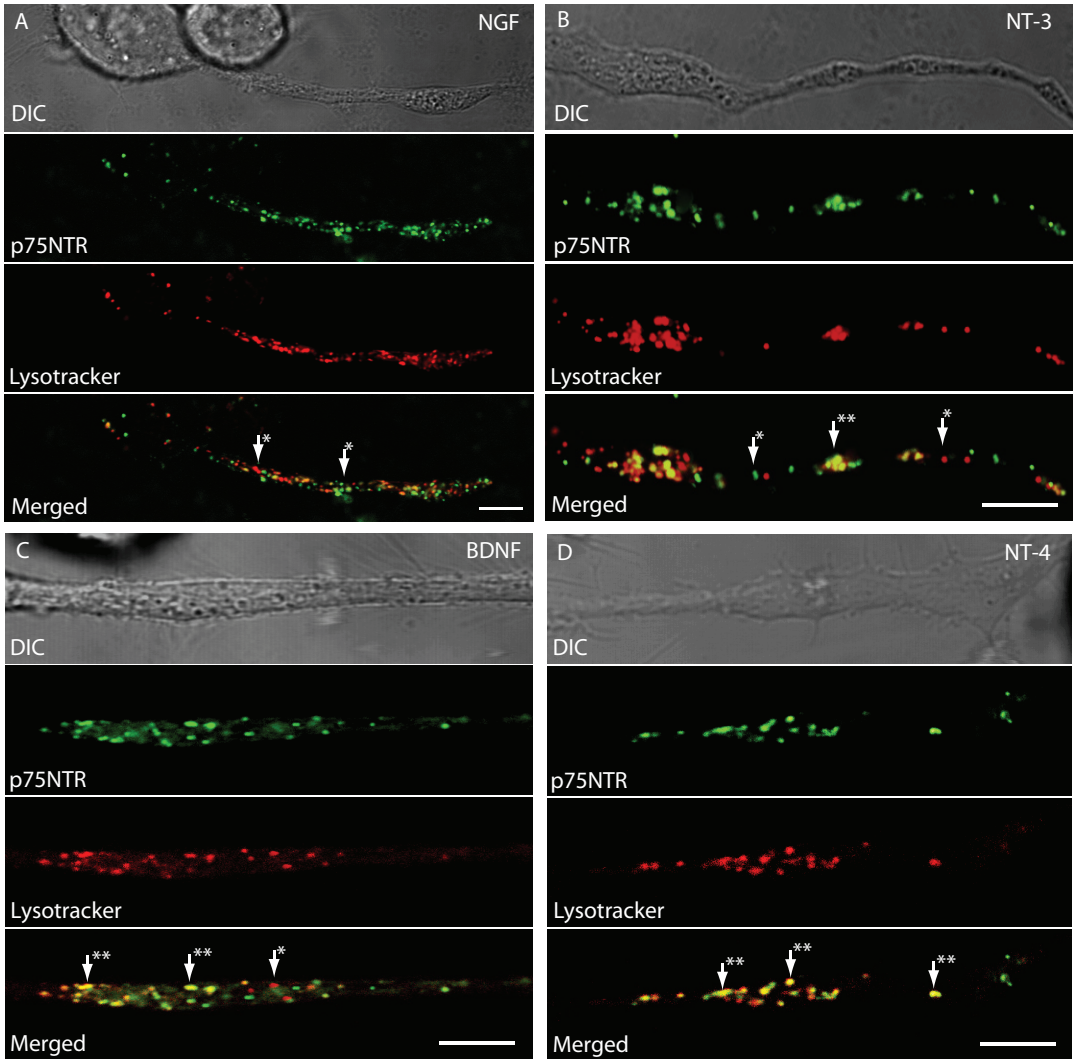


Figure 5.5d **Neurotrophins differentially regulate lysosomal fate of p75NTR.** Analysis of p75NTR colocalisation with lysotracker DND-99. (from previous page) Confocal micrographs of NSC-34 cells stained with 3µg/ml MLR-2-AF488 and 35nM Lysotracker DND-99 in the absence of neurotrophins (A, -NT), or in the presence of 100ng/ml exogenous NGF (B), NT-3 (C), BDNF (D) and NT-4 (E) following 60 minute incubation at 4°C and then 37°C. Insert in each merged image provides higher magnification of vesicular structures, arrowheads marked with (*) indicate single marker and arrowheads with (**) indicate vesicles with both p75NTR and lysotracker. The presence of acidic organelles and p75NTR organelles was also observed in cell projections and mirrored the colocalisation of the two markers in the cell body (figure 5.5d, this page) (F) Quantification of colocalisation rates at 15, 30 and 60-minute time intervals for all treatments. (Measurements represent 6-10 individual cells, n=3; *p>0.001; two-way ANOVA against treatment control at individual time points; error bars represent SEM; scale bars = 200µm (a,b,c); 20µm, d).

5.C.2 $p75^{NTR}$ enrichment of EEA-1 positive endosomes is dependent on NGF and NT-3, but not BDNF and NT-4.

EEA-1 is a vesicle-tethering factor (Mu *et al.*, 1995) with multiple binding partners (Mills *et al.*, 2001; Perret *et al.*, 2005) involved in regulating the formation of early/sorting endosomes (Miaczynska & Zerial, 2002) following cargo internalization. EEA-1 positive endosomes represent a crucial sorting step in the targeting of endosomal cargo, and this marker recently has been demonstrated to identify two early endosome populations with distinct maturation kinetics and mobility (Lakadamyali *et al.*, 2006). EEA-1 is also a Rab5 effector protein and is preferentially recruited by Rab5 positive endosomes (Callaghan *et al.*, 1999; Trischler *et al.*, 1999). Further, Rab5 positive endosomes have been implicated as signaling endosomes (Barbieri *et al.*, 2000; Varsano *et al.*, 2006). The role of EEA-1 in p75NTR internalization and endosomal sorting remains unclear, while p75NTR has been detected in EEA-1 positive endosomes in PC-12 cells exposed to exogenous NGF (Bronfman *et al.*, 2003).

Since p75NTR displays altered rates of internalization and transferrin colocalisation in the presence of neurotrophins, we investigated the role of exogenous neurotrophins in the association p75NTR with EEA-1. Markers for p75NTR and EEA-1 show consistent colocalisation in NSC-34 cells at early internalization time point of 15 minutes (confocal micrographs not shown) (Figure 5.6). At 60 minutes, when the majority of p75NTR was internalized, significant differences were observed when compared to early stages of internalization or the vehicle treatments only.

Confocal micrographs at 60 minutes show that p75NTR colocalisation with EEA-1 decreases with time in cells not exposed to neurotrophins, or cells exposed to BDNF and NT-4 (Figure. 5.6A, D and E). In contrast p75NTR trafficking to EEA-1 positive endosomes increased in NGF and NT-3 treated cells (Figure 5.6B-C).

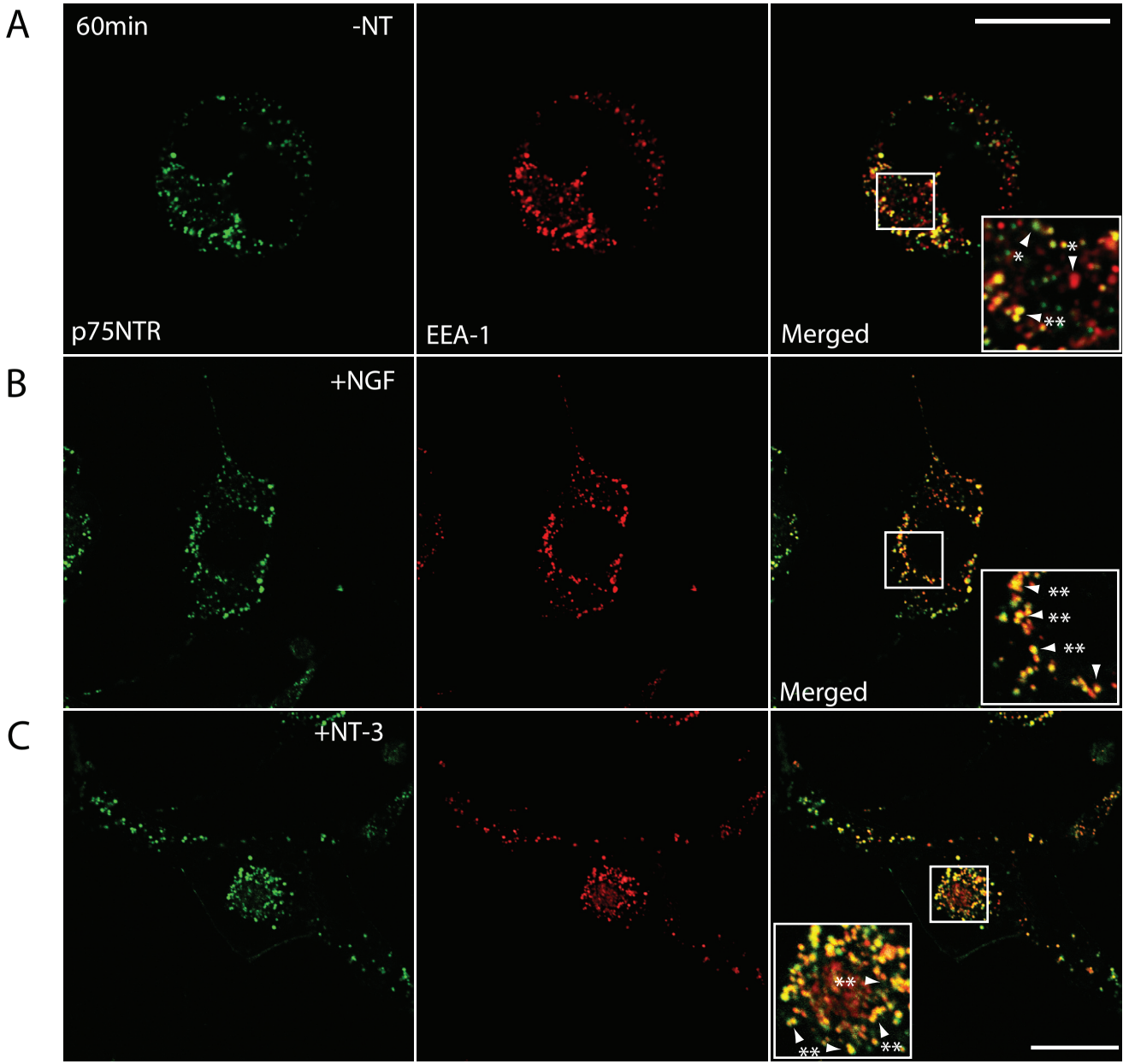


Figure 5.6*a* **NGF and NT-3 increase the association of p75NTR with EEA-1 positive sorting endosomes.**

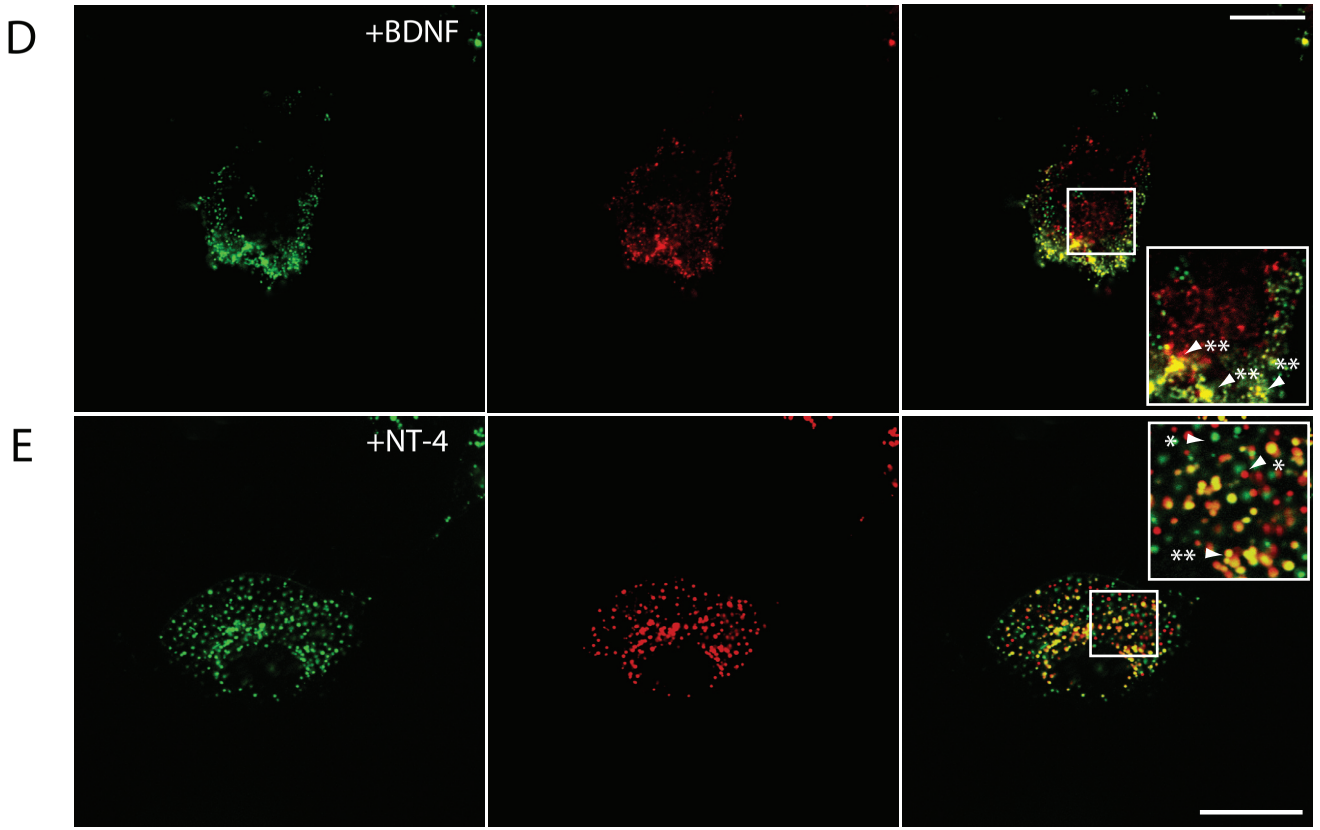


Figure 5.6*b* **NGF and NT-3 increase the association of p75NTR with EEA-1 positive sorting endosomes.**

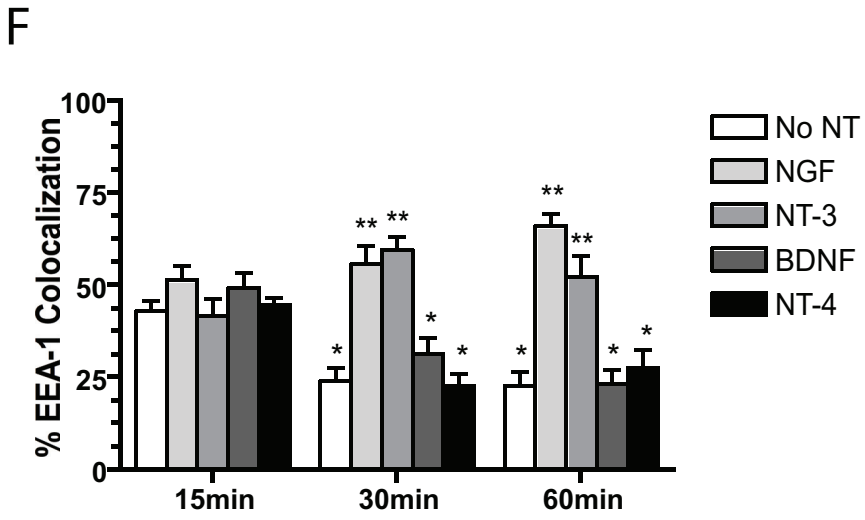


***Figure* 5.6c Neurotrophins decrease the association of p75NTR with EEA-1 positive recycling endosomes.** Analysis of p75NTR colocalisation with EEA-1 positive early/sorting endosomes. Confocal micrographs of NSC-34 cells stained with MLR-2-AF488 and EEA-1-AF594 in the absence of neurotrophins (A, -NT), or in the presence of 100ng/ml exogenous NGF (B), NT-3 (C), BDNF (D) and NT-4 (E) following 60 minute incubation at 37°C. Insert in each merged image provides higher magnification of vesicular structures, arrowheads marked with (*) indicate single marker and arrowheads with (**) indicate vesicles with both p75NTR and EEA-1. (F) Quantification of colocalisation rates at 15, 30 and 60-minute time intervals for all treatments. (Measurements represent 6-10 individual cells, n=3; *p>0.01; paired t-test against treatment control at 15minutes; **p>0.001; paired t-test against treatment control at individual time points; error bars represent SEM; scale bars = 20μm)

Quantitative analysis of different time points indicate that $p75^{NTR}$ and EEA-1 colocalise at similar rates of 40-50%, independent of treatment at 15 minutes (Figure 5.6F). At 30 and 60 minutes however, cells exposed to vehicle only, BDNF or NT-4 showing a decrease in $p75^{NTR}$/EEA-1 positive endosomes to 25-30%. In contrast, exposure to NGF and NT-3 leads to increased association of $p75^{NTR}$ with EEA-1 and this rate increases to 60% and above, remaining steady or increasing at 60 minutes.

5.C.3 NGF and NT-3 reduce $p75^{NTR}$ recycling to the plasma membrane.

Rab GTPases are necessary for efficient membrane transport and different members of the Rab superfamily are localized to distinct organelles of the biosynthetic and endosomal pathways (Miaczynska & Zerial, 2002; Jordens *et al.*, 2005). Rab4 regulates "fast recycling" from early endosomes (van der Sluijs *et al.*, 1991; van der Sluijs *et al.*, 1992) and "slow recycling" from the perinuclear-recycling compartment (Daro *et al.*, 1996), but also appears to affect trafficking along degradative pathways (McCaffrey *et al.*, 2001). $P75^{NTR}$ transport appears to be regulated by Rab5 and Rab7 in motor neurons (Deinhardt *et al.*, 2006), markers of transport/signaling endosomes (Clague & Urbe, 2001; Barbieri *et al.*, 2004), and late endosomes (Meresse *et al.*, 1995) respectively. However, the involvement of recycling compartments in neurotrophin-mediated $p75^{NTR}$ transport has not been clearly established.

To determine whether $p75^{NTR}$ recycled back to the plasma membrane following neurotrophin-dependent internalization, antibodies against the endogenous protein GTPase Rab4 were used to identify $p75^{NTR}$ containing recycling vesicles. NSC-34 cells were incubated with MLR-2-AF488 in the presence or absence of neurotrophins, fixed at time intervals up to 60 minutes, permeabilized and incubated with Rab4 primary antibodies, followed by Alexa-594 secondary antibodies.

Quantification of $p75^{NTR}$ and Rab4 in cells not treated with neurotrophins showed a significant overlap, at 50-60% of total vesicle population across 15, 30, and 60 min incubations (Figure.5.7A-F). In contrast, cells treated with neurotrophins showed reduced rates of colocalisation with Rab4, at all time points, with marginally different rates observed for the individual neurotrophins. $P75^{NTR}$ containing vesicles overlapping with Rab4 positive compartments decreased to below 40% in all treatment groups at 15 minutes. The association of Rab4 with $p75^{NTR}$ positive carriers continued to decline in cells treated with NGF, NT-3 or BDNF, but not NT-4, when observed at 30 and 60 minutes. Most significant differences in $p75^{NTR}$ colocalisation rates with Rab4 were detected at 60 minutes (Figure 5.7A-F), where the overlap between the two markers dropped to below 25% in NGF, NT-3 or BDNF. However $p75^{NTR}$ displayed colocalisation with Rab4 in NT-4 treated cells at levels at or above 37%.

These results indicate that $p75^{NTR}$ appears to enter more readily into a constitutive recycling pathway regulated by Rab4 in the absence of neurotrophins, but exposure to exogenous neurotrophins appears to reduce the sorting of $p75^{NTR}$ to Rab4 positive endosomes. The differences observed between the individual neurotrophins may indicate that each $p75^{NTR}$ may be sorted into distinct endocytic carriers depending on the type of neurotrophin bound.

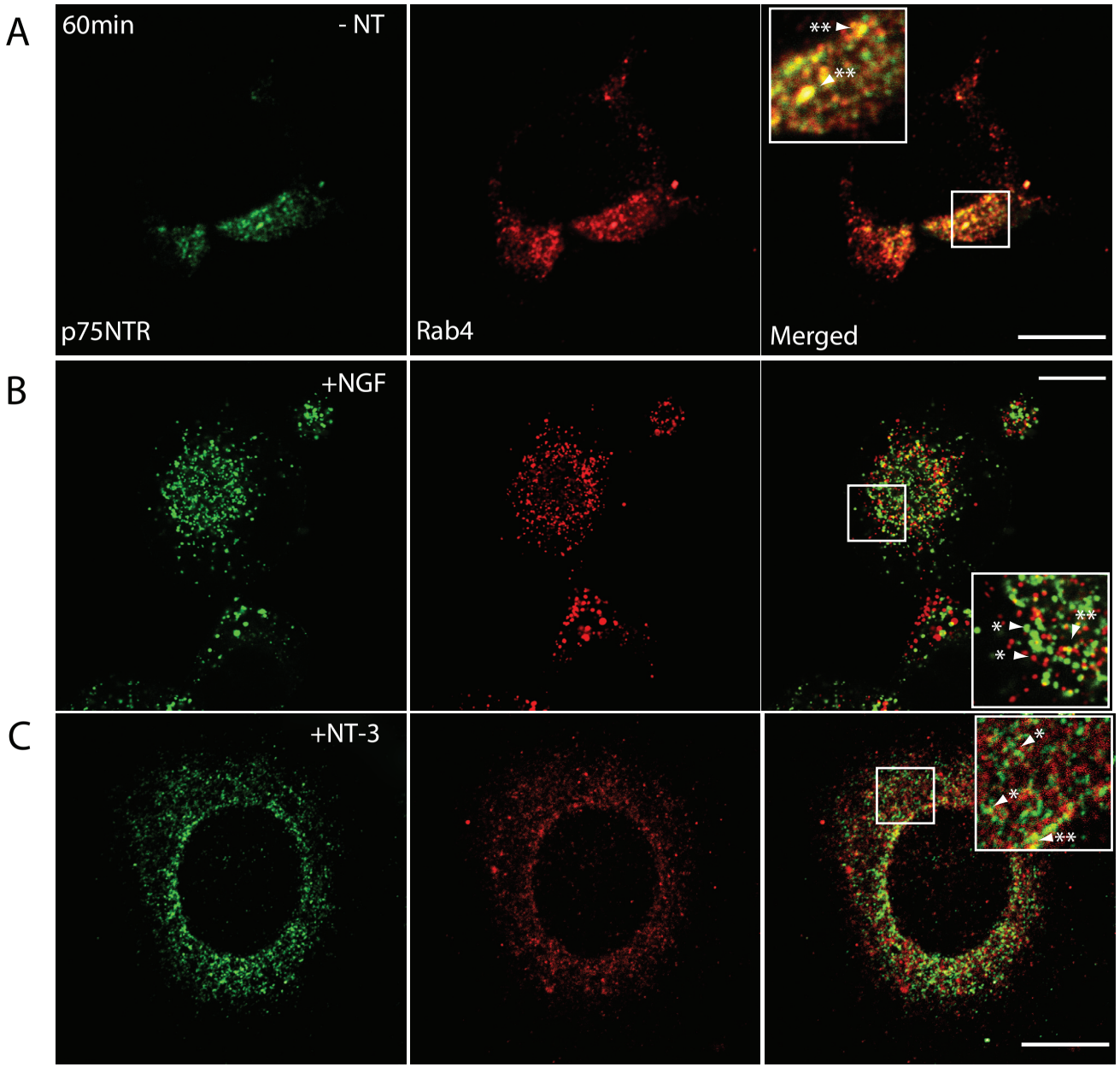


Figure 5.7*a* Neurotrophins decrease the association of p75NTR with Rab4 positive recycling endosomes.

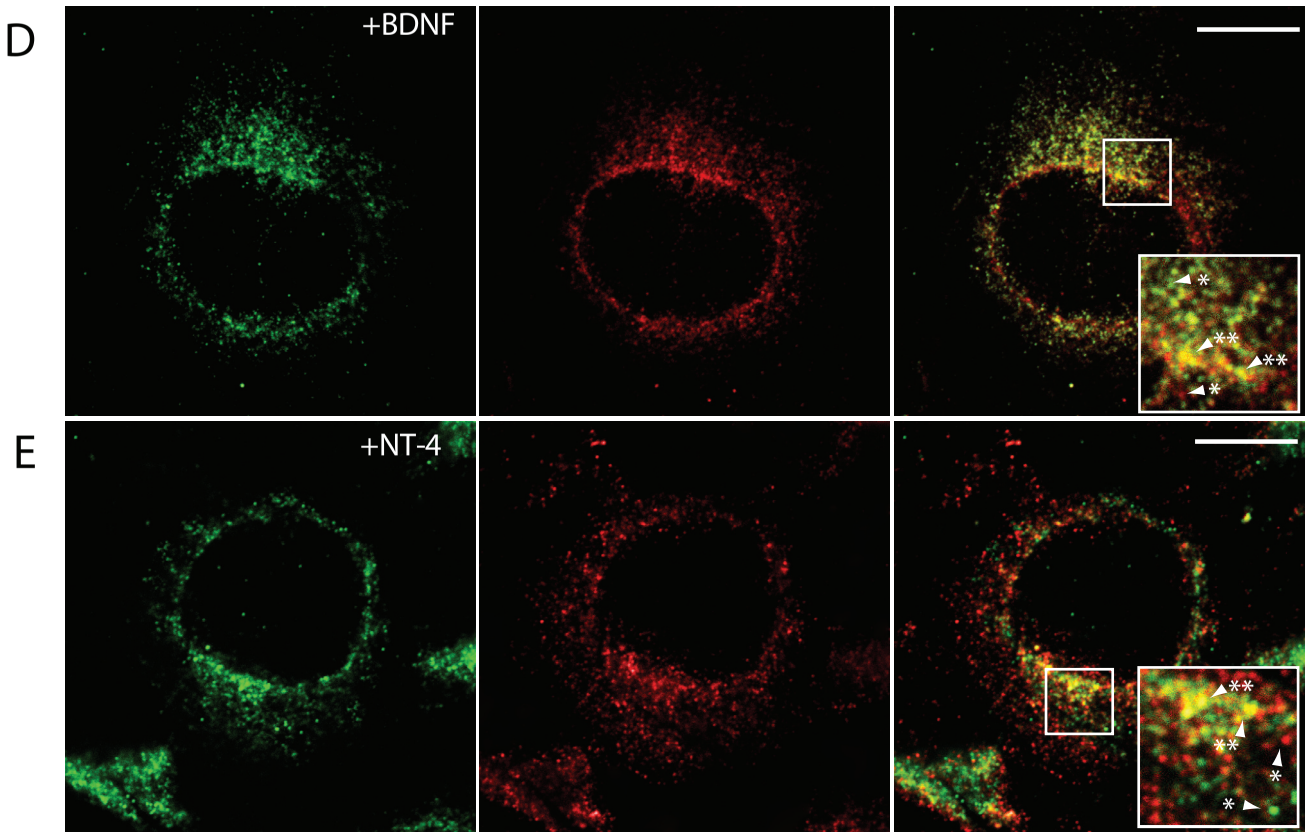


Figure 5.7b **Neurotrophins decrease the association of p75NTR with Rab4 positive recycling endosomes.**

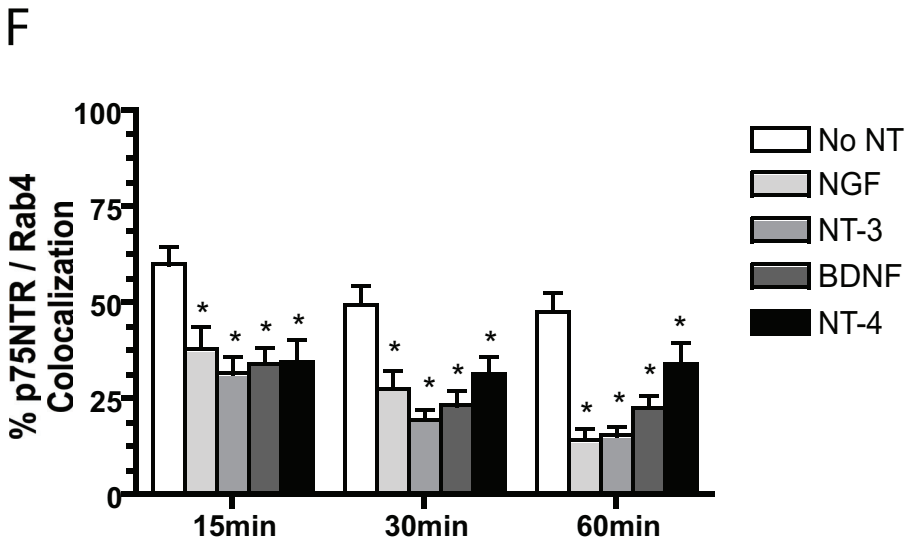


Figure 5.7c **Neurotrophins decrease the association of p75NTR with Rab4 positive recycling endosomes.** Analysis of p75NTR colocalisation with Rab4 positive recycling endosomes. Confocal micrographs of NSC-34 cells stained with MLR-2-AF488 and Rab4-AF594 in the absence of neurotrophins (A, -NT), or in the presence of 100ng/ml exogenous NGF (B), NT-3 (C), BDNF (D) and NT-4 (E) following 60 minute incubation at 37°C. Insert in each merged image provides higher magnification of vesicular structures, arrowheads marked with (*) indicate single marker and arrowheads with (**) indicate vesicles with both p75NTR and Rab4. (F) Quantification of colocalisation rates at 15, 30 and 60-minute time intervals for all treatments. (Measurements represent 6-10 individual cells, n=4; *p>0.01; **p>0.001; two-way ANOVA analysis; error bars represent SEM; scale bars = 20μm)

5.D Discussion

Evidence suggesting that $p75^{NTR}$ functions as a signaling receptor in the absence of Trks (Bronfman *et al.*, 2003) and plays a dominant role in the surface binding of neurotrophins in various cell types (Saxena *et al.*, 2004), highlights an important role this receptor plays in the action of neurotrophins. The current study investigated the internalization and subsequent subcellular localization of $p75^{NTR}$. Although numerous aspects of $p75^{NTR}$ cellular processing have been recently investigated, the endosomal sorting of this receptor remains poorly understood.

Previous reports have focused on establishing neurotrophin dependent differences in the surface binding and internalization of $p75^{NTR}$ (Saxena *et al.*, 2004). In these studies, the internalization of $p75^{NTR}$ in the absence of neurotrophins was limited. Notably, these studies used the monoclonal antibody MC192 (Chandler *et al.*, 1984), which significantly *increases* the binding of NGF to $p75^{NTR}$ (Taniuchi & Johnson, 1985). Since MC192 does not bind to mouse $p75^{NTR}$ (Taniuchi & Johnson, 1985), we used MLR-2 a new $p75^{NTR}$ antibody that recognizes mouse form of the receptor (Rogers *et al.*, 2006). MLR antibodies are suitable for $p75^{NTR}$ internalization studies as they recognize the extracellular domain of the receptor and their binding is not affected in the presence of neurotrophins or vice versa (Matusica *et al.*, 2008).

Complete internalization of $p75^{NTR}$ was observed at 60min in the presence or absence of neurotrophins. Neurotrophins altered the rate of receptor internalization depending on the neurotrophin present. Interestingly, NGF and NT-3 significantly slow down the internalization kinetics of $p75^{NTR}$, but BDNF and NT-4 do not differ significantly from the antibody control. Differential binding of neurotrophins to $p75^{NTR}$ has been suggested previously, as BDNF and NT-3 binding to the receptor was different to NGF (Bothwell, 1995; Dechant & Barde, 1997), and may be responsible for attenuating internalization rates for neurotrophin receptors. It is difficult to correlate these observations with previous in vitro studies (Bronfman *et al.*, 2003; Saxena *et al.*, 2004), as the

antibodies used to track $p75^{NTR}$ may play an important part in influencing receptor behavior. However, these observations clearly differ in that the overall rate of internalization is faster in NSC-34 cells when compared to internalization rates in PC12 cells (Bronfman *et al.*, 2003; Saxena *et al.*, 2004). In addition, the endogenous production of BDNF by the NSC-34 cells throughout the duration of the experiments described in this chapter, may have prevented NGF acting even more efficiently in killing the cell treated cultures. Furthermore, the presence of this endogenous BDNF failed to prevent the effects of NGF.

Internalization of $p75^{NTR}$ in PC-12 cells depends primarily on CME (Bronfman *et al.*, 2003; Saxena *et al.*, 2004), where as in sympathetic neurons it is sensitive to both clathrin and lipid raft disruption (Hibbert *et al.*, 2006). However, $p75^{NTR}$ is enriched in special lipid rafts linked with signaling called caveolae (Nichols, 2003), following NGF stimulation in PC-12 cells (Huang *et al.*, 1999), and caveolae-like membranes (CLM) may initiate neurotrophin signaling through both Trk and $p75^{NTR}$ receptors (Bilderback *et al.*, 1999).

Immunofluorescent localization of $p75^{NTR}$ in NSC-34 cells indicates that the receptor localizes with clathrin-HC and lipid raft ganglioside GT1b. The observation that $p75^{NTR}$ and GT1b only colocalise on the cell surface is consistent with the recent report indicating that the GT1b analogue ganglioside GD1b, which forms part of the receptor complex for tetanus toxin, doesn't undergo internalization into clathrin-coated or uncoated pits from the plasma membrane following tetanus toxin binding (Deinhardt *et al.*, 2006).

Interestingly, the non-specific blocker of endocytosis PAO effectively blocked $p75^{NTR}$ internalization in the presence of all neurotrophins. In contrast, the inhibition of CME with MDC was less effective in the presence of NGF or NT-3, than BDNF or NT-4, suggesting that NGF and NT-3 may trigger $p75^{NTR}$ to non-CME routes of internalization. This observation contrasts to similar work published as this manuscript was being finalized. Deinhardt et al., report that in primary motor neurons $p75^{NTR}$ is internalized through coated and uncoated structures (Deinhardt *et al.*, 2007), however NGF activation of $p75^{NTR}$ results in

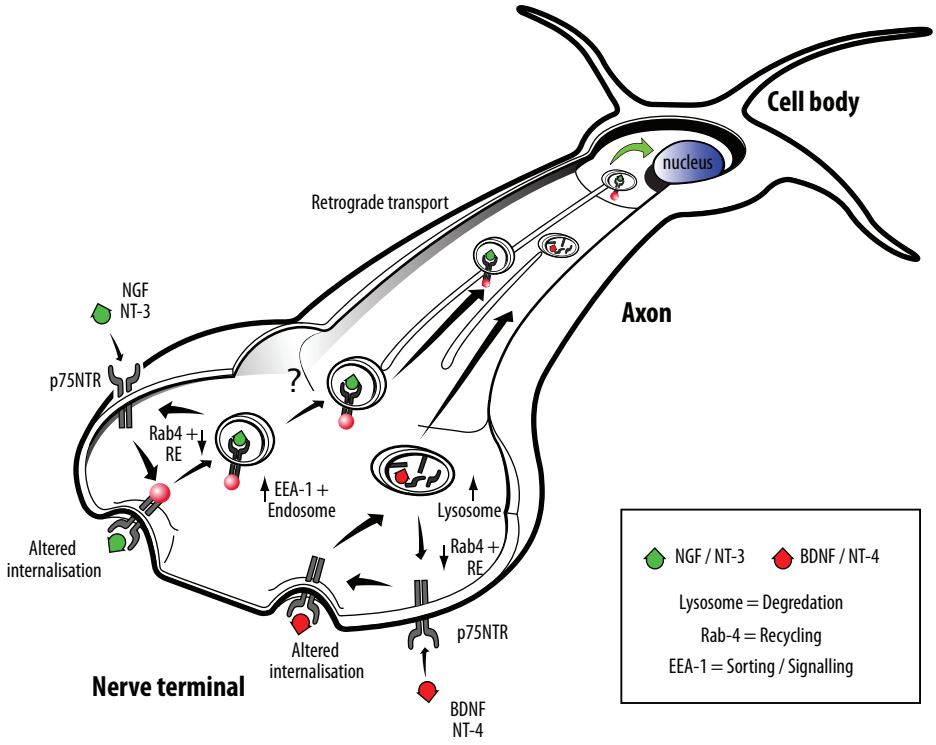


***Figure* 5.8. Neurotrophin regulation of p75NTR intracellular fate.** All neurotrophins bind to p75NTR with similar affinities. In NSC-34 cells the presence of all neurotrophins results in p75NTR internalisation via clathrin mediated endocytosis. However NGF and NT-3 may also utilize alternate pathways of internalisation, demonstrated by the ability of p75NTR to internalize in the presence of NGF and NT-3 despite pharmacological inhibition of CME. The presence of all neurotrophins results in a decrease of p75NTR recycling back to the plasma membrane, indicated by decreasing colocalization with Rab-4. However, following internalization p75NTR is rapidly sorted into the lysosomal pathway in the presence of BDNF and NT-4. In contrast, sorting of p75NTR in the presence of NGF and NT-3 is driven into an early endosomal pathway and remains associated with EEA-1 positive vesicles.

sorting of the receptor from non-CME to CME pathways coupled to axonal transport (Deinhardt *et al.*, 2007).

These results may represent differing internalization routes for the receptor between primary neurons and NSC-34 cells, or alternatively reflect the way in which neurotrophins interact with the different antibody-receptor complexes used in the two contrasting experimental paradigms.

The lack of targeting of $p75^{NTR}$ to the lysosome has been observed in the presence of NGF in cell lines and primary neuronal cultures (Saxena *et al.*, 2005b; Lalli & Schiavo, 2002; Bronfman *et al.*, 2007), however whether this property is dependent on neurotrophins, or represents a recycling observed with receptors such as transferrin (TfR) (Mayor *et al.*, 1993), remains unclear. Certainly, neurotrophin receptors recycling back to the plasma membrane (Grimes *et al.*, 1996; Zapf-Colby & Olefsky, 1998; Bronfman *et al.*, 2003), and prolonged recycling of internalized neurotrophins in the nerve terminal (Weible *et al.*, 2001) has been demonstrated. The present work provides evidence for neurotrophin-dependent differences in regulation of $p75^{NTR}$ sorting into endosomal pathways.

Three pools of $p75^{NTR}$ were observed in the current experiments in the absence of neurotrophins (Figure 5.9). One pool of vesicles positive for Rab4, suggesting that $p75^{NTR}$ is recycled to the plasma membrane, an EEA-1-positive early endosome pool, and a pool targeted for lysosomal degradation. Addition of NGF or NT-3 diverted $p75^{NTR}$ from the recycling Rab4-positive pathway, as well as the lysosomal pathway, and into EEA-1-positive sorting endosomes. In contrast, although BDNF and NT-4 treatment resulted in decreased colocalisation of $p75^{NTR}$ with Rab4, the receptor was still targeted for lysosomal degradation.

The current findings provide further support for neurotrophin-mediated spatio-temporal regulation of $p75^{NTR}$ signaling. NSC-34 cells do not express TrkA, and addition of exogenous NGF results in apoptosis, suggests that $p75^{NTR}$ is responsible for apoptotic signaling. Although NT-3 treatment also induces apoptosis, the signaling is complicated by the presence of TrkC. However, the observation that $p75^{NTR}$ is diverted away from lysosomal degradation in the presence of NGF or NT-3, which leads to apoptotic signaling, but remains present in EEA-1 positive carriers, suggest that $p75^{NTR}$ may signal from early endosomes. Further support for this concept comes from observations that the presence of Rab5 GTPase is necessary for the recruitment of EEA-1.

Taken together the results represented in this study suggest that the fate of the $p75^{NTR}$ receptor depends on which neurotrophin is bound. Further the results suggest an intracellular mechanism to explain the many observations indicating $p75^{NTR}$ activation by different neurotrophins can lead to contrasting outcomes.

Chapter 6: Discussion And Future Prospects

6.A Discussion

6.A.1 Neurotrophin Biology

Neuronal differentiation, migration, proliferation and survival in the embryonic and adult nervous system are tightly regulated by neurotrophins (Davies, 1994; Bibel & Barde, 2000; Huang & Reichardt, 2001). In addition, neurotrophins have also been implicated in different human pathologies, such as depression, eating disorders, Huntington's, Parkinson's, and Alzheimer's diseases (Eide *et al.*, 1993; Dechant & Barde, 2002; Bronfman *et al.*, 2007). The binding of neurotrophins to their receptors ultimately results in the transmission of neurotrophin signals from the cell surface to the nucleus (Heumann, 1994; Kaplan & Miller, 2000; Chao, 2003). This signal transmission is controlled by many factors including changes in the neurotrophin receptor intracellular domains, recruitment of secondary messenger proteins, intracellular scaffolding proteins, molecular motors and highly organized vesicular membrane domains of the endocytic pathway (Reichardt & Mobley, 2004; Teng & Hempstead, 2004; Yano & Chao, 2004; Arevalo & Wu, 2006).

Since the discovery of NGF some 60 years ago, a major goal of the neurotrophin biology field has been the identification of the molecular components involved in evoking the diverse array of neurotrophin signals. One of the most striking features of the action of neurotrophins is their ability to induce highly varied and often opposing effects (Schweigreiter, 2006). Even thirty years after the discovery of NGF, it remained impossible to describe the precise nature of its mechanism of action and their precise site of synthesis and subsequent transport (Bradshaw, 1978). Fortunately, this initial failure only fueled an increasing number of researchers interested in this problem, and today we understand how the specificity of neurotrophin signaling outcomes depends on precise interactions of neurotrophins with their receptors, and the type and combination of these receptors expressed on different cell types (Dechant, 2001; Segal, 2003). The last two decades of neurotrophin research have also resolved many fundamental issues in neurotrophin signaling (Whitmarsh & Davis, 2001; Arevalo & Wu, 2006). These include the identification of signal transduction

pathways for NGF (Kaplan & Stephens, 1994; Miller & Kaplan, 2001b) and the association of various signaling proteins with retrogradely transported endocytic carriers (Grimes *et al.*, 1997; Howe *et al.*, 2001). In addition, NGF studies have revealed that both Trk and $p75^{NTR}$ receptors are found at the cell membrane, clathrin coated endosomes, caveosomes, early endosomes, recycling endosomes, macropinosomes and lysosomes (Yano & Chao, 2004; Bronfman *et al.*, 2007). Despite this wealth of information, the regulation dynamics of neurotrophin receptor transport and endosomal sorting mechanisms appear complex and remain poorly defined.

The majority of studies investigating the trafficking of neurotrophin receptors have focused on TrkA and TrkB receptors (Zapf-Colby & Olefsky, 1998; Beattie *et al.*, 2000; Butowt & von Bartheld, 2001; Jullien *et al.*, 2002; Chen *et al.*, 2005a; Saxena *et al.*, 2005a). Although the retrograde transport of neurotrophin bound $p75^{NTR}$ has been studied over the last 15 years (Curtis *et al.*, 1995; Yano & Chao, 2004), the internalization mechanisms and subcellular sorting of $p75^{NTR}$ has only recently become the focus of detailed analysis (Lalli & Schiavo, 2002; Bronfman *et al.*, 2003; Saxena *et al.*, 2004; Perrone *et al.*, 2005; Hibbert *et al.*, 2006; Deinhardt *et al.*, 2007). In addition, the role of $p75^{NTR}$ in neurotrophin trafficking and signaling remains highly controversial, possibly because it interacts with multiple ligands and co-receptors, and its function is cell-type dependent.

6.A.2 The NSC-34 cell line as a motor neuron model

Cell culture models provide a powerful system for the identification of the specific features at the single cell level, and several have been essential in studies of neurotrophin trafficking and signaling (Kaplan, 1998), of which the PC-12 cell has been instrumental in establishing some of the most fundamental concepts, including interaction between neurotrophin receptors (Wolf *et al.*, 1995), internalization of neurotrophin receptors (Beattie *et al.*, 2000; Zhang *et al.*, 2000), and in recognizing $p75^{NTR}$ as a Trk-independent signaling receptor (Bronfman *et al.*, 2003). Although many of the molecular discoveries in neurotrophin signaling

have resulted from studies of PC12 cells and cultured sympathetic neurons (Kaplan & Stephens, 1994; Schweigreiter, 2006; Wooten & Geetha, 2006; Bronfman *et al.*, 2007), very little is known about these mechanisms in motor neurons.

The studies described in this thesis have established the NSC-34 cell line as suitable for investigating the trafficking of neurotrophins and their receptors, providing a motor neuron model which should complement ongoing studies in PC-12 cells and cultured sympathetic neurons. The long-term success of the PC-12 cell is a reflection of its detailed characterization over four decades, and its responsiveness to numerous growth factors, and in particular, the effects of NGF, which induces rapid changes in cell shape and gene transcription resulting in differentiation into a sympathetic neuron-like phenotype. This sympathetic neuron phenotype is characterized by the development of electrical excitability and neurotransmitter synthesis and release (Greene *et al.*, 1975).

The motor neuron properties of NSC-34 cells are well described (Cashman *et al.*, 1992). However, due to the relative lack of characterization of the NSC-34 cell line for neurotrophin related studies and documented difficulties in culturing these cells, considerable effort was necessary to establish optimal culture conditions and precisely define its neurotrophin characteristics. The culture and differentiation protocols developed provide a simple and standardized method of growing differentiated NSC-34 cells, with a time-frame and ease similar to the PC-12 cell lines. Further, the NSC-34 cells have a neurotrophin receptor profile resembling developing motor neurons, providing a cell line appropriate for neurotrophin receptor studies without the need to transgenically express the receptors as has been done with the PC-12 cell and TrkB receptors (Iwasaki *et al.*, 1997). An additional advantage results from the identification the sortilin receptor in the studies described in this thesis, which is not expressed by PC-12 cells. As the sortilin receptor has recently been confirmed as an essential co-receptor in $p75^{NTR}$ mediated pro-neurotrophin signaling (Nykjaer *et al.*, 2004; Teng *et al.*, 2005; Arnett *et al.*, 2007), its presence in NSC-34 cells provides an opportunity to investigate the mechanism of interaction with

$p75^{NTR}$ and the subsequent intracellular sorting of the multi-receptor-ligand complex.

6.A.3 Neurotrophin receptor trafficking

The shift in our understanding of endocytosis as a mechanism responsible for terminating receptor signaling, to a pivotal system regulating the delivery of specific signals to specific intracellular destinations, is primarily based on insights into the trafficking of growth factor receptors (Ceresa & Schmid, 2000; Cavalli *et al.*, 2001; Miaczynska *et al.*, 2004b). Although the formation of signaling endosomes is well supported by experimental evidence for the TrkA receptor (Grimes *et al.*, 1996; Delcroix *et al.*, 2003; Howe *et al.*, 2001; Howe & Mobley, 2005), the evidence for $p75^{NTR}$ signaling endosomes is only slowly emerging (Bronfman *et al.*, 2003; Deinhardt *et al.*, 2007).

Different experimental approaches in studies investigating $p75^{NTR}$ internalization and intracellular trafficking present a challenge when attempting to draw direct comparisons, but the obvious differences need to be discussed. In agreement with work performed in PC-12 cells by Saxena et al (Saxena *et al.*, 2004), $p75^{NTR}$ internalization in NSC-34 cells appears predominantly associated with clathrin-dependent routes of internalization. In this study, pharmacological blocking of clathrin-mediated endocytosis completely abolished the internalization of $p75^{NTR}$. However, recent work by Deinhardt and colleagues (Deinhardt *et al.*, 2007), performed in primary motor neuron cultures suggest that in the absence of neurotrophins, $p75^{NTR}$ enters the cell via both clathrin-dependent and clathrin independent mechanisms. Further, when NGF is present, $p75^{NTR}$ is shuttled into a predominantly clathrin-dependent route of internalization followed by an increased amount of the receptor targeted for retrograde transport. In contrast, our observations in the NSC-34 cells indicate the opposite scenario, where $p75^{NTR}$ internalization in the presence of NGF is increased through non-clathrin mediated endocytosis. This observation is in agreement with recent reports that $p75^{NTR}$ internalization is only partially

sensitive to disruption of the clathrin-mediated endocytosis pathway (Hibbert *et al.*, 2006).

It thus appears that NGF dependent $p75^{NTR}$ internalization in NSC-34 cells may exploit alternative routes when compared to primary motor neuron cultures and PC-12 cells. In addition, the internalization rates for $p75^{NTR}$ appear to be very different in PC-12 cells (Bronfman *et al.*, 2003; Saxena *et al.*, 2004) compared to our observations in NSC-34 cultures, and of other researchers for primary motor neuron cultures (Deinhardt *et al.*, 2007). However, in agreement with Saxena et al (Saxena *et al.*, 2004), our results also support the observation that different neurotrophins have specific internalization rates via $p75^{NTR}$. Whether the differences are cell-type specific, dependent on Trk receptor co-expression, or signaling-function specific, remains to be determined. However, the experimental protocols for experiments in all these studies were very similar, if not identical.

During the final stages of writing this thesis, Deinhardt and colleagues published a study investigating $p75^{NTR}$ internalization and trafficking in primary motor neuron cultures (Deinhardt *et al.*, 2007). Although there are overlapping similarities in experimental design and focus of their study and ours, many of the observations reported by Deinhardt et al (Deinhardt *et al.*, 2007) support our findings in the NSC-34 cell line. However, the studies in this thesis addressed a number of issues not examined by Schiavo and colleagues. One important issue is whether NGF treatment of primary motor neuron cultures results in a physiological outcome. NGF induces a pro-apoptotic effect in intact neural tube explants, but BDNF, NT-3 and NT-4 do not (Sedel *et al.*, 1999). In agreement with these results we detected apoptosis in NSC-34 cells following NGF exposure. In addition, we also observed a similar effect when the cells were treated with NT-3. This observation is interesting because NT-3 has been described as a pro-survival factor in motor neuron populations (Hughes *et al.*, 1993). NT-3 mediated cell death has been reported in medulloblastomas, but this effect was mediated via the TrkC receptor (Kim *et al.*, 1999). However, endogenous NT-3 induces cell death in adult BDNF-dependent cortico-spinal neurons, and this effect appears to

depend on $p75^{NTR}$ signaling (Giehl *et al.*, 2001). The molecular mechanisms of this action have not yet been examined.

Despite many similarities in neurotrophin trafficking between NSC-34 cells and primary motor neurons, there are also numerous differences observed between the two culture models. These differences re-emphasize the need to re-examine each finding made in the NSC-34 cell line, in primary motor neurons. However in support of the NSC-34 model, similar differences have been observed in the PC12 cell line when compared to primary sympathetic neuron cultures. Nevertheless the PC12 has been, and continues to be an extremely useful model for the study of sympathetic neuron biology.

6.A.4 Is there a distinct $p75^{NTR}$ signaling endosome?

Another unresolved issue is whether physiological outcomes mediated via $p75^{NTR}$ signaling require transport of the receptor-ligand complex to specific sites in the cell body. In recent years, $p75^{NTR}$ has been recognized as a Trk-independent signal-transducing receptor, and $p75^{NTR}$ signaling has been found to promote either survival via activation of the transcription factor NFκB (Carter *et al.*, 1996; Roux & Barker, 2002), or death via the transcription factor c-Jun (Bamji *et al.*, 1998; Coulson *et al.*, 1999). $P75^{NTR}$ is present at synapses, yet the effects of transcription factors are necessarily confined to the nucleus. Hence, $p75^{NTR}$ mediated signals initiated at the axon terminal, must be retrogradely transported to the cell body to convey survival or death signaling. Signaling might emerge either from receptors located on the cell surface or in endosomal compartments (MacInnis & Campenot, 2002). In addition, receptors internalized from the cell surface may either signal or fulfill other tasks such as subcellular trafficking of their ligands (von Bartheld *et al.*, 2001). Experimental evidence for a $p75^{NTR}$ signaling endosome is just beginning to emerge (Bronfman *et al.*, 2003; Deinhardt *et al.*, 2007).

Formation of distinct endosomal vesicles associated with signaling interactors MAGE, NRAGE and necdin has been demonstrated in PC-12 cells (Bronfman *et al.*, 2003). In addition the regulation of intracellular trafficking of

NGF bound p75^{NTR} by Rab-5 GTPase has recently been reported (Deinhardt *et al.*, 2007). The significance of Rab-5 regulation is important because this GTPase has been established as a molecular component of signaling endosomes for epidermal growth factor receptors, TrkA receptors, TGF-β receptors and G-protein coupled receptors (Barbieri *et al.*, 2000; Clague & Urbe, 2001; McPherson *et al.*, 2001; Barbieri *et al.*, 2004; Miaczynska *et al.*, 2004a; Howe & Mobley, 2005). Rab-5 is also an effector of EEA-1, and the two molecules share a high ratio of co-expression in endosomal vesicles (Simonsen *et al.*, 1998; Callaghan *et al.*, 1999).

The present study focused on determining the sorting dynamics of p75^{NTR} as a separate signaling molecule with multiple functions. Although differences in neurotrophin trafficking have been reported for Trks and p75^{NTR} (Altar & DiStefano, 1998; Bartlett *et al.*, 1998; Butowt & von Bartheld, 2001; von Bartheld *et al.*, 2001; Adachi *et al.*, 2005), this is the first study to investigate the differences in p75^{NTR} intracellular trafficking in the presence of different neurotrophins. The association of p75^{NTR} with EEA-1 has been described (Reynolds *et al.*, 2000; Bronfman *et al.*, 2003), however this observation has been interpreted in the context of routine endosomal trafficking, as most receptors internalized through the clathrin-mediated pathway are initially sorted to early endosomes (Bishop, 2003), which then serve as a platform for signaling events (Ceresa & Schmid, 2000; Gonzalez-Gaitan & Stenmark, 2003). Depending on the internalization kinetics of signaling or trafficking receptors, most are found within EEA-1 positive endosomes within 30 minutes of receptor activation (Mellman, 1996; Mousavi *et al.*, 2004; Sorkin, 2004). Association with EEA-1 after 30 minutes decreases, as the receptors and ligands undergo downstream sorting or recycling (Maxfield & McGraw, 2004).

In NSC-34 cells, p75^{NTR} exposed to NGF and NT-3 accumulates in EEA-1 positive carriers for up to 60 minutes, whereas p75^{NTR} treated with BDNF and NT-4 rapidly accumulates in lysosomes. Considering that NGF and NT-3 produce physiological effects when applied to NSC-34 cells, there appears to be a link between the sorting of p75^{NTR} into an alternate pathway, possibly linked with pro-apoptotic signal propagation. The finding that p75^{NTR} is targeted for

lysosomal degradation in the presence of a neurotrophin is a unique observation. Traditionally, $p75^{NTR}$ has been reported to escape degradation in the presence of NGF (Lalli & Schiavo, 2002; Bronfman *et al.*, 2003; Bronfman *et al.*, 2007) and BDNF (Hibbert *et al.*, 2006), leading to the suggestion that various pathogens and toxins may actively exploit this property of $p75^{NTR}$ (Butowt & Von Bartheld, 2003). Our results clearly indicate that the lysosomal fate of $p75^{NTR}$ depends on the presence of specific neurotrophins. In addition, the lysosomal fate of $p75^{NTR}$ may also depend on the cell type and expression of co-receptors.

The suggestion that $p75^{NTR}$ is a recycling receptor has received adequate experimental support (Zapf-Colby & Olefsky, 1998; Saxena *et al.*, 2005b; Deinhardt *et al.*, 2007). In agreement with this conclusion, the $p75^{NTR}$ receptor is associated with the recycling endosome marker Rab-4. However, in contrast to the observations made by Deinhardt and colleagues, suggesting that the presence of either NGF or BDNF does not alter the overall number of recycling $p75^{NTR}$ receptors, we report a decrease of Rab-4 associated $p75^{NTR}$ in the presence of any neurotrophins. This observation is interesting because Deinhardt et al (Deinhardt *et al.*, 2007) reports a redirection of $p75^{NTR}$ to a retrograde transport pathway associated with Rab-5, but no detectable changes in the receptor recycling ratio.

Although studies focusing on $p75^{NTR}$ trafficking coupled with signaling events are in their infancy, evidence for the existence of a $p75^{NTR}$ specific signaling endosome is growing. Reports describing the association of $p75^{NTR}$ endosomes with multiple signaling effectors, signaling endosome fusion proteins and the targeting of this receptor for retrograde transport, bear all the characteristics associated with other well characterized signaling endosome models (Clague & Urbe, 2001; Di Fiore & De Camilli, 2001; Howe & Mobley, 2005). The findings in this thesis provide additional support for the existence of a $p75^{NTR}$ signaling endosome. The sorting of $p75^{NTR}$ to contrasting intracellular fates support active sorting of this receptor based on specific neurotrophin binding. Although the targeting of $p75^{NTR}$ to endosomal transport was not examined, the accumulation of $p75^{NTR}$ in EEA-1 positive vesicles in the presence of neurotrophins, with associated physiological effects is an important observation. Receptors are

trafficked through the mosaic of intracellular compartments to specific destinations, however the accumulation of $p75^{NTR}$ in EEA-1 positive vesicles may represent and a significant sorting step similar to the accumulation of signaling TGF-β receptors in EEA-1 positive carriers (Hayes *et al.*, 2002). The novel concept of neurotrophins regulating the fate of the p75 receptor in a dynamic and highly specific manner needs to be investigated further both, in cell lines and primary neuronal cultures, and should bear valuable insights into the mechanisms involved when aided by the impressive range of rapidly improving tools and techniques for the study of real time receptor trafficking.

6.B Future Prospects

The NSC-34 cell line is well established as a model for motor neuron neurotoxicity studies (Durham *et al.*, 1993; Liu *et al.*, 2002; Rembach *et al.*, 2004; Babetto *et al.*, 2005; Zhai *et al.*, 2005; Atkin *et al.*, 2006; Raimondi *et al.*, 2006; Rizzardini *et al.*, 2006). The studies described in this thesis have expanded the characterization of this cell line to diversify its usefulness in not just the field of neurotrophin research, but also general motor neuron relevant biology. The neurotrophin receptor profile of the NSC-34 cell line offers broad scope to conduct investigations aimed at unraveling the complex receptor interactions that occur as a result of neurotrophin binding and that lead to multiple biological responses including survival, differentiation or programmed cell death. In particular, the presence of functional neurotrophin receptors should allow the exploitation of this cell line for studies of receptor trafficking, signaling and downstream responses including those initiated by the pro-neurotrophins through the sortilin and $p75^{NTR}$ receptor.

A number of recent and exciting discoveries, including the observation that $p75^{NTR}$ undergoes sequential cleavage through α- and γ-secretase (Kanning *et al.*, 2003; Zampieri *et al.*, 2005), the recent identification a tyrosine kinase domain on $p75^{NTR}$, and several new signaling proteins downstream of $p75^{NTR}$ (Arevalo *et al.*, 2005; Arevalo & Wu, 2006) have provided many more insights into the intricate functions and mechanisms of neurotrophin actions, and opened an

unpredictable new area of investigation to examine the function of this receptor. P75NTR cleavage has been postulated to generate an intracellular domain (ICD) fragment that appears to translocate into the cell nucleus (Frade, 2005), but whether this regulates gene expression in a way similar to other receptors remains to be determined.

Although multiple signaling functions have been attributed to $p75^{NTR}$, the verification of these pathways has been a difficult enterprise due to the weakness of signal transduction and the lack of genetic systems to verify protein-protein interactions. The constant and rapid improvement in research tools, especially highly sensitive microscopy techniques able to detect protein-protein interactions should guide future studies to (1) address the role of these new proteins in the neurotrophin signaling not only in PC-12 and NSC-34 cells, but also in primary neuron populations, (2) use genetically modified animals to verify $p75^{NTR}$ function resulting from different neurotrophin activation, (3) identify new signaling molecules, mechanisms, and regulators of $p75^{NTR}$ that can explain the puzzling diversity of functions, and (4) understand further the role of neurotrophins in human pathologies. Nevertheless, it is clear these novel findings need to be confirmed in a primary motor neuron. In conclusion, neurotrophins remain mysterious growth factors that will no doubt provide many exciting surprises for years to come.

Appendix

Appendix 1: Mapping of MLR antibodies binding domains to p75NTR.

As a integral part of characterizing the MLR antibodies, MLR-2 and MLR-3 variants were sent to Dr. Christine Tuffereau at Laboratoire de Virologie Moléculaire et Structurale, UMR 2472 CNRS-INRA, 91198 Gif-sur-Yvette, France. To map the binding site for MLR2 and MLR3, deletion mutants of the cysteine rich domains (CRDs) in human p75 with the correct folding and processing were constructed. Flow cytometry analysis (A) revealed that MLR2 and MLR3 appear to require at least CRD 3 and 4. In comparison, ME20.4 requires CRD 3. Western blot with MLR2 in non-reducing conditions shows MLR2 recognizes CRD 234, but not CRD 34 (B). The monoclonal antibodies did not recognize any CRDs under reducing conditions (data not shown). The dimmer recognized by MLR2 when CRD 1 is absent has been observed previously with ME20.4 (Lange in et al., 2002).

A

hp75	MLR1	MLR2	MLR3	Mab 20.4 (αhuman)	
CRD 1234	+++	+++	+++	+++	
CRD 234	+++	+++	+++	+++	
CRD 34	weak		+++	+++	
CRD 1	-	-	-	-	
CRD 12	-	-	-	-	
CRD 123	-	-	-	+++	

B

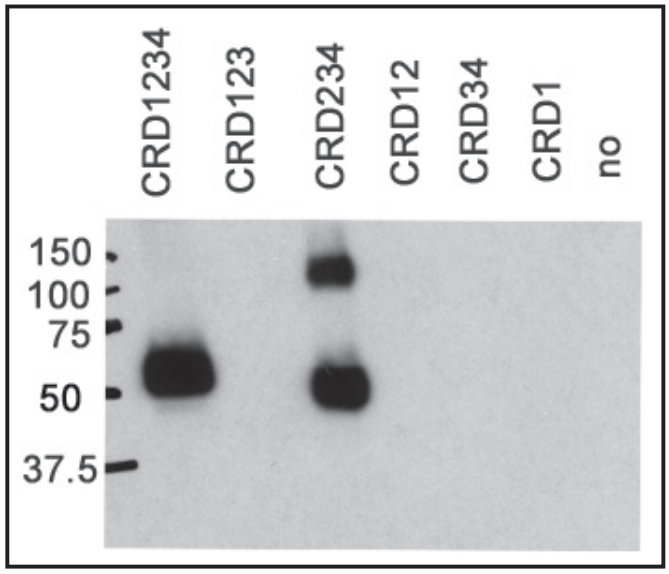


Figure A1. Mapping of MLR antibody binding epitopes of p75NTR. To map the binding site for MLR2 and MLR3, deletion mutants of the cysteine rich domains (CRDs) in human p75 with the correct folding and processing were constructed. Flow cytometry analysis (**A**) revealed that MLR2 and MLR3 appear to require at least CRD 3 and 4. In comparison, ME20.4 requires CRD 3. Western blot with MLR2 in non-reducing conditions shows MLR2 recognizes CRD 234, but not CRD 34 (**B**). The monoclonals did not recognize any CRDs under reducing conditions (data not shown). The dimmer recognized by MLR2 when CRD 1 is absent has been observed previously with ME20.4 (Lange in et al., 2002).

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Verum
est quoque

decorus futurus
vitualamen