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.

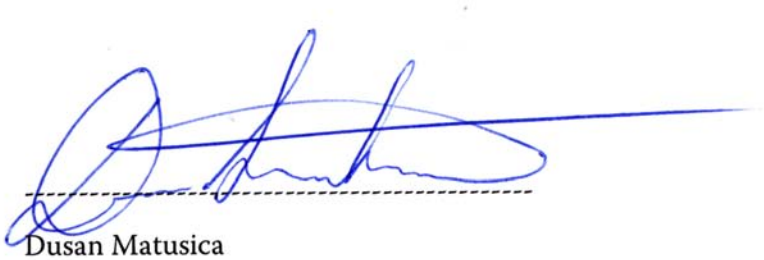
In Chapter 4, multiple experimental approaches were used to extensively screen the NSC-34 cell line for the presence of motor neuron-specific markers, neurotrophin receptors and proteins involved in regulation of endosomal transport. This characterization established the presence of a developing motor 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

ROGERS, M. L., ATMOSUKARTO, I., BERHANU, D. A., **MATUSICA, D.**, MACARDLE, P. & RUSH, R. A. (2006) Functional monoclonal antibodies to p75 neurotrophin receptor raised in knockout mice. ***Journal Of Neuroscience Methods,*** 158**,** 109-20.

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