Localisation and expression pattern of the Nogo receptor and its ligand, Nogo-A in cells of the mammalian central nervous system.

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DECLARATION

I declare that this thesis is my own work, and has never been submitted for any other academic degree or qualification.

Signature .....

On this _____ day of _____ **2007**

DEDICATION

I dedicate this book to my dearest wife **Susan M. Nyatia** and my first born daughter **Tara K. Nyatia** for the support they offered during my doctorate studies.

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LIST OF ABBREVIATIONS

AP	alkaline phosphatase
CAM	cell adhesion molecule
CAP-23	a novel myristoylated calmodulin-binding protein, abundant in nerve terminals
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
CNS	central nervous system
CSPG	chondroitin sulphate proteoglycan
CST	cortical spinal tract
DIGs	detergent-insoluble glycolipid-enriched complexes
DiI	A general fluorescent tracer dye
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
E	embryonic day
EAE	experimental allergic encephalomyelitis,
ER	endoplasmic reticulum
FCS	fetal calf serum
Fyn	a protein tyrosine kinase
g, gm	gram
GAP-43	growth associated protein-43
GDNF	growth derived neurotrophic factor
GFP	green fluorescent protein
gm/l	grams per liter
GPI	glycosylphosphatidylinositol
GST	glutathione s-transferase
HA	Haemagglutination
IgF	immunoglobulin family
IgM	Immunoglobulin M

IGSF	immunoglobulin super family
IP	Immunoprecipitation
kDa	kilodaltons
KLH	keyhole limpet hemocyanin
LRR	leucine rich repeat
MAG	myelin associated glycoprotein
min(s)	minute(s)
MS	multiple sclerosis
NCAM	neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NGF	nerve growth factor
NgR	nogo receptor
Nogo-66	novel oligodendrocyte-derived inhibitory protein
Nogo-A	novel oligodendrocyte-derived inhibitory protein-A
NTR	neurotrophin
OMgp	oligodendrocyte myelin glycoprotein
P	postnatal day
PAGE	polyacrylamide gel electrophoresis
PI-PLC	phosphatidylinositol specific phospholipase-c
PNS	peripheral nervous system
SDS	sodium dodecyl sulphate
SIB	Swiss Institute of Bioinformatics
Siglec	sialic acid-binding immunoglobulin-like lectin
Trk	tyrosine kinases
VCN	ventral cochlear nucleus

Abstract

Axon regeneration failure in the adult mammalian central nervous system is partly due to inhibitory molecules associated with myelin. The Nogo receptor plays a role in this process through an extraordinary degree of cross reactivity with three structurally unrelated myelin-associated inhibitory ligands namely; Nogo-A, myelin associated glycoprotein and oligodendrocyte myelin glycoprotein. The major aim of the study was to investigate the expression pattern of Nogo receptor and one of its ligands, Nogo-A in the mammalian nervous system, and also investigate whether Nogo receptor is located in neuronal lipid rafts by linking it to flotillins, known lipid raft markers. We therefore generated a rabbit polyclonal Nogo receptor antibody from the leucine rich repeat number 9 domain of Nogo receptor polypeptide chain. Together with a commercially available polyclonal antibody specific for Nogo receptor, and in conjunction with double labelling immunofluorescence methods on cryosections and cell cultures, Nogo receptor immunoreactivity was also observed in brain, spinal cord, and dorsal root ganglia. In cellular populations, it was confined to neuronal cell bodies and their processes. Nogo receptor was localised on the surface of extending dorsal root ganglion intact axons and growth cones in live staining experiments. Nogo-A, an important axon growth inhibitory molecule and member of the reticulon family protein, was widely distributed in the mammalian brain, spinal cord, and dorsal root ganglia. Intense Nogo-A immunoreactivity was detected in oligodendrocyte cell bodies and their myelin sheaths in nerve fibre tracts of the central nervous system. Furthermore, numerous populations of neurons in the brain and spinal cord expressed Nogo-A to a variable extent in their cell bodies and neurites, suggesting additional, as-yet-unknown, functions of this protein. In cell culture, cytoplasmic staining with anti-Nogo-A antibody was observed after fixation in

oligodendrocytes and neurones, but intracellular structures that presumably represent endoplasmic reticulum were also strongly labelled in fibroblasts. These results confirm results obtained by other researchers with different sets of antibodies. However, they also raise the question of the mechanism and circumstances under which the Nogo receptor interacts with Nogo-A, as this protein appears to be confined to the cytoplasm and can therefore not be expected to bind Nogo receptor on the axon surface. To investigate whether Nogo receptor is localised in neuronal lipid rafts, commercial and local antibodies specific for Nogo receptor, in conjunction with flotillin (a known lipid raft-associated protein) were used in double-immunofluorescence, co-immunoprecipitation and western blotting experiments. Results revealed substantial immunofluorescent colocalisation of Nogo receptor and flotillin in membranes of axons and PC-12 cells. Further more, extraction of Nogo receptor antigen from rat brain using receptor bound protein-A sepharose beads, followed by probing with anti-flotillin antibody, established the link between lipid rafts and Nogo receptor. These results indicate that rafts may provide a molecular environment that facilitates the interaction between Nogo receptor and multiple molecules involved in signal transduction downstream during neurite out-growth inhibition. Therefore, interference with lipid raft-associated signal transduction may offer interesting perspectives with regard to overcoming the failure of axon regeneration in the injured. Further studies will be required to elucidate this receptor-ligand interaction.

INTRODUCTION

1.0 INTRODUCTION

1.1 Introduction

Axonal growth is mediated by specialized structures on axon tips known as growth cones. Numerous finger-like projections known as filopodia emanate from the growth cones to scan the environment. These filopodia allow the nascent axon to respond to the external cues that guide it to the ultimate target (Purves and Williams, 2001). Axons in the mammalian central nervous system (CNS) do not spontaneously regenerate following injury and consequently there is little functional recovery, compared to injured axons in the adult peripheral nervous system (PNS) which do regenerate after injury where growth is promoted by the environment created by the presence of Schwann cells (David and Aguayo, 1981). The environment of the adult mammalian CNS is hostile to axon growth, and is a major contributor to the inability of injured neurons to regenerate (Liu *et al.*, 2002). However, axons can grow and regenerate in embryonic mammalian CNS.

In contrast to mammals, lower vertebrates, including amphibians, fish and to some extent reptiles, possess a remarkable ability to repair injuries in the CNS, resulting in the re-growth of transected axons and their reconnection with target areas. Impaired functions are usually restored in the course of a few weeks (Bernhardt, 1999). In frogs, axons from the optic nerve and tectum do regenerate (Lang *et al.,* 1995). However, CNS axons fail to regenerate in the spinal cord after metamorphosis (Beattie *et al.,* 1990; Forehand and Farel, 1982). The inability of regeneration correlates with the non permissive properties of spinal cord myelin and oligodendrocytes in co-cultures with axons, indicating the expression of inhibitory proteins in this region (Lang and Stuermer, 1996; Lang *et al.,* 1995).

Axonal regeneration after injury depends on the interplay between extrinsic cues and intrinsic properties of the lesioned neuron. Whether or not a mature neuron re-extends an

axon depends on the availability of neurotrophic factors and substrate molecules to which growing neurites may attach and extend, ability to re-express growth-related genes, the presence of growth-inhibiting molecules and the formation of a glial scar (Schwab and Bartholdi, 1996; McKerracher *et al.*, 1994).

1.2 Neurotrophic factors and substrate molecules

Since the mammalian PNS has the ability to regenerate following injury, emphasis has been directed on identifying factors that are up-regulated during PNS regeneration which could also play the role of neurotrophic factors in promoting axon regeneration in the CNS. Recent studies have revealed that neurotrophins are an important requirement for peripheral axon out-growth during development (Markus *et al.*, 2002). Expression of neurotrophins like glial-cell-line-derived neurotrophic factor (GDNF) and immunoglobulin family (IgF) family members is up-regulated in distal peripheral nerve after injury. Recently it has been shown by Markus *et al.*, 2002, that several Trk effectors and other receptor tyrosine kinases are a requirement in mediating axon growth in developing peripheral neurons *in vitro*.

1.3 Growth-related genes

Dorsal root ganglion (DRG) cells have proven particularly valuable to neuroscience research because they contain both central and peripheral processes. As expected, regeneration following a lesion to the central process is very limited. However, Smith and Skene, 1997 observed significant neurite out-growth *in vitro* when the neurons were first primed with a peripheral conditioning lesion. These results suggest that the initial lesion leads to reactivation of an intrinsic growth program. The neuron seems to increase

expression of growth associated proteins and somehow transports them to the tip of the injured central axon.

Skene, 1989 next sought to mimic the benefits of peripheral nerve injury by ectopically expressing growth cone proteins GAP-43 and CAP-23. Transgenic mice were used to study the effects of each protein on axon elongation *in vitro*. DRG neurons from mice over expressing both GAP-43 and CAP-23 exhibited an increased propensity for axon sprouting. Disappointingly, neither protein was able to recreate the transition from local arborisation to extended out-growth. However, expression of both proteins at once led to growth strikingly similar to that which follows peripheral nerve injury. Another study done using transgenic mice by Buffo *et al.,* 1997, showed that over-expression of GAP-43 substantially modifies Purkinje cell response to injury subsequent to axotomy, by inducing axonal growth processes and decreasing their resistance to injury through expression of the growth-associated gene program. However, the presence of this protein is not sufficient to enable these neurons to accomplish a full program of axon regeneration.

In an attempt to recreate Skene's results *in vivo*, the extent of regeneration in the transgenic mice was examined following spinal cord transection. A sciatic nerve graft was surgically implanted across the lesion to provide an environment more conducive to regeneration. The initiative arose from the fact that sciatic nerve being part of the PNS would promote growth due to the environment created by the presence of Schwann cells (David and Aguayo, 1981). Several months later, the fluorescent axonal tracer DiI was injected into the distal end of the nerve graft. Retrograde transport led to clear labelling of the axons that had demonstrated significant out-growth into the graft, allowing the researchers to quantify the amount of regeneration. Consistent with the *in vitro* studies, regeneration was best in the combined GAP-43 and CAP-23 transgenic mice (Bomze *et al.,* 2001). However, the

regeneration was significantly less than the level previously observed following the creation of a conditioning lesion. This implies that other factors may be involved in reactivation of growth capacity. Furthermore, regeneration of this type has not yet been demonstrated in the absence of a nerve graft that provides a permissive environment. Nevertheless, the researchers provided some evidence that overexpression of specific proteins like B-50/GAP-43 can enhance CNS regeneration.

Cell adhesion molecules are spatio-temporally expressed in the nervous tissues during development and play critically important roles for the proper cell-cell interactions (Schachner, 1990; Martini, 1994). Several characteristic proteins of cell adhesion molecules that belong to the immunoglobulin superfamily (IGSF) have been identified as regulating axonal out-growth. L1, a cell adhesion molecule, and a member of the IGSF is localised on axons and axonal growth cones in the developing nervous system and is well known to play a role in axon out-growth, fasciculation, guidance, and myelination (Beasley and Stallcup, 1987; Kamiguchi and Lemmon, 2000; Kamiguchi *et al.,* 1998). The importance of L1 in neuronal development is suggested by its conserved nature across species. L1 CAM homologues in human and mouse share 92% identity at the amino acid level and additional homologues include NILE in rat, Ng-CAM in chick, and Neuroglian in Drosophila (Bieber *et al.,* 1989; Burgoon *et al.,* 1991; Cohen *et al.,* 1997; Fransen *et al.,* 1998; Hlavin and Lemmon, 1991; Moos *et al.,* 1988). Although L1 is referred as a CAM, its expression is not restricted to the nervous system. It is also found on several non-neuronal cells, among them melanoma cells and hematopoietic tumor cells (Brümmendorf and Rathjen, 1995).

Mutations in the X-linked L1 gene can lead to major developmental errors in axonal pathfinding in both humans and mice (Dahme *et al.,* 1997). Substantial defects of the corticospinal tract and the corpus callosum have been reported in humans and mice

carrying L1 mutations (Demyanenko *et al.,* 1999; Fransen *et al.,* 1995; Kamiguchi *et al.,* 1998; Rolf *et al.,* 2002). In addition to its role in development, L1 has been proposed to function in axonal regeneration. Regenerating axons in the PNS up-regulate L1 following injury and maintain these levels during their re-growth (Martini and Schachner, 1986). Following a lesion in the entorhinal cortex, CNS axons that sprout into the denervated hippocampus are L1-positive while those failing to sprout are L1-negative (Styren *et al.,* 1995). Furthermore, when grafts of nerve growth factor (NGF) or beta-galactosidase (betaGal)-producing fibroblasts are implanted into the fimbria-fornix lesion cavity of an injured adult rat brain, there is a strong correlation between L1 re-expression and the degree of axonal regeneration (Aubert *et al.,* 1998; Chaisuksunt *et al.,* 2000; Woolhead *et al.,* 1998).

1.4 Inhibitors of axon growth in the CNS

The proposal that factors in the CNS environment could prevent regeneration was first articulated by Cajal Ramony in 1928. This observation was broadened by David and Aguayo, 1981 who made it clear that retinal neurons can form long projections in peripheral nerve grafts. Two main positions have been considered for the location of CNS factors that might inhibit axon regeneration. The first position is the scar that forms at the region of injury. Following CNS injury, the central area of necrosis prompts astrocytes to hypertrophy and reorganise to form the glial scar, that forms a barrier to regeneration, and both astrocytes and oligodendrocytes express factors and inhibitory molecules that potentially block the re-growth of axons. For instance oligodendrocytes produce NI250, myelin-associated glycoprotein (MAG), and tenascin-R, oligodendrocyte precursors produce NG2 DSD-1/phosphacan and versican, astrocytes produce tenascin, brevican, and neurocan, and can be stimulated to produce NG2, meningeal cells produce NG2 and other

proteoglycans, and acitivated microglia produce free radicals, nitric oxide, and arachidonic acid derivatives. Many of these molecules must participate in rendering the damaged CNS inhibitory for axon regeneration. (Fawcett and Asher, 1999; Fu and Gordon, 1997; Houle and Tessler, 2003). Axons do not extend through the scar, and appear to be inhibited by it, with the axon tips forming blunt ends that remain in one place for a long time. Molecular components that may contribute to this inhibitory activity include chondroitin sulphate proteoglycans (CSPG), tenascin, and semaphorins-3A which are inhibitory to axon growth in culture (Letourneau *et al.,* 1994; Davies *et al.,* 1999; Pasterkamp *et al.,* 1999).

The other major proposal is that inhibitors would be broadly distributed in the myelin that ensheaths axons in white matter tracts on the adult CNS. Supporting this idea, the loss of regeneration potential during development correlates roughly with the onset of myelination. Myelin or oligodendrocytes the cells that produce CNS myelin, are poor substrates for axon out-growth *in vitro* (Chen *et al.,* 2000). In 1988, Caroni and Schwab raised a monoclonal antibody IN-1 against a 250 kDa protein NI-250, a neurite growth inhibitor of CNS myelin, which also recognized NI-35, a smaller rat inhibitory protein. They showed that myelin can be turned into a less hostile *in vitro* axon substrate by addition of IN-1, a neutralizing antibody. Bregman *et al.,* 1995, went on to demonstrate that application of IN-1 antibodies at the lesion site as a form of treatment on rats with spinal cord injury caused an improvement in regeneration of corticospinal axons, as well as enhanced recovery of behavioural function. The rats were able to recover their specific reflex and locomotor function.

1.5 Myelin inhibitors of axon growth

To date, a number of individual proteins that inhibit axon growth have been identified. Three main inhibitory components of myelin are now known; namely myelin associated

glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgp) (Kottis *et al.*, 2002; Wang *et al.*, 2002b).

1.5.1 Myelin associated glycoprotein (MAG)

MAG is quantitatively a minor constituent, representing 1% of the total protein found in myelin isolated from the CNS and 0.1% in the PNS (Quarles *et al.*, 1979). The MAG gene spans 16 kb in length and includes 13 exons. It is located on chromosome 7 in mouse and 19 in human (Barton *et al.*, 1987; D'eustachio *et al.*, 1988). MAG has an apparent molecular mass of 100 kDa on SDS-PAGE, of which 30% is carbohydrate. MAG proteins have both a membrane-spanning domain and an extracellular region that contains five segments of internal homology that resemble immunoglobulin domains (Salzer *et al.*, 1987) and are strikingly homologous to similar domains of the NCAM and other members of the immunoglobulin gene superfamily. Both share identical extracellular and transmembrane domains but differ in their cytoplasmic domains, corresponding to the alternative splicing of exon 12 from a single RNA transcript. Nogo which I shall later expand on and OMgp tend to have a similar resemblance with MAG, in terms of extracellular and transmembrane domains. McKerracher *et al.*, 1994 purified myelin from MAG-deficient mice and separated octylglucoside extracts of myelin by diethylaminoethyl (DEAE) ion-exchange chromatography. Although they could not detect any significant difference in neurite growth on myelin purified from *MAG* knockout and wild type mice, differences were observed in the fractionated material. The major inhibitory peak that is associated with MAG in normal mice was significantly reduced in MAG-deficient mice. The *in vitro* studies, however, provided the first evidence that MAG modulates growth cone behavior and inhibits neurite growth by causing growth cone collapse. A different approach by

Filbin *et al.,* 1990, showed that Chinese hamster ovary (CHO) cells transfected with MAG inhibited neurite out-growth (Mukhopadhyay *et al.,* 1994). *In vitro*, MAG regulates growth in an age-dependant manner. MAG promotes growth of many types of embryonic and neonate neurons and, at more mature stages, strongly inhibits growth (Johnson *et al.,* 1989; McKerracher *et al.,* 1994; Mukhopadhyay *et al.,* 1994; Hasegawa *et al.,* 2004). Within the CNS, expression of MAG is restricted to paranodal loops and periaxonal membranes of myelin (Bartsch *et al.,* 1989; Martini and Schachner, 1986; Trapp *et al.,* 1989) and interacts with gangliosides GD1a and GT1b which are prominent neural cell surface sialoglycolipids, as MAG ligands (Yang *et al.,* 1996), which may serve as axonal MAG receptors. This MAG-mediated interaction between oligodendroctye and neuron is thought to initiate a bi-directional signalling system. MAG, a sialic acid-binding Ig-like lectin (Siglec), binds preferentially to carbohydrates bearing terminal α-2,3-linked sialic acids (Crocker and Varki, 2001; Vyas and Schnaar, 2001). MAG binds to neuronal cell surfaces and inhibits growth in a sialic acid-dependent, *vibrio cholera* neuraminadase-sensitive (VCN-sensitive) manner (Kelm *et al.,* 1994; DeBellard *et al.,* 1996). Though the signalling pathways for MAG, OMgp, and Nogo-66 may converge in neurons (Wang *et al.*, 2002b), there are also differences in the neuronal receptors used by these molecules, suggesting different functional activity (Vinson *et al.,* 2001, 2003; Niederost *et al.,* 2002; Vyas *et al.,* 2002). Remarkably, MAG is likely to play a role in oligodendrocyte function that may be distinct from that of Nogo or OMgp. In oligodendrocytes, the cytoplasmic region of MAG has been shown to interact with several molecules involved in cytoskeletal organisation and signal transduction. The major tyrosine phosphorylation residue was identified as Tyr-620, which was found to interact specifically with the SH2 domains of phospholipase C (PLC gamma). Myelin associated glycoprotein also specifically bound the Fyn tyrosine kinase,

suggesting that MAG serves as a docking protein that allows the interaction between different signaling molecules (Umemori *et al.*, 1994; Jaramillo *et al.*, 1994; Kursula *et al.*, 2000). Antibody engagement of oligodendrocyte cell surface MAG leads to activation of Fyn kinase, a molecule key to oligodendrocyte differentiation and survival and to myelination (Umemori *et al.*, 1994).

1.5.2 Oligodendrocyte myelin glycoprotein (OMgp)

In 1988, Mikol and Stefansson first identified OMgp as a new peanut agglutinin-binding protein in the white matter of human CNS. This protein was found only in CNS myelin preparations and on bovine oligodendrocytes in culture. OMgp appeared as a 120 kD highly glycosylated polypeptide anchored in the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI)-link. Treatments by de-glycosylating enzymes revealed the presence of several O and N-linked oligosaccharides on OMgp. This observation was consistent with its extracellular localisation. The distribution patterns of OMgp mRNA and protein in adult mouse CNS were analysed using in situ hybridisation and immunohistochemistry experiments (Habib, 1998). In situ hybridisation revealed that an important part of OMgp mRNA is in neuronal cells, the most intense signal being from the layer 5 of the neocortex, the pyramidal layer of the hippocampus and the Purkinje cells in the cerebellum. Large neurons in the spinal cord and some neurons in the hypothalamus and brainstem also express OMgp. Oligodendrocyte myelin glycoprotein (OMgp) is the most recently identified of the inhibitory components of myelin. McKerracher *et al.*, 1994 first came up with 2 major peaks of inhibitory activity while purifying myelin proteins. The first peak contained MAG, the second peak contained inhibitory proteins which were

further separated by PNA-agarose chromatography and OMgp was identified as a probable inhibitor (Kottis *et al.*, 2002).

Phosphatidylinositol specific phospholipase-C (PI-PLC), an enzyme which hydrolyses the GPI-anchor, has been used on myelin from bovine white matter. In growth cone collapse assay using dorsal root ganglia extracted from E13 chick embryo, Wang *et al.*, 2002a observed that the PI-PLC released fraction alters growth cone morphology. Wang further confirmed this hypothesis after purification of OMgp and performing functional assays, based on neurite out-growth inhibition and growth cone collapse. Arretin, a protein fraction purified from bovine brain myelin, has been identified as consisting predominantly OMgp. It is a potent inhibitor of neurite out-growth from rat cerebellar granule and hippocampal cells; from DRG explants in which growth cone collapse was observed; from rat retinal ganglion neurons; and from NG108 and PC-12 cells (Kottis *et al.*, 2002).

1.5.3 Nogo

The target of the IN-1 antibody discussed above turned out to be a novel oligodendrocyte-derived inhibitory protein (Nogo), a reticulon homologue with three isoforms. Nogo was reported independently by three groups as the long sought after high molecular weight myelin inhibitor (reviewed in Brittis and Flanagan, 2001).

1.5.3.1 Nogo isoforms

As a protein expressed by oligodendrocytes and axonal processes in mammalian brain and spinal cord tissue (Figures. 1 and 2), somewhat surprisingly, Nogo had been found earlier to have three different isoforms, termed Nogo-A, -B and -C, which are generated by alternative usage and/or splicing from a single gene, each with a transcript size of 4.6kb,

2.6kb, and 1.7kb respectively. The 4.6kb product has been postulated to correspond to NI-250, which is recognised by monoclonal antibody IN-1 with a predicted polypeptide size (1,192 amino acid residues) of 135 kDa. The predicted size of Nogo-B (373 amino acid residues, 37 kDa) suggests that it could be the NI-35 protein (GrandPre *et al.,* 2000).

The Nogo proteins differ in length but share a common 188-amino acid-long C-terminus (Chen *et al.,* 2000) consisting of two potentially membrane-spanning hydrophobic domains separated by a hydrophilic segment of 66 amino acids, termed Nogo-66 (Figure. I). The C-termini of Nogos share a high homology with the reticulon protein family, the prototype of which is reticulon 1, a neuroendocrine-specific, endoplasmic reticulum-localised protein with unknown function (Van de Velde *et al.,* 1994; Roebroek *et al.,* 1996; Moreira *et al.,* 1999).

1.5.3.2 Nogo domains and their localisation

All reticulons have two large hydrophobic domains near the C-terminus of the protein. In the case of rat Nogo proteins, these presumed transmembrane regions are 35 amino acids and 36 amino acids each respectively, long enough to span the membrane twice (Chen *et al.,* 2000; GrandPre *et al.,* 2000). In between the two transmembrane domains, the 66 amino acid loop has been found to bind the Nogo receptor (NgR) subunit, (Fournier *et al.,* 2001). Another important region for the neurite out-growth inhibitory function is located in the Nogo-A specific region in the middle of the protein chain (Oertle *et al.,* 2003). For this domain to be inhibitory, it naturally must be displayed outside the oligodendrocyte to bind and activate a receptor on the surface of neurons or fibroblasts. Specific high affinity binding of this domain to brain membranes or to live fibroblasts has been shown (Oertle *et al.,* 2003). However, the localisation and topology of Nogo-A on plasma membranes have

not been clear up to now. Our results have shown localisation of Nogo-A in structures with similar morphology to endoplasmic reticulum (ER) in fibroblasts in culture (Figure. 1A-C). Other characteristics of reticulons include a di-lysine ER retention/retrieval signal at the extreme C-terminus and a lack of a signal sequence at the N-terminus. GrandPŕe *et al.,* 2000 tried to show that Nogo could be on the cell surface, this type of topology being based on the fact that epitope tags placed at both ends of the molecule appeared to be cytoplasmically oriented. In addition, antibodies against Nogo-66 detected the Nogo-66 epitope on the surface of intact oligodendrocytes as well as transfected COS-7 cells. I therefore set out to test this, whether Nogo-A antibody can bind to antigen on the cell surface of unpermeabilized oligodendrocytes and dorsal root ganglion (DRG) axons in culture from rat species.

1.5.3.3 Nogo as a therapeutic target and Nogo knockout studies

Exciting progress has happened in trying to explain new ways to stimulate regeneration. Many of the proposed strategies either block inhibitory proteins or block signaling by inhibitory proteins. Altering the inhibitory environment of the CNS with antibodies raised against inhibitory proteins has been tested in many different injury models. I.e. the IN-1 antibody promotes axon regeneration on myelin in many different regions of the CNS (Brittis and Flanagan, 2001). Likewise, a peptide antagonist of the NgR, called NEPI-40, which binds the NgR but does not activate it, was also shown to be able to reverse inhibition by Nogo-66 (Grand Pre *et al.,* 2002). Like the IN-1 antibody, NEP1-40 promotes regeneration in the injured spinal cord, underlining the importance of NgR inhibition in blocking regeneration. Animals treated with NEPI-40 showed significant functional recovery, demonstrating its potential for therapeutic use. NEPI-40 did not, however,

completely block myelin inhibition *in vitro*, which might be explained by the activities of inhibitory proteins that do not bind NgR, such as Amino-Nogo, as well as other inhibitory proteoglycans also present in myelin. However, NEPI-40 does not reduce MAG inhibition of neurite outgrowth (Liu *et al.,* 2002). Further, it is not known if neutralizing all myelin inhibitors would promote an even more robust regeneration. Blocking components of the intracellular inhibitory signaling cascade is yet another new strategy to promote regeneration in the spinal cord, and three new studies now show that blocking inhibitory signaling can promote regeneration in the CNS (Dergham *et al.,* 2002; Neumann *et al.,* 2002; Qiu *et al.,* 2002). Another way of counteracting effects of Nogo was to take the genetic path. However, in 2003, different researchers came out with controversial results regarding mouse *nogo* knockouts. This happened when the Nogo isoforms were knocked out in different combinations (Kim *et al.,* 2003; Simonen *et al.,* 2003; Zheng *et al.,* 2003).When expression of Nogo-A and Nogo-B was lost, Strittmatter and colleagues observed limited regeneration in around half of the resulting animals (Kim *et al.,* 2003) (Table 1). The ability to regenerate spontaneously was only apparent in young mice, and was lost with age. In this study, a GENE TRAP insertion was targeted to an *exon* in Nogo-A, which had been targeted by Schwab using a more conventional strategy. Schwab had reported that when only Nogo-A is knocked out, there is compensatory upregulation of Nogo-B and only limited sprouting after injury Simonen *et al.,* 2003. The lack of extensive regeneration might have been due not only to the increased Nogo-B expression, but also to the presence of other myelin inhibitors e.g. MAG and OMgp, and this is consistent with redundancy. By contrast, in Strittmatter's gene trap strategy, expression of the Nogo-B isoform was also lost. Also, the regeneration that they observed could have been the result

of the gene trap's influence on another gene locus, close to but distinct from the *nogo* locus. If so, it might represent a gain of function rather than a loss of function phenotype.

Table I. Summary of Nogo knockouts

Targeted *Nogo* genes	Nogo isoforms expressed	Observed regeneration	Reference
Nogo-A, *-B* and *-C*	0	0	Zheng *et al.*, 2003
Nogo-A and *–B*	Nogo-C	0	Zheng *et al.*, 2003
Nogo-A	Nogo-B, Nogo-C	Limited sprouting	Simonen *et al.*, 2003
Nogo-A	Nogo-C	Limited regeneration	Kim *et al.*, 2003

1.5.3.4 Nogo-A, association with some neurodegenerative conditions.

Nogo-A has been linked to the pathogenesis of some auto immune mediated diseases like multiple sclerosis (MS). Serum IgM autoantibodies to the recombinant large amino (N) terminal inhibitory domain of Nogo-A are a frequent finding in MS, acute inflammatory (IND) and non inflammatory neurological diseases (OND), but not in neurodegenerative diseases (ND), systemic inflammatory disease and healthy patients (Reindl *et al.*, 2003). Using a MS disease like rat model, Karnezis *et al.*, 2004 observed that by targeting Nogo-A through active immunization significantly reduces clinical signs, demyelination and axonal damage associated with experimental autoimmune encephalomyelitis (EAE).

1.5.3.5 Six Hydroxy dopamine (6-OHDA) induced oxidative stress.

6-Hydroxydopamine (6-OHDA) is a hydroxylated analogue of the natural neurotransmitter dopamine (Blum *et al.*, 2001). It was originally isolated by Senoh and Witkop, 1959. Its

biological effects were first demonstrated by Porter *et al.,* 1963, who showed that 6-OHDA induces efficient and long lasting noradrenaline depletion in sympathetic nerves to the heart. Today, 6-OHDA represents one of the most common neurotoxins used in degeneration models of central catecholaminergic projections, including the nigrostriatal system, *in vivo* and *in vitro* (Ungerstedt, 1968, 1976; Sachs and Jonsson, 1975; Blum *et al.,* 2001). 6-OHDA induced toxicity is relatively selective for catecholaminergic neurons, resulting from a preferential uptake of 6-OHDA by dopamine and noradrenergic transporter molecules (Luthman *et al.,* 1989).

Inside neurons, 6-OHDA accumulates in the cytosol and induces cell death without apoptotic characteristics (Jeon *et al.,* 1995). As far as mechanisms underlying toxicity of 6-OHDA are concerned, participation of oxidative stress, is firmly established (Sachs and Jonsson 1975). It has been reported that 6-OHDA-induced neuron degeneration involves the processing of hydrogen peroxidase and hydroxyl radicals in the presence of iron (Sachs and Jonsson 1975). Furthermore, it has been shown that 6-OHDA treatment reduces striatal glutathione (GSH) and superoxide dismutase (SOD) enzyme activity (Perumal *et al.,* 1992), and increased levels of malondialdehyde (Kumar *et al.,* 1995). The prevention of neurotoxic effects of 6-OHDA and iron following pretreatment with iron chelating compounds, vitamin E, or sellegine, a monoamine oxidase B (MAO-B) inhibitor, may also be considered as indirect evidence for the production of free radicals and involvement of oxidative stress mechanisms (Knoll 1986; Cadet *et al.,* 1989; Perumal *et al.,* 1992).

In summary, the 6-OHDA model does not mimic all pathological and clinical features of human parkinsonism. It induces dopaminergic neuron death with preservation of non-dopaminergic neurons, whereas the formation of cytoplasmatic inclusions (Lewy bodies)

does not occur. 6-OHDA does not affect other brain areas involved in Parkinson Disease (PD), such as in anterior olfactory structures, lower brain stem areas or the locus coeruleus (Betarbet *et al.,* 2002; Del Tredici *et al.,* 2002). Reports on parkinsonian-like tremor are rare in studies of 6-OHDA-lesioned rodents, however, occasional akinesia, rigidity and tremor have been described (Lindner *et al.,* 1999; Cenci *et al.,* 2002). In general, this model exclusively induces acute effects, which differs significantly from the slowly progressive pathology of human PD (Betarbet *et al.,* 2002).

1.6 Nogo receptor (NgR)

An important advance in understanding the mechanism of action, and the cellular targets, of Nogo-A was the identification of NgR by Fournier *et al.,* (2001) as a leucine rich repeat (LRR), glycosylphosphatidylinositol (GPI)-linked protein with high affinity for Nogo-66. A receptor denoted the Nogo-66 receptor (NgR) now appears to play a pivotal role in conveying inhibitory signals from myelin-associated proteins to neurones of the CNS. It binds MAG and the oligodendrocyte protein (OMgp) with similar affinity as the originally discovered ligand Nogo-66 and also mediates inhibition of axonal extensions *in vitro* and *in vivo* (Fournier *et al.,* 2001; Domeniconi *et al.,* 2002; GrandPre *et al.,* 2002; Liu *et al.,* 2002; Wang *et al.,* 2002b).

1.6.1 Structure of Nogo receptor

Flanagan and Cheng, 2000 set out to identify Nogo receptor by the alkaline phosphatase (AP) fusion protein approach. In initial studies, an AP-Nogo-66 fusion protein was demonstrated to give saturable high-affinity binding to neurons, with a dissociation constant of 3nM. This fusion protein also acted as a growth cone collapse agent, with a

half-maximal response at 1nM, consistent with the binding affinity. The AP-Nogo-66 fusion protein was then used to screen pools of a mouse brain cDNA expression library transfected into COS cells, resulting in identification of a cDNA that conferred high-affinity binding activity. The encoded protein was named Nogo-66 receptor (NgR). To assess whether Nogo-66 and NgR interact directly, myc-tagged NgR was tested for binding to GST-tagged Nogo-66 in cell extracts, confirming that the two form a protein complex, although the presence of additional components could not be ruled out. Moreover, early embryonic chick retinal neurons, which are not normally sensitive to Nogo-66, become responsive upon infection with a viral vector encoding NgR cDNA. Taken together, the results provided convincing evidence that NgR is a functional cell surface receptor that can mediate inhibitory effects of Nogo-66.

The cDNA sequence for NgR encodes a protein 473 amino acids in length, with a conventional amino-terminal translocation signal sequence, since it lacks an intracellular signaling domain, and therefore must function as a part of a signaling complex (Fournier *et al.*, 2001). This is followed by eight leucine-rich repeat (LRR) motifs, and an LRR carboxy-terminal motif (LRRCT) sequence motif found in a variety of cell surface and secreted molecules (Figure. II). A likely human ortholog of mouse NgR was found, but so far no other closely homologous sequences that would suggest a family of related receptors. At the C terminus is a signal for addition of a glycosylphosphatidylinositol (GPI) lipid, which was confirmed by enzymatic cleavage to anchor NgR in the membrane. These motifs are found in a functionally and evolutionarily diverse set of proteins, including adhesion molecules and signal-transducing receptors (Kobe and Deisenhofer, 1994). By analogy with other GPI-anchored receptors, it was almost certain that NgR associates with a separate transmembrane signal-transducing polypeptide.

The question as to whether the CNS of lower vertebrates like fish express NgR, and what its function in this class of organisms that successfully regenerate their CNS axons when injured might be has not been convincingly answered. Klinger *et al.,* 2004 identified NgR and homologous genes in zebrafish. They also found out that compared to mammalian, the mRNA distribution during development and in different adult tissues is consistent with a potential role for these proteins in the regulation of axon growth and plasticity. Nogo receptor parallels the development of the nervous system; NgRH1a and NgRH2a were detected in embryos with a more mature CNS. In the adult zebrafish, the expression pattern of NgR corresponds to that in mouse, where it is also predominantly detected in brain (Fournier *et al.,* 2001). Northern blot analysis revealed low mRNA levels also in heart and kidney but not in other peripheral tissues. In zebrafish, NgR expression was also observed in heart. In addition, mRNA transcripts were detected in the eye, spinal cord, and gill. However, no functional studies were carried out to support their findings about the functional role of NgR in these lower vertebrate species.

1.6.2 Nogo receptor mediates growth inhibition by MAG, Nogo and OMgp

Nogo receptor (NgR) was first identified as a brain specific, glycosylphosphatidylinositol (GPI)-anchored membrane protein with high affinity for the soluble extracellular domain of Nogo (Nogo-66). The majority of the globular structure of NgR comprised of a leucine-rich repeat (LRR) domain capped by N-terminal and C-terminal cysteine rich molecules (LRR-NT and LRR-CT segments respectively) (Fournier *et al.,* 2001). To date, three structurally unrelated myelin inhibitory proteins have been suggested to serve as ligands for the promiscuous Nogo receptor; Nogo-A, MAG, and OMgp (Figure III), also reviewed in Oertle *et al.,* 2003. Wang *et al.,* 2002b identified NgR as an OMgp binding protein via an

expression cloning strategy. He showed that NgR transfected into neurons that would normally not be inhibited by OMgp can make these neurons sensitive to OMgp. Other investigations later showed that in addition to binding Nogo-66 and OMgp, NgR also binds MAG (Liu *et al.*, 2002; Domeniconi *et al.,* 2002). Using an alkaline phosphatase-NgR fusion protein, and in an attempt to identify a NgR co-receptor by expression cloning, they identified MAG as a ligand of NgR.

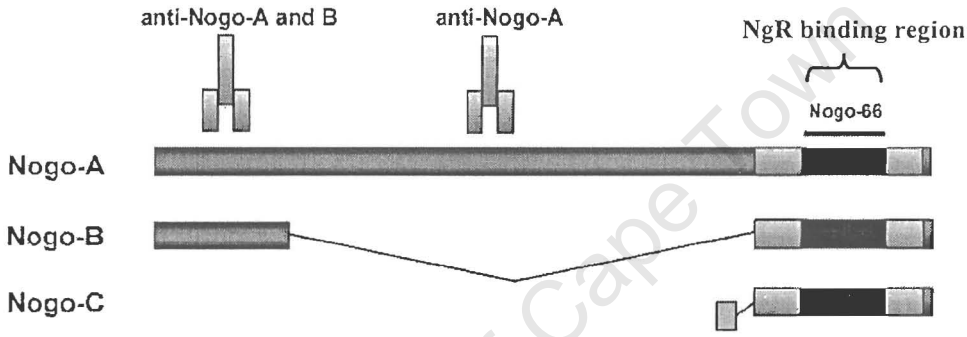


Figure. I Schematic representation of the three Nogo isoforms A, B and C.

The location of the common Nogo-66 region is labeled, as is the region associated with binding to the NgR. ***(Li et al., 2001).***

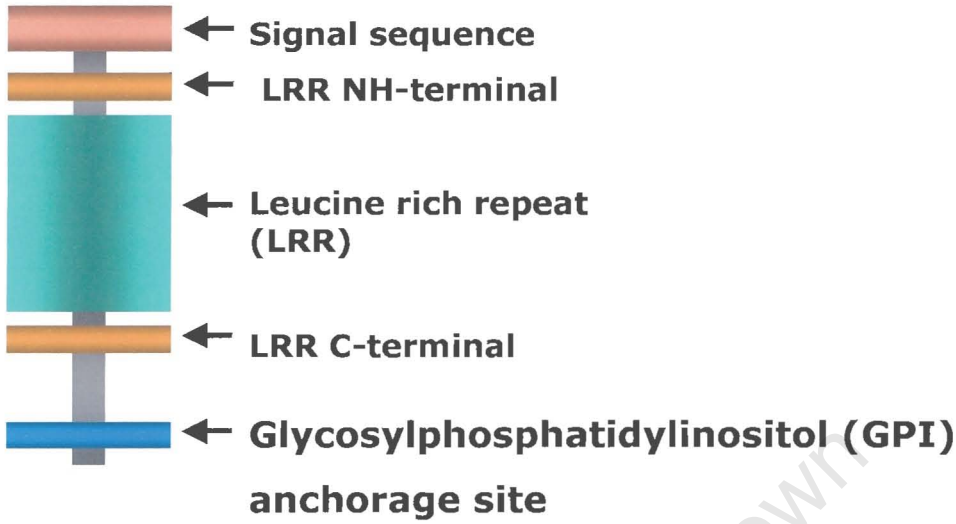


Figure. II Structure of the Nogo receptor

NgR is a functional receptor that can mediate inhibition by Nogo-66. NgR contains a translocation signal sequence (signal), eight leucine-rich repeats (LRR), an LRR carboxy terminal motif (LRRCT), and a GPI lipid anchor that tethers it to the membrane.

Figure modified from Nature Reviews Drug Discovery 2; 872-879 (2003).

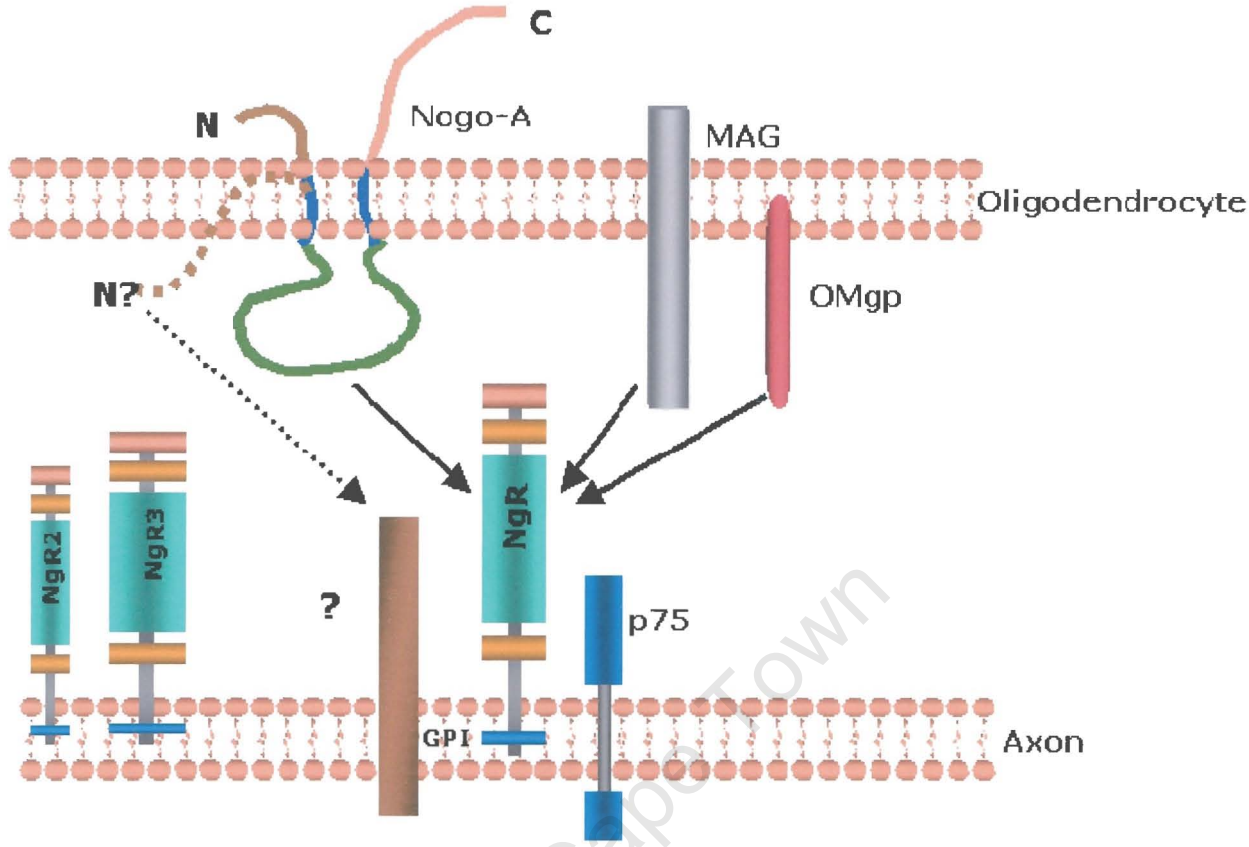


Figure. III The Nogo receptor and inhibition of axon regeneration.

The leucine-rich repeat domains of the NgR are necessary for interaction with Nogo-66, MAG and OMgp. As a GPI-anchored protein, NgR does not transduce signals directly, but instead recruits co-receptor molecules such as p75 or other unknown molecules (?). Co-receptor activation in turn activates the RHO and ROCK pathway to modulate the cytoskeleton and neurite growth. The amino-terminal domain of NogoA can be present either in the cytosol or in the extracellular space. In the latter position, it can bind to an unknown neuronal protein and thereby inhibit neurite growth.

GPI, *glycosylphosphatidylinositol;* ***MAG****, myelin-associated glycoprotein;* ***NgR****, Nogo receptor;* ***OMgp****, oligodendrocyte myelin glycoprotein.*

Figure modified from Nature Reviews Drug Discovery 2; 872-879 (2003).

1.6.3 p75 mediates inhibitory activities of MAG, Nogo and OMgp

Nogo receptor is GPI linked on the cell surface and possesses no intracellular signalling domain. It was therefore assumed that some other players appear to be part of the signalling complex. In addition, an accessory co-receptor functions to transduce the signals intracellularly. So what was this co-receptor? Wang *et al.,* 2002b, examined the possibility that p75 and NgR formed a receptor complex in mediating inhibitory activities between NgR and other neurite inhibitors including MAG. In their experiments, they over-expressed heamagglutinin (HA)-tagged rat full-length p75 in both CHO cells, and CHO cells stably expressing Flag-tagged human NgR, and found that p75 could be immunoprecipitated collectively with NgR but not with a control transmembrane protein plexin A3. A study done by Yamashita *et al.,* 2002 showed that MAG-dependant inhibition of neurite out-growth is reduced in DRG or cerebellar neurons from p75 knockout mice, suggesting the involvement p75 in MAG signalling.

However, interestingly of late the absence of NgR has been shown to have no effect on preventing neurite inhibition. Zeng *et al.,* 2005, examined *in vitro* and *in vivo* neuronal responses of neurons from p75NTR mutant and NgR-null mutant mice to inhibitory substrates, as well as the ability of CST neurons to regenerate their axons after injury. Their *in vitro* data indicated that removing NgR, in contrast to removing p75NTR, does not reduce axonal growth inhibition in the neurons that were tested. They also observed a lack of regeneration of CST axons after spinal injury in both the NgR and the p75NTR mutant mice.

1.6.4 Nogo receptor in lipid rafts

Lipid microdomains, also known as lipid rafts, are specialised membrane domains enriched in glycosphingolipids and cholesterol. They are widely seen to act as platforms for the active assembly of interacting proteins, especially of GPI-anchored cell surface and intracellular signal transduction proteins, such as non receptor tyrosine kinases of the src family, so as to increase signal transduction efficacy (Simons and Toomre, 2001). Sphingolipid–cholesterol rafts are insoluble in the detergent Triton X-100 at 4°C, in which they form glycolipid-enriched complexes (Brown and Rose, 1992; Shroeder *et al.,* 1994). Because of their high lipid content, these detergent-insoluble glycolipid-enriched complexes (DIGs) float to a low density during gradient centrifugation (Brown and Rose, 1992, which enables any associated proteins to be identified and distinguished from other detergent insoluble complexes. Milder detergents such as octylglucoside will solubilize lipid rafts (Shroeder *et al.,* 1994). Since it has already been established that the NgR is a GPI-anchored protein (Fournier *et al.,* 2001), the question still remains, as to whether NgR is localised in lipid rafts. This would have great significance in the context of pharmacologically interfering with Nogo-66 receptor signalling in order to promote regeneration in mammalian CNS. Lipid rafts promote signalling through the focal assembly of GPI-anchored proteins (Bruckner *et al.,* 1999), which on their own would not have direct access to intracellular signal transducing proteins (Simons and Ikonen, 1997; Friedrichson and Kurzchalia, 1998). Stuermer *et al.,* 2001, demonstrated co-localisation of the GPI-linked cell adhesion molecules (CAMs) F3 and Thy-1 with micropatches of anti-flotillin antibodies. And it is these flotillins which give identity to sites where activated GPI-linked CAMs preferentially accumulate, which may represent lipid rafts. In the context of signal

transduction and raft localisation of Nogo-66 receptor; there is lack of structural data. This has enabled us to undertake studies aimed at structural superimposition of Nogo-66 receptor and other known lipid raft microdomain resident proteins using rat DRG and PC-12 cells.

1.7 Aims of the study

1.7.1 To develop and establish standard techniques and methods in the laboratory for setting up neuronal and glial cell cultures.

1.7.2 To develop a synthetic peptide from mammalian and amphibian Nogo receptor sequences and inoculate rabbits to raise a new Nogo receptor antiserum. This would also be used to study the expression pattern of the receptor protein in non mammalian species like amphibians and fish.

1.7.3 Characterise the Nogo receptor antibody raised from rabbits in our laboratory.

1.7.4 Investigate the cellular localisation of the Nogo receptor using both new and commercial antisera and its ligand Nogo A. Precisely explicate the manner of expression, i.e. whether the receptor is expressed on the cell surface, in the cytosol, or on surface membrane of some cell organelles, using primary cell cultures, established cell lines and nervous tissue cryosections.

1.7.5 To determine whether the Nogo receptor is located in plasma membrane domains (lipid rafts). This would enable us to understand whether lipid rafts play a crucial role in signal transduction mediated by GPI-anchored cell surface receptors in the CNS.

1.7.6 Investigate whether neuronal cell bodies and fibres in the substantia nigra (SNC) and the caudate putamen areas of the rat brain respectively regulate Nogo-A and

Nogo-66 receptor following 6-hydoxy dopamine (6-OHDA) injury, a model for Parkinson disease-like neurodegeneration. This would enable us understand the role of these proteins in dopamine mediated oxidative stress-induced cell death, and would also be valuable in linking major inhibitors of axon regeneration like Nogo-A to neurodegenerative diseases like Parkinson.

MATERIALS & METHODS

2.0 MATERIALS AND METHODS

2.1. Animals

2.1.1. Rats

Wistar rats were used in our studies at different ages depending on the nature of the experiment. They were housed and provided by the Animal unit at the University of Cape Town (UCT). Embryos were obtained by caesarean section at E15. Other stages of development used were P1, P7 and Adult stages. All procedures, protocols involving the use of laboratory animals used in the whole project were approved by the ethics committee of the Health Sciences Faculty, and were in accordance with ethical guidelines and regulations of University of Cape Town.

2.1.2. Rabbits

Two New Zealand white (NZW) rabbits were used in our studies to raise antibodies against NgR synthetic peptide, Alpha Diagnostics International (ADI). They were housed and provided by the Animal Unit at UCT.

2.2. Production of antisera

2.2.1. Antigen preparation

Electronic BLAST searches were performed to identify conserved regions of the NgR protein. This was followed by identifying a peptide sequence that is immunogenic and highly conserved in both mammalian and amphibian species using *Spscan*. The sequence (SLQYLRLN) corresponding to amino acid 251-258 of LRR 10 was identified and later

used to design a KLH conjugated synthetic peptide *(Nyatia-1)* by Alpha Diagnostic International (ADI), USA.

2.2.2. Rabbit antisera

Rabbits (2) of 10 weeks old were initially immunised with a subcutaneous (SC) injection of 0.5ml peptide at 1mg/ml concentration + 0.5ml Freunds incomplete adjuvant [Provided by Molecular Cell Biology (MCB) lab, UCT]. The rabbits were boosted with the same amount and concentration of the peptide and adjuvant at 2 weekly intervals for 8 weeks (Appendix II). The rabbits were sedated with Torbugesic (butorphanol) (1mg/kg) and acepromazine maleate (1mg/kg) subcutaneously. Using a 19 gauge needle, rabbits were bled from a marginal vein in the ear, prior to the first injection to collect pre-immune sera. Approximately 10ml of blood were collected in sterile tubes from each rabbit prior to each boost injection throughout the whole exercise. The blood was allowed to clot, and then centrifuged at 6.72 x g to separate the serum fraction. The serum samples were sealed in sterile tubes, labeled and either frozen at -80°C for long term storage, or kept at 4°C for immediate testing. Each serum was numbered with the cumulative bleeding number, rabbit identification, and date of bleeding for record purposes.

2.3. Tissue culture equipment and medium

Cells were cultured on various tissue culture equipment, depending on the primary objective of the experiment. The equipment included 16mm 1.5mg/ml polylysine/laminin coated glass cover-slips (Marienfield, Germany) which had previously been cleaned by boiling in concentrated nitric acid, small, medium and large sized culture flasks (Greiner Bio-One, Germany).

Medium used depended on particular cells being cultured. For DRG culture; Dulbecco's Modified Eagle's Medium (DMEM, Highveld Biological (Pty). Ltd), 1% foetal calf serum (FCS) (Highveld Biological (Pty). Ltd), L-glutamate 100mg/ml, Nerve growth factor (NGF), 100 I.U each of penicillin and streptomycin: For primary glial cells; DMEM, 10% FCS, L-glutamate 100mg/ml, 100 I.U each of penicillin and streptomycin: For enriched oligodendrocyte culture; DMEM, 10% FCS, L-glutamate 100g/ml, 100 I.U each of penicillin and streptomycin: PC-12 cells differentiation stage; DMEM, 1% FCS, 1μg/ml insulin, transferrin, forskilin (1:1000), L-glutamate 100g/ml, 100 I.U each of penicillin and streptomycin.

2.4. Micro-dissection and tissue preparation

2.4.1. Dorsal root ganglion (DRG) cultures

Pregnant female rats were sacrificed by placing them in a CO_2 gas chamber, until they showed no sign of movement, and embryos obtained at E15 stage of development. Using a dissecting microscope (Zeiss), together with decapitated P1 rats, they were de-fleshed using fine iris forceps and scissors around the dorsal vertebral column, so as to expose the spinal cord. Stripping out the spinal cord resulted in the DRGs being easily visible in between transverse processes of adjacent vertebrae. The DRG were carefully harvested, stripped off their peripheral nerve pieces in a separate petri dish, chopped into tiny pieces using a tissue chopper (Brinkmann, England) and plated on 1.5mg/ml polylysine/laminin coated cover-slips. They were later placed in a 37°C/5% CO_2 incubator, set at 25% humidity. Prior to immunohistochemistry experiments on DRG cultures, some cultures were incubated with PI-PLC (Sigma Aldrich) at a concentration of 1μg/μl for 60min.

2.4.2. Primary glial cell cultures

Rats were processed as in *2.4.1* above; fleshy tissue was stripped of the vertebral column dorsally, so as to expose the spinal cord. Whole brain was recovered from the cranium by splitting open the skull. Both the brain and spinal cord tissues were placed in DMEM, 10% FCS medium on ice. Using a narrowed end glass pipette, the pieces of tissues were sucked in and out repeatedly using a suction pipette so as to free individual cells and later plated in culture flasks and placed in a 37°C/5% CO_2 incubator and 25% humidity.

2.4.3. Enriched oligodendrocyte culture

To generate an enriched oligodendrocyte culture, a glial cell culture was set up as in *2.4.2* above. It is a known fact that oligodendrocyte cells tend to settle at the top of other cells and are less adherent in a glial cell culture. To selectively remove the oligodendrocytes, flasks were washed three times with S-MEM with 0.025% EDTA and incubated in 0.01% trypsin in S-MEM for 5-7 min at 37°C. The enzymatic digestion was stopped by addition of 3ml DMEM + 10% FBS and the detached cells collected. After centrifugation at 1000 × g for 5 min, the detached cells were re-suspended in DMEM/N2 medium with 5ng/ml PDGF and plated at a density of 1.8×10^5 live cells/coverslip that were polylysine/laminin coated.

2.4.4. PC-12 cell culture

PC-12 cells with a low passage number (<30) a gift from Dr. Lang (University of Cape Town), were thawed and plated in medium culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% heat inactivated FCS (Highveld Biological (Pty), L-glutamate 100mg/ml, 100 I.U each of penicillin and streptomycin. They were incubated in a humidified atmosphere containing 5% CO_2 in air at 37°C for 5-7 days. They were later

differentiated with NGF (50 ng/ml). Medium changes and NGF additions were repeated every three days until they were fully differentiated and ready for further experiments.

2.5. Fluorescent Immunohistochemistry (FIHC)

2.5.1. Antibodies and fluorescent markers

Antibodies used in this study were: Nogo-A (ADI), a rabbit polyclonal that recognises the 66 amino acid extracellular domain of Nogo. The Nogo receptor (ADI), a rabbit polyclonal raised partly against the unique LRR 10 region of the receptor. The Nogo receptor (UCT), a rabbit polyclonal raised against the unique LRR 9 region of the receptor. Neurofilament (NF) Santa Cruz, a monoclonal antibody. Flotillin/epidermal surface antigen (ESA) (BD) a monoclonal antibody. Proteolipid protein, PLP (Santa Cruz) a monoclonal antibody that recognizes compact myelin. Tyrosine hydroxylase (TH) (Sigma), a monoclonal antibody that recognises dopaminergic neurones in the brain. Glial fibrillary acidic protein (GFAP), an astrocytic marker. The antibody O_4 (Santa Cruz) marks a specific preoligodendrocyte stage of oligodendrocyte maturation. It reacts also with sulfatides and still unidentified glycolipids (Bansal *et al.*, 1989).

2.5.2. Cell staining

Culture medium was rinsed off the cells twice with PBS, pH 7.4 and cells were fixed in 4% paraformaldehyde in PBS for 5 minutes at 25°C, and later in 100% methanol at -20°C for 5 minutes. Cells were then rinsed three times in five minute changes of PBS, pH 7.4. Cells were then blocked in 1% bovine serum albumin, BSA (Roche) in PBS for 30 minutes at 25°C. They were then incubated at 4°C overnight in appropriate primary antibodies diluted in blocking solution at varying concentrations. Nogo-A (1:1000); NgR (*both new and*

commercial) (1:1000); Flotillin (1:200); O_4 (1:50); GFAP (1:1000). The primary antibodies were rinsed off and cells washed thrice, five minutes apart with PBS, and later incubated with the appropriate secondary antibodies (Goat anti mouse Alexa-488; Donkey anti rabbit Cy3) (Molecular probes) at 1:1000 dilution in blocking solution for 2 hours at 25°C. The secondary antibodies were later rinsed off with thrice in five minute changes of PBS, and later incubated with a nuclear stain DAPI (1:1000). Cells were rinsed once in PBS and mounted in Mowiol. The Mowiol mounting medium was made up as follows; for every 2.4g Mowiol (Polyvinyl alcohol, Hoechst) 6ml glycerol was added. While stirring, 6ml of distilled water was added and left for several hours at 25°C. Twelve millilitres of 0.2M Tris (pH 8.5) was added and the solution incubated at 50°C for one hour with occasional stirring. To reduce immunofluorescence fading, approximately 20g of n-propyl gallate (Sigma) was added and dissolved at 37°C. The slides were stored in a dry environment for 24 hours at 25°C. Viewing was carried out using a Zeiss Axiovert 200M Fluorescence microscope (Zeiss, Germany).

2.6. Six-Hydroxy dopamine (6-OHDA) lesioning

2.6.1. Animals

Male Long Evans rats weighing 280-350g were used in this study. Long Evans rats were obtained from the University of Cape Town, Faculty of Health Sciences Animal Unit. The rats were maintained on a twelve hour light/dark cycle (6am–6pm) and were provided with Epol food pellets and water ad-libitum throughout the study. The rats were housed individually.

2.6.2. Unilateral 6-OHDA lesions

All surgical lesioning procedures were conducted during the dark phase of the light/dark cycle under semi-sterile conditions. The rats were injected intraperitoneally (i.p) with desipramine hydrochloride (25mg/kg) at least 25 min prior to lesioning surgery (Doucet *et al.*, 1986; Magill *et al.*, 2001). The rats were deeply anaesthetized with Halothane, (Safeline Pharmaceuticals LTD, 1 Manchester Road, Wadeville, South Africa) and were placed in a stereotaxic frame. Throughout the surgery, anaesthesia was maintained through the administration of 3% halothane/oxygen mixture. A midline incision of the skin overlying the skull was made and a burr hole was made in the skull and the infusion needle was lowered to the medial forebrain bundle (anterior-posterior with reference to the interaural line 4.7mm, 1.6mm lateral relative to the midline and 8.4mm from dura) (Paxinos and Watson, 1997). A 13.5µg/0.5ml dose of 6-hydroxy dopamine hydrochloride (6-OHDA), (Sigma Chemical Company, St Louis MO 63178, USA) containing 0.02% w/v L-ascorbic acid (Lot: F274927 726 Merck 64271 Darmstadt, Germany) in sterile saline was infused at a rate of 0.5µl/min unilaterally into the left hemisphere (Magill *et al.*, 2001) using a Hamilton syringe connected to an infusion pump. The 6-OHDA solution was prepared immediately before placing the rat on the stereotaxic apparatus. At the end of infusion period, the pump was turned off and 5 minutes was allowed for the toxin to diffuse before the needle was withdrawn slowly from the brain. The burr hole was sealed with bone wax and the skin incision was sutured with silk suture and sterilized with 70% alcohol. The animals were returned to their individual cages and allowed to recover from surgery before being returned to the animal house. Sham-operated control rats underwent the same surgical procedure but received only 4.5µl of the vehicle (sterile saline containing 0.02% w/v L-ascorbic acid).

2.6.3. Apomorphine Induced Rotations

Fourteen days following the lesioning operations, the rats underwent behavioural assessment to determine the extent of degeneration of dopamine innervation to the striatum that had occurred as a consequence of lesioning. An established technique of apomorphine induced rotations was used (Ungerstedt, 1971; Tillerson *et al.,* 2001). The assessment was conducted during the light cycle. The rats were strapped into body harnesses and were placed inside individual circular opaque plastic drums. The rats were administered with subcutaneous injections of 0.5mg/kg apomorphine (Apomorphine Hydrochloride hemihydrate A4393 Lot 41K152, Sigma Chemical Company, St Louis MO 63178, USA). Based on the recorded rotations in response to the apomorphine stimulations at least 400 contra-lateral quarter rotations during the one hour recording period following apomorphine administration qualified the rats to be selected for immunocytochemistry analysis.

2.6.4. Brain dissection and freezing

The rats were deeply anaesthetized with Halothane and perfused transcardially through a needle positioned into the left atrium. The rats were perfused with 150ml 0.15M PBS solution followed by 300ml solution of 4% paraformaldehyde in 0.15M PBS solution. Following the perfusion procedure, the brain was placed in a glass vial containing 20% sucrose for cryo-protection overnight.

The tissue was removed from the sucrose solution and embedded in tissue freezing medium (Sigma) within tin foil moulds and frozen over liquid nitrogen (-197°C) vapour. The 20µm sections of the tissue were cut on the cryostat -22°C at and the sections were placed on 3-Amino-Propyl-TriEthoxySilane (APTES) (Sigma-Aldrich) coated glass slides, that had

been prepared as follows; Hot nitric acid cleaned glass slides were rinsed twice with acetone to remove water and then immersed in a solution of 2 vol % APTES in acetone for 30 min at 25°C with stirring. Slides were then rinsed twice with acetone to remove unreacted silane and twice with de-ionized water. They were then dried and baked at 120°C for 30-60 min to anneal the silane coating and finally stored dry until use.

2.6.5. Tyrosine Hydroxylase (TH) staining using Diaminobenzidine (DAB) method

This staining was done to macroscopically visualize the sections to confirm proof of 6-OHDA lesioning using a TH antibody. The sections were initially incubated with 0.3% hydrogen peroxide solution in methanol in a humidifying chamber for 30 minutes at 25°C to quench their endogenous peroxidase activity. The sections were washed in 1% PBS, pH 7.4 thrice for 10 minutes each and later incubated with blocking solution containing 3 drops horse serum (HS) in 1% PBS solution and 0.2% Triton X-100 for 30 minutes at 25°C. The sections were then incubated with mouse monoclonal antibody against tyrosine hydroxylase (Clone TH-16) (Sigma Chemical Company, St Louis MO 63178, USA) diluted with blocking solution 1:8000 at 4°C overnight in a humidified chamber. The primary antibody was washed off in 1% PBS, pH 7.4 three times for 10 minutes, and later sections incubated with biotinylated secondary antibody at 1:400 dilution (Sigma Chemical Company, St Louis MO 63178, USA) and visualized with a Vectasatin Elite ABC kit (Avidin-Biotin-Peroxidase Complex) Kit (Vector Laboratories, Inc, Burlingame, CA 94010, USA) at 25°C for 1 hour. The secondary antibody was washed off following the same procedure as with the primary. A dark blue stain was obtained by further staining with DAB staining (3,3'-diaminobenzidine (DAB) Peroxidase Substrate Kit SK 4100 (Vector Laboratories, Inc, Burlingame, CA 94010, USA). Slides were later washed for 15

mins in tap water, air dried, cleared in xylol and mounted using clearmount mounting solution containing 0.05% sodium azide (Invitrogen).

2.7. Co-Immunoprecipitation

2.7.1. Introduction

Co-immunoprecipitation (Co-IP) is a popular technique for protein interaction discovery. In a Co-IP, the target antigen precipitated by the antibody "co-precipitates" a binding partner/protein complex from a lysate, i.e., the interacting protein is bound to the target antigen, which becomes bound by the antibody that becomes captured on the Protein A or G gel support. The assumption that is usually made when associated proteins are co-precipitated is that these proteins are related to the function of the target antigen at the cellular level. In this case study, the assumption is that NgR is linked to flotillins in plasma membrane domains called lipid rafts in cells of the nervous tissue. However, that is subject to further verification.

2.7.2. Experimental procedure

Wistar rat whole neonate brain tissue was dissected out of the cranium as described in method *(2.4.2)* above, and placed on ice cold PBS. The tissue was re-suspended in 2ml RIPA buffer (150mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50mM Tris pH 8.0, 1mM PMSF) + complete protease inhibitor (Roche) at 4°C and manually but gently ground using a pestle in a Potter homogenizer (Herveg). The mix was left on ice for 10mins, and later spun down for 10mins at 28,000 x g at 4°C. After determining the protein concentration, 500µg protein was placed into 2 (experimental and control) tubes for each condition and RIPA buffer was added until a total of 500µl was

achieved. A pre-clearing step was performed to get rid of any antibodies that could be found in the protein sample. The protein-A sepharose beads (Amersham) were first washed 3 times in RIPA buffer + Protease inhibitor followed by vortexing 2 min and spinning at 16,800 x g for 5 mins to get rid of alcohol before being added to the protein sample. Five hundred microlitre of 1µg/µl protein sample was then incubated with clean beads on a rotating wheel at 4°C for 1 hour. This was followed by spinning in a microfuge at max speed, for at least 4 mins at 4°C. The supernatant (now pre-cleared) was placed into new tubes and 5µl of NgR (new) antibody added and incubated over night at 4°C on a rotating wheel. Clean protein-A sepharose beads (25µl) were then added to bound antibody-antigen solution and incubated over night on a rotating wheel. This was followed by a spin at max speed for 4 min. After the spin, the supernatant was pipetted out and RIPA buffer (0.5ml) added to re-suspend and rinse the pellet of any salts three times in the same tube.

After the last wash, the pellet was re-suspended in 30µl of 2x SDS/SB (without β-ME), and later warmed at 85°C for 5 mins to elute the antigen. Without vortexing, the suspension was mixed gently with a pipette tip and spun down to remove the supernatant. The supernatant now with eluted protein was incubated for 37°C for 1 hour with 5% β-ME and later boiled for 5 min ready to perform a SDS-PAGE for analysis.

2.8. SDS–PAGE and Western blotting

Freshly isolated adult and neonate Wistar rat whole brain and spinal cord were homogenized in sample buffer (Amersham, Pharmacia).

A 10 % Gel was set up as follows:

Gel (Resolving)- 10 ml for 2

DDL water	4.1 ml
Acrylamide (30%)	3.3 ml
Gel buffer (Tris pH 8.8)	2.5 ml
SDS (10%)	0.1 ml
APS (10%)	50µl
TEMED	10µl

Gel (Stacking)- 3 ml for 2

DDL water	2.0 ml
Acrylamide (30%)	0.43 ml
Gel buffer (Tris pH 6.8)	0.83 ml
SDS (10%)	33µl
APS (10%)	16.7µl
TEMED	3.3µl

Twenty microlitres (1µg/µl) of lysate was run on a 10% Tris–glycine polyacrylamide gel (Bio-Rad) adjacent to molecular markers (RPN-800, Sigma Aldrich) at 100 V (stacking gel) and 180 V (resolving gel). Proteins were electroblotted onto nitrocellulose paper (Amersham Pharmacia) at 8V overnight. The filter was blocked using 5% commercial fat free milk (P'n Pay supermarket, South Africa) in PBS for 90 min. primary antibodies were diluted 1 in 5000 in 5% fat free milk and incubated with the blots for 2 hours. After being washed 3 times for 5 min with 1% PBST, depending on the primary antibody, blots were incubated with either Goat anti-Rabbit IgG-HRP (Jackson laboratories) or Donkey anti-mouse IgG-HRP (Santa Cruz) secondary antibodies, diluted 1 in 5000 for 2 hours at 25°C while shaking. The blot was washed with four changes of PBS (5 min each) and then visualized using a chemiluminescent reagent, Supersignal West Dura Substrate (Pierce Biotechnology) on a Kodak film.

RESULTS

3.0 RESULTS

3.1 Expression of Nogo-A

3.1.1 Nogo-A is expressed by dorsal root ganglion (DRG) axons in culture

Immunohistochemistry studies demonstrated that neuronal cell bodies from foetal (E15) and neonate (P1) Wistar rat DRG explants displayed similar, strong Nogo-A immunoreactivity. Their neurites, as revealed by neurofilament staining were also Nogo-A positive (Figure. 1), with the strongest expression being in axonal varicosities/ synaptic buttons, often at branch points or at sites where axons crossed other neurites (Figure. 1D-F). Characteristic cytoplasmic Nogo-A immunoreactivity was also observed in structures that morphologically bear resemblance to endoplasmic reticulum (ER) in fibroblasts which form a substrate layer for the neurites and Schwann cells to grow (Figure. 1H). Schwann cells, which myelinate axons in the PNS also expressed considerable amounts of Nogo-A (Figure. 1G) comparable to oligodendrocytes.

3.1.2 Oligodendrocytes, but not Astrocytes express Nogo-A in CNS glia cell culture.

Double immunohistochemistry with an antibody against the astrocyte marker, glial fibrillary acidic protein (GFAP) and Nogo-A did not result in any superimposition, demonstrating that Nogo-A is not present in astrocytes (Figure. 2A-C). In a primary rat CNS glial cell culture, oligodendrocytes tend to grow on top of astrocytes and other cells like fibroblasts. An enriched oligodendrocytes culture was later isolated from the rest of the CNS glia (protocol modified after McCarthy and De Vellis, 1980). After dissociation, the

oligodendrocytes were supplemented with Triodothryonine (T_3) to induce full differentiation. To confirm that oligodendrocytes expressed Nogo-A, a double immune labelling showed superimposition between Nogo-A and O_4, an oligodendrocyte marker (Figure. 2D-F). However, Nogo-A protein immunoreactivity appeared more intracellular as compared to O_4 which is expressed on the surface of oligodendrocytes (Figure. 2-inset). This observation can be substantiated by the failure to observe substantial yellow staining when the two fluorochromes; Cy3 (red) representing anti Nogo-A antibody, and Alexa 488 (green) representing anti O_4 antibody are combined together during image analysis.

3.1.3 Nogo-A protein expression in rat brain and spinal cord tissue.

It is important to understand the regional distribution of Nogo-A expression in the brain and spinal cord, since axons in these two organs do not regenerate in mammalian species following injury. By acting through the Nogo receptor, Nogo-A protein is the major inhibitor of axon regeneration in mammalian central nervous system. Therefore, understanding the expression patterns of the two proteins is crucial in finding a solution towards overcoming axon regeneration failure.

The distribution of Nogo-A protein was analysed using immunohistochemistry in the adult rat brain and spinal cord sections. Intense signal for Nogo-A protein was mostly concentrated in regions with high neuronal and oligodendrocyte cell density, plus areas with high concentration of myelinated axonal fibres. Most neurons in the cerebral cortex of adult rats exhibited only low levels of Nogo-A immunoreactivity, with highest levels seen in *Layer IV*. But smaller cells, presumptive oligodendrocytes, were strongly positive. In adult rat neostriatum, large Nogo-A immunopositive neurons were found spread throughout (Figure. 3). In the hippocampal area, no Nogo-A was detected in the dentate gyrus, but

substantial amounts were detected in hippocampal formations: CA_1-CA_4 (Figure. 4). In the cerebral peduncles, oligodendrocytes and axons were immunoreactive for Nogo-A (Figure. 5). In the cerebellum of adult rats, Nogo-A was strongly expressed in neuronal cell bodies of the deep cerebellar nuclei and in most Purkinje cell bodies and their dendrites. No considerable Nogo-A immunoreactivity was detectable in the granule cell and molecular layers. Strong Nogo-A immunoreactivity was detected in many other neurons in adult rat brain, including those of the pontine nuclei and the mesencephalic nucleus. Furthermore, axonal tracts in the caudate putamen bundles, exhibited strong Nogo A immunoreactivity especially in areas surrounding the axons (Figure. 6A-C). These structures were later revealed to be myelin, as per proteolipid protein (PLP) staining which is a myelin marker (Figure. 6G-I). On a closer look, minor superimposition of Nogo-A with neurofilament (NF) antibody which stains axonal filaments was observed at myelin sheath-axon positions which are juxtaposed (Figure. 6C).

In adult rat spinal cord sections, neurons expressed Nogo-A with the highest levels in ventral motor neurons. Oligodendrocytes (identified by their morphological appearance) which are mostly concentrated in the white matter area expressed Nogo-A, as revealed by the intense staining in the cell body and processes (Figure. 6D). In cross-section, distinctive staining encircling axons (represented by NF in green) leaving an unstained thin rim was observed at high power microscopy (Figure. 6F). Intense Nogo-A immunoreactivity was also observed in ependymal cells surrounding the lining of the central canal in the spinal cord (Figure. 7).

3.1.4. Nogo-A; likely role in oxidative stress and neurodegenerative diseases

One of the objectives of the study was to investigate whether Nogo-A, which is expressed by neurons in the brain, as observed earlier in the study is regulated in response to a neurotoxic insult, i.e. dopamine mediated oxidative stress-induced cell death. This investigation was in line with earlier studies by Karnezis *et al.*, 2004; Reindl *et al.*, 2003 which had implicated Nogo-A in a number of CNS pathological conditions. The results so obtained would be valuable in linking major inhibitors of axon regeneration like Nogo-A to neurodegenerative diseases like Parkinson. Aware that 6-OHDA elicits free radical induced oxidative stress in neurodegenerative processes (Schober, 2004), an investigation whether such a stress causes upregulation or down regulation of Nogo-A protein expression was conducted. A hemiparkinson rat model where unilateral dopaminergic toxicity with 6-OHDA selectively prompts nigral dopaminergic neurons in the substantia nigra to stop the manufacture of tyrosine hydroxylase (TH), an enzyme required for production of dopamine (Beal, 2001) was used.

The difference in TH expression in the 2 hemispheres acted both as a control, and a marker for dopaminergic neurons in the intact hemisphere. I was unable to observe suitable comparable neuronal cell bodies in the lesioned hemisphere as compared to the intact side in rats sacrificed 5 weeks after neurotoxicity (Figure. 8). There were also no noticeable differences in neuronal Nogo-A expression between the 2 hemispheres in rats sacrificed between (24-72) hours after neurotoxicity.

3.2 Expression of Nogo receptor (NgR)

3.2.1 NgR expression in DRG cultures

Dorsal Root Ganglion (DRG) explants harvested from embryonic and neonate Wistar rats were placed in a separate petri dish and stripped off any excess peripheral nerve tissue so as to minimize presence of fibroblasts and Schwann cells in the culture. Carefully chopping DRG explants into tiny pieces, followed by dissociating the cells with a pipette enabled growth of individual neurons (Figure. 9) which grew short processes after the FCS level in DMEM medium had been reduced to 1%. At the same time, long extended single axons were able to grow from neuronal cell bodies concentrated in the DRG chunks, and could be identified by their strong immunoreactivity to the NF antibody. Individual neurons in culture were identified by their morphology and also their immunoreactivity to neurofilament antibody.

Following NgR being characterised as a GPI-linked protein using non-neuronal COS-7 cells transfected with cDNA clones encoding a Nogo-binding site (Fournier *et al.*, 2001), It was of the essence to immunohistochemically reveal its physical presence on the axonal surface on an intact membrane to corroborate the above finding in cells derived from native mammalian tissue. By incubating live DRG axons with the NgR (new) antibody prior to permeabilization and fixation at 37°C, results revealed presence of NgR immunoreactivity on the axonal membrane surface (Figure. 10). On close observation, along the DRG neurites, NgR immunoreactivity was more intense at the margins of growth cones and axonal varicosities compared to other regions of the axon (Figure. 10D-F). Nogo receptor immunoreactivity was also observed in fixed DRG axons.

3.2.2 *Neither Oligodendrocytes, nor Astrocytes express NgR in CNS glial cell culture*

Nogo receptor immunoreactivity could not be detected in astrocytes and oligodendrocytes in embryonic and neonate rat enriched glial CNS cell cultures. Using known markers of oligodendrocytes (O_4) and astrocytes (GFAP) neither of the two cell types showed any immunoreactivity with NgR (commercial and new) antibody (Figure. 11).

3.2.3 *NgR expression in CNS tissue*

To provide a detailed immunohistological analysis of NgR, a NgR peptide antibody was generated locally using rabbits, and was later compared to the studies done with commercial NgR antibody (ADI). An investigation of NgR expression in the brain and spinal cord of normal intact rats revealed the following: Nogo receptor was neither detected in oligodendrocytes or the outer myelin sheath. NgR was also undetectable in astrocytes in both the brain and spinal cord. However, it was detected in neurons. This was consistent with results observed using the commercial NgR antibody. In the rat spinal cord cross section, large neurons in the grey matter showed strong NgR immunoreactivity in the cell bodies and processes (Figure. 12). In the brain, the substantia nigra (pars compacta) region, which has a high concentration of dopaminergic neuronal cell bodies, demonstrated high immunoreactivity to NgR (new) antibody (Figure. 13A-C). Using immunohistochemical techniques, further analysis was done to ascertain whether unmyelinated and myelinated axons express NgR in brain axonal tracts. Using transcardially perfusion fixed rat tissue that is meant to preserve tissue architecture, and on closer observation using fluorescent microscopic analysis, NgR was more intense in myelinated compared to unmyelinated axons. This finding was corroborated by superimposition with proteolipid protein (PLP), a protein present in compact myelin that insulates axons (Figure. 13E-G). The pre-immune

sera displayed inconsistent modest staining of individual neuronal cell bodies or axon bundles in brain and spinal cord tissue sections taken from various regions, as compared to sera after immunization.

Immunoblotting

After immunohistochemistry experiments were done showing diverse expression pattern, the specificity of the NgR (new) and Nogo-A antibodies were investigated by immunoblotting. The new NgR polyclonal antisera from UCT recognized 2 bands at different molecular weights. An unexplained band at approximately 48 kDa, and the actual NgR protein band at approximately 85 kDa from brain tissue, with a weak band from spinal cord (Figure. 14A: see arrow). Anti-Nogo-A antibody recognized a protein of molecular weight (MW) approximately 200 kDa from both neonate and adult rat CNS homogenate (Figure. 14C: see arrow). Immunoblotting was later done on the pre-immune sera as a negative control for the NgR (new) antisera. A band migrating at approximately 50 kDa in neonate brain tissue was observed (Figure. 14B: see arrow). When compared to the commercially prepared NgR purified antibody (ADI), the specific band migrates at about 85 kDa, which is within range of the NgR antibody that was raised in our laboratory at UCT. The non specific bands at approximately 48 kDa and 50 kDa seen in the NgR (new) antisera and pre-immune sera respectively could be due to lack of purification of the sera. However, there may be a possibility that the antiserum also recognizes proteins other than Nogo receptor.

3.3 Nogo receptor in lipid rafts

Although additional components of NgR complex probably exist and remain to be identified, molecules including NgR, p75, and gangliosides may be associated with lipid rafts. Lipid rafts are specialized signalling microdomains that are enriched in cholesterol and glycosphingolipids (Simons and Ikonen, 1997) existing in cell membranes of many kinds. Rafts are believed to function in cellular signalling by concentrating or separating specific molecules in a unique lipid environment (Galbiati *et al.*, 2001). With the above knowledge in mind, it was essential to link NgR to lipid rafts by co-localising the former with flotillins (known lipid raft markers). Using Wistar rat DRG axons (Figure. 15) and PC-12 cells (Figure. 16), reasonable superimposition between NgR and flotillins was observed, using immunohistochemistry technique, with yellow patches (denoting superimposition) mostly seen at particularly enlarged positions along the axonal membrane in the dorsal root ganglion axons. This method had a limitation of resolving fine detail. However, such a problem could be overcome by using other high power image resolving techniques e.g. electron microscopy (EM). Similar patches were also observed with PC-12 cells, although they were a bit smaller compared to DRG axons.

3.3.1 Co-immunoprecipitation

Results obtained above by superimpositional studies using immunofluorescence techniques needed to be verified biochemically by means of co-immunoprecipitation. Nogo receptor antigen was extracted from neonate and adult rat brain homogenate by incubating the homogenate with anti NgR (new) antibody overnight and later the antigen-antibody complex was incubated with protein-A sepharose. Following dissociation of the NgR antigen from its antibody by elution, the NgR antigen was run on a SDS-PAGE western

blot and probed with ant-flotillin antibody, results revealed bands at the estimated molecular weight of flotillin protein (49 kDa). Positive bands were detected in adult brain (AB) homogenate where the antigen was extracted using both commercial (c) and new (n) anti-NgR antibodies. No bands were detected in neonate brain (NB) under the same conditions as adult brain (Figure.17).

To harmonise the experiment, normal protein homogenate from adult and neonate brain was loaded on the same gel with immunoprecipitated samples. Results revealed bands corresponding to flotillin protein at the same molecular weight as the immunoprecipitated samples (Figure. 17), with adult brain protein displaying a stronger and larger band compared to neonate brain. There was no band detected in the negative control sample which lacked the NgR antigen.

Figure. 1. Immunofluorescence microscope images of neonate Wistar rat Dorsal Root Ganglion (DRG) axons in culture (A-F). Axons immunoreacted to Nogo-A (red) as shown by counterstaining with neurofilament (NF) protein (green) which is an axonal marker. Nogo-A is concentrated at sites where neurites cross and axonal varicosities, as shown by arrowheads (D-F), Nogo-A also stains cytoplasmic structures, probably endoplasmic reticulum in fibroblasts which form a layer where the axons grow (A). Schwann cells from neonatal peripheral nerve culture (G), immunoreacted to Nogo-A (red). Nogo-A seems to be intracellularly localised A nuclear stain, DAPI (blue) shows nuclei position(s). **Scale bars: (A-C; 10 μm, D-F; 2.5 μm, G; 10 μm).**

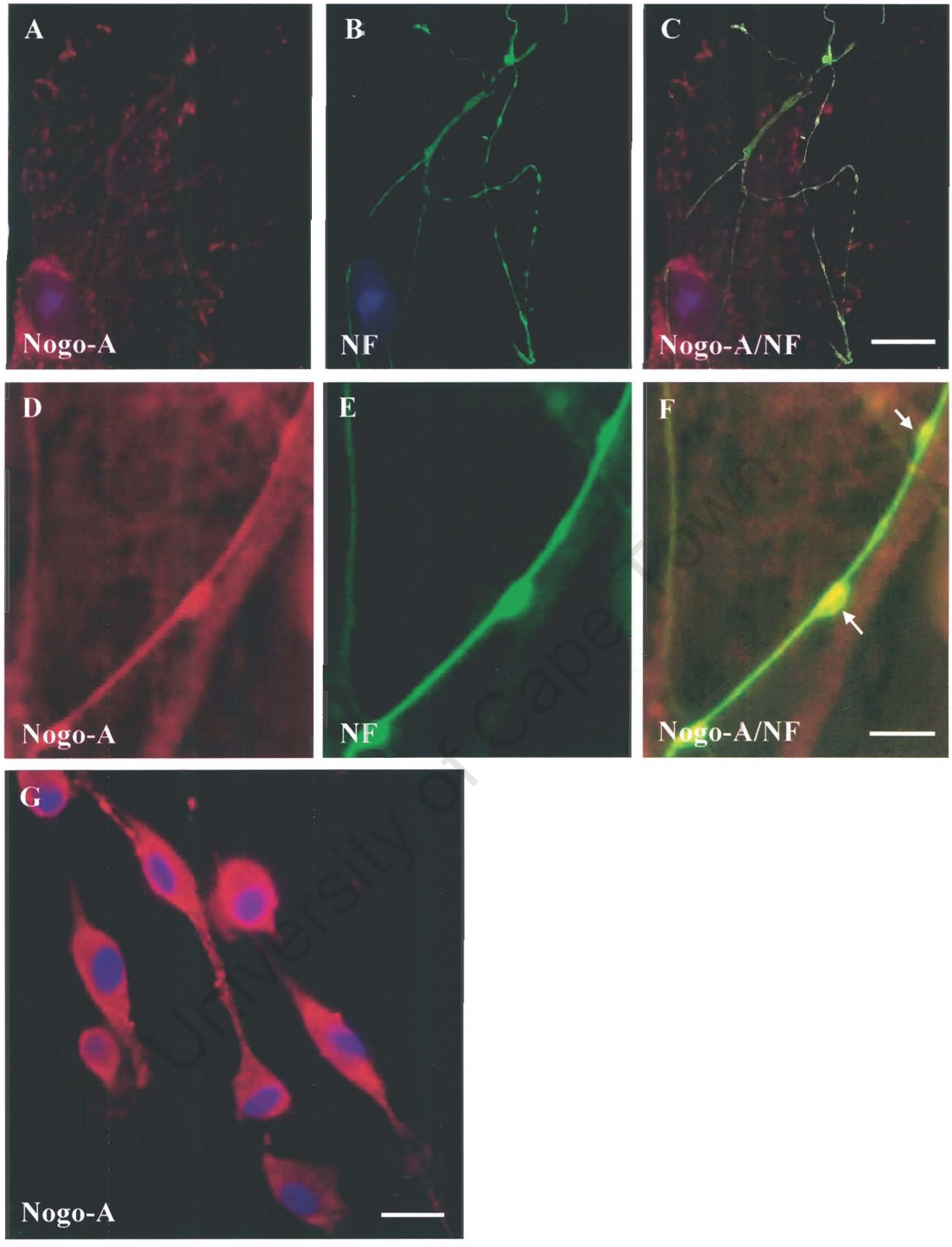


Figure. 2. Immunofluorescence microscope images of neonate Wistar rat CNS glia in culture.

In a primary glial cell culture, oligodendrocytes (A) not astrocytes (B) express Nogo-A. On merging images A & B, oligodendrocytes can be distinctly seen in layers of astrocytes expressing GFAP (C).

D-F; In an enriched culture supplemented with triiodothyronine (T_3), the existence of oligodendrocytes was confirmed by immunoreactivity with O_4 antibody (E). Note the intracellular localisation of Nogo-A when compared to O_4 which is on the oligodendrocyte cell membrane surface (insert).

Scale bars : (A-C; 25 μm, D-F; 25 μm).

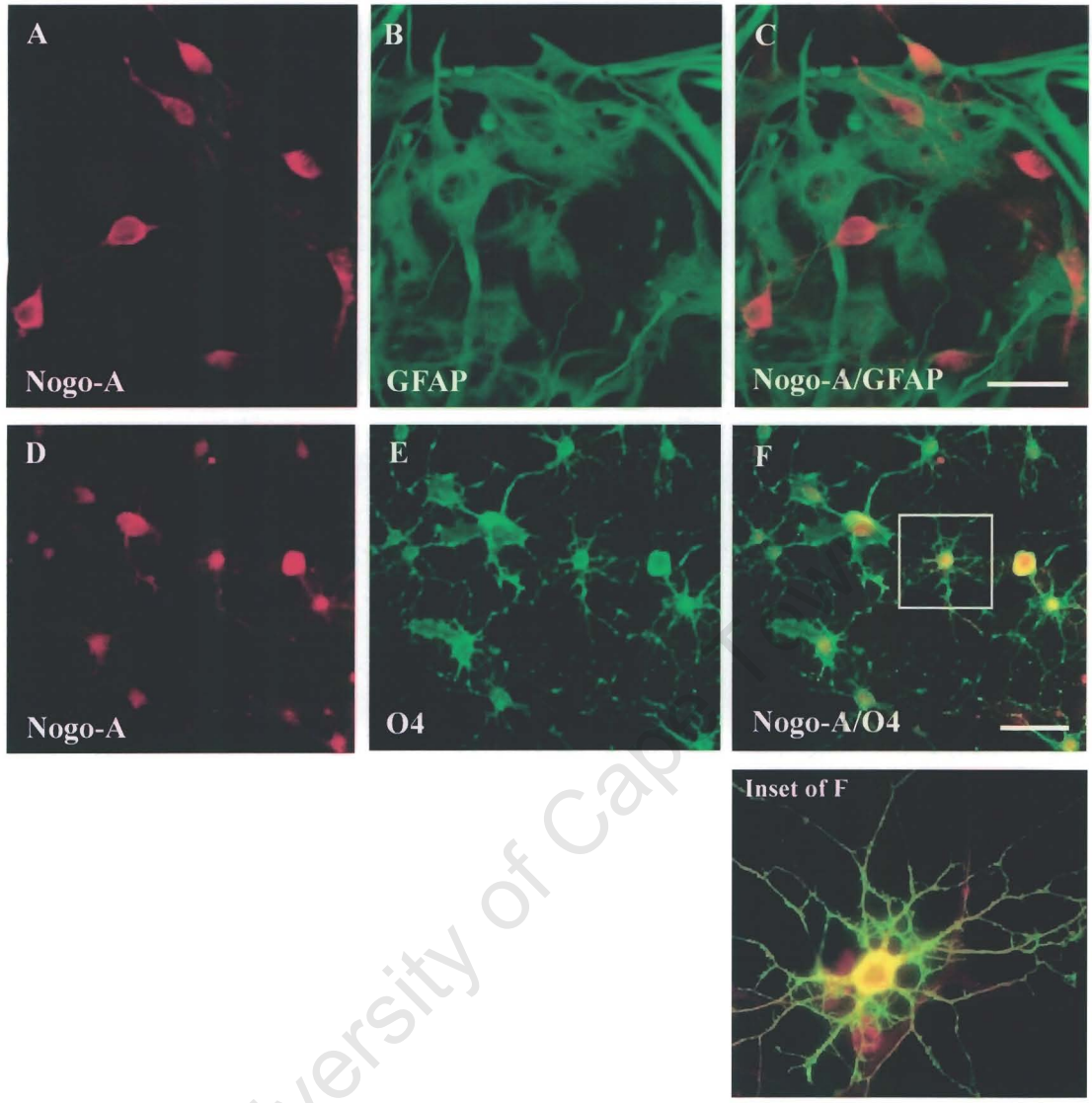


Figure. 3. Immunohistochemistry for Nogo-A in adult Wistar rat tissues: Forebrain, showing part of the deep layers of cerebral cortex: In the striatum, neurons (shown by arrows heads) can be seen expressing high levels of Nogo-A. **Scale bar: 20 μm.**

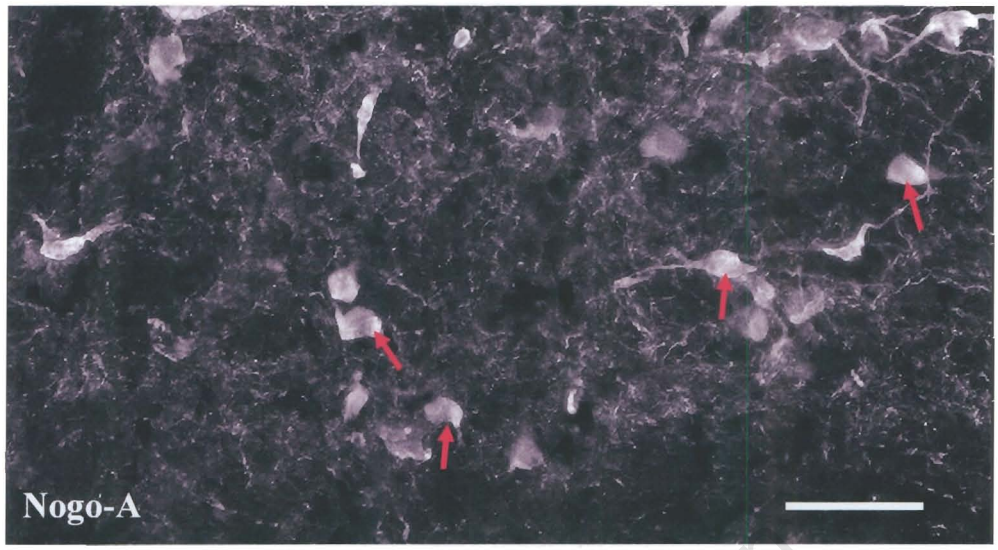


Figure. 4. Nogo-A immunoreactivity in the Wistar rat hippocampal formations: CA1-CA4.

In the hippocampus, an area with numerous neurons, the cell bodies (shown by arrows) of these neurons exhibit strong Nogo-A expression (red). **Scale bar: 20 μm.**

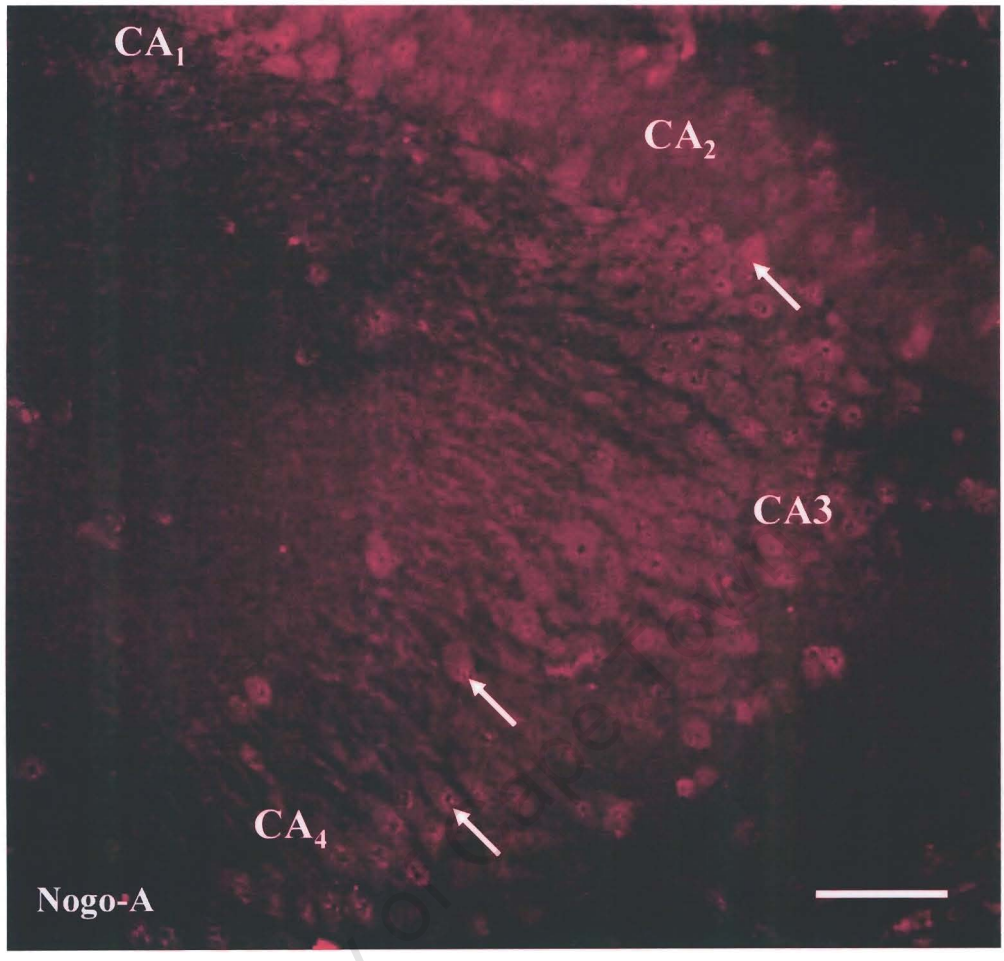


Figure. 5. Transverse section through the cerebral peduncle: Nogo-A immunoreactivity can be seen in neurons (arrow heads) and some presumptive oligodendrocytes (arrows). **Scale bar: 25 μm.**

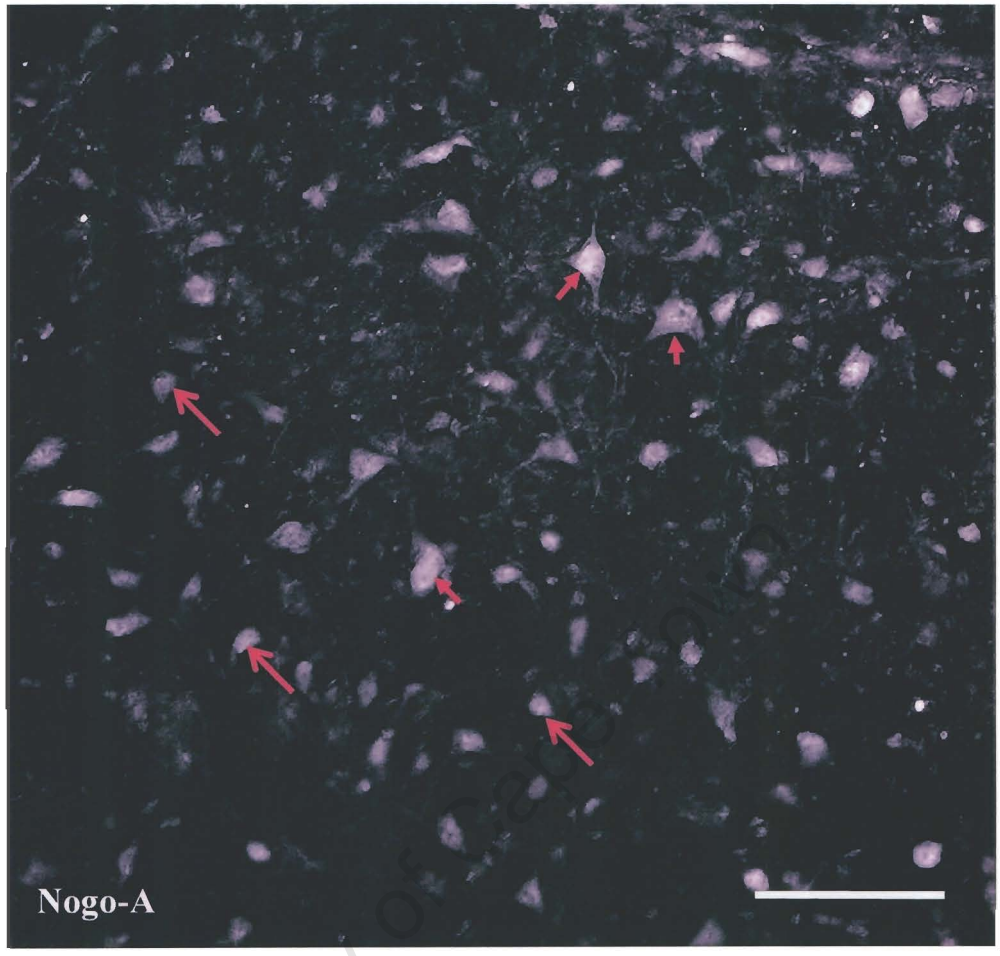


Figure. 6. Nogo-A immunofluorescence in adult Wistar rat brain white matter (A–C; G-I), and spinal cord white matter (D–F).

A-C; In the caudate putamen, an area with numerous myelinated axonal bundles, Nogo-A (red) stains the peripheral regions encircling NF immunoreactive axons (green). Notice how Nogo-A antibody stains myelin fibres surrounding the axons with slight staining on the axon (insert in C). In the spinal cord (SC) white matter area (D-F), Note the oligodendrocytes staining (arrowheads) for Nogo-A and the myelin staining (arrows) encircling myelinated axons leaving an unstained thin rim (F) suggesting absence of Nogo-A in the inner myelin sheath. G-I; Nogo-A antibody stains myelin sheath, as revealed by the myelin marker, proteolipid protein (PLP). Nuclear staining is shown by DAPI in blue. **Scale bars : (A-C; 5 μm, D-F; 5 μm, G-I; 5 μm).**

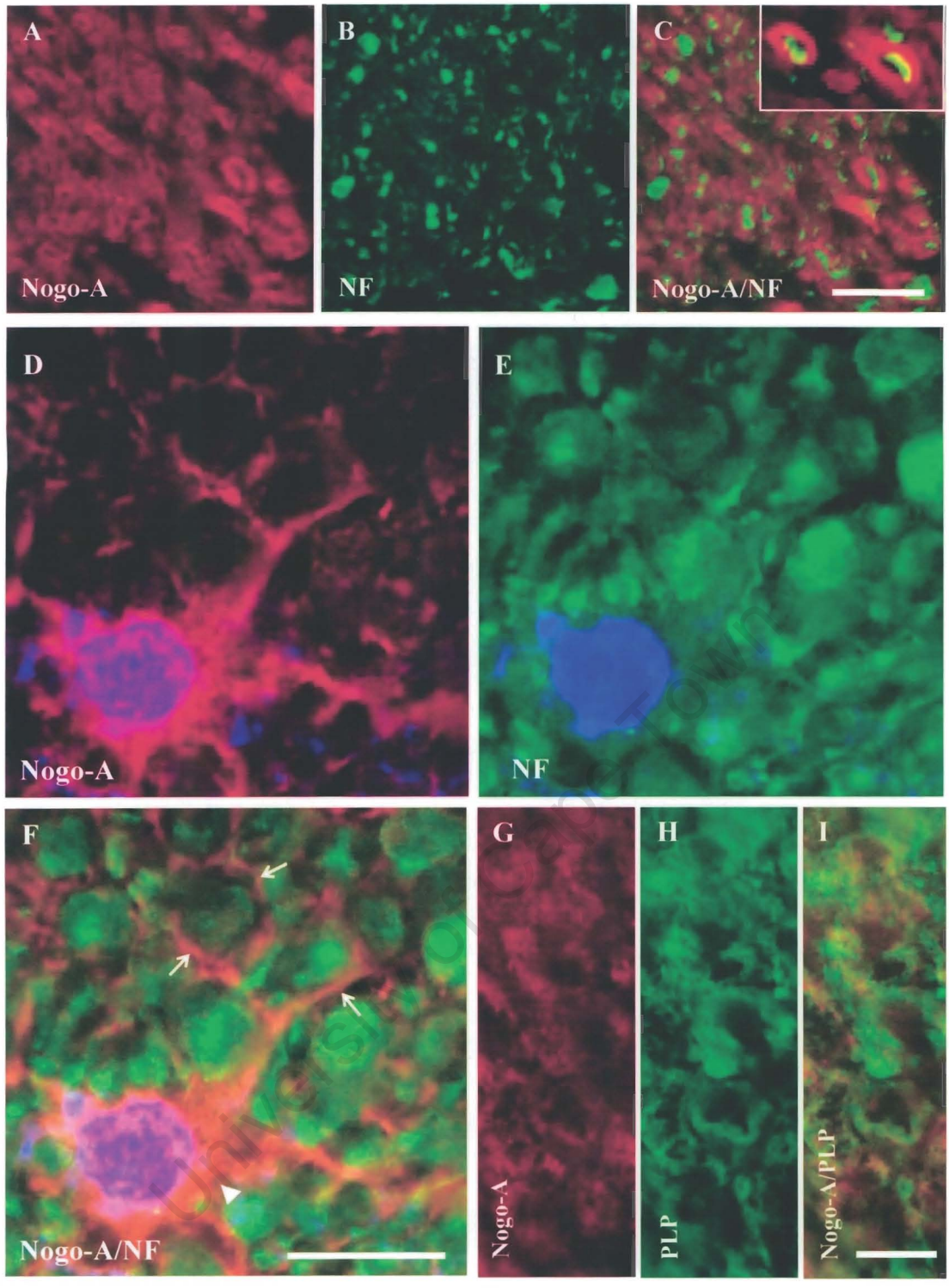

A
Nogo-A
B
NF
C
Nogo-A/NF
D
Nogo-A
E
NF
F
Nogo-A/NF
G
Nogo-A
H
PLP
I
Nogo-A/PLP

Figure. 7. Nogo-A immunoreactivity in the adult Wistar rat spinal cord (SC). Ependymal glia (shown by blue arrows) surrounding the central canal express Nogo-A protein. **Scale bar: 50 μm.**

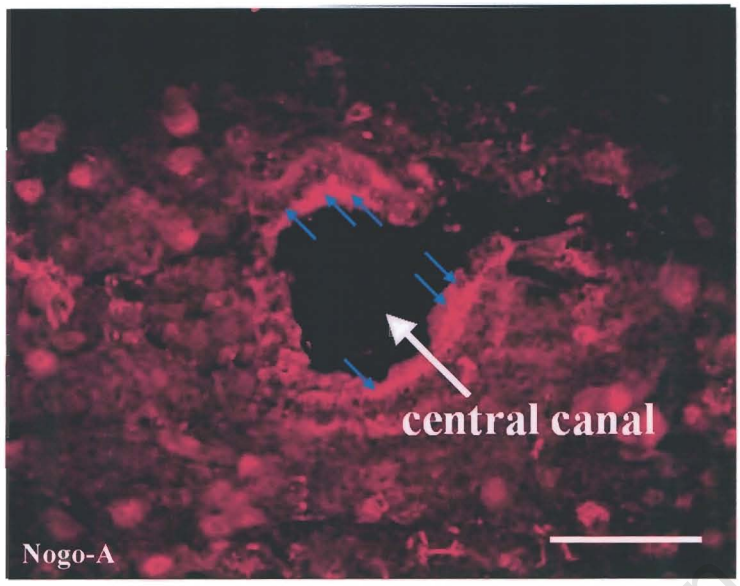


Figure. 8. Nogo-A/TH immunofluorescence in the intact (A-C) and 6-OHDA lesioned (D-F) adult rat brain. Five weeks after lesioning, dopaminergic neurons in the substantia nigra (pars compacta) were negative to TH staining (E) compared to the intact brain (B) see arrows. Due to degenerating neurons in the lesioned side, it was difficult to ascertain the extent of Nogo-A regulation. **Scale bars : (A-C; 50 μm, D-F; 50 μm).**

Intact side *6-OHDA lesioned side*

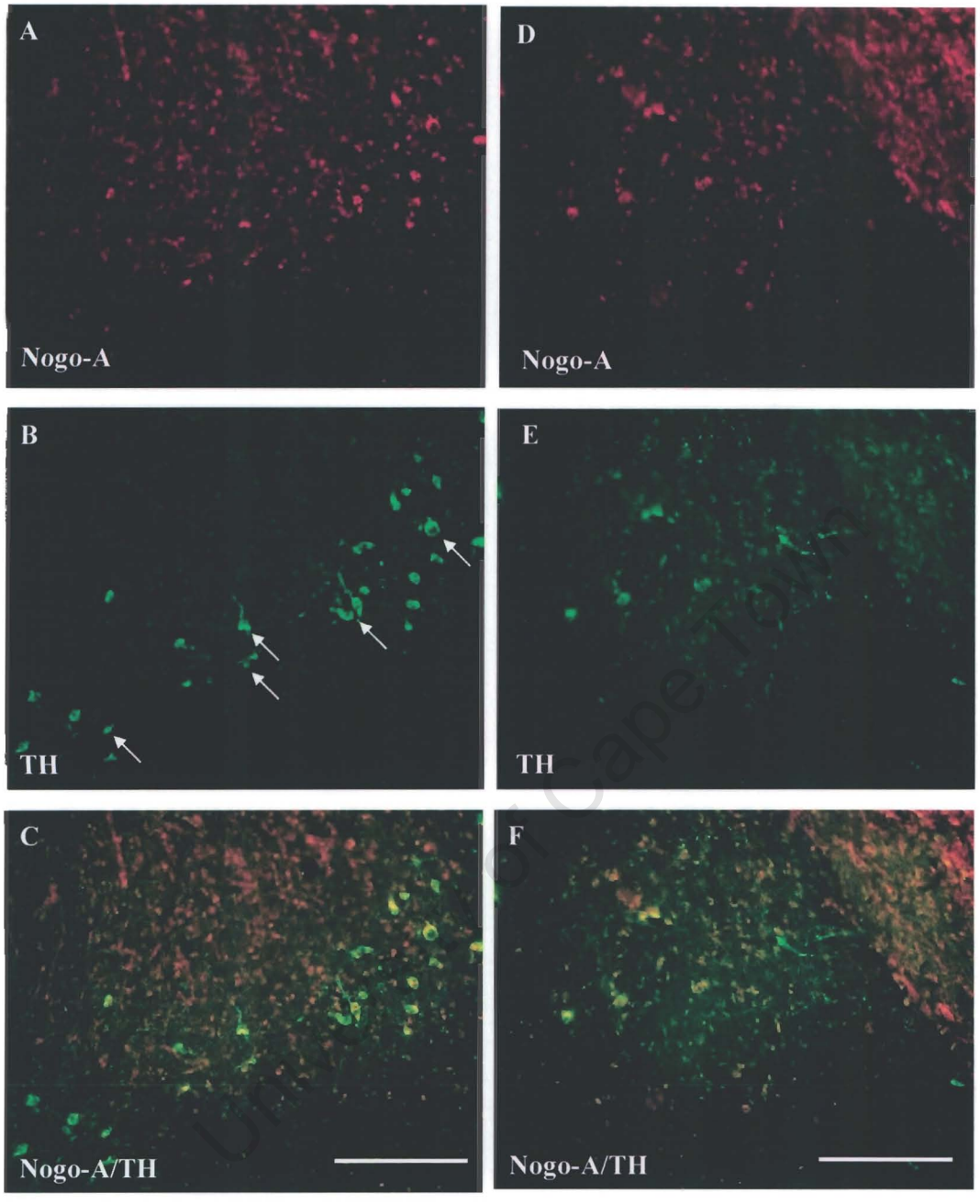
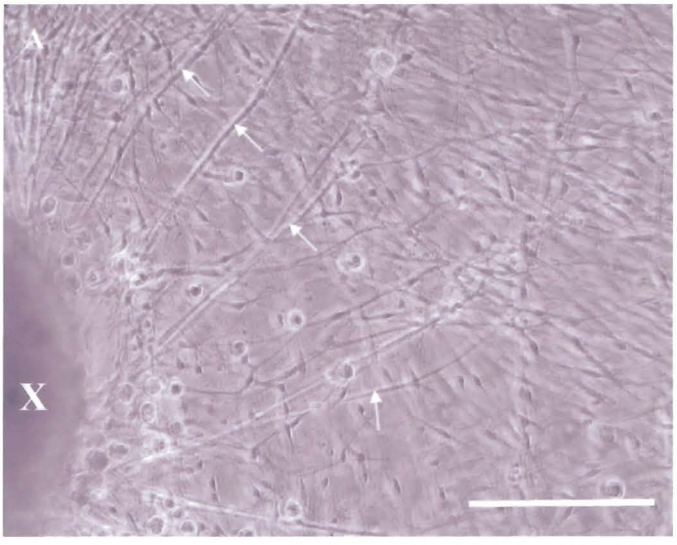


Figure. 9. Neonate Wistar rat Dorsal Root Ganglion (DRG) cell culture (Phase contrast)

A; Numerous neurites (arrows) can be seen originating from a DRG explant (X), bridging over Schwann cells and fibroblasts.

B; Neurons with their processes (arrow heads) at higher magnification can be identified by their morphology in culture. **Scale bars : (A; 50 μm, B; 25μm)**



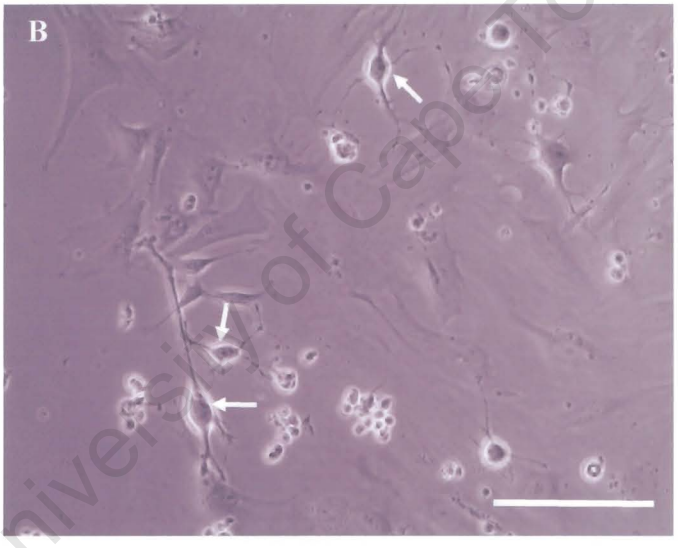


Figure. 10. Immunofluorescence microscope images of neonate Wistar rat Dorsal Root Ganglion (DRG) axons in culture (A-F). We do observe Anti NgR that bound on the axonal surface when incubated with DRG axons (arrow heads) for an hour at 37^0C in culture (live staining). At higher magnification the receptor protein tends to accumulate at the end surface of growth cones, this can be clearly observed at the extreme right in the fluorescent images (D-F). Neurofilament (green) stains axons. A nuclear stain, DAPI (blue) shows nuclear position(s) **Scale bars: (A-C; 10 μm, D-F; 2.5 μm).**

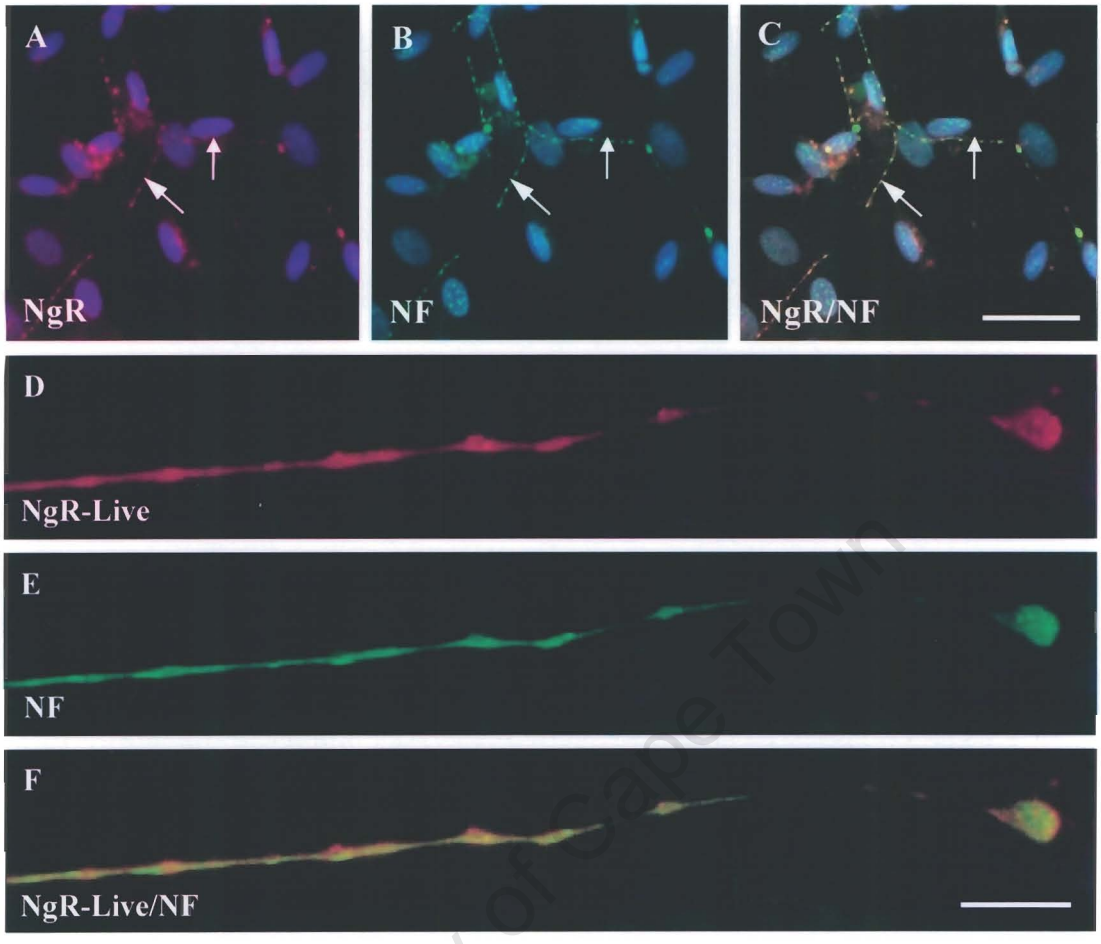


Figure. 11. Immunofluorescence microscope images of neonate Wistar rat astrocytes and oligodendrocytes in culture.

A-C; In a primary CNS glial cell culture, astrocytes do not express NgR.

D-F; In an enriched oligodendrocytes culture supplemented with triiodothyronine (T_3), oligodendrocytes do not express NgR either. (The existence of oligodendrocytes was confirmed by immunoreactivity with O_4 antibody (E). **Scale bars : (A-C; 25 μm, D-F; 25 μm).**

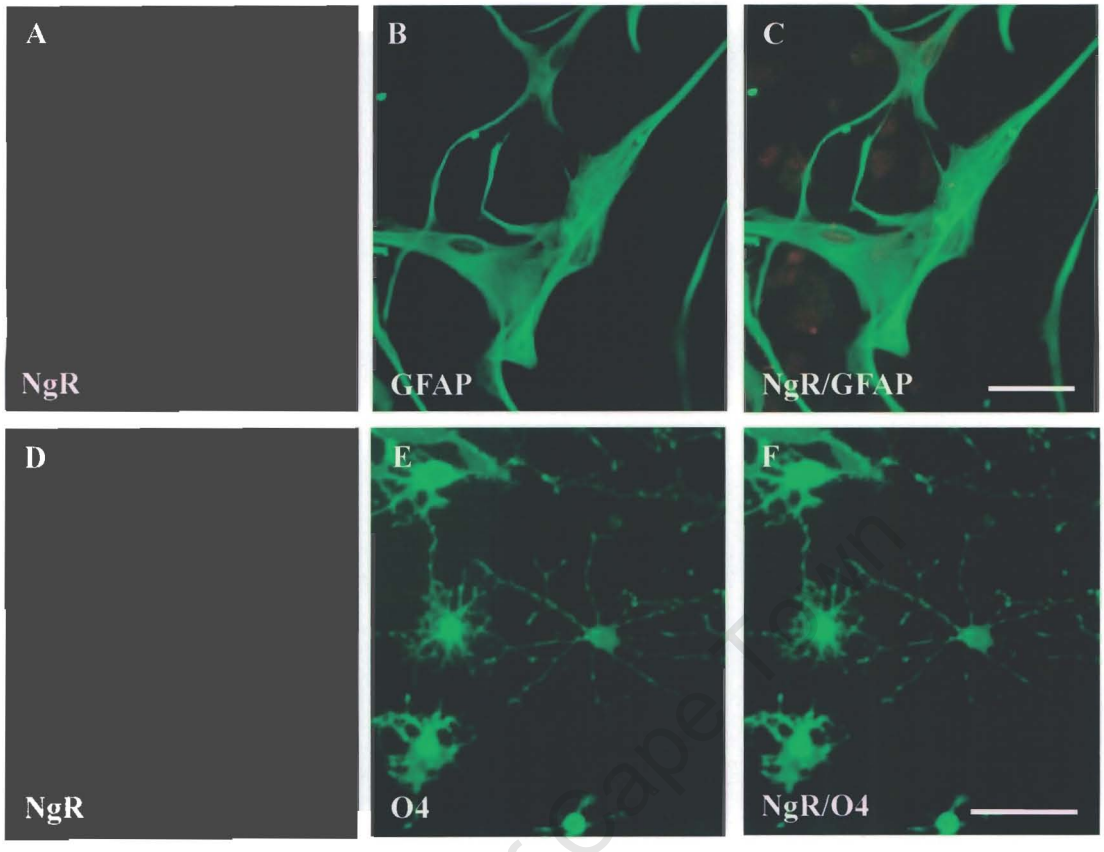


Figure. 12. Localisation of NgR in the gray matter of adult rat spinal cord (SC); Transverse sections were stained with NgR antibody (red), alongside neurofilament antibody (green). Note the strong NgR staining in neuronal cell bodies (arrowhead), and axons/dendrites (arrows). **Scale bars : (A & B; 20 μm).**

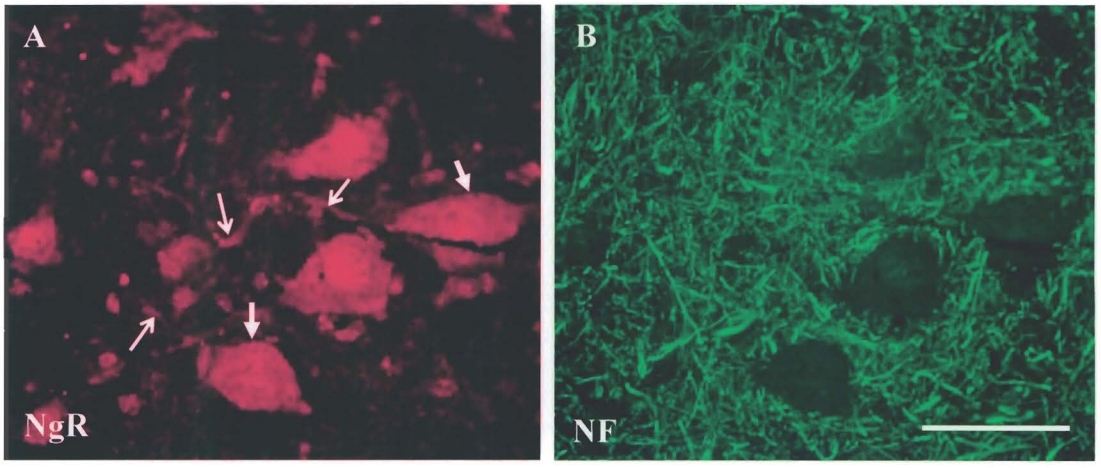


Figure. 13. NgR immunofluorescence in adult rat brain; substantia nigra (A–C), and caudate putamen (D–G).

Neuronal cell bodies in the substantia nigra (pars compacta) expressed NgR. This was apparent on superimposition with anti Tyrosine Hydroxylase (TH) antibody which labels dopaminergic neurons (A-C). In the caudate putamen, anti NgR antibody stained myelinated axonal bundles (D), which was confirmed by co-localising NgR (red) with PLP (green) (E-G).

Scale bars : (A-C; 20 μm, D; 5 μm, E-G; 10 μm).

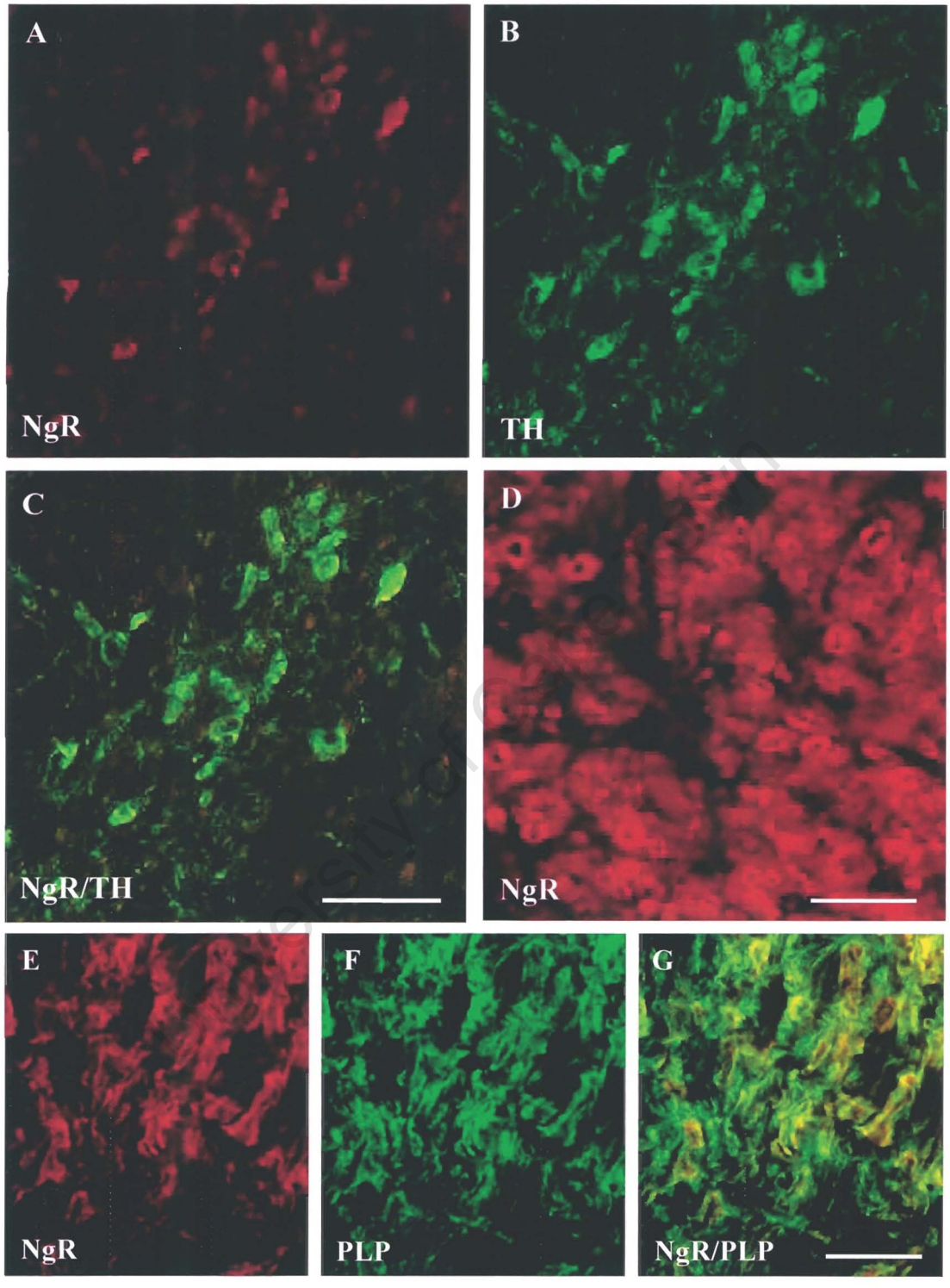

A
NgR
B
TH
C
NgR/TH
D
NgR
E
NgR
F
PLP
G
NgR/PLP

Figure. 14. Immunoblot of NgR and Nogo-A expression in rat tissues. Samples (1μg/μl) from indicated tissues were analysed by anti-NgR (h) & anti-Nogo-A immunoblots. NB, neonate brain; AB, adult brain; ASC, adult spinal cord. The positive Nogo receptor band migrated at approximately 85 kDa, with low levels in adult spinal cord (A). The Nogo-A band migrated at about 200 kDa (C). Immunoblot of pre-immune sera collected from rabbits before immunization with NgR peptide revealed a non specific band at approximately 50 kDa in neonate brain tissue (B).

MW standards in kilo Daltons (kDa)are indicated at the left side of the blots..

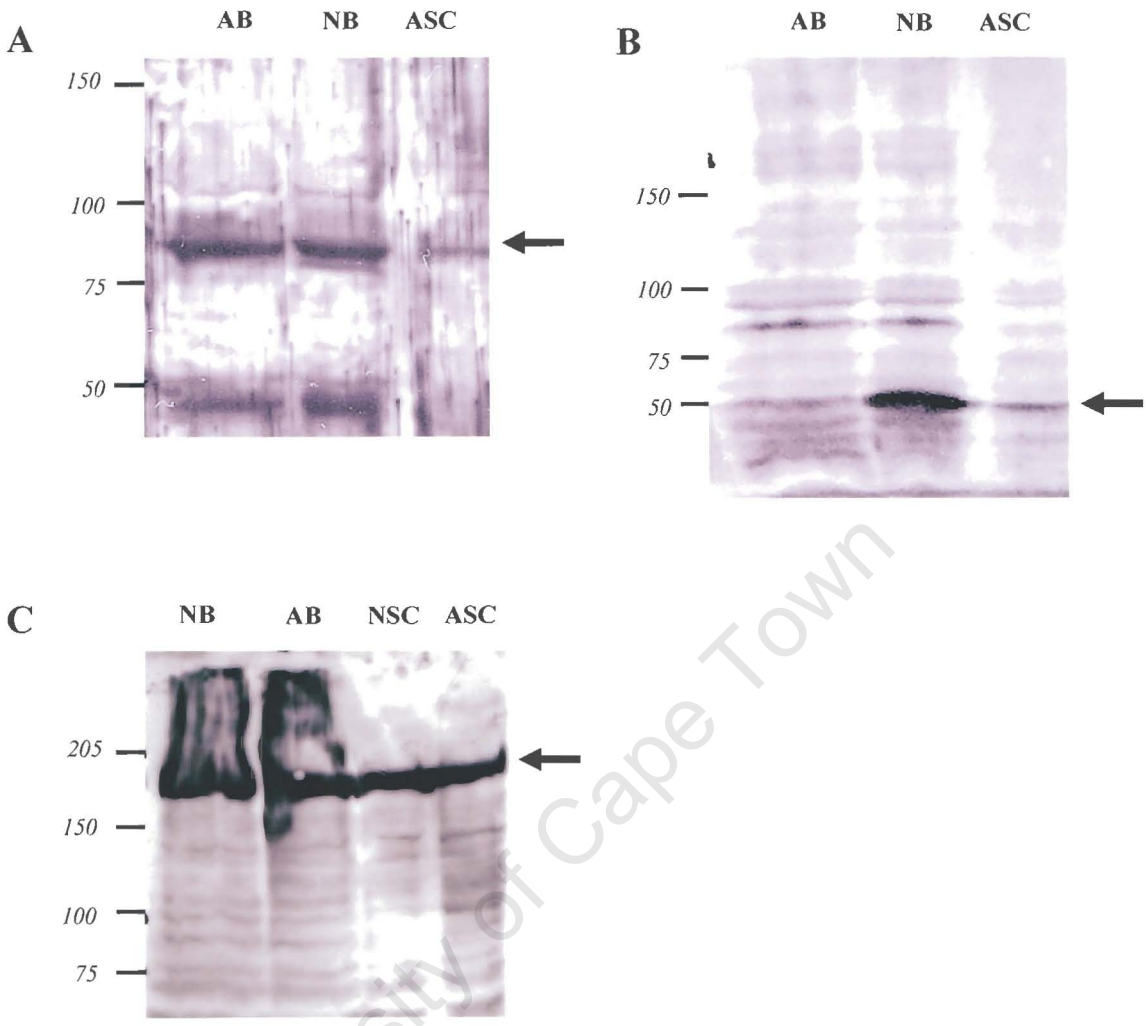


Figure. 15. An axon extending from the cell body in a Wistar rat DRG cell culture (A-C). Nogo receptor (NgR) in red associates with Flotillins (green) in lipid-raft domains. Yellow areas in merged images indicate superimposition, as shown by arrow heads (C). The yellow patches were mostly concentrated at particular enlarged spots along the axonal membrane. **Scale bar: (A-C; 12 μm).**

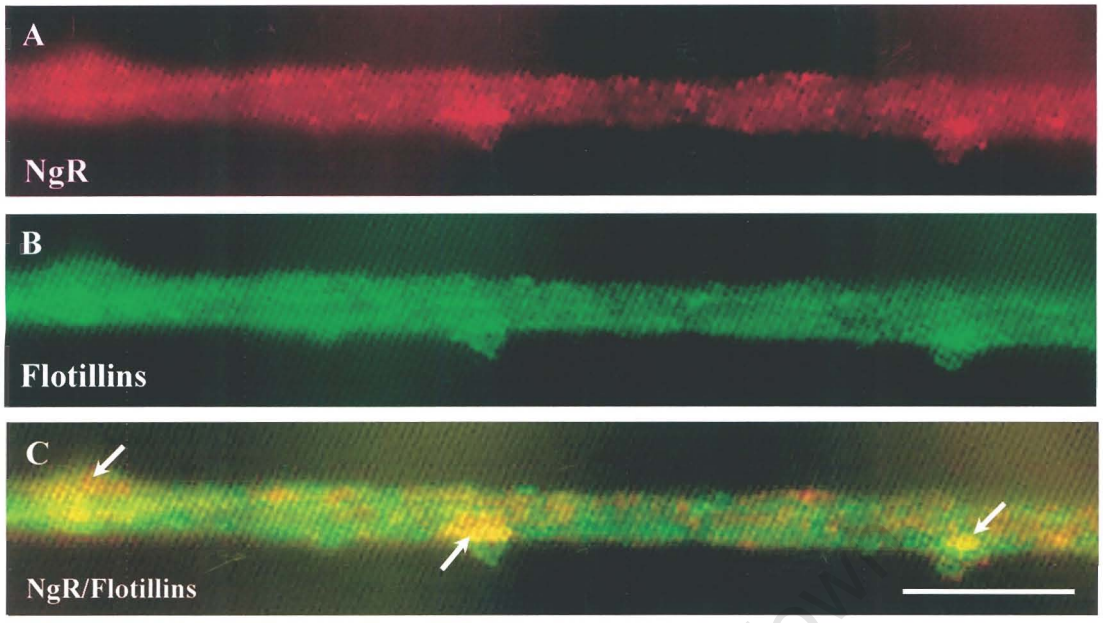


Figure. 16. PC-12 cells (A-C). Nogo receptor (NgR) in red associates with Flotillin (green) in lipid-raft domains. Yellow areas in the merged image indicate superimposition. NgR (red) partially colocalises with Flotillins (green) in lipid rafts forming yellow punctate areas as shown by arrow heads (C). The position of the nucleus is shown by DAPI in blue. **Scale bars: (A-C; 5 μm).**

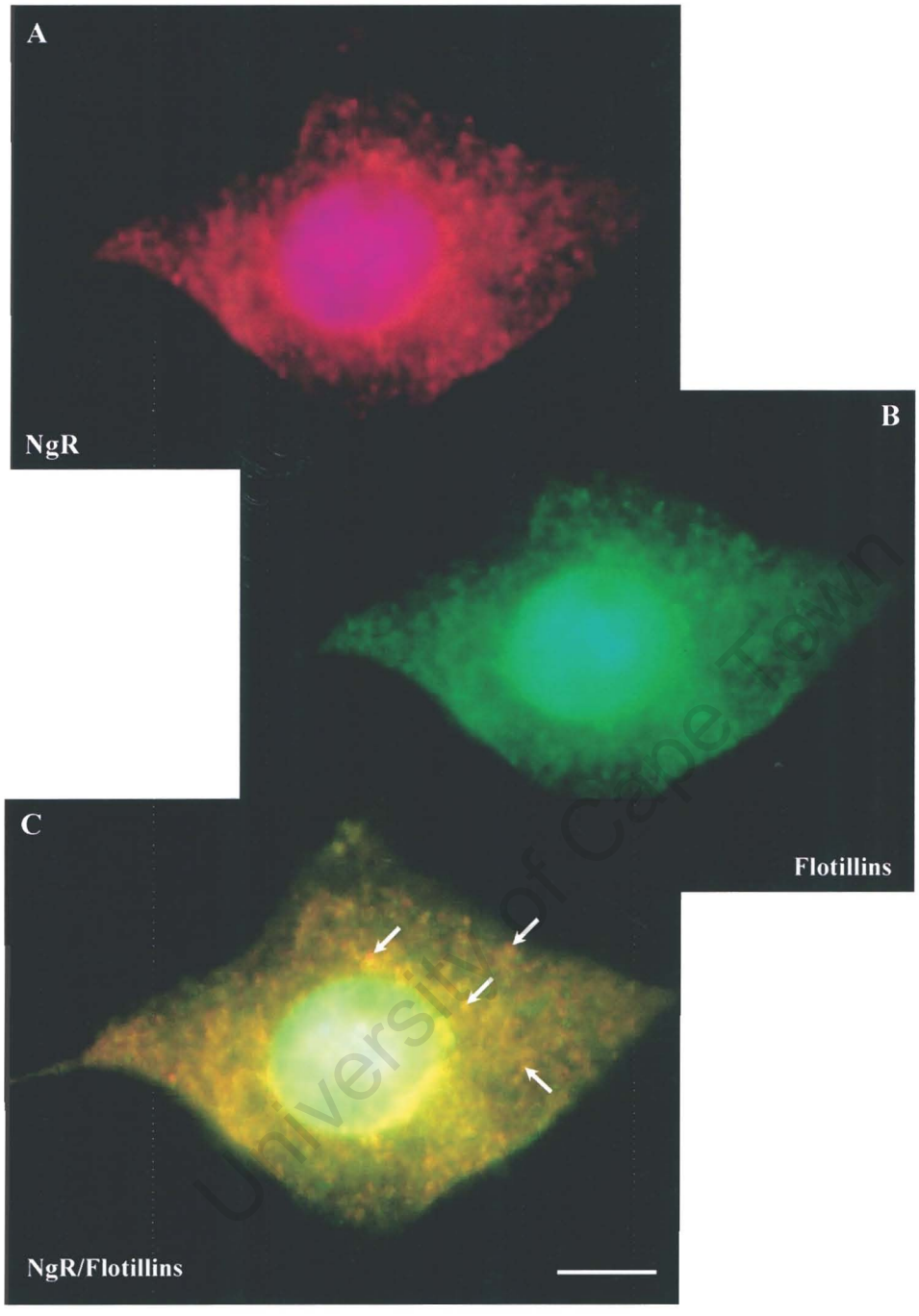
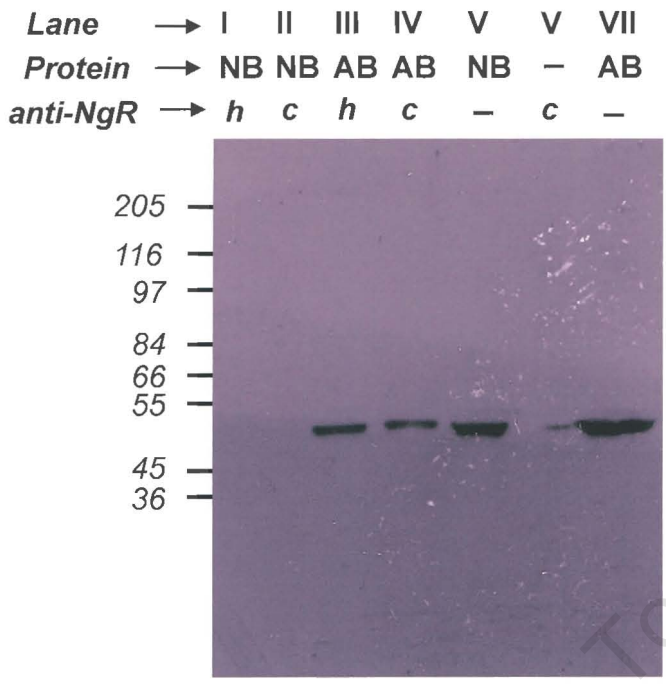


Figure. 17. Co-immunoprecipitation of NgR and flotillin in rat CNS tissues. NgR antigen was extracted from indicated rat tissues using home made (h) & commercial (c) anti-NgR antibody. Protein-A sepharose was later used to separate NgR antigen-antibody complex from the homogenate. The antigen was eluted and loaded on a SDS-PAGE western blot, and later probed with anti-flotillin antibody. Positive bands at approximately 49kDa were detected in adult brain protein extracted using both home made & commercial NgR antibody (*lane III & IV*). No bands were detected in neonate brain (*lane I & II*) and the negative control which lacked antigen (*lane VI*). Positive bands were also detected in crude protein from neonate (*lane V*) & adult (*lane VII*) brain homogenate.

NB, neonate brain; AB, adult brain; SC, spinal cord. ***MW standards in kilo Daltons (kDa)are indicated at the left of the blots..***



DISCUSSION

4.0 DISCUSSION

4.1 Nogo-A expression

The expression pattern of Nogo-A has been investigated thoroughly in the young and adult rat both in culture and in sections to obtain a clear understanding of the protein as per one of the objectives of this study. In general Nogo-A, a potent neurite out-growth inhibitor and a major ligand of NgR is extensively expressed by many cell types in the central and peripheral nervous system. Among the cells of the CNS, Nogo-A is highly expressed in oligodendrocytes in the adult CNS, with a proportion of the protein being localised to the processes that emerge from the cell body. This can clearly be seen in the spinal cord white matter, where oligodendrocytes are known to extend their processes to ensheath axon bundles that form tracts through which sensory and motor information is transmitted. The Nogo-A antibody staining forms a rim encircling individual axons leaving an unstained thin area all around (Figure. 6D-F). This observation may suggest absence of Nogo-A in the inner most loop of the myelin membrane that is adjacent to the axons. An oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon with subsequent condensation of the multispiral membrane-forming myelin (Bunge *et al.,* 1962; Bunge, 1968). On the same axon, adjacent myelin segments belong to different oligodendrocytes. The number of processes that form myelin sheaths from a single oligodendrocyte varies according to the area of the CNS and possibly the species, (Peters *et al.,* 1991). The myelin sheath is separated from the axonal membrane by a narrow extracellular cleft, the periaxonal space. Myelin is less compacted at the inner and outer end of the spiral, forming inner and outer loops that retain small amounts of oligodendrocyte

cytoplasm. Therefore it is also possible that Nogo-A could not be detected in that area because of the extracellular cleft.

Astrocytes, which are the other major supporting cells (glia) of the CNS, did not express Nogo-A (Figure. 2A-C). Although injury prompts astrocytes to hypertrophy and reorganise to form the glial scar that forms a barrier to regeneration (Fawcett and Asher, 1999), little attention has been focused on astrocytes as potential contributors in blocking axon re-growth. Astrocytes are not only involved in the formation, but also the efficiency and maintenance of synapses between neurons depends on signals from astrocytes (Barres and Smith, 2001). They also participate in the formation of the blood brain barrier (Risau and Wolburg, 1990), ion homeostasis (Bekar and Walz, 1990) as well as in the production of the extracellular matrix (Liesi and Silver, 1998).

Nogo-A protein was also found in neuronal cell bodies in CNS tissue. In comparison to oligodendrocytes, the staining of the former was less concentrated, and since the two cell types are quite dissimilar in nature, morphology, size and function, it was quite difficult to compare the staining enormity adequately.

Although in the PNS, Schwann cells are not known to be hostile to regenerating axons compared to oligodendrocyte cells (David and Aguayo, 1981) these myelinating cells of the PNS immunoreacted with Nogo-A antibody in culture (Figure. 1G), an observation that has not been reported before. Intracellularly, Nogo-A was also found in structures that morphologically resembled endoplasmic reticulum (ER) in fibroblasts (Figure. 1A). Although fibroblasts are not nervous tissue cells, Nogo-A has been reported present in other tissues like skeletal muscle (Wang *et al.,* 2002c), and therefore these findings of Nogo-A may link Nogo-A to a yet unidentified second function. Van de Velde *et al.,* 1994; Roebroek *et al.,* 1996; Moreira *et al.,* have already associated the C-terminus of all Nogos

to the reticulon protein family, the prototype of which is reticulon 1, a neuroendocrine-specific, endoplasmic reticulum-localised protein with unknown function. The nature of reticulon proteins remains obscure, but by virtue of their intracellular localisation, a di-lysine ER retention/retrieval signal at the extreme C-terminus and a lack of a signal sequence at the N-terminus, these proteins are thought to participate in endoplasmic reticulum regulation (van de Velde *et al.,* 1994; GrandPre *et al.,* 2000). Another explanation may possibly be the effects of *in vitro* environment towards cells, particularly after addition of various supplements in culture. Propagation of cells *in vitro* can induce expression not normally seen in *vivo*, this possibility is reasonably supported by Paczek and Koziorowska, 1980, who studied the behaviour of subcultures of vitamin A-treated human embryo fibroblasts. Evidence was obtained that vitamin A-induced rupture of lysosomes may lead to transient alterations in the pattern of growth, expression of certain factors, structure of the membranes and morphology in cell generations deriving from the cells injured by the action of the vitamin. Alternatively Nogo-A may regulate cellular survival in developing muscle and nerve tissues, because Nogo-B has recently been shown to modulate apoptosis in some cancer cells (Tagami *et al.,* 2000; Li *et al.,* 2001).

4.1.1 Nogo-A; likely role in oxidative stress and neurodegenerative diseases

Nogo-A has been implicated in a number of CNS pathological conditions. Reindl *et al.,* 2003, has shown that levels of Nogo-A in serum and cerebral spinal fluid (CSF) is increased in patients with autoimmune diseases like multiple sclerosis (MS), acute inflammatory (IND) and non inflammatory diseases (OND). In experimentally induced autoimmune encephalomyelitis (EAE), an MS model, targeting Nogo-A by active immunization significantly blunts clinical signs, demyelination, and axonal damage

associated with the neurodegenerative disorder (Karnezis *et al.*, 2004). These finding substantiate the extra complex role Nogo-A may possess other than neurite out-growth inhibition. With the above circumstances, it was therefore vital to investigate whether Nogo-A, which is expressed by neurons in the brain, as observed earlier in the study is regulated in response to a neurotoxic insult, i.e. dopamine mediated oxidative stress-induced cell death. The results so obtained would be valuable in linking major inhibitors of axon regeneration like Nogo-A to neurodegenerative diseases like Parkinson.

Aware that 6-OHDA elicits free radical-induced oxidative stress in neurodegenerative processes (Schober, 2004), an investigation whether such a stress causes upregulation or down regulation of Nogo-A protein expression did not yield expected results. The failure to physically observe any considerable difference in neuronal cell body Nogo-A expression, between the lesioned and the intact side in rats sacrificed 5 weeks after neurotoxicity could be explained by the fact that neurons in the lesioned hemisphere had degenerated, owing to interference with some of their biochemical functions. This is clearly supported by Sachs and Jonsson (1975), who demonstrated the mechanisms of action of 6-OHDA. It has the ability to cause neuronal degeneration through a synergistic action of respiratory inhibition and oxidative stress, induced by free radical formation. Although both toxic mechanisms are not necessarily linked, they appear to act synergistically during neuron degeneration.

Failure to physically observe any considerable results when the period between lesion application and sacrificing was shortened to (24-72) hours could also be logically explained by failure of dopaminergic neurons to experience appropriate toxicity within such a short period (24-72) hours. One way of confirming whether unilateral dopaminergic toxicity with 6-OHDA has occurred before sacrificing the rats is by performing rotational tests. However, the period (24-72) hour was too short for such a test since rats were still

recovering from surgery. One other reason why I could have failed to detect any considerable differences in Nogo-A neuronal cell body expression between the two hemispheres could also be attributed to the fixation methods that were used. Apparently anti-tyrosine hydroxylase antibody staining produces extremely better results in perfusion fixed cryo-sections than non perfused ones. However, perfusion fixed cryo-sections could still interfere with Nogo-A antibody immunostaining. This phenomenon is supported by the fact that one of the reagents used to fix the brain tissue in the experiment could be modifying a residue that is key in a particular epitope the Nogo-A antibody is supposed to recognize in the tissue. Foster *et al.,* 2006 has described how specific chemical and morphological modifications occur in tissues during fixation and can interfere with subsequent analytical techniques. For example, formaldehyde interacts with proteins by modifying lysine residues to generate intermolecular aldol cross-linkages. Therefore, immunohistochemical localisation may depend not only on the quality of the antibody, but also the method and extent of fixation.

4.2 NgR expression

4.2.1 Nogo receptor antibody design and production

Nogo receptor (NgR) was first identified by Fournier *et al.,* (2001) as an axonal surface protein predicted to contain 473 amino acids, with a conventional amino terminal membrane translocation sequence signal, which is followed by nine leucine rich repeat (LRR) domains and a carboxyl terminal motif. With that information in mind, the search was on to identify a mammalian and lower vertebrate NgR peptide sequence that was conserved and highly immunogenic to produce antibodies for the study. Using a

computation analysis programme on the NCBI website (performed at the SIB using the BLAST network service), a short peptide of eight amino acids (SLQYLRLN) corresponding to residues 251 to 258 (LRR region No.9) of rat NgR protein was identified. The peptide sequence was later conjugated with Keyhole Limpet Hemocyanin (KLH), a copper containing protein carrier.

The conjugated peptide was combined with Incomplete Freund's adjuvant (IFA) to increase the strength of the immune response and was later injected in rabbits to raise polyclonal antibodies (see Immunisation schedule Appendix II). To their advantage over monoclonal antibodies, polyclonal antibodies require less generation costs, detect a multiplicity of epitopes and therefore recognise antigen from different orientations. In addition, polyclonal reagents are relatively simple and cheap to produce in the short term compared with monoclonal reagents (Nelson *et al.,* 1997). Furthermore, the use of larger animals (such as horses, goats, and rabbits) enables the recovery of a large volume (e.g., 60ml from a rabbit) of antibody-rich serum. Successive NgR anti-sera (new) was collected and tested. There was a remarkable gradual improvement in specificity from the 1st to the 6th bleed as per immunohistochemistry studies. However, there was a general concern regarding presence of non specific bands because the NgR anti-sera (new) were raised against a short peptide which may occur in unrelated proteins. Ideally, the peptide chosen should have been aligned against all other proteins in the data base as an indication of how many others it had the potential of reacting with. But this could not be done because of logistical limitations.

4.2.2 NgR (new) immune sera

The NgR band observed with the new antisera from extracts of neonate and adult brain at approximately 85kDa on a western blot (Figure. 14A) is consistent with what other

researchers have found before (Fournier *et al.,* 2001). However, a weaker band was detected in adult spinal cord tissue, this could be due to little NgR protein in the spinal cord. A non specific band at 50 kDa similar to the band detected in pre-immune sera could be due to reaction with unrelated proteins (see *4.2.1.* above). Overall the immunoblot was not clean enough, and still this could be as a result of using unpurified sera.

4.2.3 Pre-immune sera

Pre-immune serum was collected from rabbits before immunization with the synthetic peptide was performed. It was used as a negative control alongside the NgR (new) antisera each time IHC and western blot experiments were performed. Astonishingly pre-immune sera presented related though not exact immunoreactivity like the NgR (new) antisera from both rabbits. This is a common occurrence with polyclonal antibodies. For example there are number of antisera that have unexpectedly recognized particular antigens of interest without any experimental manipulation. Reports of one antiserum that specifically stained neuronal precursors in *Drosophila* embryo have been noted before (Wagner, 2001). So it is possible that the pre-immune sera harvested from the rabbits specifically stained the same cell types that the true antibody was staining. However western blot results with the pre-immune sera did not reveal the true NgR band of 80-85 kDa normally displayed by the NgR (commercial) antibody. Nevertheless, a supposed non specific band at 50 kDa with the pre-immune sera was observed which cannot be clearly explained.

4.2.4 NgR expression in the CNS tissue

Failure to detect NgR immunoreactivity in oligodendrocytes and astrocytes (Figure. 11) is consistent with results shown by NgR antibodies raised from different domains of the protein sequence. Presence of strong NgR immunoreactivity in myelinated axons coupled with limited superimposition of anti NgR and anti PLP a myelin marker at that resolution does not ascertain localisation of NgR protein in myelin sheath. The fact that axonal tracts in the caudate putamen and some other regions in the CNS are heavily myelinated, and bearing in mind that myelin sheath wrapped around the axons is so tight, the use of fluorescent immunohistochemistry technique in cryo-sections in investigating such detailed superimposition has a limitation in resolving such a demarcation. The resolution could be improved by use of better techniques e.g. electron microscopy (EM). However, the results obtained are still consistent with what Wang *et al.*, reported in 2002, that NgR is not found in oligodendrocytes or outer myelin sheath but is detectable more in axons surrounded by myelin than naked ones as revealed by the precise matching with β-III tubilin. The expression of NgR protein by neurons in the CNS is in such a strategic distribution so as to interact with Nogo-A. This kind of localisation by these two proteins supports the concept that NgR contact with Nogo-A limits axonal regeneration after injury.

4.2.5 NgR expression in the DRG cultures

Physically showing presence of NgR on the axonal surface on an intact membrane corroborates functional studies that have been done by Fournier *et al.*, 2001. Once more these observations point to a possibility of the receptor location on the membrane surface, which mediates activities of Nogo, MAG and OMgp, recognized myelin associated neurite

out-growth inhibitors. Since growth cones are characteristic of active path-finding axons in DRG cultures, a number of axonal growth ends, resembling growth cones were identified in the DRG cultures. Growth cones contain the machinery that powers axon elongation, and also receive and interpret the extracellular cues that the regenerating axons encounter. Nogo receptor immunoreactivity was more concentrated at the periphery of growth cones when viewed at higher magnification (Figure. 10F). This probably explains why axonal growth cones collapse through retraction of microtubules when they encounter hostile molecules e.g. Nogo, MAG and OMgp that are present in mammalian CNS myelin. How the growth cone interprets signals from CNS inhibitors to halt axon growth remains largely unknown. Studies of axon guidance during development have identified the small G protein Rho as an important mediator that can translate a repulsive guidance signal in a growth cone cytoskeleton leading to collapse or repulsive turning (Patel and Van Vactor, 2002). Because some repulsive cues present during development are also expressed after CNS injury, Rho GTPases may well be important transducers of inhibitory signals from the injured CNS environment to the growing axon, impeding regeneration. Inhibition of RhoA has been shown to promote some axon regeneration after optic nerve crush (Lehmann *et al.,* 1999). It is not exactly known how Rho regulates cytoskeletal elements to stop neurite out-growth. It may do so by directly preventing actin polymerisation through LIM kinase and thus inhibiting subsequent microtubule assembly, by activating myosin II leading to axon retraction, or through as yet undefined mechanisms (Patel and Van Vactor, 2002). Signal transduction of NgR has been suggested to depend on the association with LINGO-1 and p75 neurotrophin receptor (p75), the low-affinity nerve growth factor (NGF) receptor, which may convey a signal into the cell through Rho family GTPases and consequently promote growth cone collapse and inhibit neurite extension (Wang *et al.,* 2002b).

To further back the findings as to whether indeed the NgR protein is GPI linked and thus located on the axonal surface, DRG axons were exposed to phosphatidylinositol-specific phospholipase C (PI-PLC). Treatment of cells with PI-PLC an enzyme extracted from *Bacillus cereus*, cleaves GPI-linked proteins from the cell membrane surface (Lehto and Sharom, 1998). Although Fournier *et al.,* 2001 had shown that treatment with PI-PLC renders neurons functionally insensitive to NgR, it was important to demonstrate the effect physically by immunohistochemistry. However, considerable anti-NgR (new) antibody staining could still be observed even after thorough washing off PI-PLC with laboratory culture media. This could be because the enzyme (PI-PLC) does not completely cleave the NgR protein, or probably the NgR protein may be adhering or interacting with other structures on the membrane surface via unknown biochemical bonds making it difficult to be completely detached. One molecule that the NgR could be interacting with is p75. Initially identified as a low-affinity receptor for neurotrophins, p75 was later identified as a co-receptor for NgR to transduce the signal across the axon membrane (Wang *et al.,* 2002b; Wong *et al.,* 2002).

4.2.6 Nogo receptor; localisation with lipid rafts

To further characterise the NgR protein, it was important to establish whether it is localised in lipid rafts, since a common feature of known receptor complex molecules including p75 and gangliosides, is their physical association with lipid rafts (Vinson *et al.,* 2003). Lipid rafts also called lipid microdomains are rich in sphingolipids and cholesterol, and have been proposed as regions within plasma membranes that are important for cellular signalling (Brown and London, 1998; Simons and Toomre, 2001). Lipid rafts are important

structures for signal transduction, and are enriched with signalling molecules. These signalling molecules include among others GPI-anchored receptors (of which NgR is one), as well as intracellular signalling intermediates, such as trimeric and small GTPases, Src family kinases (SFKs), lipid second messengers and a variety of cytosolic signal transducers (Anderson *et al.*, 1998).

The fact that flotillin proteins have been shown to be present in lipid rafts (Bickel *et al.*, 1997), coupled with the knowledge that molecules resident in membrane microdomain (like rafts) are associated with each other, intracellularly linking flotillin and NgR collectively by immunohistochemistry (IHC) and co-immunoprecipitation was vital to convincingly position NgR protein in lipid rafts. The link between NgR and flotillin was first observed by IHC via superimposition of the two proteins at certain locations in DRG axons (Figure. 15) and PC-12 cells (Figure. 16), and later co-precipitating the two proteins together (Figure. 17). Interestingly using the Co-IP technique, NgR demonstrated a developmental change of localisation in lipid rafts at two developmental stages examined (Figure. 17). NgR was absent in neonate brain lipid rafts, being present in higher amounts in adult brain lipid rafts. To rule out the possibility of the absence of flotillin in neonate brain rafts as being the reason for not detecting NgR, separate lanes on the western blot were loaded with normal brain tissue homogenate as a control. Both adult and neonate brain tissue gave a flotillin band at 49 kDa, although the band was stronger in adult than neonate brain rafts? I propose that NgR translocation to lipid rafts might be associated with more efficient transduction for signaling and consequently provide a cue as to why regeneration is poorer in adult than neonate rats. This kind of translocation may not be unique to NgR protein only. Yu *et al.*, 2004, were able to demonstrate developmental changes of p75 neurotrophin receptor, a co-receptor that links NgR to the cytoplasm of the cell, by illustrating its

abundance in adult than postnatal day 8 rat brain rafts. Similar developmental translocation of neural cell adhesion molecule (L1) and N-cadherin into lipid rafts has been reported previously (Nakai and Kamiguchi, 2002). Results therefore revealed by our experiments support the notion that NgR is localised in lipid rafts. It is these lipid rafts that are vital as a medium for signal transduction across the cell membrane if the receptor is to mediate neurite out-growth inhibition following injury in axons of the mammalian CNS.

Lipid rafts may not only promote, but may also interfere with neurite out-growth directly or indirectly. Rafts have also been implicated to play a major role in cell adhesion and axon guidance, which are vital if neurons are to have stable contacts with their targets during development (Giger and Kolodkin, 2001; Walsh and Doherty, 1997). One observation that suggests that rafts are required for cell adhesion is that many adhesion molecules such as TAG-1 (transiently expressed axonal glycoprotein-1), NCAM-120 (neural cell adhesion molecule), Thy-1 and F3/contactin are GPI-anchored and, thus, are located in rafts (Kasahara *et al.,* 2000; Stefanova *et al.,* 1991). Hence both cell adhesion molecules and cell guidance molecules appear to require lipid rafts for their correct localisation within the plasma membrane and for the downstream signaling events.

Lipid rafts have also been cited to play a big role in synaptic transmission. Studies have shown that rafts contribute to neuronal excitability. Two areas in which rafts contribute to synaptic transmission are in the clustering and regulation of neurotransmitter receptors and in the exocytotic process of neurotransmitter release (Martens *et al.,* 2000; Bruses *et al.,* 2001; Becher *et al.,* 2001). Lastly lipid rafts have been implicated in aiding movement of neurotoxins across cell membranes, a key pharmacological significance of lipid rafts (Herreros *et al.,* 2001).

4.2.7 Use of NgR antisera as a functional tool

After analysing the expression pattern and localisation of NgR protein in the mammalian nervous system using the NgR antisera, it would be imperative to investigate its functional properties with respect to mediating neurite out-growth inhibition. The first step would be to assess whether NgR interacts directly with one of its major ligands, Nogo-A. Using embryonic chick DRG neurons the NgR antibody should detect NgR prominently in cultures of late embryonic (day 13) neurons, which are responsive to Nogo-A, but little or none in DRG or retinal neurons of earlier embryonic stages, which are not responsive (Brittis and Flanagan, 2001).

Antibodies to a cell-surface receptor like NgR can also have a therapeutic use *in vivo*. These antibodies could be used therapeutically to block sites where major ligands (Nogo, MAG and OMgp) bind on the NgR protein thus reducing neurite out-growth inhibition in the CNS. Furthermore if NgR (new) antibody can react with a specific protein, that protein can subsequently be precipitated from solution, frequently with the help of a secondary antibody that will cross-link the antibody-antigen complexes made. Alternatively, the antigen-antibody complex can be removed by mixing the solution with either protein A or an anti-Fc antibody which has been attached to beads so that it can be easily removed from solution.

In conclusion, the results extrapolated from the whole project demonstrate that Nogo-A is mainly concentrated in CNS myelin, neurons and their axons. On the other Nogo receptor tends to be concentrated in axons, and at the same time associated with lipid rafts. These advances discussed above provide powerful tools towards a deeper understanding on the

main causes of failure of axon regeneration inhibition following injury in the mammalian CNS.

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5.0 REFERENCES

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APPENDIX

1. Nyatia-12576 peptide sequence

(SLQYLRLN) - *Leucine Rich Repeat(LRR) - **9** region (251-258)*

2. Peptide sequence (human) from Schwab group (*Invitrogen*)

EQLDLSDNAQLRSVDPA- *LRR- **3** region (108-123)*

EVPCSLPQRLAGRDLKR- *(283-300)*

GPRRRPGCSRKNRTRS- *(412-427)*

2. Peptide sequence (human) from ADALI

GCS RKNRTRSHCR *(418-430)*

KCCQPDA ADKASVLE *partly in LRR- **10** region (334-348)*

Nogo Receptor sequence from Rat

```
MKRASSGGSR LLAWVLWLQA WRVATPCPGA CVCYNEPKVT TSCPQQGLQA  50
VPTGIPASSQ RIFLHGNRIS YVPAASFQSC RNLTILWLHS NALAGIDAAA 100
FTGLTLLEQL DLSDNAQLRV VDPTTFRGLG HLHTLHLDRC GLQELGPGLF 150
RGLAALQYLY LQDNNLQALP DNTFRDLGNL THLFLHGNRI PSVPEHAFRG 200
LHSLDRLLLH QNHVARVHPH AFRDLGRLMT LYLFANNLSM LPAEVLVPLR 250
SLQYLRLNDN PWVCDCRARP LWAWLQKFRG SSSEVPCNLP QRLAGRDLKR 300
LAASDLEGCA VASGPFRPFQ TNQLTDEELL GLPKCCQPDA ADKASVLEPG 350
RPASAGNALK GRVPPGDTPP GNGSGPRHIN DSPFGTLPGS AEPPLTALRP 400
GGSEPPGLPT TGPRRRPGCS RKNRTRSHCR LGQAGSGSSG TGDAEGSGAL 450
PALACSLAPL GLALVLWTVL GPC                               473
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Appendix ii

DEPARTMENT OF HUMAN BIOLOGY

IMMUNISATION SCHEDULE CHART

Project No: **Fund No:** **Animal species/breed:** Rabbits/New Zealand white

Name of synthetic peptide: Nyatia-1 (12576) **Peptide sequence:** SLQYLRLN

Animal Identification: 452F675926

Date	Bleed date	Amt. bled (ml) /Bleed type-No.	Injection No.	Peptide (ml) / Incomplete Adjuvant(ml)
28-06-05		5/ Pre-bleed		
			1st	(0.5/0.5)
12-07-05	12-07-05	10/1	2nd	(0.5/0.5)
26-07-05	26-07-05	10/2	3rd	(0.5/0.5)
08-08-05	08-08-05	10/3	4th	(0.5/0.5)
23-08-05	23-08-05	10/4	5th	(0.5/0.5)
	01-09-05	10/5		
	06-09-05	10/6		

Animal Identification: 4530010111

Date	Bleed date	Amt. bled (ml) /Bleed type-No.	Injection No.	Peptide (ml) / Incomplete Adjuvant(ml)
28-06-05		5/ Pre-bleed		
			1st	(0.5/0.5)
12-07-05	12-07-05	10/1	2nd	(0.5/0.5)
26-07-05	26-07-05	10/2	3rd	(0.5/0.5)
08-08-05	08-08-05	10/3	4th	(0.5/0.5)
23-08-05	23-08-05	10/4		
	01-09-05	10/5		
	06-09-05	10/6		