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Localisation and Expression of a Myelin Associated Neurite Inhibitor, Nogo-A and its Receptor Nogo-Receptor by Mammalian CNS Cells

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Abstract: Axon regeneration failure in the adult mammalian Central Nervous System (CNS) is partly due to inhibitory molecules associated with myelin. The Nogo receptor (NgR) 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 (MAG) and oligodendrocyte myelin glycoprotein (OMgp). The major aim of the study was to investigate and explore the cellular localisation and expression pattern of NgR and Nogo-A in the mammalian nervous system. We therefore generated a rabbit polyclonal anti-NgR antibody from the leucine rich repeat (LRR) No. 9 domain of the NgR polypeptide chain. Together with a commercially available polyclonal antibody specific for NgR and in conjunction with double labeling immunofluorescence methods on cryosections and cell cultures, NgR immunoreactivity was observed in the CNS and Dorsal Root Ganglia (DRG). In cellular populations, it was confined to neuronal cell bodies and their processes. NgR was also localised on the surface of extending DRG intact axons and growth cones in live staining experiments. Nogo-A, a member of the reticulon family protein, was widely distributed in the mammalian brain, spinal cord and DRG. Intense Nogo-A immunoreactivity was also detected in oligodendrocyte cell bodies and their myelin sheaths in nerve fibre tracts of the CNS. 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. 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 NgR interacts with Nogo-A, as the latter appears to be confined to the cytoplasm and can therefore not be expected to bind NgR on the axon surface.

Key words: Myelin associated, neurite inhibitor, nogo-A, nogo-receptor, CNS cells

INTRODUCTION

Axonal growth is mediated by specialized structures on axon tips known as growth cones, which give off numerous finger-like projections (filopodia) to scan the environment. These filopodia allow the nascent axon to respond to the external cues that guide it to the ultimate target[1]. Axons in the 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 and where growth is promoted by the environment created by the presence of Schwann cells[2]. 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[3]. 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[4]. In frogs, axons from the optic nerve and tectum do regenerate[5]. However, CNS axons fail to regenerate in the spinal cord after metamorphosis[6,7]. The inability of regeneration correlates with the non permissive properties of spinal cord myelin and oligodendrocytes in co-cultures with axons,

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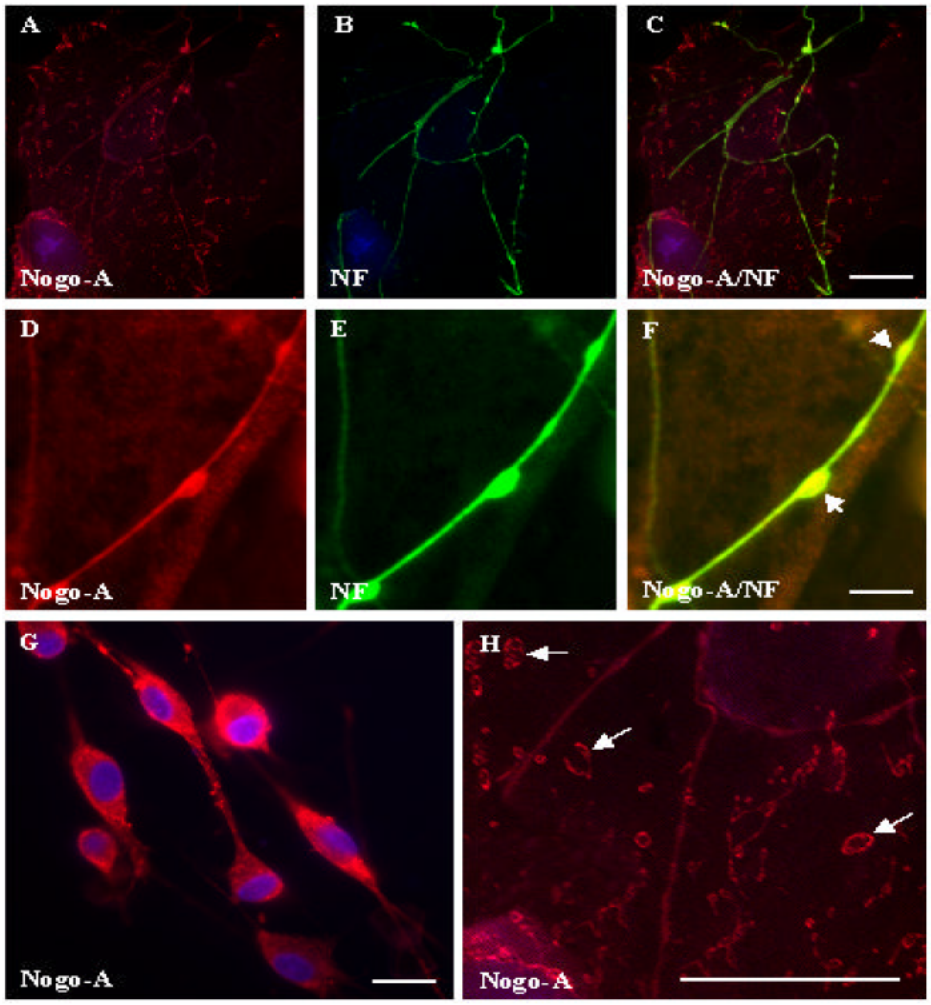

Fig. 1: Immunofluorescence microscope images of neonate Wistar rat Dorsal Root Ganglion (DRG) axons, Fibroblasts and Schwann cells in culture (A-F). Axons immunoreacted to Nogo-A (red) as shown by counterstaining with neurofilament (NF) protein (green) which is an axonal marker. Nogo-A is concentrated 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), this is more comprehensible at higher magnification in (H). 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-H; 10 μm)

indicating the expression of inhibitory proteins in this region[8,5].

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.

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[9-11].

The other most important inhibitors are broadly distributed in the myelin that ensheaths axons in white matter tracts on the adult CNS; namely MAG, Nogo and OMgp[12,13]. As a protein expressed by oligodendrocytes and axonal processes in mammalian brain and spinal cord tissue (Fig. 1and 2), Nogo exists in three different

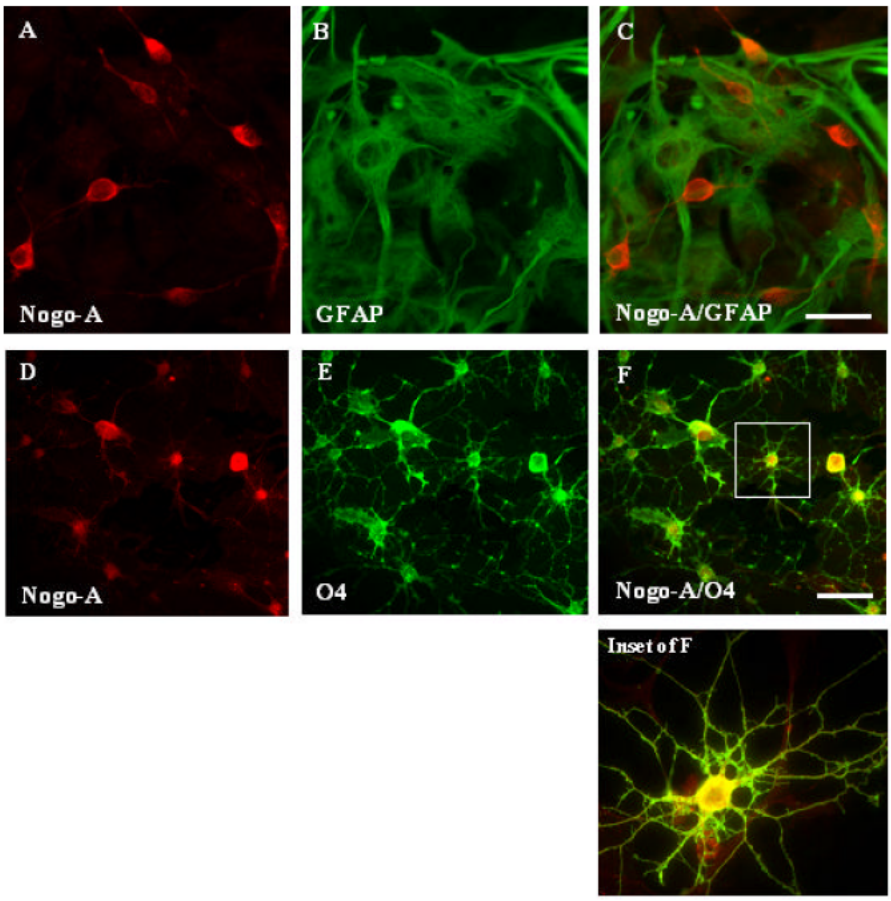

Fig. 2: Immunofluorescence microscope images of neonate Wistar rat CNS glia in culture. In a primary glial cell culture, oligodendrocytes (A) not astrocytes (B) express Nogo-A. On merging images A and B, oligodendrocytes can be distinctly seen in layers of astrocytes expressing GFAP (C).D-F; In an enriched culture supplemented with triiodothyronine (T3), the existence of oligodendrocytes was confirmed by immunoreactivity with O4 antibody (E). Note the intracellular localization of Nogo-A when compared to O4 which is on the oligodendrocyte cell membrane surface (insert). Scale bars : (A-C; 25 μm, D-F; 25 μm)

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 Nogo proteins differ in length but share a common 188-amino acid-long C-terminus[14] consisting of two potentially membrane-spanning hydrophobic domains separated by a hydrophilic segment of 66 amino acids, termed Nogo-66. 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 (ER)-localised protein with unknown function[15-17]. Nogo proteins, have presumed transmembrane regions; 35 and 36 amino acids (a.a) each respectively, long enough to span the membrane twice[14,18]. In between the two transmembrane domains, the 66 amino acid loop has been found to bind the NgR subunit[19]. 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. 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. However, the localisation and topology of Nogo-A on plasma membranes has not been clear up to now. Some of our results have shown localisation of Nogo-A in structures with similar morphology to ER in fibroblasts in culture (Fig. 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. Grand Pŕe *et al.*[18] 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. This proposed type of orientation could not be further substantiated in non transfected cells because experiments done in our laboratory illustrated failure by Nogo-A antibody to bind to antigen on the cell surface of unpermeabilized oligodendrocytes and Dorsal Root Ganglion (DRG) axons in culture from rat species.

Nogo-A activities on the axon are accomplished by NgR[19]. A 473 a.a leucine rich repeat (LRR), Glycosylphosphatidylinositol (GPI)-linked protein with high affinity for Nogo-66, appears to play a pivotal role in conveying inhibitory signals from myelin-associated proteins to neurones of the CNS. It binds MAG, OMgp with similar affinity as Nogo-A, and mediates inhibition of axonal extensions in vitro and in vivo through a neurotrophin co-receptor named p75. Fournier *et al.*[19,18,3,13].

The main objective of the study was to develop a synthetic peptide from mammalian and amphibian NgR sequences, inoculate rabbits to raise antisera followed by characterising the antibody in our laboratory. Secoundly, investigate the cellular localisation of NgR and its ligand Nogo A, elucidate the kind of cells that express the receptor. 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.

MATERIALS AND METHODS

ANIMALS

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.

Rabbits: Two New Zealand white (NZW) rabbits were used in our studies to raise antibodies against NgR synthetic peptide by immunization. They were housed and provided by the Animal unit at UCT.

All procedures involving lab animals were in compliance with protocols approved by the Ethics Committee of the Health Sciences Faculty and were in accordance with ethical guidelines in accordance with the rules and regulations of the University.

Production of antisera

Antigen preparation: Electronic blast searches on conserved regions of the NgR protein were performed to uncover a peptide sequence that is immunogenic and highly conserved in both mammalian and amphibian species. The sequence (SLQYLRLN) corresponding to amino acid 251-258 of LRR region 9 was identified and later used to design a KLH conjugated synthetic peptide (Nyatia-1) by Alpha Diagnostic International (ADALI), USA.

Rabbit antisera: Rabbits (2) of 10 weeks old were initially immunized with a Subcutaneous (SC) injection of 0.5ml peptide at 1 mg mL^{-1} concentration +0.5 mL Freuds 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.

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. 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 2000 rpm for 5 min 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.

TISSUE CULTURE

Equipment: Cells were cultured on various tissue culture equipment, depending on the primary objective of the experiment. The equipment included clean 16mm, 1.5 mg mL^{-1} polylysine / laminin coated glass cover-slips (Marienfield, Germany), small, medium and large sized culture flasks (Greiner bio-one, Germany).

Laboratory culture medium used depended on particular cells being cultured.

DRG culture: Dulbecco's Modified Eagle's Medium (DMEM, Highveld Biological (Pty) Ltd), 1% Foetal Calf Serum (FCS) (Highveld Biological (Pty) Ltd), L-glutamate 100 mg mL^{-1}, Nerve growth factor (NGF), 100 I.U each of penicillin and streptomycin.

Primary glial cell culture: DMEM, 10% FCS, L-glutamate 100 mg mL^{-1}, 100 I.U each of penicillin and streptomycin.

Enriched oligodendrocyte culture: DMEM, 10% FCS, L-glutamate 100 mg mL^{-1}, 100 I.U each of penicillin and streptomycin.

PC-12 cells differentiation stage: DMEM, 1% FCS, 1 μg mL insulin transferrin, forskilin (1:1000), L-glutamate 100 g mL, 100 I.U each of penicillin and streptomycin.

Micro-dissection and tissue preparation

DRG cultures: Pregnant female rats were sacrificed and the embryos obtained at E15 stage of development. Under the dissection microscope (Zeiss), together with decapitated P1 rats and using fine iris forceps and scissors, fleshy tissue was removed from the vertebral column dorsally, so as to expose the spinal cord. Stripping out the spinal cord, resulted in the DRG bulbs being easily visible in between transverse processes of adjacent vertebrae. The DRG bulbs 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 1.5 mg mL^{-1} polylysine/laminin coated cover-slips. They were later placed in a 37°C/5% CO_2 incubator for 2-4 weeks.

Primary glial cell cultures: Rats were processed as in the procedure above; fleshy tissue was stripped off the vertebral column dorsally, so as to expose the spinal cord. Whole brain was recovered from the cranium by splitting open the skull. Both brain and spinal cord tissues were placed in the respective culture 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.

Fluorescent Immuno Histo Chemistry (FIHC)

Antibodies and fluorescent markers: Antibodies used in this study were; Nogo-A (ADALI), a rabbit polyclonal that recognises the 66 amino acid extra cellular domain of Nogo. Nogo receptor (ADALI), 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. Neurofillament (NF) Santa Cruz, a monoclonal antibody. Flotillin/Epidermal Surface Antigen (ESA) (BD) a monoclonal antibody. Proteolipid Protein (PLP) a monoclonal antibody that recognizes compact myelin. Glial Fibrillary Acidic Protein (GFAP), an astrocytic marker. The antibody O_4 marks a specific preoligodendrocyte stage of oligodendrocyte maturation. It reacts also with sulfatides and still unidentified glycolipids[20].

Cells: Culture medium was rinsed off the cells twice with PBS, pH 7.4 and cells were fixed in 4% paraformaldehyde in PBS for 5 min at 25°C and later in 100% methanol at -20°C for 5 min. 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 min at 25°C. They were then incubated at 4°C over night in appropriate primary antibodies diluted in blocking solution at varying concentrations. Nogo-A (1:1000); NgR (1:1000); Flotillin (1:200); O_4 (1:50); GFAP (1:1000). The primary antibodies were rinsed off and cells washed thrice in five minute changes of 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 h at 25°C. The secondary antibodies were later rinsed off thrice in five min 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 2.4 g Mowiol (Polyvinyl alcohol, Hoest) 6mL glycerol was added. While stirring, 6 mL of distilled water was added and left for several h at 25°C. Twelve millilitres of 0.2 M Tris (pH 8.5) was added and the solution incubated at 50°C for one h with occasional stirring. To reduce immunofluorescence fading, small amounts of n-propyl gallate (Sigma) was added and dissolved at 37?8C. The slides were stored in a dry environment for 24 h at 25°C. Viewing was carried out using a Zeiss Axiovert 200M Fluorescence microscope (Zeiss, Germany).

Tissue: Cryo-sections from brain and spinal cord were treated the same way like cell cultures, the only difference being exclusion of paraformaldehye while performing FIHC.

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 % resolving and stacking gel was set up following the manufacturers (BIO RAD). 20il 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 super market, 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 h. 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 h at 25°C while shaking. The blot was washed with four changes of PBS (5 min each) and then visualized using a chemluscence reagent, Super signal on a Kodak film.

RESULTS

Expression of Nogo-A

Nogo-A is expressed by Dorsal Root Ganglion (DRG) axons in culture: Immunohistochemistry studies demonstrated that neuronal cell bodies from foetal (E15) and neonate Wistar rat DRG explants and foetal (E15)

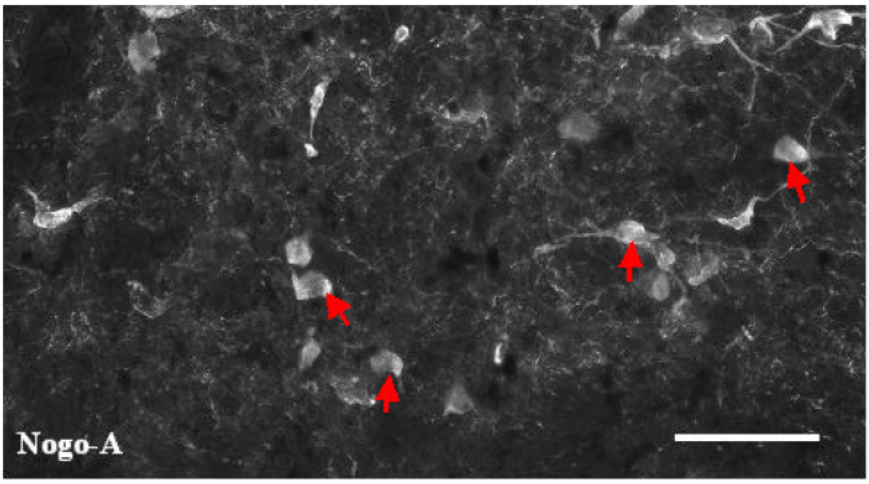

Fig. 3: Immunohistochemuistry for Nogo-A in adult wistar rat tissues: Forebrain, showing part of the deep layers of the cerebral cortex: In the striatum, neurons (shown by arrows heads) can be seen expressing high levels of Nogo-A. Scale bar: 10 μm

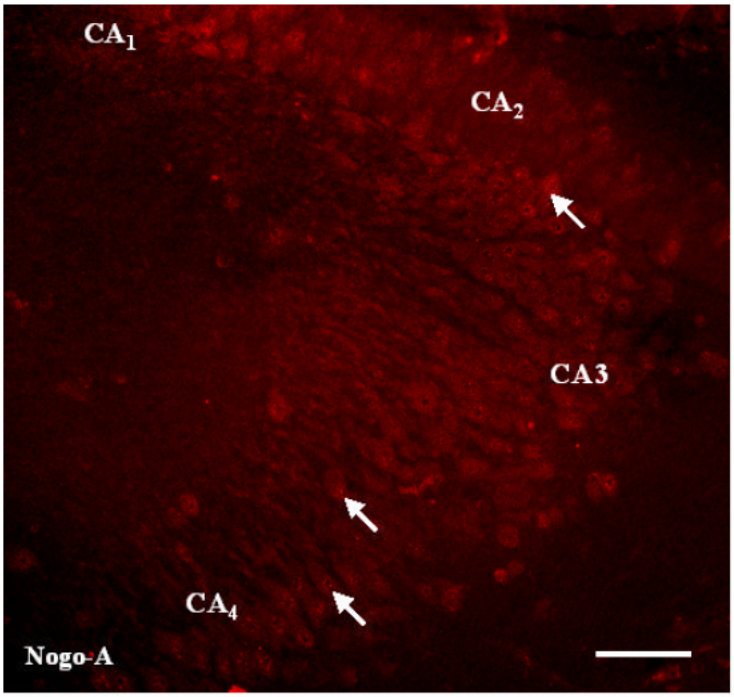

Fig. 4: Nogo-A immunoreactivity in the Wistar rat hippocampal formations: CA1-CA4. In the hippocampus, an area with numerous neurons, the cell bodies (shown by arrows) of these neurons exhibit strong Nogo-A expression (red). Scale bar: 5 μm

displayed similar, strong Nogo-A immunoreactivity. Their neurites, as revealed by neurofilament staining were also Nogo-A positive (Fig. 1), with the strongest expression being in axonal varicosities/synaptic buttons, often at branch points or at sites where axons crossed other neurites (Fig. 1d-f). Characteristic cytoplasmic Nogo-A immunoreactivity was also observed in structures that morphologically resembled Endoplasmic Reticulum (ER) in fibroblasts which form a substrate layer for the neurites and Schwann cells to grow (Fig. 1h). Schwann cells, which myelinate axons in the PNS also expressed considerable amounts of Nogo-A (Fig. 1g) comparable to oligodendrocytes.

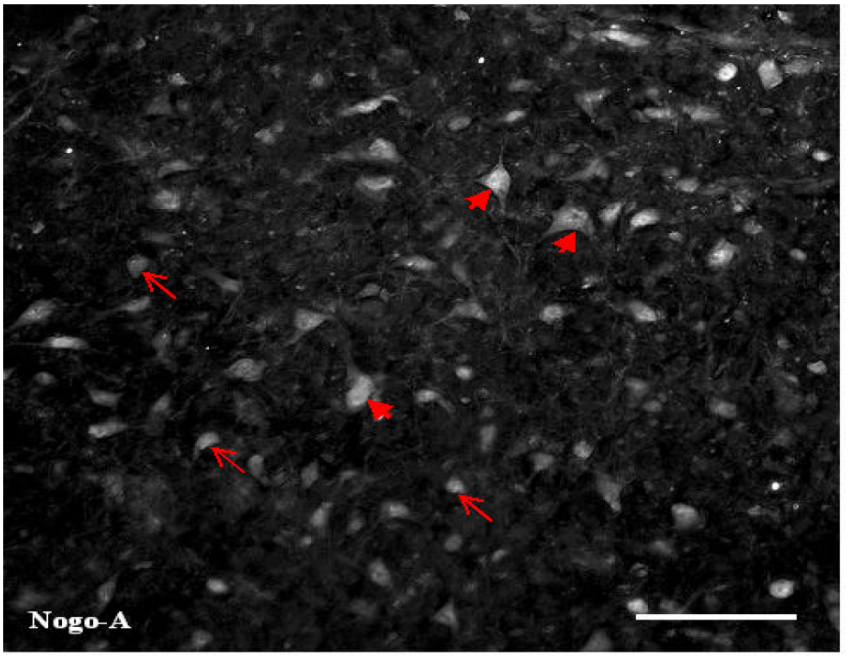

Fig. 5: Transverse section through the cerebral peduncle: Nogo-A immunoreactivity can be seen in neurons (arrow heads) and some presumptive oligodendrocytes (arrows). Scale bar: 10 µm

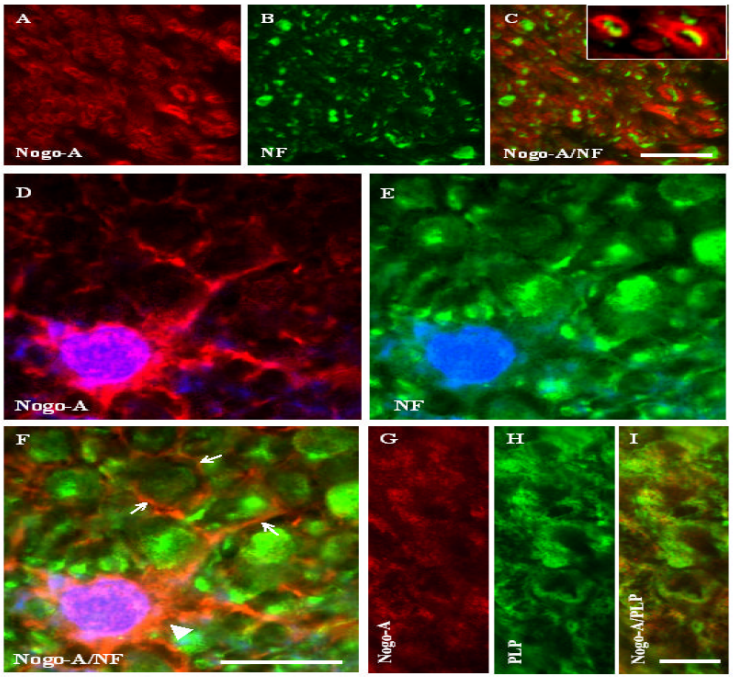

Fig. 6: Nogo-A immunofluorescence in adult Wistar rat brain white matter (A–C; G-I), and spinal cord white matter (d-f). A-C; In the caudate putamen, an area with numerous myelinated axonal bundles, Nogo-A (red) stains the peripheral regions encircling NF immunoreactive axons (green). Notice how Nogo-A 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)

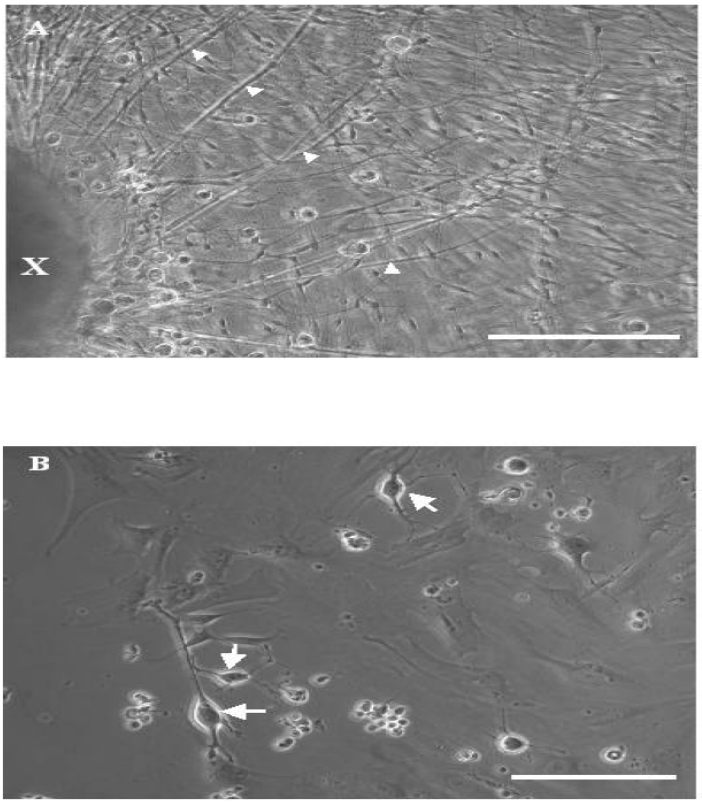

Fig. 7: Neonate Wistar rat Dorsal Root Ganglion (DRG) cell culture (Phase contrast): A; Numerous neurites (arrows) can be seen originating from a DRG explant (X), bridging over Schwann cells and fibroblasts. B; Neurons with their processes (arrow heads) at higher magnification can be identified by their morphology in culture. Scale bars : (A; 50 µm, B; 25µm)

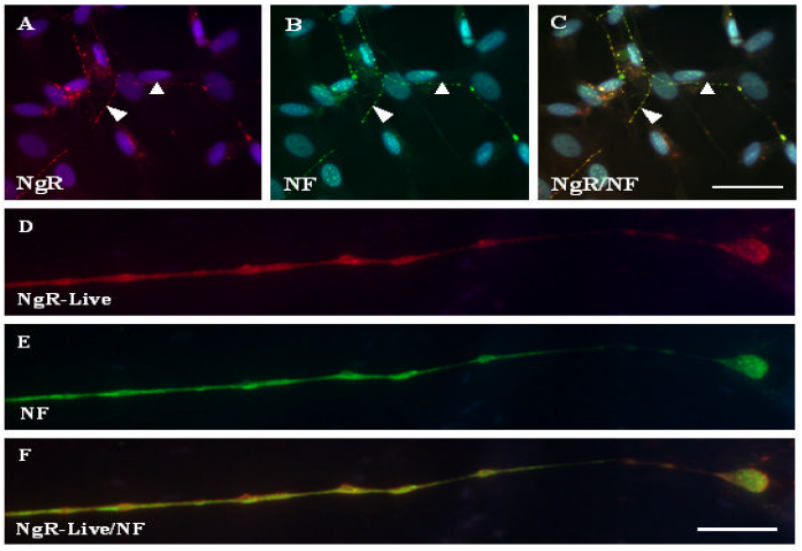

Fig. 8: Immunofluorescence microscope images of neonate Wistar rat Dorsal Root Ganglion (DRG) axons in culture (A-F). We do observe Anti NgR that bound on the axonal surface when incubated with DRG axons (arrow heads) for an hour at 370C in culture (live staining). At higher magnification the receptor 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). Neurofillament (green) stains axons. A nuclear stain, DAPI (blue) shows nuclei position(s) Scale bars: (A-C; 10 µm, D-F; 2.5 µm)

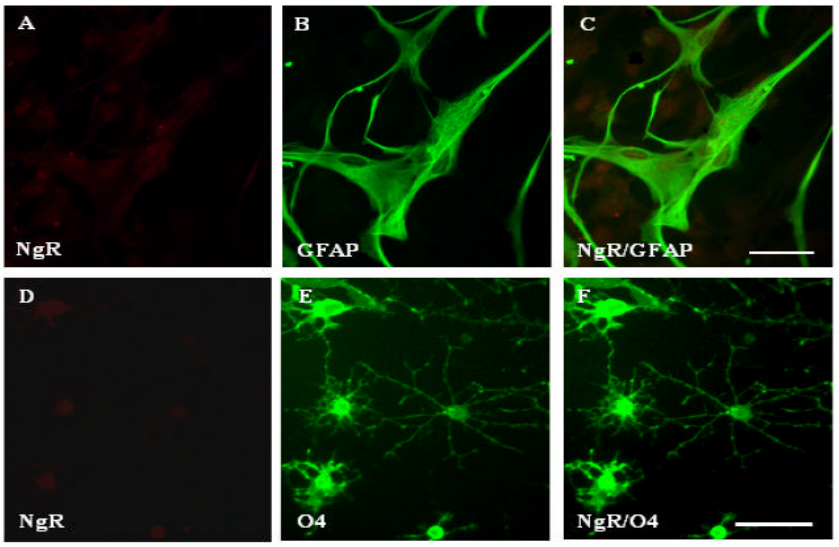

Fig. 9: Immunofluorescence microscope images of neonate Wistar rat astrocytes and oligodendrocytes in culture. A-C; In a primary CNS glial cell culture, astrocytes do not express NgR. D-F; In an enriched oligodendrocytes culture supplemented with triiodothyronine (T3), oligodendrocytes do express NgR either. (The existence of oligodendrocytes was confirmed by immunoreactivity with O4 antibody (E). Scale bars : (A-C; 25 μm, D-F; 25 μm)

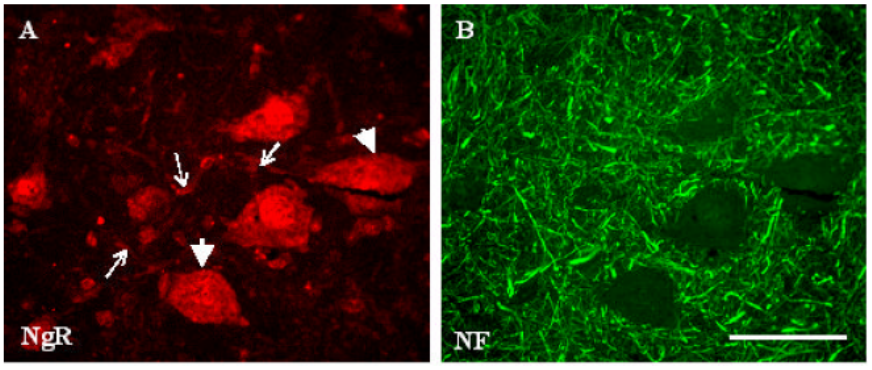

Fig. 10: Localisation of NgR in the gray matter of adult rat spinal cord (SC); Transverse sections were stained with NgR antibody (red), along side neurofilament antibody (green). Note the strong NgR staining in neuronal cell bodies (arrowhead) and axons/dendrites (arrows). Scale bars: (A and B; 20 μm)

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 co-localisation, demonstrating that Nogo-A is not present in astrocytes (Fig. 8a-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[32]. After dissociation, the oligodendrocytes were supplemented with Triodothryonine (T_3) so as to fully differentiate. To confirm that oligodendrocytes expressed Nogo-A, a double immune labelling showed co-localisation between Nogo-A and O4, an oligodendrocyte marker (Fig. 8d-f). However, Nogo-A protein immunoreactivity appeared more intracellular as compared to O4 which is expressed on the surface of oligodendrocytes (Fig. 8-inset). This observation can be substantiated by the failure to observe substantial yellow staining when the two flourochromes; Cy3 (red) representing anti Nogo-A antibody and Alexa 488 (green) representing anti O4 antibody are combined together during image analysis.

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

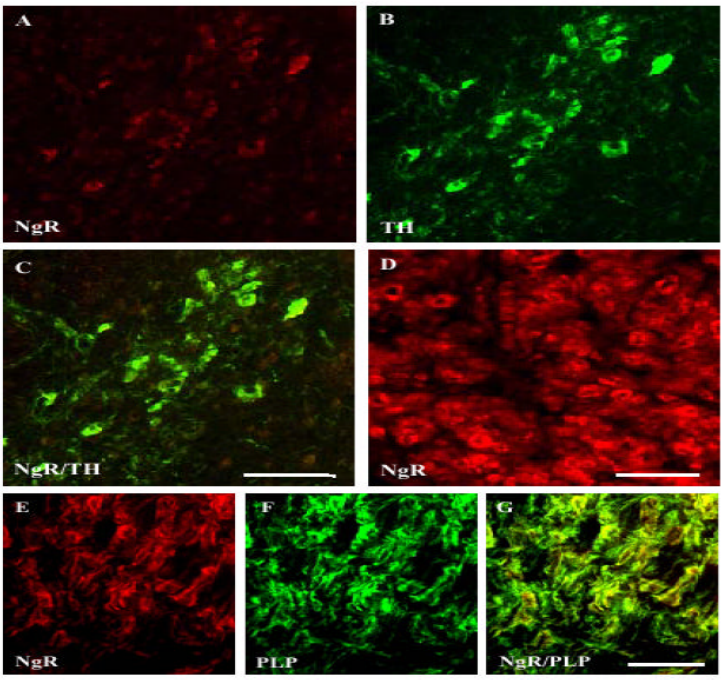

Fig. 11: NgR immunofluorescence in adult rat brain; substantia nigra (A–C) and caudate putamen (D–G). Neuronal cell bodies in the substantia nigra (pars compacta) expressed NgR. This was apparent on co-localization with anti Tyrosine Hydroxylase (TH) antibody which labels dopaminergic neurons (A-C). 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)

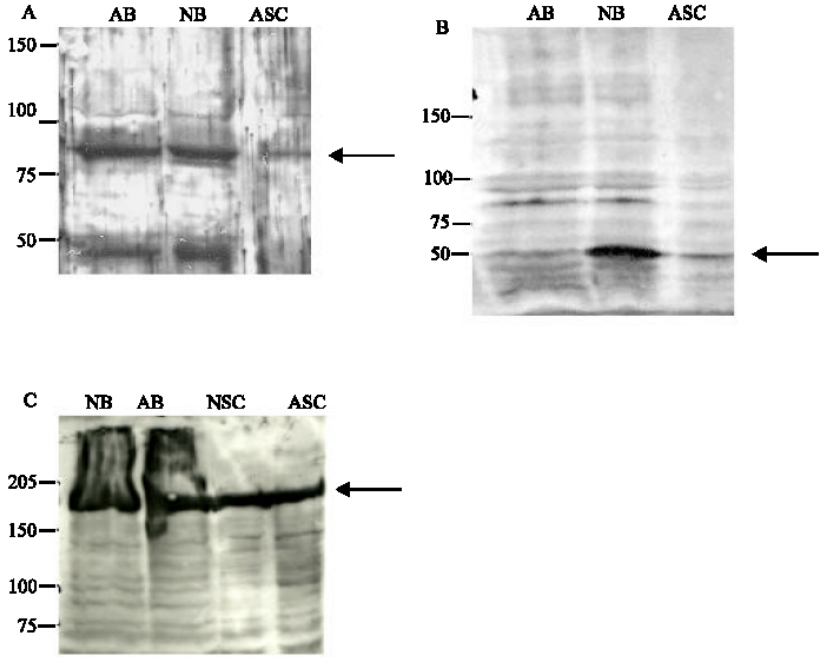

Fig. 12: Immunoblot of NgR and Nogo-A expression in rat tissues. Samples (20µl) from indicated tissues were analysed by anti-NgR (h) and anti-Nogo-A immunoblots. NB, neonate brain; AB, adult brain; ASC, adult spinal cord. The positive Nogo receptor band migrated at approximately 85 kDa, with low levels 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

of axon regeneration in mammalian CNS. 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 (data not shown). In adult rat neostriatum, large Nogo-A immunopositive neurons were found spread throughout (Fig. 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 (Fig. 11). In the cerebral peduncles, oligodendrocytes and axons were immunoreactive for Nogo-A (Fig. 10). 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 significant Nogo-A immunoreactivity was detectable in the granule cell and molecular layers (data not shown). 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 (Fig. 11a-c). These structures were later revealed to be myelin, as per proteolipid protein (PLP) staining which is a myelin marker (Fig. 11g-i). On a closer look, minor co-localisation of Nogo-A with Neurofilament (NF) antibody which stains axonal filaments was observed at myelin sheath-axon positions which are juxtaposed (Fig. 11c).

In adult rat spinal cord sections, neurons expressed Nogo-A with the highest levels in ventral motor neurons (data not shown). 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 (Fig. 11d). In cross section, distinctive staining encircling axons (represented by NF in green) leaving an unstained thin rim was observed at high power microscopy (Fig. 11f). Intense Nogo-A immunoreactivity was also observed in ependymal cells surrounding the lining of the central canal in the spinal cord (data not shown).

Expression of Nogo Receptor (NgR)

NgR expression in DRG cultures: Dorsal Root Ganglion (DRG) explants harvested from embryonic and neonate Wistar rats were placed in a separate petridish, 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 (Fig. 12) which grew short processes after reducing the FCS level to 1% in the DMEM medium. 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[19], 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 antibody prior to permeabilization and fixation at 37°C, results revealed presence of NgR immunoreactivity on the axonal membrane surface (Fig. 8). 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 (Fig. 8d-f). Nogo receptor immunoreactivity was also observed in fixed DRG axons (data not shown).

Neither oligodendrocytes, nor astrocytes express NgR in CNS glia 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 (O4) and astrocytes (GFAP) neither of the two cell types showed any immunoreactivity with NgR antibody (Fig. 9).

NgR expression in CNS tissue: To provide a detailed immunohistological analysis of NgR, an anti-peptide antibody was generated locally using rabbits and was later compared to the studies done with commercial NgR antibody (ADALI). An investigation of NgR expression in the brain and spinal cord of normal intact rat 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 (Fig. 10). In the brain, the substantia nigra (pars compacta)

region, which has a high concentration of dopaminergic neuronal cell bodies, demonstrated high immunoreactivity to NgR antibody (Fig. 11a-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 confocal microscopic analysis, NgR was more intense in myelinated compared to unmyelinated axons. This finding was corroborated by co-localisation with proteolipid protein (PLP), a protein present in compact myelin that insulates axons (Fig. 11e-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 and Nogo-A antibodies were investigated by immunoblotting. The home made 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 (Fig. 12a). Anti-Nogo-A antibody recognized a protein of Molecular Weight (MW) approximately 200 kDa from both neonate and adult rat CNS homogenate (Fig. 12b). Immunoblotting was later done on the pre-immune sera as a negative control for the NgR antisera. A band migrating at approximately 50 kDa in neonate brain tissue was observed (Fig. 12b. When compared to the commercially prepared NgR purified antibody (ADALI), the specific band migrates at about 85 kDa (data not shown) which is within range of the NgR antibody we raised in our laboratory at UCT.

The non specific bands at approximately 48 kDa and 50 kDa seen in the NgR 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.

DISCUSSION

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 outgrowth 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 (Fig. 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.

Astrocytes, which are the other major supporting cells (glia) of the CNS, did not express Nogo-A (Fig. 2a-c). Although injury prompts astrocytes to hypertrophy and reorganise to form the glial scar that forms a barrier to regeneration[21], 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[22]. They also participate in the formation of the blood brain barrier[33], ion homeostasis[23] as well as in the production of the extracellular matrix (Liesi and Silver).

Nogo-A protein was also found in neuronal cell bodies in CNS tissue, though with immunohistochemical staining with ant-Nogo-A antibody, there was slightly less intense staining compared to what oligodendrocyte cells. And since the two cell types are dissimilar in morphology, size and function, it is quite difficult to compare the staining intensity adequately.

Although in the PNS, Schwann cells are not known to be hostile to regenerating axons compared to oligodendrocytes[2], these myelinating cells of the PNS immunoreacted with Nogo-A antibody in culture (Fig. 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 (Fig. 1a). Although fibroblasts are not primarily nervous tissue cells and the fact that Nogo-A has been reported present in other tissues like skeletal muscle[24], these findings of Nogo-A in other cells and structures not associated with neurite outgrowth inhibition may link Nogo-A to yet unidentified second function. The nature of reticulon proteins remains obscure, but by virtue of their intracellular localisation, these proteins are thought to participate in endoplasmic reticulum regulation[15,18]. 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[25].

NgR expression

Nogo receptor antibody design and production: Nogo receptor (NgR) was first identified by Fournier *et al.*[19] 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, 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 Freuds 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. This is particularly important as far as NgR protein is concerned since it binds three different ligands (Nogo-A, MAG and OMgp) and the precise binding sites for the three distinct ligands have not yet been defined. In addition, polyclonal reagents are relatively simple and cheap to produce in the short term compared with monoclonal reagents. Furthermore, the use of larger animals (such as horses, goats and rabbits) enables the recovery of a large volume (e.g., 60 mL from a rabbit) of antibody rich serum. Successive NgR anti-sera was collected and tested. There was a remarkable gradual improvement in specificity from the 1st to the 6th bleed as per immunohistochemistry studies.

NgR immune sera: The NgR band observed with the home made antisera in neonate and adult brain at approximately 85kDa on a western blot (Fig. 12a) is consistent with what other researchers have found before. 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 not be accounted for. It could probably be as a result of using non purified sera. Overall the immunoblot was not clean enough and still this could be as a result of using unpurified sera.

Pre-immune sera: This was serum collected from rabbits before immunization with the synthetic peptide was performed. It was used as a negative control alongside the NgR antisera each time IHC and western blot experiments were performed. Astonishingly pre-immune sera presented related though not exact immunoreactivity like the NgR 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[26]. 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 antibody. Nevertheless, a supposed non specific band at 50 kDa with the pre-immune sera was observed which cannot be clearly explained.

NgR expression in the CNS tissue: Failure to detect NgR immunoreactivity in oligodendrocytes and astrocytes (Fig. 9) 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 colocalisation 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 colocalisation 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.*[13,24], 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 distribution that it may receive a Nogo-A signal. This kind of localisation by these two proteins supports the concept that NgR interaction with Nogo-A limits axonal regeneration after injury.

NgR expression in the DRG cultures: By physically showing presence of NgR on the axonal surface on an

intact membrane corroborates functional studies that have been done by Fournier *et al.*[19]. Again these observations position the receptor on the membrane surface to enable it mediate activities of Nogo, MAG and OMgp, which are recognized myelin associated neurite outgrowth inhibitors. Since growth cones are characteristic of active path-finding axons in DRG cultures, a number of axonal growth ends in the DRG cultures, which resemble growth cones were identified. Growth cones contain the machinery that powers axon elongation and also receive and interpret the extra cellular cues that the regenerating axons encounter. Nogo receptor immunoreactivity was more concentrated at the periphery of growth cones when viewed at higher magnification (Fig. 8f). 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[27]. 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[28]. It's not exactly known how Rho regulates cytoskeletal elements to stop neurite outgrowth. 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[27]. 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[13].

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[29]. Although Fournier *et al.*[19] had shown how treatment with PI-PLC renders neurons functionally insensitive to NgR, It was fundamental to demonstrate the effect physically by immunohistochemistry. However, significant anti-NgR antibody staining could still be observed even after thorough washing 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[13,30].

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 outgrowth inhibition. First would be to access 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 non in DRG or retinal neurons of earlier embryonic stages, which are not responsive[31].

Antibodies to a cell-surface receptor like NgR cans 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 outgrowth inhibition in the CNS. Further more if NgR 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 incubating 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. The approaches discussed above provide a powerful criterion for studying the function of antibodies.

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