The Expression and Functional Analysis of Neurite Outgrowth Inhibitors in the Nervous System of *Xenopus Laevis*

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Thesis Presented for the Degree of

Doctor of Philosophy

in the Department of Human Biology

University of Cape Town

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February 2007

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DECLARATION

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ACKNOWLEDGEMENTS

I am grateful to have such a wonderful supervisor, Dr Dirk Lang. Without his guidance, encouragement and patience, this work would not have been possible. I appreciate the liberty he has entrusted me with for the project development. He is also very understanding who listens and supports through the worst of times. It is truly a privilege to work with him.

I would like to thank the head of the department, Prof Sue Kidson, and a few Human Biology staff members Mrs Toni Wiggins, Mrs Liz van der Merwe, Mr Bruce Dando, Mr Charles Pelston and especially to Mrs Barbara Mohr. They have provided endless technical support, advices and making the working environment enjoyable. My gratitude also goes to the pass and present Human Biology postgraduate students in Kidson's lab: Dr Thandi Mgwebi, Dr Paula Sommer, Purity Macheru, Dheshnie Govender; and my colleagues in Dirk's lab: Ruth McBride, Dr Edward Nyatia, Bryony Dobson and Regina Lindau for their invaluable friendships and expertise.

I would also like to acknowledge the Medical Research Council (MRC) and the University of Cape Town (UCT) for providing financial support in parts of this study. International Brain Research Organization (IBRO), UCT and the department of Human Biology have provided stipends so that I had the opportunities to attend conferences in South Africa and aboard.

My affection goes to my parents, my brother, and family of Lee for their unconditional support. My sincere gratitude goes to my friends, Crystal, Tian, and especially Tzu-Ting Lee and David Chu for having me and cheering me in wine and braai when there is up-and-down. Mostly, I am deeply grateful to my best friend, Dr Tzu-Dai Yeh, who supports me with love and care, without you, this would not be possible. It is greatly appreciated.

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PUBLICATIONS

Full Length Paper

Pesheva, P., Probstmeier, R., Lang, D.M., McBride, R., Hsu, N.J., Gennarini, G., Spiess, E. and Peshev' Z. (2006). Early coevolution of adhesive but not antiadhesive tenascin-R ligand-receptor pairs in vertebrates: a phylogenetic study. *Mol. Cel. Neurosci.* **32**: 366-386.

Hsu, N.J., Dobson, B. and Lang, D.M. Nogo-A/RNT4-A expression in the intact and injured nervous system of *Xenopus laevis. J. Neurobiol.* (Submitted)

Poster Presentation in International Conferences

Hsu, N.J., Lang, D.M., Pesheva, P. and Schwab, M.E. (2001). The expression of neurite outgrowth inhibitory proteins in the amphibian central nervous system.
At the XVIth International Symposium on Morphological Sciences in Sun City, RSA

Hsu, N.J. and Lang, D.M. (2003). Expression of Nogo-A in the amphibian central nervous system.
At the VI European Meeting on Glial Cell Function in Health and Disease in Berlin, Germany

Hsu, N.J. and Lang, D.M. (2003). Expression of Nogo-A in the amphibian central nervous system.
At the Spier Psychopharmacology and Neurosciences Congress 2003 in Stellenbosch, RSA

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LIST OF ABBREVIATIONS

APS	ammonium persulphate
APTES	3-aminopropyltriethoxysilane
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	cyclic AMP
CNS	central nervous system
CSPG	chodroitin sulphate proteoglycan
Cy-3	cyanin-3
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCC	deleted in colorectal cancer
DMSO	dimethyl sulphoxide
DRG	dorsal root ganglion
ECL	enhanced chemiluminescence
ECM	extracullular matrix
EGF	epidermal growth factor
RHD	reticulon-homology domain
ER	endoplasmic reticulum
FG	fibrinogen
FN	fibronectin
GAP-43	growth-associated protein 43kD
GFAP	glial fibrillary acidic protein
GPI	glycosylphosphatidyl-inositol
GTPase	guanosine triphosphatase

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IgSF	immunoglobulin superfamily
kD	kilodalton
LRR	leucine-rich-repeat
LRRCT	LRR C-terminal
mAB	monoclonal antibody
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
mg	milligram
ml	millilitre
mM	millimolar
N-CAM	neural cell adhesion molecule
NF	neurofilament
NgR	Nogo-66 receptor
OMgp	oligodendrocyte myelin glycoprotein
ON	optic nerve
PBS	phosphate buffered saline
PFA	paraformaldehyde
PNS	peripheral nervous system
RAM-HRP	rabbit-anti-mouse horse-radish peroxidase
RGC	retinal ganglion cell
RT	room temperature
Rtn	reticulon
SAR-HRP	swine-anti-rabbit horse-radish peroxidase
SC	spinal cord

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SDS	sodium dodecyl sulphate
TEMED	N, N, N',N-tetramethylethylenediamine
TM	transmembrane
TN-R	Tenascin-R
µg	microgram
µl	microlitre
V	volt

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ABSTRACT

Neurons of mammalian central nervous system (CNS) fail to regenerate their axons after injury, leading to permanent loss of function. In contrast, axotomised CNS neurons in fish, amphibians and some reptile species can perform functional axon regeneration after injury. Generally, the factors contributing to success or failure of axon regeneration lie in the intrinsic properties of injured neurons, as well as the surrounding microenvironment of the transected axon. Mammalian neurons may lack the intrinsic ability to survive after trauma, or to re-express genes required for axonal regrowth. Moreover, several proteins inhibitory to neurite growth, such as Tenascin-R (TN-R) and Nogo-A, have been identified in mammals. These proteins are associated with oligodendrocytes and myelin and are considered major inhibitory components of the CNS microenvironment.

Anuran amphibians, e.g. *Xenopus,* present a particularly interesting model for CNS axon regeneration: in tadpoles, the optic nerve (ON) and spinal cord (SC) are regenerating successfully, while axon regeneration only occurs in the ON, but not the SC of frogs after metamorphosis. In this study, the distribution and functional aspects of TN-R and Nogo-A were analysed in *Xenopus* frogs and tadpoles by means of immunological detection methods and *in vitro* functional assays.

Western blots and immunohistochemical results revealed that Nogo-A and TN-R are present in tadpole as well as post-metamorphic *Xenopus* ON and SC tissues. Using double-labelling techniques with cell-type specific marker antibodies, both proteins were found to be associated with oligodendrocytes and CNS myelin, as described in mammals. However, expression of Nogo-A was also found in the

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axons and somata of non-myelinated neurons throughout the CNS, where it was particularly prominent during development, as well in peripheral nervous tissues - thus diverging from the findings in mammals. These cell-type specific expression patterns of TN-R and Nogo-A were confirmed by immunocytochemical results obtained from *Xenopus* glial and neuronal cultures. Therefore, no direct correlation exists between levels of TN-R and Nogo-A expression on the one hand, and regenerative success in the different parts and developmental stages of the *Xenopus* CNS on the other.

Analysis of the post-lesion expression of TN-R and Nogo-A revealed no down-regulation of either protein, thus excluding the possibility that reduced levels or absence of these putative neurite growth inhibitors following injury could account for successful axon regeneration in *Xenopus*.

In vitro functional assays using purified TN-R or Nogo-A proteins showed that the mammalian homologues of these proteins are poor substrates for the growth of *Xenopus* neurons – demonstrating that the signaling pathways mediating growth inhibition by TN-R and Nogo-A are conserved. However, it is not possible to state currently whether *Xenopus* TN-R and Nogo-A are as inhibitory as their mammalian counterparts. Moreover, differences might reside in the intrinsic sensitivity of *Xenopus* neurons to neurite growth inhibitors and thus influence their regenerative capacity - highlighting the need for further studies to address these questions.

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Chapter 1

Introduction: Central Nervous System Regeneration

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1.1 General Introduction

The central nervous system (CNS) integrates and coordinates all nervous impulses from the body, as well as all impulses following stimuli originating outside the body. This complex function requires a highly organized and sophisticated structure. In higher vertebrates, damage of this structure leads to irreversible loss of functions due to its limited capability to regenerate. On the clinical level, the impaired CNS could lead to paraplegia or blindness, if the injury occurs in spinal cord (SC) or optic nerve (ON) respectively.

In the peripheral nervous system (PNS), the axonal injury leads to the Wallerian degeneration, which is a series rapid breakdown and phagocytosis of the nervous tissue and myelin sheath by macrophages and Schwann cells. Following Wallerian degeneration, Schwann cells in the distal nerve stump de-differentiate, proliferate, align and express molecules to guide and promote axonal regeneration. Different phases can be distinguished in the regenerative response: sprouting of the proximal axon stump, axon elongation, target innervations and reformation of functional synapses (Fig. 1.1A).

However, CNS responds differently to injury (Fig. 1.1B). Degenerated tissue is removed mostly and slowly by the endogenous microglia. The microglia then become activated to form macrophages that are responsible for the phagocytosis of the debris. Following degeneration, the axonal regeneration in the CNS is mechanically and physiologically unfavorable.

The reasons for the difference between CNS and PNS regenerative abilities in mammals could be (i) due to changes in the glial environment, or (ii) changes in the neurons and axons themselves, which make them unable to react to injury with a substantial regenerative response. Thus, identifying the factors that contribute to the changes after nerve lesion will lead to a better understanding of axonal regeneration.

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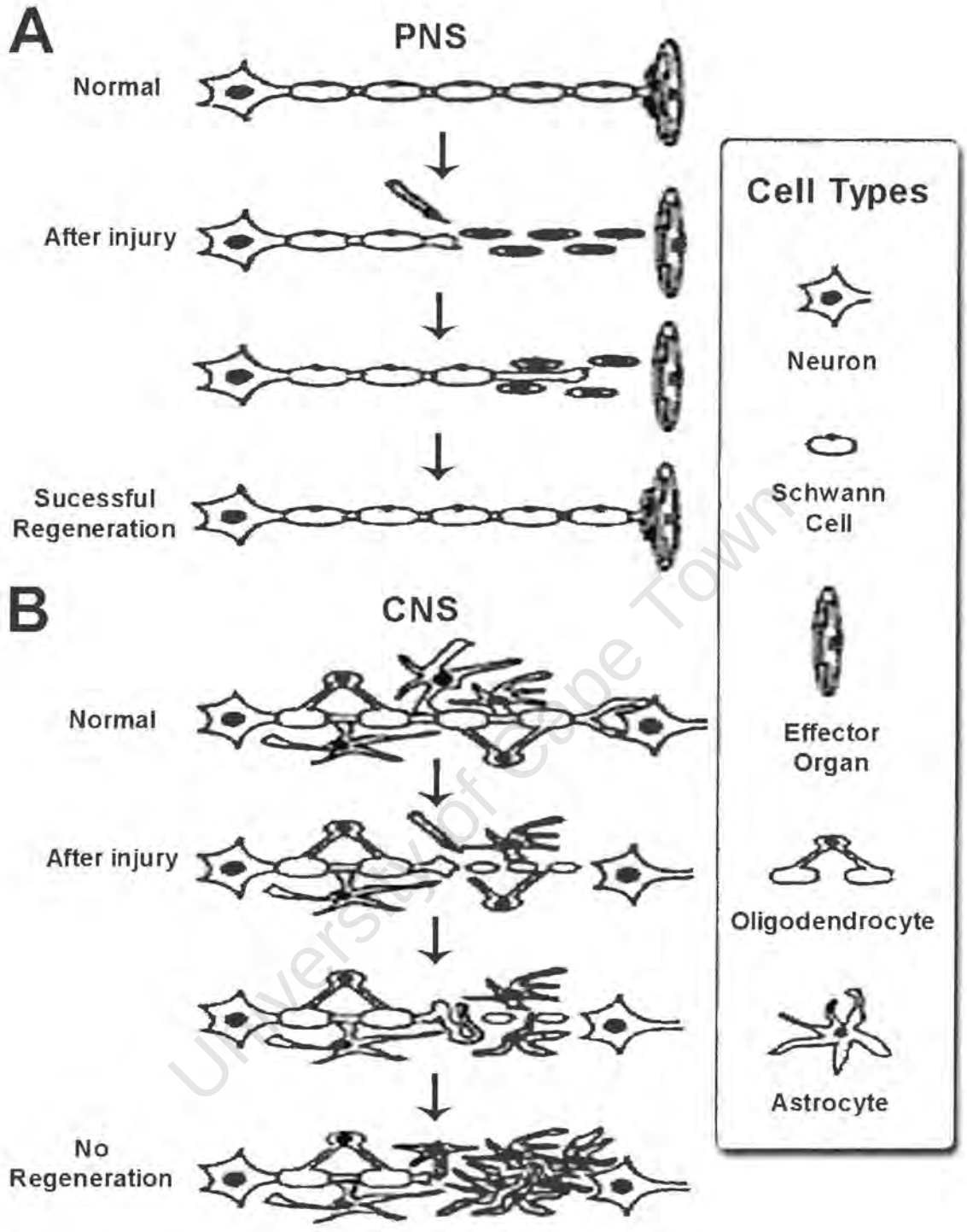

Figure 1.1. Schematic diagram representing the main cellular reactions in the events of PNS and CNS injury. **(A)** In the PNS, after injury, there is sprouting from the proximal axon stump, and the Schwann cells lines up forming channels at the distal nerve stump. Guided by Schwann cells, the regenerating axons reach the effecter organ and reconnect the synapses, thus complete the regeneration process. **(B)** In the CNS, active astrocytes invade the site of injury and form a dense scar. The transected axon fail to grow across and reform the connection, thus leads to the unsuccessful regeneration.

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1.2 Factors Contributing to the Axonal Regeneration

A CNS lesion triggers metabolic and morphological changes in the axotomized neurons and their immediate or subsequent surrounding microenvironment. However, before axonal regeneration can commence, the injured neurons must first be able to survive after lesion. The intrinsic ability of neuronal growth should then be re-established during development. Secondly after regeneration commences, the extrinsic factors in the surrounding environment can alter the result of regeneration in such a way that when a favorable substrate is provided, growing axons can elongate through an obstacle-free environment. Lastly, the guidance cues are crucial to the growth cone pathfinding and making appropriate contact with their targets so the functional connection can be restored (for reviews, see Bregman, 1998; Schwab and Bartholdi, 1996).

Accordingly, the following factors are likely to be the major influences on the axonal regenerating capacity: (i) neuronal survival, (ii) intrinsic property of axon growth, (iii) extrinsic environmental factors, and (iv) the molecular guidance of axon outgrowth.

1.2.1 Neuronal Survival

In the lesioned CNS, necrosis can lead to massive neuronal death. The initial wave of necrotic cell death may be followed by a wave of apoptotic cell death. Apoptosis, or programmed cell death plays a key role in the developing nervous system, and also contributes to cell death after CNS injury (Liu et al., 1997). Apoptotic cell death is mediated by either gene expression or regulation of proteins already present in the cell, such as the pro and anti- apoptotic proteins. The injured neurons after axotomy can be protected from the apoptotic death by the expression of anti-apoptotic proteins, such as Bcl-2 (Bonfanti et al., 1996). However, the anti-apoptotic activity of Bcl-2 may be altered by proteins other than known apoptotic regulators. For example, overexpression of Nogo-B (also know as RTN-X, a member of reticulon family) could sequester the Bcl-2

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members on the endoplasmic reticulum membrane, thereby reducing their anti-apoptotic function (Li et al., 2001).

The survival of neurons also depends on the continuous inhibition of apoptosis by trophic signals from the environment. During development, neurons compete for substances called neurotrophic factors that are supplied by target cells. The nerve cells that do not make proper connections with the target cells die by apoptosis (for review, see Goldberg and Barres, 2000; Bähr, 2000).

In addition, Schwann cells are capable of secreting a variety of neurotrophic factors to promote neuronal survival after PNS injury (for review, see Goldberg and Barres, 2000). However, it is uncertain whether CNS glia play a similar role to promote the survival of an injured neuron. Thus, blocking intrinsic cell suicide programs by the supply of neurotrophic stimuli might allow some cells to survive the initial trauma of CNS injury. In fact, studies have indicated that neurotrophic factors play a role in helping the survival of injured CNS by preventing the atrophy of axotomized neurons, and consequently enhancing the ability to regenerate (Kobayashi et al., 1997; for review, see Goldberg and Barres, 2000).

1.2.2 Intrinsic Property of Axonal Growth

Successful axonal regeneration requires axon growth. The axotomized neurons need to be induced or signaled to extend their axons by the re-expression of proteins, which are responsible for the formation of neural network in embryonic and early post-natal life. Several molecules present during development have been identified and shown to govern the neuronal regenerative response to axotomy. Within these molecules, a number of cell surface proteins directly mediate interactions with others in the surrounding environment of the axon, such as growth-associated protein 43kD (GAP-43) and a variety of cell adhesion molecules (CAMs).

Although the function of GAP-43 is poorly understood at present, the expression of GAP-43 is found on the inside of the growth cone membrane. It is highly

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expressed in PNS neurons as well as in the developing CNS. Therefore, GAP-43 is a well known marker of axonal growth. In response to axonal lesions, re-expression of high GAP-43 levels that correlated with successful regeneration was observed (Becker et al., 1998). Stimulation or overexpression of GAP-43 expression at the lesion site promoted axonal sprouting and regeneration (Aigner et al., 1995; Kobayashi et al., 1997).

Homophilic – self binding
Hetrophilic – binding to other molecules

Another example of growth-associated surface proteins is a group of cell adhesion molecules (CAMs) of immunoglobulin superfamily (IgSF). These CAMs capable of both homophilic and hetrophilic binding play an important role in the development of the PNS and CNS. They function in axon outgrowth, elongation and fasciculation. The expressions of cell adhesion molecules such as NCAM (neural cell adhesion molecule), L1 and N-cadherin have been shown to correlate with axonal growth after axotomy (Becker et al., 1998; Roonprapunt et al., 2003). Among these CAMs, L1 in particularly, can stimulate neurite outgrowth in culture and promote axon regeneration after spinal cord injury (Roonprapunt et al., 2003).

There are also intracellular molecules that affect the processes of growth cone advance and axon motility, such as the cytoskeletal proteins as well as the intracellular machinery involved in the signaling pathways of axonal growth. Most of the cytoskeletal proteins observed during axonogenesis are re-expressed during axon regeneration, for instance Tα1, a tubulin isoform (Kobayashi et al., 1997; Miller et al., 1989). Members of Rho family of small guanosine triphosphatases (GTPases) are also of importance in the transduction of extracellular signals into changes in the cytoskeleton. Manipulating the activity state of Rho and its effectors may either inhibit or promote neurite outgrowth (Fournier et al., 2003). High level of the second messenger, cAMP (cyclic AMP), is associated with growth cone attraction and axon regeneration (Qie et al., 2002).

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1.2.3 Extrinsic Factors in the Environment

In higher vertebrates, the injured CNS axons are irreversibly impaired due to the lack of successful regeneration. In contrast, PNS axons regenerate well. Increasing evidence suggests that there are differences in the PNS and CNS environment in which axons are located. Generally, the extrinsic environmental factors contributing to the different regenerative capacities lie in the surrounding glial environment and extracellular matrix (ECM) of transected axons (for review, see Fawcett and Asher, 1999).

The glial cells (originated from the word "nerve glue") surrounding the neurons in the CNS environment, were termed in 1859 by Rudolph Virchow, who believed glia to be a passive supporting tissue that held the neurons together. In the late 19th century, Cajal employed metallic staining techniques, which revealed the different types of glia in the CNS: astrocytes, oligodendrocytes, microglia and ependymal cells. In the PNS, the major glial component is Schwann cell (Zigmond et al., 1999). As the biotechnology has advanced, new findings have changed the understanding of glial cells. Glial cells are found no longer to be inactive supporting cells; rather they are active participants in many nervous functions under physiological and pathological conditions (for reviews, see Fawcett and Asher, 1999; Fields and Stevens-Graham, 2002).

Santiago Ramón y Cajal (1852-1934) is considered to be the great pioneer of neuroscience. He was awarded Nobel Prize for Medicine in 1906.

The ECM is an intricate network composed of a variety of proteins and polysaccharides that are secreted and organized into a meshwork in extracellular spaces, in close association with the local cells. The cells produce all the ECM molecules and are at the same time influenced by them. The matrix plays a complex role in regulating cellular behavior, such as proliferation and migration. Thus, variations in different types of ECM molecules and organization give rise to a vast diversity of forms to meet the functional requirements of the particular tissue (Zigmond et al., 1999). In the CNS, the ECM contains several types of molecules, such as glycoproteins, proteoglycans and hyaluronan, with which

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neurons and glia interact. Many ECM molecules have been identified along axonal pathways during development or after lesion in the nervous system that may have axon growth promoting or axon growth inhibitory effects (Zigmond et al., 1999).

When the CNS environment is replaced by that of peripheral nerves at the transected CNS, the axons from the injured neurons can elongate into the peripheral nerve grafts. Although the regenerating neurites eventually bridge the injury site, function is not restored because the neurites stop growing after re-entering the CNS environment (David and Aguayo, 1981; Benfey and Aguayo, 1981).

It has been established that in the mammalian CNS microenvironment that oligodendrocytes, the myelin sheaths formed by oligodendrocytes and the glial scar formed by reactive astrocytes at the site of a CNS lesion can prevent axon growth (Fig. 1.2). This is achieved by exposing potent neurite outgrowth inhibitors or depositing inhibitory ECM molecules at the site of injury that prevent axon growth (Davies et al., 1997; Liuzzi and Lasek, 1987; Schwab and Caroni, 1988). More details on neurite outgrowth inhibitors are shown below.

1.2.4 Molecular Guidance of Axon Outgrowth

One of the aspects considered in axonal regeneration is whether the re-growing axons can find their targets and establish functionally meaningful connections. During development, the pathfinding mechanism of nerve growth ensures that growing axons can elongate along "specific" pathway and innervate appropriate targets. The axon pathfinding depends on the guidance cues, which serve either as attractants or repellents, thereby resulting different types of axonal behavior (for review, see Stoeckli et al., 1998). The growth cone receptors, acting as sensors, can detect and navigate toward or away from the source of substrate-bound or diffusible guidance cues presented by their environment (Fig. 1.3).

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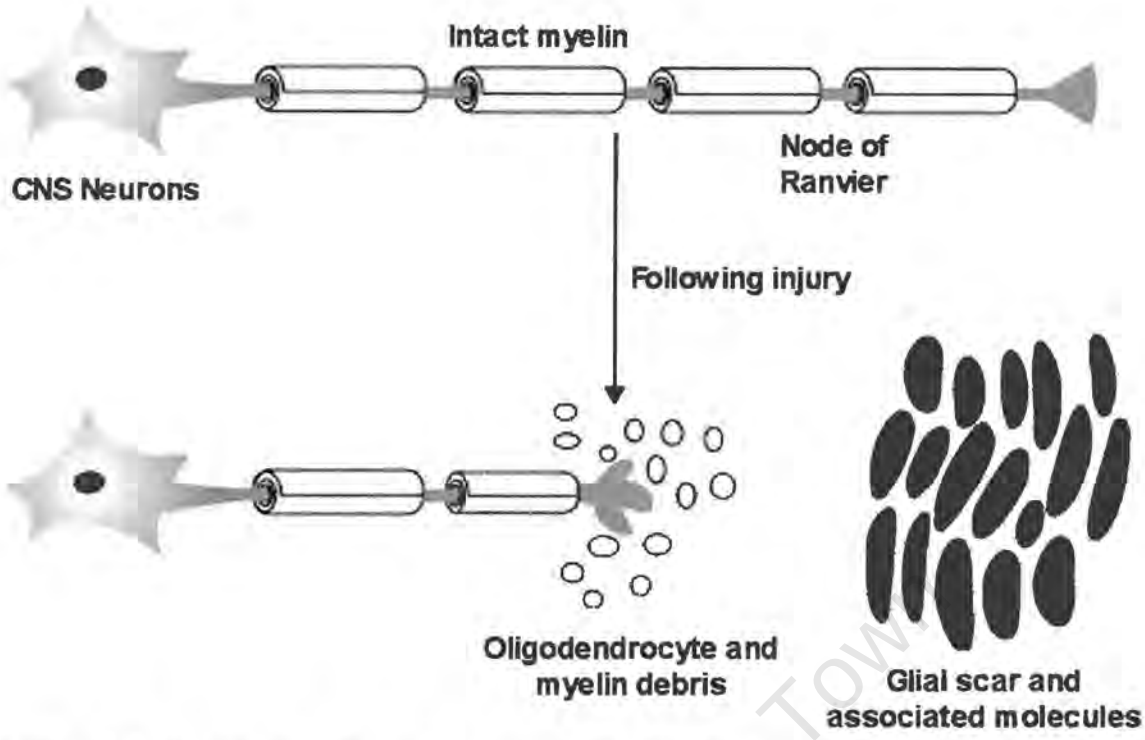

Figure 1.2. Schematic diagram representing the microenvironment at the site of lesioned CNS axon. Following injury, a regenerating axon will encounter neurite outgrowth inhibitors which are associated with oligodendrocytes and myelin sheath. The glial scar is not only a mechanical barrier, also contains inhibitory molecules that can inhibit axonal extension. In general, these are believed to be the extrinsic factors in the surrounding environment of axotomized axon contributing to the failure of CNS regeneration. (Adapted from Spencer et al., 2003)

The netrins are a group of guidance cues for developing axons. *In vitro* studies showed neurites from the *Xenopus* and mouse embryonic retinal ganglion cell (RGC) exhibit a chemoattractive response toward a point of netrin-1 source, mediated by the receptor DCC (de la Torre et al., 1997). At the optic disc of the developing eye, null expression of netrin-1 disorientates the pathfinding of RGC axon, leads to optic nerve hypoplasia (Deiner et al., 1997). In the injured optic nerve of fish, the coordinated expression of netrin-1 and its receptors correlates with the successful regeneration (Petrausch et al., 2000). In addition, netrin-1 promote neurite outgrowth from embryonic mouse retinal explants in a dose dependant manner (Deiner et al., 1997).

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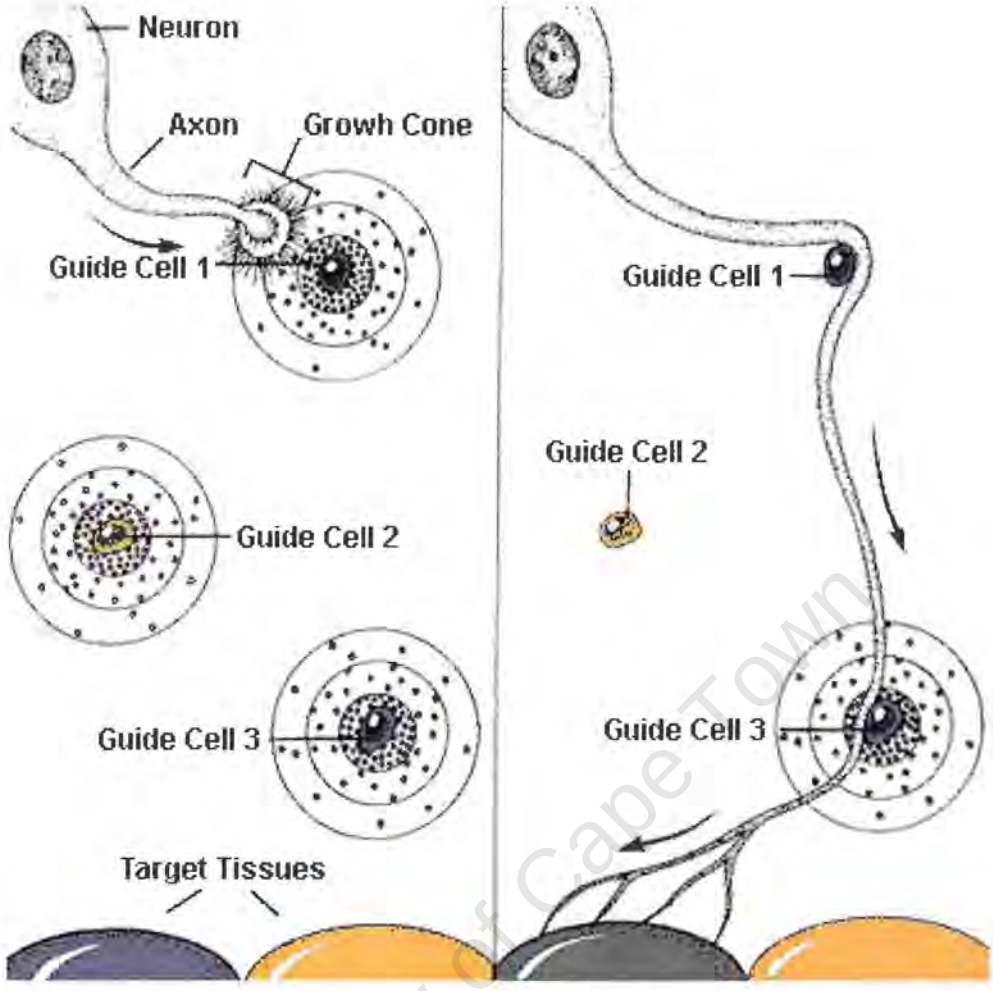

Figure 1.3. Schematic diagram representing the effects of environmental cues on axon guidance. The growth cone of the axon is attracted by the secretion of guide cell 1, but is repelled by the guide cell 2. Eventually the growth cone passes through guide cell 3 and reaches its target tissues.
(Adapted from http://web.sfn.org/content/Publications/BrainBriefings/axon.html)

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The large semaphorins family, including secreted, transmembrane and GPI-linked proteins, has been strongly associated with axon guidance. Similarly, a variety of ephrins and their Eph receptors have been implicated in guiding axons and topographic mapping. These molecules are involved in the formation of axon projections during nervous system development, acting through bidirectional signalling (for reviews, see Cook et al., 1998; Holland et al., 1998). Furthermore, they are capable of inducing growth cone collapse. The upregulation of these molecules after injury have implicated their role of neurite outgrowth inhibition that contributes to the unfavourable environment for axonal regeneration (Miranda et al., 1999; Moreau-Fauvarque et al., 2003).

1.3 Neurite Outgrowth Inhibitors

Differentiated oligodendrocytes in culture and CNS myelin exert a strong inhibitory effect on adhesion and outgrowth for primary neurons as well as spreading of non-neuronal cells (Caroni and Schwab 1988; Mckerracher et al., 1994). It is further known that growth cones interacting with differentiated oligodendrocytes could not extend across these cells and growth cone collapse was induced in contact with the surface of oligodendrocytes (Fawcett et al., 1989; Lang et al, 1996). In addition to those surface expressed inhibitory molecules, there are others in the surrounding ECM, especially at the site of injury.

The search for oligodendrocyte and myelin associated neurite growth inhibitors has led to the characterization and identification of several activities and molecules such as myelin-associated glycoprotein (MAG) (Mckerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte myelin glycoprotein (OMgp) (Wang et al, 2002b), chondroitin sulfate proteoglycans (CSPGs) (Niederöst et al., 1999), tenascin-R (Pesheva et al., 1989; Rathjen et al., 1991) and Nogo-A (Caroni and Schwab, 1988; Chen et al., 2000). In the present study, the emphasis is placed on two of the neurite outgrowth inhibitors; tenascin-R (TN-R), a cell surface ECM glycoprotein; and Nogo-A, an oligodendrocyte and myelin associated inhibitor.

1.3.1 Tenascin-R

Most of the ECM proteins, which consist of multidomain glycoproteins as the structural basis, are involved in a variety of cellular interactions. Some proteins have both cell adhesive and anti-adhesive properties, while some have both neurite inhibitory and promoting effects within the same protein. TN-R is a typical example of such a multipurpose molecule (for reviews, see Pesheva and Probstmeier, 2000; Pesheva et al., 2001).

TN-R belongs to the tenascin family of ECM glycoproteins. This family comprises of five known members, TN-C, TN-R, TN-X, TN-Y and TN-W respectively. Only

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three of these, TN-C, TN-R and TN-X, are expressed spatiotemporally in the CNS (Deckner et al., 2000). TN-C and TN-R called J1 initially and collectively are the most structurally related members of this family. Each tenascin polypeptide chain is characterized by a series of common structural motifs (Fig. 1.4). These comprise of a cysteine-rich N-terminal, followed by a variable number of epidermal growth factor (EGF)-like domains and fibronectin (FN) type III-like repeats. The C-terminal regions are homologous to fibrinogen (FG) chains (for reviews, see Chiquet-Ehrismann, 2004; Joester and Faissner, 2001).

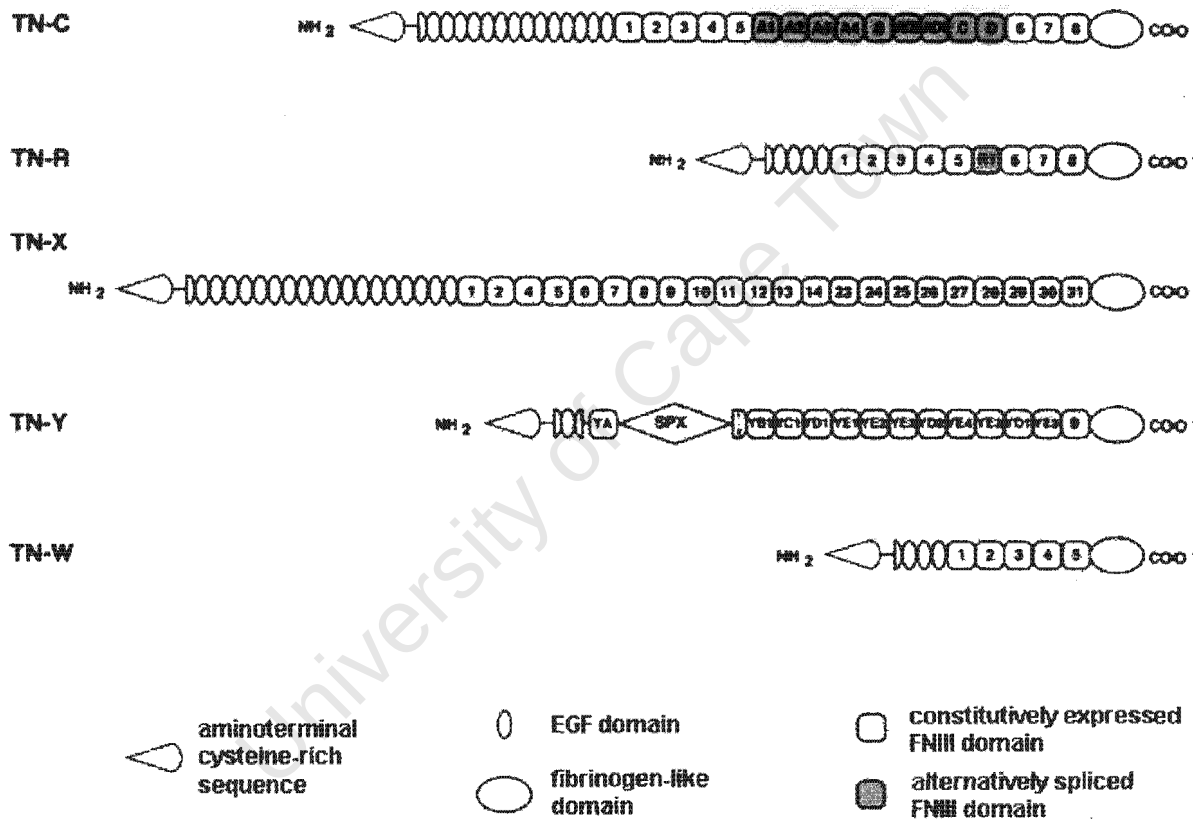

Figure 1.4. Structures of tenascin family. The polypeptide sequence analysis of each tenascin members displays a common arrangement of structural motives. (Adapted from Pesheva and Probstmeier, 2000)

TN-R protein was originally isolated from a membrane fraction of an adult mouse brain with molecular weights of 160 and 180kD, and then later identified as J1-160/180 (Kruse et at., 1984, 1985). Although the functional differences between these two isoforms are not clear at present, they are generated from alternative splicing (Fig. 1.4). TN-R sequence is phylogenetically highly conserved, and is

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called janusin (in rodents) and restrictin (in chicken). The predicted amino acids sequences of chicken and rat TN-R show a homology of more than 80% (Fuss et al., 1993; Pesheva et al., 1989; Rathjen et al., 1991). TN-R protein is also found in the lower vertebrates, such as amphibians and fish, whose predicted amino acids sequence is ~60% to those of TN-R in amniotes (Becker et al., 1999, 2003).

In the CNS, TN-R proteins are predominately expressed by O-2A linage cells (oligodendrocyte and type-2 astrocyte), and are most abundant during the period of myelination. The expression of TN-R was mostly associated with the surface of oligodendrocytes, myelinated axons and Nodes of Ranvier (Fuss et al., 1993; Nörenberg et al., 1996; Pesheva et al., 1989; Rathjen et al., 1991). It has been shown to accumulate in perineuronal nets of interneurons and motoneurons, of which is often found in a co-expression with TN-C or CSPG (Angelov et al., 1998; Brückner et al., 2003; Xiao et al., 1997). Apart from the CNS tissue, TN-R is transiently expressed by Schwann cells during PNS development (Probstmeier et al., 2001).

Many *in vitro* studies have clearly demonstrated the neurite outgrowth inhibitory effects of TN-R on different population of neuronal explants (Fig. 1.5) (Becker C.G. et al., 1999, 2004; Becker T. et al., 2000; Probstmeier et al., 2000a). The repulsive property of TN-R on neurite outgrowth is mediated by a neuron-specific immunoglobulin superfamily glycoprotein F3/11/contactin (Probstmeier et al., 2000b), which anchors on the cell surface membrane by glycosylphosphatidyl-inositol (GPI) linkage and is implicated in axonal growth and synaptogenesis (For review, see Falk et al., 2002). In the context of inhibitory substrate, TN-R was investigated as a neurite outgrowth inhibitor in part of CNS axonal regeneration. It is believed that the continued expression of TN-R protein after CNS injury contributes to the failure of axonal regeneration in mammal (Becker et al., 2000; Probstmeier et al., 2000b).

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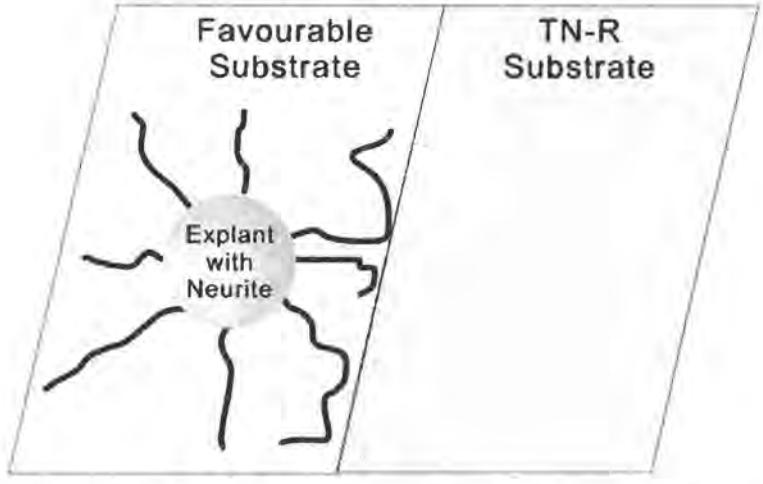

Figure 1.5. Schematic diagram representing the neurite outgrowth inhibition of TN-R. In the *in vitro* substrate border assay, the neurites are allowed to exit the neuronal explants on the favourable substrate, such as poly-lysine or laminin. When the neurites grow into a sharp border of TN-R protein, neurites steered away and avoided the border.

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1.3.2 Nogo-A

Initially, Caroni and Schwab were testing the nonpermissive components for neurite extension in the optic nerve of rats. The neurite growth and fibroblast spreading assay revealed that an important part of the neurite outgrowth inhibition was exerted in membranes of differentiated oligodendrocyte and in CNS myelin (Caroni and Schwab, 1988; Schwab and Caroni, 1988). Two inhibitory protein fractions of 35 kD and 250 kD were recovered from electrophoresis gel, and identified as neurite growth inhibitors NI-35/250 by their molecular weights (Caroni and Schwab, 1988). A monoclonal antibody, mAB IN-1 (for inhibitory neutralization), raised against myelin fraction was able to reduce the myelin-derived inhibitory activity *in vitro* (Chen et al., 2000; Lang et al., 1995). The administration of IN-1 antibody after SC lesion of rats could neutralize myelin-associated neurite outgrowth inhibitors, and enhance axonal regeneration leading to some degree of functional recovery (Bregman et al., 1995: Brösamle et al., 2000; Merkler et al., 2001; Schnell and Schwab, 1990; Thallmair et al., 1998).

The bovine homologue of rat NI-250, bNI-220, was purified by biochemical characterization. Purified bNI-220 exhibited potent neurite outgrowth inhibitory activity that could be fully neutralized by the mAB IN-1 (Spillmann et al., 1998). Results of microsequencing of bNI220 protein were used to screen cDNA libraries and databases, led to the isolation of *nogo* gene. The protein products of this *nogo* gene, Nogo-A, -B and –C resulted from both alternative splicing and promoter usage and showed strong C-terminal homology to reticulon (Rtn) protein family (Fig. 1.6) (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). Since the identification of Nogo/Rtn4, most of the Rtn researcher have set emphasis on Nogo-A/Rtn4-A which corresponds to NI-250 (for review, see Oertle and Schwab, 2003).

Expression analysis of Nogo-A revealed Nogo-A is highly expressed in oligodendrocytes, myelin and various types of neurons (Huber et al., 2002; Hunt et al., 2003; O'Neill et al., 2004; Taketomi et al., 2002; Tozaki et al., 2002). The application of mAB IN-1 and the antisera generated against different peptide sequences of Nogo-A have been shown to neutralize the activity of the myelin-derived inhibitory proteins in several *in vitro* bioassays, such as cell spreading and dorsal root ganglion (DRG) or RGC neurite outgrowth assay (Chen et al., 2000; Oertle et al., 2003b). The structural and functional studies suggest that the regions of Nogo-A exert these nonpermissive effects lie in both Nogo-A specific N-terminal domain and the C-terminal 66-amino acid loop (Nogo-66) within the two putative transmembrane domains (Fig. 1.6) (Fournier et al., 2001; Oertle et al., 2003). Furthermore, the inhibitory N-terminal was shown to exhibit two different active sites: a region involved in fibroblast spreading, and a stretch limited cell spreading and neurite outgrowth (Oertle et al., 2003).

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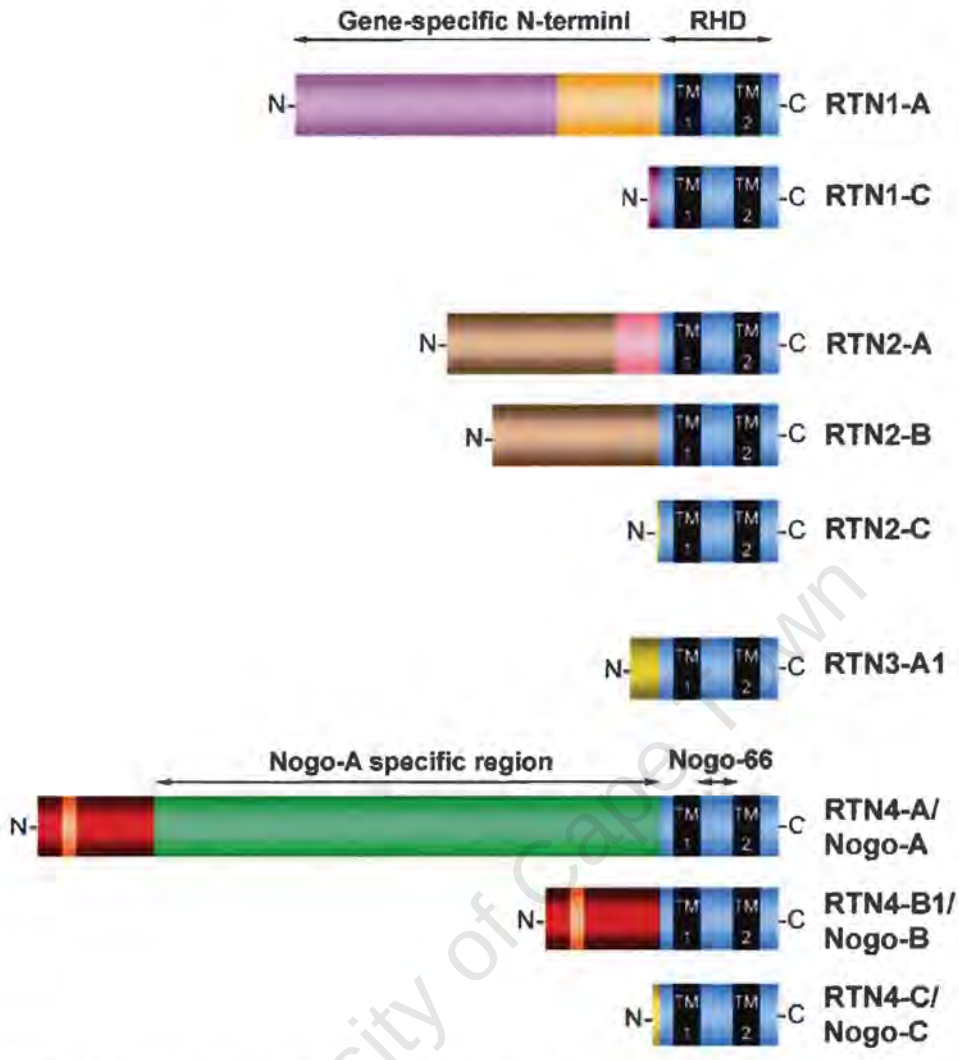

Figure 1.6. The sequence structures of Nogo/Rtn-4 and other paralogues of the Rtn proteins. Rtn family members share the homology of C-terminus, called reticulon-homology domain (RHD), consisting of two putative transmembrane (TM) domains. Both Nogo-66 and the Nogo-A specific N-terminus of Nogo-A/Rtn4-A were shown to be inhibitory. (Adapted from Oertle and Schwab, 2003)

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1.3.2.1 Nogo-66 Receptor

Although the identity of the binding receptors for Nogo-A specific N-terminus is still not clear, the Nogo-66 receptor (NgR) has been identified and shown to express in neurons and axons (Fournier et al., 2001; Wang et al., 2002). The predicted protein contains a conventional N-terminal signal sequence followed by leucine-rich-repeat (LRR) domains, and an LRR C-terminal (LRRCT) motif that is GPI-linked to the plasma membrane (Fournier et al., 2001).

1.4 Regeneration in Lower Vertebrates

In the CNS of mammals and birds, the poor regeneration capacity after an injury is well recognized. In contrast, regeneration of injured CNS axon occurs in the lower vertebrates, such as fish, urodeles and anurans (Gaze, 1970). The underlying mechanisms and differences are not yet fully understood. However, extensive behavioral recovery and axonal growth across spinal cord (SC) and optic nerve (ON) lesions in fish and certain amphibians have been studied and observed in many post-lesion studies.

Transection of the ON in goldfish is followed by a high degree of axonal regeneration. One of the factors that contributes to the successful regeneration is the growth cone of regrowing RGC which is capable of making contacts with the myelin and glial scar, thus successfully reestablishing the visual pathway (Strobel and Stuermer, 1994). In the urodele amphibian animal models, lesioned SC can be regenerated completely even in adulthood. However, there is not yet any evidence for such successful regeneration. There are only indications supporting this regeneration process, such as retention of embryonic character and the response of ependymal glia (for review, see Chernoff et al., 2003).

In anurans, the cellular substrate, like ependymal processes, was suggested to be supportive for the axonal regeneration of the *Xenopus* tadpole SC (Michel and Reier, 1979). Reier proposed that the regenerating ON of *Xenopus* tadpole was able to ignore the mechanical barrier formed by glial scar, and penetrate through it (Reier, 1979). Further studies showed regeneration of fiber tracts in the injured SC does occur in the larval tadpole stage, but not after metamorphosis (Beattie et al., 1990). However, ON regeneration is possible in both tadpole and adult frogs (Gaze et al., 1990; Reier, 1979). Thus, different CNS regeneration capacities in anuran, which are dependent on the metamorphosis, make it a particularly interesting model in which to study axonal regeneration.

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1.4.1 Expression of Neurite Inhibitors in Lower vertebrate

Early results suggest that the absence or low expression of neurite outgrowth inhibitory proteins in the CNS of lower vertebrates plays a major role in the success of axonal regeneration (for review, see Bernhardt, 1999). However, tenascins (Fuss et al., 1993; Pesheva et al., 1989; Rathjen et al., 1991) and Nogo/RTN4 (Oertle et al., 2003a) proteins are evolutionary conserved, and therefore their homologs may exist in amphibians.

In the salamander, an *in vitro* study using adult RGC, TN-R was shown to exhibit similar inhibitory substrate property as the mammals (Becker et al., 1999). The expression study revealed the correlation of TN-R with myelination during metamorphosis and in the adult ON. However, in the case of ON crush, TN-R is shown to be down-regulated during axonal regeneration (Becker et al., 1999). In anurans, there have been no studies done regarding TN-R up to date. However, *Xenopus* homologue of the TN-R receptor, contactin (also known as F3 in mammal, or F11 in chicken), has been identified (Nagata et al., 1996). Similar to that in the nervous system of higher vertebrates contactin has also been shown to be essential for *Xenopus* nervous development (Fujita et al., 2000). Thus, the presence of *Xenopus* contactin provides an indication for the possible existence of TN-R in the *Xenopus laevis*.

Similarly in mammals, Nogo-A is an antigen for mAB IN-1 and also a myelin-associated neurite inhibitor (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). Previous studies in *Xenopus* have indicated the presence of IN-1 antigen in *Xenopus* CNS. Furthermore, the neurite outgrowth inhibitory effects of *Xenopus* oligodendrocytes and myelin have been neutralized by IN-1 antibody (Lang et al., 1995). Against this background, two *nogo/rtn4* genes were identified in *Xenopus laevis* recently (Klinger et al., 2004), though the detail of expression and function for the existence of the *Xenopus rtn4* genes was not clear.

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1.5 Aim and Objectives

Many of the studies on neurite outgrowth inhibitors have been done in the mammalian CNS. However, detailed studies regarding the expression pattern and roles of TN-R and Nogo-A in lower vertebrates are primarily lacking. Thus, the analysis of these two proteins' expression and function in the CNS of lower vertebrates appears clearly warranted. Moreover, no data is presently available concerning putative receptors for either Nogo-A or TN-R in amphibians. This novel investigation on these two proteins will shed more insight into the mechanisms governing axonal regeneration, as well as into the evolution of glial cells and functions of these molecules in lower vertebrates.

The aim of this study was to establish the expression pattern and the role of TN-R, Nogo-A and their receptors in the CNS of *Xenopus laevis* frog and tadpole. Several objectives are outlined below:

(i) To carry out a full expression study of TN-R, Nogo-A and their receptors in the *Xenopus* nervous system.

(ii) To study the post-injury regulation of these molecules, i.e. the expression pattern of TN-R and Nogo-A in the lesioned nerve of *Xenopus*.

(iii) To analyze the response of axonal growth cones and glia cells encountered with TN-R and Nogo-A in the *in vitro* functional assays.

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1.6 Experimental Design

1.6.1 Expression Studies

In order to carry out a full expression study of TN-R, Nogo-A and their receptors, immunohistochemistry / immunocytochemistry were employed in the *Xenopus* frog and tadpole. A panel of cross-species reactive poly- and monoclonal antibodies against the protein of interest were used together with the specific cell-type markers as the double immunostaining in the whole mount tissue or frozen and/or paraffin sections. In addition, to study a detailed cellular expression, the double immunostaining was also performed in the cell cultures.

1.6.2 Post Lesion Studies

In anurans, metamorphosis is the critical point for the axonal regeneration; therefore the post-lesion study was done in both larval and adult *Xenopus*. The optic nerves were crushed in the anesthetized animals. The injured animals were allowed for recovery and then sacrificed at different time intervals. Immunohistochemistry assays were performed in the injured tissues, using double immunolabeling on the whole mount tissue or cryosections.

1.6.3 Functional Studies

Is regenerative success in the CNS of amphibians linked to permissive substrate properties of the microenvironment? Or, are these mammalian neurite outgrowth inhibitors simply not inhibitory to the CNS axons of amphibian? To address these questions, the responses of the growth cones was monitored using the TN-R or Nogo-A as the *in vitro* culture substrate.

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Chapter 2

Materials and Methods

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2.1 Animals

2.1.1 Housing and Breeding

The adult Southern African clawed frogs (*Xenopus laevis*) were bought from the Zoology Department of the University of Cape Town, and kept in large concrete tanks at the Anatomy Building of Human Biology Department in compliance with animal welfare legislation (Ethics approval number: Ref. No. 13/01). The frogs were fed on a diet of pieces of liver, mince and dry food pellets. For breeding purposes, both male and female frogs were kept in the lab tank and injected with the human chorionic gonadotrophin hormone (Profasi, South Africa or Pregnyl, Netherlands) to stimulate the spawning and fertilization of eggs. The tadpoles were reared on suspensions of yeast powder, and cared for in the lab tank with aeration.

2.1.2 Dissection

Both *Xenopus* frog and tadpole were terminally anesthetized with an overdose of 0.1% solution of MS222 (3-aminobenzoic acid ethylester; Sigma-Aldrich, Seelze, Germany) in tap water for tissue preparation. The tissue was dissected free with the aid of surgical instruments (listed below) in phosphate buffered saline (PBS) on ice under the dissection microscope (Olympus C011, Japan), and further processed using different protocols depending on the experiments carried out.

Surgical Instruments	Brand
Dissection scissors	Martin
Iridectomy scissors	Martin
Surgical blades	Swann-Morton
Surgical forceps	Martin
Watchmaker's forceps	Dumont

Table 2.1. List of surgical instruments.

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2.1.3 Optic Nerve Lesions

The *Xenopus* tadpoles were selected at the stage of 50-58 according to the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1956) prior to the operation. They were anesthetized in a 0.05% solution of MS222, and then placed in a glass Petri dish containing wet paper towel. Due to the transparency of the embryonic tissue (Fig. 2.1A), the pigmented optic nerves (ON) were easily found under the dissection microscope. At the midpoint of the ON between the eye and the cranium, the skin is opened to expose the nerve. The ON crush was made using the watchmaker's forceps (Fig. 2.1B). The ON lesion was only performed unilaterally so that the animal is not completely blind and the other side of the ON was used as a control. The post-lesioned animals were separated from the normal ones, and monitored in different tanks. The animals were sacrificed after different survival time intervals. Both injured and uninjured optic nerves were removed, and either embedded in tissue freezing medium (Jung, Germany) for cryosection or used as whole mount.

For nerve lesions in frogs, all the procedures were similar to those above, except the ON was approached through a small opening made with razor blade in the roof on the mouth. The surrounding ocular muscle and blood vessels were gently pull away to expose the embedded ON. After nerve crushed, the frogs were kept in the lab tank and fed normally the next day.

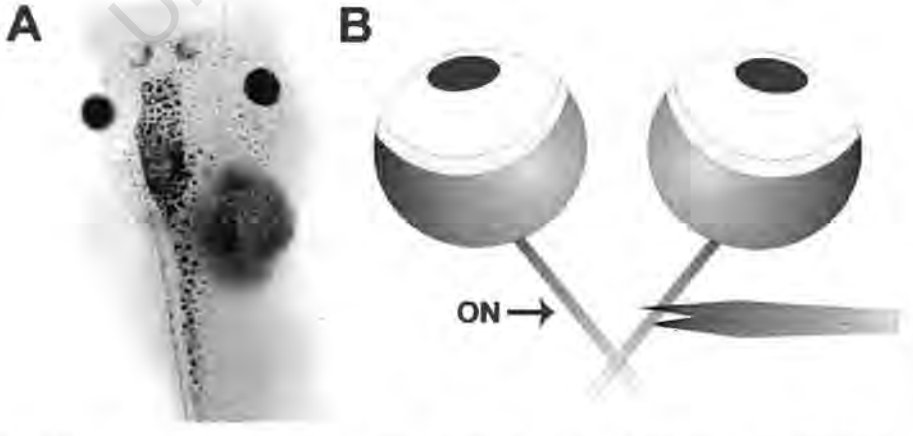

Figure 2.1. *Xenopus* tadpole and optic nerve crush. **(A)** *Xenopus* tadpole. Most of the tissues are transparent, that makes the guts and pigmented melanocytes visible. **(B)** Schematic diagram of performing optic nerve (ON) crush with watchmaker's forceps.

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2.2 Immunoblot

2.2.1 SDS Gel Electrophoresis

Freshly dissected tissues were transferred into a glass homogenizer with complete extraction buffer (freshly prepared from Aprotinin, PMSF and extraction buffer which contains 0.1M Tris-HCl (pH 7.2), 1% Nonidet P-40, 0.01% sodium dodecyl sulphate (SDS)). After homogenizing the tissues thoroughly, the samples were centrifuged for 5 to 7 minutes at 4°C or 2 min at room temperature (RT). The resulting pellet was discarded, and the aliquots of supernatant could be stored at –80°C.

The protein extracts were heated to 95°C for 2 min in reducing sample buffer containing 0.125M Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue. Aliquots of protein extracts and protein molecular weight marker were loaded onto a 7.5% or 10% SDS-polyacrylamide vertical gel (Bio-Rad, Italy) depending the molecular weight of the protein of interest. Gel electrophoresis started with 150V in the stacking gel, switched to 180V in the resolving gel. The resulting gel could be checked by Coomassie-Blue stain.

2.2.2 Western Blot

The proteins separated by SDS-gel were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Bioscience) in a tank blot apparatus. For blocking, the membrane was incubated in blocking solution (3% (W/V) non-fat dry milk, 0.3M NaCl and 0.05% (V/V) Tween-20 in PBS) at RT for at least 1 hr with agitation. After which the membrane was incubated with the primary antibody in blocking solution overnight at 4°C or for at least 2 hours at RT with continuous agitation. Blots were rinsed twice in the above washing solution (0.3M NaCl and 0.05% (V/V) Tween-20 in PBS) for 10 min each, and incubated with either anti-rabbit HRP-conjugated or anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology or Jackson Immuno Research) in blocking

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solution at room temperature for 2 hours. Control blots were labeled without primary antibody, but secondary antibody only. Thereafter, blots were rinsed four times in washing solution for 10 minutes each, and developed using either the ECL Detection kit (Amersham Bioscience) or the Super Signal kit (Pierce, USA). The chemiluminescence signal was detected by Kodak medical film, which was developed according to standard procedures.

Primary Antibody	Dilution Factor	Source
Nogo (Bruna, polyclonal)	1:10000	Gift of Dr. M. Schwab, Zurich, Switzerland
Nogo (702, polyclonal)	1:10000	Produced by Dr. D. Lang
Nogo receptor (commercial, polyclonal)	1:5000	Alpha Diagnostic International, USA
Nogo receptor (antisera raised in rabbit) and pre-immune control sera	1:5000	Own production
Tenascin-R (polyclonal)	1:5000	Gift of Dr. P. Pesheva, Bonn, Germany
Janusin (polyclonal)	1:5000	Gift of Dr. P. Pesheva, Bonn, Germany

Table 2.2. List of primary antibodies employed in the western blot.

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2.3 Antibodies and Immunohistochemistry

2.3.1 Tissue Preparation

For cryosections, the tissue was placed in aluminium molds embedded in the freezing medium. The specimen was allowed to equilibrate with the freezing medium for few minutes at RT or 1 hr at 4C, then snap-frozen in liquid nitrogen. Twelve to twenty-micrometer fresh-frozen sections were cut directly onto APTES (3-aminopropyltriethoxysilane) -coated slides using the cryostat (Leica CM1850,

Germany). The sections were air dried, then fixed for 5 min in methanol at -20°C and 5 min in 4% paraformaldehyde (PFA; Merk, Germany) in PBS. The sections were washed three times for 5 min in PBS, and there after incubated in blocking solution containing 1% bovine serum albumin (BSA; Roche Diagnostics, Germany) in PBS for 1 hour at room temperature. Subsequently, the treated sections were immunostained as described below.

For whole mounts, the fresh tissue was fixed for 15 min in 4% PFA, then 15 min in methanol at -20°C. The whole mounts were washed three times for 5 min in PBS, and then incubated in the above blocking solution with extra 1% dimethyl sulphoxide (DMSO) overnight at 4°C. After that, immunostaining was carried out according to the procedure below.

2.3.2 Immunostaining and Imaging

For immunohistochemical analysis on either the sections or whole mounts, the tissues were incubated with primary antibodies in blocking solution overnight at 4°C (two nights for whole mounts). The tissues were then rinsed twice in PBS and incubated with the appropriate secondary antibodies for 2 hours at RT (overnight at 4°C for whole mounts). The antibodies are listed in Table 2.3.

For membrane-bound proteins, such as O4 marker or GPI-anchored proteins, the culture live cells were incubated with the antibody in the medium at room temperature for 30-45 min, then rinsed with fresh medium and fixed in methanol at -20°C for one minute only. The cells were washed and immunolabeled as described above. The live-staining protocol was established in the laboratory and no internalization of the antibodies under these labeling conditions was observed previously (Lang, pers. comm.).

The specimens were washed four times with PBS, and then the nuclei were labeled with DAPI (4',6-diamidino-2-phenylindole; Sigma) at 1:50 dilution for 5min in the dark. The tissue was mounted with Mowiol (polyvinylalcohol; Hoechst, Germany) with anti-fading agent (n-propylgalate; Sigma), and viewed with either

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the conventional fluorescence microscope (Zeiss Axiovert) or confocal imaging system (Leica, TCS-SP). All the photos were taken with automatic exposure time determined by the camera/computer, unless specified for comparison of intensities. The images were further processed for clarity using Adobe Photoshop Without changing the information content.

Few of the thick specimens were analyzed using Axio Imaging System with ApoTome slider (Fig. 2.2) (Zeiss Germany). The ApoTome slider is bearing grid pattern and imaging is based on the fundamental principle of "gird projection" or "structured illumination" (Bauch and Schaffer, 2005). Therefore, the resulted ApoTome image is an optic section through the sample without the out-of-focus information from other focal planes.

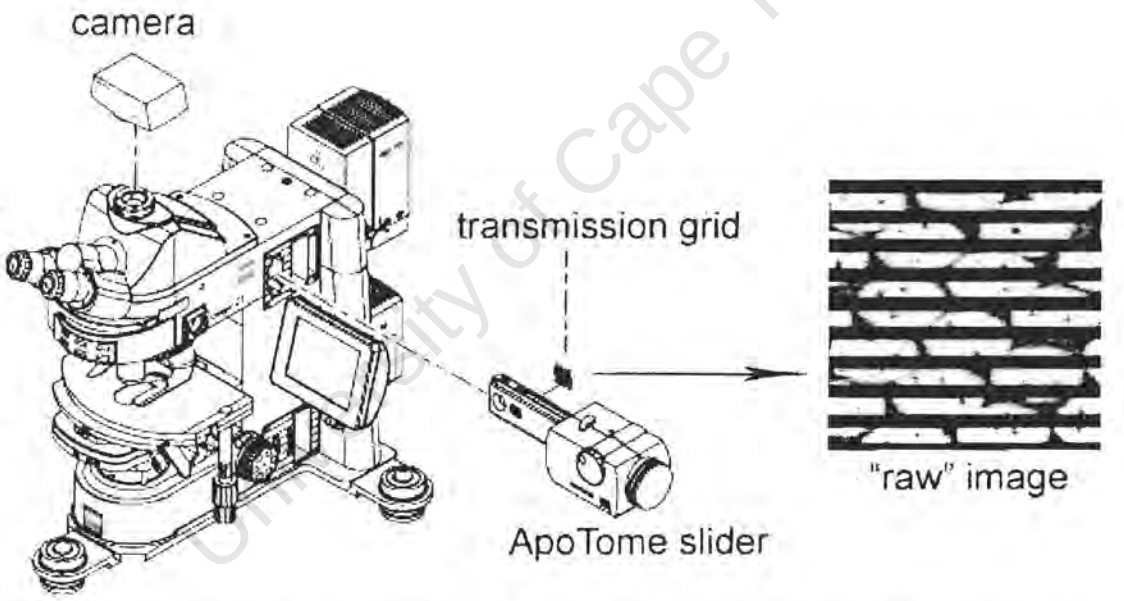

Figure 2.2. Axio Imaging System. The system consists of the ApoTome slider with a transmission grid inserted into the fluorescence beam path of the microscope. Three "raw" images at different Z-position are acquired with grid projection and calculated using a mathematical algorithm to reconstruct an optical plane. (Adapted from http://www.zeiss.com/apotome)

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Primary Antibody	Dilution Factor	Source
F3 (pAB, rabbit)	1:2000	Gift of Dr. G. Gennarini, Bari, Italy
glial fibrillary acidic protein (GFAP, mAB GA5, mouse)	1:500	Sigma, Germany
GFAP (pAB, rabbit)	1:2000	Sigma, Germany
myelin basic protein (MBP) hybridoma supernatant (mAB, rat)	1:100	Gift of Dr. C. Linington, Munich, Germany
neurofilament (NF, mAB SMI31, mouse)	1:1000	Sternberger-Meyer Monoclonals, Lutherville, USA
Nogo-A (702, pAB rabbit)	1:2000	Produced by Dr. D. Lang
Nogo receptor (commercial, pAB rabbit)	1:1000	Alpha Diagnostic International
Nogo receptor (antisera raised in rabbit) and pre-immune control sera	1:1000	Own production
O4 hybridoma supernatant (mAB mouse)	undiluted	Gift of Dr. J. Trotter, Mainz, Germany
Peripheral myelin protein P0 (mAB mouse)	1:500	Gift of Dr. J. Archelos
Tenascin-R (pAB rabbit)	1:1000	Gift of Dr. P. Pesheva, Bonn, Germany
Janusin (pAB rabbit)	1:1000	Gift of Dr. P. Pesheva, Bonn, Germany
Tenascin-R (mAB mouse)	1:500	Gift of Dr. P. Pesheva, Bonn, Germany

Secondary Antibody	Dilution Factor	Source
goat anti-mouse conjugated to alexa-488	1:1000	Jackson Immuno Research West Grove PA, USA

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goat anti-rabbit conjugated to alexa-488	1:1000	Jackson Immuno Research West Grove PA, USA
goat anti-rat conjugated to alexa-488	1:1000	Jackson Immuno Research West Grove PA, USA
donkey anti-mouse conjugated to Cy3	1:1000	Jackson Immuno Research West Grove PA, USA
donkey anti-rabbit conjugated to Cy3	1:1000	Jackson Immuno Research West Grove PA, USA
donkey anti-rat conjugated to Cy3	1:1000	Jackson Immuno Research West Grove PA, USA
donkey anti-mouse conjugated to cy5	1:1000	Jackson Immuno Research West Grove PA, USA

Table 2.3. List of primary and secondary antibodies employed in the immunostaining.

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2.3.3 Diaminobezidine (DAB) Staining

The activity of endogenous peroxidase of the tissue was quenched by 30 min incubation with 0.3% hydrogen peroxide in methanol at RT. The sections were then washed three times 5 min in TBS, and then incubated with the blocking solution containing 0.5% BSA in TBS for 30 min at RT.

After blocking, the sections were incubated with primary antibody at 4°C overnight. Followed by four 10 min washes with TBST (1% Tween-20 in TBS), the sections were incubated with biotinylated secondary antibody for 1 hr at RT, then washed four times for 10 min with TBS. While washing, Avidin-Biotin-Perosidase complex from Vectasatin Elite ABC kit (Vector Laboratories, USA)

was pre-reacted for 30 min at RT. The complex solution was applied onto the sections for 30 min at RT, and then washed off by TBS. The sections were briefly rinsed with 0.05M Tris solution. The colour stain was achieved by 5 min colour reaction of DAB Peroxidase Substrate Kit (Vector Laboratories, USA). The sections were later washed for 15 min with tap water, and dehydrated with alcohols. Lastly, the sections were cleared in xylol and mounted with Entellen.

2.3.4 Generation of Nogo-Receptor Antibody

- This part of the work was done together with and mostly by Dr. Edward Nyatia.
- All procedures were in compliance with protocols approved by the Animal Ethics Committee (Ethics approval number: REC REF: 03/009).

The mouse and fish *nogo-A/rtn-4* receptor protein sequences were aligned using the William Pearson's *lalign* program to locate the overlapping amino acids (aa). A short sequence of 8 aa (SLQYLRLN), that was conserved from mouse to fish was selected. A synthetic peptide with KLH (keyhole limpet hemocyanin) conjugation was made from Alpha Diagnostic International (see Appendix for data sheets), and introduced into two New Zealand White rabbits (animal identification: 452F675926 and 4530010111) to induce immuno-responce. The rabbits were kept in the Animal Unit at the University of Cape Town.

Five inoculations were scheduled at time intervals to induce and boost immuno-reaction. Before each administration of immunogen, serum was collected from both rabbits, including pre-immune serum, i.e. before the first inoculation. When collecting the blood, a portion of tail was swabbed with alcohol and a scalpel blade used to nick the site to expose the vein. The animals were anaesthetized using carbodioxide inhalation or sodium pentobarbitone injection. The desired amount of immunogen was injected into the rabbits subcutaneously at several places.

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2.4 Cell Culture and Functional Analysis

2.4.1 Glial Cell Culture

Glial cell cultures were obtained from the brain and spinal cord of tadpoles or adult frogs. The only difference found between glial cells obtained from frogs or tadpoles was that more mature oligodendrocytes grew from the adult tissue. In addition, the neurite outgrowth was observed in the cultures of tadpole CNS explants.

The brain and spinal cord were dissected in Leibovits medium (L15, GibcoBRL) and the meninges were removed carefully. The tissue was chopped into small fragments with a McIlwain tissue chopper (The Mickle Laboratory). The fragments were rinsed in L15 containing 10% fetal calf serum (FCS, Highveld), then centrifuged briefly. The tissue was resuspended in glial culture medium, consisting of L15/F12 (1:1, Highveld) supplemented with 10% FCS, 0.4% methyl cellulose, 2mg/ml $NaHCO_3$, 50µg/ml gentamycin and 50µg/ml amphotericin B. The suspension was placed on poly-D-lysine coated glass coverslips (Marienfeld, Germany) in a sandwich culture (Fig. 2.3) as described by Lang et al (1995). The cultures were kept in a humidified chamber at 28°C in an incubator and maintained over several weeks.

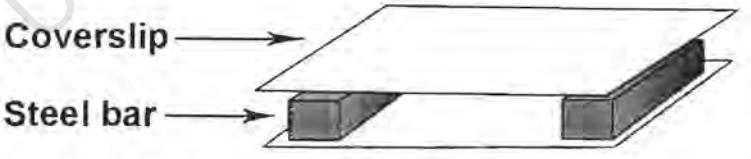

Figure 2.3. Schematic diagram of sandwich culture. Two stainless steel bars were placed between two glass coverslips. The bottom coverslip was coated with protein substrate, but the top coverslip was uncoated.

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2.4.2 Dorsal Root Ganglion Cell Culture

Dorsal root ganglion (DRG) cell cultures were prepared from young adult frogs. The vertebral column was removed from the dorsal of the frog. The muscle and connective tissues were stripped to expose the dorsal root ganglia in between the vertebrates. The DRG was carefully dissected out in L15 medium, freed of connective tissue as well as nerve roots and finally chopped into smaller fragments on the tissue chopper. The fragments were rinsed in L15 containing 10% fetal calf serum (FCS, Highveld), then centrifuged briefly. The tissue was resuspended in neuronal culture medium, and placed on poly-D-lysine coated glass coverslips (Marienfeld, Germany) in a sandwich culture. The cultures were kept in a humidified chamber at 28°C in an incubator.

2.4.3 Analysis of Substrate Property

The coverslips were first coated with 0.1% poly-lysine for 1 hour at room temperature. After a brief rinse with ddH_2O and air dry, the coverslips were then coated with test proteins diluted in PBS to create a homogenous substrate. DRG microexplants were prepared from neonate rat or young adult frog, and cultured on the coated coverslips. The mammalian and amphibian cultures were kept in a humidified chamber at 37°C and 28°C incubator respectively.

Substrate	Concentration	Source
Mouse laminin protein	100ug/ml	Invitrogen
Bovine serum albumin protein	100ug/ml	Roche Diagnostics
Nogo-P4 peptide	100ug/ml	Alpha Diagnostic International
purified mouse Tenascin-R protein	70ug/ml	kind gift from Dr Pesheva, Bonn Germany
purified fish Tenascin-R protein	70ug/ml	kind gift from Dr Pesheva, Bonn Germany

Table 2.4. List of purified proteins or peptides substrates.

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2.4.4 Stripe Assay

Stripe assays were prepared following established procedures (Bastmeyer et al., 1991). Striped substrates were created with the aid of a special silicone matrix containing parallel bars separated by channels in its central area (purchased from Dr S. Lang, Max-Planck-Institute, Tübingen Germany). The matrix was sterilized by washing and autoclaving in ddH_2O, and then oven dried at 37°C overnight. The matrix was placed on a polylysine- or laminin-coated coverslip, and the channels were filled with the test protein or peptide by injection. The test protein or peptide was diluted in PBS to have the same concentration as stated in Table 2.4. The matrix with protein/peptide on the coverslip was incubated for an hour at RT. After the removal of the matrix, this procedure gave a patterned substrate of alternating lanes of polylysine and test protein/peptide. The *Xenopus* tadpoles were selected at the stage of 50-58 for the CNS primary culture. The tadpole CNS was dissected out according to Section 2.1.2 and cut into smaller pieces by using iridectomy scissors. Small fragments of neuronal explant were placed onto the coverslip perpendicular to the parallel lanes and cultured in a humidified chamber at 28°C incubator for 2-3 days (Fig. 2.4).

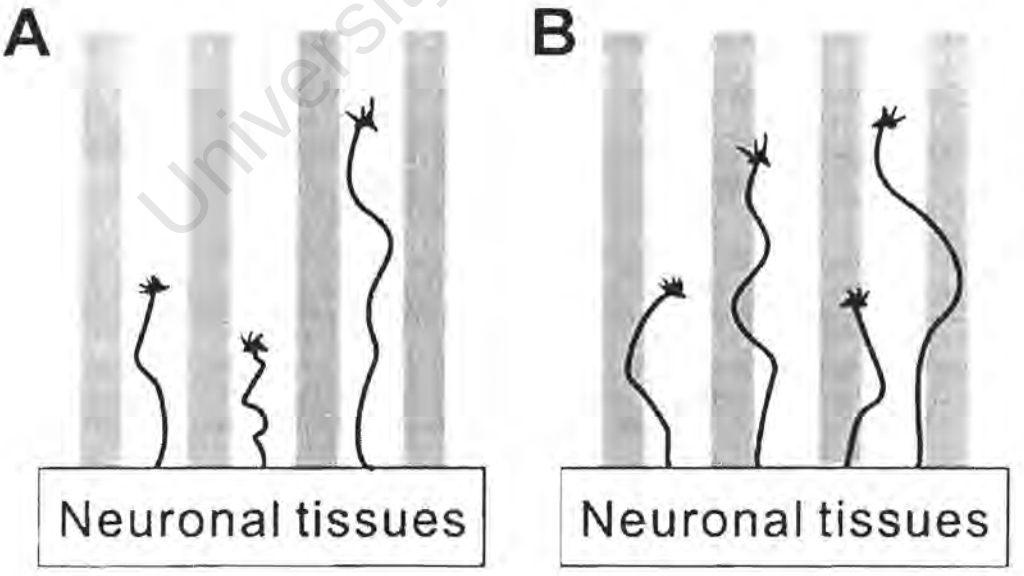

Figure 2.4. Schematic diagram of stripe assay. A fragment of neuronal explant was placed perpendicular to the alternating lanes of polylysine and test protein/peptide (shaded lanes). Neurite outgrowth was either **(A)** elongating on the permissive lanes only, or **(B)** not effected by the test substrate and crossing over the lanes.

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Chapter 3

Expression Analysis of Tenascin-R

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3.1 Identification

Tenascin-R (TN-R) is an ECM protein expressed by mammalian oligodendrocytes. It has been shown to be expressed at high levels in the lesioned mouse optic nerve (ON) and to repel axon growth *in vitro*, and it is therefore suggested to be an important component influencing mammalian axonal regeneration (Becker et al., 2000). The isoforms of TN-R protein were shown to migrate at different molecular weights of 160/180kD (Pesheva et al., 1989). To verify the expression of TN-R in *Xenopus* and compare it to that in mammals, two polyclonal TN-R (pAB janusin and pAB TN-R) antibodies were used in the western blotting. These antibodies were kind gifts from Dr Pesheva who has shown the specificity and cross-reactivity of the TN-R antibodies in the phylogenetic analysis by western blotting (see Appendix for the data sheet, Pesheva et al., 2006).

Results of immunoblotting revealed that pAB janusin antibody recognized double bands representing a protein with two isoforms of molecular weights above 230kD in rat brain (Fig. 3.1, lane 1), as well as in *Xenopus* frog brain (Fig. 3.1, lane 2) and ON (Fig. 3.1, lane 3). The pAB janusin was not cross-reactive with any other non-nervous tissues, as shown by the absence of immunoreactive band in *Xenopus* frog liver (Fig. 3.1, lane 4). The immunoblot using pAB TN-R also showed a similar staining pattern in both frog and tadpole CNS (data not shown). Although the apparent molecular weights of TN-R are higher than the expected 160/180kD, the protein has a high number of charged residues that may play a role in altering its mobility in the gel electrophoresis. Both polyclonal antibodies specifically recognized doublet protein bands in the immunoblots. The immunoblotting pattern of anti-TN-R in the *Xenopus* CNS is similar to that in various vertebrate species (Pesheva et al., 2006). This indicates that the TN-R antibodies specifically recognize the two isoforms of TN-R protein which are expressed in the CNS of *Xenopus* frog and tadpole.

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Figure 3.1. Immunoblot analysis of TN-R expression in the rat and *Xenopus*. The pAB janusin detected double bands (two arrows) in: rat brain (lane 1), *Xenopus* frog brain (lane 2) and ON (lane 3), but not in *Xenopus* liver (lane 4). The doublet has a molecular weight above 230kD. *M*, molecular weight marker; *kD*, kilodaltons.

3.2 Expression of TN-R in *Xenopus* CNS

During the mammalian CNS development and in the pathological conditions, the expression of TN-R has so far been associated with oligodendrocytes and subsets of neurons (Fuss et al., 1993; Nörenberg et al., 1996; Pesheva et al., 1989: Rathjen et al., 1991). From the immunoblotting results in this study (Section 3.1), TN-R immunoreactivity occurs in the CNS *Xenopus* frog and tadpole. To study the localization and developmental regulation of TN-R in the *Xenopus*, the nervous systems of the frog and tadpole were analyzed using immunohistochemistry. Double immunostaining technique was performed using the monoclonal or polyclonal antibodies against TN-R in combination with various cell type-specific markers to visualize the distribution and expression pattern of TN-R.

3.2.1 In Spinal Cord

To analyze whether TN-R immunoreactivity is localized in CNS myelin, myelinated tracts in the spinal cord (SC) of *Xenopus* frog were identified by myelin specific marker MBP. In the longitudinal section of *Xenopus* frog SC, staining of MBP antibody revealed labeling of fiber tracts in the white matter (Fig. 3.2B). The staining of monoclonal antiserum against TN-R (mAB TN-R) revealed a nearly identical pattern to anti-MBP staining which indicates TN-R immunoreactivity in the myelinated area of the frog SC (Fig. 3.2A-C).

In different CNS regions of higher vertebrates, TN-R is also associated with perineuronal nets of interneurons and motoneurons (Angelov et al., 1998;

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Brückner et al., 2003). Thus, frog SC section was examined and stained with the polyclonal antibody against an IgSF glycoprotein F3, which is also known to be the receptor mediating the inhibitory effects of TN-R in mammals (Probstmeier et al., 2000b; Pesheva et al., 2006). The anti-F3 staining revealed the labeling of large neuronal somata in the gray matter of frog SC (Fig. 3.2D). Although it was difficult to visualize the meshwork of perineuronal net, the labeling of mAB TN-R was found surrounding the F3-positive somata (Fig. 3.2D-E). This finding implicates the immunoreactivity of TN-R in the perineuronal nets of neurons.

The different CNS regeneration capacity in anurans depends on the stage of metamorphosis, i.e. the tadpole SC can regenerate after injury (Beattie et al., 1990). To study the developmental regulation of TN-R in relation to myelin, the whole mount of *Xenopus* tadpole SC were double stained with TN-R (pAB janusin) and MBP antibodies and analyzed using the confocal microscopy (Fig. 3.3). The high magnification image revealed the same labeling of MBP antibody in the myelinated tract of tadpole SC as in frog. The staining of pAB janusin showed a very similar pattern to that of anti-MBP. The immunoreactivity of TN-R was found in the MBP-positive fiber tract which indicates an association of TN-R with myelinated axons in the *Xenopus* tadpole SC, similar to that in the adult SC (Fig. 3.2A-C).

On the other hand, in order to analyze the immunoreactivity of TN-R in relation with CNS glia, polyclonal GFAP (glial fibril acidic protein) antibody was counter-stained with mAB TN-R in the cross section of tadpole SC (Fig. 3.4). The staining of pAB GFAP displayed a fibril pattern, which extended from the spinal central canal into the white matter. The GFAP immunoreactivity was also found in the cell bodies (arrows in Fig. 3.4), which presumably were astrocytes with branched processes. The staining of mAB TN-R was predominantly found in the white matter labeled by anti-MBP (data not shown) but the distribution, however, was different from that of GFAP (Fig. 3.4A-D). Thus, the results suggest that TN-R immunoreactivity does not associate with astrocytes or astrocytic processes in the *Xenopus* tadpole SC.

Figure 3.2. (next page) Immunohistochemical localization of TN-R in the *Xenopus* frog spinal cord. **(A-C)** The myelinated nerve fibers of white matter were labeled by both TN-R and MBP antibodies in the longitudinal section of frog SC. **(D-F)** The TN-R immunoreactivity was also found in the perineuronal net of F3-positive neurons (arrows) in the gray matter of SC. Scale bar: C, 100μm; F, 20μm.

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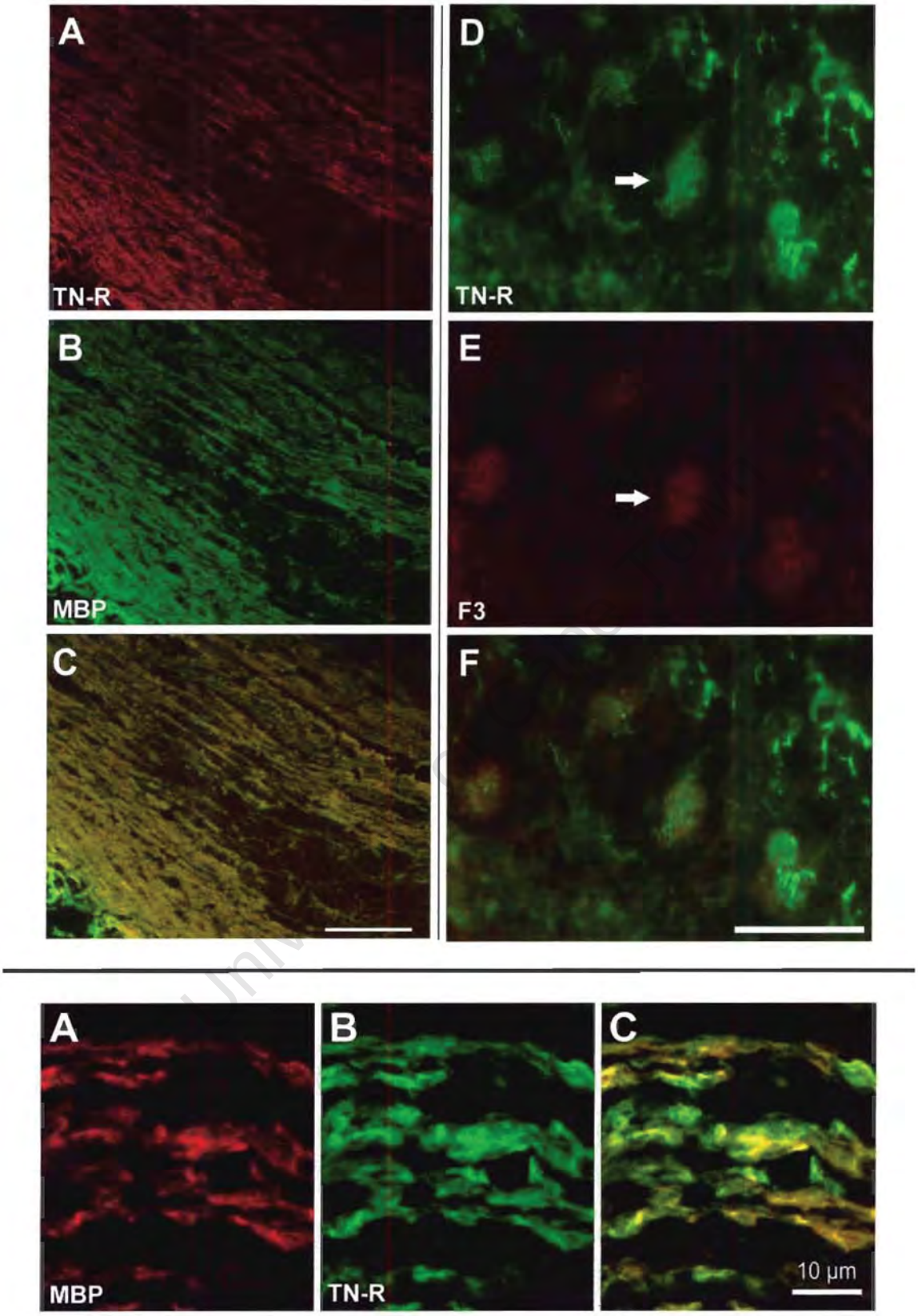

Figure 3.3. The confocal images of immunolocalization of MBP and TN-R in the *Xenopus* tadpole spinal cord. **(A)** The myelinated fibers in whole mount of SC were labeled by anti-MBP. **(B)** The nerve fibers were labeled by pAB janusin. **(C)** When two channels merged, the MBP and TN-R immunoreactivities were colocalized in the myelinated axon. Scale bar: C, 10µm

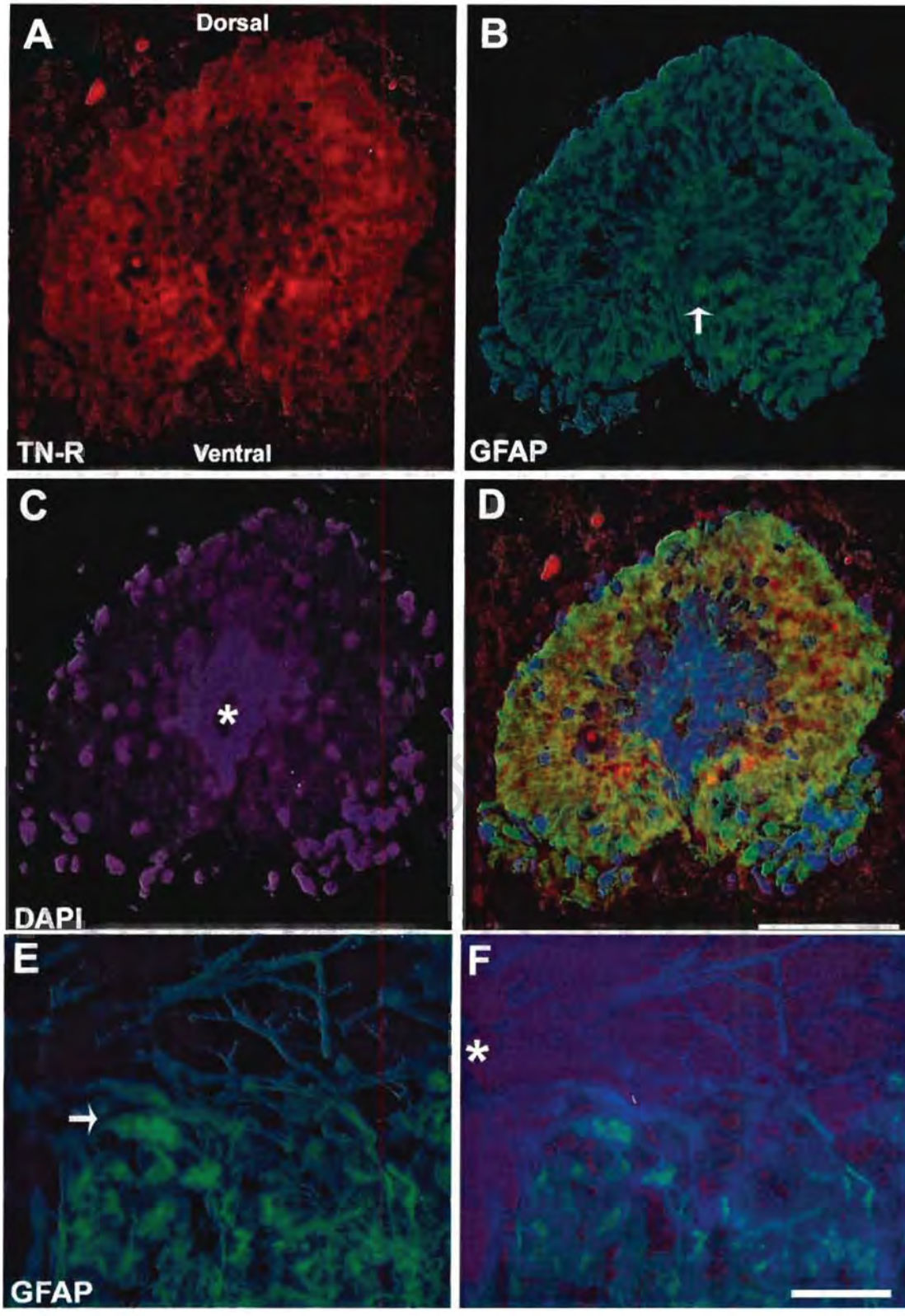

Figure 3.4. Immunohistochemical localization of TN-R and GFAP in the *Xenopus* tadpole spinal cord. **(A-D)** The mAB TN-R was used together with polyclonal GFAP antibody in the cross-section of SC. The nuclei were labeled with DAPI. The multichannel image was shown in (D). The GFAP-positive cells were indicated by arrows (B, and higher magnification in E), and the spinal central canal was indicated by (*). **(F)** The image of E combined with DAPI stain. Scale bar: D, 50μm; F, 10μm.

It has been shown that TN-R is an intrinsic autocrine factor for oligodendrocyte differentiation in mammals (Pesheva et al, 1997). Thus, the expression of TN-R was examined in the early developing SC of *Xenopus* tadpole. At this early stage of development, the spinal cord was not fully developed; the white matter and the gray matter were ill-defined, and consisted only of a few cells. The absence of MBP immunoreactivity in the section of developing tadpole SC indicates that myelination is yet to commence (data not shown).

At this stage of development, the immunoreactivity of GFAP was found in the cross-section of tadpole SC, in which a single large cell with process extension was stained intensively with pAB GFAP and mAB TN-R (Fig. 3.5). This finding of GFAP and TN-R co-expression at early developmental stage is contrary to the results obtained in tadpole SC at later developmental stage, where GFAP and TN-R immunoreactivities were not colocalized (Fig. 3.4A-D). However, it has been shown that the *Xenopus* oligodendrocytes express GFAP in culture (Lang et al., 1995) and it is likely that TN-R expression is found in the $GFAP^+$ oligodendrocyte of developing tadpole SC before myelination.

3.2.2 Expression of TN-R in the Optic Nerve and Retina

Both *Xenopus* tadpole and frog are capable of regenerating optic nerve throughout life. To analyze the expression pattern of TN-R in the *Xenopus* visual system, whole mounts or sections of optic nerve (ON) and eye were stained with either mAB TN-R or pAB TN-R in combination with cell type-specific markers.

The whole mounts of tadpole ON double stained with pAB janusin and MBP antibodies was analyzed using the confocal microscopy (Fig. 3.6). The MBP marker labeled the myelinated fiber tract in the tadpole ON. The immunoreactivity of TN-R was also found in the nerve fibers, co-localized with the labeling of MBP antibody. In addition, TN-R (Janusin) antibody stained in the presumptive oligodendrocyte (arrows in Fig. 3.6), which was not yet fully differentiated as indicated by the absence of MBP immunoreactivity.

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Similar expression patterns of MBP and TN-R antibodies were also found in the adult frog ON. Staining of the longitudinal section of frog ON with MBP antibody revealed dense labeling of myelinated fiber tract, which were also labeled by pAB TN-R (Fig 3.7A-C). In the consecutive section stained by mAB TN-R and GFAP antibody, the staining patterns of mAB TN-R and GFAP were however, different from each other (Fig 3.7D-F). The immunoreactivity of GFAP appeared to be in the glial processes, as well as in the glial somata (arrow in Fig. 3.7E), whereas TN-R immunoreactivity was not detectable in these presumable astrocytic structures. Thus, these findings demonstrate the association of TN-R with oligodendrocyte and myelin in the optic nerves of tadpole and frog.

In the avian and mammalian eye, the immunoreactivity of TN-R has been found at the outer plexiform layer of the retina (Bartsch et al., 1993; Pesheva et al., 1989; Rathjen et al., 1991). Therefore, the distribution of TN-R in the *Xenopus* retina was analyzed. The retinal sections from *Xenopus* frog eyes were stained with various antibodies against TN-R, GFAP, and TN-R interaction partner CSPG (Fig. 3.8). The staining of both mAB TN-R and pAB TN-R showed intensive TN-R immunoreactivity in the outer plexiform layer, which was not labeled by GFAP or CSPG antibody. The GFAP immunoreactivity was found predominately in the cell bodies of glia in the RGC layer, as well as glial processes which dispersed in retinal layers (Fig. 3.8B). The immunostaining of CSPG was mutually exclusive from that of TN-R immunoreactivity in the adjacent retinal layers (Fig. 3.8D-F). In the control experiment without antibodies, fluorescent signals were detected in the photoreceptor layer due to autofluorescence (data not shown). This rules out any non-specific immunoreactivity of CSPG in the photoreceptor layer. The CSPG immunoreactivity was found only in the inner nuclear and RGC layers. These findings indicate the expression of TN-R is localized in the outer plexiform layer of frog retina but not associated with GFAP-positive glial structures, whereas CSPG expression is found in the neighboring inner nuclear layer.

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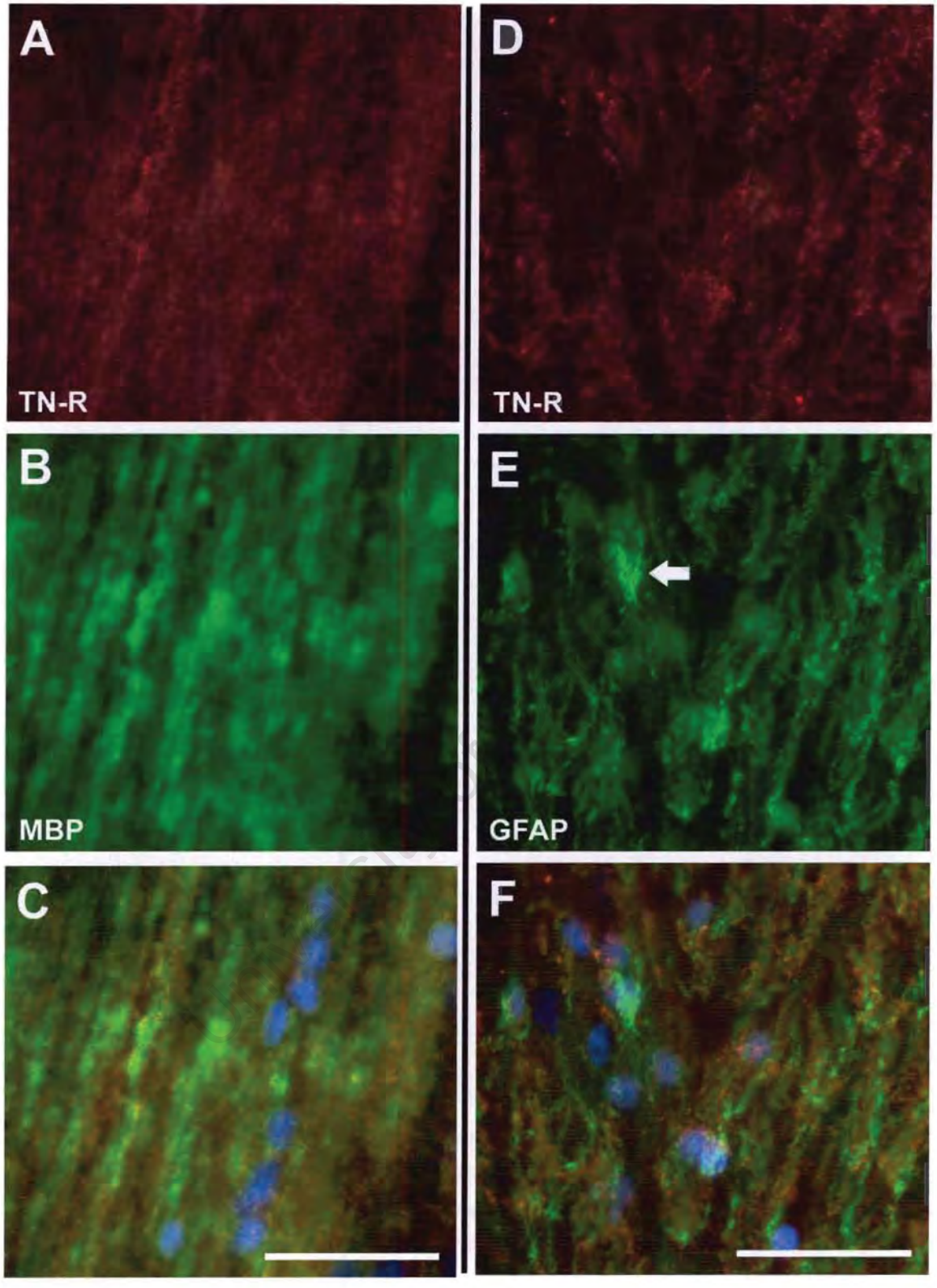

Figure 3.7. Immunohistochemical localization of TN-R in the longitudinal section of *Xenopus* frog optic nerve. **(A-C)** The pAB TN-R and MBP antibody stained the myelinated nerve fibers of frog ON. **(D-F)** The mAB TN-R was counter-stained with GFAP antibody, which labeled astrocytic fibers and cell bodies (indicated by arrow). The images were overlaid with DAPI (C, F). Scale bar: C, F, 20µm.

3.2.3 Post-lesion Expression of TN-R

As previously shown, the obtained immunohistochemistry results from the intact *Xenopus* ON (Section 3.2.2) indicate the expression of TN-R, a mammalian neurite outgrowth inhibitor, in the region of high regenerative plasticity. The regulation of TN-R expression after ON lesion has been studied in adult rat, fish and salamander (Becker C.G. et al., 1999, 2004; Becker T. et al., 2000). To compare the post-injury regulation of TN-R in the *Xenopus* with that in other animals, the expression of TN-R was investigated following ON crush in *Xenopus* frog. The ON crush was performed unilaterally in the anaesthetized frog, thereby setting the unlesioned nerve as control.

Two weeks (Fig. 3.9A) and three weeks after lesion (Fig. 3.9B), the whole mounts of injured ON were double stained with pAB TN-R and MBP marker. The unlesioned nerves were also processed with the same procedure (Fig. 3.9C). No difference was observed in the two weeks and three weeks control nerves (data not shown). Apart from the injury site, the distribution of TN-R immunoreactivity in the lesioned ON appeared to be similar to that in unlesioned nerve at all post-lesion time intervals studied. However, looking at the injured site under higher magnification, the intensity of TN-R immunoreactivity appeared to be slightly higher at the distal end than that at the proximal end (c in Fig. 3.9A-B).

By examining the immunolabeling of MBP two weeks post-lesion nerve, the staining pattern at the proximal end differed from that at the distal end (Fig. 3.9A). Unlike the intact nerve, the staining of MBP at the distal end dramatically changed from a fibril to irregular and granular pattern. The intensity of MBP immunoreactivity at the distal end was also higher than that of the proximal end. When comparing the distribution of MBP immunoreactivity between two weeks and three weeks (b & d in Fig. 3.9A-B), the granular staining pattern became less evident. In addition, the MBP staining was still slightly higher at the distal end three weeks post-lesion.

Although the morphology of MBP-positive structures changed at different post-lesion time intervals, no significant difference in TN-R expression level was

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evident in the corresponding changes of MBP immunoreactivity. These findings show that in the course of frog ON degeneration/regeneration, changes take place in the myelin distribution concerning both morphology and expression of the myelin marker. In contrast, the distribution of TN-R persists in the frog ON after nerve crush.

3.2.4 Expression of TN-R in Oligodendrocyte Culture

Immunohistochemical characterization of TN-R in the CNS of *Xenopus* frog and tadpole indicate that the expression of TN-R is associated with oligodendrocyte and myelin (Section 3.2.2). This was further characterized by double staining with early oligodendrocyte marker O4 and TN-R antibody in the glial culture, which was prepared from frog SC. The cells which migrated from the fragments of spinal cord gave rise to heterogeneous cell populations with distinct morphology, such as the shapes of elongated bipolar, irregular polygonal and highly branched (Fig. 3.10A-B). Occasionally, there were enormously large cells i.e. up to 100μm with extremely long processes extending to unaccountable distance (arrow in Fig. 3.10B). Amongst the different cell populations, the immunoreactivity of O4 was found specifically on the membrane of highly branched cells (Fig. 3.10D), which was also labeled by pAB TN-R (Fig. 3.10C). Thus, the immunoreactivity of TN-R was associated with $O4^+$ oligodendrocyte in CNS glial culture.

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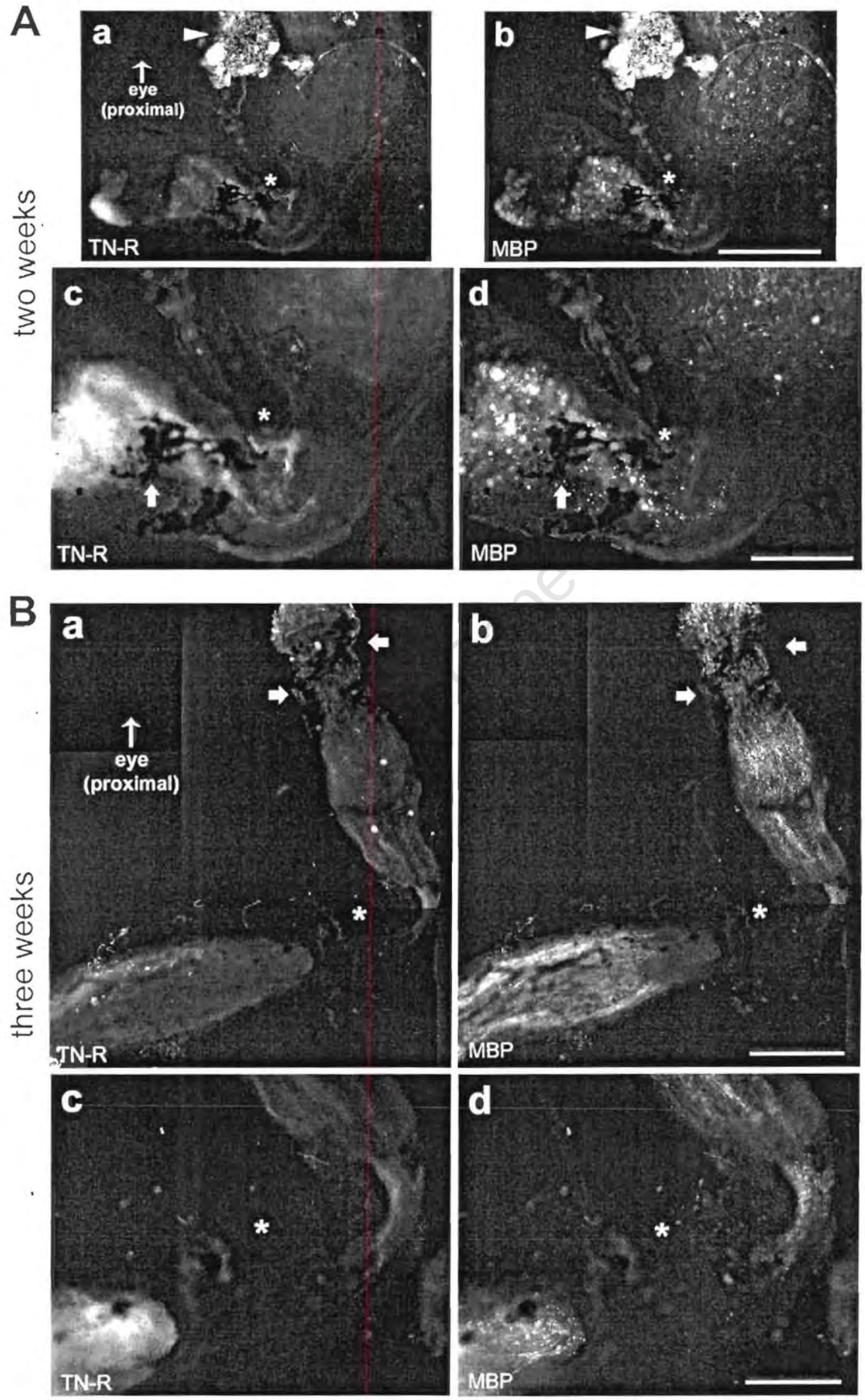
A
a
eye
(proximal)
TN-R
b
MBP
two weeks
c
TN-R
d
MBP
B
a
eye
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b
MBP
three weeks
c
TN-R
d
MBP

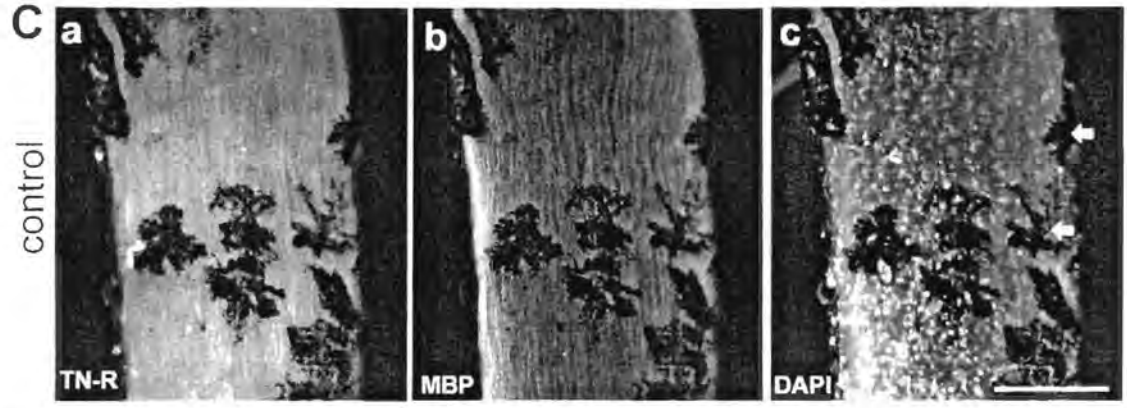

Figure 3.9. Post-lesion expression of TN-R in the whole mounts of frog optic nerve. **(A)** Two weeks post-jury: (a-b) Both TN-R and MBP immunoreactivities were found in the injured ON and weakly at the injury site (*). The arrowheads indicate the non-specific signal from a cluster of dust and crystal. (c-d) Higher magnification images at the injured site (*). **(B)** Three weeks post-injury: (a-b) Both TN-R and MBP immunoreactivities were found in the injured frog ON at the proximal and distal end, but not at the injury site (indicated by *). (c-d) Higher magnification at the injured site (*). **(C)** Control: In the unlesioned control ON, TN-R and MBP antibodies labeled the myelinated nerve fibers. The nuclei were stained by DAPI. In all specimens the pigmented melanocytes were found and indicated by arrows. Scale bars in panels (A) and (B): b, 200μm; d, 100μm. Scale bar in panel (C): c, 100μm.

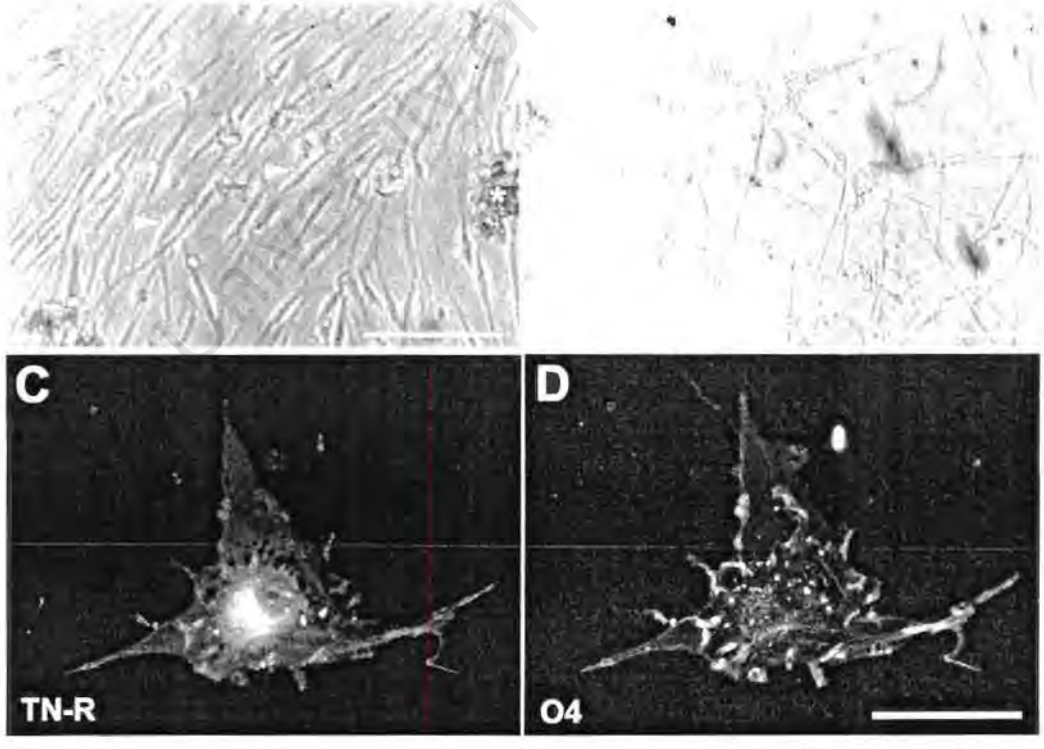

Figure 3.10. Immunohistochemical localization of TN-R in the *Xenopus* glial culture. **(A-B)** Phase contrast images of the glial culture. The different cells migrated from the fragments of frog SC (indicated by *), including elongated bipolar cells (arrowhead) and large branched cells (arrow). **(C-D)** Both pAB TN-R and O4 marker labeled the oligodendrocyte in culture. Scale bar: A, B, 200μm; D, 50μm.

3.3 Expression of TN-R in PNS

The distribution of TN-R was initially thought to be CNS-specific in mammals. Recent findings have also shown the TN-R expression by Schwann cells in the developing mammalian PNS, suggesting TN-R may play a similar role in myelination as in CNS (Probstmeier et al., 2001). To analyze whether TN-R immunoreactivity is present in *Xenopus* PNS as in mammals, the section of *Xenopus* frog DRG with peripheral nerve was stained with pAB TN-R (Fig. 3.11). Weak TN-R immunoreactivity was found in the somata of the ganglia. Around each individual neuron, intensive TN-R immunoreactivity was found in the satellite cells, which were special type of Schwann cell (arrows in Fig. 3.11E). In order to judge whether TN-R immunoreactivity is also localized in PNS myelin, sections were counter-stained PNS myelin marker P0 (Fig. 3.11F-H). The myelinated peripheral nerve fibers exiting the frog DRG were labeled by P0, colocalizing with TN-R immunoreactivity. On the other hand, the connective tissues found in the peripheral nerve section, possibly perineurium or epineurium, were not stained by either P0 or TN-R antibodies (* in Fig. 3.11H). This verified the specificities of these two antibodies. These findings show that TN-R is also expressed in the *Xenopus* frog PNS and associated with myelin and Schwann cells.

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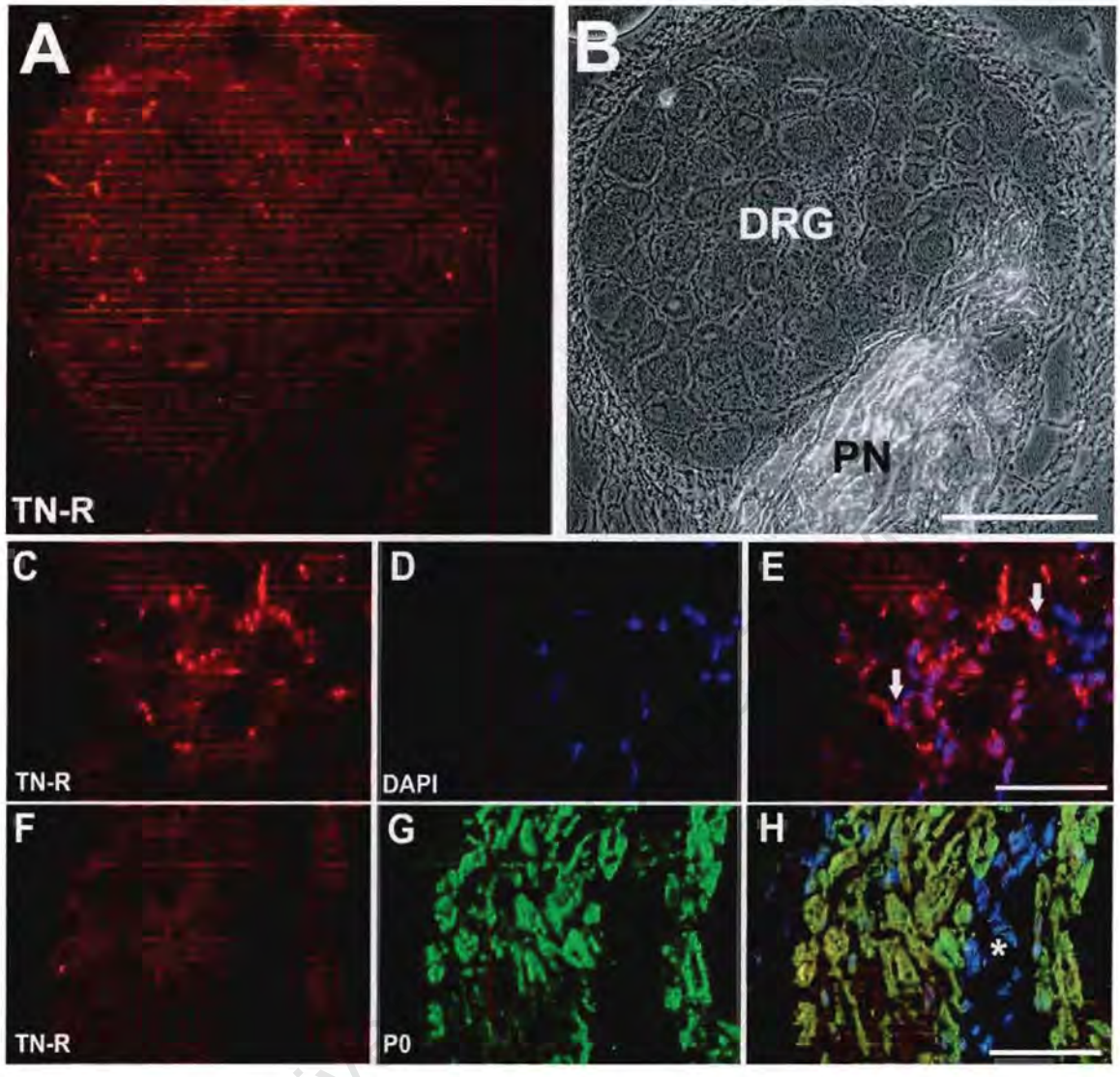

Figure 3.11. Immunohistochemical localization of TN-R in the section *Xenopus* frog DRG. **(A-B)** The section of frog DRG and peripheral nerve fibers were labeled by pAB TN-R. Compare with phase contrast image. **(C-E)** In the higher magnification image of DRG, the immunoreactivity of TN-R was shown in the satellite cells (arrows), together with DAPI stain. **(F-H)** TN-R and P0 labeling were colocalized in the peripheral nerve fibers, but not in the connective tissues (*).The blue nuclear stain was included in multichannel images. *DRG*, dorsal root ganglion; *PN*, peripheral nerve. Scale bar: B, 100μm; E, H, 50μm.

Chapter 4

Expression Analysis of Nogo-A and Nogo-Receptor

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4.1 Identification of Nogo-A in *Xenopus*

Nogo-A is generally accepted as a mammalian neurite outgrowth inhibitor. The expression of Nogo-A have been studied extensively in higher vertebrates (Chen et al., 2000; Huber et al., 2002; O'Neill et al., 2004). To compare the distribution of Nogo-A in *Xenopus* with others, the specificity of the mammalian Nogo antiserum was first tested in the rat and frog material. The Nogo (Bruna) antiserum was generated against a 45kD recombinant rat Nogo-A fragment that recognized all mammalian forms of Nogo. Nogo (Bruna) antiserum has been shown to recognize a protein of about 220kD in bovine myelin and spinal cord extract in immunoblots (Chen et al., 2000). In the present study, anti-Nogo (Bruna) detected a band in the rat brain with molecular weight close to the literature results (Fig. 4.1A). However, no Nogo immunoreactivity was observed in *Xenopus* tissues with this mammalian specific antiserum (data not shown).

In order to recognize the *Xenopus* form of Nogo-A, the Nogo-A (702) antiserum was used instead of Nogo (Bruna) antiserum thereafter. The Nogo-A (702) was generated against a conserved region of Nogo-A peptide sequence in *Xenopus* and shown to be specific in *Xenopus* by previous western blots (Klinger et al., 2004). In the current study, immunoblotting revealed that Nogo-A (702) antiserum recognized a protein just below 230kD in *Xenopus* frog ON (Fig. 4.1B, lane 1), brain (Fig. 4.1B, lane 2) and SC (Fig. 4.1B, lane 3). In the same blot, a band with low molecular weight was also present in the frog ON and SC and was shown later to be non-specific. In addition, Nogo-A (702) also detected a band in the *Xenopus* tadpole CNS (data not shown), similar to that in frog CNS.

The control experiments were performed by omitting primary antibodies, the results showed secondary antibody SAR-HRP (Santa Cruz Biotechnology) did not show any non-specific binding in the brains of frog and rat (Fig. 4.1C, lane 1 and lane 2 respectively). It was however cross-reactive with the frog ON (Fig. 4.1C, lane 3) and SC (Fig. 4.1C, lane 4) shown by the presence of immunoreactive band with low molecular weight. Thus, Nogo-A (702) antiserum specifically recognized Nogo-A protein in the *Xenopus* frog and tadpole with molecular weight close to that in mammals of 220kD.

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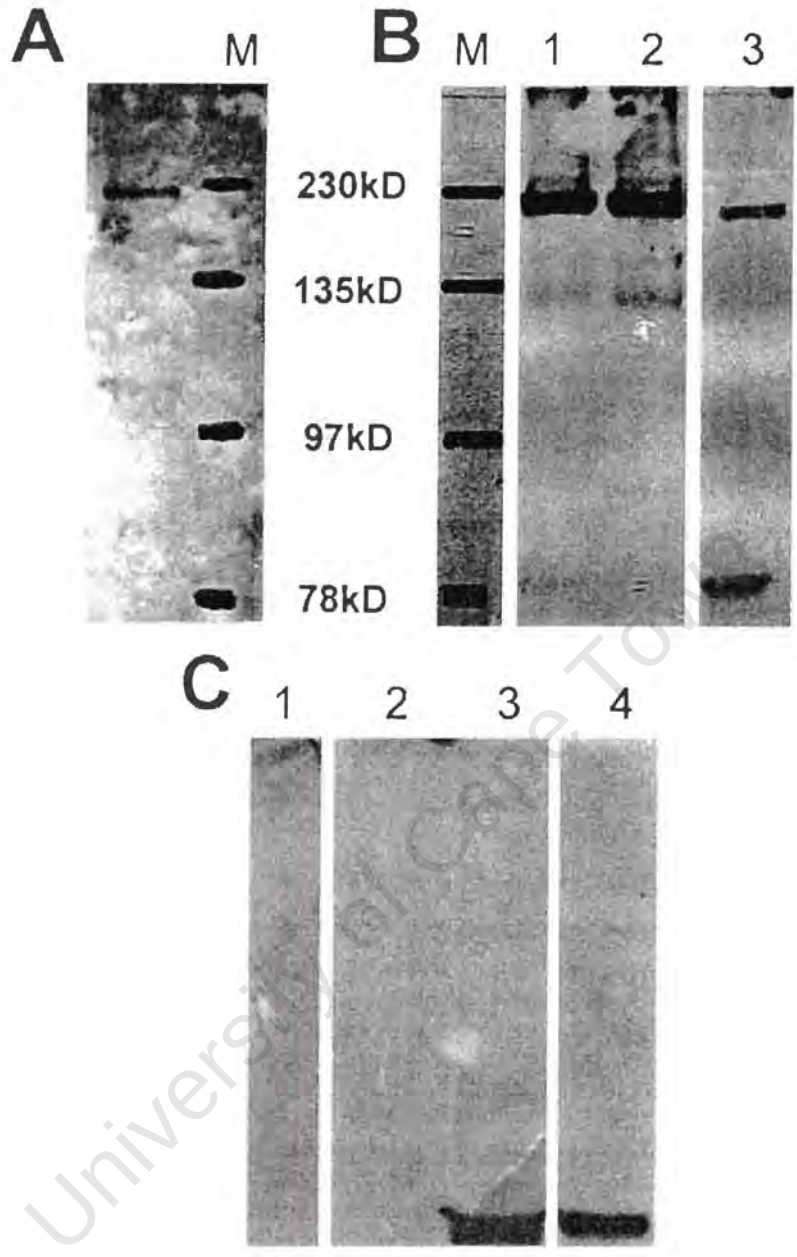

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Figure 4.1. Immunoblot analysis of Nogo-A expression in the rat and *Xenopus* CNS. **(A)** pAB Nogo (Bruna) detected a band slightly below 230kD in rat brain. **(B)** pAB Nogo-A (702) detected bands with molecular weight below 230kD in *Xenopus* frog CNS; ON (lane 1), brain (lane 2), and SC (lane 3). The results also showed a band present with a low molecular weight both in the ON and SC. **(C)** Experiments performed without primary antibodies: frog brain (lane 1), rat brain (lane 2), frog ON (lane 3) and frog SC (lane 4). *M*, molecular weight marker; *kD*, kilodaltons.

4.2 Expression of Nogo-A in Nervous System

Nogo-A is shown to be associated with mammalian CNS myelin and oligodendrocytes (Huber et al., 2002; Hunt et al., 2003; Taketomi et al., 2002). It is also believed to contribute to the failure of mammalian CNS axonal regeneration. However, as previously described in Section 4.1, the immunoblotting results strongly indicate the immunoreactivity of Nogo-A in *Xenopus*. To further analyze the expression pattern of Nogo-A protein in the *Xenopus* frog and tadpole, immunohistochemistry was performed using the same polyclonal Nogo-A (702) antiserum as tested in the immunoblots.

4.2.1 Distribution of Nogo-A in Brain and Spinal Cord

To establish an overview of Nogo-A expression in the *Xenopus*, staining of Nogo-A (702) antiserum in conjunction with DAB detection method was performed in the longitudinal section of frog SC. The immunoreactivity of Nogo-A was found in both white matter (W in Fig. 4.2) and gray matter (G in Fig. 4.2). In the white matter, intensive labeling of Nogo-A (702) antiserum was found in different diameters of axon fibers and scattered cell bodies. In the gray matter, the immunoreactivity of Nogo-A was detected strongly in the neuronal somata, judging from the morphology and localization of the frog SC.

To clearly outline the white matter, the myelin marker MBP was used together with Nogo-A (702) antiserum in the cross-section of the frog SC (Fig. 4.3). The staining of MBP revealed dense labeling of myelinated fiber tracts in the white matter, while Nogo-A (702) antiserum labeled in both gray and white matter similar to that in the longitudinal section. In the ventral horn of the gray matter, strong Nogo-A expression was found in the somata of motoneurons. In the white matter, Nogo-A immunoreactivity showed intensive labeling of presumable oligodendrocytes and axons.

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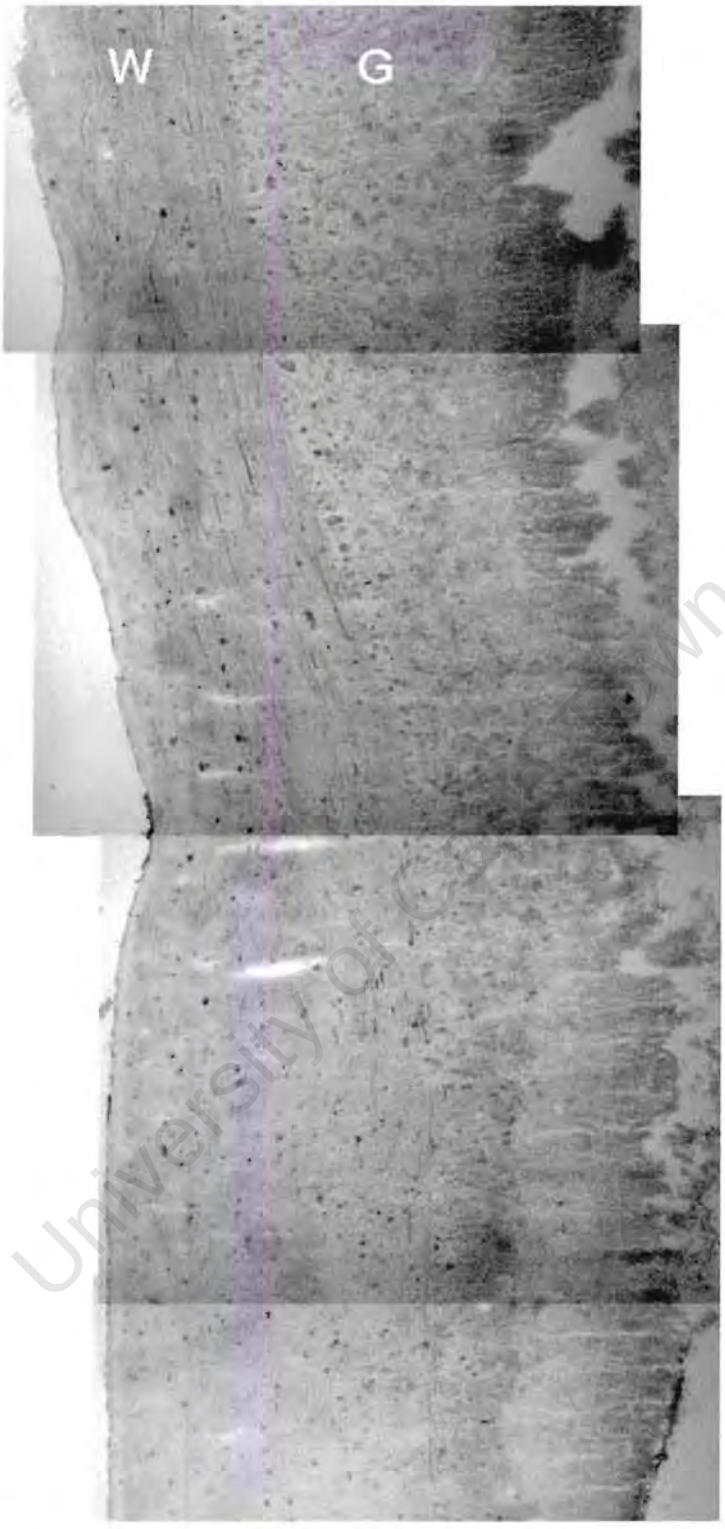

Figure 4.2. An overview of immunolocalization of Nogo-A in the *Xenopus* frog spinal cord. The DAB detection method has shown the immunoreactivity of Nogo-A in both gray matter (indicated as **G**) and white matter (indicated as **W**) of longitudinal frog SC section.

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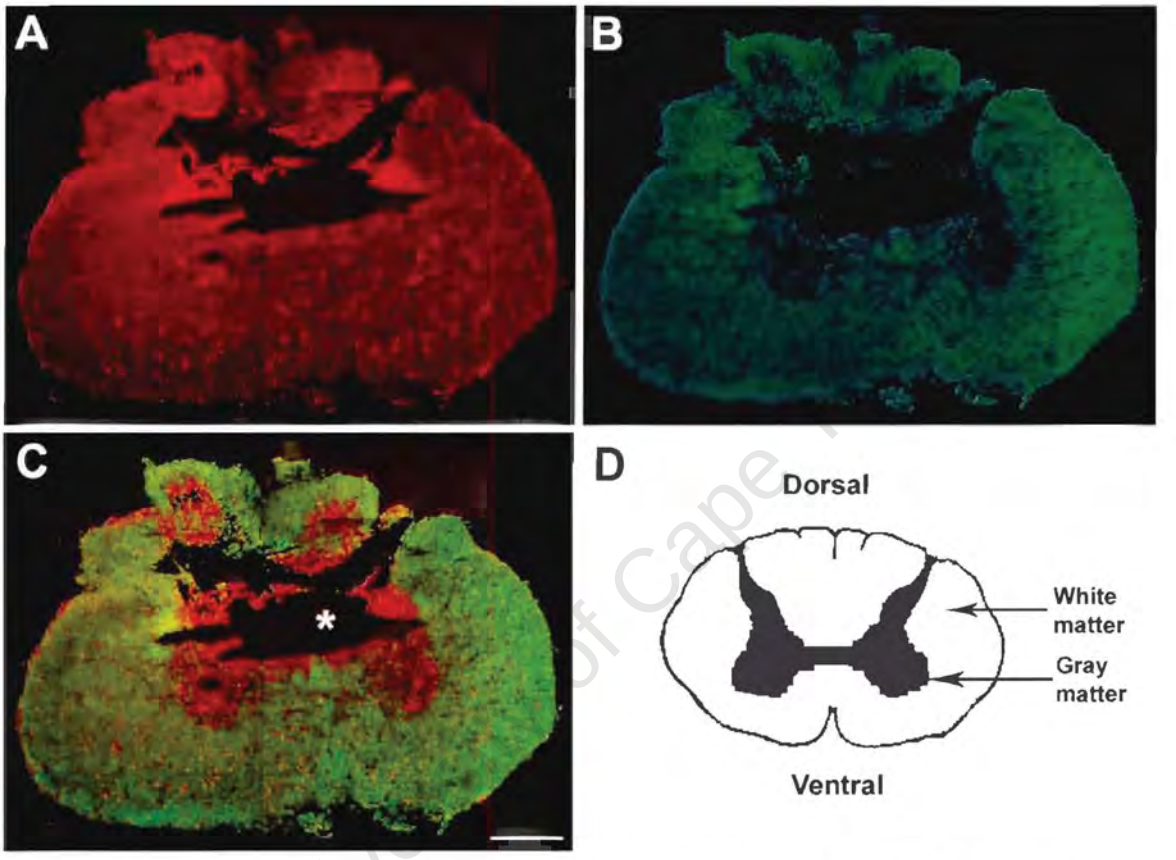

Figure 4.3. An overview of immunolocalization of Nogo-A and MBP in the spinal cord of *Xenopus* frog. **(A)** Labeling of Nogo-A (702) antiserum and **(B)** MBP in the cross-section of frog SC. **(C)** The merged image of two fluorescent channels. The space was a technical artifact indicated by (*). **(D)** Schematic diagram of cross-section SC. Scale bar: C, 200μm.

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To further analyze the expression of Nogo-A in the *Xenopus* frog, Nogo-A (702) antiserum was used in parallel with various cell-type specific markers (Fig. 4.4). In the longitudinal section of the frog SC, double staining of the myelin marker MBP and Nogo-A (702) revealed that the immunoreactivity of Nogo-A was found in the myelinated nerve fibers labeled by anti-MBP (Fig. 4.4A-C). The staining of the neurofilament NF antibody also revealed dense labeling of nerve fibers in the white matter of SC, but less intense in the gray matter (Fig. 4.4E). However, in the ventral region of SC gray matter, large neurons were found which were strongly labeled by Nogo-A (702) antiserum (arrow in Fig. 4.4D). Among these fiber tracts, Nogo-A immunoreactivity showed up intensively in the scattered cells, which exhibit the morphology of oligodendrocyte with processes (arrowheads in Fig. 4.4). Although these presumable oligodendrocytes were MBP negative, no NF immunoreactivity was found either (Fig. 4.4G-I). The high power confocal images of whole-mount SC, containing 3-D information, revealed that the immunoreactivity of Nogo-A was found in the highly branched cell not labeled by anti-NF (Fig. 4.4M). This finding verified the glial expression of Nogo-A. In addition, the ependymal cells at the spinal central canal were also labeled by Nogo-A (702) antibody (Fig. 4.4J-L), but not by anti-MBP or anti-NF (data not shown).

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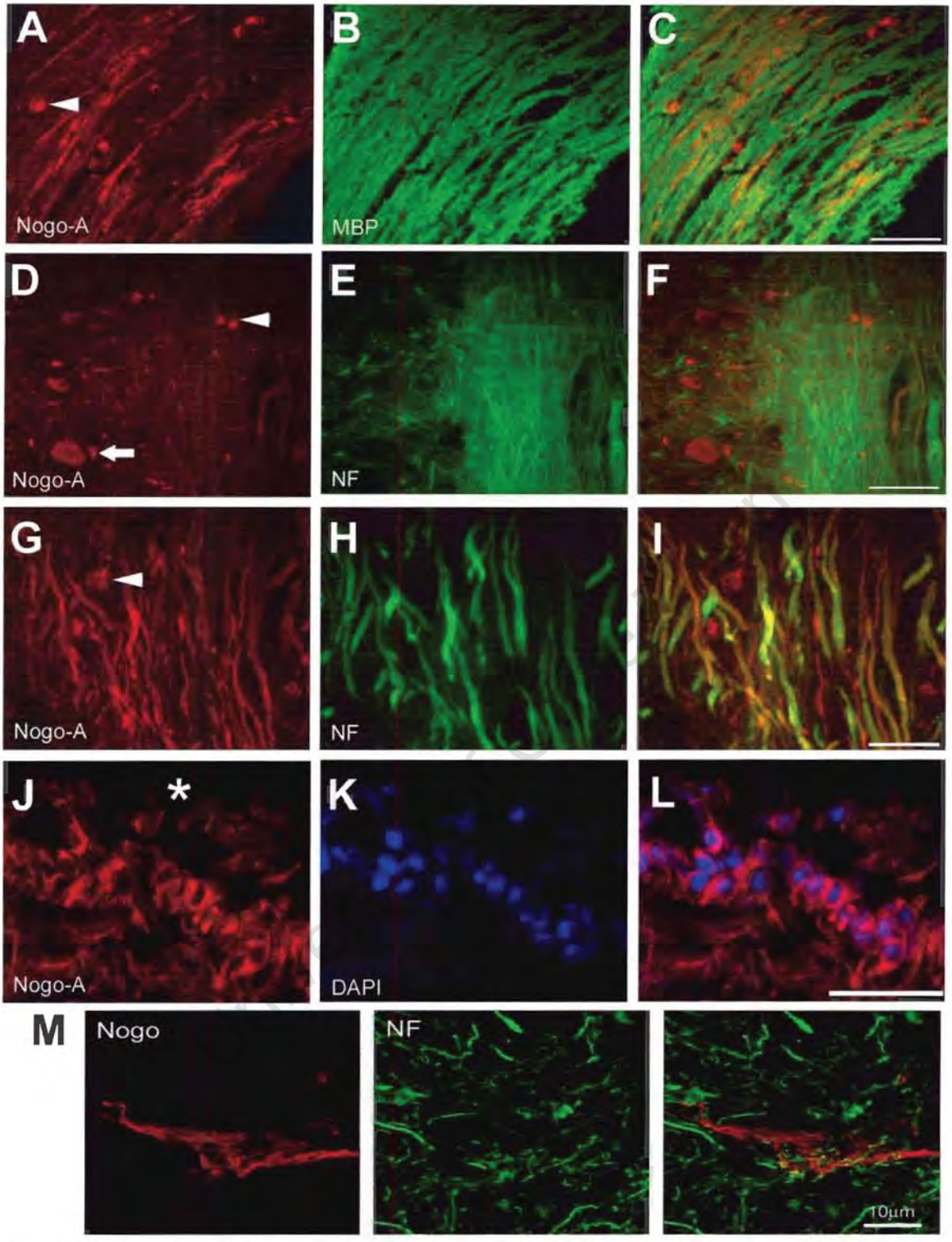

Figure 4.4. Immunohistochemistry for Nogo-A and cell-type specific markers in adult *Xenopus* spinal cord. In the longitudinal section of frog SC, **(A-C)** the staining patterns of Nogo-A (702) antiserum and myelin marker MBP; **(D-I)** the staining patterns of Nogo-A (702) antiserum and the neurofilament marker NF. The immunoreactivity of Nogo-A was found in the oligodendrocytes (arrowheads) and the neuronal somata (arrow in D). **(J-L)** At the spinal central canal (indicated by * in J), Nogo-A expression was found in the ependymal cells, whose nuclei were labeled with DAPI. **(M)** Confocal images of the Nogo-A and NF expressions in the whole-mount of SC. Scale bar: C, F, 50μm; I, L, 20μm; M, 10μm.

In *Xenopus*, metamorphosis alters the regenerative ability (Beattie et al, 1990), and therefore the expression pattern of Nogo-A was also studied in the cross-section of tadpole SC (Fig. 4.6). These sections were obtained by cutting through the tail of the tadpole, in which the DAPI stain labeled the nuclei of the nervous tissues and the surrounding connective tissue. Nogo-A immunoreactivity was shown exclusively in the SC, but not in the surrounding connective tissues. Thus this rules out non-specific binding of Nogo-A (702) antiserum. High level of Nogo-A immunoreactivity was found in the large neurons (arrow in Fig. 4.5D) and smaller scattered cells, as well as the dorsal root ganglion (DRG) adjacent to the SC (Fig. 4.5G-I). Staining of MBP revealed partial labeling of the tadpole SC, where presumptive white matter was found (Fig. 4.5B). MBP immunoreactivity was found mostly in the fiber tracts of ventral region and gradually decreased to the dorsal region. Despite the intense MBP labeling, the fiber tracts were weakly stained by NF (Fig. 4.5K). However, high NF expression was found in the cross-section of the Mauthner axon, which was also labeled by Nogo-A (702) antiserum (Fig. 4.5M-O). Moreover, the oligodendrocytes with their processes around the Mauthner axon were strongly Nogo-A positive (arrowheads in Fig. 4.5).

The above results described the immunostaining in the tadpole SC and indicate that the expression of Nogo-A correlates only partially with myelin. Thus, the expression of Nogo-A was further investigated in the non-myelinated regions of tadpole brain (Fig. 4.6), where MBP immunoreactivity was absent (data not shown). The staining of Nogo-A (702) was much weaker in the optic tectum and fourth ventricle than in the rest of the brain (Fig. 4.6B-D). This difference could be due to the high density of nuclei in the regions labeled by DAPI. Staining of Nogo-A (702) and NF antibodies in the whole mount of tadpole brain revealed the colocalization and labeling of neuronal fibers and somata in the confocal images (Fig. 4.6E).

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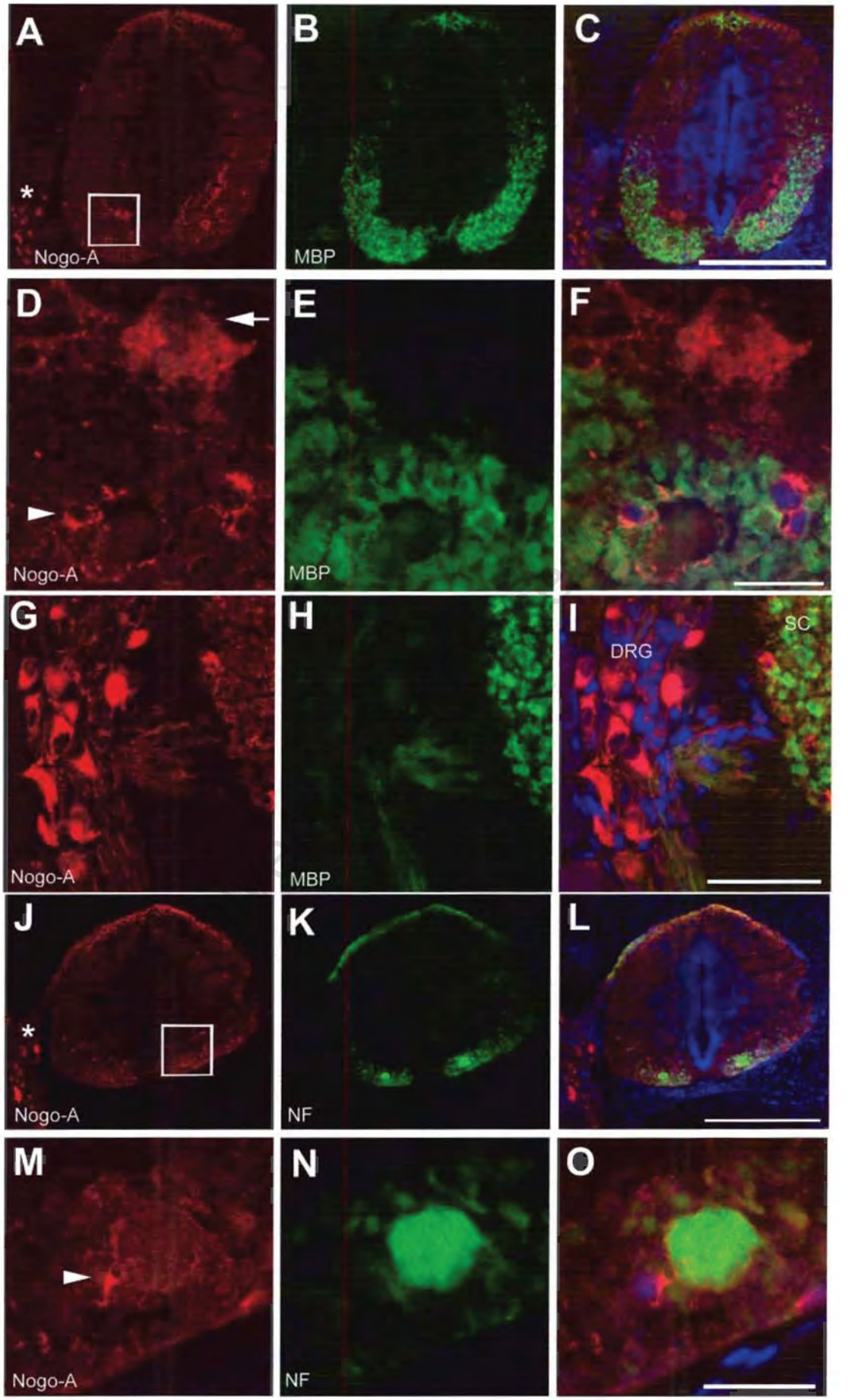
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Figure 4.5. (previous page) Nogo-A immunoreactivity in the transverse sections of *Xenopus* tadpole spinal cord. All multichannel images include nuclear stain. **(A-C)** The Nogo-A (702) antiserum was counter-stained with anti-MBP. **(D-F)** High magnification images of boxed region in (A). Nogo-A (702) antiserum stained strongly in the motoneuron (arrow) and oligodendrocytes (arrowheads). **(G-I)** High magnification images of tadpole DRG (from * in A). Apart from the SC, the DRG was also strongly labeled by anti-Nogo-A. Anti-MBP labeled weakly the nerve fibers at the DRG. **(J-L)** The Nogo-A (702) antiserum was counter-stained with NF antibody. **(M-O)** High magnification image of boxed region in (J). The Mauthner axon was labeled by anti-NF. Scale bar: C, L, 200μm; I, 50μm; F, O, 20μm.

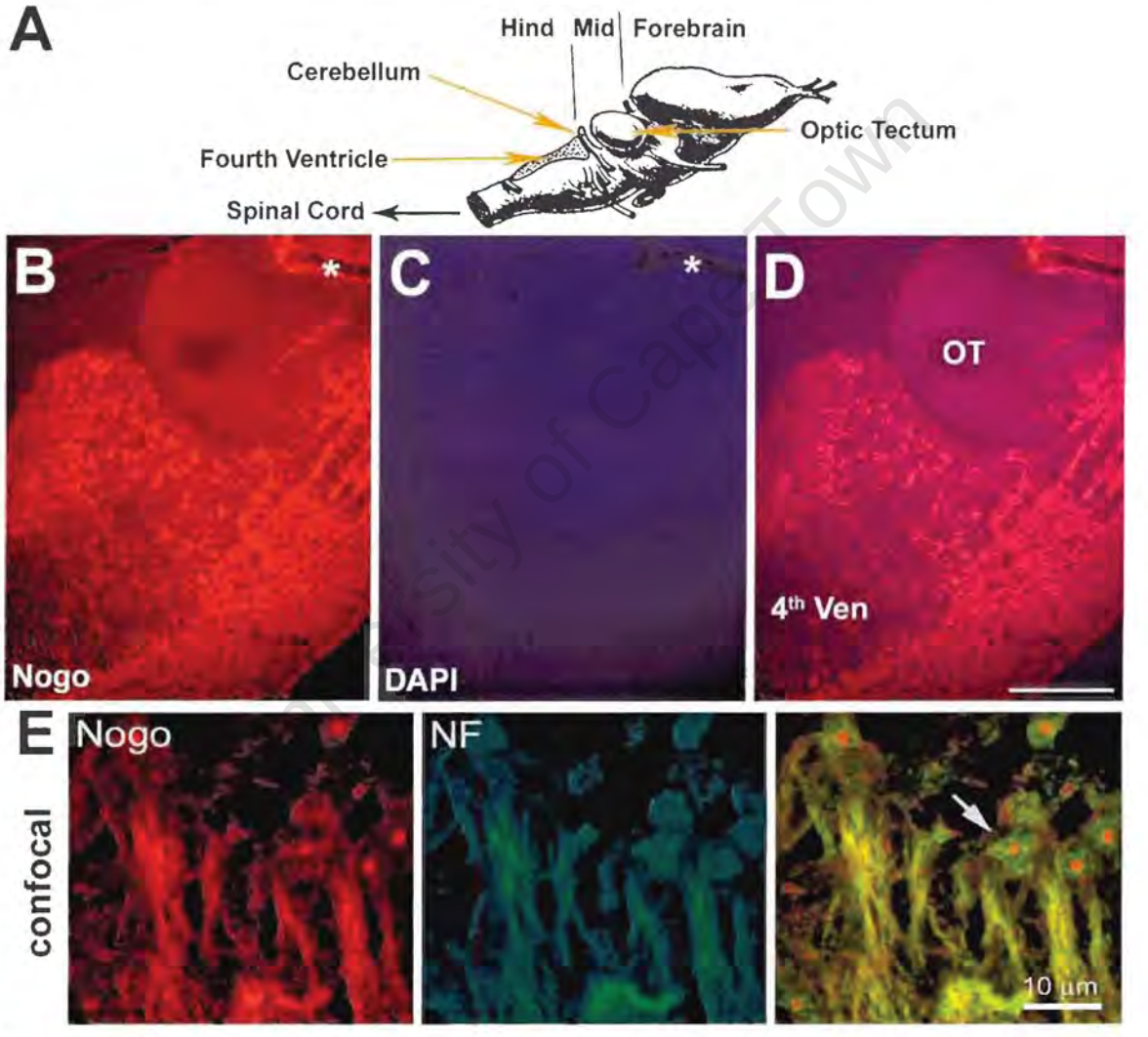

Figure 4.6. Immunolocalization of Nogo-A in the *Xenopus* tadpole brain. **(A)** Schematic diagram of lateral view of brain regions. **(B-D)** The longitudinal section of tadpole brain was labeled Nogo-A (702) antiserum. The expression of Nogo-A is found in the OT and hindbrain, where the nuclei were densely labeled by DAPI. There was non-specific fluorescent signal from a piece of thread (indicated by *). **(E)** Confocal image of Nogo-A and NF immunoreactivities in the whole mount of tadpole forebrain. The colocalization was found in the cell bodies (arrow) and fibers of the neurons. *4th Ven*, fourth ventricle; *OT*, optic tectum. Scale bar: D, 10μm; G, 50μm.

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The immunoreactivity of Nogo-A in the tadpole CNS has so far indicated the neuronal expression of Nogo-A in the developing nervous system. Therefore, it is of interest to investigate the expression of Nogo-A in the much earlier developmental stage where the myelination has not yet commenced. This can be shown by the absence of MBP immunoreactivity (data not shown). The transverse section of the early developing tadpole SC consisted of only few cells. The immunoreactivity of Nogo-A was found strongly among these cells demonstrating the morphology of neurons (Fig. 4.7), either presumptive neurons or neural progenitor cells. These findings suggest the Nogo-A expression is found even at the very early stage of nervous system development and associated with neurons before the myelination.

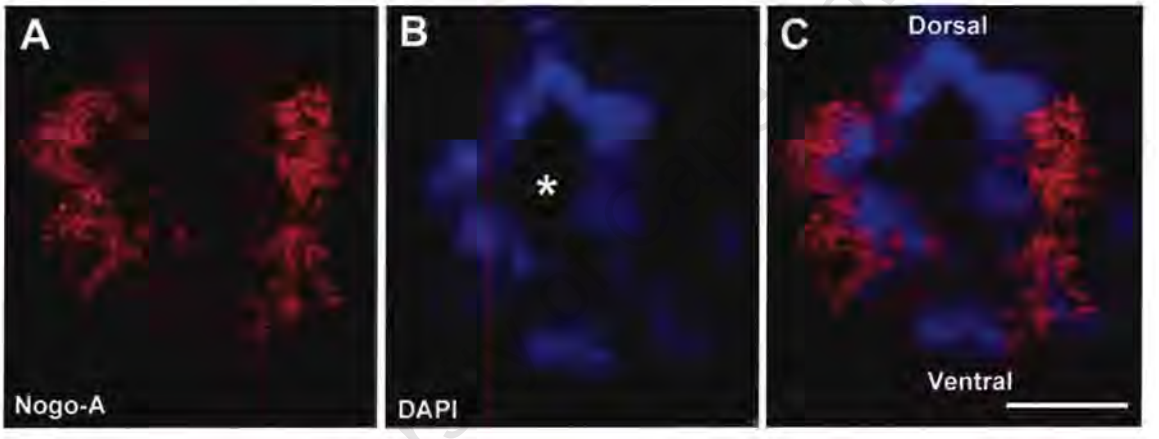

Figure 4.7. Immunoreactivity of Nogo-A in the early developing spinal cord. **(A)** The cross-section of developing SC was labeled by Nogo-A (702) antiserum, and analyzed using ApoTome Imaging System. **(B)** The image of nuclear DAPI stain was captured using the conventional fluorescent microscope. Spinal central canal was indicated by (*). **(C)** The merged image. Scale bar: 20μm.

4.2.2 Nogo-A in Myelinated Tract of Optic Nerve

Both *Xenopus* frogs and tadpoles are capable of regenerating the optic nerve (ON) after injury, hence the expression patterns of Nogo-A were examined in both frog and tadpole ONs. The immunostaining results described in the frog SC (Section 4.2.1) indicate the expression of Nogo-A in the myelinated fiber tracts. To study whether this association is also reflected in the frog ON, the myelinated fiber tracts were identified by myelin marker MBP as well as neurofilament NF antibody in the longitudinal sections of frog ON (Fig. 4.8).

Both MBP and NF antibodies labeled densely in the myelinated fiber tract throughout the section. The immunoreactivity of Nogo-A was colocalized with MBP and NF immunoreactivity in frog ON (Fig. 4.8A-F). In addition, the staining of Nogo-A (702) antiserum also revealed intensive labeling of cell bodies with foot processes representing oligodendrocytes (arrows in Fig. 4.8A-F). These presumable oligodendrocytes were not labeled by either MBP or NF antibodies. To further study these Nogo-A expressing cells in the frog ON, whole mounts were immunostained with Nogo-A (702) and anti-NF, and then analyzed using confocal microscopy (Fig. 4.8G). The immunoreactivity of Nogo-A was found in the cells scattered among the fibers and their processes were extended onto the nerve fibers (arrows in Fig. 4.8G). The NF antibody stained strongly in the nerve fibers, but not in these Nogo-A expressing cells presumed oligodendrocytes. Therefore, these findings have indicated that Nogo-A immunoreactivity is found in the oligodendrocytes and myelinated nerve fibers of frog ON.

Similarly, the staining patterns of Nogo-A (702) and myelin marker MBP were found in the whole-mount of tadpole ON (Fig. 4.9). The staining of anti-MBP labeled the myelinated tracts of tadpole ON and colocalized with Nogo-A immunoreactivity. However, unlike the frog ON, the immunoreactivity of MBP was detected in the oligodendrocytes of the tadpole ON colocalizing with the Nogo-A (702) staining (arrow in Fig. 4.9). This result supports the identity of Nogo-A expressing cells in the ON as oligodendrocytes. Therefore, the expression of Nogo-A is associated with myelinated nerve fibers and oligodendrocytes in the tadpole ON.

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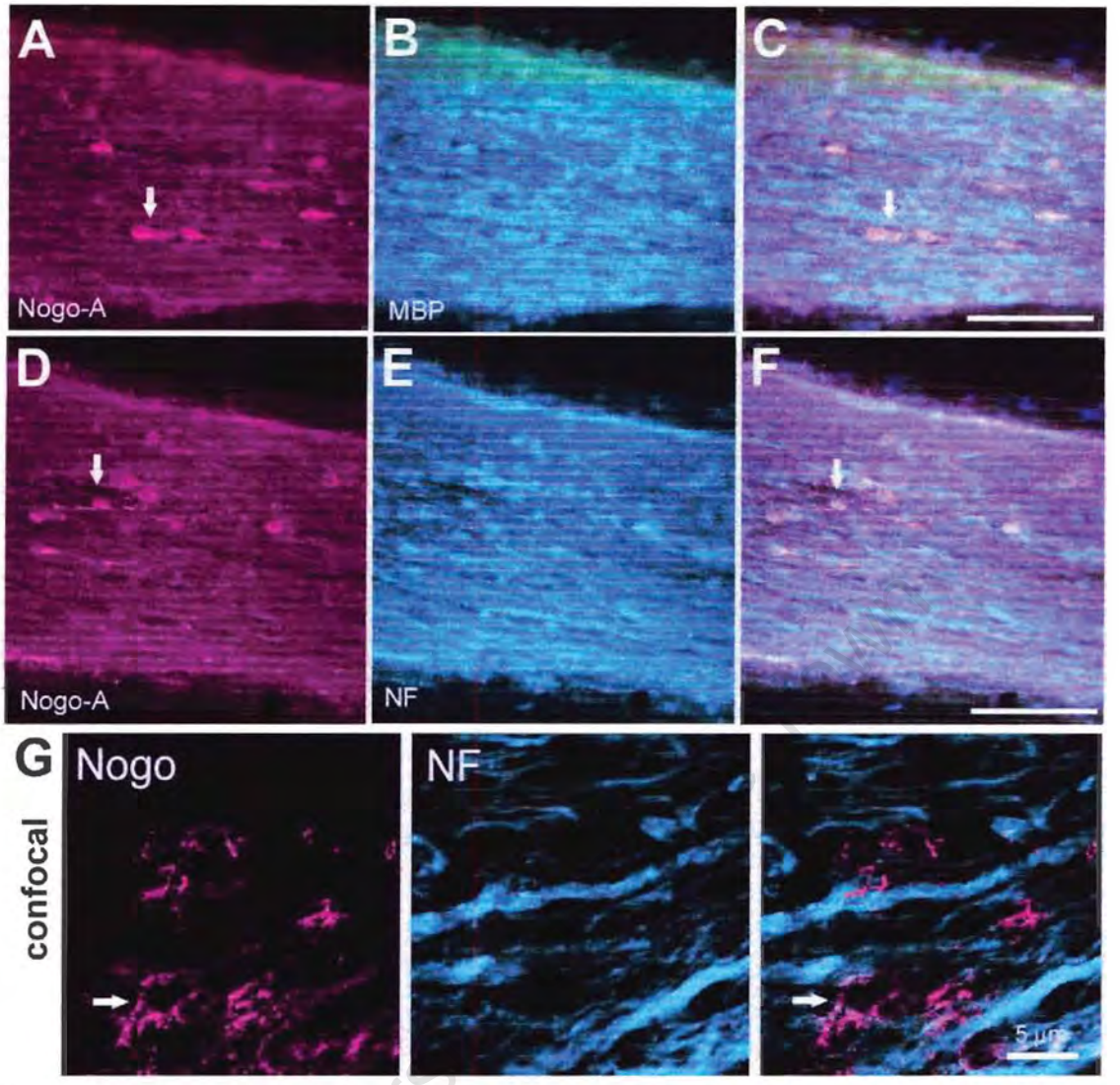

Figure 4.8. Immunohistochemistry for Nogo-A, MBP and NF in *Xenopus* frog optic nerve. **(A-C)** In the longitudinal section of frog ON, myelin marker MBP labeled the myelinated fibers, while Nogo-A (702) labeled the oligodendrocytes (arrow) and the fibers. **(D-F)** Both Nogo-A and NF immunoreactivities were found in the nerve fibers. The Nogo-A (702) also stained the oligodendrocyte (arrow). **(G)** In the confocal images of Nogo-A and NF in the whole mount of *Xenopus* frog ON, Nogo-A was expressed in the NF-negative cells with processes (arrows). (C and F) Multichannel images with blue nuclear stain. Scale bar: C, F, 50µm; G, 5µm.

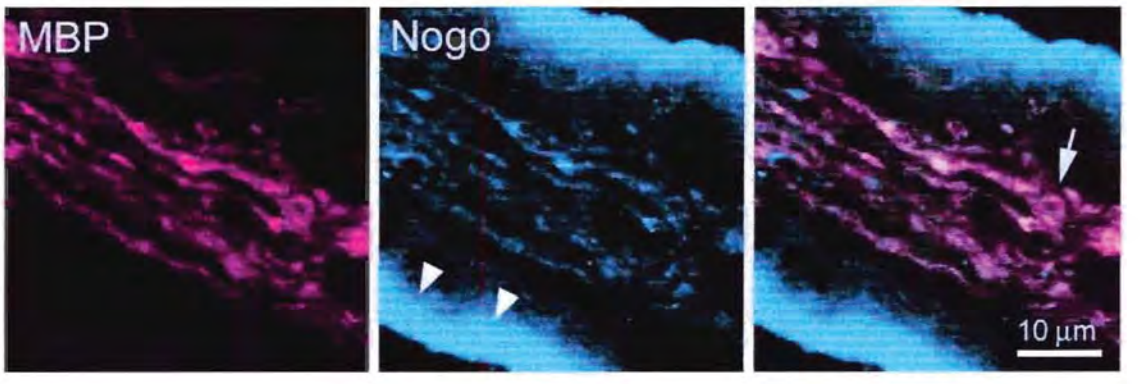

Figure 4.9. (previous page) Confocal images of Nogo-A and MBP in the *Xenopus* tadpole optic nerve. The immunoreactivity of Nogo-A was colocalized with MBP-positive myelinated fibers in the whole mount of tadpole ON. Both MBP and Nogo-A (702) antibodies also labeled oligodendrocyte (arrow). The nerve sheath was labeled non-specifically (arrowheads).

4.2.3 Comparison of Nogo-A Expressions in Optic Nerve and Spinal Cord

The results of the immunostaining experiments described in Section 4.2.1 and Section 4.2.2 indicate that Nogo-A is associated with myelin tracts and expressed in both SC and ON of frogs. However, the regenerative plasticity of the frog ON is different from the SC (Lang et al., 1995). To verify whether the difference would be reflected in the intensity of the Nogo-A immunoreactivity, the data of immunostaining experiments carried out in frog SC was compared side by side with that of frog ON (Fig. 4.10).

Though the immunoreactivity may depend on the amount of protein present and many vary from tissue to tissue, the camera attached on the fluorescent microscope will automatically adjust the exposure time to detect the fluorescent signals from the antibody. Since the absolute values for the intensity of immunoreactivities cannot be measured, the camera exposure time can be used as an indication of immuno-intensity, i.e. longer the exposure time, the weaker fluorescent signal, which indicates the weaker intensity of the immunoreactivity.

In the immunostaining experiment, sections of frog SC and ON were immunolabeled with both Nogo-A (702) and MBP antibodies and their camera exposure time was recorded as a reference of immuno-intensity. In an attempt to have a direct comparison of the immunoreactivity, the time of exposure for the staining of Nogo-A (702) in the sections of frog SC and ON was set at the same time (Fig. 4.10A&C). The immunoreactivity of Nogo-A was detected in the oligodendrocytes (arrows in Fig. 4.10) and myelinated tracts of both SC and ON and immuno-intensity at the same exposure time was stronger in the SC (Fig. 4.10A-D). Moreover, the recorded auto-exposure time for Nogo-A immunoreactivity was also shorter in the SC, i.e. higher intensity in the SC (Fig.

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4.10 E-F). Similarly, MBP immunoreactivity was also more intense in the frog SC. To sum up, these findings implicate that frog SC showed higher intensity of Nogo-A immunoreactivity than the ON. Furthermore, the comparison of MBP immunoreactivity intensities demonstrates the myelin is more compact and denser in the SC than in the ON.

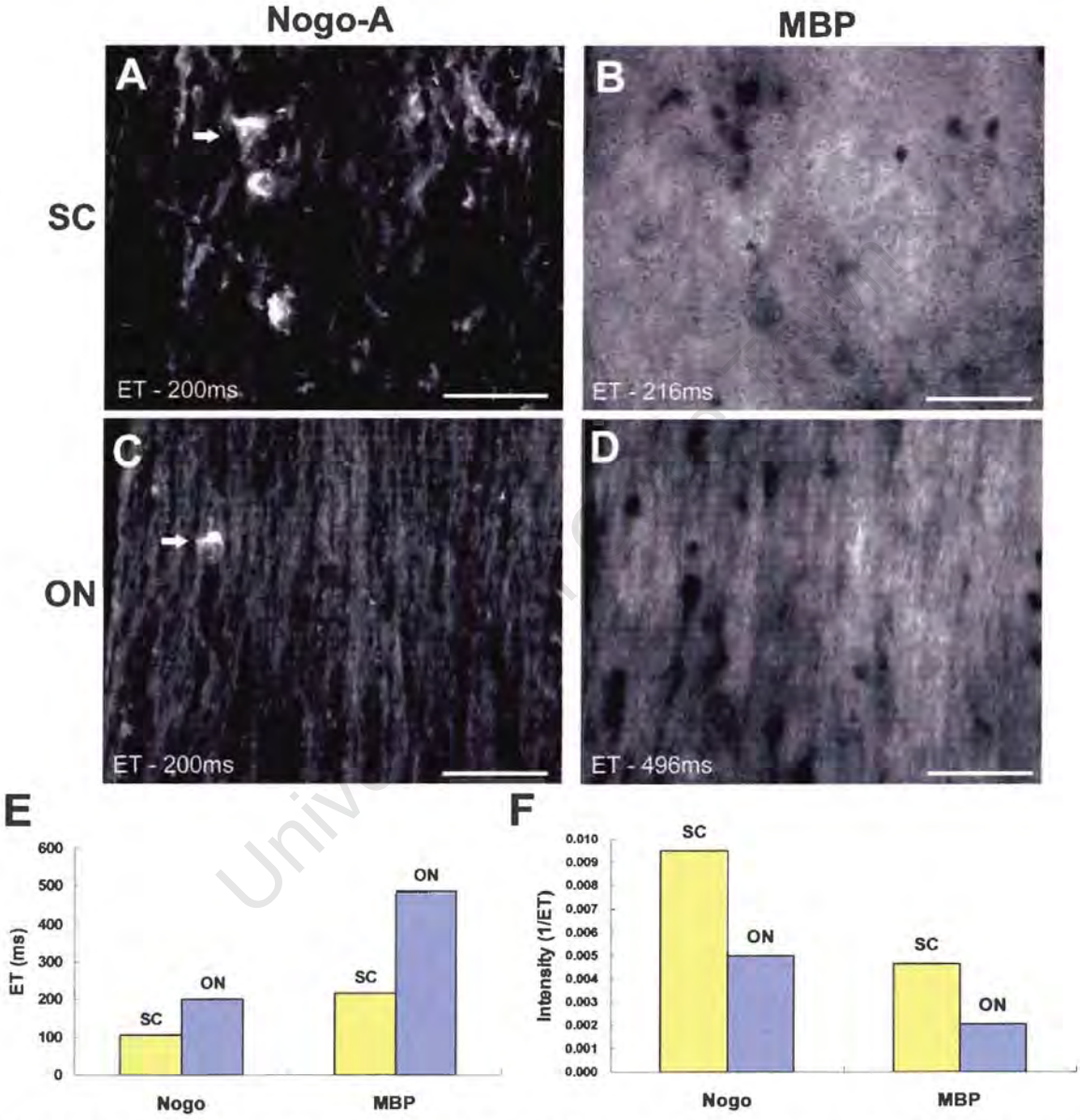

Figure 4.10. Comparison of Nogo-A and MBP immunoreactivities in *Xenopus* frog optic nerve and spinal cord. **(A-D)** Both Nogo-A (702) antiserum and myelin marker MBP labeled the myelinated fiber tracts in the longitudinal sections of frog SC and ON. The camera exposure time for detecting Nogo-A and MBP immunoreactivities was set for 200ms and auto-exposure respectively. High level of Nogo-A immunoreactivity was found in the oligodendrocytes (arrows). **(E)** The auto-exposure time for Nogo-A and MBP immunoreactivity was compared in the graph. **(F)** Bar chart representing relative Nogo-A and MBP immunoreactivity intensity. *ET*, exposure time; *SC*, spinal cord; *ON*, optic nerve. Scale bars: A-D, 50μm.

4.2.4 Post-lesion expression of Nogo-A

The results of mammalian post-lesion experiments have demonstrated that the application of the antibody against Nogo-A enhances the axonal regeneration in the mammalian CNS (Brösamle et al., 2000; Merkler et al., 2001; Thallmair et al., 1998). In the present study, the immunostaining results indicate the expression of Nogo-A in *Xenopus*. In particularly, the adult and tadpole optic nerves are both capable of regeneration after injury (Gaze, 1970). Therefore, the success of axonal regeneration in frog and tadpole ON may be due to the downregulation of Nogo-A after injury. To examine whether the post-injury expression of Nogo-A was regulated and correlated with the *Xenopus* regenerative capacity, the distribution patterns of Nogo-A were analyzed in the lesioned nerves of both *Xenopus* tadpole and frog.

The tadpoles received an ON crush unilaterally. After 5 days post-injury, the whole-mount of lesioned ON was double labeled with Nogo-A (702) and MBP antibodies (Fig. 4.11 A-B). The immunoreactivities of Nogo-A and MBP were found on the eye-side and brain-side of ON. However, the anti-MBP staining was hardly found around the injured site (indicated by *) where the Nogo-A (702) staining was weakly labeled.

In order to achieve better details on the morphological changes, higher magnification images were taken with the aid of ApoTome system on the fluorescent microscope. This system eliminates out-of-focus information and enhances the sharpness of the image at each individual focal plane. When a stack of ApoTome images were taken at different depths of optical section and combined, the image of extended focus demonstrated clearer overviews of Nogo-A or MBP immunoreactivity in the specified regions of ON (Fig. 4.11C).

At the eye-side of the injured ON, the myelinated nerve fibers were stained by the anti-MBP (Fig. 4.11C, panel-I). The fibers were also strongly labeled by Nogo-A (702) antiserum. However, these fibers, especially the MBP-positive nerve fibers disappeared before the lesion site (Fig. 4.11C, panel-II). This indicates the disappearance of myelin or myelinated axons at the lesion site. In

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contrast to the staining patterns at the proximal end, the staining of Nogo-A (702) and MBP antibodies demonstrated a much weaker and irregular labeling of fibers around the injury site (Fig. 4.11C, panel-III). Moreover, numbers of Nogo-A positive cells with processes were found around the lesion site. At the distal end, MBP immunoreactivity reappeared in the fibers (Fig. 4.11C, panel-IV). The staining pattern of Nogo-A (702) antiserum persisted in the presumed oligodendrocytes (arrow in Fig. 4.11C) and much less in the fibers.

These findings indicate that there is demyelination of the lesioned nerve and an activation of Nogo-A expressing oligodendrocytes following tadpole ON crush. Therefore, this post-lesion distribution of Nogo-A immunoreactivity does not implicate a downregulation of Nogo-A expression after tadpole ON injury.

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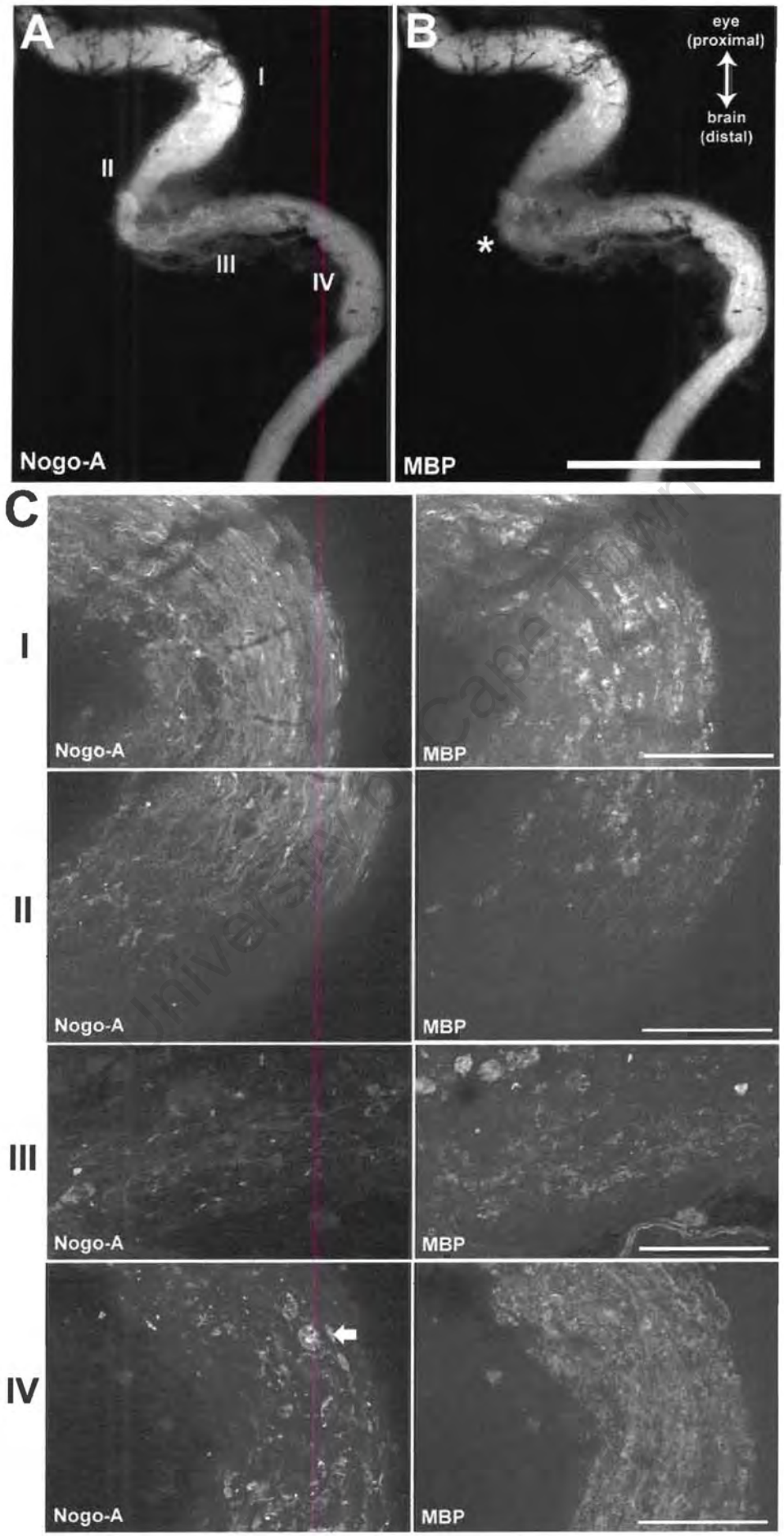

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Figure 4.11. (previous page) Five days post-injury expression of Nogo-A in the tadpole optic nerve. **(A-B)** both Nogo-A and MBP immunoreactivities were weak at the injured site (indicated by *). **(C)** High magnification images of Nogo-A expression from proximal to distal end (indicated as I-IV in A). The immunoreactivity of Nogo-A was found in the presumable oligodendrocytes (arrows). Scale bar: B, 200μm; C, 50μm.

The post-lesion situation in the frog ON differs considerably from that in the tadpole ON, as the adult tissues require a longer period to regenerate. The frogs were therefore allowed to recover a longer time interval after receiving an ON crush. To analyze the Nogo-A expression in nerve fibers and myelin, the longitudinal sections of 2 weeks post-lesion ON were double stained by Nogo-A (702) and anti-NF, or Nogo-A (702) and anti-MBP. Weak NF immunoreactivity was found in the injured nerve when analyzed using conventional microscope (data not shown). The intensities resulted from these two immunostaining experiments cannot be directly compared (Fig. 4.12A-B), since the enhanced nerve fiber images were analyzed using ApoTome Imaging System (Fig. 4.12A).

In the injured nerve section stained by neurofilament NF antibody (Fig. 4.12A), the intensity of the staining was less at the eye-side than the brain-side. The distribution of NF immunoreactivity was found weakly in the central core of the ON and predominantly in the nerve fibers at the periphery of the ON. In contrast, the immunoreactivity of Nogo-A was found evenly throughout the ON section, and partially colocalized with the labeling of anti-NF in the fibers (arrows in Fig. 4.12A).

Similarly, the staining of anti-MBP was weaker at the proximal end than the distal end (Fig. 4.12B). However, the distribution of MBP immunoreactivity was found predominantly in the central core of the ON. The staining of MBP in the lesioned nerve did not reveal dense fibrous pattern parallel to the orientation of axons as did the intact nerve.

Although there was no significant difference in the observed intensity of the Nogo-A (702) staining at both sides of the injured ON, there was a change in the predominant distribution of Nogo-A immunoreactivity (Fig. 4.12C). Despite the weak MBP immunoreactivity at the proximal end, a large number of cells were

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labeled by Nogo-A (702) throughout the nerve section. The population of Nogo-A expressing cells in the injured ON increased dramatically compared to that in the intact nerve. In terms of morphology, these cells possessing Nogo-A immunoreactivity were smaller and rounder than the Nogo-A-positive oligodendrocytes observed in the intact nerve. It is noteworthy that these cells were also highly branched with processes extending to myelinated fibers and exhibited MBP immunoreactivity (arrowheads in Fig. 4.12). Therefore, they are likely to be oligodendrocytes expressing both Nogo-A and MBP in the frog ON after two weeks of injury.

In the longitudinal section of injured ON 3 weeks following nerve crush, the immunoreactivity of MBP was barely detected around the lesion site (shown by *), but found in the fibers at the distal end (Fig. 4.13A-F). Apart from the ON tissue, a piece of peripheral nerve was found adjacent to the ON (PN in Fig. 4.13). Intensive labeling of MBP antibody showed the densely myelinated axons in the peripheral nerve, where the immunoreactivity of Nogo-A was also found. This result indicates the expression of Nogo-A in the myelinated tracts of PNS.

The similar distribution of NF immunoreactivity was observed in the injured ON, but more fibers were labeled at the distal end (Fig. 4.13G-L). The staining of Nogo-A antiserum labeled weakly at the lesioned site (indicated by *) and increased gradually towards the distal end, where it colocalized with the staining of MBP and NF antibodies. The immunoreactivity of Nogo-A was found in both nerve fibers and cell bodies with processes representing oligodendrocytes (arrows in Fig. 4.13) and colocalized with anti-MBP labeling. These MBP-positive oligodendrocytes extended their processes onto the nerve fibers, this indicates the remyelination of the axons by the Nogo-A expressing oligodendrocytes.

Taken together, these observations demonstrate that Nogo-A expression is found in the regenerating and remyelinating processes of frog nerve fibers. Moreover, the ON lesion induces an activation of Nogo-A expressing oligodendrocytes. Therefore, this distribution of Nogo-A immunoreactivity does not demonstrate a downregulation of Nogo-A following frog ON crush to support the axonal regeneration.

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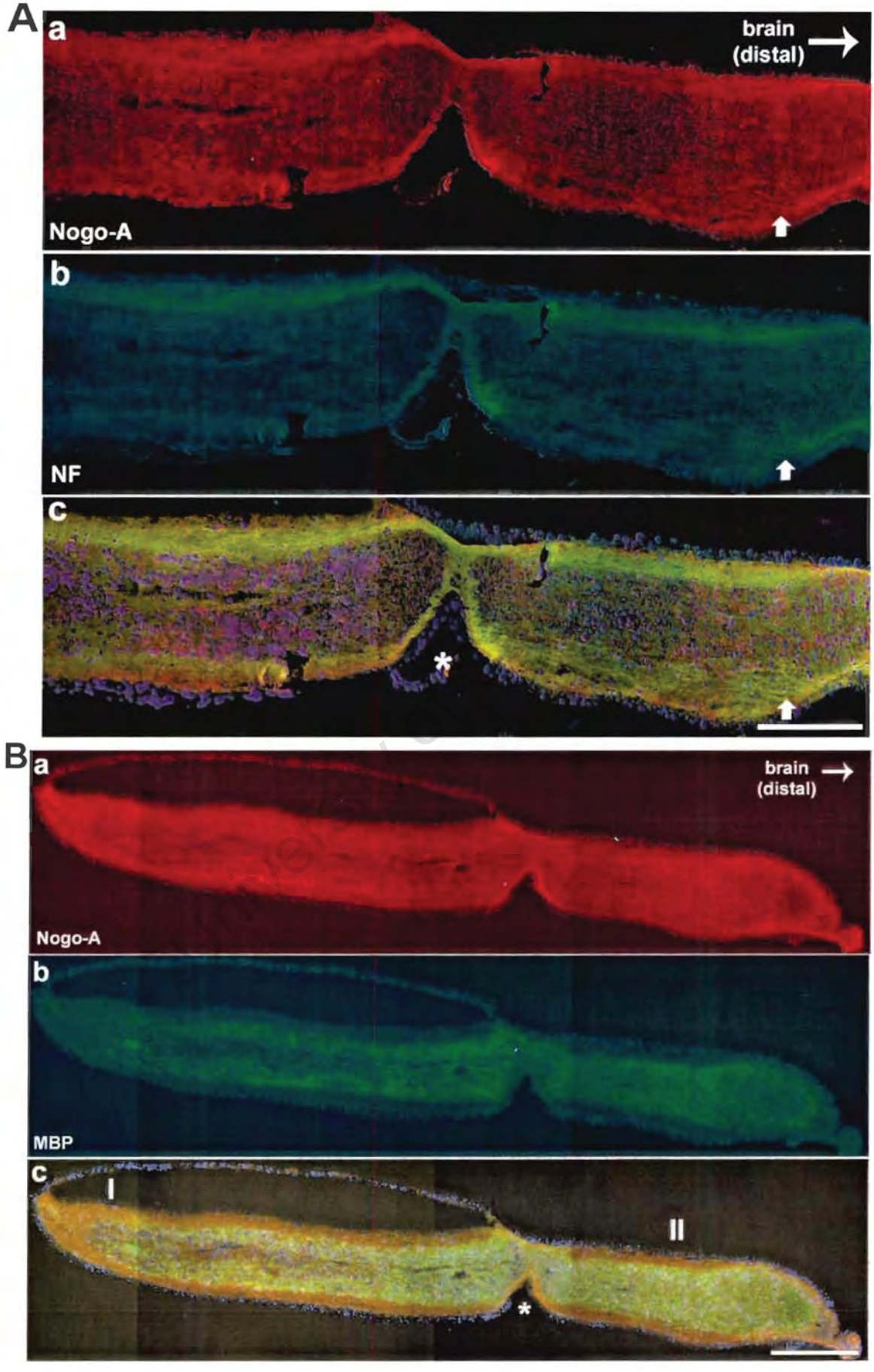
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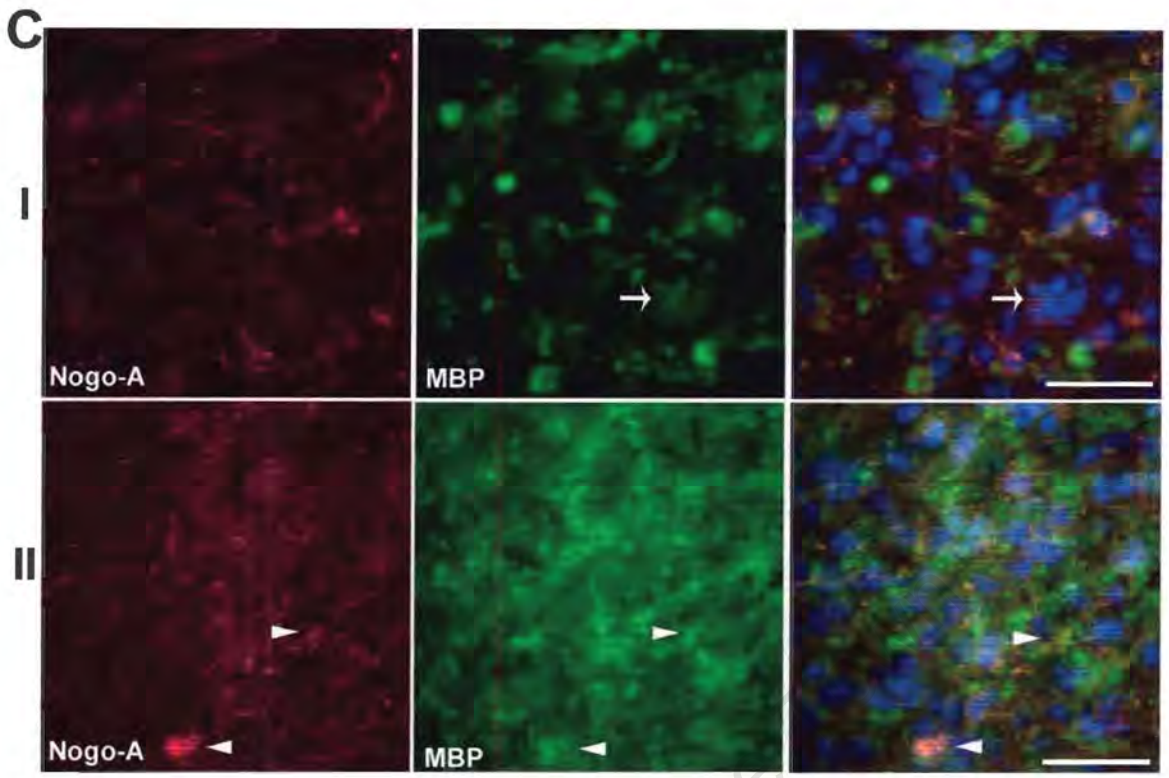

Figure 4.12. Nogo-A immunohistochemistry analysis of after 2 weeks post-lesion frog optic nerve. **(A)** An overview of Nogo-A and NF immunoreactivities in the longitudinal section of frog ON analyzed using ApoTome imaging system. The NF and Nogo-A expressions was colocalized in the nerve fibers (arrows). The lesioned site is indicated by (*). **(B)** An overview of Nogo-A and MBP immunoreactivities in the longitudinal section of frog ON analyzed using conventional fluorescent microscope. The lesioned site is indicated by (*). **(C)** The high magnification images at the eye-sided and brain-sided of the injured nerve (indicated by I and II in B). The immunoreactivities of Nogo-A and MBP were colocalized in the myelinated fibers and oligodendrocytes (arrowheads). The signal of nuclear stain breached through the green-alexa channel (indicated by arrows). The multichannel images were combined with blue DAPI stain. Scale bar: A, B, 200μm; C, 20μm.

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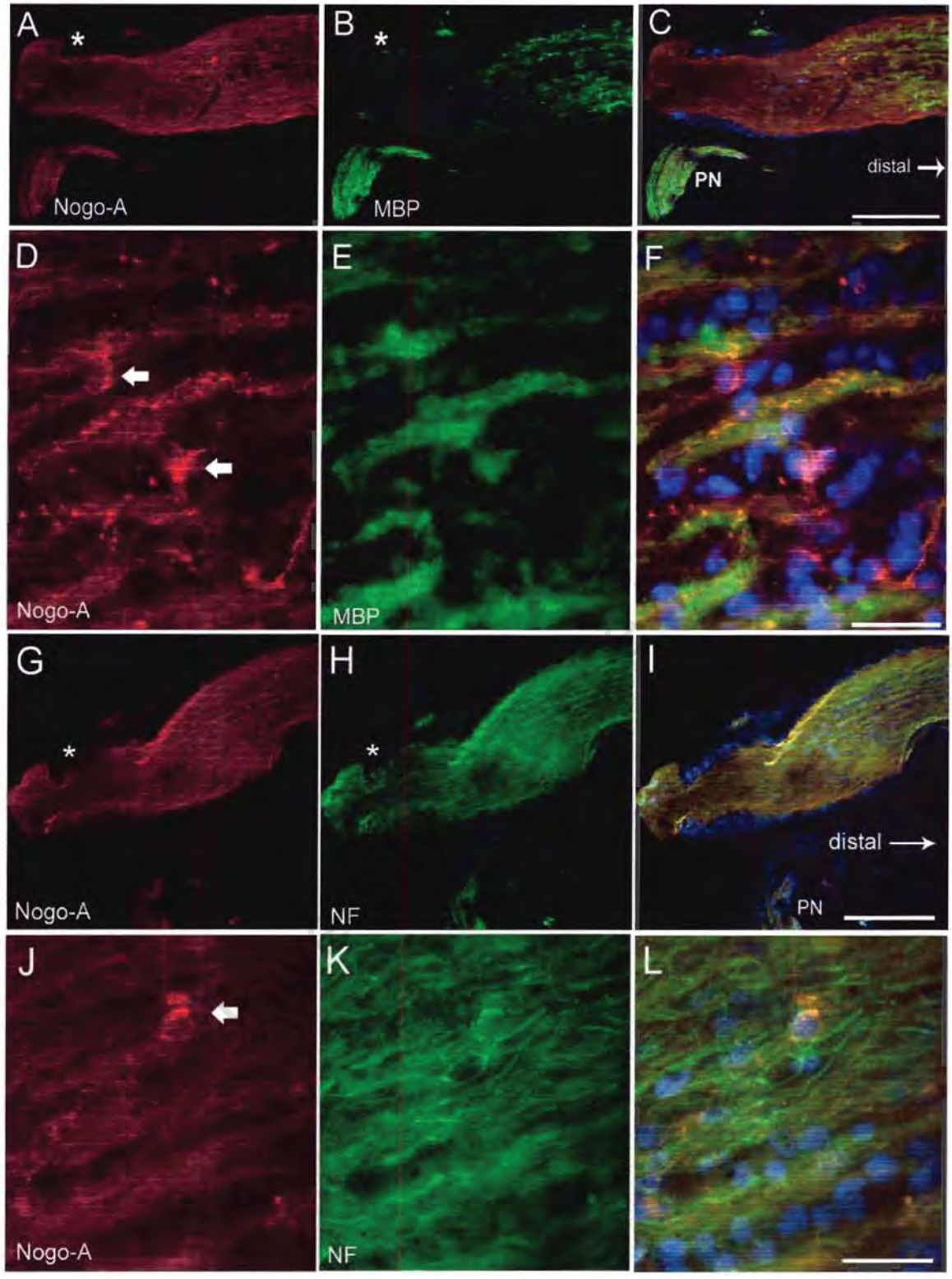

Figure 4.13. Nogo-A immunohistochemistry analysis of after 3 weeks post-lesion frog optic nerve. **(A-C)** staining of Nogo-A and MBP in the longitudinal section of injured frog ON. The adjacent peripheral nerve (PN) was labeled densely by both antibodies. The lesioned site was indicated by (*). **(D-F)** High magnification images at the distal end showed the colocalization of Nogo-A and MBP immunoreactivities in the nerve fibers and oligodendrocytes (arrows). **(G-I)** staining of Nogo-A (702) and anti-NF labeled the injured ON. The adjacent peripheral nerve (PN) was also labeled.

The lesioned site was indicated by (*). **(J-L)** High magnification image at the distal end showed the colocalization of Nogo-A and NF immunoreactivities in the nerve fibers. Staining of Nogo-A antiserum also labeled the oligodendrocytes (arrow). All the multichannel images were combined with blue DAPI nuclear stain. *PN*, peripheral nerve. Scale bar: C, 200μm; F, I, 20μm.

4.2.5 Expression Analysis of Nogo-A in Culture

Several structural studies have predicted that the two hydrophobic regions of Nogo-A protein sequence, are the two putative transmembrane domains. Furthermore, Nogo-A protein has been shown to express on the surface of the Nogo-A transfected cells (Chen et al., 2000; GrandPré et al., 2000). These expression and functional studies suggest that Nogo-A is expressed on the surface of the oligodendrocyte, thereby acting as contact inhibition on the mammalian neurite outgrowth. Results of the immunohistochemical characterization of the Nogo-A in this study indicate that the expression of Nogo-A is associated with oligodendrocyte in the CNS of both frog and tadpole. To further characterize the ectopic expression of Nogo-A in oligodendrocytes, immunohistochemical analysis was performed on the live primary cell cultures.

The primary culture was prepared from tadpole CNS explants, which gave rise to heterogeneous cell populations with distinct morphology and antigens. Cells migrated out of the explants were observed after 1 day. The cell populations consisted mainly of irregular polygonal cells, elongated bipolar cells, highly branched cells and flat cells. Occasionally, neurite outgrowth extended from the explant. If the cells had been cultured for a long period, the population of large flat cells resembling fibroblasts became the predominant cell type, and the neurites fasciculate.

To identify the surface expression of Nogo-A, both live and fixed cultures were subjected to immunostaining of Nogo-A (702) antiserum and compared to each other. In addition, in order to recognize the oligodendrocytes among the cell populations, immunostaining of the oligodendrocyte marker O4 was applied to the live culture. The immunoreactivity of O4 was found specifically on the membrane of oligodendrocytes which were the highly branched cells. However,

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the results obtained from the live-staining of both Nogo-A (702) and O4 antibodies did not reveal any immunolabeling of anti-Nogo-A on the membrane surface of the $O4^+$ oligodendrocytes (data not shown). The immunoreactivity of Nogo-A was observed only in the oligodendrocytes from fixed culture after permeabilisation (Fig. 4.14A-C). Therefore, the expression of Nogo-A in *Xenopus* glial culture is associated with $O4^+$ oligodendrocyte, but not on the membrane surface of the oligodendrocytes.

In addition, the Nogo-A antibody also labeled the neuronal cell body with neurite extension, which was not stained by O4 marker (Fig. 4.14D-F). When the culture was subjected to immunostaining of neurofilament NF antibody, the immunoreactivity of NF was found together with that of Nogo-A in the neurites (Fig. 4.14G-I). This finding demonstrates the neuronal expression of Nogo-A in *Xenopus*.

4.2.6 Nogo-A in PNS

Initially, Nogo-A was identified in the CNS myelin in mammals and thought to be CNS-specific (Caroni and Schwab, 1988; Schwab and Caroni, 1988). Nevertheless, the immunostaining results in the present study have indicated otherwise. The Nogo-A immunoreactivity was found in the peripheral nerve of *Xenopus* frog (Section 4.2.4) as well as the spinal ganglia of tadpole (Section 4.2.1). Recent mammalian studies have demonstrated high expression of Nogo-A in the PNS neurons and axons after peripheral nerve injury (Hunt et al., 2003). This correlation between Nogo-A expression and regenerative potential suggests that Nogo-A may be beneficial to regeneration by function within the axoplasm (Hunt et al., 2003). Therefore, Nogo-A may be present in the *Xenopus* PNS in a similar distribution to that in the mammalian PNS, thus correlates with high plasticity.

To characterize the Nogo-A distribution in the neuron and axon of *Xenopus* PNS, immunostaining of Nogo-A was done together with antibodies against neurofilament NF and peripheral myelin protein P0 in the longitudinal sections of *Xenopus* frog DRG (Fig. 4.15). The staining of Nogo (702) antiserum revealed

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intensive labeling of DRG neurons as well as the peripheral nerve attached to the DRG (Fig. 4.15A-C). The peripheral nerve fibers were also stained by anti-NF (Fig. 4.15D-F) and P0 (Fig. 4.15G-L), colocalized with the Nogo-A immunoreactivity, indicating the expression of Nogo-A in the myelinated axons. Moreover, the Nogo (702) antiserum appeared to stain a thin layer at the edge of P0-positive myelin, indicating the immunoreactivity of Nogo-A is in the outer layer of the myelin sheath (arrowheads in Fig. 4.15). These findings strongly suggest that the distribution of Nogo-A in the *Xenopus* PNS is associated with neuron, myelinated axon and the outer layer of the myelin sheath.

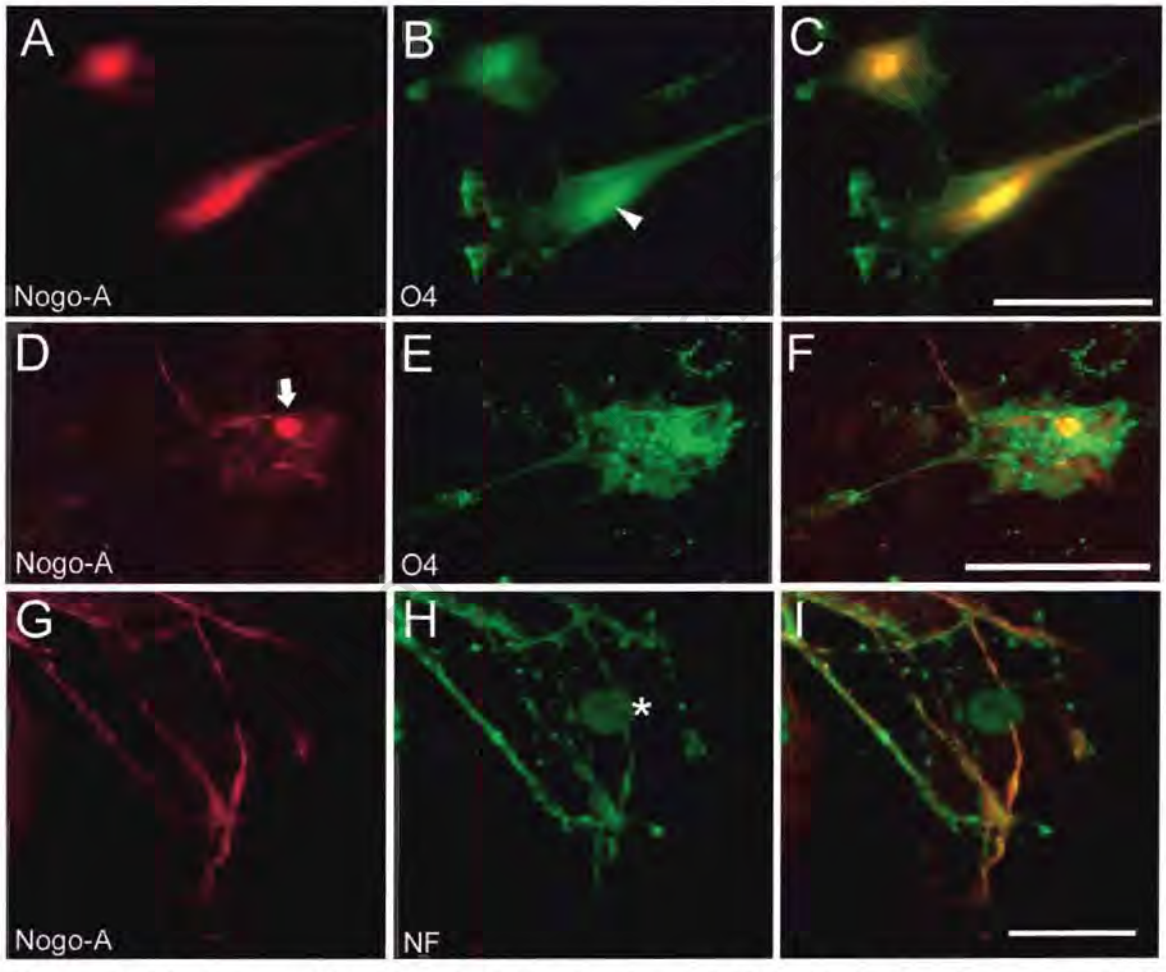

Figure 4.14. Immunohistochemical localization of Nogo-A in the *Xenopus* CNS culture. **(A-C)** Two individual oligodendrocytes were labeled by Nogo-A (702) and O4 marker. The fluorescent signal from Nogo-A staining was so strong to breach through to green-channel (indicated by arrowhead). **(D-F)** Immunoreactivity of Nogo-A was found in the neuronal cell body with neurite extension (arrow), which was not labeled by O4. **(G-I)** The neurites were stained by Nogo-A and NF antibodies. The nucleus was labeled non-specifically by anti-NF (*). Scale bar: C, 25μm; F, 20μm; I, 25μm.

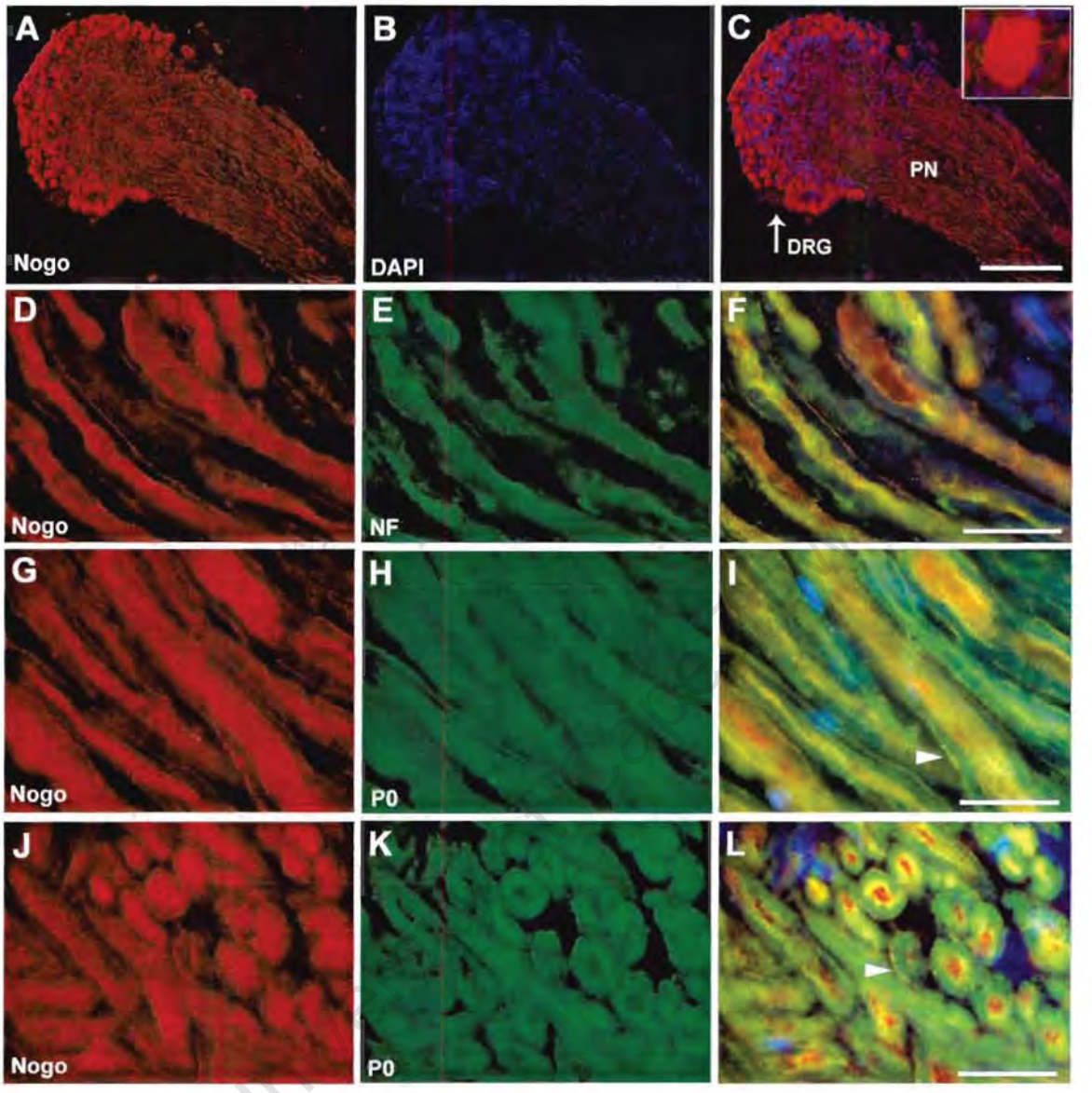

Figure 4.15. Immunoreactivity of Nogo-A in the frog DRG and peripheral nerve sections. **(A-C)** Under lower magnification, Nogo-A (702) antiserum labeled the neuronal cell bodies in the DRG (the insert), as well as PN. **(D-F)** Under higher magnification, immunoreactivity of Nogo-A was found in the NF-positive axons. **(G-I)** The P0 antibody labeled the myelinated nerve fibers. (J-L) The cross-sections of the myelinated axons were stained by anti-Nogo-A and P0. The immunoreactivity of Nogo-A is also found at the edge of the myelin sheath (arrowheads). *DRG*, dorsal root ganglion; *PN*, peripheral nerve. Scale bar: C, 200μm; F, I, L, 20μm.

4.3 Generation of Nogo-Receptor Antiserum

In vitro experiments with the mammalian protein suggest that Nogo-A exerts the inhibitory effect in the C-terminal 66-amino acid loop (Nogo-66) which lies within the two putative transmembrane domains (GrandPré et al., 2000; Fournier et al., 2001; Oertle et al., 2003). The characterization of the Nogo-66 loop has led to the identification of the Nogo66-receptor (NgR) from a mouse brain cDNA expression library (Fournier et al., 2001). With the availability of the sequence, NgR homologs and NgR-related genes in many animals have been identified (Barton et al., 2003; Fournier et al., 2001). There are antibodies raised against mammalian sequences of NgR, such as those in rat, mouse and human (Fournier et al., 2001; Wang X. et al., 2002) and those are commercially available. However, in an attempt to allow a comparative analysis of NgR expression and function in mammals and lower vertebrates, such as *Xenopus*, a new antibody was raised against a region of NgR the sequence which is conserved across different species.

More recent studies have identified the sequence of a NgR homolog in fish (Klinger et al., 2004b). As no NgR protein sequences are available for *Xenopus*, NgR sequences alignment and phylogenetic analysis were done across mammals and fish using the blast algorithms of bioinformatics (Table 4.1). A short sequence of 8 aa (SLQYLRLN) is thought to be conserved from mouse to fish, thus an antibody was raised against this region (highlighted in Table 4.1).

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[ISREC-Server] Date: Tue Feb 22 11:18:59 Europe/Zurich 2005
LALIGN finds the best local alignments between two sequences version 2.0u66 September 1998 Please cite: X. Huang and W. Miller (1991) Adv. Appl. Math. 12:373-381

```
 Comparison of:
(A) ./wwwtmp/lalign/.23300.1.seq mouse
- 473 aa
(B) ./wwwtmp/lalign/.23300.2.seq fish
- 479 aa
 using matrix file: BL50, gap penalties: -14/-4

  45.9% identity in 464 aa overlap; score: 1286 E(10,000): 2.9e-107

               10         20         30         40         50         60
mouse  MKRASSGGSRLLAWVLWLQAWRVATPCPGACVCYNEPKVTTSCPQQGLQAVPTGIPASSQ
       ::     :.:::  ..:::.   : . ::. ::::.:::.:..: :::: ..:: ::. ::
fish   MKTLIVEGGRLLCLMFWLNLVPVINSCPAKCVCYSEPKATVACQQQGLFSIPTEIPVRSQ
               10         20         30         40         50         60

               70         80         90        100        110        120
mouse  RIFLHGNRISHVPAASFQSCRNLTILWLHSNALARIDAAAFTGLTLLEQLDLSDNAQLHV
       :::::.:... : ..:::.: .:::.::..:: ...:.:.:: ::  ::.:::..::..:..
fish   RIFLQSNKLTVVRSTSFSSVHNLTVLWMYSNNISHIEAGAFYGLERLEELDIGDNSNLRI
               70         80         90        100        110        120

              130        140        150        160        170        180
mouse  VDPTTFHGLGHLHTLHLDRCGLRELGPGLFRGLAALQYLYLQDNNLQALPDNTFRDLGNL
       ..::.:.:: .:::::: :::: :: :.:::: .:::::::::: :: ..:: ::.::
fish   ISPTAFRGLTKLHTLHLHRCGLSELPVGVFRGLFSLQYLYLQDNNLLALHEDTFLDLANL
              130        140        150        160        170        180

              190        200        210        220        230        240
mouse  THLFLHGNRIPSVPEHAFRGLHSLDRLLLHQNHVARVHPHAFRDLGRLMTLYLFANNLSM
       :.::::.:.  : .: .::: .:::::::::.:.:. .:: :::.: ::.:: :::.:
fish   TYLFLHNNKIKVVTDHMLRGLVNLDRLLLHQNRIVHVQQQAFNDLSKLTTLFLFFNNLTM
              190        200        210        220        230        240

              250        260        270        280        290        300
mouse  LPAEVLMPLRSLQYLRLNDNPWVCDCRARPLWAWLQKFRGSSSEVPCNLPQRLADRDLKR
       : .: . :: :::::::: : :.:::::::: :...:.:::.. :.:: : .::::
fish   LTGESMNPLVSLQYLRLNGNQWICDCRARPLWDWFKRFKGSSSDLECHLPASLNGKDLKR
              250        260        270        280        290        300

              310        320        330        340
mouse  LAASDLEGCA-----VASGPFRP-IQTSQ---LTDEELLSLPKCCQPDAADKASVL----
       : ..::::::.     : .. :   .....   : :  . :.:.::  :  ::.:..
fish   LKSDDLEGCVDSPSQVQTSIFNSKVHSGKFLSLDDPLVESIPRCCLSDN-DKSSIISSKS
              310        320        330        340        350

         350        360                370        380        390
mouse  --EPGRPAS---AGNALKGRVPPGDTP-------PGNGSGPRHINDSPFGTLPSSAEPPL
         .:.  :   ..: :: .   . :          ..  . . .:::.:::. .. .  :
fish   IPDPSSYNSRQITNNPLKEKENISKTKFREVERTKNETRNKQSLNDGPLGTMSNNLDQSL
     360        370        380        390        400        410

         400        410        420        430
mouse  TALRP---GGSEPPGLPTTGPRRRPGCSRKNRTRSHCRLGQAGS
       . :   :. ::   ::   ...  ::.: .. ..:  :....
fish   DRIDPELLGNLEPSTAPT---KKKKKCSKKPKSDQNCLKGHGST
     420        430        440        450        460
```

Table 4.1. Comparison of mouse and fish *nogo-A/rtn4-A* receptor peptide sequences. The sequences were aligned using the William Pearson's *lalign* program. The selected sequence of interest was highlighted in red. (Alignment result was adapted from http://www.ch.embnet.org/software/LALIGN_form.html)

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4.4 Analysis of Nogo-Receptor Expression

The expression of NgR has so far been described in the axons and cell bodies of neurons (Fournier et al., 2001; Wang X. et al., 2002). In an attempt to study the expression of NgR in *Xenopus*, the staining pattern of the NgR antiserum was analyzed in the *Xenopus* neuronal cultures.

Primary cultures containing heterogeneous cell populations were prepared from the tadpole CNS explants. There was numerous neurite outgrowth and even fasciculation, which was ideal for immunohistochemical analysis of NgR in association with axons. The immunoreactivity of NgR antiserum was found to colocalize with that of neurofilament marker NF in the neurites (Fig. 4.16A-B). However, the NgR antiserum also labeled the NF-negative cells. To verify the immunoreactivity of NgR antiserum in the neurites, control experiments were performed with pre-immune serum. The immunostaining of pre-immune serum also labeled the NF-negative cells from the explant, but not the neurites stained by NF marker (Fig. 4.16C-D). Additional control experiments were performed without primary antibodies to verify the specificity of the secondary fluorescent antibodies. No signals were detected when staining with secondary antibodies alone (data not shown). In summary, though the newly raised NgR antiserum labels non-neuronal tissues non-specifically, it stains the neurites from the tadpole CNS culture.

Results of immunoblotting revealed that NgR antiserum detected numerous of bands in the molecular weights range of 180kD and 60kD (Fig. 4.17A). One of the protein bands in frog spinal cord and weakly in frog brain may be of interest with an estimated molecular weight of 90kD (arrow in Fig. 4.17A). On the other hand, the pre-immune serum recognized numerous protein bands, but none of them bear similar molecular weights to the NgR protein (Fig. 4.17B). The control blot without primary antibodies did not show any non-specific binding by the secondary antibody SAR-HRP (Jackson Immuno Research, data not shown). This NgR antiserum has not yet been immuno-purified, and the normal serum from the rabbit exhibits non-specific binding to components of *Xenopus* tissues, which at this stage makes the use of this antiserum as a tool for specific NgR-labelling across species boundaries impossible.

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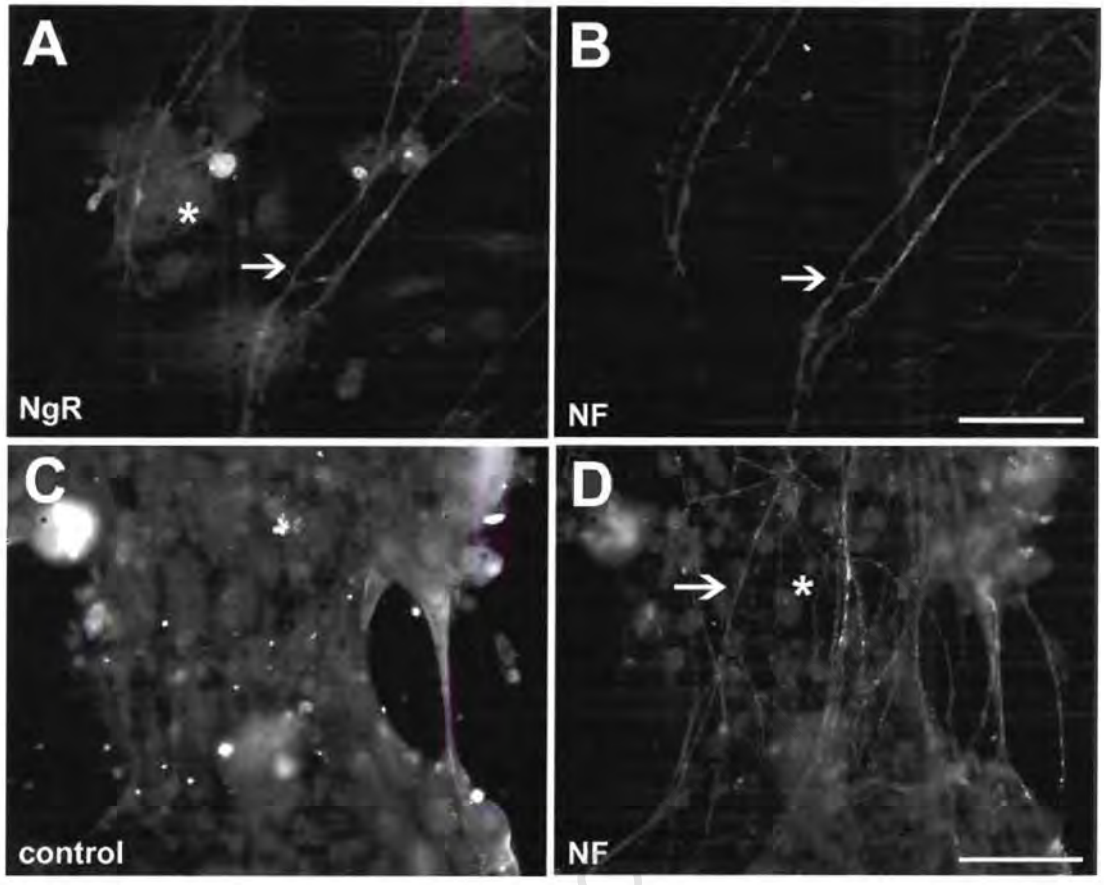

Figure 4.16. Immunoreactivities of NgR antiserum in the *Xenopus* tadpole CNS culture. **(A-B)** Staining patterns of new NgR antiserum and NF antibody. The NgR antiserum labeled the NF^+ neurites (arrows) specifically and other tissues (*) non-specifically. **(C-D)** Staining patterns of pre-immune serum and NF antibody. The NF antibody stained neurites specifically (arrow) and the nuclei non-specifically (*). Scale bar: B, D, 50μm.

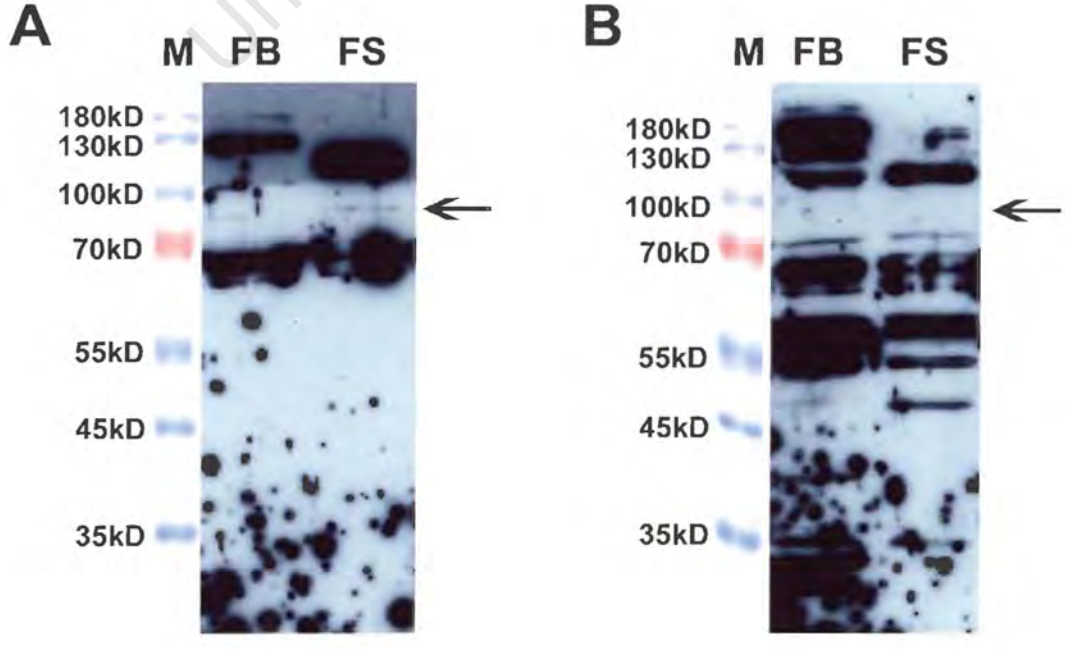

Figure 4.17. (previous page) Immunoreactivities of pre-immune serum in the *Xenopus*. **(A)** The pAB NgR antiserum detected numerous bands in the immunoblot of *Xenopus* CNS, and a band of approximately 90kD (arrow) in *Xenopus* frog brain (FB) and spinal cord (FS). **(B)** The immunoblot of pre-immune serum did not show a corresponding band (arrow) as that in the NgR immunoblot. *M*, molecular weight marker; *kD*, kilodaltons.

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Chapter 5

In Vitro Functional Analysis

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5.1 *In Vitro* Analysis

In *in vitro* experiments using the mammalian homologues, tenascin-R (TN-R) and Nogo-A have been shown to possess inhibitory effects on neurite outgrowth. The results of the immunostaining experiments described in the present study confirm the expression of TN-R and Nogo-A in *Xenopus*. Thus, functional analysis was carried out to investigate whether these proteins exhibit similar inhibitory properties in *Xenopus* as in mammals.

5.1.1 Substrate Properties of TN-R

It has been shown that TN-R protein extracted from mammals inhibits neurite extension when provided with neuronal culture substrates (Becker C.G. et al., 1999, 2004; Becker T. et al., 2000). To test whether mammalian TN-R exhibits the same substrate property in *Xenopus* neuronal tissues, the purified mouse TN-R protein was applied as a homogenous substrate and compared to non-inhibitory control substrates. The native mouse TN-R protein was kindly provided by Dr Pesheva who has shown the axon growth inhibitory effects of the mouse TN-R protein on the mammalian neurons (Fig. 5.1A, adopted from Pesheva et al., 2006).

In primary cultures of the *Xenopus* tadpole CNS, the substrate properties of mouse TN-R were compared to the favourable substrate laminin and the neutral substrate BSA. Substantial neurite outgrowth and cell migration were found after 3 days of culture on BSA and particularly on laminin substrate (Fig. 5.1B). On the laminin substrate, the neurites were long extended and substantial cell migration out of the explants occurred. Neurite extension was also observed on the mouse TN-R protein substrate, together with a heterogeneous population of cells migrated out of the explant (Fig. 5.1C). Neurite outgrowth, however, was observed much later than on the neutral or favourable substrates. Moreover, these neurites appeared to project from the explant onto the cells which had migrated out of the explant prior to the neurites. In order to differentiate the neurites from the other cell populations, the culture was subjected to

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immunostaining with anti-neurofilament (NF) antibodies. Immunoreactivity of the NF marker was occasionally found in the neurites which extended directly from the explant to the mouse TN-R substrate (Fig. 5.2A). The majority of the anti-NF labelled neurites were found on top of a layer of cells that had migrated and flattened out from the explant (Fig. 5.2B). The neurite outgrowth from the tadpole CNS explant did not appear to be inhibited by the mouse TN-R protein substrate, though there was a higher occurrence of growing neurite on the cells than on the homogenous TN-R protein substrate, indicating that the cellular substrate was preferred over the TN-R by the axons.

Experiments *in vitro* initially demonstrated that the axons have no preference for CNS myelin on a patterned substrate, consisting of alternating lanes of myelin and poly-lysine/laminin, in the so-called stripe assay (Bastmeyer et al., 1001). When mammalian neuronal cultures are provided with alternating stripes of TN-R and favourable substrates, the neurites choose to grow only on the favourable substrates and avoid the TN-R protein boundary (Xiao et al., 1997). Thus, to study the cellular responses of *Xenopus* neurons to a choice of mammalian TN-R and a known favourable substrate, the stripe assay was performed using mouse TN-R protein and laminin. The purified mouse TN-R protein was applied as a substrate in a stripe pattern onto a homogenous layer of laminin substrate, thereby creating alternative lanes of laminin and TN-R protein substrates. However, the fish TN-R homolog might not exhibit the same inhibitory effects as mammalian TN-R protein. Thus fish TN-R protein was compared with that of mouse TN-R protein in the stripe assay. As a control, combination of BSA protein stripes on laminin substrate was tested.

Both mouse TN-R/laminin and fish TN-R/laminin stripe assays supported vigorous outgrowth of cells and neurites from the explants of *Xenopus* tadpole CNS (Fig. 5.3). Due to the heterogeneity of the cell populations from the primary culture, it was extremely difficult to quantify and compare the numbers. The comparison between the stripe assays of mouse TN-R/laminin and fish TN-R/laminin could only be done by observation and estimation. The spreading of cells covered the entire coverslip within 2 days of cultivation. However, there were also cells, predominantly elongated cells with long process, migrating

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together with neurite outgrowth in a stripe pattern (arrowheads in Fig. 5.3). This phenomenon occurred more frequently on mouse TN-R/laminin patterned substrates (Fig. 5.3B) than on the fish TN-R/laminin substrate (Fig. 5.3A). In the control stripe assay, no substrate preference was observed. The cells/neurites grew freely on the BSA/laminin patterned substrate (data not shown).

In order to visualize the association of neurite outgrowth to the patterns of TN-R protein substrates, the cultures of stripe assays were subjected to immunostaining with pAB TN-R and anti-NF mAB. TN-R immunoreactivity was found to occur in stripe patterns in both fish (Fig. 5.4) and mouse TN-R (data not shown) stripe assays. This confirms the presence of the patterned substrate of TN-R protein as well as the integrity of the protein in the stripe assay. In addition, there were pAB TN-R labelled cells on the stripes of TN-R substrate (Fig. 5.4B). These cells appeared to extend their processes onto the stripes of laminin substrates indicated by the negative TN-R immunoreactivity (arrows in Fig. 5.4B). However, no conclusive results were derived from the anti-NF staining due the loss of cells/neurites during the processes of fixing and washing (data not shown). These findings indicate that when the TN-R and laminin proteins were used as culture substrates for the CNS of *Xenopus* tadpole, the outgrowth of the cells and neurites in general did not show significant avoidance of the TN-R protein. However, the comparison of axon growth on TN-R proteins derived from mouse and fish indicated that the substrate properties of mouse TN-R may be less permissive than those of fish TN-R, that at least mouse TN-R is capable of channelling growth of amphibian axons and that the signalling mechanisms through which TN-R exerts its function are conserved in these lower vertebrates.

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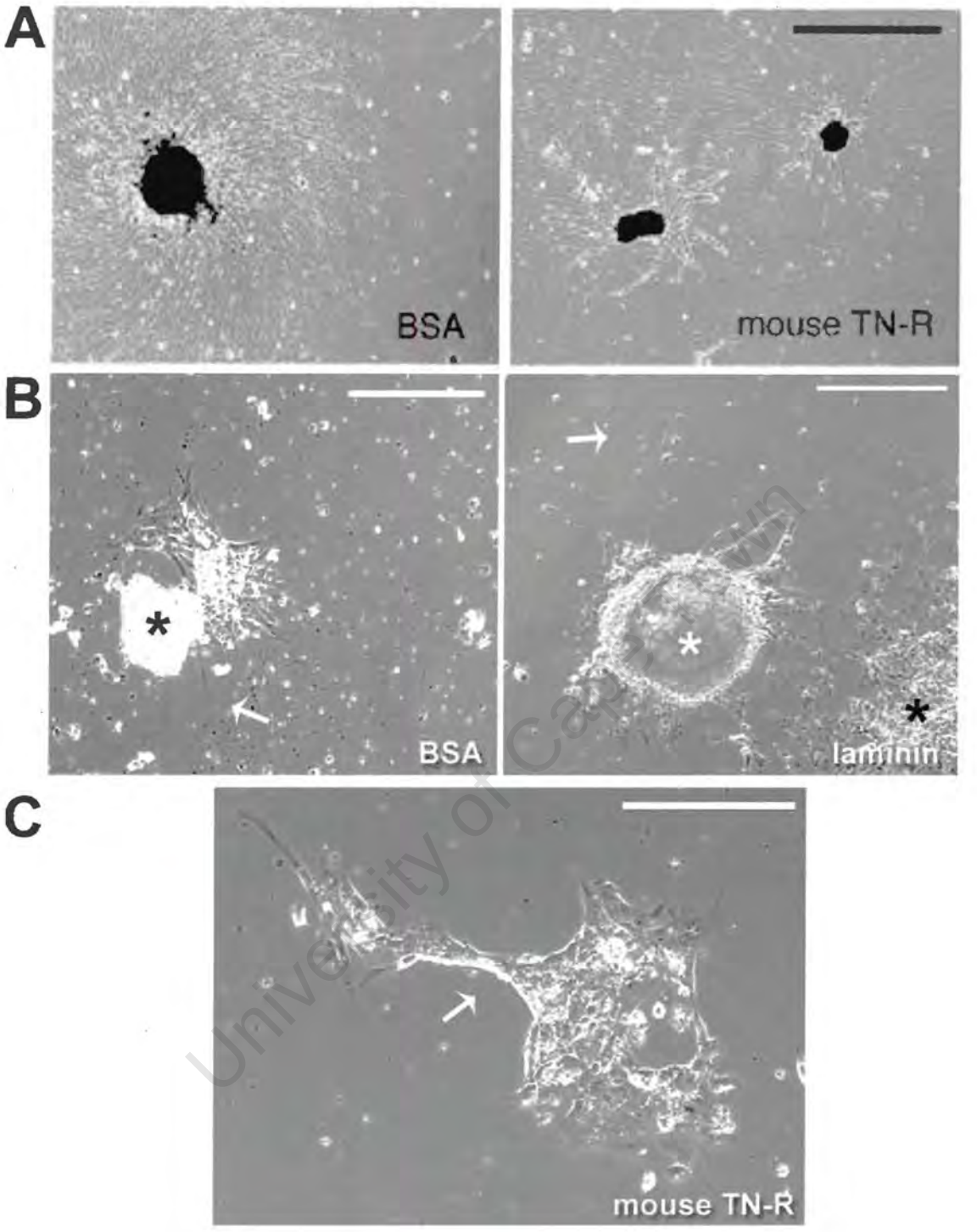

Figure 5.1. TN-R action on the neurite outgrowth. **(A)** Axon growth pattern of mouse cerebellar neurons on homogeneous BSA or mouse TN-R protein substrata (adopted from Pesheva et al., 2006). **(B)** Tadpole CNS culture after 3 days on homogenous substrate of either BSA or laminin protein. There were outgrowth of neurites (arrows) and cells from the explants (*). **(C)** Tadpole CNS culture after 3-4 days with neurite outgrowth (arrow) on homogenous mouse TN-R protein substrate. Scale bar: A, 200µm; B, C, 100µm.

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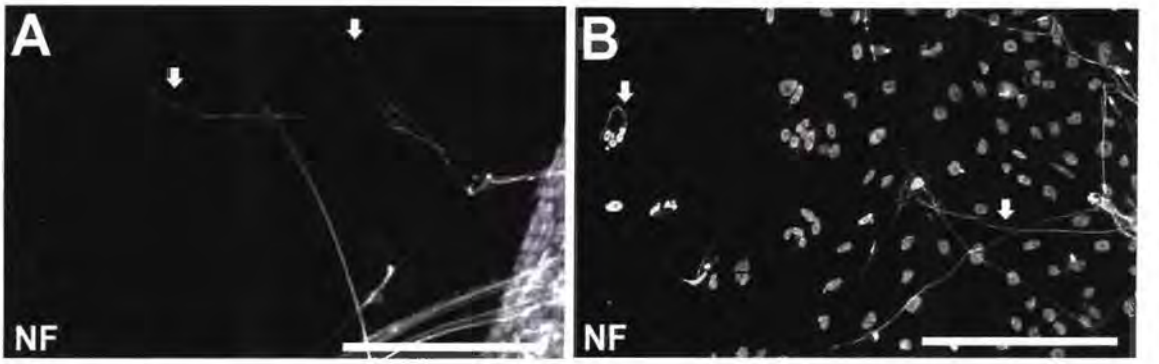

Figure 5.2. Immunohistochemical analysis of NF in tadpole CNS culture on homogenous mouse TN-R protein substrate. **(A)** Neurite outgrowth (arrows) on homogenous mouse TN-R protein substrate. **(B)** Neurite outgrowth (arrows) on the cells migrated from the explant growing on a homogenous mouse TN-R protein substrate. The NF antibody labelled all the nuclei non-specifically. Scale bar: A, 50µm; B, 100µm.

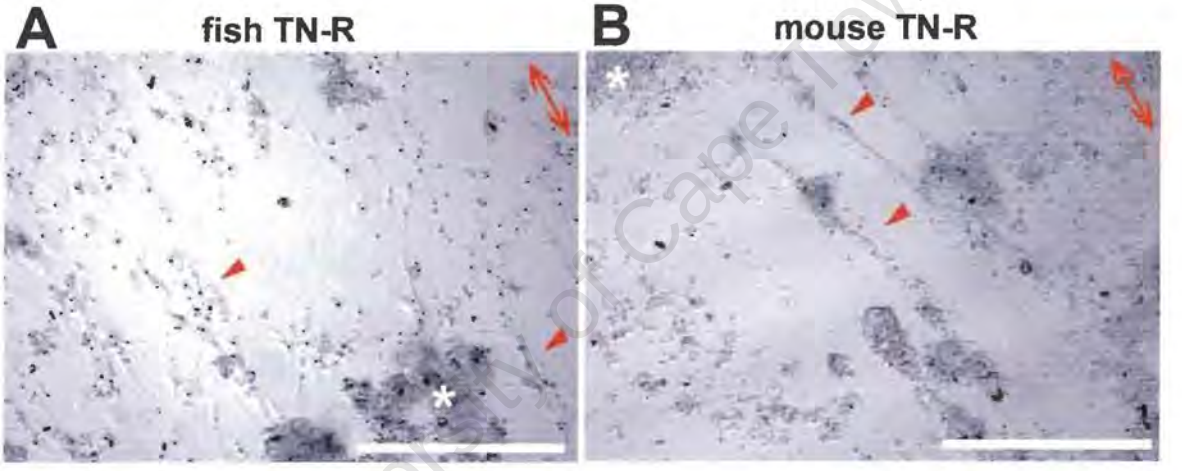

Figure 5.3. Primary culture of tadpole CNS on pattern substrates of TN-R proteins. **(A)** Phase contrast image of culture on pattern substrates of fish TN-R protein and laminin. **(B)** Phase contrast image of culture on pattern substrates of mouse TN-R protein and laminin. Explants of tadpole CNS were indicated by (*). The cells/neurites from the explants formed a pattern (arrowheads) in line with the direction of the substrate pattern (arrows). Scale bar: A, B, 200µm.

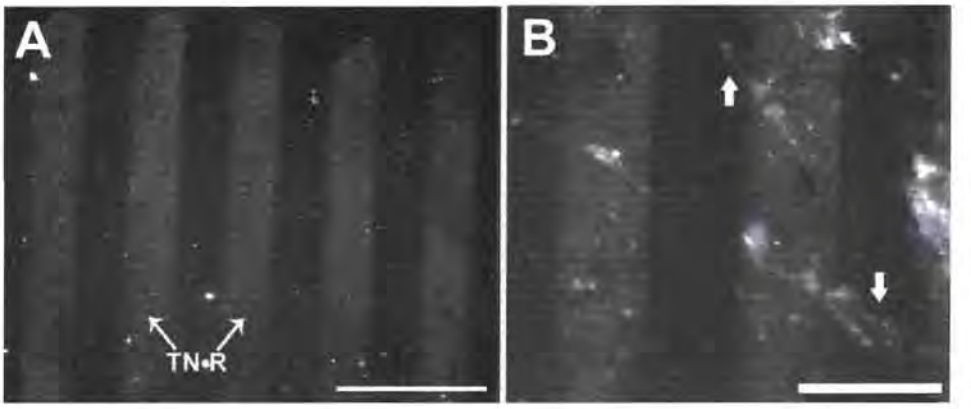

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Figure 5.4. (previous page) Immunohistochemical analysis in TN-R stripe assay. **(A)** Staining with pAB TN-R labelled the stripes substrate of fish TN-R protein. **(B)** TN-R-positive cells on the TN-R protein substrate with processes extended to the laminin substrate (arrows). Scale bar: A, 100µm; B, 50µm.

5.1.2 Substrate Properties of Nogo-A

Functional experiments have demonstrated that Nogo-A exerts the strongest inhibitory effect in a hydrophilic region of 66-aa between two putative transmembrane domains, so called, Nogo-66 (GrandPré et al., 2000). Within the same study, the application of a 25-aa peptide from the rat Nogo-66 region (designated as Nogo-P4) was sufficient to induce growth cone collapse and inhibit neurite outgrowth from chick DRG neurons and NGF-differentiated PC12 cells (GrandPré et al., 2000).

To determine whether Nogo-A substrate property is non-permissive in *Xenopus* neuronal culture as described in the neuronal cultures derived from higher vertebrates (GrandPré et al., 2000), primary cultures of the *Xenopus* tadpole CNS was cultured on the substrate properties of Nogo-P4 and compared to the favourable substrate laminin (Fig. 5.5). Striking difference on the outgrowth of cells/neurites was observed in the primary cultures on the laminin and Nogo-P4 substrates. The neurite outgrowth was extensive and cells migrated out of the explants were massive on the laminin substrate, but not on the Nogo-P4 substrate.

The stripe assay was also performed using Nogo-P4 peptide together with laminin as pattern substrate (Fig. 5.6). In the assay, the cells/neurites appeared to be exploring the surrounding environment and changing directions (insert in Fig. 5.6C). After 2 days of cultivation, the outgrowth of cells and neurites from the explants of *Xenopus* tadpole CNS had already been channelled and formed a pronounced pattern of stripes. In order to visualize and verify the Nogo-P4 peptide substrate pattern, the cultures of stripe assays were subjected to immunostaining by polyclonal Nogo (Bruna) antibody, which was raised against

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mammalian Nogo (Chen et al., 2000). Although there were no cells/neurites left after processing, the staining pattern of Nogo (Bruna) antibody revealed lanes of Nogo immunoreactivity (Fig. 5.7) corresponding to the stripes pattern seen in culture. Therefore, the Nogo-P4 peptide was exerting a channelling effect on the cells and neurites from the explants of *Xenopus* tadpole CNS, thereby producing a stripe pattern of cell migration and neurite extension in the stripe assay. This indicates that, as for TN-R, the mammalian Nogo-A protein is capable of eliciting a response in the frog cells, pointing to a conserved signalling mechanism.

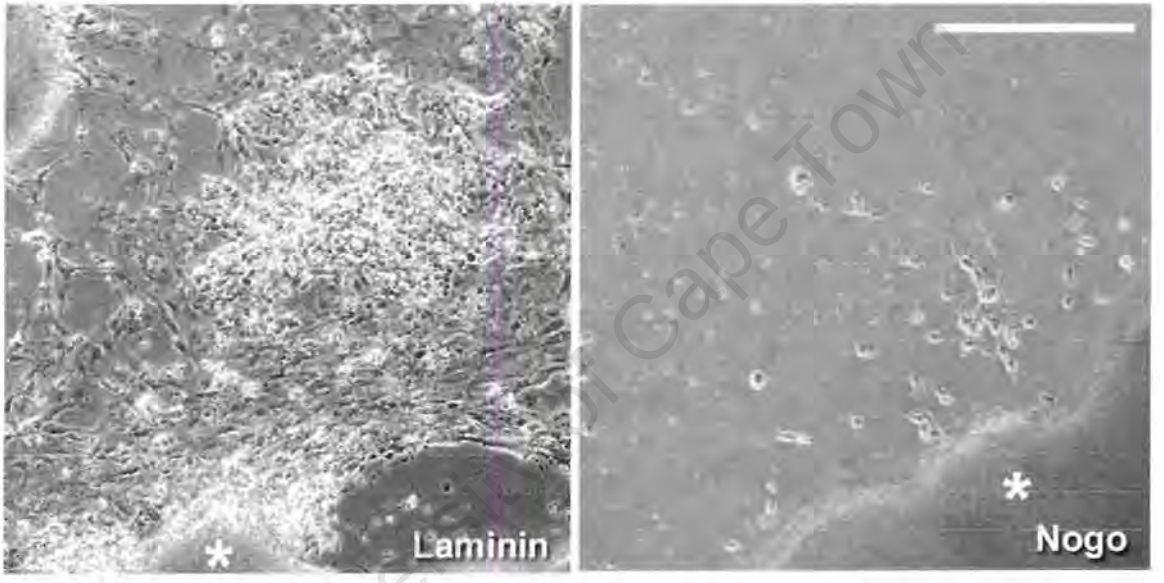

Figure 5.5. Nogo action on the neurite outgrowth. Phase contrast image of culture on homogenous substrates of Nogo P4 peptide and laminin. Explants of tadpole CNS were indicated by (*). Scale bar: A, 200µm.

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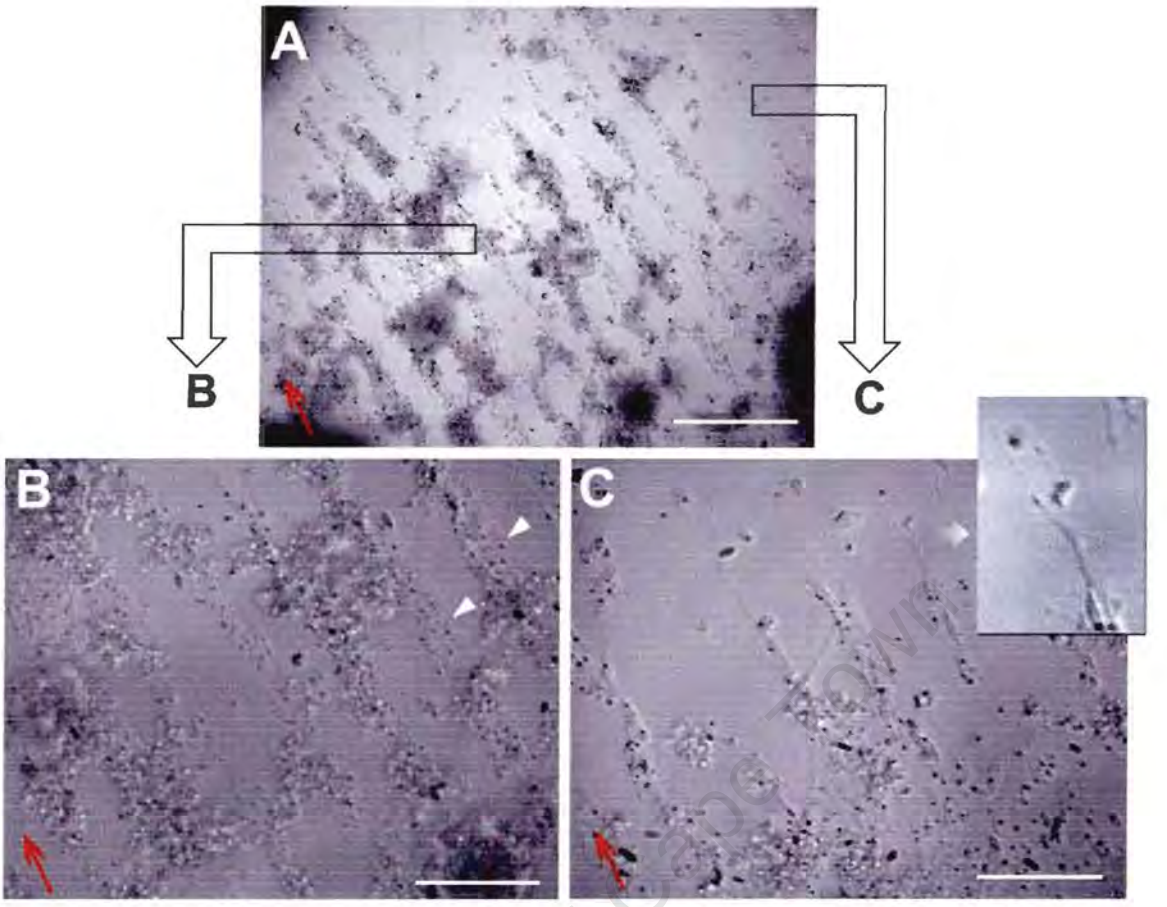

Figure 5.6. Primary culture of tadpole CNS on pattern substrates of Nogo P4 peptide. **(A)** Phase contrast image of culture on pattern substrates of Nogo P4 peptide and laminin. **(B-C)** Enlarged images of regions as indicated in (A). The cells/neurites from the explants were forming a pattern (arrowheads) in line with the direction of the substrate pattern (arrows). Scale bar: A, 200μm; B, C, 100μm.

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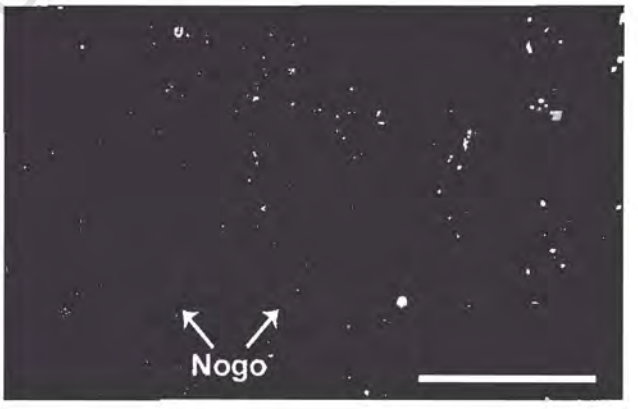

Figure 5.7. Immunohistochemical analysis in Nogo stripe assay. Staining with Nogo (Bruna) antibody labelled the Nogo P4 peptide substrate. Scale bar: 100μm.

Chapter 6

Discussion and Conclusion

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6.1 Discussion

Neurons in the injured mammalian central nervous system (CNS) are unable to perform functional regeneration of their axons. This has been attributed to the unfavourable substrate properties of the microenvironment of the injured neurons, and in particular to the presence of neurite outgrowth inhibitory molecules, such as Nogo-A and tenascin-R (TN-R). Most studies on neurite outgrowth inhibitors are focused on mammalian CNS. Since lower vertebrates have an outstanding capability of regenerating CNS axons, detailed studies on the putative neurite growth inhibitors TN-R and Nogo-A in these animals will lead to a better understanding of the role of these molecules in axon growth and regeneration. In the present study, expression patterns of TN-R and Nogo-A were analyzed in *Xenopus* frogs and tadpoles. Since down-regulation of neurite growth inhibitors is a possible mechanism of facilitating axon regeneration after injury, the post-lesion regulation of TN-R and Nogo-A was also studied in the optic nerve of both larval and adult *Xenopus*. Furthermore, the substrate properties of these proteins were investigated in order to gain insight into their functional role in lower vertebrates.

6.1.1 Tenascin-R in *Xenopus*

Similar expression patterns of TN-R in Xenopus and higher vertebrates

Initially, TN-R was identified from the mouse brain with molecular weights of 160 and 180kD. In the present study, the presence of TN-R in *Xenopus* CNS is biochemically verified. From the immunoblotting results (Section 3.1), both two polyclonal antibodies against TN-R recognized a protein with two isoforms bearing high molecular weights in the *Xenopus* CNS. Previous sequence analysis attempts have been made at identifying the TN-R gene in *Xenopus tropicalis*, close related to *Xenopus laevis*. Unfortunately, only a partial sequence, encoding for fibronectin (FN) type III domain, was found in the genomic database (Tucker et al., 2006). On the other hand, recent phylogenetic studies have demonstrated that the TN-R proteins of different vertebrates, including *Rana*

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frogs, have two major 160 and 180kD forms (Pesheva et al., 2006). Although there are no sequence data available yet, the unexpected high molecular weights of TN-R protein bands in current study are likely the result of high number of charged residues that may alter its mobility in the gel electrophoresis. The specificity of the antibodies was confirmed in the results of two TN-R isoforms in both mammalian and *Xenopus* CNS together with the similar staining patterns of both polyclonal and monoclonal TN-R antibodies. Moreover, these antibodies were also shown to recognize the TN-R protein of various species, such as mammals, avian, reptiles, amphibians and fish, in a phylogenetic analysis (Pesheva et al., 2006). Therefore, the TN-R antibodies specifically recognize TN-R protein with two isoforms in *Xenopus* in the present study.

The TN-R protein has been shown to be associated with the myelin and oligodendrocytes in the CNS of higher vertebrates (Kruse et al., 1984, 1995; Pesheva et al., 1989; Rathjen et al., 1991). The present immunohistochemical studies in the sections and whole mount tissues revealed that the immunostaining of TN-R was localized in the myelinated fiber tract in both spinal cords (SCs) and optic nerves (ONs) of *Xenopus* tadpole and frog (Section 3.2.1 and 3.2.2). Moreover, the expression of TN-R in *Xenopus* was also found in the presumable oligodendrocyte that was further verified by the immunostaining of the oligodendrocyte marker in the cell culture (Section 3.2.4). Therefore, the association of the TN-R with CNS myelin and oligodendrocytes correlated with the reported results on higher vertebrates (Fuse et al., 1993; Kruse et al., 1984, 1995; Pesheva et al., 1989). However, the expression patterns of TN-R in *Xenopus* raise the question about the functional role of a putative neurite outgrowth inhibitor in an animal capable of CNS axon regeneration.

The sequence of TN-R is phylogenetically highly conserved (Pesheva et la., 2006; Tucker et al., 2006), and the protein is found in many species, including mammals (Fuss et al., 1993; Pesheva et al., 1989), avians (Rathjen et al., 1991), fish (Becker et al., 2003) and amphibians (Becker et al., 1999). Moreover, the same TN-R antibodies used in the current and previous studies (Pesheva et al., 2006) recognize the TN-R protein, possibly by the same epitope, in all vertebrate species. Taken together with the fact that expression patterns are similar in the

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different species, it may imply for likely functions, other than neurite inhibitory effects, of TN-R across species. For instance, based on the association of the TN-R expression patterns with CNS myelin and oligodendrocytes, TN-R may play a role in myelination and oligodendrocyte function in *Xenopus*, as has been suggested in mammals (Fuss et al., 1993).

TN-R expression persists in the optic nerve of Xenopus after lesion

In vitro functional studies have demonstrated the inhibitory effects of TN-R on the neurite outgrowth in neuronal cultures (Becker C.G. et al., 1999; Becker T. et al., 2000). The role of TN-R in poor axonal regeneration has also been suggested from the results of animal studies on CNS injury. The expression of TN-R after CNS injury has been investigated in adult rat, zebrafish *Danio rerio* and the salamander *Pleurodeles Waltl* (Becker C.G. et al., 1999, 2004; Becker T. et al., 2000). After ON transection, TN-R expression in rat is upregulated at the lesion site where TN-R is assumed to contribute to axon growth inhibition and glial scar formation (Becker et al., 2000). In contrast to mammals, after ON crush in the salamander, TN-R expression was downregulated at the lesion site, which could possibly contribute to successful regeneration (Becker et al., 1999). In the present study, the post-injury expression patterns of TN-R were investigated in the *Xenopus* ON. In contrast to the downregulation of TN-R in salamander, TN-R expression persisted at the lesion site following frog ON crush. Reduced TN-R protein expression was also observed in the zebrafish, but the mRNA expression was surprisingly increased (Becker et al., 2004). This may have resulted from the post-lesion response of oligodendrocyte dedifferentiation and redifferentiation (Becker et al., 2004). The expression of TN-R protein appeared again in the nerve with progressing remyelination of regenerated axons (Becker et al., 1999, 2004).

It was suggested in zebrafish that TN-R acted as a repellent guidance molecule by forming a boundary and channel for growing axons in TN-R free regions (Becker et al., 2003, 2004). However, the distribution of TN-R immunoreactivity in *Xenopus* crushed nerve did not demonstrate any downregulation of TN-R after lesion, especially at the distal end to the lesion site where morphological

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changes of oligodendrocytes were observed (Section 3.2.3). These oligodendrocytes may indicate a change into an immature phenotype that no longer expresses myelin marker proteins such as MBP. Previous post-lesion studies of *Xenopus* frog ON have shown the possibility of oligodendrocyte dedifferentiation into immature oligodendrocytes after lesion and redifferentiation during remyelination (Lang et al., 1996), as has been demonstrated in goldfish (Ankerhold and Stuermer, 1999).

Interestingly, an upregulation of TN-R after ON crush also occurs in other fish and reptile species that regenerate axons, such as goldfish, cichlid fish and lizard (Lang, pers. comm.). In fact, TN-R has been shown to act as an intrinsic autocrine factor for the differentiation and maturation of oligodendrocytes (Pesheva et al., 1997). Thus, the persistent expression of TN-R after nerve crush may be the result of a post-lesion response of oligodendrocyte dedifferentiation and redifferentiation in the animals that successfully regenerate axons. Hence, the success of the axonal regeneration in *Xenopus* ON is unlikely to be due to the TN-R downregulation at the lesion site as suggested in either salamander or zebrafish ON (Becker et al., 1999, 2003, 2004).

TN-R as a glial marker

During mammalian CNS development, TN-R protein and mRNA are abundant in the presumptive white matter within the pathways of oligodendrocyte precursor migration (Pesheva et al., 1989). Furthermore, TN-R promotes oligodendrocyte differentiation and maturation in an intrinsic autocrine manner (Pesheva et al., 1997). In mammalian CNS cultures, TN-R antibodies labeled $O4^+$ and/or $A2B5^+$ oligodendrocyte progenitors and type-2 astrocytes (O-2A cells), but not $GFAP^+$ type-1 astrocytes (Jung et al., 1993; Pesheva et al., 1989, 1997). The *in vitro* immunostaining results from the present study showed that TN-R protein was found in $O4^+$ oligodendrocytes in *Xenopus* glial cultures (Section 3.2.4). In *Xenopus* CNS sections, TN-R immunoreactivity was associated with presumptive oligodendrocytes (Fig 3.6), but not the $GFAP^+$ astrocytes and astrocytic processes (Fig 3.4, 3.6, 3.8). Taken together, the immunohistochemical results

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suggest that TN-R may be expressed by O-2A linage cells, but not astrocytes in *Xenopus* CNS.

On the other hand, TN-R may also be expressed by immature oligodendrocytes which are GFAP$^+$ in mammals. However, in *Xenopus* glial culture, it has been shown that the undifferentiated oligodendrocytes were A2B5$^-$, while the differentiated oligodendrocytes retained GFAP (Lang et al., 1996). The results from the current immunolocalization of TN-R, there were occasionally GFAP$^+$ cells labeled by TN-R antibodies, though the expression of TN-R did not appear to be associated with astrocytes. Since the oligodendrocytes could retain GFAP in *Xenopus* glial culture, these GFAP$^+$ TN-R$^+$ cells in the *Xenopus* CNS may very well be either immature or differentiated oligodendrocytes. Thus, even though these differential marker expressions of *Xenopus* oligodendrocytes in different differentiation stages may not be the same as in mammals, TN-R can be considered an established marker for *Xenopus* oligodendrocyte linage cells. This also implies that TN-R possibly plays a role in amphibian oligodendrocyte differentiation and myelination similar to that in mammals.

Apart from the CNS glia and myelin tissues, TN-R expression was found in the Schwann cells of both mammalian (Probstmeier et al., 2001) and *Xenopus* PNS (Section 3.3). While the expression of mammalian TN-R has been found during PNS development and downregulated at later developmental stages (Probstmeier et al., 2001), the immunoreactivity of TN-R in *Xenopus* was found to persist in the adult. In the same study, TN-R immunoreactivity was associated with Schwann cells in the degenerating nerve stump after adult sciatic nerve injury. A functional role of TN-R has thus been suggested in PNS myelination as well as PNS pattern formation in terms of axon pathfinding (Probstmeier et al., 2001). Although more functional analyses are required, it is possible that TN-R in *Xenopus* PNS is playing a similar role as in the mammalian PNS. Moreover, Schwann cells are able to produce neurotrophic factors and neurite outgrowth promoting cell adhesion molecules, thereby creating a permissive environment in the PNS especially during regeneration (for review, see Goldberg and Barres, 2000). Therefore, an association of *Xenopus* TN-R with Schwann cells suggests a potentially growth-permissive effect of this molecule in the *Xenopus* PNS, or an

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overriding of the inhibitory effects of TN-R by the high levels of axon growth-promoting components.

TN-R and its interaction partner CSPG in Xenopus

In the avian, mammalian and fish retina, TN-R has been found in the horizontal cells on the outer plexiform layer (Bartsch et al., 1993; Becker et al., 2003; Pesheva et al., 1989; Rathjen et al., 1991). In *Xenopus* retina, TN-R was also found on the outer plexiform layer. Thus, the interspecies comparison of TN-R neuronal expression is not very different between *Xenopus* and the other vertebrates. Interestingly, the immunoreactivity of CSPG was found in the inner nuclear layer adjacent to that of TN-R in the frog retina (Fig. 3.8).

CSPGs are a heterogeneous group of molecules with distinct core proteins. During CNS development, the migrating cells and growing axons avoid CSPG-expressing areas in ECM (Friedlander et al., 1994). In a CSPG substrate border study, neurite growth of chick DRG explants did not grow over the border into the CSPG containing area (Niederöst et at., 1999). Additionally, the presence of CSPGs in the injured mammalian CNS, mostly in the glial scar tissues, contributed to the axon growth-inhibitory environment (Asher et al., 2002; Lemons et al., 1999). Thus it might also contribute to the failure of CNS axonal regeneration.

Biochemical studies have demonstrated that TN-R is capable of forming complexes with CSPGs by protein-protein interaction (Asperg et al., 1997). Members of CSPG have been shown to interact and co-express with TN-R in oligodendrocytes, demonstrating inhibitory effects on neurite outgrowth in mammals (Asher et al., 2002; Probstmeier et al., 2000a, 2000b). Moreover, an upregulation of TN-R at the lesion site possibly promotes glial scar formation in association with CSPGs (Probstmeier et al., 2000b). Given that both TN-R and CSPG have been shown to inhibit axon growth, the association of TN-R and CSPG could constitute a particular non-permissive substrate. Thus, it is of interest to find the expressions of both putative mammalian neurite outgrowth inhibitors, TN-R and CSPG, in *Xenopus* visual system, which is capable of

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regenerating optic nerve throughout life. Alternatively, the CSPGs could modulate the repulsive activity of TN-R by forming complex with TN-R, thus hindering the inhibitory domains and thereby neutralizing the effects. Although further functional analysis is required, the immunostaining results from the *Xenopus* retina sections may imply an association or interaction of TN-R and CSPG in *Xenopus*.

Possible interactions of TN-R with F3/11 and other unidentified receptors

Apart from the expression of TN-R by horizontal cells in the retina, the neuronal expression of TN-R in mammals has been shown to accumulate in perineuronal nets of interneurons and motoneurons in different parts of the CNS (Angelov et al., 1998; Brückner et al., 2003, 2006). Perineuronal nets appear as mesh-like structures on the cell surface as a result of ECM molecular composition. The presence of TN-R, a neurite repulsive substrate, proposes that the perineuronal nets may serve as a barrier for the formation of new synaptic contacts. Although the functions of the perineuronal net are not fully understood, knowledge of the molecular components leads to the postulation of its functional roles, such as maintenance of synaptic plasticity (for review see Celio et al., 1998).

Similar expression was observed in that TN-R was found in perineuronal nets of F3-posivite neurons in the *Xenopus* frog SC. In mammals, F3/11 is expressed on the cell surface membrane and is implicated in both axonal growth and synaptogenesis (for review, see Falk et al., 2002). The *Xenopus* F3/11 homologue, contactin, was shown to be expressed in both adult and embryonic CNS of *Xenopus* and it is essential for *Xenopus* nervous development (Fujita et al., 2000; Nagata et al., 1996), similar to the nervous system of higher vertebrates. Although the repulsive property of TN-R for neurite outgrowth was shown to be mediated by this neuron-specific glycoprotein F3/11, this neurite outgrowth inhibition was also shown to be F3/11-independent by its interaction with an as yet unidentified receptor (Probstmeier et al., 2000b). Furthermore, fish TN-R protein was shown to be more permissive than the mammalian TN-R protein and the neurite outgrowth inhibition was suggested to be partial independence of F3/11-mediated by fish TN-R protein (Pesheva et al., 2006).

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This difference on the substrate properties between the mammalian and lower vertebrates TN-R proteins was also observed in the functional analysis of the present study (Section 5.1.1). Thus, it seems reasonable to assume a similar association of F3 with TN-R in the roles of synaptogenesis and axon pathfinding in *Xenopus* frog as in mammals. However, TN-R protein is more permissive in the lower vertebrates and less independent on the F3/11/contactin-mediated neurite inhibition. Therefore, *Xenopus* TN-R protein may interact with other unknown receptors and partially inhibit the neurite outgrowth in *Xenopus* frog.

TN-R may perform functions other than inhibiting neurite outgrowth in Xenopus

Based on the expression pattern in mammals, TN-R was implicated in cell interactions, which play a vital role in development of the nervous system. A number of *in vitro* functional studies were done to investigate the substrate properties of TN-R (Angelov et al., 1998; Liao et al., 2005; Pesheva et al., 1989, 1994, 1997; Probstmeier et al., 2000a; Rathjen et al., 1991). Sulfatide-mediated cell adhesion was demonstrated in the oligodendrocyte cultures (Pesheva et al., 1997). FN-mediated cell adhesion and neurite outgrowth could be interfered with by TN-R (Pesheva et al., 1994; Probstmeier et al., 2000a). On the other hand, the expression of the TN-R interaction partners FN and laminin is upregulated together with that of TN-R in the lizard after ON crush (Lang, pers. comm.) and thus these ECM proteins may act in concert as instructive signals for axon growth. TN-R expression also persists after ON crush in *Xenopus*. Although the post-lesion expression patterns of FN and laminin have not yet been examined in *Xenopus*, an increase intensity of laminin has been shown in the CNS of axotomized *Rana* frog (Matesz et al., 2005). These effects suggest that TN-R possibly plays an important role in myelination, as well as having a role in an interaction between TN-R and its interaction partners that may imply a role of TN-R as a modulator in ECM interactions. TN-R substrate has also been shown to be anti-adhesive for activated microglia (Angelov et al., 1998; Liao et al., 2005) and neurons (Pesheva et al., 1989). In a mixture of TN-R and adhesive substrates, neural cell attachment was not stimulated and furthermore neurite extension was inhibited (Pesheva et al., 1989; Rathjen et al., 1991). It would be

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interesting to investigate whether TN-R is also anti-adhesive for microglia in Xenopus, as activation and migration of these macrophage-like cells is considered crucial for successful axon regeneration (Angelov et al., 1998).

To investigate its substrate properties, TN-R was used in neurite outgrowth assays of *Xenopus* neuron and glia cell cultures. Using the tadpole CNS primary culture in the assay it was difficult to visualize the direct effect of TN-R upon the neurites among the heterogeneous cell population. Based on the distribution of the anti-NF immunoreactivity, most of the neurite outgrowth on the homogenous substrate of mammalian TN-R avoided making direct contact with the protein substrate but extended onto the cells instead (Fig. 5.1). The substrate property of mammalian TN-R has also been shown to be non-permissive for neurite outgrowth of mammalian, fish and salamander neurons (Becker et al., 1999, 2004; Pesheva et al., 1989, 2006). This suggests that the mammalian TN-R, as a sole substrate, may exhibit a high degree of inhibitory effects on the outgrowth of *Xenopus* neurons.

Different form of substrate preparation, such as homogenous and patterned, give rise to different types of *in vitro* cellular responses. The stripe assay is ideal for the studies on substrate choice and guidance of extending axon. Thus, the *in vitro* effect of the TN-R protein on *Xenopus* neurite outgrowth was further analyzed by offering the neurons alternating parallel lanes of TN-R and control substrate. Ideally, different sets of assay should be done to compare neurite outgrowth on the combination of inhibitory, or favourable and "neutral" protein substrates. Yet, simply by comparing the *in vitro* effects of mammalian and fish TN-R, neurite outgrowth from the *Xenopus* neurons did not appear to be inhibited substantially by either mammalian and to an even lesser extent by fish TN-R substrates (Fig. 5.3). This could be attributed to the presence of laminin, one of the TN-R interaction partners (Brückner et al., 2003; Pesheva et al., 1989), as positive substrate it may counteract the inhibitory effects of TN-R. This could also possibly be attributed to a possibly different inhibitory action of TN-R from different organisms (Pesheva et al., 2006), since most of the studies on neurite inhibition have been done and described with mammalian TN-R (Becker C.G. et al., 1999; Becker T. et al., 2000). The responsiveness of the *Xenopus* neurites,

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such as the existence of different receptor(s) or different conformation of the receptor-ligand binding sites resulting in different interactive responses, may be different from that of mammalian neurites. Alternatively, it can be argued that the TN-R substrates may not be non-permissive substrates in *Xenopus*.

6.1.2 Nogo-A in *Xenopus*

Nogo-A/RTN4-A ortholog in Xenopus

CNS myelin and oligodendrocytes of the *Xenopus* spinal cord (SC) are known to be nonpermissive for neurite outgrowth, however, the inhibition for neurite growth is neutralized by the monoclonal inhibitor-neutralizing antibody mAB IN-1 (Lang et al., 1995). Furthermore, in mammalian spinal cord injury model, neutralization of non-permissive substrate property by mAB IN-1 has lead to promote axonal regeneration as well as functional motor recovery (Bregman et al., 1995; Brösamle et al., 2000; Merkler et al., 2001; Spillmann et al., 1998; Thallmair et al., 1998). Since Nogo-A is shown to be an antigen of IN-1 (Chen et al., 2000), it can be postulated that Nogo-A in part contributes to the hostile environment for CNS axon growth in *Xenopus* SC. Together with the phylogenic analysis of reticulon gene family (Oertle et al., 2003a), the Nogo-A/Rtn4-A ortholog in *Xenopus* frogs was identified (Klinger et al., 2004a). In the same study, two independent *rtn4.1* and *rtn4.2* genes were identified and the resulting three major transcripts, rtn4-A, -B and -C were related to those in mammals. However, the molecular weight of *Xenopus* Nogo-A protein was shown to be slightly lower than the mammalian Nogo-A (Klinger et al., 2004a).

In the present immunoblotting analysis, the same antiserum against *Xenopus* Nogo-A used by Klinger et al. (2004a) revealed a protein band in *Xenopus* CNS of a similar molecular weight to the band in rat CNS recognized by mammalian specific Nogo-A antibody. The current immunoblotting results are consistent with previously published results (Chen et al., 2000; Klinger et al., 2004a). In addition, there were bands with low molecular weight in the *Xenopus*, however, these were shown to be non-specific binding from the secondary antibody in the control

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experiment. Thus, the specificity of the Nogo-A antiserum is confirmed in current work and the Nogo-A immunolabelling in the *Xenopus* CNS can be considered specific.

On the other hand, there remains some uncertainty of Nogo-A as an antigen of original mAB IN-1 which could suggest functions of Nogo-A other than neurite inhibition. The IN-1 antibody was raised against NI-250 which was initially identified from mammalian CNS myelin extract (Caroni and Schwab, 1988). In mammalian expression studies, staining patterns of IN-1 antibody in the adult rat CNS were identical to those of mammalian Nogo-A antibodies (Chen et at., 2000). However, the lack of IN-1 immunoreactivity in the *Xenopus* ON (Lang et al., 1995) is in contradiction to the results obtained in this study, which strongly demonstrated Nogo-A immunoreactivity in both tadpole and frog ON. This controversy has raised the question on recognition of Nogo-A by IN-1, since no direct evidence has been presented so far and the biochemical analysis of this aspect has turned out to be difficult. This is due to the nature of mAB IN-1, which belongs to the IgM isotype family. Another challenge lies within the full purification of the original IN-1 antigen, NI-35/250, thus no direct comparison between the NI-35/250 and Nogo-A could be elucidated. Therefore, one could argue that the inhibitory activity concluded by many studies of axonal regeneration using IN-1 neutralization was the result of IN-1 recognizing a different epitope of Nogo-A. This could also lead to clarification of a variety of unknown functions of Nogo-A.

Interestingly, the mammalian specific Nogo-A antibody did not recognize Nogo-A in the *Xenopus* CNS in present study. Thus, one could argue the degree of conservation of Nogo-A structure and function in different species. On the other hand, the mammalian Nogo-A P4 peptide has demonstrated a high degree of repulsive effects on the outgrowth of *Xenopus* axons in the functional assays, indicating conservation of the signalling pathways mediating the inhibitory action of Nogo-A. In fact, high variability of Nogo/RTN4 sequence conservation has been observed in vertebrates (Oertle et al., 2003a). Furthermore, some inhibitory domains of Nogo-A are only present with weak similarity in amphibians (Oertle et al., 2003a). Therefore, one may speculate that the substrate property of *Xenopus*

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Nogo-A is not inhibitory to the *Xenopus* neurite outgrowth. It is possible that the evolution of Nogo/RTN4 may lead to protein structural divergence which may result in functional divergence, but more thorough studies are needed on this issue.

Nogo-A is associated with oligodendrocytes and myelin

Nogo-A is strongly expressed in oligodendrocytes and myelinated tracts of the mammalian CNS (Chen et al., 2000; Huber et al., 2002). In the *Xenopus* frog and tadpole CNS, the immunoreactivity of Nogo-A was found in the highly branched oligodendrocytes and myelinated nerve fibers of the SC and optic nerve (ON). In addition, the expression of Nogo-A was found in the myelinating oligodendrocytes of tadpole ON as indicated by the high expression level of myelin protein. These findings imply that the expression of Nogo-A in *Xenopus* CNS is associated with oligodendrocytes and myelin similar to that in mammals. However, this expression pattern in *Xenopus* CNS does not support the notion that Nogo-A is a neurite outgrowth inhibitor because *Xenopus* is capable of CNS regeneration after injury. The permissive substrate property of *Xenopus* frog optic nerve was demonstrated by earlier functional assays (Lang et al., 1995). Furthermore, Nogo-A is already expressed in the tadpole CNS, where both the ON and SC are capable of regeneration after injury (Beattis et al., 1990).

The immunostaining data of post-lesioned nerves revealed the expression of Nogo-A persisted after the ON injury in tadpole and frog (Section 4.2.4). This is an important finding because Nogo-A is believed to be an inhibitor for axon outgrowth in mammals, yet it is expressed in the regenerating nervous system of amphibians. The expression patterns of Nogo-A in the injured nerves were, however, different from those in the intact nerves. Findings revealed that more Nogo-A expression was found in the oligodendrocytes than in fibers. Results also indicated that after lesion the Nogo-A expressing cells had a similar morphology to the myelinating oligodendrocytes found in the tadpole ON. The close association of Nogo-A with the oligodendrocyte in the regenerating nerve may provide an indication of the active state of oligodendrocytes. Furthermore, it may also indicate the putative function of Nogo-A in relation to oligodendrocyte

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dedifferentiation and redifferentiation, when *Xenopus* glia adapt the plasticity after lesion (Lang et al., 1996).

Possibility of different membrane topology of Nogo-A

In vitro studies have demonstrated that Nogo-66 is a potent inhibitory region of the Nogo-A protein causing growth cone collapse in different models (Fournier et al, 2001). The results of stripe assay from the present study have also demonstrated some degree of inhibitory effects by Nogo-66 peptide on the *Xenopus* neurons (Section 5.1.2). This implies that all Nogo proteins have inhibitory properties (Oertle et al., 2003; Fournier et al., 2001). In addition, Nogo-A contains a Nogo-A specific region in the N-terminal that results in potent neurite growth inhibition, thereby making Nogo-A the most potent inhibitor of the Nogo proteins (Chen et al., 2000; GrandPré et al., 2000). So far, the membrane topology is still not clear. Two possible membrane topologies of Nogo-A have been postulated in mammals (Fig. 6.1). When the surface expression of Nogo-A was studied in *Xenopus* oligodendrocyte cultures, the immunoreactivity of Nogo-A antiserum was detected after permeabilisation as described in the literature (Klinger et al., 2004a; Taketomi et al., 2002). This did, however, not occur in the live-staining of Nogo-A antiserum. Although one cannot neglect the possibility of culture artifacts, these results do not indicate the *in vitro* surface expression of Nogo-A on the oligodendrocyte cultures. This controversy leads to a question regarding the membrane topology of *Xenopus* Nogo-A. One possibility is that *Xenopus* Nogo-A may exhibit a different membrane topology from that in the mammals and consequently it does not play a role in neurite inhibition as in the mammalian form. Another hypothesis is that the expression of *Xenopus* Nogo-A is not expressed on the plasma membrane of oligodendrocytes at all, hence the presence of Nogo-A in the *Xenopus* may be not acting through contact inhibition of axon growth as occurs in mammals (Chen et al., 2000; Oertle et al., 2003b).

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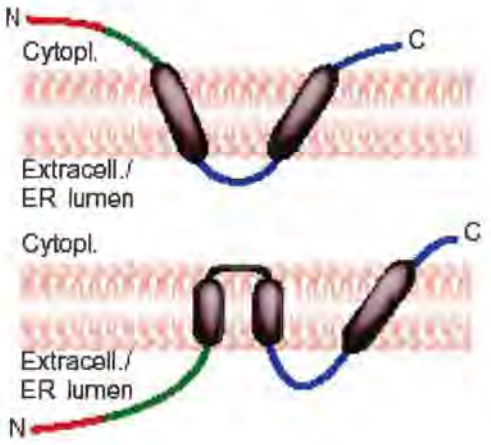

Figure 6.1. The two membrane topologies for Nogo-A protein. Two putative transmembrane domains may span the membrane once or twice. Thus, the N-terminus of Nogo-A may either be in cytoplasmic compartment or in extracellular / ER lumen space. (Adapted from Oertle and Schwab, 2003)

Neuronal expression of Nogo-A

In mammals, the expression of Nogo-A is not only described in oligodendrocytes and the myelinated tract, but in neurons as well, especially the motoneurons in the ventral horn of SC (Huber et al, 2002; Hunt et al, 2003) and the growing axons during nervous system development (Tozaki et al., 2002). In *Xenopus*, immunoreactivity of Nogo-A was also found in the neurons, in particular the motoneurons in the ventral horn of SC and the neurons in the tadpole forebrain. Although these results indicate that the neuronal expression patterns of Nogo-A in *Xenopus* are similar to those in mammals, the functions of Nogo-A in neurons are still awaiting characterization.

In both human and chick embryonic developmental stages where SC regeneration is possible, Nogo-A expression is also associated with neurons (O'Neill et al, 2004). In the same study, both the Nogo-A protein and transcript were detected before myelination began at neurogenesis commence (O'Neill et al, 2004). Similar developmental stages were examined in the *Xenopus* tadpole SC, and indeed, the spatiotemporal expression of Nogo-A is associated with neurons as described in chicks and humans earlier. Based on the expression of Nogo-A in the mammalian developing and injured nervous system, it has been

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postulated that Nogo-A may be a marker for growing axons and may possibly promote effects axon growth (Hunt et al., 2003; Tozaki et al., 2002). In the present study, post-lesion expression of Nogo-A was found in the nerve fibers of regenerating frog ON colocalized with the neurofilament expression in the absence of myelin protein (Section 4.2.4). Due to the similarity in Nogo-A expression between *Xenopus* CNS and amniotic CNS, one could hypothesize that during development the functions of Nogo-A in *Xenopus* and higher vertebrates neurons are likely to be the same. Moreover, Nogo-A may also be a marker for regenerating axons in *Xenopus*.

Nogo and apoptosis

When the *nogo* gene was initially shown to have high homology to cDNA of bNI-220, the bovine homologue of NI-250, the sequence analysis also revealed that the Nogo shares similar C-terminal domains with the reticulon group of proteins (Chen et al., 2000; GrandPré et al., 2000). Nogo is thus one of the most recently identified members of the reticulon family (Oertle et al., 2003; Oertle and Schwab, 2003). The expression of Rtn proteins is widely found in most eukaryotes. Although a role of Rtn has been suggested in endoplasmic reticulum (ER) membrane transport, cytoskeleton linkage of the ER network and in cell division, the functions of Rtn proteins are still poorly understood. Nonetheless, the Nogo-A/RTN4-A has been widely described as a potent neurite outgrowth inhibitor in mammals (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). One could hypothesise that Nogo-A/RTN4-A may have functional relatedness with its Rtn protein family.

One of the Nogo spliced isoforms, Nogo-B is also known as RTN-Xs which bind to anti-apoptotic Bcl-2 family proteins, and is likely to modulate their anti-apoptotic ability. In fact, an apoptosis inducing gene (ASY) has been identified to be the same as Nogo-B, and its ectopic expression has been shown to induce extensive apoptosis of cancer cells (Li et al., 2001). Another link between the function of Nogo and apoptosis was implicated by the interaction between the Nogo-66 and mitochondrial proteins, namely Nogo-interacting mitochondria protein (NIMP) and two of the components of ubiquinol-cytochrome c reductace

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complex, UQCRC1 and UQCRC2 (Hu et al., 2002). These mitochondrial proteins are known to be part of the trigger of programmed cell death in mammalian cells. Furthermore, evidences from expressional and functional studies in the mammalian CNS have demonstrated that $p75^{NTR}$, neurotrophin receptor and also co-receptor of NgR, mediates the cell death via Rho signaling under physiological and pathological conditions (Dubreuil et al., 2003; Grade and Barde, 1999).

Thus, one could speculate that the persisted expression of Nogo-A in the injured nerve of mammalian CNS has a role in programmed cell death leading to the failure of regeneration. On the other hand, the persisting post-lesion expression of Nogo-A in the *Xenopus* could possibly mean the modulation of apoptosis after injury and during regeneration. However, there is a high level of Nogo-A expression in retinal ganglion cell axons after *Xenopus* ON crush, and these cells in the frog and lizard show only relatively low levels of apoptotic cell death after axotomy (Lang et al., 2002; Tennant et al., 1993). Thus, one could also question the connection between Nogo-A and apoptosis in *Xenopus*. In fact, the validity of the Nogo-A isoform, Nogo-B, as a physiological pro-apoptotic protein has also been questioned in one of the mammalian studies (Oertle et al., 2003c). Although more functional assays, such as cell-count study or BrdU labeling, are required to demonstrate the validity of Nogo playing a role as a promoter of apoptosis, the link between the function of Nogo and apoptosis remains an interesting possibility.

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Nogo-Receptor expresses in Xenopus

Hints on the possible roles of Nogo-A in *Xenopus* may come from the distribution of the Nogo66-Receptor, NgR, which mediates the axon inhibitory effects through binding to the Nogo-66 loop. Extensive NgR expression studies have been done in mammals (Fournier et al, 2001; Wang et al, 2002), while in lower vertebrates the NgR homologous gene has been identified in fish (Klinger et al., 2004b). However, no studies have been done on amphibians to date. Although the *Xenopus* NgR sequence is yet to be identified, a new polyclonal NgR antiserum has been generated from the highly conserved region of the NgR

sequence across species. Although this new antiserum may recognize the protein of interest in the neurites of *Xenopus* culture, as well as in the immunoblots, it is at present not specific enough to be used as a tool in further expression studies or functional assays. This is mainly due to unacceptable levels of non-specific binding of pre-immune serum that has been shown by the multiple intensive bands in the Western-blots. In order to obtain a more specific antiserum, affinity purification can be done with the antigen, however, there can be inherent problems with this purification due the use of a short peptide as an antigen. The immunogenic peptide only consisted of 8 amino acids which may give rise to problems due to potential cross-reactivity of the antiserum raised against it. Moreover, further studies such as the pre-incubation of the antiserum with the immunizing peptide need to be carried to obtain the final proof of such specificity. Only once all these issues have been addressed will it be legitimate to state that this new antiserum does actually recognize NgR in *Xenopus.*

Even though the mechanism behind the interaction of *Xenopus* NgR and Nogo-A is not clear, there is little doubt that NgR is present in the *Xenopus*, based on the fact that Xenopus neurons are sensitive to the inhibitory influence of Nogo-A *in vitro*. It cannot be ruled out that an intrinsic neuronal factor may modulate the inhibitory signal transduction in the same way that intracellular molecules mediate the axonal growth, or overcome the inhibitory effects of the surrounding environment. NgR protein was characterized as a glycosyl-phosphadidylinositol (GPI) anchor protein (Fournier et al, 2001) that was found to be attached to the plasma membrane of neurons and axons (Wong et al., 2002). Due to the nature of GPI-anchor of NgR which has no transmembrane domain, other membrane proteins have been described to form a receptor complex with NgR: $p75^{NTR}$ (Wang et al., 2002a; Wong et al., 2002), LINGO-1 (Mi et al., 2004) and recently TROY/TAJ (Park et al., 2005; Shao et al., 2005). Functional studies have shown that the enhancement of axon regeneration is induced by manipulating the Nogo receptor complex (Wang et al., 2002a; Wong et al., 2002; Yamashita et al., 2002) or the molecules downstream of the signal transduction cascade, such as Rho (Fournier et al, 2003; Yamashita et al., 2002) and cAMP (cyclic AMP) (Neumann et al., 2002; Qiu et al., 2002). Furthermore, inactivation of Rho (Bertrand et al., 2005; Fournier et al., 2003; Lehmann et al., 1999) or elevation of cAMP (Cai et

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al., 1999; Neumann et al., 2002; Qiu et al., 2002) attenuates the inhibition of axon growth and promotes axonal regeneration in the presence of myelin-associated inhibitors. Taken together, it is possible that *Xenopus* neurons may exhibit different intrinsic properties within the signal transduction or the Nogo receptor complex in which the *Xenopus* p75 homologue has also been identified (Hutson and Bothwell, 2001). However, the lack of inhibitory activity cannot be explained by the absence of NgR as this receptor is most likely also expressed in *Xenopus* as shown by this study.

6.2 Conclusion

The inhibitory activity of TN-R and Nogo-A towards neurite outgrowth has been shown in *in vitro* studies of mammalian cells. Thus, it could be responsible for the failure of axonal regeneration in the mammalian CNS. From the immunohistochemical experiments of this study, it is clear that the putative neurite growth inhibitory proteins, Nogo-A and TN-R are expressed in the amphibian CNS, which can perform axonal regeneration after CNS injury. The expression patterns of TN-R and Nogo-A in *Xenopus* CNS was found to be associated with myelin and oligodendrocytes. This is in line with published findings in the mammalian CNS, suggesting their roles in myelination and oligodendrocyte functions. In addition, expression of these proteins was also observed in neurons, similar to mammals. Conflicting with their postulated role in inhibition of axon growth, however, both molecules were present at the injury site of the ON, which can be regenerated in *Xenopus*. These post-lesion expression patterns of TN-R and Nogo-A could imply a role in oligodendrocytes dedifferentiation and redifferentiation. Moreover, the outgrowth of cultured *Xenopus* neurons was not compretely inhibited by the TN-R and Nogo-A. Thus, the TN-R proteins from different classes of vertebrates might differ in their ability to inhibit axon growth.

In conclusion, these results do not provide evidence of a correlation between the presence (or absence) of mammalian neurite outgrowth inhibitors and the ability

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for axon regeneration in the CNS of *Xenopus*. One could argue that there is an intrinsic difference in the neuron properties between amphibians and the mammals. Alternatively, TN-R and Nogo-A may exhibit different functions during development, adulthood and regeneration in different vertebrate classes.

6.3 Future Work

Although TN-R and Nogo-A are known potent neurite outgrowth inhibitors, other functions are yet to be discovered that lead to future studies:

- An investigation on the origin of the protein synthesis may provide an indication of where and how the proteins are synthesized. This can be achieved by considering mRNA expression in TN-R- and Nogo-A-expressing cells, such as *in situ* hybridization. It will also be interesting to measure the 2nd messenger levels of the signal transduction in an attempt to understand the change in the intrinsic neuronal properties.
- Normally, the transgenic or knock-out animals are the models for discovering the functions of a protein. It is, however, technically challenging to perform such experiments in *Xenopus*. To reveal the yet unknown functions of these proteins, various *in vivo* and *in vitro* analyses of TN-R and Nogo-A can be performed in this regenerating system. For instance, RNA-interference technique can be performed at the embryonic stage of the animal, or the application of function-blocking antibodies can be done in the *in vivo* or *in vitro* models. Another example of functional study will be the intravital microscopic imaging and analysis of axon growth and guidance under physiological and pathological conditions.

Only with greater clarity on the functions of TN-R and Nogo-A it will be possible to assess the correlation between the expression of these proteins and the regenerating ability among the different classes of vertebrates and thus shed light on the role of TN-R and Nogo-A in development and regeneration.

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Chapter 7

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Appendix

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Data Sheet of Tenascin-R Antibodies

Phylogenetic analysis of tenascin-R in the vertebrate CNS

1. Vertebrate species (brain tissue) analyzed by Western blotting:

Squalus acanthias (shark)

Cyprinus carpio (carp), Salmo irideus (trout), Carassius aureus

Triturus cristatus (newt), Salamandra salamandra (salamander), Rana ridibunda (frog)

Natrix natrix (water snake), Lacerta viridis (lizard), Testudo hermanni (tortoise)

Galus domesticus (chick), Columba livia (pigeon)

Erinaceus concolor (hedgehog), Spermophilus citellus, Mus (Mus musculus, Microtus arvalis, Apodemus fl.), Rattus (rat), Oryctolagus cuniculus (rabbit), Bos (cattle), Sus scrofa domestica (pig), Homo sapiens (man)

Species	TN-R forms*	tn-R1	tn-R2	tn-R3	tn-R4	tn-R5	tn-R6	pTN-R
Squalus	200-220 kD	+	-	-	-	+	+	+
Cyprinus, others	170 kD	+	-	-	-	+	+	+
Salamandra, Rana	180-160 kD	+	+	-	+	?	+	+
Natrix, others	160-180 kD	+	+	-	+	?	+	+
Galus	180-160 kD	+	+	+	+	+	+	+
Mammals (diverse)	160-180 kD	+	+	+	+	+	+	+
Homo sapiens	160-180 kD	+	+	+	+	+	+	+

* Major molecular forms detectable; the order of description reflects the predominant appearance of either TN-R 160 or TN-R 180 isoform.

2. Polyclonal (pTN-R) and monoclonal antibodies tn-R1-6 to TN-R

Antibody	Species	[conc.]	Immunoblot (ECL)	Immunocytochemistry
1. pTN-R	rabbit IgG	4.2 mg/ml	1:2000 - 1:5000	1:500 - 1:1000
2. tn-R1*	mouse IgG	2.0 mg/ml	1:1000 - 1:2000	1:300 - 1:700
3. tn-R2	mouse IgG	3.0 mg/ml	1:1000 - 1:3000	1:100 - 1:500
4. tn-R4*	mouse IgG	4.0 mg/ml	1:1000 - 1:2000	1:500 - 1:2000
5. tn-R6*	mouse IgG	4.8 mg/ml	1:1000 - 1:3000	1:500 - 1:2000

All antibodies represent affinity purified IgG fractions (**1 ml** each) in PBS + 0.2% sodium azide.

* most suitable for immunocytochemistry

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Data Sheet of Synthetic NgR Peptide

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Animal Derived/Byproducts Health Certificate

I, Undersigned certify that **Alpha Diagnostics Intl. Inc.,** located at 5415 Lost Lane, San Antonio,TX,78238,USA is a United States Drug Agency (USDA) registered and periodically inspected animal research facility **(USDA Reg.#:74-R-140.)** All antibodies included with this shipment have been derived from our own healthy animals that have no clinical signs of any infectious diseases. In addition, the antibodies have been affinity purified.

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Masarrat Ali, Ph.D
Director, Animal Resources
Dated: 05/02/05

Personally Appeared Masarrat Ali this Date:

Mohammad Ali
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Specification Sheet for Nyatia-1 Peptide-KLH Conjugation

Name of peptide:	**Nyatia-1 (12756)**
Form supplied in:	**Lyophilized (Reconstitute in 5 ml 1X PBS)**
Amount of peptide Coupled:	**10 mg**
Concentration:	**2 mg/ml**
Volume:	**5 ml**
Amount of KLH Coupled:	**5 mg**
Peptide Solubility:	**Soluble in DMSO**
Coupling Method:	**GLUT**

Recommended Storage for peptide-Conjugate: 4°C for short term;Freeze in suitable aliquots.

The peptide-conjugate may be cloudy in appearance upon storage. If that occurs, mix well before use. This does not affect the antigenicity of the conjugate.

Please note that no preservative was added to the conjugate. Appropriate preservative can be added if the conjugate will be used for non-immunization purposes. Do **NOT** add any preservative if it is used for immunization.

Prepared and Checked by:

Date 05/01/05

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PEPTIDE DATA SHEET

LOT NO:	28532/12561
PEPTIDE NAME:	Nyatia-1
PEPTIDE SEQUENCE:	SLQ YLR LN
EXPECTED MASS:	1006
AVERAGE MASS:	1006.8
SOLUBILITY:	Soluble in DMSO
AMOUNT DELIVERED:	5 mg
PURITY:	100%
COMMENTS:	
DATE:	4/20/2005
RELEASED BY:	
VERIFIED BY:	

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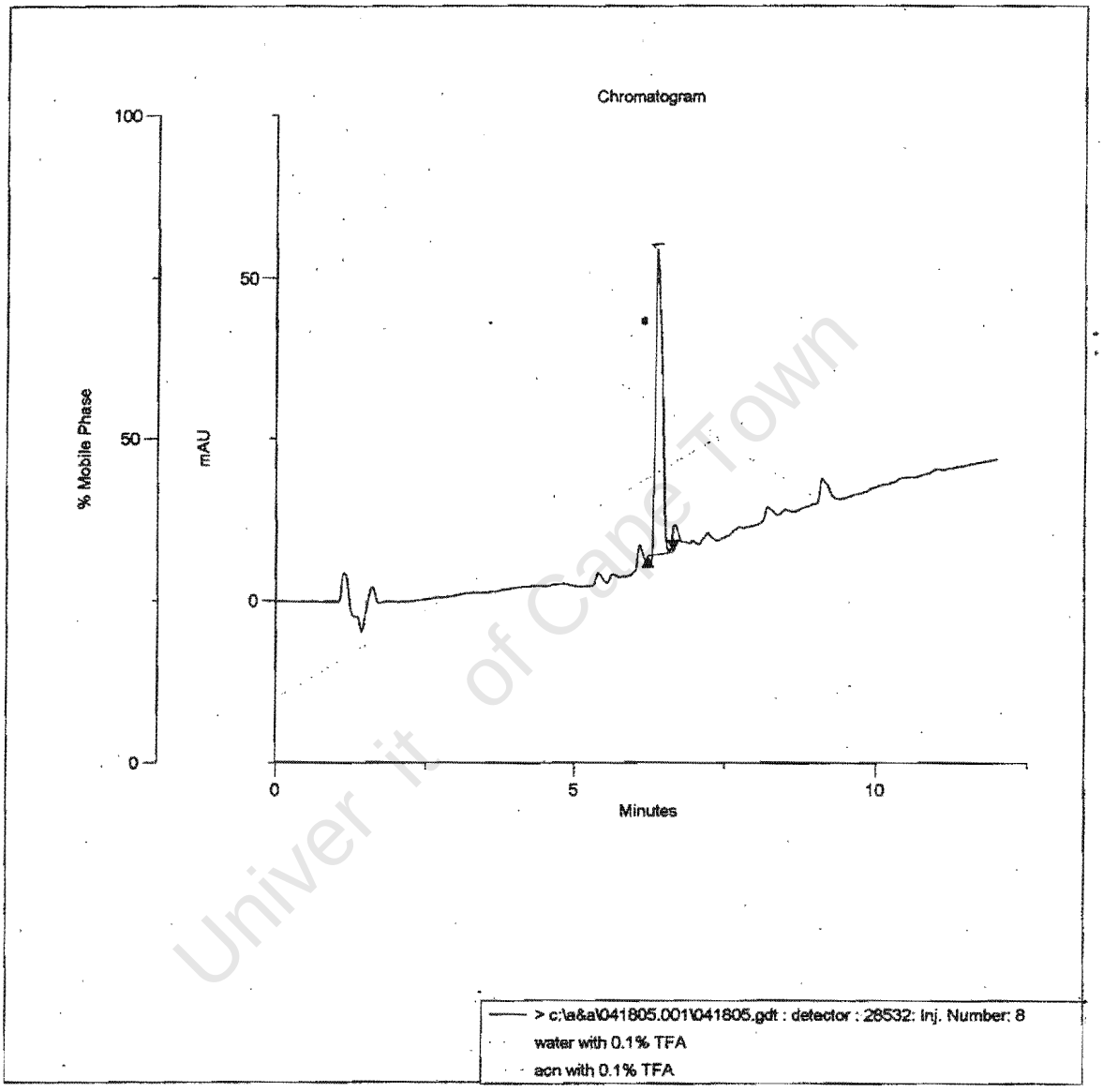
Data Sheet of Synthetic NgR Peptide

Mon Apr 18 2005 17:19:10 Results Report Page



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	Sample Descrip.	R. Time	Area	Area %					
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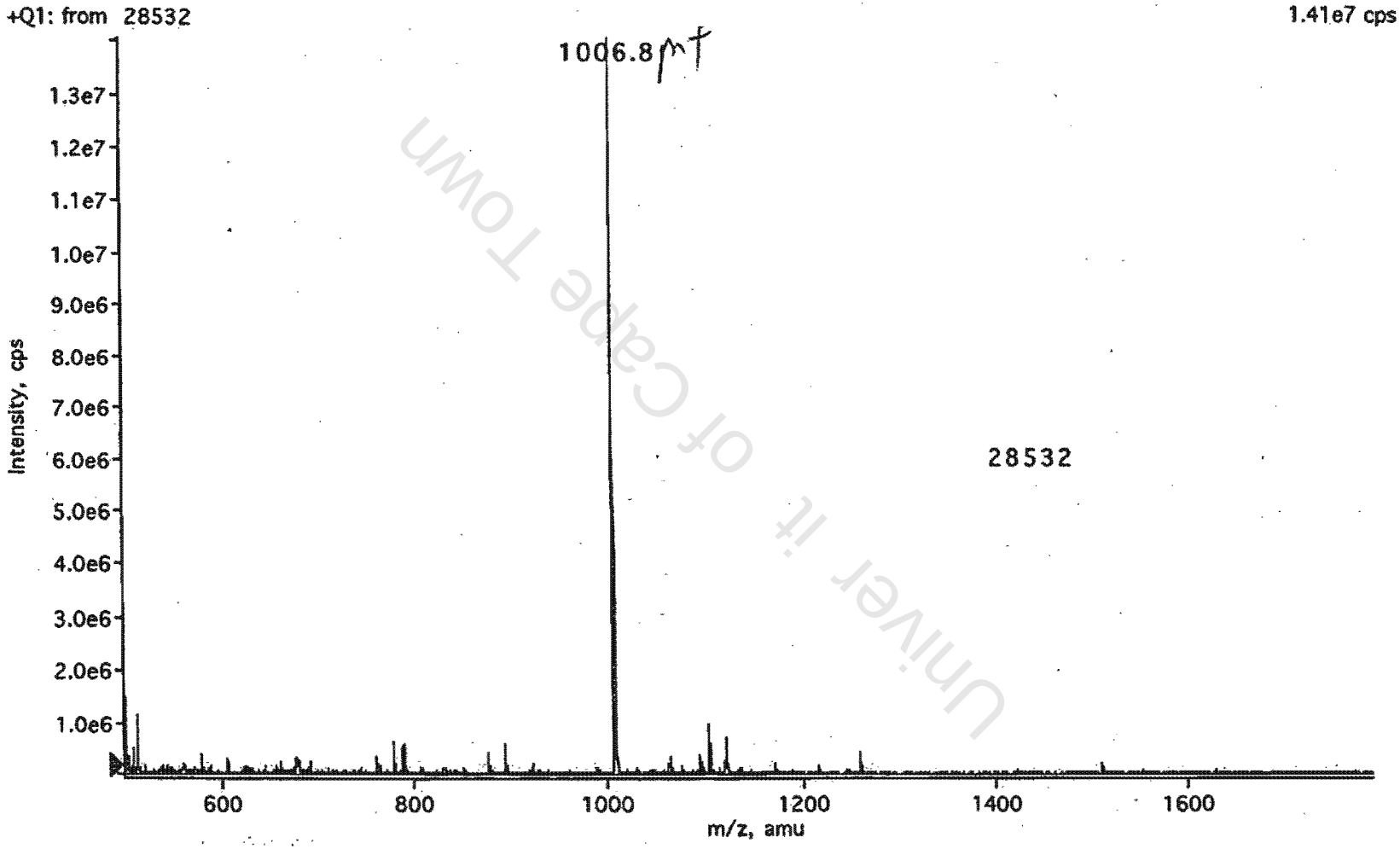
Data Sheet of Synthetic NgR Peptide



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Data Sheet of Synthetic NgR Peptide

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Reconstitution and storage of Peptides

To ensure that our material retain their integrity, the following guidelines are suggested for storage and use.

To determine the suitable method of dissolving your peptide, utilize only a small amount of appropriate solvents. Dissolve the peptide in Distilled or deionized sterile (and if possible, oxygen free) water. Only when the peptide has been fully dissolved should required buffer salts/solution be added and the solution diluted to its final concentration, that is, dissolve the peptide first and add the buffer afterwards.

Bacterial degradation can occur in peptide solutions. Always use sterile water to dissolve peptides. If required, the peptide solutions may be filtered through a 0.45 um or 0.2 um filter. Do not keep peptide solutions at room temp.

Amino acids such as cysteine, Methionine or Tryptophan are susceptible to oxidation and should be dissolved and stored in oxygen free water. This may be prepared by degassing under reduced pressure by sparing with an inert gas such as Nitrogen, Helium or Argon.

If peptide is insoluble in pure water, sonication may help in breaking up any particles and increase the rate of dissolution. Caution: Sonication can cause warming of the solution and degradation of the peptide.

If the peptide contains many basic amino acids (K, R etc), use an aqueous acetic acid solution (1 to 10%) with or without sonication. For very hydrophobic peptides, use 50% aqueous acetic acid.

If the peptide has many acidic amino acids (E, D etc), use an aqueous ammonia solution (1 to 10%) or a volatile basic buffer (up to pH 8) such as N-Ethymorpholine Acetic or Bicarbonate with or without sonicaton. The pH may have to be adjusted before chromatography.

Propanol or acetonitrile may be used to dissolve some medium sized peptides. If the peptide is to be injected onto a column, the amount of organic solvent, especially propanol must be kept small, or retention time will be greatly affected.

If the peptide is highly hydrophobic with aromatic or hydrocarbon-like side chains, such as Val, Leu, Ile, Met, Phe, Try, Ala or if the peptide is neutral, use a chaotropic agent such as DMF/DMSO-

a) High concentration of chaotropic salts to help dissolve the peptide by breaking up the secondary structures.
b) Chaotropic agents are suitable for preparing solution for analysis, but may interfere with a biological system used for study of the peptide
c. The best agent are DMF or DMSO (up to 30%) added drop until the peptide dissolves.
d. On reverse phase chromatography, the DMF will elute with the buffer front. The peak can be vary high, depending on how much was injected. Most peptides are retained longer than the few minutes it takes for a large DMF peak to elute. If the peptide is very small and elute early, the starting % may have to be decreased when the peptide is injected.

Storage Of Lyophilized Peptides

All peptides should be stored refrigerated, preferably at –20 °C for maximum stability. Most peptides when stored below –20 °C will remain stable for several months. This applies to many amino acid derivatives, lipids, enzymes and other proteins as well as peptides. The only exception to this is immobilized proteins and peptides.

When using a refrigerated product, the bottle or the vial should be allowed to warm to room temp. in a desiccators containing fresh desiccant before opening. From -20 °C this can take an hour or more, depending on the pack size. Failure to do this can cause condensation to form on the product when the bottle or vial is opened and will greatly reduce the stability of the material. Once opened, the required quantity should be weighed out to prevent water uptake. This is a particular problem with some hydroscopic peptides.

Storage Of Peptides in Solution

Peptide in solution are much less stable than lyophilized peptides. For best result please follow the following guidelines.

1. Avoid repeated thawing and freezing. It is recommended that the stock solution be aliquoted Thaw only what is needed. DO not store diluted Solutions.
2. For maximum stability, store at –20 °C in a sterile, pH 5 to 7 buffered solution.
3. Amino acid such as Cysteine, Methionine or Tryptophan are susceptible to oxidation and should be dissolved/stored in oxygen free water.
4. Bacterial contamination degrades peptides, so use sterile water or buffer.

Peptide recon 40107

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