The Role of Epidermal Growth Factor Receptor (ErbB1) in Axon Regeneration

MARY TEENA JOY

Department of Cell and Developmental Biology

University College London

Supervisor: Professor Patrick N. Anderson

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Declaration

I, Mary Teena Joy confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed_____ Date_____

Abstract

The thesis describes experiments on the role of Epidermal Growth Factor receptor (ErbB1) in limiting axonal regeneration. Pharmacological inhibitors of ErbB1 are known to improve neurite outgrowth from neurons grown *in vitro* on various inhibitory substrates such as CNS myelin, chondroitin sulphate proteoglycans (CSPGs) and fibrinogen (Koprivica et al., 2005). However, the hypothesis that ErbB1 is involved in the signalling mechanisms from various CNS inhibitory molecules has been challenged in papers form the Logan laboratory (Ahmed et al., 2010) which concluded that pharmacological blockers disinhibit neurite outgrowth by acting off-target to ErbB1. The first stage of this project was to use primary sensory neurons from ErbB1 knockout mice to test the hypothesis that ErbB1 is involved in inhibition of neurite outgrowth *in vitro* in the presence of CNS myelin, a Toll-Like Receptor 3 ligand (Poly I:C) and CSPGs. Through a series of experiments that have been described in this thesis, it was shown that ErbB1 antagonists act on-target and that ErbB1 signalling causes inhibition of neurite outgrowth of neurons cultured in the presence of Poly I:C, CNS myelin or CSPGs. Moreover, buffering calcium was shown to abolish the inhibitory effects caused by ErbB1 signalling, indicating that calcium is essential for the activation of the receptor. Other molecules that may be associated with ErbB1 signalling leading to inhibition of neurite outgrowth include PTEN and novel Sulfatase enzymes- Sulf1 and Sulf2. Using immunohistochemistry, Sulf1 and Sulf2 were shown to be expressed by a large variety of neurons. Also, ErbB1 expression detected immunohistochemically was predominantly localised to neurons. The thesis also describes preliminary observations on the efficacy of an ErbB1 antagonist or a dominant-negative ErbB1 lentivirus in improving axonal regeneration following an optic nerve crush or spinal cord injury in adult rats. In conclusion, ErbB1 signalling following injury is associated with regeneration failure.

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will support you no matter what"; "Everything will be fine" and "Shall I book tickets for our next holiday?"

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Abbreviations

CGRP- Calcitonin Gene Related Peptide

cAMP -cyclic adenosine monophosphate

cGMP- Cyclic guanosine monophosphate

CGC- Cerebellar Granule Cells

CNS –Central Nervous System

CNTF- Ciliary neurotrophic factor

CSPG- Chondroitin Sulphate Proteoglycan

CST- Corticospinal tract

DLK- Dual leucine zipper kinase

DREZ- Dorsal root entry Zone

DRG- Dorsal Root Ganglia

DLK- Dual leucine zipper kinase

EGFR- Epidermal Growth Factor Receptor (also known as ErbB1)

GAG- Glysosaminoglycan

GDNF- Glial-cell line derived neurotrophic factor

GPI- glycosphingoinositol

GSK-3 – Glycogen synthase kinase-3

HSPG- Heparan Sulphate Proteoglycan

HB-EGF –Heparin-binding epidermal growth factor

IB4- Isolectin B4

KLF- Kruppel-like factor

LAR- Leukocyte Antigen Related

LIF- Leukemia inhibitory factor

LPS- Lipopolysaccharide

LRR - Leucine rich repeat

MAG-Myelin Associated Glycoprotein

mTOR- Mammalian Target of Rapamycin

MyD88- Myeloid Differentiation factor 88

NF- Neurofilament

NGF- Nerve growth factor

NgR- Nogo receptor

OMgp- Oligodendrocyte Myelin protein

PirB- Paired Immunoglobulin-like receptor

PKA- Protein Kinase A

PTEN- Phosphatase and Tensin homolog

PI3-K- Phosphoinositide 3-kinase

PTP- Protein tyrosine phosphatise

RAGs- Regeneration-associated genes

SOCS-3- Suppressor of Cytokine Signalling-3

STAT- Signal transducer and activator of transcription

SOCS-3- Suppressor Of Cytokine Signalling-3

TLR- Toll-like receptor

Trk- Tropomyosin receptor kinase

TSC- Tuberous Sclerosis Complex

TGFα - Transforming Growth Factor α

CHAPTER-1 INTRODUCTION

1.1 General features of axonal regeneration

Axons regenerate following injury to peripheral nerves but the extent to which they regenerate varies from short distances, e.g. into a lesion site, to long distance functional regeneration, depending on the region along the axon which is injured and the nature of the injury. In contrast, for many decades it has been known that axons of the Central Nervous System (CNS), once injured, fail to regenerate. One of the first and most detailed accounts for regeneration failure in the adult CNS was given by Rámon y Cajal (Ramon y Cajal, 1928) who concluded 'But the functional specialization of the brain imposed on the neurones two great lacunae; proliferative inability and irreversibility of intraprotoplasmatic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably."

1.11Axons of the injured CNS are normally incapable of regeneration

Axon regeneration refers to the re-growth of axons whose integrity has been destroyed at some point. What people refer to as regeneration varies from local sprouting into undamaged tissue or a lesion site to long distance regeneration leading to recovery of function (Tuszynski and Steward, 2012). However, the term regeneration differs from sprouting which refers to the initial growth from the tip of an injured axon. Although there have been reports on sprouting of CNS axons after injury, dating from the time of Rámon y Cajal, regeneration of injured axons in the CNS of adult mammals beyond the lesion site without any experimental intervention is very uncommon. Various axonal systems in the CNS and their ability to regenerate after injury have been studied in the last few decades. These include dorsal column axons, corticospinal axons, rubrospinal axons, cerulospinal axons, raphespinal, spinocerebellar and propriospinal axons in the spinal cord and axons from retinal ganglion cells in the optic nerve.

1.111 The responses to injury of different CNS tracts

How easy it is to investigate axonal regeneration in the CNS *in vivo* depends on the type of lesion used and the tracts which are injured. Complete transection of the optic nerve or spinal cord is the gold standard model for eliminating the possibility of spared fibres, whereas crush injuries have the inherent problem that it is difficult or impossible to rule out the presence of axons which have not been destroyed by the lesion. Partial transection injuries have an intermediate possibility of spared fibres. If partial injuries to the spinal cord are used, it is much easier to reduce the risk of the presence of spared fibres if some tracts are lesioned rather than others. For example, in the hands of an experienced operator, almost all sensory axons of the ascending dorsal column can be lesioned without transecting the entire spinal cord. The completeness of the lesion can be confirmed by the lack of sensory axon terminals in the nucleus gracilis. After a transection injury, the injured axons form retraction bulbs and no regeneration beyond the lesion can be seen even after one year post injury. However, axonal sprouts may be seen proximal to the lesion (Neumann and Woolf, 1999). In contrast to the results of transection injuries, it has been shown that mice that receive a crush injury at T9 can regenerate their ascending sensory axons into the connective tissue matrix formed at the site of injury (Inman and Steward, 2003). Hence implying that the connective tissue matrix found in mice is a slightly more conducive environment for regenerating axons.

Corticospinal (CST) axons are responsible for relaying instructions for voluntary motor functions and their functional importance makes them an important axonal system for studying regeneration failure. Corticospinal axons descend into three tracts in the spinal cord- the dorsal corticospinal tract (dCST) which forms the main tract in rodents, the dorso-lateral corticospinal tract (dlCST) which forms the main tract in humans and the ventral corticospinal tract (vCST) which is present in rats and humans but may be seen only in a few strains of mice. Assessing regeneration of axons from these tracts is not trivial as it becomes difficult to distinguish between regeneration and sprouting of spared axons. The most reliable means of achieving a complete lesion of corticospinal axons is by complete transection of the spinal cord or transection of the pyramids. However, as cord transection disables the animal and poses an immense barrier for regenerating axons, other injury models are in common use. These include

dorsal hemisection, and contusion injuries. Regeneration of corticospinal axons after a complete transection without experimental interventions has not been described, but regeneration following some interventions has been claimed. Such findings have proven difficult to replicate (Ramon-Cueto et al., 2000;Cheng et al., 1996).

Studies have shown that injury to the corticospinal tract causes dying back of injured axons for a short distance, while the regenerative response is limited to a few sprouts (Kerschensteiner et al., 2005;Li and Raisman, 1995). There is a growing volume of literature on the existence of natural anatomical plasticity of injured corticospinal axons. Complete cervical transection of the dorsal corticospinal tract encourages sprouting of axons across the midline from the ventral corticospinal tract, which leads to some functional recovery (Weidner et al., 2001;Bareyre et al., 2005). Moreover, lesioned CST axons are capable of sprouting into grey matter to form an intraspinal circuit with propriospinal neurons (Bareyre et al., 2004;Lang et al., 2012). It is not quite clear whether there is death of CST neurons after spinal injury. Although the older literature reported no CST cell death, one study in recent years has claimed that lesions to the corticospinal tract causes death of many of the neurons (Hains et al., 2003). However, more recent studies have not supported the findings of Hains et al. Although there is shrinkage in the size of cell bodies after axotomy, there is no loss or death of cortical motor neurons as demonstrated after dorsal column lesion at T9 or lateral hemisection at C5 (Nielson et al., 2011). Similar to dorsal hemisection, contusion injuries and lesions of the dorsal, lateral and ventral CST (Liebscher et al., 2005) may also spare fibres in the ventral and/or dorsolateral CST. While there may be some functional recovery as a result of sprouts from spared fibres following partial lesions, axon regeneration is absent in the overwhelming majority of studies of animals that have received a complete transection injury to the spinal cord.

Raphespinal axons are serotonergic projections that arise from cells in the midline raphe and descend into the spinal cord. Not all serotonergic neurons arise from the raphe nuclei (Deumens et al., 2005). To achieve a complete lesion of raphespinal axons, a bilateral lesion that extends below the central canal would be required (reviewed Tuszynski and Steward, 2012). It is not generally believed that injured raphespinal tract axons can regenerate across lesion sites in the spinal cord and enter the caudal host tissue (Ramon-Cueto et al., 2000) although this has been

claimed in at least one study (Cheng and Olson, 1995) . Rubrospinal axons arise from the red nucleus in the brain stem, cross the midline and project into the lateral column of the spinal cord. The rubrospinal tract can be completely lesioned by means of a lateral funicular lesion and hence is an attractive injury model to investigate axon regeneration. Rubrospinal axons show a greater tendency to regenerate when compared to corticospinal axons (Liu et al., 1999).

Other axonal tracts that project into the spinal cord include cerulospinal axons that can be identified by immunostaining for tyrosine hydroxylase or dopamine beta hydroxylase and propriospinal axons which project up and down the spinal cord but for which no immunohistochemical markers have been identified. Like most of the other axonal tracts, lesioning of the specific tract and avoiding sparing of fibres has proven difficult. Amongst the ascending tracts, the dorsal spinocerebellar tract originating from Clarke's column in the thoracic spinal cord and situated in the lateral white column, has been used in some studies of axonal regeneration (Yick et al., 2000;Yick et al., 2003;Yick et al., 2004) and has the advantage that it is possible to perform a complete unilateral axotomy.

The optic nerve has served as another injury model to study axonal regeneration. Similar to axons in the spinal cord, most injured axons from retinal ganglion cells are incapable of regenerating through the lesion although some injured axons can grow into the lesion site (Campbell et al., 1999). Even more traumatic is the occurrence of extensive cell death among axotomised retinal ganglion cells (Villegas-Perez et al., 1988). Cell death does not occur immediately after injury but after 5-6 days post injury to the intraorbital region. At day 14, only 10% of the injured RGCs survive. The rate of survival depends on where the lesion has been made. In the case of an intracranial lesion 8-9mm behind the eyeball of a rat, cell death progresses more slowly and 80% of the RGCs survive 4 weeks after injury (Berkelaar et al., 1994;Fischer et al., 2004).However, the optic nerve which is a pure axonal tract is less complex in nature when compared to the spinal cord which contains axons from various tracts and consisting of both gray and white matter.

1.2 Reasons for the failure of injured CNS axons to regenerate

The regenerative failure of damaged CNS axons has been attributed to several reasons. These factors may either be extrinsic or intrinsic to neurons. The predominant extrinsic cues include the presence of the glial scar formed around the site of injury, the injury site itself with its content of macrophages and meningeal cells, and inadequate neurotrophic support, whereas intrinsic reasons include the reduced growth potential of the damaged neuron compared with those in the PNS.

1.21 Extrinsic factors

1.211 The Injury Site and Glial Scar

There are at least two regions to the scar which forms at sites of injury to the CNS, a glial scar, comprising various cells which include oligodendrocytes, microglia, astrocytes, oligodendrocyte precursors and multipotential progenitor cells (Fawcett and Asher, 1999), and the lesion site itself which is characterised by a lack of glial cells but an influx of macrophages, meningeal and other cells from outside the CNS (Zhang et al., 1995). The glial scar and lesion site not only act as a physical barrier to regenerating axons but also as a storehouse of various molecules that prevent regeneration. The changes seen following lesions to the CNS are the result of the activity of various cells which migrate to or are present in the damaged area.

At the first instance of spinal cord injury, all cells die in the region directly exposed to mechanical stress. Initially, this region is small but it progressively becomes larger in size as a secondary injury develops in which neurons, oligodendrocytes, astrocytes and other CNS glia near the lesion site die because of a cascade of events leading to necrotic and apoptotic cell death (Cao and Dong, 2012;Priestley et al., 2012). Hypertrophic astrocytes form the boundary of the lesion site and microglia and haematogenous macrophages are recruited to the site of injury where they clear debris before progressively disappearing starting at about 2 weeks post injury, leaving a cavity or fibrotic scar.

The glial scar which surrounds the cavity consists predominantly of reactive astrocytes and activated microglia (Dusart and Schwab, 1994) and oligodendrocyte precursors that express

NG2 (Rezajooi et al., 2004). It has been reported that there is an increase in NG2 expression 24hrs post lesion in adult rats with a puncture lesion in the cerebellum. This peaks at 7 days, after which there is a decline in expression levels (Levine, 1994). A similar increase in NG2 expression in the injured rat spinal cord and sciatic nerve has also been reported (Rezajooi et al., 2004). Astrocytes which otherwise act as supporting cells in the CNS change their morphology when CNS axons are injured. They take on a reactive, hypertrophic phenotype where their processes are tightly packed leaving little extracellular space and the astrocyte processes abutting onto the lesion develop a basal lamina to form a partial glia limitans (Zhang et al., 1997).

In addition to the changes seen at the lesion site, degenerative events occur in all regions to which the injured axons project. When an axon is lesioned, the distal part undergoes Wallerian degeneration, causing the degeneration of myelin and the formation of myelin debris. Macrophages and microglia which become activated migrate to the site of axonal degeneration in order to clear myelin debris. However, this process is rather slow compared to debris removal in damaged peripheral nerves and may take many weeks (Lawson et al., 1994;Perry et al., 1987).

1.212 Inhibitory molecules in the glial scar

Damaged CNS tissue contains various molecules that inhibit neurite outgrowth or repel neurites *in vitro* and may prevent the course of regeneration. Over the years, some molecular players and their receptors have been identified. These can be broadly classified as myelin-derived inhibitors (MAG, Nogo, OMgp), Chondroitin sulphate proteoglycans and axon guidance molecules (semaphorins, netrins, ephrins).

CNS Myelin

The idea that CNS myelin was inhibitory for axonal regeneration was first proposed by Berry 30 years ago (Berry, 1982). The first breakthrough in the molecular characterization of the inhibitory factors in myelin came from Schwab's laboratory (Caroni et al., 1988;Caroni and Schwab, 1988a). Since that time an abundance of inhibitors of axonal elongation have been

found in CNS myelin. The classical inhibitors present in CNS myelin include Nogo, Myelin associated glycoprotein (MAG) and Oligodendrocyte myelin glycoprotein (OMgp).

Myelin associated glycoprotein (MAG)

MAG is a member of the immunoglobin gene superfamily and is expressed selectively by myelin-forming cells. It is present in periaxonal Schwann cell and oligodendroglial membranes of PNS and CNS myelin internodes and in the Schmidt-Lanterman incisures (Trapp et al., 1989). It has been shown to participate in axon-glial interactions (Quarles, 2002).

MAG acts as a bifunctional molecule by promoting neurite outgrowth from foetal DRG neurons where as it inhibits outgrowth from mature neurons (Johnson et al., 1989;McKerracher et al., 1994). Dorsal root ganglia neurons from postnatal day 1 rats cultured on CHO cells expressing MAG had significantly longer neurites than in the absence of MAG, but outgrowth from DRG neurons from animals older than P6 was severely inhibited (Mukhopadhyay et al., 1994). In the case of cerebellar granule neurons, inhibition of neurite outgrowth was persistent irrespective of age. The mechanism involved in the molecular switch from growth promotion to inhibition of sensory neurite outgrowth may be related to the higher cyclic AMP levels in younger neurons (Cai et al., 2001). Why MAG should be stimulatory to neurite outgrowth in young sensory neurons when sciatic nerves from new born rats lack MAG expression is not clear (Martini and Schachner, 1988).

An *in vivo* study on MAG-induced inhibition of neurite outgrowth (Sicotte et al., 2003) showed that mice immunised with myelin and MAG/Nogo had extensive sprouting and regeneration of dorsal and ventral CST axons in the injured spinal cord. However, the role of MAG as a potent inhibitor of axonal regeneration has been questioned. It has been reported that MAG knockout mice did not show any improvement in regeneration of the injured optic or CST in adult mice (Bartsch et al., 1995). Additional evidence was also provided that deleting MAG reduced CST sprouting (Lee et al., 2010), implying that MAG may be less important in preventing axonal regeneration in vivo and may play a more important role in axonal protection (Nguyen et al., 2009).

Nogo

In an attempt to investigate factors that contribute to the inhibitory nature of CNS white matter, Caroni and Schwab (1988) used an *in vitro* spreading assay with 3T3 cells grown on various myelin fractions. Their studies revealed that 35kDa and 250kDa protein fractions were necessary for CNS myelin inhibition of cell spreading and neurite outgrowth *in vitro*. Later, they raised antibodies against both fractions. Application of the IN-1 antibody to rats with complete CST lesion allowed injured CST axons to regenerate 7-11mm caudal to the lesion (Schnell and Schwab, 1990;Bregman et al., 1995; Brosamle et al., 2000;Liebscher et al., 2005). However, it is worth noting that there are very few reports of IN-1 producing axonal regeneration in other types of axons in the CNS.

The Nogo (reticulon 4) gene is transcribed as three splice variants-Nogo A, Nogo B and Nogo C. Nogo A is the largest of the three variants and shares a common carboxy terminus of 188 amino acids with Nogo B and C and an amino terminus of 188 amino acids with Nogo B. Nogo A was cloned by three independent groups (Chen et al., 2000;GrandPre et al., 2000;Prinjha et al., 2000) and shown to be a potent inducer of growth cone collapse and inhibitor of neurite outgrowth. Nogo A has two inhibitory domains- Nogo-66 and Amino Nogo which may function through different mechanisms. Nogo-66 seems to mainly affect neurons and can act in the single monomeric form whereas amino Nogo requires clustering to function as an inhibitor of neurite outgrowth and spreading of non-neuronal cells (Fournier et al., 2001;Fournier et al., 2003).

In situ hybridisation studies have shown that Nogo A mRNA is expressed in most neurons (Hunt et al., 2002;Hasegawa et al., 2005). Nogo A is widely expressed in peripheral and central nervous system neurons such as spinal motor, DRG, sympathetic neurons, retinal ganglion cells, Purkinje cells and also oligodendrocytes but is absent from astrocytes, Schwann cells and NG2+glia (Hunt et al., 2003;Josephson et al., 2001;Dodd et al., 2005;Peng et al., 2010). Hunt et.al, (2003) reported the expression of Nogo A in cultured mouse foetal neocortical neurons and its presence in the perikarya and growth cones of cultured rat DRG neurons. In the injured CNS, Nogo A is expressed in lesioned dorsal column fibres and regenerating sprouts of the injured spinal cord and proximal and distal stumps around the lesion site of crushed optic nerve (Hunt et al., 2003). Increased neuronal Nogo A expression was seen in rat DRG neurons *in*

vivo when their dorsal roots near the dorsal root entry zone (DREZ) were crushed and *in vitro* when exposed to myelin inhibitors (Peng et al., 2010). However, in the injured sciatic nerve, only some of the regenerating axons expressed Nogo A (Hunt et al., 2003).

Nogo B has been reported to be strongly expressed by cultured endothelial cells, smooth muscle cells and blood vessels and may play a role in vascular remodelling (Acevedo et al., 2004). Nogo C was seen to be widely expressed in muscle fibres and in the adult rat brain particularly Purkinje cells, the hippocampus, cerebral cortex and cell bodies of oligodendrocytes (Huber et al., 2002). However, it has been shown by Dodd et.al, (2005) that the predominant proteins that interact with Nogo A are Nogo B and Nogo C and that all three isoforms interact to form a high molecular mass complex.

Oligodendrocyte Myelin Glycoprotein (OMgp)

Oligodendrocyte Myelin glycoprotein (Ji et al., 2008) was initially described as a 120kDa peanut agglutinin binding protein isolated from human CNS myelin that is highly glycosylated and found predominantly on the white matter of CNS and on cultured oligodendrocytes, hence the name oligodendrocyte–myelin protein (Mikol and Stefansson, 1988). OMgp is not only present on oligodendrocytes but also pyramidal cells of the hippocampus, Purkinje cells, motor neurons in the brainstem and anterior horn of the spinal cord (Habib et al., 1998). It has also been shown to be present on structures encircling the nodes in the CNS (Huang et al., 2005;Ji et al., 2008). OMgp null mice showed abnormally wide nodes of Ranvier and collateral sprouting from these disrupted nodes (Ji et al., 2008). However, the nodal localisation of OMgp and its proposed function in maintaining nodal architecture was challenged by a more recent study (Chang et al., 2010). According to this study, anti OMgp antiserum used in previous studies exhibited strong cross reactivity with a type of CSPG namely versican and also stained OMgp null nodes. Also, they were unable to detect abnormal nodal collateral sprouting in OMgp knockout mice.

OMgp was considered similar to arretin, a protein fraction of CNS myelin capable of inhibiting neurite outgrowth. It was later reported that arretin was high in OMgp content. OMgp

has been reported to inhibit neurite outgrowth *in vitro* from cultured rat cerebellar granule cells, hippocampal neurons and causes growth cone collapse of DRG neurons (Kottis et al., 2002;Wang et al., 2002b).

Receptors for myelin associated inhibitors

Nogo Receptors

The discovery of Nogo and its first inhibitory domain, Nogo-66, led to the identification of its receptor namely Nogo receptor1- NgR1. The disruption of NgR1 renders axons insensitive to Nogo-66-mediated inhibition of neurite outgrowth (Fournier et al., 2001). Subsequently it was reported that both MAG (Domeniconi et al., 2002;Liu et al., 2002) and OMgp (Wang et al., 2002a) bind to NgR1 indicating that it forms a point of convergence for three myelin-associated inhibitors, although they are structurally different.

The Nogo receptor gene family consists of three members –NgR1, NgR2 and NgR3. Nogo receptors are leucine rich repeat (LRR) proteins that are linked to the surface by means of a glycosphingoinositol (GPI) anchor. The LRR cluster of NgR1 is connected to the cell membrane by means of a 100 amino acid stalk and GPI anchor. The stalk is necessary for growth cone collapse of retinal ganglion cells whereas the LRR cluster is necessary for binding of Nogo, MAG and OMgp (Fournier et al., 2001;Fournier et al., 2002;Barton et al., 2003). As NgR1 lacks a transmembrane domain, it is most likely that the receptor transduces a signal intracellulary by means of co-receptors. The low-affinity neurotrophin receptor and transmembrane protein, p75 has been shown to act as a co-receptor with NgR1 (Wang et al., 2002a;Wong et al., 2002). Activation of NgR1 through its co-receptor leads to activation of small GTPases such as RhoA and its downstream targets ROCK and LIMK1 (Fournier et al., 2003;Niederost et al., 2002;Montani et al., 2009). It was reported that the p75/NgR1 complex alone was insufficient to activate Rho signalling. Hence, came the discovery of LINGO, a nervous system specific receptor that interacts with p75 to enable NgR1 to transduce a signal into the cytoplasm (Mi et al., 2004). However, as the expression pattern of p75 does not overlap with the expression of NgR1 in most cells, an alternative co-receptor was required and this has been suggested to be the TNF alpha orphan receptor TROY (Park et al., 2005;Shao et al., 2005).

NgR2 is another member of the NgR family that has shown to be a high affinity receptor for MAG and binds with greater affinity than NgR1 (Venkatesh et al., 2005). NgR1 and NgR2 are expressed by myeloid dendritic cells (McDonald et al., 2011). Although NgR1, NgR2, NgR3 share similar structural architecture, NgR2 and NgR3 do no interact with p75, TROY and LINGO (Barton et al., 2003).

Two independent studies were pursued to assess the functional significance of the NgR1-MAG association for neuronal growth inhibition. One study showed that inhibition of neurite outgrowth from cerebellar granule neurons plated on CHO-MAG substrate is blocked in the presence of anti-NgR1 antibody (Domeniconi et al., 2002). A second study found that ectopic expression of NgR1 in embryonic chick DRG neurons is sufficient to confer MAG responsiveness (Liu et al., 2002). However, experiments on neurons including cerebellar granule neurons isolated from *NgR1*null mice showed that NgR1 is not necessary for MAG elicited neurite outgrowth inhibition (Chivatakarn et al., 2007;Venkatesh et al., 2007). Moreover, NgR1 null DRG neurons were also tested for OMgp responsiveness. It was found that neurite outgrowth inhibition on substrate bound OMgp is NgR1 independent (Chivatakarn et al., 2007). However, growth cone collapse of postnatal-day 21 DRG neurons in response to acutely applied OMgp or MAG is attenuated in NgR1 mutants compared to age-matched wild-type controls (Chivatakarn et al., 2007). These results demonstrate that NgR1 is not necessary for neurite outgrowth inhibition of substrate-bound inhibitor.

Paired Immunoglobulin-like Receptor B (PirB)

As deletion of NgR1 does not completely overcome inhibition of neurite outgrowth by myelin, it was hypothesised that additional receptors would be present. In an screen to identify other receptors for soluble Alkaline Phosphatase-Nogo-66 fusion protein (AP-Nogo-66), Paired Immunoglobulin-like Receptor B (PirB), a major histocompatibility class 1 receptor, was shown to bind to Nogo-66, MAG and OMgp (Atwal et al., 2008).

PirB is expressed by corticospinal neurons in layer V of the motor cortex and also by rubrospinal neurons in the magnocellular part of the red nucleus of adult mice (Omoto et al., 2010). In culture, PirB was expressed by neurons from mouse embryonic as well as P0 neocortex and was reported to be localised at or near synapses (Syken et al., 2006). However, PirB

expression was undetectable in both the white and gray matter of spinal cord in adult mice (Omoto et al., 2010). Moreover, immunoblots from total brain extracts of adult mice suggest low protein levels of PirB when compared to NgR1, Nogo, MAG and OMgp (Lee et al., 2010).

Atwal and colleagues (2008) showed that PirB plays a role in inhibiting neurite outgrowth *in vitro*. They showed that either genetic deletion or blocking PirB in cerebellar granule neurons improved neurite outgrowth in the presence of Nogo-66, MAG or OMgp. When both NgR1 and PirB were blocked, near complete disinhibition of neurite outgrowth was achieved in the presence of myelin, suggesting that PirB and NgR are the two functional receptors for myelin associated inhibitors. PirB plays a role in limiting ocular dominance plasticity in the visual cortex and it has been reported that mice lacking PirB displayed more robust plasticity at all ages (Syken et al., 2006).

Gangliosides

MAG binds to two main brain gangliosides GD1a and GT1b that are expressed on axons (De Vries and Zmachinski, 1980;Sun et al., 2004). It has been shown that mice lacking complex brain gangliosides or the key enzymes involved in ganglioside biosynthesis have decreased MAG expression and abnormalities in the PNS and CNS. Also neurons in which gangliosides have been altered show reduced MAG-mediated inhibition of neurite outgrowth and neuritogenesis (Sheikh et al., 1999;Vyas and Schnaar, 2001). However, the importance of ganglosides in MAG-mediated inhibition of neurite outgrowth varied with cell type. In cultured dorsal root ganglia neurons, MAG binding to gangliosides played a small role in mediating inhibitory signals and inhibition of neurite outgrowth was predominantly via NgRs (Mehta et al., 2007). However, in the case of cerebellar granule neurons and hippocampal neurons, MAG inhibition of neurite outgrowth was exclusively via gangliosides.

Chondroitin Sulphate Proteoglycans

CSPGs are another class of inhibitory molecules that are present in the glial scar and lesion site. They consist of a core protein covalently linked to one or more glycosaminoglycan

(Tuszynski et al., 1996) chains. The synthesis of the core protein-chondroitin sulphate (CS) begins in the cytosol and the nascent protein undergoes post-translational modifications in the endoplasmic reticulum (Wei and Hendershot, 1996). Sulphation of chondroitin sulphate mainly occurs at C-4 or C-4 and C-6 of the GAG chain. It has been reported that sulphation of CS chains occur while the nascent protein grows but ceases after protein elongation (Sugumaran and Silbert, 1990).The differences in the patterns of sulphation of GAGs lead to the high heterogeneity of CS chains.

There are 16 different CSPGs that have been identified (Herndon and Lander, 1990). Most of the chondroitin sulphate proteins expressed in the CNS belong to the lectican/ hyalectan family. These include aggrecan, versican, neurocan and brevican.

Neurocan

Neurocan which has a molecular size of ~500kDa has been reported to be the most abundant chondroitin sulphate proteoglycan present in the developing rat brain particularly in the molecular layer and fibre tracts of developing cerebellum (Rauch et al., 1991;Friedlander et al., 1994). Neurocan readily binds to cell adhesion molecules (CAMs) such as N-CAM and Ng-CAM (Grumet et al., 1993) but does not bind to collagen IV, laminin, fibronectin or ErbB1 (Friedlander et al., 1994). It has been shown that neurocan has a direct inhibitory effect on cell adhesion as well as neurite outgrowth from chick embryonic brain cells (Friedlander et al., 1994), newborn chick dorsal root ganglia neurons (Ughrin et al., 2003), rat retinal ganglion cells (Inatani et al., 2001) and cerebellar neurons (Asher et al., 2000). Also, transforming growth factor β (TGFβ) and EGF upregulate expression of neurocan in lesion sites of the injured CNS as well as in cultured astrocytes (McKeon et al., 1999;Asher et al., 2000).

Aggrecan

Aggrecan is a component of perineuronal nets which surround neuronal cell bodies and proximal dendrites forming a mesh-like structure with holes at the point of synaptic contacts (Asher et al., 1995;Yamaguchi, 2000). It has been reported that aggrecan is widely expressed by neurons in the adult rat as well as embryonic chick brain (Hennig et al., 1992;Schwartz et al., 1996). In the adult CNS, aggrecan is expressed in the cerebral cortex, deep cerebellar nuclei, brain stem nuclei, the colliculi of the midbrain, dentate gyrus, CA1, CA2, CA3 regions of the

hippocampus and in the spinal cord (Matthews et al., 2002). Aggrecan has been shown to be inhibitory to neural crest migration in avian embryo (Perris et al., 1996) and aggrecan extracted from human intervertebral disc acts as a non-permissive substrate for neurite outgrowth from chick DRGs (Johnson et al., 2002;Borisoff et al., 2003).

Versican

Versican is the largest member of the lectican family. It was initially identified as a fibroblast proteoglycan with a carboxy terminal consisting of two EGF-like repeats, a lectin-like sequence and a complement regulatory-like domain (Zimmermann and Ruoslahti, 1989). In versican, the CS attachment domain is encoded by GAGα and GAGβ. Hence, there are four splice variants for versican- V0 consists of both GAGα and GAGβ domains; V1 and V2 consists of either GAGβ or GAGα and V4 does not have any attachment domains (Dours-Zimmermann and Zimmermann, 1994;Naso et al., 1994). It has been reported that the domain α-containing splice variant of versican is least expressed in embryonic stages and increases 7 fold at 100 days when compared to postnatal day 10. However, the concentration of the domain β-containing splice variants of versican doubles in the developing rat brain between E14 and time of birth and then declines with age (Milev et al., 1998).

Versican splice variant –V2, which is highly expressed by oligodendrocytes in optic nerve and other myelinated fibre tracts of adult mouse brain, has been shown to be a potent inhibitor of neurite outgrowth from E7 chick retinal explants as well as E14 chick dorsal root ganglia neurons (Schmalfeldt et al., 2000). Versican V0/V1 has shown to be a negative regulator of cell attachment *in vitro* (Yamagata et al., 1989).

Brevican

Brevican is a neural proteoglycan present in perineuronal nets (Hagihara et al., 1999;Bruckner et al., 2000). A significant amount of brevican in the brain lacks GAG chains (Yamaguchi, 1996). Yamaguchi, (2000) reported that the presence of the GAG chains is essential for the ability of brevican to inhibit neurite outgrowth, since the core protein had been found to promote neurite outgrowth. The core protein exists as two distinct isoforms, of which the shorter isoform harbours a GPI (glycosylphosphatidylonositol) anchor in place of the C-terminal domain. The GPI anchor aids in the attachment of brevican to the cell membrane (Seidenbecher

et al., 1995;Rauch et al., 1997). Quantitative analysis of brevican by means of slot blot immunoradioassay using a monoclonal antibody, has shown that levels of brevican in the embryonic rat brain are relatively low compared to postnatal brain and its expression is maximum after terminal differentiation (Milev et al., 1998). Brevican is present in myelinated tracts and has been reported to contribute to the non-permissive nature of CNS myelin (Seidenbecher et al., 1998;Niederost et al., 1999). Surface staining of brevican on cultured hippocampal neurons have been reported (Seidenbecher et al., 2002). Brevican knockout mice display reduced synaptic plasticity (Brakebusch et al., 2002).

Phosphacan

Phosphacan, which is not a member of the lectican/hyalectan family, occurs in the CNS as a large CSPG with a molecular size of ˜800kDa (Faissner et al., 1994). It is secreted as a splice variant of the receptor- Receptor Protein Tyrosine Phosphatase β (RPTPβ) and corresponds to the entire extracellular domain of RPTPβ (Maurel et al., 1994).The receptor itself exists as two splice variants -the long form, and the short form which is devoid of 850 amino acids in the interstitial space of the extracellular domain which contains many GAG binding sites. This accounts for the existence of various isoforms for phosphacan.

The expression of phosphacan increases with age, with rapid increase in the late embryonic and early postnatal stages and high levels of expression in the adult brain (Meyer-Puttlitz et al., 1996). In the embryonic stage, phosphacan mRNA is confined to active areas of proliferation such as the ventricular zone of the brain and the ependymal layer surrounding the central canal of the spinal cord. Its expression during development overlaps with regions related to the formation of axonal trajectories and hence may play a role in promoting neurite outgrowth or inhibiting it depending on the neural lineage (Garwood et al., 1999). Although it was initially suggested that CSPGs play a role in limiting axonal outgrowth (Snow et al., 1990), there is evidence that shows that certain types of developing neurons such as thalmocortical neurons are capable of growing in CSPG enriched regions of the brain (Miller et al., 1995;Bicknese et al., 1994). In the case of E18 hippocampal neurons, phosphacan promotes neurite outgrowth (Garwood et al., 1999;Faissner et al., 1994) whereas it inhibits neurite outgrowth from chick DRG explants grown on laminin substrate (Garwood et al., 1999). In the case of chick brain cells

plated on Ng-CAM, phosphacan has been shown to be inhibitory to neurite outgrowth (Grumet et.al., 1993). It has been reported that although phosphacan promotes morphological differentiation of E18 rat cortical neurons, it acts as a repulsive substrate for these neurons *in vitro* (Maeda and Noda, 1996). Different neuronal populations have shown varied ability to regenerate into the CSPG-rich glial scar in the injured spinal cord (Inman and Steward, 2000).

Regenerating CST axons are not capable of entering the lesion but sprout into the region that is adjacent to the lesion whereas sensory axons grow into the lesion site, and this response can be enhanced when the neurons are stimulated by a conditioning injury (Bavetta et al., 1999). Nonetheless, it is rare for axons to be shown to be capable of regenerating through the CSPG-rich scar and beyond the lesion site in the spinal cord without experimental intervention.

Expression of CSPGs in the injured CNS

There is ample evidence of increased expression of CSPGs in and around injury sites in the injured CNS, such as in the case of cortical stab wounds (Asher et al., 2000;Asher et al., 2002), cerebellar injury (Levine, 1994b), transection of the fornix (Lips et al., 1995), spinal cord injury (Tang et al., 2003;Jones et al., 2003;Iaci et al., 2007;Fitch and Silver, 1997), and the DREZ after dorsal root lesions (Pindzola et al., 1993). An increased expression of CSPGs is shown by reactive astrocytes present in the chronic glial scar (Canning et al., 1993;McKeon et al., 1999;Smith-Thomas et al., 1994). An upregulation in the levels of phosphacan and neurocan in the injured cortex and its localisation to reactive astrocytes 30 days post- lesion has been reported (McKeon et al., 1999). Moreover, it has also been shown that NG2, phosphacan and brevican mRNA levels are upregulated after CNS injury (Levine, 1994;Snyder et al., 1996;Jaworski et al., 1999). The most comprehensive account of the expression of CSPGs in the injured spinal cord was reported by Tang et al. (2003) who used Western blotting and immunohistochemistry to show robust increases in neurocan, tenascin-C, and NG2 levels by 24 hours. Peak levels of 245/130 kD neurocan, NG2, and 250/200 kD tenascin-C were reached at 8 days but phopshocan and brevican peaked at one month (Tang et al., 2003).

Receptors for CSPGs

PTPσ

Protein tyrosine phosphatases (PTPs) are members of the LAR (Fisher et al., 2011) sub-family which act as receptors for Heparan Sulphate Proteoglycans (Johnson et al., 2006) and may be involved in controlling growth cone morphology, axon guidance and synapse formation (Aricescu et al., 2002;Johnson et al., 2006;Chagnon et al., 2004). The identification of PTPσ as a receptor for HSPGs, combined with studies on enhanced regeneration in axons from injured sciatic, facial and optic nerves in PTPσ knockout mice (McLean et al., 2002;Thompson et al., 2003;Sapieha et al., 2005) led to the investigation of PTPσ and its role in CSPG-mediated inhibition of axonal growth. Shen et al., (2009) first reported that PTPσ acts as a receptor for CSPGs. It was shown that PTPσ binds with high affinity to neural CSPGs such as neurocan and that gene disruption of PTPσ caused neurons to be less affected by CSPG-mediated inhibition of neurite outgrowth (Shen et al., 2009). This discovery coincided with the observation that CST axons apparently regenerate vigorously in the spinal cord of PTPσ defficient mice (Fry et al., 2010).

NgR1 and NgR3

There is recent evidence on the role of NgR1 and NgR3 as receptors for CSPGs (Dickendesher et al., 2012). It has been shown that knocking out the classical receptors for myelin-associated inhibitors (NgR1, NgR2, and NgR3) improves axonal regeneration in the crushed optic nerve and that double mutants for NgR1 and NgR3 were less affected by CSPGs *in vitro* when compared to mutants with genetically deleted NgR1 and NgR2. Moreover, it was shown that NgR1 and NgR3 bound CS-GAGs with high affinity, and that NgR1, NgR3 and PTPσ had cumulative effects on axonal regeneration, leading to the conclusion that NgR1 and NgR3 act as receptors for CSPGs (Dickendesher et al., 2012).

Axon guidance molecules

The growth cones of neurites of developing or regenerating neurons detect various environmental cues that may be attractive or repulsive, in order to navigate to their targets. The

path finding of an axon during development is normally guided by molecules which include ephrins, netrins, semaphorins, slits and Wnts. There are indications that certain axon guidance molecules which may facilitate growth cone collapse and inhibition of neurite outgrowth are upregulated in the injured CNS. These include the following.

Ephrins and their receptors

The ephrins comprise two subfamilies: the A-subclass (A1-A6) that are glycosylphosphatidylinositol (GPI)-coupled and the B-subclass (B1-B3) which are integral membrane proteins. Ephs are receptor protein tyrosine kinases which bind ephrins (Josephson et al., 2001) at points of cell contact (Pasquale, 2005). 15 vertebrate receptor Ephs have been identified, divided into A- and B-subclasses on the basis of ligand affinity and sequence similarity. Ephrins and ephs bind promiscuously with high affinity to most members of the corresponding subclass (Zhou, 1998) and even between subclasses. For example, EphA4 binds ephrinB molecules with high affinity (Kullander et al., 2001; Yokoyama et al., 2001). Under some conditions reverse signalling, in which downstream secondary messengers are activated in cells expressing ephrins when they bind Ephs, has also been reported (Cowan and Henkemeyer, 2002). Receptor Ephs are expressed in both developing as well as in the adult nervous system (Tuzi and Gullick, 1994). Ephs are expressed in developing axon tracts (Pasquale et al., 1992;Henkemeyer et al., 1994) and it was initially considered that Eph signalling mediated repulsive responses in neurite retraction, but was later reported that they also mediate attractive/adhesive responses (Holmberg et al., 2000). Changes in Eph expression occur following a spinal cord injury and may be significant in the responses to injury. The expression of ephrin receptors (Josephson et al., 2001) is upregulated following CNS injury. In contusion injuries to the thoracic spinal cord, an increased expression of EphB3 in motor neurons in the grey matter and astrocytes in the white matter has been reported (Miranda et al., 1999). Other ephrin receptors that are upregulated in the injured spinal cord include EphA3, Eph4A4, EphA6, EphA7 and EphA8 (Willson et al., 2002;Figueroa et al., 2006). EphA4 accumulates in corticospinal axons after injury while its ligand ephrin B2 is upregulated in astrocytes near the lesion site, posing as a barrier to regenerating corticospinal axons (Fabes et al., 2006 ;Bundesen et al., 2003). Apart from ephrin B2, ephrinB3 has also been reported to be inhibitory towards axonal outgrowth. Ephrin B3 is present in CNS myelin and expressed by postnatal myelinating

oligodendrocytes. The inhibitory effects of ephrin B3 on neurite outgrowth from neocortical neurons are equivalent to the combined effects of Nogo, MAG and OMgp (Benson et al., 2005). EphB3 upregulation in astrocytes has been implicated in the astrogliosis in adult rats (Goldshmit et al., 2004), which in turn has implications for regeneration of axons.

Semaphorins

Semaphorins are a large family of axon guidance molecules that elicit both attractive as well as repulsive cues. The first identified semaphorin, Sema3A was previously called collapsin because it induced growth cone collapse in embryonic chick DRG neurons (Luo et al., 1993). The semaphorin family consists of 8 classes and these differences are based on sequence similarity and structural features (Pasterkamp and Verhaagen, 2006;Mark et al., 1997). Class 1 and 2 semaphorins are expressed in invertebrates whereas classes 3-7 are expressed in vertebrates. Semaphorins are either secreted or exist in the membrane-bound form whereby they are linked to the membrane by means of a GPI anchor.

Of all semaphorin classes, the functions of class 3 semaphorins have been most widely studied. Sema3A has been reported to be repulsive towards many different type of axons which include axons from sensory (Shepherd and Raper, 1999), sympathetic (Kobayashi et al., 1997), olfactory (de Castro F. et al., 1999),pontocerebellar (Rabacchi et al., 1999), cortical (Bagnard et al., 1998) and hippocampal (Chedotal et al., 1998) neurons. Semaphorins can not only act as repellents but also attractants, eg: Sema3B and Sema3C have shown to attract olfactory bulb axons as well as cortical axons (Bagnard et al., 1998;de et al., 1999;de Castro F. et al., 1999). Sema3A can exert differential effects on growth cones of cortical axons and dendrites of the same neuron depending on the levels of intraneuronal cyclic guanosine monophosphate (cGMP). Hence, in the presence of elevated cGMP levels, apical dendrites of pyramidal neurons are attracted by Sema3A, whereas its axons are repelled in the presence of low cGMP levels (Song et al., 1998;Polleux et al., 2000).

The first identified receptors for class 3 semaphorins were neuropilins- neuropilin1 and neuropilin 2 (Nrp1, Nrp2) (Kolodkin et al., 1997;Chen et al., 1997a;He and Tessier-Lavigne,

1997). However, as the cytoplasmic domain of Nrp was found not to participate in Sema3 signalling, it was suggested that other receptors were involved in signalling from Sema3(Nakamura et al., 1998).The main co-receptors identified for Npr were Plexins (class A plexin) (Takahashi et al., 1999). Other co-receptors discovered include LI CAM, Nr CAM , PlxnB1 and PlxnD1 (Castellani et al., 2000;Falk et al., 2005;Usui et al., 2003;Gitler et al., 2004).

In the intact CNS, Sema3A mRNA is expressed in cerebellar Purkinje cells, sub-populations of spinal and cranial motor neurons and distinct nuclei of the amygdaloid complex (Giger et al., 1998). However, only low levels of expression were seen in the leptomeningeal sheet and glial cells showed no detectable level of semaphorin mRNA when the nervous system was intact (Giger et al., 1996). In the injured CNS, meningeal fibroblasts around the lesion site express Sema3 A-C and Sema3E-F (Pasterkamp et al., 1999). There is evidence that injured axons are repelled by class 3 semaphorins e.g. corticospinal axons and rubrospinal axons that sprout around the area of the lesion avoid regions expressing class 3 semaphorins. Moreover, ascending dorsal column axons subjected to a conditioning lesion that enables them to regenerate into the glial scar, avoid areas expressing Sema3A (Pasterkamp et al., 2001). Also, it has been suggested that certain semaphorins are co-expressed with CSPGs and that the inhibitory effects of CSPGs may be produced by their associated semaphorins (de Wit et al., 2005;de Wit and Verhaagen, 2007;Tannemaat et al., 2007) (but see above for receptors on CSPGs).

Semaphorins that have a transmembrane domain such as Sema4D have been shown to be expressed by oligodendrocytes and are present in CNS myelin. After injury, the expression of Sema4D is strongly upregulated by oligodendrocytes. Sema4D acts as a potent inhibitor of neurite outgrowth from DRG and cerebellar granule neurons (Moreau-Fauvarque et al., 2003).

Netrins

Netrin-1 was first identified as a chemoattractant for commissural axons from explants of embryonic rat spinal cord (Serafini et al., 1994;Kennedy et al., 1994). Other members of the netrin family that have been identified include Netrin-3, Netrin-4, Netrin G1 and Netrin G2 (reviewed by (Cirulli and Yebra, 2007) and (Barallobre et al., 2005). Netrin-1 is the most widely studied mammalian netrin. Netrin-1 is expressed in the midline of the developing CNS and binds to receptors belonging to the UNC5 and DCC families. UNC5 has shown to be expressed by

rubrospinal and corticospinal axons. Netrin-1 is expressed by mature oligodendrocytes and present in paranodal loops of myelin in the nodes of Ranvier (Low et al., 2008). Netrin-1 acts as a potent myelin-associated inhibitor of neurite outgrowth from spinal motor neurons *in vitro* and axonal regeneration *in vivo* as demonstrated by the reluctance of rubrospinal axons to regenerate into netrin-1 enriched grafts in the injured spinal cord (Low et al., 2008).

Slits

Slits are large extracellular glycoproteins with a molecular weight of about 200kDa. There are three known members of the family identified in mammals, namely slit1, slit2, slit3. Slits bind to Robo (roundabout) receptors-Robo-1, Robo-2, Robo-3, and Robo-4.

Slit-1 and slit-2 act as repulsive molecules for growth cones of retinal axons. Gene deletion of both slit-1 and slit-2 causes the formation of a second optic chiasm and hence these molecules play a role in directing retinal axons to their true targets (Plump et al., 2002). Slit-2 is also known to repel embryonic spinal motor neurons (Brose et al., 1999) and cortical axons (Shu and Richards, 2001) *in vitro*. However, slit-2 promotes neurite elongation from DRG neurons depending on the substrate and levels of intraneuronal cGMP. Using slit-2 in stripe assays on a laminin or fibronectin substrate, it was demonstrated that slit-2 repelled neurites from rat DRG neurons, whereas as slit-2 N terminal fragment acted as a chemoattractant (Nguyen-Ba-Charvet et al., 2001).

In the injured brain, slit-1, slit-2 and slit-3 are expressed 7 days post injury particularly in cells surrounding the necrotic tissue. Slit-2 mRNA is expressed by GFAP positive astrocytes (Hagino et al., 2003a). Neuroprotective and anti-inflammatory functions have been attributed to slits in the injured brain (Altay et al., 2007). However, in injured spinal cord slit-1 and slit-3 but not slit-2 was seen in the centre of lesion, presumably expressed by meningeal fibroblasts and macrophages (Wehrle et al., 2005).

1.213 Neurotrophic factors

Inadequate neurotrophic support may be one factor limiting the regeneration of axons within the CNS. Neurotrophins comprise a family of polypeptide neuronal growth-promoting

factors related to NGF (Nerve Growth Factor). NGF was first identified by Levi-Montalcini and colleagues (Cohen and Levi-Montalcini, 1957;Angeletti et al., 1968) and since then there has been a large body of evidence that shows that NGF promotes survival, differentiation and neurite outgrowth from sympathetic neurons and neural crest-derived neurons but not from cranial sensory, parasympathetic and spinal motor neurons (Barde et al., 1980;DiStefano et al., 1992;Lindsay, 1988). Later on, it was shown that homozygous mutants with a loss of function mutation for NGF exhibited a profound loss of neurons in sympathetic and sensory ganglia particularly medium and small sized peptidergic neurons and basal forebrain cholinergic neurons (Crowley et al., 1994;Chen et al., 1997b).

The second neurotrophic factor identified after NGF was brain-derived neurotrophic factor (BDNF), first isolated from pig brain (Barde et al., 1982). The molecular structure of BDNF revealed a high degree of similarity in its amino acid sequence (~55%) to that of NGF and this led to the concept of a neurotrophin family. The emergence of this concept led to the discovery of other family members such as NT-3 (Hohn et al., 1990;Rosenthal et al., 1990) and NT-4/5 (Berkemeier et al., 1991;Hallbook et al., 1991). Other polypeptides that posses neurotrophic properties but do not belong to the neurotrophin family include ciliary neurotrophic factor (CNTF) (Ip and Yancopoulos, 1996), leukemia inhibitory factor (LIF) (Leibinger et al., 2012), glial derived neurotrophic factor (GDNF) (Lindsay and Yancopoulos, 1996), insulin-like growth factor (IGF-1) (Dore et al., 1997) , basic fibroblast growth factor (Walicke et al., 1986) , transforming growth factor β (TGF-β) (Farkas et al., 2003) and sonic hedgehog (Miao et al., 1997).

Neurotrophins bind to two different classes of receptors- tropomyosin receptor (Trk) and $p75^{NTR}$. Although, $p75^{NTR}$ lacks a catalytic domain, it interacts with various other proteins that convey signals important for neuronal growth, differentiation and plasticity (He and Garcia, 2004). Different members of the neurotrophin family bind specifically to different Trk receptors. eg: NGF binds to TrkA (Klein et al., 1991a), BDNF binds to TrkB which also binds NT4/5 (Klein et al., 1991b), NT-3 binds to TrkC (Lamballe et al., 1991) and also TrkA and TrkB but with less affinity (Squinto et al., 1991). The binding of Trks to their ligands causes dimerisation of the receptor and subsequent transactivation of its tyrosine residue in the cytoplasmic domain.

Neurotrophins are widely expressed in the CNS. The expression patterns of BDNF, NGF and NT-3 exhibit spatial and temporal differences except in the adult hippocampus where all three neurotrophins are expressed at high levels (Maisonpierre et al., 1990). NT-3 and BDNF show reciprocal expression patterns during development with highest levels of NT-3 expression in the newborns which decline with age and lowest expression levels of BDNF in the embryonic stages progressively increasing with age. However, neither NT-3 nor BDNF are expressed in the newborn striatum (Maisonpierre et al., 1990) although BDNF and NT-3 promote survival and morphological differentiation of embryonic striatal neurons *in vitro* (Ventimiglia et al., 1995). NGF expression is relatively constant during development. NT-3 expression is high in regions involved in neurogenesis, particularly in the cerebellum (Altman and Bayer, 1982;Altman and Bayer, 1984). Hence, it has been suggested that NT-3 plays a role in acting as a mitogen to developing neurons whereas BDNF maintains and regulates morphological and neurochemical properties of mature neurons (Sofroniew et al., 1993).

Apart from neurotrophins, the receptors to neurotrophins are widely expressed in the CNS and PNS. TrkA mRNA is expressed in all most all PNS ganglia and by cholinergic neurons in the nucleus basalis of Meynert, septal nuclei and striatum (Holtzman et al., 1992). DRG neurons express all three different Trks-A, B and C in varying combinations (McMahon et al., 1994) whereas neurons in the neocortex and hippocampus express both TrkB and TrkC mRNA (Martin-Zanca et al., 1990;Lamballe et al., 1994;Valenzuela et al., 1993).

Expression of neurotrophins in the injured nervous system

There have been relatively few published studies of neurotrophin levels in the injured CNS. In what is probably the original study on the subject, it was reported that mRNA levels for NGF, BDNF, NT3 and NT4 varied little following injury to the spinal cord (Funakoshi et al., 1993). BDNF and NT3 mRNA have been reported to be elevated, beginning 6 hrs after spinal injury and remained elevated for the duration of the experiment (72 hours) (Hayashi et al., 1997;Hayashi et al., 2000). Excitotoxic injury to the hippocampus, which kill neurons but not glia, resulted in a decline in NGF, BDNF and NT3 mRNA and protein expression in the affected region, suggesting that most neurotrophin expression in the brain is in neurons and that reactive glia do not constitute such a strong source of neurotrophins as do Schwann cells in injured

peripheral nerve (Sofroniew et al., 1993). The absence of major changes (comparable to those in injured peripheral nerve) in neurotrophin production after stroke injury in rat cerebral cortex was demonstrated in several more recent studies (Quirie et al., 2012;Cui et al., 2010). The general view is therefore that neurotrophins are synthesised in the CNS, both before and after injury, mainly by neurons and that there is no dramatic increase in neurotrophin expression by glia following injury. It is not surprising; therefore, that exogenous neurotrophins have been reported in many studies to have a beneficial effect on axonal regeneration in the CNS (Zhang et al., 1998;Ramer et al., 2000;Taylor et al., 2006).

Expression studies of mRNA from receptors to neurotrophins after facial axotomy showed an increase in the levels of TrkB in motor neurons two days post injury, which increased three-fold at 7 days and remained 1.5 fold higher than baseline levels at 21 days after injury (Kobayashi et al., 1997). A similar increase in TrkB expression was observed in motor neurons after sciatic nerve transection (Hammarberg et al., 2000). It has been reported that TrkA is not expressed by motor neurons and the expression of TrkC remains altered after sciatic nerve injury (Funakoshi et al., 1993a;Hammarberg et al., 2000). However, when the peripheral nerve tissue is removed from proximally lesioned motor axons (Hammarberg et al., 2000), downregulation in the expression of TrkB and TrkC are seen, accompanied by extensive cell death of motor neurons (Hammarberg et al., 2000).

The $p75^{NTR}$ receptor, known as the low affinity receptor for neurotrophins, is expressed in very low amounts in motor neurons. However, after axotomy, the levels of $p75^{NTR}$ increases by 12-fold and only reaches baseline levels after 30 days post lesion. Greater expression levels of $p75^{NTR}$is seen in motor neurons when the sciatic nerve is crushed as opposed to transected (Rende et al., 1992;Raivich and Kreutzberg, 1987;Ernfors et al., 1989;Koliatsos et al., 1991;Yan and Johnson, Jr., 1988). In the absence of TrkA, p75 activation seems to have harmful effects on neurons -see review by Ichim et al. (2012).

1.22 Intrinsic factors

It has been known for many years that neonatal CNS neurons subject to axotomy possess greater potential to regenerate when compared to adult CNS neurons. The capacity of injured axons in the corticospinal tracts and visual tract to regenerate declines with age (Schwab and Bartholdi, 1996;Harvey et al., 1986;Kalil and Reh, 1979). For example, in the neonatal rat spinal cord, developing corticospinal axons that have not reached the site of injury can grow across the lesion site and innervate appropriate targets (Bregman et al., 1989). However, such is not the case with mature corticospinal axons which not only fail to regenerate beyond the glial scar but usually retract away from the lesion (Bregman et al., 1989;Li and Raisman, 1995;Hill et al., 2001). Similarly, axonal regeneration of Purkinje cells *in vitro* has been reported to be dependent on age (Dusart et al., 1997). Hence, the switch from regenerating to non-regenerating phenotype in mature axons of the CNS could be attributed to changes in its intrinsic growth programme, although the environment of the injured axons also changes during development and could limit regeneration.

1.221 The cell body response

After axotomy, various morphological changes, termed chromatolysis, occur in the neuronal cell body. These changes include displacement of the nucleus to the cell's periphery, dispersal of Nisl substance, swelling of the cell body and retraction of synaptic terminals (Lieberman, 1971). The intensity of these changes depends on the type of axon and distance of the cell body from the injury site. In the case of spinal motor neurons that are capable of regeneration, axotomy induces the cell body to become hypertrophic causing elevated levels of metabolism and protein synthesis whereas, in the case of non-regenerating (CNS) neurons, the cell bodies become atrophic characterised by decreased cell body size, low levels of protein synthesis and lack of dendritic arborisation (Lieberman, 1971). Both rubrospinal neurons (Kwon et al., 2002) and corticospinal neurons have cell bodies that become atrophic after injury. In both cases, a proximal axotomy may cause cell death (Liu et al., 2003;Giehl and Tetzlaff, 1996) but a

more distal axotomy, in the spinal cord, does not do so (Nielson et al., 2011;Brock et al., 2010;Barron et al., 1990).

1.222 Axonal response to injury

After axotomy, most CNS axons retract from the lesion site and some axons may sprout to a distance of one millimetre or less (Windle, 1980;Tom et al., 2004;Erturk et al., 2007). The axons that retract form retraction bulbs characterised by swollen ends and dystrophic growth cones. In the case of sensory neurons, injured axons in the ascending dorsal columns retract 200-300µm from the lesion site and only one third of the axons regenerate by forming new growth cones or sprouts at the nodes of Ranvier (Erturk et al., 2007;Kerschensteiner et al., 2005;Ylera et al., 2009).

1.223 Molecular events after axotomy

Calcium influx

Axotomy causes the disruption of the cell membrane exposing the axoplasm to extracellular calcium ions. Calcium influx is necessary for the production of new growth cones in injured sensory and sympathetic neurons and it has been shown that new growth cones cannot be formed in a calcium-free environment (Ziv and Spira, 1997;Kulbatski et al., 2004;Chierzi et al., 2005). The increase in intracellular calcium in both the distal and proximal stumps i) causes the formation of a membrane seal ii) reorganisation of cytoskeletal components to either form a new growth cone or a retraction bulb iii) retrograde signalling and iv) protein translation (Ambron and Walters, 1996;Vogelaar et al., 2009). As well as by entering the cell through the cut end, axotomy leads to membrane depolarisation thereby activating voltage-gated calcium channels causing elevation of calcium concentrations. An overall increase in calcium levels causes the reversal of sodium-calcium exchange pump leading to the release of calcium from intracellular stores. Hence this causes prolonged and elevated levels of calcium in both the cell body as well as injured axon (Ziv and Spira, 1997;Mandolesi et al., 2004).

Membrane sealing

Ionic homeostasis is essential for the survival of an axon after axotomy. This can be achieved by means of sealing the ruptured membrane. Membrane sealing involves collapse of the plasma membrane at the cut end and transport of vesicles to the injured membrane, causing the formation of multivesicular structures that fuse with the plasma membrane to form a sealing patch. The formation of the sealing patch prevents further influx of calcium through the cut end (Fishman and Bittner, 2003;McNeil and Kirchhausen, 2005).

Formation of a growth cone

Once the membrane has been sealed, the next step in regenerating an axon is the formation of a growth cone. The events that take place in growth cone formation have been well demonstrated using cultured neurons from Aplysia (Ziv and Spira, 1997).Approximately 100µm proximal to the cut end, active depolymerisation of microtubules take place depending on the levels of calcium. Moreover, actin-containing adhesion complexes depolymerise, freeing the plasma membrane from its extracellular surroundings and giving it more mobility to collapse, seal and form a growth cone. At the same time, spectrin which is a cytoskeletal structure lining the cytoplasm is proteolytically cleaved by calpain. Calpain-mediated proteolysis facilitates the radial polymerisation of microtubules and extension of the lamellipodium (Ziv and Spira, 1997;Erez et al., 2007;Sahly et al., 2006).

Formation of retraction bulbs

In the presence of low calcium influx at levels sufficient to form a membrane seal, retraction bulbs are formed in place of growth cones. Low calcium levels cause microtubule depolamerisation to occur at a very slow rate. Moreover, Golgi vesicles that move towards the cut end are unable to fuse with the plasma membrane due to spectrin that has not been proteolytically cleaved. It has been shown that growth cone formation can be reactivated by locally increasing calcium levels by applying ionomycin which triggers the cleavage of spectrin (Kamber et al., 2009;Erturk et al., 2007).

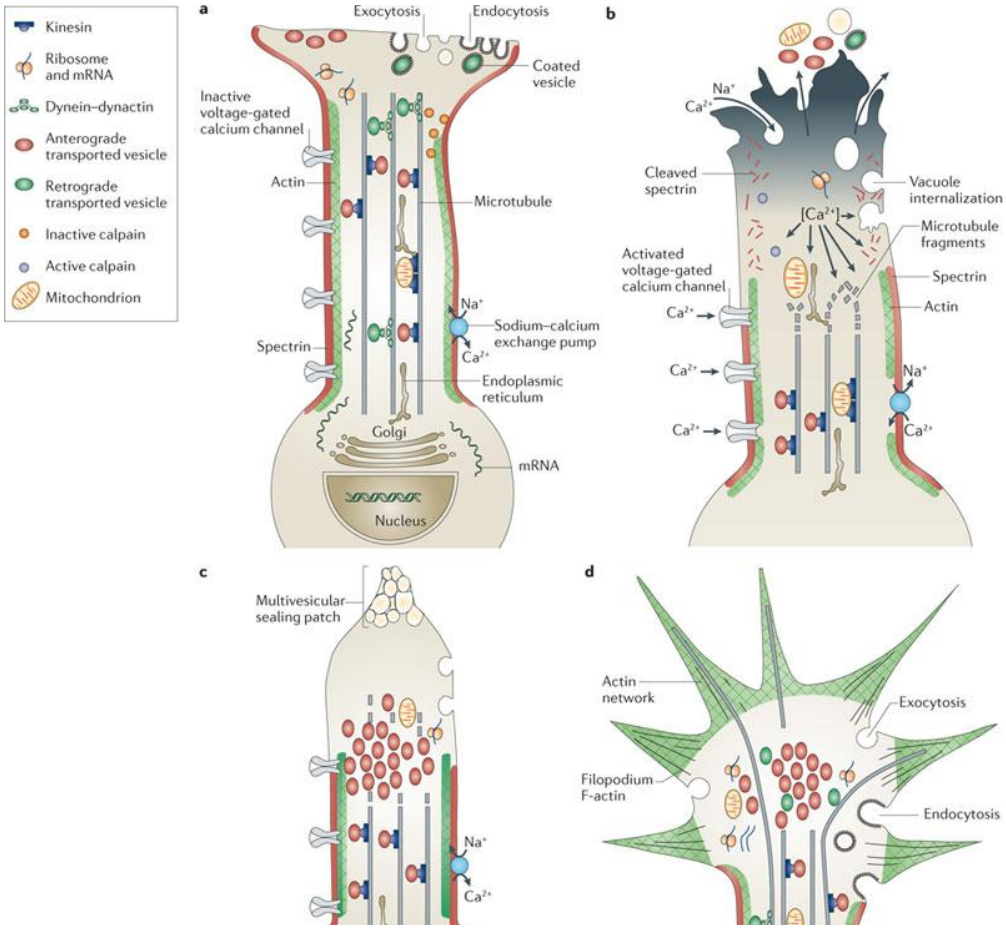

Figure1.1 : Events in the transformation of the proximal tip of a cut axon into a growth cone. (Bradke et al., 2012) a | Various components of the intact neuron take part in the transformation of a cut axonal end into a growth cone. **b** | Membrane rupture leads to elevation of the free intra-axonal calcium concentration (shown by the grey shading) by diffusion from the cut end, membrane depolarization, activation of voltage-gated calcium channels and release of calcium from intracellular stores such as the endoplasmic reticulum. Among many other processes, the elevated intracellular calcium concentration ($[Ca^{2+}]_i$) leads to the following: microtubule and actin depolymerization, membrane retrieval, activation of calpains (which proteolytically cleave submembrane spectrin), stimulation of vacuole internalization and activation of numerous other enzymes (not shown). These processes are confined to an axonal segment at the tip of the cut axon and lead to a small volume of the axoplasm being lost before the membrane at the cut end collapses. **c** | In most cases, the membrane of the cut end reseals by membrane collapse and accumulation of vesicles to form a sealing patch. Once a seal is formed, calcium removal mechanisms lower the $[Ca^{2+}]_i$, and this process is followed by microtubule and actin repolymerization. Anterogradely transported vesicles accumulate at the tips of the undamaged microtubule tracks. In *Aplysia californica* neurons, the vesicles fuse with the plasma membrane at regions in which the spectrin skeleton is cleaved. **d** | In the final phase of growth cone reconstruction, actin filaments assemble to generate the mechanical force at the leading edge of the lamellipodium. Microtubules polymerize and point their plus ends towards the plasma membrane. Note that proteins are translated both in the cell body (not shown) and at the growth cone.

1.224 Signalling in response to injury

Nuclear transport proteins

There are reports suggesting that axotomy induces certain proteins containing a nuclear localisation signal (NLS) to be transported to the nucleus, in turn activating various transcription factors. Three different classes of nuclear transport proteins have been identified. These include importins (Hanz et al., 2003), RanGTP (Yudin et al., 2008) and JNK interacting proteins (JIP).

It has been shown that importins are present in axons at a considerable distance from the cell body. Importin α is expressed in uninjured axons whereas importin β is present in the form of mRNA. After injury, importin β mRNA is translated into its protein form in the axons and forms an importinα/β high affinity NLS complex which binds the motor protein-dynein, thus facilitating retrograde transport (Hanz et al., 2003). This high affinity complex can then interact with various other proteins which get transported to the nucleus.

The transport of cargo using the importin system is regulated by a GTPase, Ran, which can either be found in the GDP-bound form in the cytosol or in the GTP-bound form in the nucleus (Weis, 2003). However, it has been reported that Ran-GTP is found in the axonal cytoplasm of the sciatic nerve (Yudin et al., 2008). Importins cannot bind to Ran in the GDP bound form but Ran-GTP can bind imporin β directly, and indirectly importin α via the adaptor protein CAS (Kalab and Heald, 2008;Yudin et al., 2008). In the uninjured sciatic nerve, RanGTP is found in association with importin α, dynein and CAS. After injury, $RanBP_1$ is locally synthesised and competitively binds to importins stimulating the release of RanGTP which gets hydrolysed by RanGAP to form RanGDP and hence cannot rebind to importins. This facilitates synthesis of importin β which then binds to importin α and dynein (Yudin et al., 2008). This complex which binds cargo is then transported to the nucleus in a retrograde fashion.

The different cargos that are transported to the nucleus in response to injury are under investigation. Examples of proteins transported to the nucleus include vimentin, Smad1and dual leucine zipper kinase. It has been reported that vimentin, a type III intermediate filament protein, is upregulated and is cleaved by calpain after sciatic nerve injury. Vimentin associates with phosphorylated Erk1 and Erk2 in the presence of elevated calcium levels and facilitates

retrograde transport of these proteins by directly binding to importin β. Phosphorylated Erk upon reaching the cell body activates the transcription factor Elk1 (Perlson et al., 2005).

A recent study has been made suggesting the role of Smad1 in acting as a transcription switch that enhances the growth state of injured dorsal root ganglia (Zou et al., 2009). Under conditions of peripheral nerve axotomy, there is increased expression of Smad1 which gets transported to the nucleus activating various downstream target genes. It has been shown that the effects of conditioning lesions on axon regeneration are severely compromised when Smad1 is knocked down (Zou et al., 2009). Smad1 acts as a mediator of BMP signalling via retrograde transport and hence regulates neuronal differentiation and synaptic maturation (Hodge et al., 2007).

Another candidate for cargo that has recently been reported is the dual zipper kinase (Xiong et al., 2010; Shin et al., 2012). DLK is a MAP kinase kinase kinase (MAPKKK) which activates JNK and p38MAP. DLK has been implicated in responses to injury after axotomy in Drosophila and C.*elegans* (Hammarlund et al., 2009;Xiong et al., 2010). Also, DLK has been shown to promote Wallerian degeneration in the distal segment of the injured sciatic nerve (Miller et al., 2009). There is recent evidence on the role of DLK in retrograde signalling in response to axotomy in mice (Shin et al., 2012). Regeneration of axons is severely impaired in DLK-/- mice and the effects of a conditioning lesion are completely abolished in such mice. Moreover, Shin et.al. (2012) showed that DLK is necessary for retrograde transport of STAT3 to cell bodies of DRGs in response to injury.

Regeneration-associated transcription factors

The considerable changes in gene expression in regenerating neurons must be dependent on changes in transcription factors. Various microarray studies have been performed to investigate changes in gene expression under regenerating and non-regeneration conditions, such as DRG neurons with and without a conditioning lesion, or retinal ganglion cells (RGCs) with and without lens injury, RGCs from lower organisms such as Zebrafish and neurons from rodents in their early developmental stages. (Costigan et al., 2002;Nilsson et al., 2005;Fischer et al., 2004;Veldman et al., 2007;Goldberg et al., 2002;Schmitt et al., 2003). Based on these studies, various transcription factors whose expression is positively correlated with axonal

regeneration have been identified and these include-c-Jun, ATF3, STAT3, Smad1, CREB, c-EBP and Sox11.

STAT3, a member of the JAK/STAT signalling pathway has shown to be involved in initiating axonal outgrowth, although it does not play a role in continuing axonal regeneration. STAT3 is activated after injury to the peripheral branch but not the central branch of DRG axons (Schwaiger et al., 2000) and it has been reported that conditional deletion of STAT3 in neurons impairs sprouting after dorsal column lesions (Bareyre et al., 2011). Moreover, STAT3 over-expression improves neurite outgrowth from cultured cerebellar granule neurons (Smith et al., 2011).

c-Jun is a member of the AP-1 (activator protein-1) family of transcription factors. c-Jun is upregulated in DRGs after peripheral nerve lesion and comparatively lower expression of c-Jun is seen in DRGs after a lesion of their central axon (Broude et al., 1997). c-Jun has been shown to play a dual role in regeneration. It is necessary for peripheral nerve regeneration but also for cell death of motor neurons after facial axotomy (Raivich et al., 2004;Ruff et al., 2012). ATF3 has been shown to be expressed by injured CNS neurons regenerating into a peripheral nerve graft in the thalamus (Campbell et al., 2005). Unlike c-Jun, ATF3 is not normally expressed in uninjured neurons but shows marked increase in expression in sensory and motor neurons after peripheral axotomy (Tsujino et al., 2000). ATF3 has also been shown to be expressed in corticospinal axons after an intercortical lesion but not after a spinal cord lesion (Mason et al., 2003). There are suggestions that ATF3 dimerizes with c-jun predominantly because of the concurrent expression of both c-Jun and ATF3 after injury (Lindwall and Kanje, 2005; Nakagomi et al., 2003). However, *in vivo* data in support of this view is lacking.

Apart from transcription factors that are positively correlated with axon regeneration, there has been recent evidence suggesting that Krüppel-like-factor 4 (KLF-4) is an inhibitor of axonal regeneration active at the transcriptional level. In a screen to identify developmentally-regulated genes in RGCs, it was observed that KLF-4 acts as a potent inhibitor of neurite outgrowth (Moore et al., 2009). RGCs not only express KLF-4 but all 17 members of the KLF family. Mature RGCs express growth-suppressing KLFs at higher levels in comparison to

growth-enhancing KLFs (Moore et al., 2009). A similar pattern of expression was observed in corticospinal neurons (Arlotta et al., 2005).

Nuclear factor IL-3 (NFIL3) is a transcription factor that shows increased expression after injury and has been shown to regulate regenerative responses. NFIL3 has been reported to regulate regeneration in sensory neurons by suppressing CREB and members of the C/EBP family that activate regeneration associated genes such as GAP-43 and arginase (MacGillavry et al., 2009;MacGillavry et al., 2011).

Cyclic nucleotides

Raising levels of cyclic adenosine monophosphate (cAMP) has been associated with promoting axonal regeneration. There are elevated levels of cAMP in embryonic and postnatal younger neurons (<P5) which may explain why these neurons are less inhibited by CNS myelin (Cai et al., 2001). There is a reduction in the levels of neuronal cAMP after spinal cord injury (Pearse et al., 2004). In the presence of high neuronal cAMP levels, growth cones exhibit chemoattractive responses whereas in the presence of less cAMP, the effects on the growth cone are that of chemorepulsion (Ming et al., 1997;Song et al., 1997;Cai et al., 1999). However, it not clear whether the effects of cAMP *in vivo* are on the tip of the axon or on the cell body or both. In the injured retina, cAMP regulates the expression of SOCS molecules which are involved in reducing cytokine induced outgrowth of neurites (Park et al., 2009).

Regeneration-associated genes

Axotomy to the peripheral processes of DRGs causes the upregulation of hundreds of genes which may be associated with growth and regeneration. However in neurons within the injured CNS, few or no regeneration-associated genes (RAGs) are upregulated (Mason et al., 2003;Fernandes et al., 1999). Injury to the peripheral nerve causes the upregulation of RAGs such as GAP-43 and α-tubulin (Skene and Willard, 1981). In the CNS, it has been shown that injured CNS neurons regenerating axons into a peripheral nerve graft increase their expression of RAGs (Anderson et al., 1998;Mason et al., 2002). Also, the exogenous application of

neurotrophic factors such as BDNF to the cell bodies of rubrospinal axons that are regenerating into a peripheral nerve explant in the injured spinal cord increases expression of GAP-43 (Kwon et al., 2002). However, over-expression of GAP-43 does not enhance axonal regeneration but only induces sprouting (Aigner et al., 1995;Buffo et al., 1997). Overexpression of both GAP-43 and CAP-23 facilitates regeneration of DRG axons into nerve grafts after spinal cord transection (Bomze et al., 2001). Other RAGs that have limited effects on axonal regeneration include Sprr1a and itga7 (Bonilla et al., 2002;Werner et al., 2000).

Growth suppressors

PTEN (Phosphatase and Tensin homologue deleted on chromosome 10) is a tumour suppressor that regulates Akt signalling. Most signalling events are mediated via receptor tyrosine kinases. The activation of receptor tyrosine kinase leads to the activation of PI3K (phosphoinositide 3-kinase). PI3K causes the conversion of PIP2 (bisphosphate) to PIP3 trisphosphate which in turn induces PDK1/2 (phosphatidylinositol-dependent kinase ½) to activate Akt. PTEN antagonises the conversion of PIP2 to PIP3 by inhibiting PI3 kinase (Song et al., 2005;Park et al., 2010). PTEN is also known to negatively regulate mammalian target of rapamycin (mTOR). Activation of mTOR increases protein synthesis by activating ribosomal protein S6 kinase and also eukaryotic transcription factor eIF4E (eukaryotic initiation factor 4E) (Guertin and Sabatini, 2007). Another effecter of axonal regeneration that is regulated by PTEN is glycogen synthase kinase (GSK). GSK-3 is a key signalling molecule that integrates various extracellular cues to regulate microtubule assembly (Zhou and Snider, 2005). It controls CRMP-2 (collapsin response mediator protein 2) as well as other α/β tubulin dimer binding proteins thereby regulating microtubule polymerisation (Fukata et al., 2002). GSK-3β is known to control growth cone initiation and elongation. GSK-3β compromises microtubule stabilisation at the tip of axons by inducing phosphorylation of APC (adenomatous polyposis coli) which is a microtubule plus end binding protein. The application of NGF causes the inactivation of GSK-3β via PI3K pathway and enhances binding of APC to microtubules (Zhou et al., 2004). Apart from inactivation of GSK-3β by NGF, it has been reported that axon guidance molecules such as Sema3A activates GSK-3β and hence mediates its inhibitory effects on axonal outgrowth

(Eickholt et al., 2002a). Hence, GSK-3β regulates microtubule assembly in response to extracellular cues in a complex fashion.

1.3 Experimental interventions to improve axonal regeneration in the injured CNS

Classical experiments conducted by Aguayo and colleagues in 1981, have shown that injured CNS axons are capable of regenerating in an altered environment. When peripheral nerve grafts were placed between the spinal cord and medulla, injured axons of CNS neurons were capable of regenerating 30mm into the graft. It was concluded, that by changing the glial environment of CNS axons to that seen in the peripheral nervous system, regeneration could be encouraged (David and Aguayo, 1981). Aguayo's laboratory carried out similar experiments in the injured optic nerve. A peripheral nerve (PN) graft was placed at the ocular stump after intraorbital transection. It was observed that in the absence of a PN graft, 90% of the RGCs underwent cell death. In the presence of the graft, 20% of surviving RGCs extended long neurites into the graft (Villegas-Perez et al., 1988). However, when the injured optic nerve is transplanted into PNS tissue, there is occasional sprouting up to a few millimetres, but the axons fail to extend long neurites (Aguayo et al., 1981). Since then, several experiments which have made use of a peripheral nerve bridge have shown that many types of CNS axons can regenerate in a changed glial environment. However, there are various other classes of neurons such as Purkinje cells, thalamocortical and striatal neurons that do not regenerate into peripheral nerve grafts (Anderson et al., 1998a). Apart from peripheral nerve grafts, grafts from foetal spinal cord have been shown to induce axonal regeneration from certain types of injured CNS neurons (Bregman, 1987; Howland et al., 1995; Miya et al., 1997). However, axons that regenerate into bridge grafts tend to stop regenerating once they reach the distal host-transplant border (Jakeman and Reier, 1991).

1.31 Combinatorial treatments using grafts and neurotrophins

In the quest to achieve long distance regeneration, various types of grafts that were thought to provide superior environment when compared to peripheral nerve grafts have been tested. These include grafts made of fibroblasts, olfactory ensheathing cells (Ramon-Cueto et al., 2000; Lu et al., 2006; Richter and Roskams, 2008), bone marrow stromal cells (Ankeny et al., 2004), Schwann cells (Duncan et al., 1981; Li and Raisman, 1994; Guest et al., 1997; Tuszynski et al., 1998) and stem cells (Pfeifer et al., 2004; Lepore and Fischer, 2005). However, most cellular grafts (other than those of Schwann cells) are incapable of mimicking the environment in the PNS, with regard to neurotrophin production. For successful regeneration in PNS, Schwann cells upregulate the expression of NGF, BDNF and GDNF. Once the regenerating nerve grows back into the distal stump, the expression levels of neurotrophins return to baseline levels (Funakoshi et al., 1993; Trupp et al., 1997). In order to overcome this drawback, grafts were made of genetically modified cells capable of producing neurotrophic factors. Tuszynski and colleagues showed that cells producing GDNF or NGF improve regeneration from dorsal column axons and certain noradrenergic neurites and encouraged some sprouting from motor neurons (Tuszynski et al., 1996b;Blesch and Tuszynski, 2003). Reticulospinal, propriospinal and cerulospinal axons have shown to respond to treatment with cellularly delivered NT4/5 (Blesch et al., 2004) and regeneration of rubrospinal axons is augmented by BDNF-expressing fibroblasts (Liu et al., 1999). While it has been reported that NT-3 induces sprouting of corticospinal axons in the injured spinal cord (Blits et al., 2000), corticospinal axons fail to extend neurites into cellular grafts producing elevated levels of NT-3. Even the axons from the various tracts that do grow into grafts expressing neurotrophins rarely regenerate beyond the graft.

1.311 Enhancement of axonal regeneration by creating growth factor gradients

It is known that neurotrophins can strongly stimulate neurite outgrowth. The expression of neurotrophins is believed to be an essential requirement for regenerating neurons in the PNS. Expression of neurotrophin gradients may aid in chemotropic guidance of PNS axons, leading to the innervation of appropriate targets. Similar functions of neurotrophins in the injured CNS have been reported. Following injury to the dorsal columns of the spinal cord at C1, axons from the fasciculus gracilis have been shown to regenerate into grafted marrow stromal cells and

reinnervate the nucleus gracilis, only if the nucleus had received injections of NT-3-expressing lentivirus. The effects of the NT-3 were dose-dependent (Alto et al., 2009). However, it is to be noted that in this particular study, the lesion was made at a high cervical level and hence regenerating axons would be required to travel a comparatively short distance to reinnervate its target. When the lesion was made at C3 and a gradient of NT-3 created at the rostral end of the lesion, it was observed that although there was axon bridging beyond the lesion, axons did not regenerate any further even in the presence of a growth factor gradient (Taylor et al., 2006).

Neonatal dorsal root ganglia neurons transplanted into the corpus callosum can be induced to extend neurites using growth factor gradients. Such neurons can regenerate their axons up to the area of the lesion, with some axons growing beyond the lesion and towards the target in the contralateral hemisphere by the presence of either NGF/FGF or NGF gradients (Jin et al., 2008). Also, a combinatorial approach using grafts of neural progenitor cells into the lesion site and lentiviral delivery of BDNF to the target has shown that injured dorsal column axons grow through the grafts and towards the BDNF-expressing dorsal column nuclei and form synaptic connections. Such connections are formed only if the lesion is placed at C1, not further from the target (Bonner et al., 2010; Bonner et al., 2011).

The drawback of using cellularly-delivered neurotrophins is that sustained production of neurotrophins leads to increased size of the graft, partly because of invasion of the grafted region by Schwann cells (Blesch and Tuszynski, 2003). Moreover, increased production of neurotrophins at the site of injury causes axons to extend into the lesion but not beyond, presumably because the axons are reluctant to extend into regions where lower neurotrophin levels are present (McCall et al., 2012). However, growth beyond the site of lesion and reinnervation of targets can only be achieved when lesions are close to target nuclei.

1.312 Modulating the effects of neuropoietic cytokines

The regenerative effects of CNTF

Following injury to the optic nerve, about 90% of retinal ganglion cells die (Berkelaar et al., 1994). Intravitreal delivery of the cytokine CNTF improves cell survival following axotomy. Repeated application of CNTF has also shown to improve regeneration of retinal ganglion cell

axons either into a peripheral nerve graft or into and through the lesion (Mey and Thanos, 1993; Cui et al., 1999; Cui and Harvey, 2000; Muller et al., 2007). Adenoviral delivery of CNTF has shown to induce long distance regeneration after crush injury with some axons regenerating up to the optic chiasm (Leaver et al., 2006). Moreover, in the presence of a peripheral nerve graft, regeneration was further enhanced with increased cell survival even at 7 weeks post-injury (Leaver et al., 2006). A deep penetrating lens injury has shown to exert neuroprotective effects on injured neurons as well as stimulating the regeneration of their axons after injury (Fischer et al., 2000). It has been suggested that lens proteins, crystallins, mediate the effects of lens injury on retinal ganglion cells (Fischer et al., 2000). Similarly, injecting β/γ crystallins into the eye ball mimics the effects of a lens injury (Fischer et al., 2008). Lens injury, intravitreal injections of crystallins or application of the inflammatory yeast extract, zymosan, stimulate the release of CNTF from astrocytes which induces regeneration and neuroprotection (Muller et al., 2007). Moreover, it has been suggested that CNTF does not exert it s effects directly, instead its effects are mediated via activation of macrophages (Yin et al., 2003; Cui et al., 2008). Oncomodulin, a calcium binding protein, has been identified as a macrophage-derived growth factor which binds to retinal ganglion cells with high affinity. It has been shown to exert a greater regenerative effect when compared to CNTF (Cui et al., 2008). When oncomodulin is immune-depleted from media conditioned by macrophages, the growth promoting effects of that medium are reduced. Intravitreal delivery of microspheres that release both oncomodulin and cAMP analogue, greatly enhances the regeneration of axons from retinal ganglion cells *in vivo* as well as neurite outgrowth from DRG neurons *in vitro* (Yin et al., 2006).

The regenerative effects of LIF

Leukaemia inhibitory factor (LIF) is not expressed in the intact CNS but has been reported to be upregulated after sciatic nerve injury and is transported retrogradely by sensory and sympathetic neurons (Hendry et al., 1992; Curtis et al., 1994). Treatment with LIF improves axonal regeneration and recovery of skeletal muscle function following injury to the sciatic nerve (Tham et al., 1997). LIF has been implicated in conditioning responses following lesion to the peripheral branch of DRGs. The effectiveness of the conditioned lesion response is significantly

blunted in axons from LIF-/- mice (Cafferty et al., 2001). Following contusion injury to the spinal cord, there is strong upregulation of LIF mRNA in and around the region of contusion between 3 and 24 hour. However, this signal disappears 2 days after injury (Pineau and Lacroix, 2007). LIF is expressed by astrocytes and some microglial cells in the injured spinal cord (Pineau and Lacroix, 2007). LIF also plays an important role in the infiltration of macrophages after injury to the spinal cord and sciatic nerve, hence mediating the initial inflammatory responses (Sugiura et al., 2000). Increased expression of LIF has shown to exert positive effects on CNS regeneration. Cellular delivery of LIF by modified fibroblasts grafted into the injured spinal cord reduces the retraction response of corticospinal axons and improves growth of corticospinal fibres into the tissue bridge. Moreover, LIF-secreting grafts increased the production of NT-3 at the lesion site (Blesch et al., 1999). Hence, cytokines can modulate cellular responses after injury and also augment production of neurotrophins.

1.32 Modulation of growth-inhibitory molecule signalling to promote axonal regeneration

As seen with experiments using peripheral nerve grafts, CNS axons can be induced to regenerate in a suitable environment. Pioneering work by Davies and colleagues has shown that the glial scar formed near the lesion acts as a major impediment to regenerating axons. DRG neurons implanted into the intact CNS can extend their neurites. After a lesion to the dorsal column, DRG neurons transplanted several millimetres rostral to the lesion can grow neurites into degenerating white matter, up to the region of the lesion. However, when these neurites encounter the glial scar, their growth cones become dystrophic and regeneration is stopped (Davies et al., 1997; Davies et al., 1999). Various extracellular molecules present near the lesion site that discourage axonal elongation have been identified. These include: myelin associated inhibitors, CSPGs and axon guidance molecules. Various genetic knockout models have been used to assess the inhibitory nature of these molecules, the ultimate aim being to find ways to create an environment conducive to regenerating axons. Experimental approaches to ameliorate the effects of extrinsic factors on regeneration are discussed below:

1.321 Myelin associated inhibitors

Targeting NOGO, MAG and OMgp to enhance axonal regeneration

The pioneering work of Caroni and Schwab established the presence of inhibitory molecules in CNS myelin (Caroni and Schwab, 1988b). Antibodies namely IN-1 and IN-2 were made against the isolated fractions from CNS myelin (Caroni and Schwab, 1988a). The efficiency of the antibody in abolishing the effects of CNS myelin on neurite outgrowth in *vitro* led investigators to use these antibodies *in vivo*. Schwab and colleagues have shown that the administration of the antibody after spinal cord injury improved regeneration, plasticity and functional recovery following injuries to the corticospinal tracts (Buchli and Schwab, 2005). However, it remains difficult to explain the actions of Nogo-A blocking antibodies (such as IN-1) because when Nogo is genetically deleted, there is not always improvement in axonal regeneration of injured corticospinal axons. Although, robust regeneration of corticospinal axons in Nogo KO mice has been reported in one study (Kim et al., 2003), only modest amounts of regeneration was seen in another (Simonen et al., 2003), and no regeneration seen in other studies(Zheng et al., 2003; Lee et al., 2009). Hence clear and reproducible data is lacking. The differences between these observations can be attributed to differences in genetic background of the mice used in these studies and labelling artefacts (Steward et al., 2007; Cafferty et al., 2007). Genetic deletion of other myelin associated inhibitors such as MAG or OMgp have proven even less effective in enhancing regeneration. MAG-deficient mice failed to regenerate their axons when either the optic nerve or corticospinal tract was injured (Bartsch et al., 1995). In the case of OMgp KO mice, enhanced regeneration from 5HT serotonergic and dorsal column but not corticospinal axons has been reported (Ji et al., 2008). However, it not clear whether regeneration was a result of elongation of injured axons or growth of sprouts from spared fibres (Lee and Zheng, 2012).

Immunological approaches have been used to ameliorate the effects of myelin-derived inhibitors of axonal regeneration. Immunisation of mice against CNS myelin apparently causes the production of antibodies against myelin and subsequent increase in axonal regeneration from

corticospinal axons (Huang et al., 1999). Immunization with MAG/ Nogo-66 produced regeneration to a lesser extent (Sicotte et al., 2003). Also immunisation against Nogo A apparently elicits a T- cell response which accounts for neuroprotection (Hauben et al., 2001). However, immunisation against a component of myelin thought not to be inhibitory elicits similar neuroprotective responses. As neuroprotection leads to enhanced survival of spared axons, it can be often confused with enhanced regeneration (Lee and Zheng, 2012).

Targeting receptors for myelin-derived inhibitors to enhance axonal regeneration

Another strategy to overcome inhibition caused by myelin-derived inhibitors is to target their receptors. Strittmatter and colleagues have shown that blocking NgR1 by administering a blocking peptide, NEP-1 in rats with mid-thoracic injury to the spinal cord, significantly improves regeneration from corticospinal axons and enhances functional recovery (GrandPre et al., 2002; Li and Strittmatter, 2003). However, these results have not been reproducible in other laboratories. A more recent study has shown that the effects of NEP-1 on regenerating corticospinal axons were modest and no improvements in functional recovery were observed (Steward et al., 2008). Another strategy was to use an NgR1 antagonist. It has been reported that treatment with the NgR1 antagonist induced axonal sprouting of serotonergic and tyrosine hydroxylase-positive axons into the dorsal horn following dorsal rhizotomy (MacDermid et al., 2004). However, regeneration of corticospinal axons was not found after treatment of animals with the NgR1 antagonist following contusion injury to the spinal cord but raphespinal regeneration was reported (Wang et al., 2006). Also, combination treatments which include administration of a soluble NgR1antagonist and a glucocorticoid, methylprednisolone, did not enhance regeneration, although sprouting of corticospinal axons was induced (Ji et al., 2005). Experiments with NgR1 knockout mice did not show the same effects on corticospinal regeneration that were apparent in the early experiments with NEP-1. In general corticospinal regeneration beyond the lesion did not occur (Kim et al., 2004; Zheng et al., 2005) although raphespinal regeneration was enhanced in one study (Kim et al., 2004).

As it remains certain that deleting NgR1 does not enhance axonal regeneration from corticospinal neurons, the next step was to target its co-receptors which include $p75^{NTR}$ and

LINGO. After dorsal hemisection to the spinal cord of $p75^{NTR}$ knockout mice, there was no improvement in axonal regeneration from corticospinal axons or ascending sensory axons (Song et al., 2004). However, immunohistochemical data produced by the same study has shown that $p75^{NTR}$ is not expressed by corticospinal axons and that only a subset of ascending sensory axons express $p75^{NTR}$. The lack of expression may account for the ineffectiveness of deleting $p75^{NTR}$ on axon regeneration, at least from these types of neurons and calls into question the role of p75 as an NgR co-receptor. LINGO-I is another co-receptor for NgR1. Adult rats that have received a dorsal or lateral hemisection injury to the spinal cord when treated with LINGO-1 antagonist show decreased RhoA activation and increased sprouting of corticospinal and rubrospinal axons, but axonal regeneration from either of the tracts was absent (Ji et al., 2006). If p75 and LINGO-1 are not critical for inhibition of axonal regeneration in the CNS, then either there are other NgR1 co-receptors or NgR1 is not a major cause of the failure of axonal regeneration in the CNS. TROY has been suggested as a replacement for p75 as a co-receptor, but like p75 its expression is restricted and it is not present in corticospinal neurons (Barrette et al., 2007).

More recently, PirB has been discovered as a receptor for Nogo, MAG and OMgp (Atwal et al., 2008). Studies using PirB knockout mice have shown that neither axonal plasticity nor regeneration from injured corticospinal axons could be improved in these mice even when treated with the NgR1 antagonist, NEP-1 (Omoto et al., 2010; Nakamura et al., 2011). Hence, it can be said that targeting both NgR-1 and PirB may not be sufficient to improve axonal regeneration.

Outcomes of using triple knockouts on axonal regeneration

As single mutants for MAG and OMgp did not produce any significant increase in regeneration of injured axons and also, as there are discrepancies in the literature about the outcomes of deleting Nogo on regeneration, researchers were led to investigate the synergistic effects of deleting all three of the NgR-binding myelin-associated inhibitors-Nogo, MAG and OMgp. Two different laboratories tested the hypothesis that these myelin-derived inhibitors are associated with hindering axonal regeneration. The results in the different laboratories were dissimilar. Cafferty et.al. (2010) showed that Nogo knockout mice can regenerate their

corticospinal axons after dorsal hemisection and that these effects were enhanced when combined with MAG and Nogo mutations. However, double mutants for MAG and OMgp did not show any improvement in regenerating their axons. Hence, it was implied that MAG and OMgp synergise with Nogo to inhibit axonal growth. They also reported regeneration from serotonergic axons after dorsal hemisection in triple knockout mice (Cafferty et al., 2010). In contrast to these findings, Lee et.al. (2010) reported the lack of corticospinal regeneration in both Nogo single mutants and triple KO mice. Serotonergic neurons did not regenerate their axons after complete transection of the spinal cord. However, after lateral hemisection, sprouting of seretonergic axons was observed (Lee et al., 2010).

The differences in results obtained by the two groups can be attributed to differences in the mutant alleles used to generate triple KO mice, differences in the genetic background of the mice and also differences in the interpretation of results (whether there was regeneration or sprouting from spared fibres) (Lee and Zheng, 2012). While Cafferty et.al. (2010) used a Nogo A/B gene trap mutant allele which includes retroviral insertion that targets Nogo-A isoform also affecting expression of B isoform, Lee et.al. (2010) used a null allele that lacks all the three Nogo isoforms. Moreover, Cafferty et.al. (2010) used triple KO mice that were bred using a more pure line of C57BL/6 mice whereas Lee et.al. (2010) outbred their mice in a mixed background of C57BL/6 and 1297S strains. Because both groups agree that inactivating both MAG and OMgp does not improve axonal regeneration, the explanation of the differences may lie in the mutated Nogo alleles. There were also differences in assessing axonal regeneration from serotonergic axons. When using an incomplete injury model such as lateral or dorsal hemisection, regeneration from serotonergic axons was observed by the two groups. However, the regenerative response of serotonergic fibres was classified as sprouting by Lee et.al (2010) because there was a complete absence of regeneration in the fully transected spinal cord. Moreover, a separate study conducted by the same group showed a lack of regeneration across the lesion site from 5-HT and corticospinal axons in NgR1/Nogo/MAG triple mutants (Lee et al., 2010).

Summing up all the evidence from various studies, it can be inferred that there is little reason to believe that the classical myelin-associated inhibitors and their receptor are important targets for studies seeking to achieve long distance regeneration in the injured CNS.

1.322 Targeting chondroitin sulphate proteoglycans to enhance axonal regeneration

After injury to the CNS, reactive astrocytes and meningeal cells upregulate the expression of CSPGs in the region of the glial scar (Davies et al., 1997). Many studies have shown that CSPGs act as potent inhibitors of axonal outgrowth *in vitro*. The inhibitory nature of CSPGs can be attributed to its GAG side chains and in some cases, also to the core protein. Evidence for the importance of GAGs comes from studies that used the bacterial enzyme, chondroitinase ABC (Lee et al., 2010) to separate most of the GAG chains from the core protein (Oohira et al., 1991). As removal of GAG chains decreases the inhibition produced by CSPGs, several investigators tested the effects of digesting CSPGs using chABC on axonal regeneration *in vivo.*

Enzymatic digestion of CSPGs as a means to improve axonal regeneration

Several studies have shown that administering chABC at the lesion site evokes a regenerative response in various types of injured axons. Intrathecal administration of chABC induced growth of lesioned dorsal column axons into the epicentre of the lesion (Bradbury et al., 2002). Moreover, an increase in GAP-43 expression was seen in DRG neurons that received treatments with chABC, particularly DRGs with larger diameter cell bodies. The authors also claimed regeneration of corticospinal axons in the dorsal columns. More corticospinal axons were seen at and below the site of lesion in the treated animals and collateral sprouting into grey matter and functional recovery was reported (Bradbury et al., 2002). However, the lesion model used by Bradbury et al. was not ideal for assessing axonal regeneration because it involved a crush injury with no precise boundaries. More recent studies have reported modest amounts of regeneration from sensory fibres treated with chABC after injury to the dorsal funiculus using a vibraknife (Steinmetz et al., 2005; Shields et al., 2008). In fact, regeneration was so meagre that

the authors did not feel the need to carry out behavioural studies to analyse functional recovery (Shields et al., 2008). Neurons from Clarke's nuclei that project their axons into the spinocerebellar tract have shown to regenerate when treated with chABC (Yick et al., 2003). Following hemisection at T11 on the right side of the spinal cord, administration of a single dose of chABC promoted regeneration of axons past the glial scar into the site of lesion. The authors reported that regenerating axons could be seen at C7 at four weeks post injury, indicating that the distance over which the axons re-grew would be approximately 30mm (Yick et al., 2003). However, there have been no further studies from other laboratories with such spectacular results. Nigrostriatal axons have also been reported to regenerate into the lesion site and innervate its targets when treated with chABC (Moon et al., 2001). More recently, treatment with chABC has shown to improve regeneration of injured axons from RGCs across the site of lesion in the crushed optic nerve (Brown et al., 2012).

There is some evidence that regenerative response of corticospinal axons to chABC varies with the type of injury. In a dorsal hemisection model of spinal cord injury, a single dose of chondroitinase injected into the spinal cord was reported to promote axonal growth from injured corticospinal fibres around the site of lesion (Iseda et al., 2008). However, following contusion to the spinal cord, injections of chABC failed to promote axonal growth around the site of lesion. It must be pointed out that the quality of the corticospinal labelling in that paper was poor and that the result may just show that the contusion injuries produced a larger lesion. Various studies have shown that treatment with chABC induces sprouting of corticospinal axons towards the lesion site and encourages traversing around the site of injury, but rarely do these axons enter the lesion (Bradbury et al., 2002; Barritt et al., 2006). Failure of robust axonal regeneration when treated with chABC could be due to incomplete digestion of GAG chains and also due to residual inhibition by the core protein +/- the residual GAG stubs which may be inhibitory by itself (Lemons et al., 2003; Ughrin et al., 2003).

A DNA enzyme capable of reducing GAG chains by targeting mRNA responsible for glycosylation, has shown to improve regeneration of DRG neurons transplanted at the lesion site following spinal cord injury (Grimpe and Silver, 2004). However, the efficacy of this enzyme is yet to be tested on other injured axonal systems. A transgenic approach to digest CSPGs using a

chABC transgene expressed in astrocytes has shown to improve sensory axon regeneration after dorsal rhizotomy and allows corticospinal neurons to enter the glial scar but does not allow regeneration across the site of lesion (Cafferty et al., 2007). Although, the efficacy of enzymatic disruption of CSPGs in producing regeneration of corticospinal axons across lesions may have been exaggerated, various studies have reported improved functional recovery as a result of such treatment (Caggiano et al., 2005; Garcia-Alias et al., 2009; Jefferson et al., 2011). This probably reflects increased functional plasticity.

Antibodies against CSPGs to improve regeneration

Other approaches to reduce or neutralize the expression of CSPGs include the use of neutralizing antibodies to NG2. After a dorsal over-hemisection at T8, administration of NG2 antibody induces the regrowth of mechanosensory afferents into the site of lesion (Tan et al., 2006). However, NG2 knockout mice fail to show any improvements in axonal regeneration in the PNS or CNS (de Castro R. et al., 2005; Hossain-Ibrahim et al., 2007). Recently, characterisation of a sugar epitope on CSPGs namely CS-E has shown it to be a potent inhibitor of axonal outgrowth *in vitro* as well as *in vivo* (Brown et al., 2012). Attenuation of CS-E activity using a function blocking antibody has been shown to improve axonal regeneration in the crushed optic nerve. Axons regenerated across the lesion site up to a distance of one millimetre. The extent of regeneration in the crushed optic nerve was similar when retinal ganglion cells were treated with either chABC or CS-E antibody. When CS-E antibody was used in combination with cAMP, the distance covered by regenerating axons was greater than when compared to treatments with either CS-E, chABC, CS-E +chABC or CPT-cAMP alone (Brown et al., 2012).

Receptors for CSPGs as molecular targets

CSPGs mediate their inhibitory responses by means of binding to their receptors which long proved elusive. Recently, the LAR subfamily of PTPs has shown to interact with CSPGs. These include PTPσ and LAR. Genetic knockout models for these receptors have been used to study their role in axonal regeneration.

It has been recently shown that sensory neurons from PTPσ-deficient mice are less inhibited by CSPGs *in vitro*. In an *in vivo* spinal cord injury model where the dorsal columns were cut, it was shown that PTPσ-deficient sensory axons regenerated into the glial scar but not beyond the core of the lesion, whereas those in wild type animals did not approach the lesion. (Shen et al., 2009). In contrast, it has been reported that modest number of axonal fibres from the corticospinal tract regenerated up to 6-12mm after a dorsal hemisection and 7mm after contusion injuries to the spinal cord in PTPσ-deficient mice (Fry et al., 2010). Similarly, in the optic nerve that received a microcrush injury, modest amounts of regeneration was observed in PTPσ-deficient mice, although the deletion did not have any effect on cell survival (Sapieha et al., 2005). Hence, PTPσ does not seem to be so important for the regeneration of ascending dorsal column axons as it does for corticospinal axons or, alternatively, the greater difficulty in transecting all the corticospinal fibres rather than transecting all the ascending dorsal column fibres may have led to the presence of more axons spared by the lesion. An enhanced rate of regeneration in the injured sciatic nerve of PTPσ knockout animals has been reported (McLean et al., 2002).

Recently, LAR has shown to act as a receptor for CSPGs (Fisher et al., 2011). Drosophila LAR has also shown to interact with GAGs from HSPGs (Fox and Zinn, 2005). Recent studies have shown that blocking LAR enhances axonal regeneration and functional recovery. Following dorsal over-hemisection at T7, subcutaneous injections of LAR antagonist peptides (Quintanar-Audelo et al., 2011) for 10 days, caused a high density of 5-HT fibres to regenerate across the site of lesion and 5-7mm caudal to the lesion.

Recently, it has been shown that NgR1 and NgR3 bind with high affinity to CSPGs (Dickendesher et al., 2012). Genetic deletion of NgR1, NgR2 and NgR3 showed enhanced regeneration of retinal ganglion axons after intraorbital optic nerve crush. Single mutants or NgR1/NgR2 double mutants showed little enhancement of regeneration but regeneration was seen in NgR1 and NgR3 double mutants. Moreover, regeneration was further enhanced when NgR1, NgR3 and PTPσ were ablated. Even more robust regeneration was observed in the optic nerve of NgR1, NgR3, PTPσ -negative mice were treated with intravitreal zymosan

(Dickendesher et al., 2012). Hence, NgR1 and NgR3 function in synergy with PTPσ to inhibit axonal regeneration.

1.323 Targeting axon guidance molecules to enhance regeneration

Various axon guidance molecules are expressed by the adult CNS and are upregulated following injury. The fact that mature axons express receptors for guidance molecules indicates that they still respond to guidance cues. However, emerging evidence on the role of these molecules suggest that the very same molecules that were important for growth may also be responsible for some aspects of regeneration failure. Various approaches to block the activity of axon guidance molecules which include using function-blocking antibodies/peptides and genetic knockout models have been made.

Targeting semaphorins

The expression of semaphorin 3 is upregulated following various types of insults to the adult CNS such as dorsal column lesions, transections of the lateral olfactory tract, stab wound lesions of cerebral cortex and transections of the thoracic spinal cord (Pasterkamp et al., 1998; Pasterkamp et al., 1999; Pasterkamp et al., 2001). In addition to the increased expression of semaphorin 3 in the scar tissue, its receptor (neuropilin-1) shows increased expression in regenerating olfactory neurons following axotomy (Pasterkamp et al., 1998). SM216289 isolated from *Penicillium* sp. has been identified as an inhibitor of Sema3A. Following axotomy to the olfactory nerve, local and continuous administration of SM216289 between the cribriform plate and olfactory bulb significantly improves axonal regeneration (Kikuchi et al., 2003). However, when SM216289 was delivered via a mini osmotic pump, there was no regeneration of corticospinal axons or ascending dorsal column axons following transection of the spinal cord, although a few serotonergic, CGRP+ve and parvalbumin+ve axons were seen regenerating into the lesion site (Kaneko et al., 2006). In organotypic slice cultures, severed entorhinal-hippocampal neurons fail to regenerate. However, when a specific inhibitor to Sema 3A namely SICHI (a stable N-alkyglycine peptoid) was applied, entorhinal-hippocampal axons regenerated into the denervated hippocampus in organotypic culture preparations (Montolio et al., 2009).

Targeting Ephrins

Ephrin B3 is strongly expressed in CNS myelin and acts as a potent inhibitor of neurite outgrowth of cultured cortical neurons (Benson et al., 2005). Following lesion of the spinal cord, there is increased expression of EphA4 in severed corticospinal axons, a suitable receptor for the ephrin B3 in CNS myelin or ephrin B2 present in scar tissue (Fabes et al., 2006). Infusion of a peptide EphA4 antagonist induced sprouting of corticospinal axons rostral to the lesion but did not encourage regeneration across the lesion (Fabes et al., 2007). In contrast to these findings, Eph4A knockout mice have been reported to show increased regeneration of corticospinal and seretonergic axons across the lesion in the hemisected spinal cord (Goldshmit et al., 2004), associated with a reduction in scar formation. However, it is not sure whether regeneration was a result of sprouting of spared fibres or elongation of axotomised fibres and the observations on scar formation have been questioned (Herrmann et al., 2010). A more recent study by the same group has shown that treatment with either EphA5-Fc or EphA4-Fc following hemisection to the spinal cord increases the regenerating axons growing towards the glial scar and more axons were seen at the proximal edge although, no axons crossed the lesion site (Goldshmit et al., 2011). More recently, myelin derived ephrinB3 has been implicated in inhibition of axonal regeneration (Duffy et al., 2012). Following a crush injury to the optic nerve, significant numbers of regenerating axons were seen in ephrinB3 knockout mice. However, in the near complete transected spinal cord, no regeneration was observed, although there was some functional recovery. Following dorsal hemisection, there was increased regeneration of corticospinal and raphespinal axons in ephrinB3 knockout mice (Duffy et al., 2012).

Targeting Wnts

Wnts are expressed in a rostro-caudal decreasing gradient along the cervical and thoracic cord (Lyuksyutova et al., 2003; Liu et al., 2005). Wnts attract somatosensory axons whereas they repel corticospinal axons. Repulsion of CST axons is mediated via its high affinity tyrosine kinase receptor Ryk as well as Frizzled receptors (Li et al., 2009). Ryk antibodies have been reported to block the caudal growth of corticospinal axons. Following spinal cord injury, there is

increased expression of wnt-5a in the astrocytic scar and Ryk expression in corticospinal axons. Following contusion to the thoracic spinal cord, infusion of Ryk-neutralising antibodies induced sprouting of CST axons and functional recovery (Miyashita et al., 2009). Curiously, DRG neurons also upregulate Ryk expression after peripheral nerve injury (Li et al., 2008) even though they regenerate vigorously.

1.33 Increasing the intrinsic growth response of injured neurons

Perhaps the best-characterised way to increase the growth state of an injured axon involves the use of a conditioning lesion in primary sensory neurons (Richardson and Issa, 1984). Regeneration is normally absent following injury to the central processes of DRG axons in the dorsal columns of the spinal cord. However, if injury to the peripheral branch precedes injury to the central branch, there is increased sprouting and regeneration into the lesion or into a peripheral nerve graft (Richardson and Issa, 1984; Neumann and Woolf, 1999; Bavetta et al., 1999). The exact signalling mechanisms that lead to an increased growth state of such neurons have not been well characterised, although elevated cAMP levels, cytoskeletal signalling, upregulation of growth factors and cytokines have been implicated in these responses (Hyatt-Sachs et al., 2010;Qiu et al., 2002).

1.331 Elevation of cAMP levels

Elevated levels of cAMP have been associated with increased axonal regeneration. Increased intracellular cAMP levels can be achieved by administering cAMP analogs (Neumann et al., 2002; Qiu et al., 2002; Hannila and Filbin, 2008), phosphodiesterase inhibitors such as rolipram (Nikulina et al., 2004) or priming with neurotrophins(Cai et al., 1999). Elevated cAMP levels as well as GAP-43 expression has been detected in DRGs after a conditioning peripheral nerve lesion or sciatic nerve lesion (Qiu et al., 2002a). Microinjections of dibutyryl cAMP into the lumbar DRG one week before lesion significantly increases regeneration of its central processes into the site of lesion after a dorsal hemisection. The regenerative response seen with

injecting dibutyryl cAMP were similar to effects produced by a conditioning lesion (Neumann et al., 2002). The greatest drawback of this approach is that neurons have to be primed with cAMP prior to the lesion, making it less therapeutically viable. Neuronal responses to CNS myelin following a conditional lesion in the presence of elevated cAMP levels depend on protein kinase A (PKA) activity. Blocking PKA activity *in vivo* blocks regenerative response of axons induced by a conditioning lesion (Qiu et al., 2002a).

An alternate approach to elevate cAMP levels is to use pharmacological blockers, such as Rolipram, that prevent degradation of cAMP by its phosphodiesterase. Enhanced regeneration was reported following administration of rolipram two weeks after hemisection of the spinal cord into which fetal spinal cord tissue was implanted at the time of lesion.(Nikulina et al., 2004). Following contusion injury, the levels of cAMP drop in the sensorimotor cortex, brain stem and the region of the cord rostral to the lesion. When dibutyryl cAMP is administered in combination with Rolipram near the lesion site into which Schwann cells have been grafted, there is increased axonal sprouting and increased growth of serotonergic fibres into and beyond the graft (Pearse et al., 2004).

1.332 Inhibition of growth suppressors-PTEN and SOCS3

It has recently been suggested that the growth promoting effects of cAMP are mediated by suppressing suppressor of cytokine signalling-3(SOCS3) (Park et al., 2009). Deletion of SOCS3 leads to robust regeneration of retinal ganglion cell axons following injury to the optic nerve (Smith et al., 2009). Regeneration is further enhanced in the presence of cilliary neurotrophic factor (CNTF) administered by intraocular injections. When both SOCS3 and gp130 are deleted, previously observed regenerative effects were abolished, indicating that SOCS3 deletion increases outgrowth in a gp130-dependant manner (Smith et al., 2009). These findings have been supported by a more recent study, which shows over-expression of SOCS3 via an adeno-associated viral vector results in almost complete regeneration failure in retinal ganglion cells stimulated with CNTF (Hellstrom et al., 2011).

Phosphatase and Tensin homolog (PTEN) is preferentially expressed by Purkinje neurons, olfactory mitral neurons and large cortical pyramidal neurons (Lachyankar et al., 2000). Work from He's lab has shown that PTEN deletion facilitates axonal regeneration from injured retinal ganglion cells as well as corticospinal axons. In adult retinal ganglion cells, robust regeneration was reported in conditional PTEN knockouts following an optic nerve crush (Park et al., 2008). The predominant pathway through which PTEN elicits its effects is by suppressing mammalian target of rapamycin (mTOR). Deleting Tuberous Sclerosis Complex 1 (TSC1) which is another negative regulator of mTOR also enhanced regeneration of retinal ganglion cell axons (Park et al., 2008). The effects of deleting PTEN on regeneration from corticospinal axons were assessed using three different injury models- pyramidotomy, dorsal hemisection and complete crush injury to the spinal cord (Liu et al., 2010). Following unilateral left pyramidotomy, increased sprouting of axonal fibres was seen on the right side in PTEN conditional knockout mice. However after dorsal hemisection at T8, a large cohort of PTEN-/- corticospinal axons were seen growing towards the lesion, with substantial numbers growing past the lesion while some fibres circumvented the site of lesion through the spared ventral matter. In a crush injury model, more PTEN deleted axons were seen regeneration past the lesion, up to a distance of 3mm distal to the lesion (Liu et al., 2010).

Regeneration as a result of either blocking PTEN activity or SOCS3 signalling results in robust outgrowth from injured retinal ganglion cell axons, but such effects tapered off two weeks after injury. In an attempt to promote sustained axonal regeneration, He and colleagues co-deleted PTEN and SOCS3 in retinal ganglion cells that were later subjected to an optic nerve crush (Sun et al., 2011). There was robust regeneration of PTEN-/-; SOCS3-/- axons with more axons regenerating past the lesion for longer distances when compared to the effects produced by deleting either PTEN or SOCS3 alone. Moreover, STAT3 deletion abolishes the regenerative effects of SOCS3 deletion whereas rapamycin, an inhibitor of mTOR abolishes majority of the effects produced by PTEN deletion. Hence, suggesting that STAT3 and mTOR are key signalling pathways required for sustained axonal regeneration (Sun et al., 2011).

1.333 Targeting Kruppel-like factors (KLFs)

KLFs are zinc-finger like transcription factors that have recently been shown to be involved in axon regeneration. It was initially noted that KLFs such as KLF6a and KLF7a were upregulated following optic nerve crush in Zebrafish and it was suggested that their expression was necessary for regeneration of retinal ganglion cells (Veldman et al., 2007). Later on, an independent study that analysed differences in expression of developmentally regulated genes in retinal ganglion cells from rats aged between E17 to P21 revealed that KLF4 acted as a transcriptional repressor of axonal regeneration (Moore et al., 2009). It was shown that over-expression of KLF4 greatly reduced neurite outgrowth from retinal ganglion cells and embryonic hippocampal neurons *in vitro*. KLF4 -/- mice with crushed optic nerves showed an increase in the number and length of regenerating retinal ganglion cell axons, compared to control animals (Moore et al., 2009).

1.334 Targeting Dual leucine zipper kinase

More recently, dual leucine zipper kinase (Xiong et al., 2010) has been identified as a key molecule involved in the retrograde transport of injury induced signals (Shin et al., 2012). DLK promotes robust regeneration of peripheral axons. Neurons from DLK knockout mice have been shown not to respond to a conditioning lesion. DLK is involved in transporting pSTAT3 and c-JUN to the cell body following injury (Shin et al., 2012). Hence, modulating the expression of DLK such that it mimics a preconditioning lesion in CNS neurons may prove useful in enhancing regeneration.

1.335 Targeting Anaphase promoting complex (APC)

APC initiates the destruction of inhibitor of DNA binding 2 (Id2) and the transcriptional repressor SnoN, both of which promote axonogenesis and axonal growth (Gieffers et al., 1999; Stegmuller et al., 2006; Yu et al., 2011). Loss of APC causes activation of a gene expression programme in neurons that leads to elongation of axons (Iavarone and Lasorella, 2006). The activation of APC during late mitosis is initiated by the regulatory protein Cdh1. APC-Cdh1

complexes are highly expressed in post-mitotic neurons and they play a role in limiting the intrinsic growth potential of neurons. Moreover, knock down of Cdh1 completely overrides the inhibitory effects of CNS myelin on neurite outgrowth from cerebellar granule neurons *in vitro* (Konishi et al., 2004). Recently, it has been reported that a mutated form of Id2 that is resistant to APC-Cdh1 mediated degradation can enhance axonal regeneration of sensory fibres in the dorsal columns into lesion sites in the spinal cord (Yu et al., 2011).

1.34 Combinatory approaches to promote axonal regeneration

A central problem with axonal regeneration in the CNS is that multiple factors seem to contribute to the failure of regeneration. Consequently, in order to maximise regenerative responses, various strategies that involve combining blockade of inhibitory molecules and over-expression of growth promoting factors have been tested.

Degradation of the GAG chains of CSPGs using chABC has shown to induce axonal sprouting and promote modest amounts of regeneration, as discussed previously. However, when grafts are implanted in parallel with chABC treatment, there is further enhancement of regeneration with more regenerating axons crossing the lesion site and growth beyond the lesion into the caudal region of injured cord. When Schwann cell-seeded channels were implanted into hemisected thoracic cord, chABC treatment at the distal end of the graft improved axonal regeneration of descending NF200+ve fibres past the graft-host interface and into the distal stump up to a distance of 5mm (Chau et al., 2004). Moreover, when Schwann cell bridges were combined with olfactory ensheathing cells (OECs) and chABC treatment, there was functional recovery of forelimb, hind limb and open-field locomotion, although serotonergic but not corticospinal and reticulospinal axons regenerated (Fouad et al., 2005). Apart from Schwann cells, the use of peripheral nerve grafts in conjunction with chABC delivery to the lesion has also shown to improve axonal regeneration with increased number of axons passing through the host-graft interface (Houle et al., 2006;Tom and Houle, 2008). In addition to the effectiveness of combined treatments in promoting regeneration from acutely injured neurons, the efficacy of such treatments has also been tested in chronic injury models. Regeneration following chronic

contusion injury to the cervical cord can be induced when growth factors such GDNF are delivered in parallel with chABC infusion into the lesion site containing a peripheral nerve graft (Tom et al., 2009). In order to prolong the effects of chABC, a more thermostable chABC was made and when used in combination with NT-3, sprouting of serotonergic fibres and well improved locomotor function were observed (Lee et al., 2010). The same combination of NT-3 and chABC has shown to induce growth of neurites from transplanted DRG into the denervated dorsal column nuclei (Massey et al., 2008). As shown by Davies et.al. (1999), microtransplanted DRGs can extend neurites in degenerating white matter tracts following lesion to the spinal cord. However, these neurites stop extending when they encounter dorsal column nuclei. Treatment with either NT-3 or chABC has shown to increase their regenerative response, but to a modest extent. However, combining both chABC as well as NT-3 significantly improves growth of neurites into the dorsal column nuclei by about 10- fold (Massey et al., 2008).

Degradation of CSPGs or blocking receptors to CSPGs in conjunction with deletion of receptors to myelin-derived inhibitors has been shown to further enhance regeneration when compared to targeting individual receptors. NgR1 knockout mice display modest amounts of regeneration following an optic nerve crush but intraocular zymosan was synergistic and produced a greater regenerative response (Wang et al., 2012). Deleting NgR1, NgR2 and PTPσ produced more robust regeneration of injured retinal ganglion cell axons than deleting individual receptors. The regenerative response can be further enhanced by treatment with zymosan resulting in increasing numbers of regenerating axons growing further into the distal nerve (Dickendesher et al., 2012). However, combinatorial treatments are not always successful. In the injured spinal cord that received a dorsal column crush, regeneration of sensory axons as result of treatment with chABC and NgR1-Fc decoy did not significantly differ from treatment with individual drugs. But when a conditioning lesion to the sciatic nerve preceded spinal cord injury and subsequent treatment with NgR1 decoy and chABC, a greater regenerative response was observed (Wang et al., 2012). In contrast, deleting NgR1 as well as two of the receptors to semaphorins- PlexinA3 and PlexinA4 did not improve axonal regeneration from serotonergic or corticospinal axons after complete transection to the spinal cord (Lee et al., 2010).

1.4 The other molecules associated with this project

1.41 Toll-like receptors

Toll-like receptors (Olson and Miller, 2004) are transmembrane glycoproteins that can recognize pathogen-associated molecular patterns of bacteria, viruses, yeast, fungi and parasites (Janeway, Jr., 1992). TLRs consist of an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Rock et al., 1998). 13 different TLRs have been identified in mammals of which TLRs1-9 are expressed in mice and humans and TLRs10-13 are expressed only in mice (Takeda and Akira, 2007;Chuang and Ulevitch, 2000).

The intracellular domains of TLRs interact with intracellular adaptor proteins that relay various signals. Five such adaptor proteins that have been identified, of which myeloid differentiation factor 88 (MyD88) has been most well characterized. MyD88 induces NF-κB-mediated activation of genes such as tumour necrosis factor α (TNFα), chemokine C-C motif ligand 5 (CCL-5), interleukin-1β (IL-1β) and IL-8. Other adaptors include MyD88-like protein (MaI), TIR domain-containing adaptor inducing interferon-β (Carty et al., 2006), TRIF-related adaptor protein (Risling et al., 1983) and sterile-α and HEAT/Armadillo motifs-containing protein (Carty et al., 2006; Verstrepen et al., 2008;Carty et al., 2006). However, as Trudler et al. (2010) have pointed out, because all cells do not express the same set of adaptors, TLR signalling exerts different effects in different types of cell. For example: MyD 88 expression in neurons leads to TLR-mediated JNK signalling that results in apoptosis whereas in glial cells, it induces NF-κB signalling pathway that mediates inflammatory responses (Trudler et al., 2010).

1.411 TLR ligands

Components of various pathogens act as ligands to TLRs. TLR2 recognizes components of gram-positive bacteria, fungi and mycobacteria. It forms dimers with either TLR1 or TLR6. TLR1-TLR2 dimers bind to triacylated lipopeptides whereas TLR2-TLR6 binds to diacylated lipopeptides (Takeuchi et al., 2001;Wyllie et al., 2000). Bacterial components such as lipopolysaccahride is recognized by TLR4 (Poltorak et al., 1998) whereas TLR5 recognizes

flagellin (Hayashi et al., 2001). TLR7 and TLR8 can bind to single-stranded RNA (Diebold et al., 2004;Heil et al., 2004) whereas TLR9 can recognize DNA that is either host or pathogen-derived (Hemmi et al., 2000;Tabeta et al., 2004). TLR3, which is the subject of experiments described in this thesis, binds to double-stranded RNA (Alexopoulou et al., 2001).

1.412 Expression of TLRs in the nervous system

In the healthy CNS, expression of TLRs is hardly detectable. In the event of an infection, the predominant types of cells in the CNS that recognize pathogens and elicit appropriate responses are microglia and astrocytes. When stimulated, microglia expresses TLRs1-9 (Olson and Miller, 2004). Gene expression studies comparing the levels of TLR transcripts in the resting and infected CNS report an upregulation of TLR3 expression in response to RNA viruses (McKimmie and Fazakerley, 2005;McKimmie et al., 2005). The sub-cellular expression of TLRs in astrocytes and microglia vary. TLR3 and TLR4 are both expressed in vesicular structures in microglia. However in astrocytes, TLR3 and TLR4 are present on the cell surface. These differences reflect differences in phagocytic and antigen processing properties of the two types of cells (Bsibsi et al., 2002).

The TLR members that are dominantly expressed in microglia are TLRs 1-4, with TLR2 being most highly expressed in human microglia (Bsibsi et al., 2002). In the case of murine astrocytes, TLRs1-9 has been reported to be expressed on the cell surface with TLR3 having the highest expression. In human astrocytes, the expression of TLRs 1-5 and TLR9 have been reported. However, a 300-fold increase in TLR3 expression has been reported in cultured human astrocytes stimulated with chemokines such as TNFα, IL-12, 4, 6 and IFNγ and IL-1β but not TGFβ or IL-10 (Bsibsi et al., 2006). Following ischemic brain injury, the levels of TLR2 and TLR4 are upregulated in microglia (Lehnardt et al., 2007). TLR2 expression has also been localized to circumventricular organs and meninges, which are areas with no blood/brain/barrier and cells in these regions can respond to circulating TLR ligands, in this case bacterial lipopolysaccharide (Laflamme et al., 2001).

There is accumulating evidence for the expression of TLRs by neurons. Microarray analysis of TLR transcripts expressed in cultured mouse cortical neurons have shown relatively low expressions of TLRs1,3,6,7,8 ; intermediate levels of expression of TLRs 2, 4 and high levels of expression of TLR5 and TLR9. Following ischemic brain injury or reperfusion injury, the levels of TLR2 and TLR4 are upregulated by neurons (Tang et al., 2007). In the developing CNS, TLR-8 has been reported to be expressed in peripheral sensory and sympathetic ganglion. In late embryonic stages, TLR-8 is expressed in axonal tracts including olfactory fibre tracts, cortical intermediate zone, fimbria of hippocampus and optic chiasm (Ma et al., 2006). Cultured motor and primary sensory neurons mainly express TLRs3-5(Goethals et al., 2010). TLR3 is present in growth cones of DRG neurons (Cameron et al., 2007), and in primary mouse cortical neurons *in vitro* (Tang et al., 2007) and Purkinje cells when subjected to viral infections (Jackson et al., 2006).

Peripheral nerves express relatively high levels of TLRs including TLR3; and TLR4 are expressed by Schwann cells (Goethals et al., 2010). All TLRs appear to be functional in cultured Schwann cells but a ligand for TLR1/TLR2 receptors produced the strongest response. Basal levels of TLRs in sciatic nerve were similar to those seen in cultured cells (Goethals et al., 2010).

1.413 Functions of TLRs in the nervous system

TLR signalling has been associated with neurotoxicity as well neurogenesis. Microglial cells that are activated by various TLR ligands produce neurotoxic substances such pro-inflammatory cytokines, nitrous oxide, reactive oxygen species (ROS) and peroxynitrite (Xie et al., 2002). However, TLR signalling in astrocytes have been reported to produce neuroprotective and anti-inflammatory mediators (Bsibsi et al., 2006).

Most TLRs expressed in glial cells signal via MyD88, which induces NF-κB mediated responses. However in neurons, TLRs signal via a MyD88 variant which associates with JNK and regulates cell death under conditions of oxygen and glucose deprivation (Trudler et al., 2010). These signalling mechanisms operate independently of NF-κB (Trudler et al., 2010). Examples of such signalling include ligand-induced signalling via TLR3 which causes growth

cone collapse in DRG neurons (Cameron et al., 2007), TLR4 which causes apoptosis (Tang et al., 2007) and TLR8 that inhibits neurite outgrowth and induces apoptotic death (Ma et al., 2006). The TLR1/2 ligand bacterial lipopeptide Pam3CysSK4, when injected into the CNS causes meningeal inflammation and apoptosis of neurons in the dentate gyrus (Hoffmann et al., 2007). Also, injection of LPS can cause severe loss of dopaminergic neurons in the substantia nigra and even low doses result in a reduction of these neurons (Castano et al., 1998;Monje et al., 2003). Infusion of the TLR3 agonist, Poly I:C which is artificial dsRNA, into the lateral ventricle causes neuronal loss in regions of the brain which include the septal nuclei, hippocampus and neocortex (Melton et al., 2003).

The two major regions in the brain associated with neurogenesis are the sub-ventricular zone (SVZ) and the dentate gyrus of the hippocampal formation. TLRs have been shown to be associated with neuronal proliferation and differentiation. TLR2 and TLR4 are expressed by neural progenitor cells (NPCs) but have opposing functions. Mice deficient in TLR2 show impaired hippocampal neurogenesis whereas TLR4 knockout mice display enhanced neuronal proliferation (Rolls et al., 2007). TLR3 has shown to negatively regulate proliferation of neural progenitor cells, although it induces the production of anti-inflammatory and neuroprotective factors and hence promotes cell survival (Lathia et al., 2008;Bsibsi et al., 2006).

1.42 Heparan Sulphate Proteoglycans

Heparan Sulphate Proteoglycans (Johnson et al., 2006) are sulfated carbohydrate polymers consisting of glycosaminoglycan (Tuszynski et al., 1996) chains attached to a core protein. They are expressed on the cell surface or secreted into extracellular space (Kraemer, 1971). The formation of the heparan sulphate chains involves polymerization of repeated disaccharide units made of glucuronic acid and N-acetyl glucosamine onto a tetrasaccharide linker which is covalently bound to the serine residues in the core proteoglycan. The polymer undergoes various modifications that include epimerization, de-acetylation and sulfation reactions which lead to the formation of a numerous isoforms (Whitelock and Iozzo, 2005). These isoforms vary in their tissue-specific distribution (Dietrich et al., 1983) and functional interactions (Esko and Lindahl, 2001). The HSPG core proteins belong to four different families-

GPI-linked glypicans, transmembrane syndecans, prelicans and agrins. The latter two are both secreted (Bernfield et al., 1999).

1.421 Ligands for HSPGs

HSPGs bind to a wide range of molecules such as growth factors, cytokines, chemokines, and extracellular matrix (ECM) molecules. They can form signalling complexes with growth factor receptors resulting in the formation of a tri-molecular complex consisting of the growth factor, its receptor and HSPG. Also, the immobilization of proteins by HSPG-binding can lead to their internalization, followed by a variety of fates within the cell. These fates depend on whether the ligand is soluble or insoluble, its interactions with receptors and also on whether the ligands bind to the GAGs or to the core proteins. HSPGs are also capable of shedding their ectodomains, a process which rapidly changes surface receptor dynamics and generates soluble ectodomains that can function as paracrine or autocrine effectors, or competitive inhibitors (Manon-Jensen et al., 2010). Moreover, the shed ectodomain can interact with any extracellular matrix molecule and can have activities that are not characteristic of surface expressed forms (Bernfield et al., 1999). Examples of insoluble ligands include N-CAM (Reyes et al., 1990), neutrophil integrin-Mac-1(Diamond et al., 1990), DCC- a receptor to Netrin-1(Bennett et al., 1997;Keino-Masu et al., 1996), fibronectin and Herpes Simplex viral glycoproteins (Feyzi et al., 1997). Soluble ligands include FGF-2 (fibroblast growth factor-2) (Rapraeger et al., 1991), hepatocyte growth factor(Zioncheck et al., 1995) heparin-binding EGF (HB EGF) (Aviezer and Yayon, 1994;Prince et al., 2010) cytokines such as IL-2,IL-3, GMCSF (Najjam et al., 1998) and chemokines such as IL-8, MCP-1 (monocyte chemoattractant protein-1) and RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted) (Hoogewerf et al., 1997).

1.422 Receptors for HSPGs

RPTPs (Receptor protein tyrosine phosphatase) have shown to interact with HSPGs. The heparan sulfate proteoglycan, agrin has shown to strongly associate with PTPσ (Aricescu et al., 2002;Ledig et al., 1999). However, PTPσ has also shown to interact with CSPGs (Shen et al., 2009). Signalling through CSPGs and HSPGs exert opposing effects on neurite outgrowth,

yet they function through the same receptor. It has recently been shown that PTPσ acts bimodally to either inhibit neurite outgrowth through interaction with CSPGs or promote neurite outgrowth by interacting with HSPGs (Coles et al., 2011). This can be achieved by competitive binding of heparin sulphate GAGs or chondroitin sulphate GAGs whereby HSPGs cause PTPσ oligomerisation leading to neurite outgrowth while CSPGs have the opposite effect. Hence, the outcome with regard to the effects on axon outgrowth is determined by the relative amounts rather than individual effects of CSPGs or HSPGs.

1.423 Functions of HSPGs in the nervous system

There is ample data suggesting the role of HSPGs in promoting outgrowth of neurons *in vitro* (Chernoff, 1988;Verna et al., 1989;Kaneda et al., 1996;Akita et al., 2004;Baerwald-de la Torre et al., 2004;Kim et al., 2003). Moreover, HSPGs modulate central and peripheral axon path finding and have shown to be required for specific directional cues (Irie et al., 2002). Enzymatic digestion of heparan sulphate prevents the growth repellent, slit from binding to the cell surface and hence attenuates its repellent activity (Hu, 2001). Sema5A has shown to act as a bifunctional molecule. Sema5A acts as a chemoattractant when HSPGs are expressed on the cell surface whereas it mediates repulsion in the presence of CSPGs in the extracellular matrix (Kantor et al., 2004). HSPGs also play a role in axon guidance at the CNS midline. Midline attractants such as Netrin/UNC6 attracts axons to the contralateral side whereas midline repellents such as slit prevent inappropriate crossing over. It has been reported that slit/Robo requires HSPGs to function *in vivo* (Serafini et al., 1994;Liang et al., 1999;Bennett et al., 1997;Kappler et al., 2000;Geisbrecht et al., 2003;Johnson et al., 2004). Moreover, syndecan-1 expressed in the CNS has shown to play a role in the slit pathway (Rothberg et al., 1990;Minniti et al., 2004).

There are implications that HSPGs regulate regenerative responses of injured axons. The expression of glypican-1 increases following injury to PNS and CNS axons particularly in the region surrounding necrotic tissue and is co-expressed with slit-2 in reactive astrocytes (Bloechlinger et al., 2004;Hagino et al., 2003a;Hagino et al., 2003b). Similarly, syndecan-2 is also expressed in the lesion site between 7-14 days after nigrostriatal axotomy and is particularly expressed by macrophages (Moon et al., 2002). It has been suggested that certain types of

HSPGs negatively regulate axonal outgrowth following injury. Enzymatic digestion of HSPGs with Heparinase1 improves axonal sprouting of common fibular nerves by two-fold (Groves et al., 2005). Also, treatment with Heparinase III, which cleaves closer to the peptide linkage, increases the number axons regenerating into the lesion or a peripheral nerve graft (Groves et al., 2005).

1.424 Sulfatases

Sulf1and Sulf2, are endosulfatases that modify the 6-0-sulphation patterns on HS molecules expressed on the cell surface. They regulate many signalling pathways by removing sulphates from HSPGs which are required by some growth factors for signalling. QSulf1 (Quail ortholog) was first identified in a screen for hedgehog target genes and has been shown to be expressed in muscle and neural progenitor cells during development as well as in the adult (Dhoot et al., 2001). QSulf1 contains 4 structural domains- an N-terminal signal peptide that directs secretion onto the cell surface, a catalytic domain, central hydrophilic domain that aids in attachment to the cell surface and a C terminal domain (Dhoot et al., 2001). Sulf1 orthologs have been identified in Drosophila, C.*elegans*, chick, mouse and humans. A closely related family member, Sulf2 has been identified in vertebrates (Morimoto-Tomita et al., 2002;Ohto et al., 2002;Braquart-Varnier et al., 2004).

Sulfs can act as tumour suppressors and regulate HSPG-mediated extracellular signalling (Lai et al., 2003;Lai et al., 2004;Uchimura et al., 2006). As both Sulf1 and Sulf2 can remove 6-0-sulphate groups from HS chains and hence can act as repressors or promoters of various signalling pathways. For example, FGF requires 6-0-sulphate groups in order to form ternary complexes with the HS-receptor; Sulfs act as repressors for such signalling (Schlessinger et al., 2000;Wang et al., 2004;Lamanna et al., 2008). In contrast, Sulfs reduce ligand binding of Wnts and GDNF to HSPGs and promote signalling presumably by increasing their availability to receptors (Ai et al., 2003;Nawroth et al., 2007;Ai et al., 2007). In Drosophila, Sulf1 expression is temporally regulated and dependant on ErbB1 signalling (Wojcinski et al., 2011). Also, there are implications of Sulf1 and Sulf2 in Alzheimer's disease (AD). An upregulation of heparan

sulphate domains degraded by Sulfs in the neocortex and hippocampus has been observed in conjunction with AD pathogenesis (Hosono-Fukao et al., 2012).

Despite its functional significance in regulating various signalling pathways, the effects of targeted disruption of either Sulf1 or Sulf2 in mice were small. Sulf1 or Sulf2 knockout mice have reduced weight with subtle skeletal and renal defects. In contrast, deletion of both Sulf1 and Sulf2 causes perinatal lethality and the embryos display kidney hypoplasia and skeletal defects, indicating the functional redundancy of Sulf1 and Sulf2 (Ai et al., 2007;Lum et al., 2007a;Holst et al., 2007;Nagamine et al., 2012).

1.5 Work leading to this project

There is evidence that Epidermal Growth Factor receptor (ErbB1) plays a role in limiting axonal regeneration. A small molecule screen of over 400 different molecules revealed that the pharmacological inhibitors of ErbB1 improved neurite outgrowth from neurons grown *in vitro* on various inhibitory substrates such as CNS myelin and CSPGs, (Koprivica et al., 2005) and also improved optic nerve regeneration *in vivo* (Koprivica et al., 2005). It was proposed that ErbB1 activation was downstream of receptors for inhibitors of axonal growth. The effects of ErbB1 antagonists on neurite outgrowth in the presence of CNS myelin have been confirmed (Ahmed et al., 2009) and it has been shown that they also enhance outgrowth in the presence of fibrinogen (Schachtrup et al., 2007). Moreover, the ErbB1 blockers enhanced functional recovery after spinal cord injury (Erschbamer et al., 2007). However, the hypothesis that ErbB1 is involved in the signalling mechanisms from various CNS inhibitory molecules has been challenged more recently in papers from the Logan laboratory (Ahmed et al., 2010;Ahmed et al., 2009) which concluded that pharmacological blockers act off-target rather than on ErbB1. The aim of this project is to investigate the role of ErbB1 in axon regeneration.

1.51 Epidermal growth factor receptor

ErbB1 is a 170 kDa protein that belongs to the ErbB family of tyrosine kinase receptors. The other members of this family include ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). The known ligands for ErbB1 include EGF (Epidermal Growth Factor), TGFα (Transforming

Growth Factor α), betacellulin, epiregulin, amphiregulin and heparin binding EGF (Carpenter, 1987;Yarden, 2001). In the CNS, the predominant ligand that binds to ErbB1 is TGFα, owing to a paucity of EGF in the brain (Probstmeier and Schachner, 1986;Lazar and Blum, 1992;Kaser et al., 1992). Apart from EGF, there are various proteins that contain EGF-like domains such as laminin (Engel, 1992), tenascin-C (Swindle et al., 2001), versican (Xiang et al., 2006), slit (Rothberg et al., 1990) and netrin (Yin et al., 2000). Of these, versican and tenascin-C have shown to act as ligands for ErbB1 (Swindle et al., 2001;Yin et al., 2000;Xiang et al., 2006).

1.511 ErbB1 signalling

The binding of ligands to ErbB1 results in the dimerisation of the receptor leading to the autophosphorylation of its tyrosine residues and subsequent activation of its downstream targets (Schlessinger and Ullrich, 1992). Although, ErbB1 can form homodimers, it prefers to heterodimerise with other members of family, particularly ErbB2. ErbB1-ErbB2 herterodimers can prolong the duration of signalling by increasing responsiveness to ErbB1 ligands such as EGF, amphiregulin and betacellulin but not TGFα (Novak et al., 2001). Apart from the receptor being activated directly by its ligands, ErbB1 can be transactivated by various other membrane-bound proteins, such as G-protein coupled receptors (GPCRs) (Fischer et al., 2006), angiotensin-II receptors (Flannery and Spurney, 2006) , beta-II andrenergic receptors (Snider and Meier, 2007)and insulin-like growth factor recptor-I (El-Shewy et al., 2004).

The phosphotyrosine residues on ErbB1, upon transactivation or ligand binding, form high affinity docking sites for signalling molecules that contain Src homolog-2 (SH-2) domains. Proteins that contain SH-2 domains include phospholipase γ (PLCγ), Ras-GTPase-activating protein (Ras-GAP), Grb2, SHC and SHP-2 (van der Geer et al., 1994). Activation of PLCγ induces conversion of PIP2 to diacylglycerol (Hoffmann et al., 2007) and inositol 1,4,5 triphosphate (IP3) (Cockcroft and Thomas, 1992). DAG activates protein kinase C (PKC) whereas IP3 induces release of calcium from intracellular stores. Grb2 which lacks a catalytic domain acts as an adaptor protein and facilitates the conversion of Ras-GDP to its GTP bound form and subsequent activation of MAP kinase and Raf pathways (Yamada et al., 1997). EGF stimulates phosphatdylinositol 3-kinase (PI3-K) activation. PI3-K is a heterodimer that has a

catalytic subunit and a regulatory subunit. PI3-K is activated by binding of its regulatory subunit to phosphotyrosine residues (Yamada et al., 1997;Mattoon et al., 2004). Activated PI3-K in turn activates Akt and PKC (Protein kinase C) (Cockcroft and Thomas, 1992). Another pathway induced by EGF binding is the JAK-STAT pathway. Janus kinase (JAK) is a non-receptor tyrosine kinase which activates cytoplasmic Signal transducer and activator of transcription (STAT). This results in the movement of STAT to the nucleus where it acts as a transcription factor for various target genes (Ihle, 1994;Darnell, Jr. et al., 1994).

1.512 Expression of ErbB1 in the CNS

ErbB1 has been reported to be expressed by both glia and neurons, although the quality of the published immunohistochemical evidence is not always high. In the case of neurons, ErbB1 immunoreactivity has been localised in cerebellar cortical neurons that have the morphology of basket cells in adult rats (Gomez-Pinilla et al., 1988). Double labelling in situ hybridization demonstrated that ErbB1 is synthesised by a subpopulation of GABAergic neurons of the rat striatum (Kornblum et al., 1995).Western blotting and immunohistochemistry has shown the presence of ErbB1 in adult retinal neurons of rat, mouse and human (Chen et al., 2007). The presence of ErbB1 has been demonstrated in adult and developing human dorsal root ganglia by means of immunoblotting and immunohistochemistry (Huerta et al., 1996). These authors also observed heterogeneity in its expression by DRG neurons, suggesting that different DRG neurons may have different responsiveness to pathways involving ErbB1 activation. ErbB1 controls the phenotypic characteristics of astrocytes, whereby in the developing CNS it is involved in astrocytic differentiation, but it is absent in astrocytes of the mature, intact CNS. There is substantial literature showing the upregulation of ErbB1 in astrocytes at the site of injury to the CNS, particularly from the Neufeld laboratory (Zhang and Neufeld, 2005;Zhang and Neufeld, 2007;Liu and Neufeld, 2003;Liu and Neufeld, 2004;Liu et al., 2006;Liu and Neufeld, 2007). Other authors also observed ErbB1 immunoreactivity in neurons, satellite glial cells and Schwann cells (Ahmed et al., 2010;Ahmed et al., 2009).

1.513 Functions of ErbB1 in the nervous system

ErbB1 plays an important role in development of the CNS. ErbB1 knockout mice, apart from having skin and lung defects, suffer from severe neurodegeneration in frontal cortex after birth and have reduced brain size (Sibilia and Wagner, 1995).The mutation subsequently proves lethal within three weeks of birth (Sibilia and Wagner, 1995;Sibilia et al., 1998). Unpublished data from Threadgill shows that ErbB1 deletion by Cre driven by the nestin promoter causes similar perinatal neurodegeneration to that in the conventional mutant, although deletion by Cre driven by the GFAP promoter does not produce neurodegeneration, despite both promoters being active in neural progenitor cells. EGF acts as a mitogen for neural progenitor cells (Yamada et al., 1996). There is evidence that the presence of EGF and the activation of ErbB1 promotes neurite outgrowth from cultured neurons under some circumstances (Morrison et al., 1987;Kornblum et al., 1990;Yamada et al., 1995;Goldshmit et al., 2004a;Tsai et al., 2010). ErbB1 is not only present on reactive astrocytes but also its phosphorylation is part of the process by which astrocyte hypertrophy is achieved following various types of insult to the CNS (Liu and Neufeld, 2003;Liu and Neufeld, 2004;Liu et al., 2006).

1.514 Implications of ErbB1 signalling in CNS axonal regeneration

There has been an emerging body of evidence suggesting that ErbB1 antagonists improve axonal regeneration in the injured optic nerve and functional recovery in the contused spinal cord *in vivo* (Koprivica et al., 2005;Erschbamer et al., 2007;Li et al., 2011;Robinson et al., 2011). There is a single report on the inadequacy of ErbB1 antagonists for improving functional outcome following spinal cord injury (Sharp et al., 2012), and another report showing that ErbB1 signalling can promote the formation of a growth-permissive environment in the contused spinal cord (White et al., 2011). There is more substantial evidence that ErbB1 antagonists improve neurite outgrowth in the presence of inhibitory substrates *in vitro* (Kaneko et al., 2007;Povlsen et al., 2008;Schachtrup et al., 2007;Ahmed et al., 2009;Leinster et al., 2012). However, the hypothesis that ErbB1 activation in neurons inhibits neurite outgrowth has been challenged directly by three studies (Ahmed et al., 2009;Ahmed et al., 2010;Douglas et al., 2009). The evidence comes from studies on siRNA knockdown of ErbB1 in neurons plated in the presence

of CNS myelin. The authors showed that the knockdown of the receptor did not improve neurite outgrowth on CNS myelin but increased neurotrophin expression by glia and interpreted this as showing that the effects of ErbB1 blockers act off-target and on glial cells rather than neurons. Since the role of ErbB1 in axonal regeneration has been questioned and because the majority of the effects of ErbB1 would, theoretically, be expected to be to promote neurite outgrowth it became of interest to use genetically deleted ErbB1 neurons and study their response to CNS inhibitors. This has formed the major goal of this project.

1.6 Objectives of this Thesis

The main objective of this thesis was to investigate the role of epidermal growth factor receptor (ErbB1) in limiting axonal regeneration. Most of the work described in the thesis was carried out *in vitro* on cultured dorsal root ganglia neurons (DRG). Preliminary objectives included confirmation of previously published data on the neurite-enhancing effects of pharmacological blockers of ErbB1 on neurons cultured in the presence of CNS myelin or CSPGs (Koprivica et al., 2005;Ahmed et al., 2009). It was most important to investigate if the pharmacological blockers were acting off-target (i.e. not acting on ErbB1) as previously established by Ahmed et.al. 2009. Hence, the effects of ErbB1 antagonists on neurite outgrowth from ErbB1-/- and wildtype neurons were assessed, because if the drugs were acting off-target, they should still have effects on ErbB1 -/- cells.

As it had been shown recently that TLR3 activation brings about inhibition of neurite outgrowth (Cameron et al., 2007), it was also an objective to investigate if ErbB1 was involved in inhibition of neurite outgrowth by TLR3. Since it had been claimed that pharmacological blockers of ErbB1 produced their effects on neurite outgrowth by promoting neurotrophin secretion by glial cells, we wanted to investigate if it was signalling via neuronal or non-neuronal ErbB1that mediated inhibition of neurite outgrowth. To investigate other molecules that may be involved in neurite inhibition by ErbB1, the involvement of calcium and PTEN in ErbB1 signalling was also tested.

Another objective was to study the expression of ErbB1 in the intact and injured nervous system. In addition it was of interest to test the effects of blocking ErbB1 on axonal regeneration *in vivo*. We proposed to use two models to assess regeneration – optic nerve crush and dorsal section to the spinal cord of adult rats. We aimed to block activation of ErbB1 in these injury models by using a pharmacological blocker in the case of animals that received an optic nerve crush and by injecting a dominant-negative ErbB1 lentivirus into the red nucleus of rats that received an injury to their spinal cords.

In the course of our investigation, we were given the opportunity to investigate the role of novel sulfatase enzymes- Sulf1 and Sulf2 on neurite outgrowth. It was proposed to investigate the expression of Sulfs in the nervous system. We then aimed to study the effects on neurite outgrowth of either blocking or enhancing Sulf activity in cultured DRG neurons using function-blocking antibodies and conditioned media from Sulf-secreting cells.

Chapter-2 Materials and Methods

2.1 Primary cell culture

Dorsal Root Ganglia (DRG) neurons

Preparation of substrate

Normal substrate:

The culture vessels used for primary cell cultures were eight-chambered glass slides (VWR). To each well of the slide, 100µl of 100µg/ml Poly-l-lysine (Sigma) were added. The slides were placed overnight in the incubator after which the excess poly-l-lysine was removed and wells were washed three times each with 500µl of distilled water. The slides were allowed to dry. 100µl of laminin (Invitrogen) at a concentration of 2µg/ml were added to the wells and left in the incubator for 2 hrs. At the time of plating cells, excess laminin was removed.

Inhibitory substrate:

Preparation of CNS myelin:

Rat CNS myelin extract used for experiments was made in accordance with the protocol by Mckerracher et al, 1994 and Li et al, 1996. Briefly, brains were dissected out from adult rats and homogenised in ice cold 0.3M sucrose(5g of wet tissue per 100ml of 0.3M sucrose) containing protease inhibitors (cocktail from Sigma- comprising AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin, Pepstatin A) with a Dounce Homogeniser. The entire procedure was carried out on ice. 18mls of the homogenate was then overlaid on 18 mls of 0.83M sucrose and centrifuged for 30 mins at 75000 g. The interface material collected and resuspended in ice-cold Tris-Cl buffer and centrifuged for 25 mins at 25000 g. The pellet was resuspended in ice-cold Tris-Cl buffer and centrifuged for 15 mins at 10000 g. The last step was repeated and the pellet was resuspended in 0.3M sucrose and then overlaid on 0.83M sucrose and centrifuged for 1 hour at 75000 g. Purified myelin which separates at the interface was collected and washed

twice with ice-cold Tris-Cl buffer by centrifugation for 15 mins at 10000g.The final pellet was re-suspended in sterile 1XPBS followed by protein estimation using Pierce BCA Protien Assay kit. Aliquots of CNS myelin extract at 200µg/ml were maintained at -20°C and used at a working concentration of 100µg/ml.

Coating slides with myelin: Aliquots of rat CNS myelin extract was diluted to the appropriate concentration (1:1; myelin extract and 0.01M PBS) and added to the culture slide pre-coated with poly-l- lysine which was then left in the hood for four hours. It was made sure that the myelin extract was not allowed to dry onto the slide. The excess volume of myelin was drained and laminin was added to the myelin coat and incubated at 37°C for two hours. Excess laminin was removed and cells were plated at appropriate cell densities.

Culture procedure

The protocol was adapted from (Gavazzi et al., 1999;Shortland et al., 2006). The vertebral columns from mice aged between P7-P11 were dissected out and cut longitudinally into two equal halves. The ganglia were then pulled out using a pair of fine tipped forceps and placed in cold Hank's buffer (PAA). The ganglia were cleaned of any excess connective tissue and the roots were trimmed using microsurgical scissors.

Ganglia were subjected to centrifugation for 10 min. at a speed of 1000rpm. The supernatant was discarded and the enzymes Collagenase (5mg/ml) (Sigma), Dispase (2mg/ml)(Invitrogen) and DNAase (1000U/ml) (Invitrogen) were added. The cells were incubated in a water bath at 37°C for 40 min with gentle shaking every 10 min. To stop the activity of the enzyme, 7mls of pre-warmed growth medium containing 10% fetal bovine serum (FBS)(Invitrogen) was added to the cells which were then centrifuged for 10 min. at 1000rpm.

After centrifugation, the supernatant was discarded and 1ml of growth medium was added, triturated and centrifuged for 5 min at 400 rpm. The resultant supernatant was discarded and 1ml of growth medium was added and triturated to form the cell suspension. In order to reduce the number of non-neuronal cells, a BSA cushion (15%) was prepared in growth medium. The cell suspension was slowly added through the sides of the tube such that the suspension

rested on the BSA (Sigma) solution. It was then subjected to centrifugation at 1000rpm for 10 min. The neuronal cells formed the pellet at the bottom of the tube and the non-neuronal cells were seen as a white layer at the interface. The pellet was then resuspended in 1 ml of growth medium and triturated gently.

10µl of the cell suspension was placed on a haemocytometer and the number of cells in each quadrant were counted and averaged. The cell density of the suspension was calculated using the formula: Average number of cells counted x 10^4/ Total volume of cell suspension. Cells were then plated at a cell density of 1,500 cells/well onto culture slides pre-coated with poly-l-lysine and laminin. Growth medium was added, cells were incubated at 37°C and grown for a fixed period of time, either 24hrs or 48 hrs.

Protocol for culturing DRGs from neonatal rats

DRGs were extracted from P7-P8 rat pups and cultured as described above.

Protocol for culturing DRGs from adult mice

The culture procedure was the same as above with the exception that 3mg/ml collagenase (Sigma) and 2.5mg/ml trypsin (Sigma) were used to ezymatically dissociate cells.

Preparation of conditioned media from non-neuronal cells of DRGs

Preparation of substrate

300µl of poly-l-lysine (100µg/ml) was added to 24-well culture dishes (Nunc) and incubated overnight at 37°C. Excess poly-l-lysine was drained and dishes were washed three times with 500µl of distilled water.

Culture procedure

Non-neuronal cells from DRGs were cultured to obtain conditioned media for experiments described in chapter 4. DRGs were dissected and subjected to enzymatic dissociation as described previously. The reaction was stopped using 7mls of growth medium containing 10%FBS. The supernatant was discarded and the pellet was resuspended in 1ml of growth medium. The cells were then centrifuged at 400 rpm for 5 min. In the meanwhile, a BSA

cushion was prepared using 1ml of 30%BSA and 1ml of growth medium which were gently mixed forming a 15%BSA solution. Following centrifugation, the cell pellet was resuspended in 1 ml growth medium and gently added to 15% BSA through the sides of the tube such that the cell suspension was layered on top of the BSA solution. This was followed by centrifugation at 1000rpm for 10min. As a result of centrifugation, 3 layers were formed- the top layer that was BSA solution, the intermediate layer containing non-neuronal cells and the bottom layer containing a cell pellet enriched in neurons. The intermediate layer was harvested using a 1000µl pipette and centrifuged for 5min at 1000rpm. The cell pellet was then resuspended in 1ml growth medium. 10µl of the cell suspension was placed on a heamocytometer and cells in each quadrant counted. Counts from four quadrants were averaged and cell density of the cell suspension calculated using the formula: average number of cells counted x 10^4/ Total volume of cell suspension. Cells were plated on coated 24-well plates at a density of 30,000 cells/well in 500µl of growth medium and grown at 37°C for 72 hours. At the end of the growth period, 300µl of medium from each of the wells were collected and used for experiments at concentration of 1:10. Conditioned medium was generally collected on the day of experiments but some was stored at -20°C for later use. Non-neuronal cell were cultured three days before conducting experiments using conditioned media.

Cerebellar Granule Cells (CGCs)

Preparation of substrate

Poly-l-lysine at a concentration of 100µg/ml and volume of 300µl were added to sterile glass cover slips (13mm, VWR) placed in 24-well plates (Nunc). The glass cover slips with poly-l-lysine were incubated for 24hrs. Excess poly-l-lysine was drained and glass cover slips were washed three times with sterile distilled water or sterile PBS (0.01M). 150µl of Laminin (2µg/ml) was then added to the cover slips and incubated at 37°C for 2hrs. At time of plating cells, excess laminin was drained off.

Dissection

Cerebellum was extracted from mice aged between P5-P7. The pups were decapitated. A pair of coarse forceps was used to hold the head by the jaws and the skin was cut using a fine pair of scissors starting from the neck and proceeding along the midline. The skin was pulled aside and the skull was cut laterally along the back of the olfactory bulb and laterally along the cerebral hemispheres to minimize any damage to the cerebellum. The skull was then gently peeled off. The connections to the cerebellum that attaches it to the brain stem were cut on the left and right sides and place in a 35mm petri dish containing ice cold PBS (0.01M). The meninges and large blood vessels were removed.

Culture procedure

The cerebellum was transferred to the top of a clean 35mm petri dish (BD Biosciences) and excess buffer was drained. The tissue was then chopped with a 10A blade while rotating the lid to get small pieces. The chopped tissue was then transferred using a Kwill tube attached to the tip of a syringe into a 15ml centrifuge tube containing 10 mls of PBS (0.01M). The tissue was centrifuged at 1000rpm for 2 min. Enzymatic digestion was carried out using 2mls of 0.05% trypsin which was added to the pellet and incubated at 37°C for 12 min. To stop the reaction at the end of 12 min, 7mls of growth media (F12 or neurobasal media) (Invitrogen) containing 10%FBS was added and centrifuged at 1000rpm for 2 min.. The supernatant was removed and 5µl of DNAase (2000 units/ml) was added to the pellet. Cells were triturated up and down ten times using a 5ml pipette and 6 times using a 1ml Gilson avoiding air bubbles. The large undissociated pieces were allowed to settle at the bottom of the tube and the supernatant was harvested and added to 4mls of neurobasal media growth media. This was followed by centrifugation at 1000rpm for 5min. The supernatant was removed and the cell pellet was resuspended in 1ml of pre-warmed Neurobasal growth period. 10µl of the cell suspension was placed on a heamocytometer and cells in each quadrant counted and averaged. The number of cells in the cell suspension was calculated using the formula: average number of cells counted x 10^4/ Total volume of cell suspension. Appropriate volumes were added to coated cover slips such that the cells were plated at a density of 65,000cells/well and 500µl of growth media were added to each of the wells. They were allowed to grow for a period of 16-18 hrs at 37°C.

Schwann cells

Preparation of substrate

Plastic petri dishes (10cm) (BD Biosciences) were treated with 3mls of poly-l-lysine at a concentration of 100µg/ml. The petri dishes containing poly-l-lysine were placed in the incubator overnight. Excess poly-l-lysine was drained and petri dishes were washed three times in 3mls of distilled water. 2mls of laminin at concentration of 10µg/ml were added and incubated for 2 hours. Excess laminin was removed at the time of plating cells.

Culture procedure

The protocol was adapted from (Dong et al., 1999). Sciatic nerves were dissected from mice aged P7. The dissected sciatic nerves were transferred to a petri dish containing ice cold L15 medium. The epineural sheath was carefully removed by running no. 5 forceps up and down the nerve. The nerves were then dissociated using 3mg/ml collagenase and 2.5mg/ml trypsin and incubated for a period of 35min. at 37°C. Following incubation, the cells were triturated 5 times using a 1ml pipette and 10 times using a 200µl pipette. The cells were then re-incubated for 5 min and further triturated 15 times using a 200µl pipette. To stop the enzymatic reaction, 7 mls of growth media (DMEM) containing 10%FBS was added to the cell suspension. This was followed by centrifugation at 1000rpm for 10min. The supernatant was discarded and the pellet was resuspended in 1ml of growth medium. The cells were then plated on plastic pteri dishes. A mitotic inhibitor, AraC (10µM) (Sigma) was added to the cells at the time of plating in order to minimize the proliferation of fibroblasts and cells were incubated at 37°C. The medium was replaced after 48 hrs and the total culture period was 3 days.

Neurite outgrowth inhibitors added to cultures

Poly I:C

Preparation of stock- PolyI:C (Sigma) purchased as a sodium salt was reconstituted in sterile 0.01M PBS. 1ml of PBS was added to the bottle containing 10mg of PolyI:C and heated on a water bath at 50°C for 10 min. The solution was allowed to cool at room temperature. 100µl aliquots were prepared and stored at -20°C.

Working concentration- 5µl of the stock solution (10mg/ml) was added to 500µl of growth media to achieve a working concentration of 100µg/ml and was then added to cells at the time of plating.

Chondroitin sulfate proteoglycans –CSPGs

Stock- Commercially available (Chemicon) CSPGs that were isolated from embryonic chicken brains was contained in a vial at a concentration of 1mg/ml. Aliquots of 5ul were made and stored at -20°C.

Working concentration-CSPGs were used at a working concentration of 0.5µg/ml made by diluting stock in appropriate volumes of media and directly added to cells at the time of plating.

Treatment with drugs

ErbB1 antagonist-PD168393

Preparation of stock- PD168393 (Calbiochem) was available in the powdered form. 2.7mls of DMSO was added to 1mg of PD168393 yielding a stock solution of 1mM and stored at -20°C.

Working concentrations- PD168393 was used at concentrations of 10nM and 100nM. 1µl of the stock solution was dissolved in 10mls of growth media yielding a concentration of 100nM. 1ml of 100nM solution was further diluted in 10 mls of growth media in order to obtain a concentration of 10nM.The growth media containing PD168393 at appropriate concentrations were added to the cells at the time of plating.

VO-OHpic (PTEN antagonist)

Preparation of stock- 10mg of VO-OHpic trihydrate (Sigma) was dissolved in sterile distilled water to obtain a 10mM stock. Aliquots of 5µl were made and stored at -20°C.

Working concentrations- VO-OHpic was used at concentrations of 10µM, 1µM, 100nM and 50Nm by diluting stock in appropriates volumes of growth media. VO-OHpic was added to the cells at the time of plating.

BAPTA-AM [*1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester]* (Calcium chelating agent)

Reagents were freshly made up before the start of each experiment.

Preparation of stock- BAPTA-AM (Invitrogen) is available as 1mg aliquots. BAPTA-AM was dissolved in 2.5% pluronic F-127 which is a detergent. 25mg of pluronic F-127 was added to 1ml of DMSO (invitrogen) and vortexed. To achieve complete dissolution, the mixture was placed in a drying cabinet at 40°C for 10min. 654µl of pluronic F-127-DMSO was added to 1mg aliquot of BAPTA-AM and vortexed. The resulting solution had a concentration of 2mM which formed the stock. A sub-stock of 2µM was made by diluting 1µl of the stock in 1ml of growth media.

Working concentrations-BAPTA-AM was used at working concentrations of 10nM and 100nM by diluting the sub-stock in appropriate volumes of growth media and was added to the cells at the time of plating.

BCECF-AM [*2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein*]

BCECF-AM acted as control to BAPTA-AM as both drugs are metabolised by the cells in the same manner but BCECEF-AM has no effect on calcium homeostasis. All reagents were freshly made prior to the start of each experiment.

Preparation of stock- BCECF-AM (Invitrogen) is available as 50µg aliquots. BCECF-AM was dissolved in pluronicF-127 as in the case of BAPTA-AM. 30µl of pluronic F-127-DMSO was added to 50µg aliquot of BCECF-AM and vortexed. The resulting solution had a concentration of 2mM which formed the stock. A sub-stock of 2µM was made by diluting 1µl of the stock in 1ml of growth media.

Working concentrations-BCECF-AM was used at working concentrations of 10nM and 100nM by diluting the sub-stock in appropriate volumes of growth media and was added to cells at the time of plating.

CFSE [Carboxyfluorescein diacetate succinimidyl ester] (Cell tracker)

Preparation of stock-CFSE (Life technologies) was provided in single-use vials along with DMSO stored at -20°C. 18µl of DMSO was added to a single vial of CFSE yielding a concentration of 5mM. The stock was made before the start of the experiment.

Working concentration- CFSE was used at a working concentration of 25µM.

Treatment of cells with CFSE- 5µl of the stock solution was added to 495µl of pre-warmed F12 media yielding a sub-stock at a concentration of 50µM. 200µl of the sub-stock was added to 200µl of the cell suspension. Hence, reducing the concentration of CFSE to 25µM. The mixture was incubated at 37°C for 30 min. This was followed by centrifugation at 1000rpm for 5 min. The cell pellet was then re-suspended in 1ml of pre-warmed F-12 media and incubated at 37°C for 30 min. After incubation, the cell suspension was centrifuged at 1000rpm for 5 min. The resultant pellet was resuspended in 1ml of pre-warmed growth media and cells were plated on CNS myelin-coated substrate. The cells were grown at 37°C for 48 hrs.

Immunocytochemistry

Preparation of fixative

4g of paraformaldehyde (VWR) was weighed and added to 50mls of distilled water. The mixture was heated to 60°C with constant stirring using magnetic stirrer. When the temperature rose to 60°C, drops of 1M NaOH were added until a clear solution was obtained. The solution was then allowed to cool to room temperature and filtered using a Whatman filter paper to get rid of any undissolved paraformaldehyde granules. The solution was made up to 75 mls using distilled water. 25mls of 0.4M PBS was added to the solution. Hence, the resultant solution contained 100mls of 4% paraformaldehyde in 0.1M PBS. The pH of the solution was tested before use.

Preparation of blocking solution

Blocking solution was made using 0.01M PBS, 1%BSA, 0.05% Tween-20, 0.01% Triton-X and 5% goat serum.

Triton-X solution

0.5% Triton-X solution served as detergent to facilitate antibody penetration into cells. This was made by adding 100µl of Triton-X to 20mls of 0.01M PBS. The mixture was thoroughly vortexed.

Primary antibodies

Primary antibodies were diluted in blocking solution at the time of use. The appropriate dilutions for various antibodies are listed below:

Primary antibody	**Working concentration**
Polyclonal anti-βIII Tubulin produced in rabbit (recognises amino acids 441-450 of human βIII Tubulin which is conserved in all mammals; purchased from Sigma).	1:1000
Monoclonal anti-neurofilament produced in mouse (recognises medium and heavy neurofilament -molecular weight:160kDa and 200KDa of mouse, rat and human, purchased from Sigma).	1:500
Polyclonal anti-neurofilamnt 200 produced in rabbit (antibody from IgG fraction of anti-serum from bovine spinal cord, purchased from Sigma)	1:800
Polyclonal anti-calcitonin gene receptor peptide (CGRP) produced in rabbit (recognises both rat and mouse CGRP, purchased from Peninsula Labs).	1:500
Isolectin B_4 – Biotin conjugate (purchased from Sigma)	1:100
Polyclonal anti- NG2 produced in rabbit (Kindly donated by Dr. Stallcup).	1:1000
Monoclonal anti- S100 produced in mouse (purchased from Sigma)	1:1000
Sulf1antibody produced in rabbit (recognises exon6 and detects full length Sulf1)	1:200
Sulf2 antibody produced in rabbit (recognises exon 6 and detects full length Sulf2)	1:200
Anti- Sulf1isoform produced in rabbit(recognises exon 20/21 and detects all sulf1 isoforms)	1:00
Anti- Sulf2 isoform produced in rabbit (recognises exon 23 and detects all sulf2 isoforms)	1:100

Table 2.1: List of primary antibodies and the concentrations at which they were used to immunostain cultured cells.

Note: The antibodies against Sulf1, Sulf2 and its isoforms were kindly given to us by Dr. Gurtej Dhoot, Royal Veterinary College, London.

Secondary antibodies

Florescent-labeled secondary antibodies were used and diluted in appropriate volumes of blocking solution. The secondary antibodies used are listed below:

Secondary antibody	**Working concentration**
Anti-rabbit IgG-Atto 594 produced in chicken (Sigma)	1:600
Anti-rabbit IgG-Atto 488 produced in goat (Sigma)	1:600
Anti-rabbit IgG-Alexa fluor 488 produced in goat (Life technologies)	1:600
Anti-mouse IgG-Alexa fluor 594 produced in goat (Life technologies)	1:400

Table 2.2: List of secondary antibodies and concentrations at which they were used to immunostain cultured cells.

Antibody staining technique:

Primary cells after grown for the stipulated period of time, were fixed using 4% paraformaldehyde. 300 µl of growth media was removed from each well containing cells in 500 µl of media. 300µl of 4%paraformaldehyde was added to each well, hence diluting it from 4% to 2.4%. The cells were left in fixative for 10min. At the end of 10min, the solution containing a mixture of growth media and paraformaldehyde was aspirated gently causing minimal damage to cells. The cells were then washed in 500µl of 0.01M PBS three times, each wash lasting 5 min.

After the washes were completed, 100µl of 0.5% Triton-X was added to each well and left for 10min. This was followed by a single wash in 0.01M PBS for 5 min. 200µl of primary antibody diluted in blocking solution was added to each well. The cells were incubated in primary antibody overnight at 4°C. When βIII tubulin antibody was used, cells were incubated for 2 hours at room temperature. For double labeling to detect more than one protein, cells were incubated in blocking solution containing antibodies from different species at appropriate concentrations. Following incubation with primary antibody, the cells were washed three times for 5 minutes each with 0.01M PBS. The cells were then incubated for 2 hours in blocking solution containing secondary antibody and the nuclear stain, Hoechst. For double labeling, cells

were incubated with secondary antibodies from two different species with different detection wavelengths. This was followed by three washes in 0.01M PBS for 5 min each.

When 8-well chambered slides were used, the chambers were carefully removed using a removal device provided by the manufacturer. In the case of coverslips, these were gently removed using a pair of foreceps and placed on glass slides. The slides or coverslips were mounted using mounting media (PBS, glycerol, DABCO).

Data analysis

Microscopy

Images were acquired using a Zeiss Axiophot microscope, Hamamatsu C4742 camera and Improvision software. Photomicrographs of cells that were both βIII tubulin and Hoechst positive were taken. Images of at least 30 neurons were taken .Two separate stacks of images of which one stack contained images of βIII tubulin positive neurons and another stack that contained images of their corresponding Hoechst positive nuclei were created. These were stored as .TIFF files.

Analysis of neurite length

In order to analyse neurite lengths from photographed neurons, image stacks were run through the software, Image J (Java based image processing programme developed at the National Institute of Health). Neurite tracer, a plug-in by Image J was used to analyse the total lengths of neurites. The measurements given by the software was processed using the formula:

$$(\,length\ of\ neurites\ per field\ of\ view\ measured\ by\ neurite\ tracer)$$
$$\times\ conversion\ factor\ specific\ to\ the\ microscope\ in\ use\ (i.e.\ to\ convert\ pixels\ to\ \mu m)$$
$$=\ neurite\ length\ of\ neurites\ \boldsymbol{in}\ \mu\boldsymbol{m}\ per\ field\ of\ view$$

$$Average\ length\ of\ neurites\ from\ all\ neurons\ photographed\ from\ one\ experiment = \frac{Sum\ of\ \ neurite\ lengths\ in\ \mu m\ from\ all\ fields\ of\ view}{Sum\ of\ the\ number\ of\ neurons\ from\ all\ fields\ of\ view}$$

Data collected from cell counts

An experiment had various conditions, for example, cells treated with poly I:C, cells treated with poly I:C and ErbB1 antagonist etc. An experiment was referred to as a 'single experiment' when cells grown under different conditions were derived from a single animal. Experiments were repeated from 5-6 different animals. Cells positive for the antibody against the protein under investigation were counted from different fields of view. Cells were counted to assess the following:

Assessment of cell-attachment

To assess the effects of drugs/ inhibitors on attachment of cells to the substrate, total number of Hoeschst+ve nuclei in one field of view were counted (unattached cells were washed off). Counts were taken from 15 different fields of view from wells containing cells grown under the same condition. This method of counting was repeated for all conditions in a single experiment.

Assessment of neuritogenesis

To assess the effects of drugs/ inhibitors on neuritogenesis, two types of counts were taken:

1) Total number of βIII tubulin+ve neurons (with and without neurites).
2) Total number of βIII tubulin+ve neurons that extended neurites.

Both the counts were taken from the same well containing cells grown in the same condition. The same was repeated for all conditions of an experiment.

Effects of genetic deletion of ErbB1 or treatment with inhibitor on non-neuronal cells in neuronal cultures

Non-neuronal cells were immunostained for S100 and NG2 . The following were counted from a single field of view:

1) Number of NG2+ve cells.

2) Number of S100+ve cells.

3) Number of Hoechst nuclei.

Counts were taken from 10-15 different fields of view from wells containing cells grown in the same condition. The relative numbers of non-neuronal cells that were either S100+ve or NG2+ve were calculated as follows:

$$= \frac{Total\ number\ of\ S100 + ve\ or\ NG2 + ve\ cells\ counted\ from\ all\ fields\ of\ view}{Total\ number\ of\ Hoechst + ve\ cells\ counted\ from\ all\ fields\ of\ view}$$

The same was repeated for all conditions in a single experiment.

Computing statistical significance

All experiments were repeated from 5-6 different animals, hence N=5 or N=6. Values pertaining to measurement of neurite length or cell counts from all experiments were averaged. Based on the mean value, standard deviation and standard error of mean (SEM) were calculated. Statistical significance was computed by the software, Graph Pad Prism by using student's t test as the statistical method.

2.2 Techniques to detect ErbB1, Sulf1 and Sulf2 transcripts in primary cells and CNS tissue

Two types of PCRs were carried out to assess the expression of various transcripts in cultured cells and neural tissue. These include- RT (reverse transcriptase) PCR and q (quantitative) PCR.

RT PCR

Reverse transcriptase polymerase chain reaction was used to detect the expression of ErbB1 and Sulf transcripts in cell and tissue samples. The following steps were carried out:

1) Extraction of RNA from samples using a Qiagen kit.

2) Synthesis of cDNA from reversely transcribed RNA by means of using a reverse transcriptase enzyme.

3) PCR reaction that uses synthesized cDNA as template and a set of primers specifically designed to anneal to the target sequence.

Extraction of RNA from cultured primary cells (Qiagen kit)

(RNAase free conditions were ensured by cleaning the work bench and equipments with bleach or RNAaseZAP purchased from Sigma)

DRG neurons (P7-8) or Schwann cells were cultured on 10cm dishes (BD biosciences) for three days. Cells were pooled from 5-8 animals. On the day of extraction, growth media was aspirated and 700µl of chilled Trizol (Invitrogen) was added to the culture dishes. A cell scraper was used to spread Trizol to all the sides of the dish and cells were scraped at the same time. The dish was tilted and a mixture of cells in Trizol were collected and transferred to an eppendorf tube. Cell suspension was rigorously pipetted up and down to ensure lysis.700µl of chilled ethanol was added to the tube and mixed thoroughly. The contents were then transferred to a spin column.

The steps that follow were carried out in using reagents and the protocol supplied by Qiagen. The spin column was centrifuged at 8000g for 15sec at 4°C. The flow-through was discarded and 700 µl of buffer RW1 was added to the spin column. The spin column was then centrifuged at 8000g for 15 sec at 4°C. The flow-through was discarded and 500µl of buffer RPE was added, followed by centrifugation at 8000g for 15 sec at 4°C. The flow-through was discarded and once again 500µl of buffer RPE was added. This was followed by centrifugation at 8000g for 1 min. After having discarded the flow-through, the spin column was placed in a new collection tube and centrifuged at 8000g for 1 min to get rid of remaining buffer. The spin column was then placed in an RNAase free 1.5ml microfuge tube. To elute RNA present in the spin column, 30µl of RNAase-free water was added directly into the spin column without touching the sides of the tube. The spin column was centrifuged at 8000g for 1 min. at 4°C. The microfuge tube containing RNA was placed on ice for further use or stored at -80°C.

The protocol on extraction of RNA from tissue samples has been described later in this section.

Measurement of RNA concentration:

RNA concentration in the sample was measured using a nanodrop (Thermo Scientific).

DNAase treatment of RNA sample:

To digest DNA that may have contaminated the RNA sample, the following reaction was set up:

RNA - 29µl

Buffer (Invitrogen) -4µl

DNAase (Invitrogen) - 6µl

Water-1 µl

Total-40µl

The mixture was incubated at 37°C for 20 min.

Analysis of degradation of RNA sample:

To check for RNA degradation, samples were run on a 1.8%gel.

Preparation of gel: 0.45g of ultrapure agarose (Invitrogen) was added to 25mls of 1X TBE buffer. The solution was heated in a microwave for 1 min. A drop of ethidium bromide was added and the agarose solution was gently swirled to for even distribution. It was then poured into a gel tank in which the combs were appropriately positioned. The gel tank was placed in a fume hood and the gel was allowed to set.

Preparation of sample: 1µl of the sample was mixed with 1µl of RNA loading buffer. It was then placed on a heat block at 90°C for 2 min to denature RNA. At the end of 2 min, the sample was quickly placed on ice.

The samples were loaded onto the gel and run at 180V for 20 minutes. In a non-degraded RNA sample, all three bands corresponding to 28S, 18S and 5S ribosomal RNA would be visible. The band corresponding to 28S would be most intense and 5S would be least intense, although at times the 5S band is hardly visible.

cDNA synthesis

The reagents for cDNA synthesis from RNA were purchased from Promega. RNA stock was diluted to 1µg in 10µl using RNAase free water. 1µl of random primers (0.5µg primer per µg RNA) were added such that the total volume does not exceed 11µl. It was then heated to 70°C for 5 min toachieve annealing of the primer to the template . The tube was then chilled on ice for 5 min. The following components were added to the primer/template :

5X AMV RT buffer	5µl
dNTP mix (10mM)	2.5µl
RNAsin (40 units)	1µl
AMV RT (30 units)	3µl
RNAse free water	2.5µl

Total volume of reaction mix- 25µl

The contents in the tube were then mixed by flicking the tube and incubated at 37°C for 60min. The cDNA thus obtained, was aliquoted and stored at -20°C.

Primers

The primers were purchased from Sigma.

ErbB1 primers

Primer sequences were designed from mouse genome ; accession number- NM_207655.2.

The following primers were used:

Primers that anneal to regions between exon 1 and exon7. These sequences have been previously published (Threadgill et al., 1995)

Forward primer

5'-GGGGCGTTGGAGGAAAAGAA

Reverse primer

5'-ATGAGTGGTGGGCAGGTG

Product size-736bp

Primers that anneal to regions between exon2 and part of exon3 (from 371bp-562bp):

Forward primer

5'TGCCAAGGCACAAGTAACAG

Reverse primer

5'GTTGAGGCAATGAGGACAT

Product size-191bp

Sulf1 and Sulf2 primers

The primers for Sulf 1 and Sulf 2 were kindly given to us by Dr. Gurtej Dhoot, Royal Veterinary College, London. The primer sequences are as follows:

Mouse Sulf1:

Forward primer

5'- AGAG GGA TCC ATG AAG TAT TCC CTC TGG GCT CTG

Reverse primer

5'-CAA TGT GGT AGC CGT GGT CC

Size of PCR product= 953 bp

Mouse Sulf2:

Forward primer

5'- AGAG GGA TCC ATG GCA CCC CCT GGC CTG CCA CTA T

Reverse primer

5'-CAT AGA CTT GCC CTT CAC CAG CCC

Size of PCR product=998bp.

It is to be noted that the letters in red indicate restriction sites within the primer as these primers were originally designed to clone sequences into plasmids. The restriction sites simply overhang and do not interfere with priming.

Restriction site sequence- AGAG GGA TCC

PCR reaction used to detect ErbB1 transcripts expressed in cultured DRG and Schwann cells

The following contents were added to a 0.5ml eppendorf tube:

10x Taq buffer	5 µl
10mM dNTPs	1µl
50mM MgCl2	1.5µl
Primers sense	2.5µl
Antisense	2.5µl
Taq polymerase	0.5µl
d.H20	12µl
Total	25µl

0.5µl of cDNA was added to the reaction mixture.

Before loading samples, the temperature on the PCR machine was set to 95°C indefinitely . The reaction was as follows:

94°C - 2min

94°C - 1min.
58°C - 15sec. X 32 cycles
72°C - 30sec.

72°C - 5min

4°C - ∞

PCR reaction to detect Sulf1 and Sulf2 transcripts in CNS tissue and cultured cells

A ReadyMix Taq PCR reaction mix consisting of Taq polymerase, $MgCl_2$ and dNTPs was purchased from Sigma. A 30µl reaction was set up.

ReadyMix Taq PCR reaction mix	15µl
Double distilled water	14µl
Primers (sense+antisense)	1µl
Total volume	30µl.

1µl of cDNA was added to the reaction mix.

The PCR reaction was as follows:

95°C - 5min

94°C - 30 sec.
52°C - 45 sec. X } 40 cycles
72°C - 1 min.

72°C - 10min.

4°C -∞

To visualize PCR products, a 1.8% agarose gel in 1X TAE buffer was made. The samples were mixed with loading dye and loaded onto the gel. A 1kb plus ladder was also loaded.The gel was run at 80V.

Quantitative PCR (qPCR)

qPCR was used to detect changes in expression levels of ErbB1 transcripts in various types of CNS tissue isolated from at various time points. The table below shows the time points at which different types of CNS tissue were isolated.

CNS tissue	**P0** (day pups were born)	**P8**	**Adult**
Cortex	+	+	+
Spinal cord	+	+	+
DRGs	+	+	+
Sciatic nerve	+	+	-

Table 2.3: The types of CNS tissue and age at which they were isolated for RNA extraction.

Extraction of RNA from tissue samples *(RNAeasy plus mini kit from Qiagen)*

Dissection instruments were autoclaved and cleaned with RNAaseZAP. Cortex, spinal cord, DRGs and sciatic nerve were dissected from P0, P8 and adult mice. As soon as the tissue was extracted, it was placed in RNAlater (Ambion) to prevent RNA degradation and stored at -20°C. DRGs and sciatic nerve were pooled from 3-8 animals. Prior to the day of extraction, the Dounce homogenizer was oven baked at 180°C for 4 hours and allowed to cool. On the day of extraction, excess RNAlater was drained off the tissue which was directly placed into a Dounce homogenizer cooled in liquid nitrogen. The tissue was allowed to freeze in liquid nitrogen and crushed by stroking a tight-fitting pestle up and down several times. Once the tissue was crushed to form a powder, the homegeniser was placed on ice and 600µl of lysis buffer RLT plus was added. The tissue in lysis buffer was further homogenized by another 10 strokes with the pestle. The tissue homogenate was transferred to a Qia shredder (Qiagen) by mean of a sterile narrow Pasteur pipette and centrifuged for 2min at maximum speed. The Qia shredder ensures thorough lysis of the sample. The supernatant was transferred to a gDNA eliminator which eliminated genomic DNA present in the sample. This was followed by centrifugation at 8000g for 30 sec. The supernatant was collected and 700µl of chilled ethanol was added in a tube. The contents were thoroughly mixed. 700µl of the sample was then added to a RNAeasy spin column and centrifuged at 8000g for 30 sec. The flow-through was discarded and the reamaining sample was added to the spin column and centrifuged at 8000g for 30 sec. After discarding the flow-through, 700µl of buffer RW1 was added and centrifuged at 8000g for 30sec. Flow-through was discarded. 500µl of buffer RPE was added to the spin column and centrifuged at 8000g for 30 sec. This was followed by a second addition of 500µl of RPE and centrifuged at 8000g for 2 min.

Following disposal of the flow-through, the spin column was place in a new collection tube and centrifuged at maximum speed at 1 min. This was done to drain residual buffer that may be present in the spin column. The spin column was then placed on an RNAase free micrfuge tube . RNA in the spin column was eluted using 40µl of RNAase free water . The spin column was centrifuged at 8000g for 1 min.The spin column was discarded and the RNA that collects in the microfuge tube was placed on ice.

RNA concentration was measured using a nanodrop.

Assessment of RNA integrity

The integrity of RNA was analysed using Experion automated electrophoresis system (Bio-Rad). The system requires as a little as 100pg of RNA and electrophoresis is conducted in channels of microchips. The electropherogram produces two distinctive peaks that correspond to 28S and 18S rRNA. The software then calculates RQI (RNA quality indicator), a term introduced by Bio-Rad to standardize and quantitate RNA intergrity. RQI values range from 10 (Hunt et al., 2002) to 1(highly degraded). Samples that had an RQI of less than 7 were not used for cDNA synthesis.

<u>cDNA synthesis</u>

All reagents were purchased from Invitrogen. The following were added to a microfuge tube:

Reaction I

Oligo Dt	1µl
RNA	11ul (500ng)
10mM dNTPs	1µl

The mixture was heated at 65°C for 5 min and then kept on ice for 1min.

In a separate tube the following were added:

Reaction II

5X first-strand buffer 4µl

0.1M DTT 1µl

RNAase OUT 1µl

The contents from reaction I were mixed with contents from reaction II. 1µl of SuperScript III reverse transcriptase enzyme was added to the tube. The mixture was thoroughly mixed by pipetting up and down, vortexed and spun down using a table top centrifuge. The mixture was then incubated at 50°C for 1 hour followed by incubation at 70°C for 15 min to inactivate the reaction.

To check for genomic DNA contamination, separate reactions were set up using the same set of RNA samples. The tubes were labeled as RT negative samples. The contents of the reaction mixture were the same as mentioned above ,with the exception of reverse transcriptase enzyme which was replaced with water.

cDNA synthesized were aliquoted and stored at -20°C.

Primers

Primers for ErbB1 were designed from mouse genome, accession number - NM_207655.2.Primers for mouse housekeeping genes, Rpl3 and GAPDH used as internal references were designed using accession numbers NM_013762 and NM_008084 respectively.

Primer sequences for mouse ErbB1

Forward primer

3'-AGAGGAGACATATAGACA

Reverse primer

3'-TTCATTGCTACAGTTAGA

T_m (melting temperature of primer) = 57.1°C

Product size-124bp

Primer sequences for mouse Rpl3 (Ribosomal protein L3)

Forward primer

3’-CAGAGATTAACAAGAAGATTTAC

Reverse primer

3’-TTGAGCATGATGAAGTCA

T_m= 57°C

Product size-169bp

PCR reaction

Ready-made master mix - Kapa SYBR Fast Universal qPCR Kit (cat no. KK4602) was purchased from Anachem. 5µl of cDNA was used for each reaction.

The following reaction was set up using real-time PCR machine (Bio-Rad CFX96) :

1 95.0 C for 3:00

2 95.0 C for 0:15

3 56.0 C for 0:30

4 72.0 C for 0:30 + Plate Read

5 GOTO 2, 39 more times

6 Melt Curve 65.0 to 90.0 C, increment 1.0 C,0:05 + Plate Read

END

2.3 Detection of ErbB1, Sulf1 and Sulf2 proteins in CNS tissue and cultured cells by means of Western blotting

Extraction of protein from tissue

Cortex, cerebellum, spinal cord, DRGs and sciatic nerve were dissected from adult rats and stored at -80°C. On the day of extraction, samples were placed in FastPrep tubes on ice. The composition and volume of buffer used depended on the type of sample. Details are as follows:

Tissue sample	Type of lysis buffer	Volume of buffer (µl)
Cortex	RIPA (Sigma)	500

Cerebellum	RIPA (Sigma)	500
Spinal cord	5M Urea buffer	400
DRGs	5M Urea buffer	200
Sciatic nerve	5M Urea buffer	200

(RIPA buffer was supplemented with protease inhibitor cocktail-Pierce and 0.5MEDTA-ThermoScientific)

Table 2.4 : List of lysis buffers used to extract protein from different CNS tissue.

Specially designed FastPrep beads (8-10 beads) were placed into each tube containing the sample and lysis buffer. The samples were then homogenized on a high speed homogenizer (Thermo Savant) at 4°C. Two cycles on the homogenizer, 30 seconds each were carried out. The samples were then transferred to fresh tubes and centrifuged at 10,000rpm for 2 min at 4°C. The supernatant was collected and transferred to another microfuge tube. If the supernatant was too viscous, it was further diluted with lysis buffer and centrifuged again. The procedure was repeated until the supernatant attained a liquid consistency. Samples were placed on ice or stored at -80°.

Extraction of protein from cultured cells

DRGs from P8 mice were cultured and grown for 24 hours. Cells were pooled from 6 animals. Cells were grown under different conditions such as in the presence of Poly I:C, CSPGs PTEN antagonist etc. On the day of extraction, 80µl of chilled RIPA buffer was added to the culture dish and cells were scarped using a cell scrapper. The cell extract was transferred to an eppendorf tube placed on ice and piptted up and down several times to facilitate lysis. Protein lysate was placed on ice.

Estimation of protein concentration- *Bradford assay*

Preparation of BSA standards:

BSA was diluted to a working concentration of 1% with distilled water. To a 96-well plate, the following volumes (µl) of BSA were added - 0, 0.5, 1,2,4,6,8,10. To each of the wells,1µl of lysis buffer was added. The total volume in each well was made up to 50µl using

distilled water. 200µl of Bradford reagent was added to each well and allowed to stand for 10 min.

Preparation of sample:

The following are added to each well:

Sample 2µl

Distilled water 48µl

Bradford reagent 200 µl

The plate was kept for 10 minutes at room temperature to allow the colorimetric reaction to take place.

Following 10min, the plate containing samples and BSA were placed in a plate reader that measures optical density. Concentration of the protein was obtained by plotting standard curve.

Denaturation of protein

Depending on the amount of sample loaded onto the gel, the appropriate volumes equivalent to 10-40 µg of protein were added to a separate tube. 5X Lamelli buffer was added to the sample. The mixture was heated to 95°C for 5 min.

Preparation of gel for SDS PAGE

Composition of resolving gel :

10% gel was prepared for proteins below a size of 200kDa.

	10%gel	**10%, 2 gels**
H20	6.1mls	12.2mls
1.5M Tris,pH8.8	3.2mls	6.4mls

40% 19:1 Acr:Bis	3.2mls	6.4mls
10%SDS	125ul	250ul
10%APS	100ul	200ul
TEMED	10ul	20ul

Table 2.5 : Composition of resolving gel.

Composition of Stacking gel:

	5%gel	**5%, 2 gels**
H20	3.1mls	6.2mls
1.5M Tris,pH8.8	1.25mls	2.5mls
40% 19:1 Acr:Bis	0.625mls	1.25mls
10%SDS	50ul	100ul
10%APS	37.5ul	75ul
TEMED	7.5ul	15ul

Table 2.6 : Composition of stacking gel.

Casting of gels:

Gels were cast using equipment from BioRad. 7.5mls of 10% resolving gel were poured into the gel casting unit (1.5mm thick). 2mls of isopropanol were added to avoid air bubbles. Once the gel was set, excess isoproponal was drained and washed three times with distilled water. 2.5mls of stacking gel was poured into the gel casting unit and allowed to sit on top of the resolving gel. The gel combs were appropriately placed into the gel plates and the stacking gel was allowed to set. Once the gel was set, the comb was removed and gel casting unit was transferred into the electrophoresis. The samples and protein ladder (SeeBlue plus 2,Invitrogen) were loaded onto the gel. The gel was run in Tris-glyciene buffer at 80V.

Transfer

The transfer of proteins on the gel to a nitrocellulose membrane (Hybond ECL, Amersham) was carried out using a semi-dry transfer blotting unit (BioRad) in accordance with manufacturer's instructions. Briefly, two thick botting sheets (BioRad) were soaked in 200mls of transfer buffer containing 20% methanol. The thick blotting sheet was placed on a flat surface. The nitrocellulose membrane pre-soaked in transfer buffer was placed on top of the sheet ensuring that no air bubbles were trapped. The gel which was removed from the casting unit was placed on top of the nitrocellulose membrane. It was important to ensure that no air bubbles were trapped between the membrane and the gel. Another sheet pre-soaked in transfer buffer was placed on top the gel. This was then transferred to the blotting unit. The transfer took place at 25V for 45min.

Antibody staining of the membrane

Following transfer, the nitrocellulose membrane was stained with Ponceau which stains all proteins on the membrane. This was done to ensure efficient transfer of proteins to the nitrocellulose membrane. The membrane was washed with 1XPBS and then blocked using 5% skimmed milk (Marvel) for 30 min with agitation. The blocking solution was drained and membranes were incubated in primary antibody diluted in 5% milk or 3%BSA. Membranes were

incubated in primary antibody overnight at 4°C with gentle shaking. The table shows the concentration at which different primary antibodies were used.

Primary antibody	**Working concentration**
Polyclonal anti-EGFR produced in rabbit (recognises C-terminus of EGFR of human origin, purchased from Santa Cruz)	1:200
Sulf1antibody produced in rabbit (recognises exon6 and detects full length Sulf1)	1:3000
Sulf2antibody produced in rabbit (recognises exon6 and detects full length Sulf2)	1:3000
Monoclonal anti-GAPDH produced in mouse (purchased from Abcam)	1:2000

Table 2.7: List of primary antibodies used for western blotting.

Following incubation with primary antibody, the membranes were washed with 1X PBS three times for 10 min each with gentle agitation. The membranes were then incubated with secondary antibody diluted in 5% milk for 1 hr with agitation. The secondary antibodies used were:

Secondary antibody	**Working concentration**
Anti-mouse IgG HRP conjugate (Promega)	1:2000
Anti-rabbit IgG HRP conjugate (Promega)	1:2000
Anti-goat IgG HRP conjugate (Santa Cruz)	1:20,000

Table 3.8 : List of secondary antibodies used for western blotting.

Following incubation with the secondary antibody, the membranes were washed in 1XPBS three times for 10 minutes each.

Detection

Chemiluminescence was used to visualise protein bands. The nitrocellulose membrane was placed on a flat surface and excess PBS was drained. The membrane was then treated with ECL or SuperSignal ECL (Piercenet) for 1 min. Excess ECL was drained and place in a cassette. The protein bands were developed on an X-ray film (ThermoScientific) using an XO graph machine.

After detection of the protein of interest, membranes were re-probed by treatment with 1X re-blotting buffer (Pierce) for 5 minutes with agitation. The membranes were then blocked in 5% milk for 30 min and incubated with antibody usually GAPDH which served as the loading control.

2.4 Cloning of dominant negative ErbB1 (dn ErbB1) lentiviral vector

Cloning strategy

Mutant ErbB1 (K721A) which was inserted into pcDNA3.1 (dnErbB1-pcDNA3.1) was kindly given to us by Dr. Inger Helene Madchus, University of Oslo. The mutation in ErbB1involved replacement of the lysine residue at 721 with alanine which led to the inactivation of its kinase domain. DnErbB1 contained in pcDNA3.1 was restricted using the enzymes Hind III and Xba I. It was then sub-cloned into the commercially available plasmid, pmCherry-N1. The sub-clone, dnErbB1-pmCherry-N1 was restricted using the restriction enzymes, Hpa1 and Nhe 1. HRSX Lentiviral vector containing GMCSF served as the lentiviral backbone into which dnErbB1 fragment was ligated. Prior to ligation, GMCSF-HRSX was restricted using the enzymes, Spe I and Pme I. The reaction resulted in the separation of the GMCSF fragment from the rest of the backbone. dnErbB1 was then ligated into the lentiviral backbone.

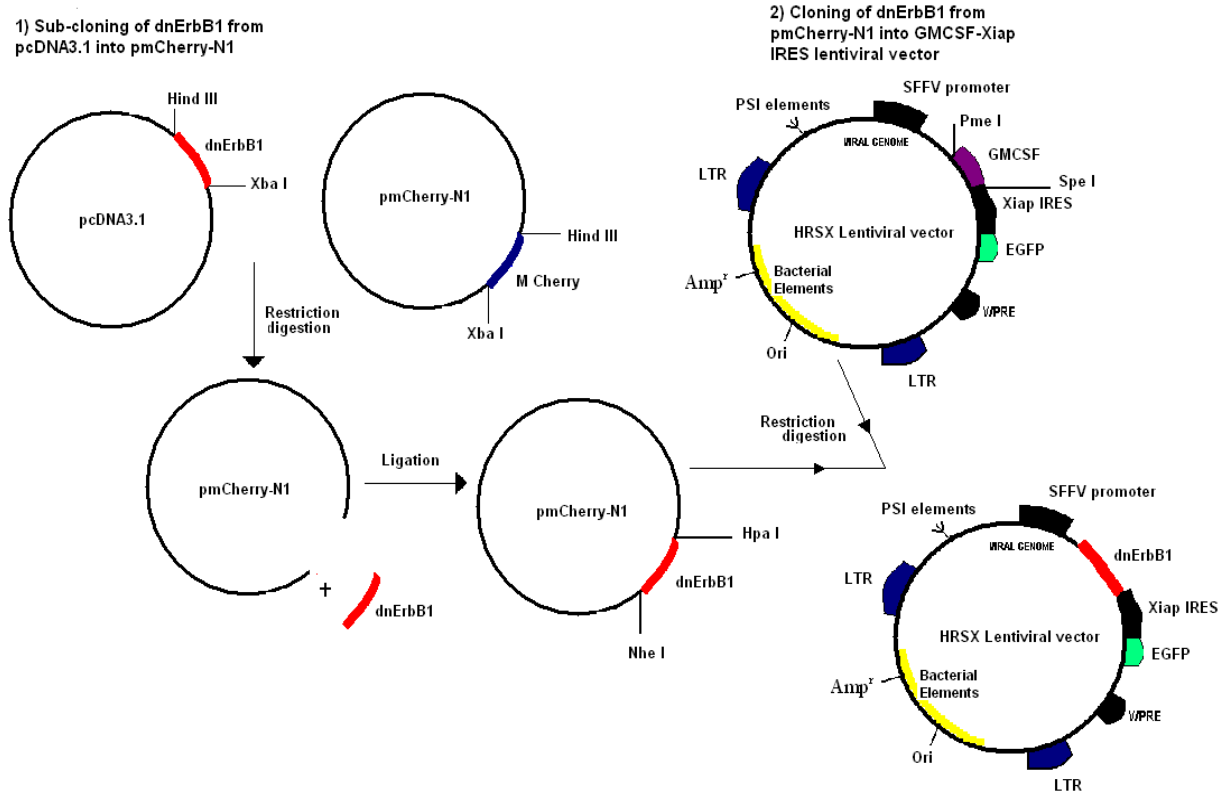

Figure 2.1: Cloning strategy. Diagram illustrates the cloning strategy used to clone dnErbB1 lentiviral vector.

The protocol for cloning dnErbB1 lentiviral vector consisted of the following steps:

1) Preparation of plasmids (Mini preps)
2) Digestion of plasmids with restriction enzymes
3) Analysis of fragments using gel electrophoresis
4) Gel elution of DNA from gel fragments
5) Ligation of restricted bands
6) Diagnostic digest to confirm orientation of the insert
7) Sequencing of plasmid DNA

8) Preparation of virus

Cloning of dnErbB1 fragment into pmCherry-N1 vector

Preparation of plasmids:

Transformation

Competent cells (*E.coli* , Mach1-Invitrogen) were transformed with plasmid DNA (dnErbB1-pcDNA3.1). 2µl of dnErbB1-pcDNA3.1 was added to 25 µl of competent cells placed on ice. The tube containing competent cells and plasmid DNA were incubated at 42°C for 45 sec followed by incubation in ice for 2 min. Cells were then incubated with 700µl of LB medium for 1 hour in a shaker incubator (to recover from heat shock). After incubation, cells were centrifuged at 5000rpm for 3 min and the pellet was resuspended in 100µl of LB media. The cell suspension was then streaked on agar plates that contained ampicillin and incubated overnight at 37°C.

Harvesting and extraction of plasmid DNA

The following day various colonies were picked using the ends of micropipette tips. 5 mls of LB media containing ampicillin were inoculated and cultures were incubated for 16-18 hours with rigorous shaking at 37°C. 1.5mls of each of the cultures were added to microfuge tubes and centrifuged at 8000rpm for 3 min. DNA was extracted from the cell pellet by using QIAprep spin Miniprep kit (Qiagen) in accordance with manufacturer's instructions. DNA was eluted in 50µl of distilled water and the concentration was measured using a nanodrop.

Restriction digestion of dnErbB1 and pmCherry-N1:

(All restriction enzymes and buffers were purchased from New England Biolabs)

pmCherry -N1 and dnErbB1-pcDNA3.1 were restricted using restriction enzymes HindIII and XbaI. The reaction was as follows:

DNA 2µl

HindIII	1.3µl
XbaI	1.3µl
BSA (1X)	3µl
Buffer 2	3µl
Water	19.4µl
Total volume	30µl

The mixture was incubated in a water bath for 30 min. The digests were then electrophoresed on a 2% agarose gel.

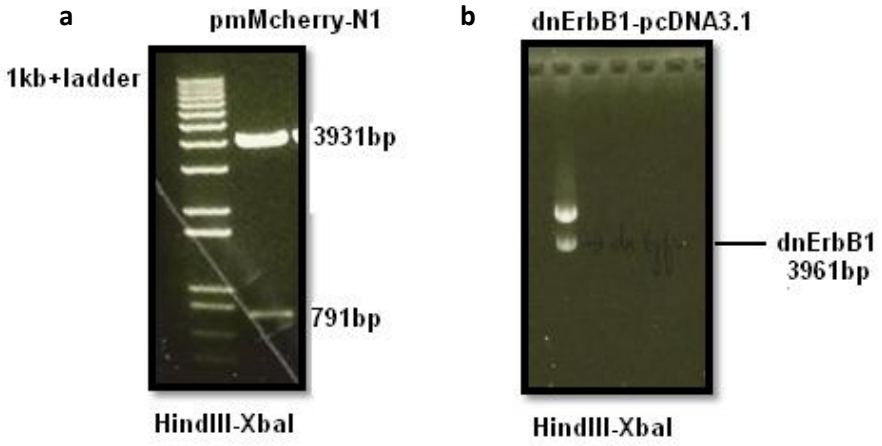

Figure 2.2: Restriction digestion of pmCherry-N1 and dnErbB1. Images of gels showing restriction products of a) pmCherry-N1 plasmid b) dnErbB1-pcDNA3.1 plasmid digested with enzymes Hind III and Xba I.

Gel elution:

The desired bands on the agarose gel were excised using a clean scalpel. The gel slice was weighed and 3 volumes of diffusion buffer (Qiagen- QIAquick gel extraction kit) to 1 volume of gel was added (i.e, if the weight of the gel was 150 mg, the volume of buffer added was 450µl). It was then heated to 50°C for 10 min and was vortexed every 2-3 minutes until the gel dissolved. 1 volume of isoprpanol (150µl) was added to the sample and mixed. DNA was

then extracted from the sample using QIAquick gel extraction kit (Qiagen) in accordance with manufacturer's instructions.

Ligation:

dnErbB1 was inserted into pmCherry-N1 by means of ligation. The enzyme and its buffer were purchased from New England Biolabs. The reaction was as follows:

2X Quick ligase reaction buffer	10µl
dnErbB1	7.5µl
pm-Cherry-N1 (Vector)	2.5µl
Quick ligase	1µl

The reaction mixture was incubated 10 min at room temperature.

A separate reaction was set up, where the addition of insert (dnErbB1) was replaced by water. This reaction served as control.

2X Quick ligase reaction buffer	10µl
Water	7.5µl
pm-Cherry-N1 (Vector)	2.5µl
Quick ligase	1µl

Transformation and plasmid preparation:

Following ligation, 5µl of DNA was used to transform 25µl of competent cells using the protocol as described earlier. The transformed cells were recovered from heat shock by treatment with 700µl of LB media for 1 hour at 37°C with rigorous shaking. The cell suspension was then centrifuged at 8000 rpm for 10 min. The cell pellet was resuspended in 100µl of LB media. Cells from the ligation reaction as well the control reaction were streaked onto separate agar plates containing kanamycin. Plates were incubated at 37°C for 24 hrs. The following day, both plates were checked for colonies. DNA from a successful ligation reaction would produce more colonies when compared to colonies on a control plate as competent cells preferentially uptake

circular DNA as opposed to linearised DNA. Colonies were picked and cultures inoculated. Plasmids were prepped as described previously using Qiagen spin Miniprep kit.

Cloning of dnErbB1 into HRSX lentiviral vector

Restriction digestion of dnErbB1-pmcherry-N1 and the lentiviral vector- GMCSF-Xiap-IRES

The reactions set up were as follows:

Recation 1

GMCSF-Xiap-IRES	5µl
PmeI	1.2µl
SpeI	1.2µl
BSA (1X)	3µl
Buffer 4	3µl
Water	16.6µl
Total volume	30µl

Reaction 2

dnErbB1-pmCherry-N1	5µl
Hpa1	1.2µl
NheI	1.2µl
BSA (1X)	3µl
Buffer 4	3µl
Water	16.6µl
Total volume	30µl

The reaction mixtures were incubated in a water bath for 30 min in a water bath. The digests were then electrophoresed on a 2%agarose gel.

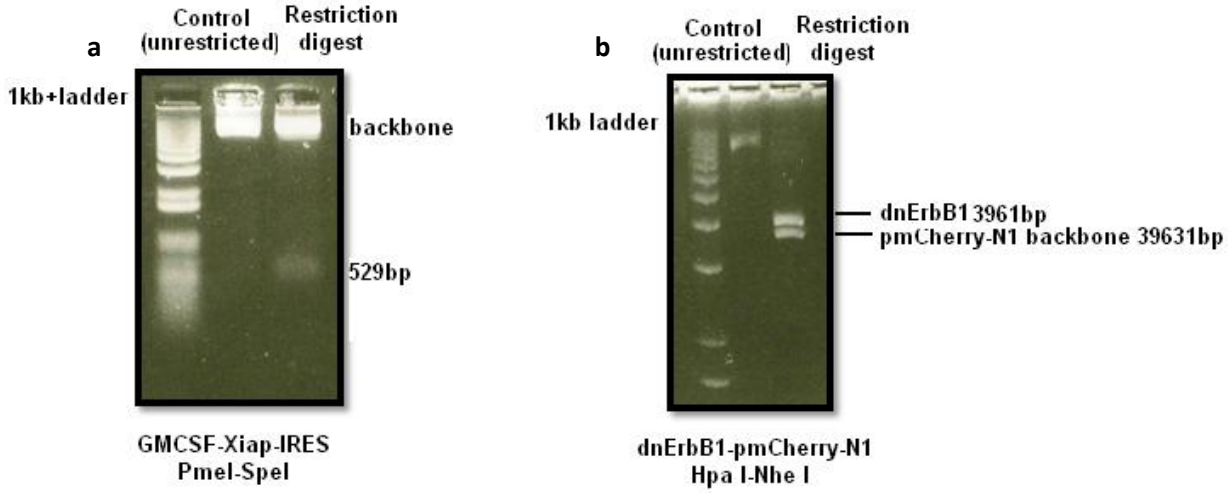

Figure 2.3 : Restriction digestion of HRSX lentiviral vector and dnErbB1-pmCherry-N1. a) HRSX lentiviral vector containing GMCSF-Xiap-IRES was digested with enzmes Pme1 and Spe1. GMCSF (529BP) is excised from the lentiviral backbone. b) dnErbB1 is restricted from its pmCherry-N1 backbone by enzymes Hpa1 and Nhe 1. Control refers to undigested DNA.

Gel elution:

The desired bands were excised and DNA was eluted using QIAquick gel extraction kit as discussed previously.

Ligation:

dnErbB1 was ligated into the lentiviral vector using the enzyme Quick ligase (New England Biolabs). 4 diffrent reactions were set up of which 2 of the reactions served as controls

Reaction 1

2x Quick ligase buffer	10µl
dnErbB1	7µl
GMCSF –Xiap-IRES (Vector)	3µl
Quick ligase	1µl

Control reaction:

2x Quick ligase buffer	10µl
Water	7µl
GMCSF –Xiap-IRES (Vector)	3µl
Quick ligase	1µl

Reaction 2

2x Quick ligase buffer	10µl
dnErbB1	9µl
GMCSF –Xiap-IRES (Vector)	2µl
Quick ligase	1µl

Control reaction:

2x Quick ligase buffer	10µl
Water	9µl
GMCSF –Xiap-IRES (Vector)	2µl
Quick ligase	1µl

The reaction mixtures were incubated for 10 min at room temperature. As previously described, competent cells were transformed with the ligation products and bacteria were streaked on ampicillin resistance plates. The following day at least 10 colonies were picked and plasmids prepped using Qiagen spin Miniprep kit.

Diagnostic digests to confirm presence and orientation of insert

a) Diagnostic digest using Sal1:

Sal1 does not have a site in dnErbB1 but restricts the lentiviral backbone at 9008 bp. GMCSF-Xiap-IRES and the ligation product- dnErbB1-Xiap-IRES were restricted by the following reaction:

dnErbB1-Xiap-IRES/ GMCSF-Xiap-IRES	2µl
Sal1	1.3µl
BSA (1X)	3µl
Buffer 3	3µl
Total volume	30µl

The reaction mixtures were incubated at 37°C in a water bath for 30 min. The digests were electrophoresed on a 2% agarose gel.

Figure 2.4: Restriction digestion of various dnErbB1 lentiviral clones with Sal 1. Gel image shows digests of samples that previously underwent two types of ligation reactions. Sal1 restricts the lentiviral backbone but does not restrict dnErbB1. Hence successfully ligated samples will produce two bands when restricted with Sal 1. It can be inferred that samples 2 and 4 (bottom half) from reaction 2 have been successfully ligated.

b)Restriction digestion using enzymes EcoR1 and Not1

EcoR1 has 3 sites in ErbB1 and Not1 has a site after egfp in GMCSF-Xiap-IRES. The reaction was as follows:

dnErbB1-Xiap-IRES/ GMCSF-Xiap-IRES	2µl
EcoR1	1.3µl
Not1	1.3µl
BSA (1X)	3µl
Buffer 3	3µl
Water	19.4µl
Total volume	30µl

The reaction mixtures were incubated at 37°C in a water bath for 30 min. The digests were electrophoresed on a 2% agarose gel.

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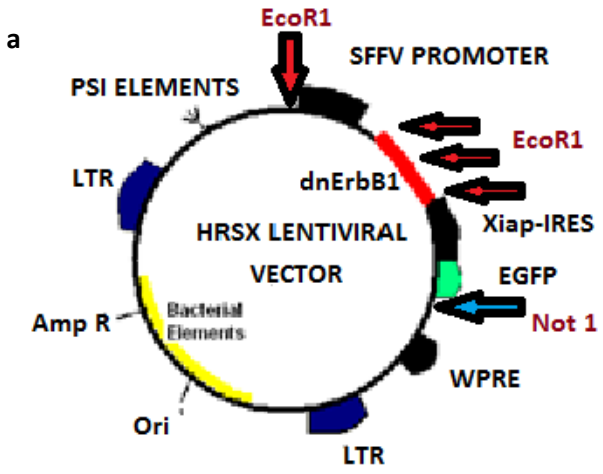
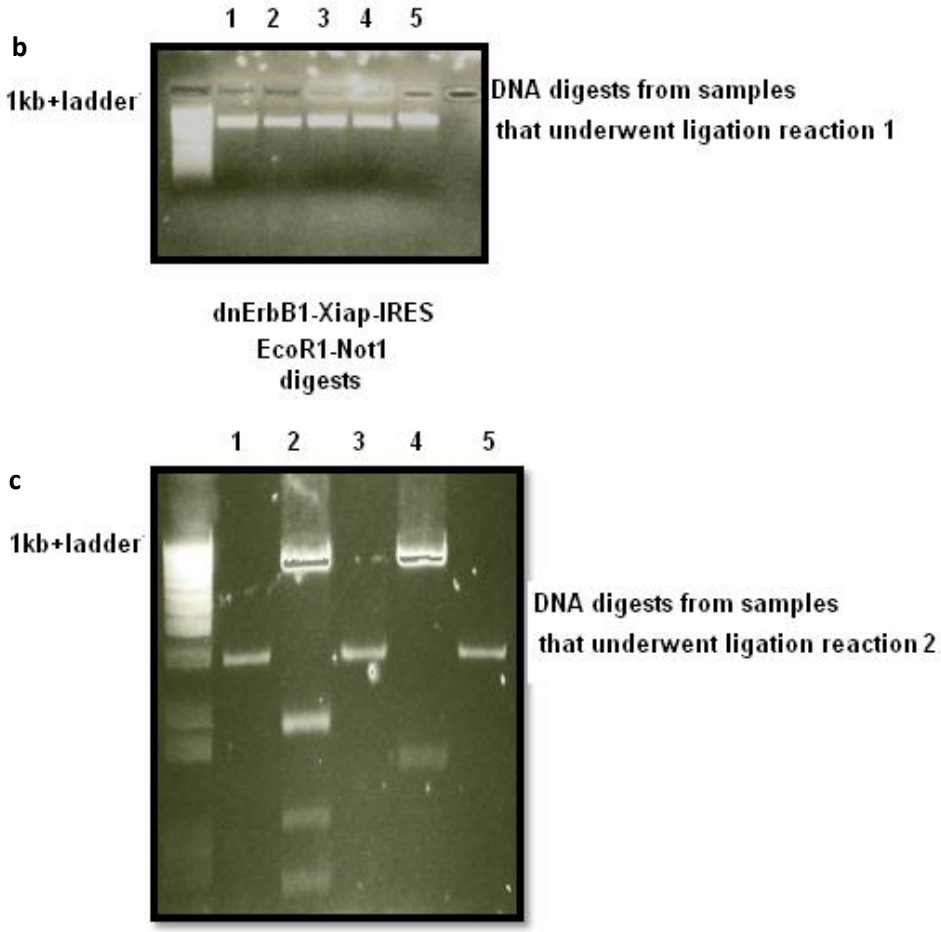


Figure 2.5: Diagonostic digests with EcoR1 and Not1. a)Diagram illustrating restriction sites on dnErbB1 lentiviral vector. EcoR1 has 3 restriction sites within dnErbB1 and one site before the promoter. Not 1 has one site after EGFP. Hence, using EcoR1-Not1, the orientation of the insert can be assessed. Gel images of b) samples from ligation reaction1 which are not restricted by EcoR1-Not1 indicating unsuccessful ligation and cloning c) Sample 2 from from reaction 2 produces expected bands indicating successful cloning.



Sequencing

Following diagnostic digests, successful clones were sequenced at the Wolfson Institute of Biomedical Research, UCL. Primers that primed various regions within ErbB1 were used for sequencing. Following were the primers used:

5'-AGCAGGTCCTCCATCCCAACAGC

5'-GTGGTGGATGCCGACGAGTACCT

5'-CCACTCATGCTCTACAACCCCAC

5'-ACGGATGCACTGGGCCAGGTCTT

Preparation of Lentivirus

293T cells grown at 60%-80% confluency were transfected with the following plasmids:

Packaging plasmid-non-integrating (pCMVdR8.74 D64V)

Envelope plasmid (pMD.G2)

Vector construct (dnErbB1-Xiap-IRES EGFP)

Cells were transfected with transfection reagent PEI (Sigma). PEI was diluted in serum-free OPTI MEM media and used at working concentration of 2µM in. The following quantities of constructs were prepared in serum-free OPTI MEM media:

Packaging plasmid- 32.5µg

Envelope plasmid- 17.5µg

Vector construct- 50µg

1 volume of PEI was added to 1 volume of media containing plasmids. The DNA-PEI mixture was filter sterlised using 0.22µm filters and incubated at room temperature for 20min. 10 mls of the DNA-PEI mixture was added to one flask of 293T cells and grown at 37°C. After 4 hours the media was changed and fresh growth media added. The cells were allowed to grow for 3 days. The viral supernatant was collected and filtered through a 0.22µm filter. This was followed by ultracentrifugation at 4°C for 90min.The pellet was collected and resuspended in growth media. Aliquots were prepared and stored at -80°C.

2.5 *In vivo* experiments

Surgery

All surgical procedures were carried out by Prof. Patrick Anderson. Adult Sprague-Dawley rats were used for surgery. Various injury models were used to analyse the expression of Sulf1 and Sulf2 and to assess the effects of blocking ErbB1or PTEN *in vivo*. These include:

1) The optic nerve crush in adult rats which were either treated with the ErbB1orPTEN antagonist. Regeneration was assessed 14 days after surgery.

2) The sciatic nerve transection in adult rats to analyse changes in expression of ErbB1 after 7 days after axotomy.

Prior to surgery, the animal was anaesthetised with a mixture of halothane, nitrous oxide and oxygen. Hair was shaved in the region to be operated and the skin was sterilised by wiping with alcohol. The animal was placed on a heating pad throughout surgery. After surgery, the animals were given injections of analgesic and antibiotic.

Optic nerve crush as an injury model to assess the effects of blocking ErbB1 or PTEN on regeneration:

A midline incision to the scalp was made and the skin on either side was pulled aside. An incision was made along the orbital rim. The Harderian gland was moved to the side and the levator palpebrae and superior rectus muscles incised. The retractor bulbii muscle was opened. Using a pair of spring-bow scissors, an incision was made to the dura surrounding the optic nerve. The dura was retracted and the optic nerve was crushed using a fine pair of watchmakers' forceps. Care was taken to avoid injury to the ophthalmic artery and vein.

Treatment with drugs

PD168393:

A strip of gel foam was soaked in PD168393 (10µM) and placed adjacent to the area of crush. In the case of a control animal, the gel foam was soaked in vehicle (0.1% DMSO).

VO-OHpic (PTEN antagonist)

A strip of gel foam was soaked in VO-OHpic (1µM) and placed in the region of the crush. In the case of a control animal, the gel foam was soaked in sterile distilled water.

Following treatment with drugs, the incised skin was sutured using sterile silk. The animals were given sub-cutaneous injections of analgesic and antibiotic.

Eyeball injections

6 µl of PD168393 (10µM) or VO-OHpic (1µM) was injected into the eyeball using a Hamilton syringe. Control animals received injections with 6µl of either 0.1%PD168393 or

sterile distilled water. A second round of injections was given 7 days post surgery. Treatment paradigm has been summarised below:

DAY	EXPERIMENTAL PROCEDURE	ANIMAL	
		EXPERIMENT (n=2)	CONTROL (n=2)
DAY 1	OPTIC NERVE CRUSH	1) Gel foam soaked in PD168393 (10µm) or VO-OHpic (1µl) placed at crush site. 2) 6µl of PD168393 or VO-OHpic injected into eyeball.	1) Gel foam soaked in 0.1% DMSO or water placed at crush site. 2) 6µl of 0.1%DMSO or water injected into eyeball.
DAY 7	EYE BALL INJECTIONS	6µl of PD168393 or VO-OHpic injected into eyeball.	6µl of 0.1%DMSO or water injected into eyeball.
DAY 14	PERFUSION	Animals sacrificed.	

Table 2.9 : Treatment paradigm of adult rats that received optic nerve injuries.

Sciatic nerve transection

The right hindquarter of the animal was shaved and the skin was incised parallel to the femur. The muscle was pulled apart to expose the sciatic nerve. The sciatic nerve was transected using a fine pair of scissors and 3mm of nerve was removed. The skin was then sutured. The animal was killed 7 days after surgery. The sciatic nerve was transected only in the experimental group (n=2) whereas the control group (n=2) did not receive any surgical intervention.

Spinal cord section

The animal was anaesthetized. After laminectomy at C3 level, the dura mater was opened longitudinally and the left side of the spinal cord, including the left dorsal horn and the left lateral funiculus, was cut using microsurgical scissors(n=2). Control animals (n=2) did not have surgical intervention. The experimental animals were killed 7 days post surgery.

Viral injections into the red nucleus

Adult Sprague-Dawley rats received an injection of 4µl of a 50% mixture of the lentivirus and the axonal tracer BDA (Molecular Probes, Oregon, USA; 10% solution in dissolved in 0.1 M PBS) into the right red nucleus. The lentivirus vectors carried with dominant-negative ErbB1-IRES-EGFP or EGFP.

Immunohistochemistry

Transcardial Perfusion

After the stipulated time period of the experiment, animals were perfused transcardially with 2% fixative. 2% Paraformaldehyde which served as fixative was made as described previously. The animal was killed with an overdose of Halothane and was placed on a dissection tray. An incision was made to the lower abdomen and cut along the midline up to the diaphragm. The ribs were cut on either side exposing the chest cavity. The tip of the right atrium was cut to allow the blood to exit. The needle of the perfusion pump was then inserted into the left ventricle and the animal was initially perfused with 200 mls of 0.1M PBS followed by perfusion with 400mls of 2% paraformaldehyde.

Post-fixation

Brain, optic nerve , spinal cord, DRGs and sciatic nerve were dissected and immersed in 10mls of 2% fixative for 1hr. Tissue were then transferred to 10 mls of 30% sucrose in 0.1MPBS and stored at 4°C overnight.

Tissue embedding and sectioning

The tissue was embedded in Tissue-Tek (Sakura) placed in plastic moulds of appropriate sizes. These were then stored at -20°C. When required, 12µm thick sections of tissue were cut using a cryostat and sections were adhered to glass slides that were subbed with chromium potassium sulphate and gelatin . The sections were allowed to dry and was then stored at -20°C.

To obtain free-floating sections, tissue were not embedded, instead they were frozen in dry ice. Freezing microtome sections were cut at 40µm. Sections were collected in 0.1M PBS in 24-well plates.

Antibody staining

Blocking to prevent non-specific binding

The sections were treated with blocking solution. The components of blocking solution included 1% BSA, 0.1M PBS, 0.05% Tween-20, 0.01% Triton-X and 5% goat serum. 600µl of blocking solution were added to the slides and incubated for 1 hr at room temperature. Free floating sections were incubated with 500µl of blocking solution at room temperature for 1 hr with agitation.

Primary antibodies

Primary antibodies were diluted in blocking solution. Concentrations of various primary antibodies used are given below.

Primary antibody	**Working concentration**
Monoclonal anti-GAP-43 produced in mouse (generously provided by D.Schreyer Saskatoon, Canada)	1:400
Polyclonal anti-GFAP produced in rabbit (isolated from bovine spinal cord and recognises rat, mouse, sheep, cow and dog GFAP, purchased from DAKO).	1:500
Monoclonal anti-S100 produced in mouse (purchased from Sigma)	1:1000
Monoclonal anti-neurofilament produced in mouse (recognises medium and heavy neurofilament - molecular weight:160kDa and 200KDa of mouse, rat and human, purchased from Sigma).	1:1000
Monoclonal anti-Nogo A produced in mouse (generously provided by RK Prinjha, GSK)	1:400
Polyclonal anti-EGFR produced in rabbit (recognises C-terminus of EGFR of human origin, purchased from Santa Cruz)	1:50

Monoclonl anti- phospho EGFR produced in rabbit (recognises EGFR when phosphorylated at Tyr 1173, purchased from Cell Signalling)	
Sulf1antibody produced in rabbit (recognises exon6 and detects full length Sulf1)	1:200
Sulf2antibody produced in rabbit (recognises exon6 and detects full length Sulf2)	1:200
Anti- Sulf1isoform produced in rabbit(recognises exon 20/21 and detects all sulf1 isoforms)	1:100
Anti- Sulf2 isoform produced in rabbit (recognises exon 23 and detects all sulf2 isoforms)	1:100

Table 2.10: List of primary antibodies and the concentrations at which they were used to immunostain tissue.
Note: Antibodies against Sulfs were kindly donated by Dr. Gurtej Dhoot, Royal Veterinary College, London.

Sections were incubated in 400µl of antibody overnight at 4°C. Free floating sections were incubated with 250µl for 1 hr at room temperature with agitation followed by incubation at 4°C overnight.

Secondary antibodies

After incubation with primary antibody, sections were washed with 0.1M PBS three times, each wash lasting 5 minutes. 400µl of secondary antibody and Hoechst stain diluted in blocking solution were added to slides. The sections were then incubated at room temperature for 2 hrs. Free floating sections were incubated at room temperature in 250 µl of antibody with agitation. The concentrations of secondary antibodies used are given below.

Anti-rabbit IgG-Atto 594 produced in chicken (Sigma)	1:600
Anti-rabbit IgG-Atto 488 produced in goat (Sigma)	1:600
Anti-rabbit IgG-Alexa fluor 488 produced in goat (Life technologies)	1:600
Anti-mouse IgG-Alexa fluor 594 produced in goat (Life technologies)	1:400

Table 2.11: List of secondary antibodies and the concentrations at which they were used to immunostain tissue.

Mounting of sections

Following incubation in secondary antibody, sections were washed three times with 0.1M PBS for 5 min. each. Free floating sections were mounted onto a microscope glass slide and sections were allowed to dry. Sections were mounted in mounting media (PBS, glycerol, DABCO) and stored at 4°C.

Microscopy

Photomicrographs of sections were captured using a Zeiss Axiophot microscope, Hamamatsu C4742 camera and Improvision software.

Chapter-3 Role of ErbB1 in limiting neurite outgrowth *in vitro*

Introduction

ErbB1 may play an important role in regulating axonal regeneration. In some studies, stimuli that increase ErbB1 phosphorylation have been shown to promote axon outgrowth (Evangelopoulos et al., 2009;Tsai et al., 2010b;Goldshmit et al., 2004). However, other studies indicate that ErbB1 may be a critical mediator of outgrowth-inhibitory cues in the adult CNS. Inhibitors of ErbB1 kinase activity allow axons to grow over an inhibitory substrate of CNS myelin or CSPG *in vitro* and through a crush injury of the optic nerve *in vivo* (Koprivica et al., 2005). In contrast, experiments using siRNA to knock down ErbB1 expression *in vitro* have yielded results inconsistent with the hypothesis that blocking ErbB1 improves axonal regeneration. Cultures in which ErbB1 expression had been dramatically reduced by treatment with siRNA showed undiminished inhibition of axon outgrowth by CNS myelin, and the ErbB1 kinase inhibitor, AG1478 retained its ability to rescue axon outgrowth. On the basis of this and other evidence it was suggested that AG1478 exerted its axon-promoting effect through an action on a protein other than ErbB1 (Ahmed et al., 2009 and Douglas et al., 2009). However, siRNA rarely eliminates the target protein completely. We therefore re-examined this question by using neurons from ErbB1 knockout mice in which the protein is completely absent.

The first stage was to confirm if the pharmacological blockers improve neurite outgrowth on CNS myelin and then use neurons from ErbB1 knockout mice which completely lack the protein in order to test the hypothesis that ErbB1 is involved in inhibition of neurite outgrowth *in vitro*.

Methods

For a detailed account of the methods, please refer Chapter-2.

Briefly, dorsal root ganglia neurons from ErbB1-/- and wildtype/ heterozygous mice aged between P7-P9 were cultured on a poly-l-lysine/laminin substrate in 8-well glass

chambered slides. To test the effects of CNS myelin on neurite outgrowth, DRG cells were plated on substrate coated with CNS myelin for a period 4 hours. The ErbB1 antagonist, PD168393 (Calbiochem) used at a concentration of 10nM or 100nM or 0.1% DMSO (vehicle control) were added to the cells at the time of plating. Cells were incubated at 37°C for 24 or 48 hours. Following the culture period, cells were fixed in 4% paraformaldehyde and immunostained with βIII tubulin. Photomicrographs of βIII tubulin-positive neurons cultured under various conditions were captured and neurite length was measured using the software Image J. Experiments were repeated from 5-6 different animals. Statistical significance was computed using Student's t test.

Results

CNS myelin acts as an inhibitory substrate for neurite outgrowth.

It has been known for a long time that CNS myelin inhibits neurite outgrowth *in vitro* (Schwab and Caroni, 1988). Initial experiments confirmed that CNS myelin is a strong inhibitor of neurite outgrowth. When DRG neurons from wild type (WT) mice were plated on a myelin substrate they grew shorter neurites than neurons plated on a control substrate (Figure 3.1).

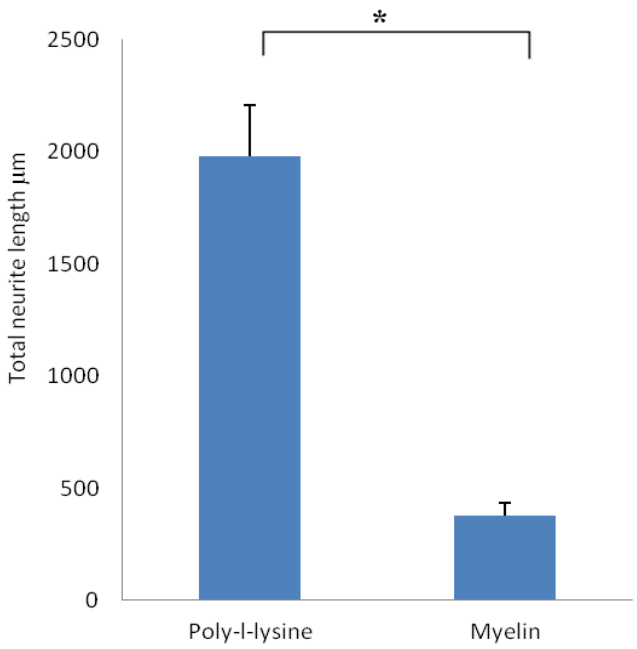


Figure 3.1: CNS myelin inhibits neurite outgrowth. DRG neurons from 7-11 day old wildtype mice were cultured for 48hrs on Poly-l-lysine and laminin alone or with additional CNS myelin. The mean neurite length for cells grown on myelin was significantly shorter. (*$p<0.001$ Student's t test All error bars are SEM, n=5 experiments).

Addition of PD168393 improves neurite outgrowth on CNS myelin substrate.

The pharmacological inhibition of ErbB1 using an irreversible inhibitor, PD168393, did not affect neurite outgrowth of ErbB1-/- DRG neurons or cells from littermate controls grown on poly-l-lysine and laminin (i.e. in the absence of inhibitors of axonal growth; Figure 3.2). However, PD168393 increased neurite outgrowth of ErbB1 wildtype/heterozygous neurons plated on a myelin substrate (Figure 3.3). These results are consistent with previous studies (Koprivica et al., 2005;Ahmed et al., 2009;Kaneko et al., 2007) and are compatible with the hypothesis that ErbB1 or another molecule targeted by PD168393 is part of the signalling pathway from myelin molecules leading to inhibition of neurite outgrowth. There was no evidence that PD168393 is a general promoter of neurite outgrowth.

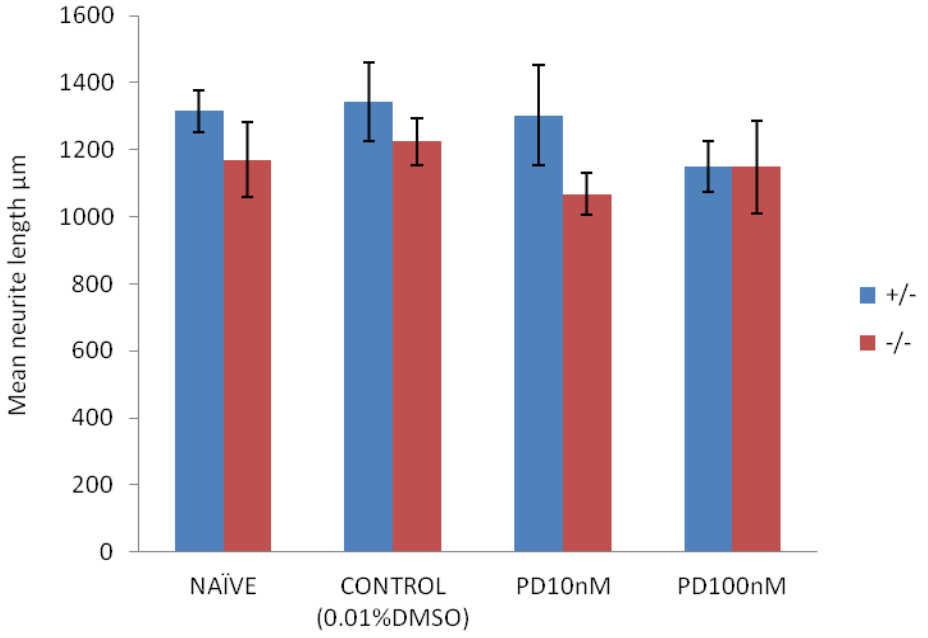


Figure 3.2: PD168393 has little effect on DRG neurons grown on non-inhibitory substrates. There were no statistically significant differences (n=3).

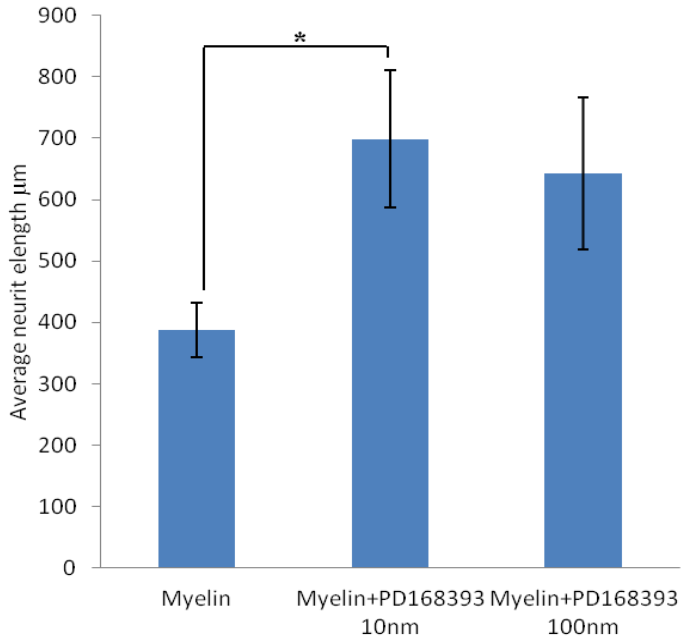


Figure 3.3: DRG neurons from +/+ mice were cultured for 48hrs on myelin. PD168393 enhanced neurite outgrowth (*p<0.05 compared to neurite outgrowth on myelin alone, Student's t test, n=5).

PD168393 does not have an effect on cell adhesion.

CNS myelin is not only a strong inhibitor of neurite outgrowth but also acts as a non-permissive substrate for adhesion of neurons and other cells. The ErbB1 blocker PD168393, although it improved neurite outgrowth on myelin, did not increase the number of neurons adhering to a substrate coated with CNS myelin (Figure 3.4).

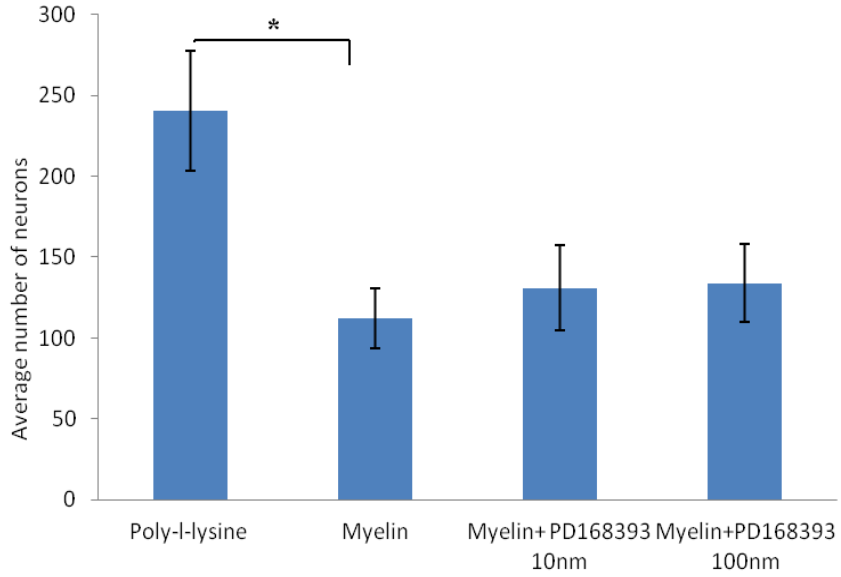


Figure 3.4: CNS myelin acts as non-permissive substrate for cell adhesion through a mechanism independent of ErbB1. PD168393 did not increase the number of wildtype DRG neurons adhering to the substrate after 48hrs culture on CNS myelin. ($p<0.05$, Student's t test, n=6).

ErbB1 -/- neurons are less inhibited by CNS myelin substrate.

ErbB1 -/- DRG neurons cultured on CNS myelin showed a two fold increase in neurite length when compared to wild type neurons (Figure 3.5). This supports the hypothesis that ErbB1 plays a role in the signalling from inhibitory molecules in CNS myelin. However ErbB1-/- non-neuronal cells are also present in the cultures and could be supporting neurite growth.

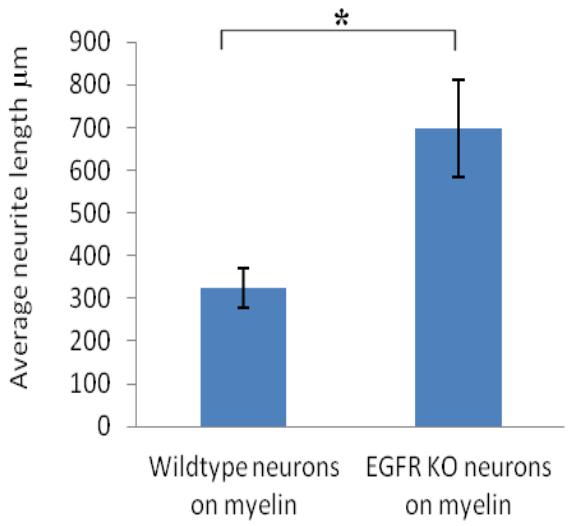


Figure 3.5: DRG neurons from ErbB1 KO mice, cultured for 48hrs. on CNS myelin, grew longer neurites than neurons from wild type /heterozygous mice ($p<0.01$, Student's t test, n=5).

Enhanced neurite outgrowth following pharmacological inhibition of ErbB1 is not the result of off- target interactions.

The addition of PD168393 improved neurite outgrowth from wildtype/heterozygous neurons on myelin but did not improve neurite outgrowth from ErbB1 -/- neurons (Figure 3.5). This is not compatible with the hypothesis (Ahmed et al., 2009a) that the ErbB1 blocker PD168393 acts off-target. However, this experiment does not prove that PD168393 acts only on neurons.

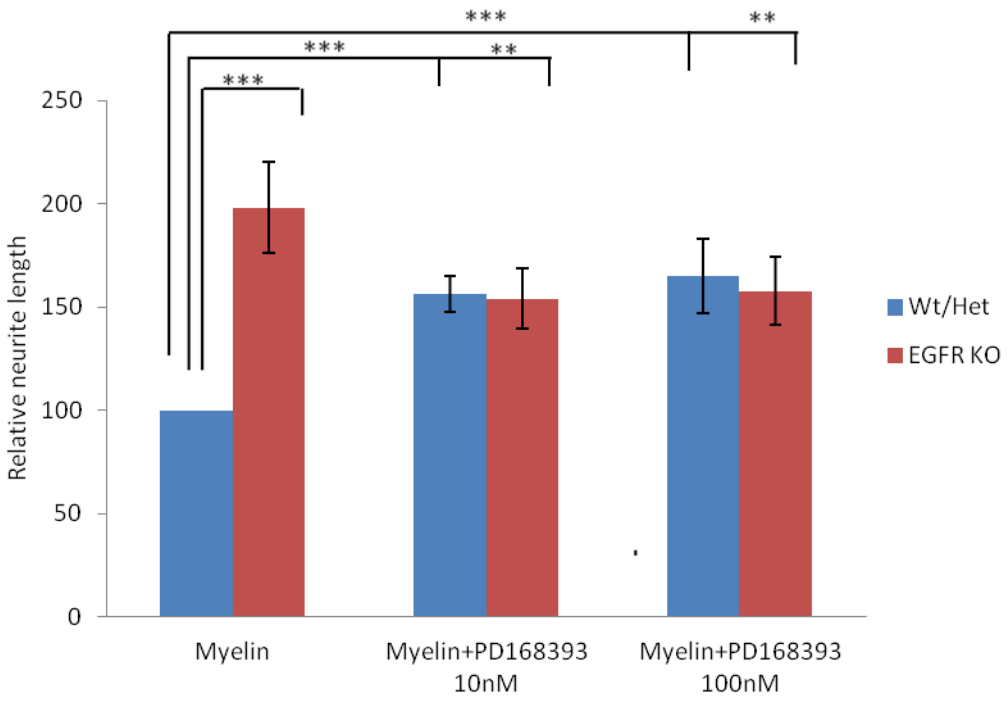


Figure 3.6: PD168393 increased neurite outgrowth from wildtype/heterozygous neurons on CNS myelin but not from ErbB1 KO neurons. DRG neurons were cultured for 48hrs. Neurite outgrowth was normalised to that of wildtype/heterozygous neurons on CNS myelin (Moreau-Fauvarque et al., 2003) (**p<0.01 compared to neurite length on myelin,***p<0.001 compared to neurite length on myelin, Student's t test, n=7).

Addition of PD168393 improves neurite outgrowth from DRG neurons in the presence of CSPGs:

Apart from CNS myelin, chondroitin sulphate proteoglycans (CSPGs) are known inhibitors of axonal regeneration. When CSPGs were added to DRG cultures at a concentration of 0.5µg/ml, neurite outgrowth was significantly inhibited (Figure 3.6). Neurite outgrowth was diminished by approximately 50% when compared to control. However, the addition of the ErbB1 blocker, PD168393 at 100nM significantly improves neurite outgrowth in the presence of

CSPGs (Figure 3.6). It is to be noted that the addition of PD168393 did not produce any effects on neurite outgrowth from DRG neurons grown in the absence of CSPGs.

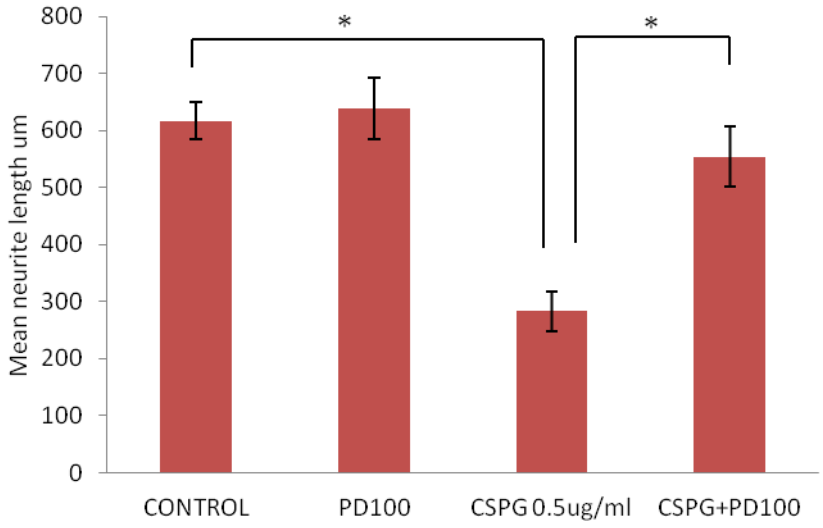


Figure 3.7: CSPG inhibits neurite outgrowth from DRG neurons grown for 24hrs. PD168393 (100nM) improves neurite outgrowth in the presence of CSPGs. *p<0.005 when compared to neurite outhgrowth from DRG neurons in the presence of CSPGs; Student's t test, n=5.

Discussion

The effects of myelin-derived inhibitors in axonal regeneration have been widely studied but the mechanisms through which these inhibitors signal are still unclear even though a number of signalling pathways have been proposed. This study has shown that an inhibitor of ErbB1, PD 168393 and genetic deletion of ErbB1 both increase neurite outgrowth from DRG neurons in the presence of CNS myelin. PD 168393 does not improve adhesion of neurons on myelin substrate, probably indicating that different mechanisms are involved in this process and that a reduction in cell adhesion is not the principle way in which myelin reduces neurite outgrowth.

Multiple signalling pathways are involved in the inhibition of axonal growth by CNS myelin and CSPGs.

Neither elimination of the ErbB1 gene nor the inhibition of its product by PD168393 completely overcomes the inhibition of neurite outgrowth by CNS myelin. My finding that PD168393 does not fully rescue neurite outgrowth on myelin seems to contradict some previous observations. Koprivica et.al, (2005) showed almost complete rescue of neurite outgrowth from cerebellar granule cells and DRG neurons on myelin by ErbB1 inhibitors and (Ahmed et al., 2009a) reported that adult DRG neurons treated with PD168393 grew longer neurites on myelin than untreated cells on a non-inhibitory substrate. This could theoretically have been because we were less successful in inhibiting ErbB1 but experiments on cerebellar granule cells in our laboratory (Leinster et.al., 2012) showed that the concentrations of PD168393 used in the present study effectively blocked ErbB1 activation. In the present study the effects of PD168393 and of the absence of ErbB1 were comparable. Neuronal ErbB1 signalling is not required for all of the inhibitory effects of myelin on neurite growth in our laboratory. It should be noted, however, that cells of different ages and slightly different culture conditions were used in the experiments by Koprivica et al. (2005) and Ahmed et al. (2009).

In the case of CNS myelin, it is not surprising that PD168393 failed to completely rescue neurite outgrowth in my experiments since many molecules capable of inhibiting neurite outgrowth or repelling neurites have been identified on CNS myelin (reviewed by Anderson et al., 2007). For some of these molecules expressed on CNS myelin e.g. Sema4D, Ephrin B3, Netrin1, there has been no suggestion as yet that they exert their effects via ErbB1. In addition, there are multiple receptors for some inhibitors of axonal regeneration and it is likely that not all the receptors for the known inhibitors present on myelin have been identified. These multiple receptors may signal through different pathways, some of which presumably do not involve ErbB1. None the less, the inhibition of ErbB1 or its genetic deletion reduces the ability of a number of molecules, including those present on CNS myelin, CSPGs and Poly I:C (Chapter-4) to inhibit neurite outgrowth. The ability to attenuate signalling from all these molecules may prove useful in encouraging axonal regeneration *in vivo*.

Pharmacological inhibitors of ErbB1 do not improve axonal regeneration by acting off-target.

The proposal that ErbB1 blockers act off -target to ErbB1 (Ahmed et al., 2009;Ahmed et al., 2010;Douglas et al., 2009), was addressed in this study. We investigated the effects of myelin on genetically deleted ErbB1 neurons. Some of the most important findings in our study were related to the effects of PD168393 on -/- and control cells. The effects of genetic deletion of ErbB1 on neurite outgrowth on CNS myelin were similar to the results obtained with the ErbB1 blocker i.e. ErbB1-/- neurons were less inhibited by CNS myelin. Of particular significance is the observation that the addition of PD168393 to ErbB1-/- neurons did not lead to a further increase in neurite outgrowth, thus proving that ErbB1 blockers do not act off-target to increase neurite outgrowth. Although Ahmed et.al. 2009 showed that neurite outgrowth was still inhibited by myelin when there was a 90% knock down of ErbB1 with siRNA, it is possible that residual ErbB1 activity was sufficient for signalling from CNS myelin inhibitory molecules. There was a slight, not statistically significant, decrease in neurite length of ErbB1 -/- neurons in the presence of PD168393 (Figure 3.5). If this should eventually prove a real effect, it will suggest that any off-target effects of PD168393 are to inhibit neurite outgrowth rather than enhance neurite outgrowth.

Conclusion

With regard to the role of ErbB1 in limiting neurite outgrowth, there were at least 4 classes of explanations of the published data at the beginning of this study (Figure 3.7).

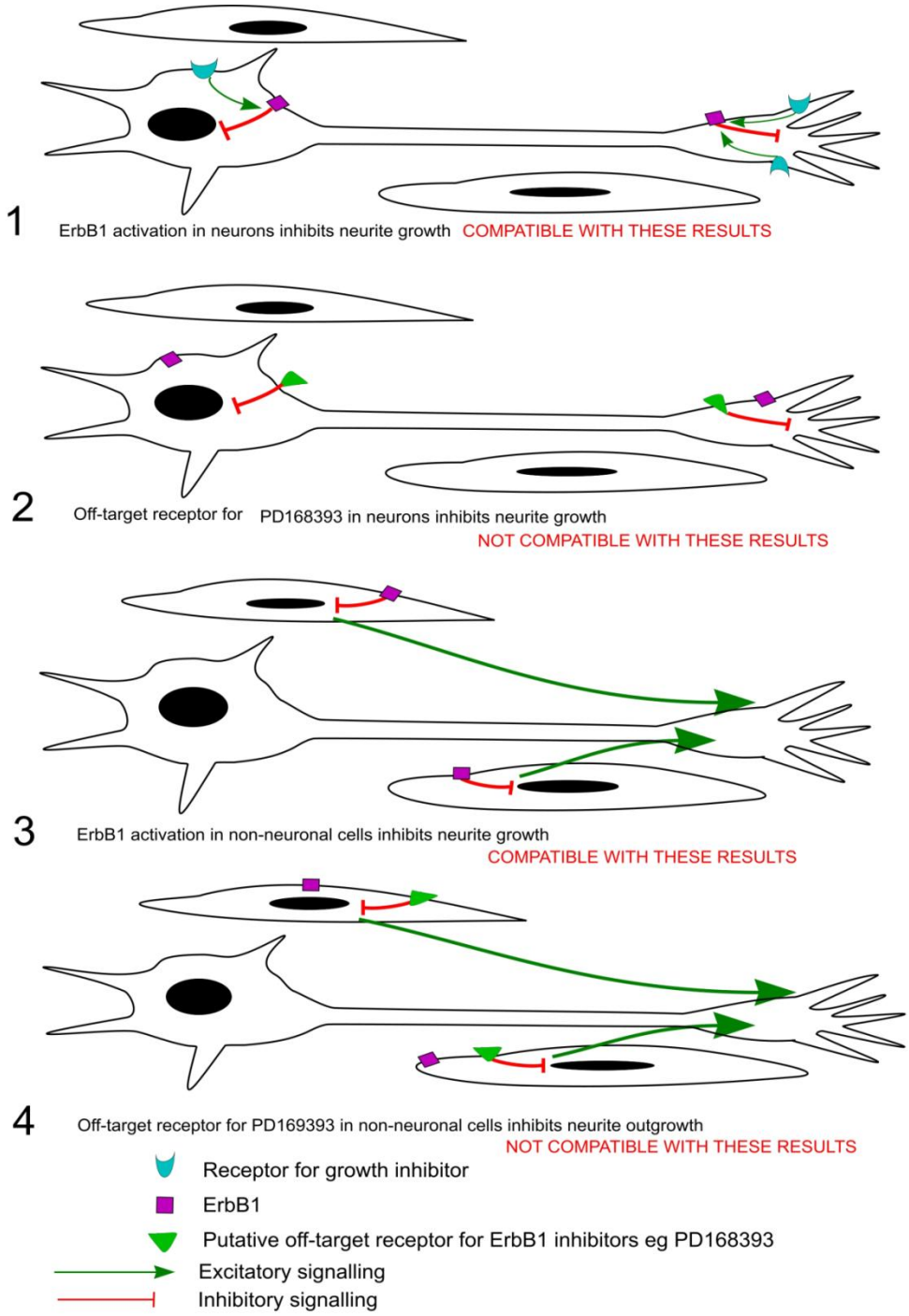


Figure 3.8: Models for the regulation of axonal growth by ErbB1 and ErbB1 inhibitors.

It was originally proposed that neuronal ErbB1 was on a pathway from receptors for inhibitory molecules leading to the inhibition of axonal growth (Fig 3.7, 1; Koprivica et. al.

2005). This pathway involved calcium signalling upstream of ErbB1 activation. A second possibility was that another neuronal target of ErbB1 inhibitors was involved in the signalling pathway (Fig 3.7, 2). A third possibility was that non-neuronal cell ErbB1 was important for regulating neurite outgrowth, perhaps by reducing the expression of neurotrophins (Fig 3.7,3) and a final possibility was that non-neuronal expression of an alternative target of ErbB1 inhibitors limited neurite outgrowth by reducing production of neurotrophins (Fig 3.7,4; Ahmed et al., 2009). The results of the present experiments are compatible with the hypothesis that neuronal ErbB1 is on one of the signalling pathways by which CNS myelin inhibits neurite outgrowth (Fig 3.7, 1) and with the hypothesis that ErbB1 on non-neuronal cells inhibits neurite outgrowth (Fig 3.7,3). They are incompatible with the hypothesis put forward by Ahmed et al 2009 that PD168393 produces its effects on neurite outgrowth through off-target interactions with other receptors (Fig 3.7,2 and 3.7,4). The cells in which ErbB1 activation is important for neurite outgrowth and the mechanism of ErbB1 activation from receptors for CNS inhibitory ligands will be discussed in following chapters.

Chapter-4 The effects of a Toll-Like receptor 3 agonist on neurite outgrowth

Introduction

Toll like receptors function as initiators of innate immune responses against pathogens (Olson and Miller, 2004). These molecules are also responsible for recognition of specific ectodomains on pathogens thereby shaping adaptive responses. Intensive investigation of TLRs as molecular players in immune responses was initiated by the discovery of Toll in Drosophila, in which species, a loss of Toll function led to defective innate immunity and aggravated susceptibility to fungal infection (Lemaitre et al., 1996). Currently, 13 different members of the TLR family have been identified in mice and 11 family members in humans. The different types of TLRs are capable of recognizing different motifs on pathogens (reviewed by Hanke and Kielian, 2011). Of these, the Toll like receptor-TLR3 is activated by double-stranded (ds) viral RNA. In addition to ds RNA of viral origin, RNA released from necrotic cells can have sufficient secondary structure to activate TLR3 (Kariko et al., 2004). As cell necrosis is of common occurrence in brain and spinal cord injury, it became relevant to investigate the effects of TLR3 activation in neurons. Poly I:C, which is artificial double-stranded RNA used as a common agonist to activate TLR3, was shown to stimulate growth cone collapse and be a potent inhibitor of neurite outgrowth (Cameron et al., 2007). These authors also showed that brain-derived Poly(A) mRNA acts as a potent inhibitor of neurite outgrowth only in the presence of RNAase inhibitors whereas the absence of RNAase inhibitors led to loss of secondary structures in mRNA rendering it an impotent ligand to TLR3. Cameron et.al.(2007), showed that TLR3 is expressed by dorsal root ganglia neurons, particularly in the growth cones, with some TLR3 expression also seen in endosomes.

The mechanisms through which TLR3 activation leads to inhibition of neurite outgrowth is unknown. ErbB1 is believed to be a mediator of axonal growth inhibition in response to a variety of cues. Hence, we investigated whether ErbB1 also plays a role in TLR3-mediated inhibition of neurite outgrowth. For this purpose, cultured DRG neurons from both wildtype as

well ErbB1-/- mice were treated with Poly I:C and neurite outgrowth from both types of neurons was measured.

The main goals of this study were to investigate the following:

- To confirm previous observations that Poly I:C acts as a negative regulator of neurite outgrowth *in vitro*.
- To investigate whether ErbB1 plays a role in TLR3-mediated inhibition of neurite outgrowth.
- To investigate whether Poly I:C and the ErbB1 blocker, PD168393 differentially affect various sub-populations of DRG neurons.
- To determine the effects of blocking ErbB1 on neurons and non-neuronal cells in DRG cultures treated with Poly I:C.
- To investigate whether calcium signalling plays a role in ErbB1 signalling from activated TLR3.

Methods

(For a detailed account on methods used, please refer Chapter-2)

Neonatal mouse DRG cultures:

DRG neurons from neonatal mice aged between P7 and P11 were cultured from wildtype and ErbB1-/- animals. The cells were plated at a density of 1,500 cells per well of an 8 well chambered slide that was pre coated with Poly lysine (100µg/ml) and laminin (2µg/ml). When sub-populations of DRGs were to be identified, cells were plated at a density of 3000 cells/well. Cultures were treated with either 0.01M PBS; Poly I:C that was dissolved in 0.01M PBS and used at a concentration of 100µg/ml; DMSO (0.01%- vehicle control for ErbB1 blocker, PD168393) and/or PD168393 at 10 or 100nM. DRG cultures were grown for 24 hrs at 37°C and then fixed using 4% paraformaldehyde for 10 min. DRG cultures were immunostained using various primary antibodies:

βIII Tubulin 1:1000; Sigma

NF200 1:800; Sigma

CGRP 1:500 ; Peninsula laboratories

IB4 (Biotin conjugated) 1:100; Sigma

NG2 1:1000 (kind donation from Dr. Stallcup).

S100 (Mouse monoclonal) 1:1000; Sigma

Neonatal rat DRG cultures:

DRG neurons from neonatal rat pups aged between P7-P8 were isolated and dissociated using enzymes collagenase (2mg/ml) - dispase(3mg/ml). Cells were plated at a density of 1,500 cells per well for 24 hrs at 37°C and then fixed using 4% paraformaldehyde for 10 min and immunostained using rabbit polyclonal βIII tubulin.

Adult mouse DRG cultures:

DRG neurons from adult mice (> 3months of age) were isolated. Connective tissue was removed and roots trimmed using spring bow scissors. Cells were dissociated using enzymes Collagenase (3mg/ml)-trypsin(2.5mg/ml). Cells were plated at density of 1,500 cells per well and grown for 24 hrs at 37°C after which they were fixed and immunostained using antibodies against βIII tubulin (1:1000, Sigma).

Data analysis:

Analysis of neurite length:

Photomicrographs of βIII tubulin and corresponding Hoechst cells were taken from different fields of view. Neurite length was quantified using the software Image J.

Analysis of cell counts:

1) Data on total number of cells:

The number of Hoechst+ve cells was counted from 15 different fields of view from each condition of an experiment. The mean number of Hoechst+ve cells per field of view from each

condition was calculated. Experiments were repeated from 6 different animals (n=6). The average mean from all experiments were calculated.

2) Data on the total number of neurons:

The total number of βIII tubulin-positive neurons and neurons that extended processes was counted from an entire well per condition of an experiment. Experimental conditions were repeated from 3 different animals (n=3) and the mean was calculated in each case.

3) Data on non-neuronal cells:

The number of NG2+ve cells and S100+ve cells were counted from 10 different fields of view per condition of an experiment. The corresponding number of Hoechst+ve cells in the field of view was also counted. Mean number of cells was calculated in each case. Experiments were repeated from 4 different animals (n=4). The numbers of NG2 +ve and S100+ve cells are represented as percentage of the total number of Hoechst positive cells per condition.

In each of the above cases, statistical significance was computed using Student's t test. Error bars on the graphs are represented as SEMs.

Results

Poly I:C inhibits neurite outgrowth but ErbB1 -/- neurons are less affected by Poly I:C.

The addition of Poly I:C at various concentrations ranging from 10µg/ml to 100µg/ml to neonatal mouse DRG neurons causes inhibition of neurite outgrowth. The extent to which neurite outgrowth was inhibited was dependant on the concentration of Poly I:C added to the media (Figure 4.1). The observation that Poly I:C inhibits neurite outgrowth is consistent with previous observations by Cameron et al. (2007) and show that Poly I:C is a potent inhibitor of axonal elongation by neonatal mouse DRG neurons .

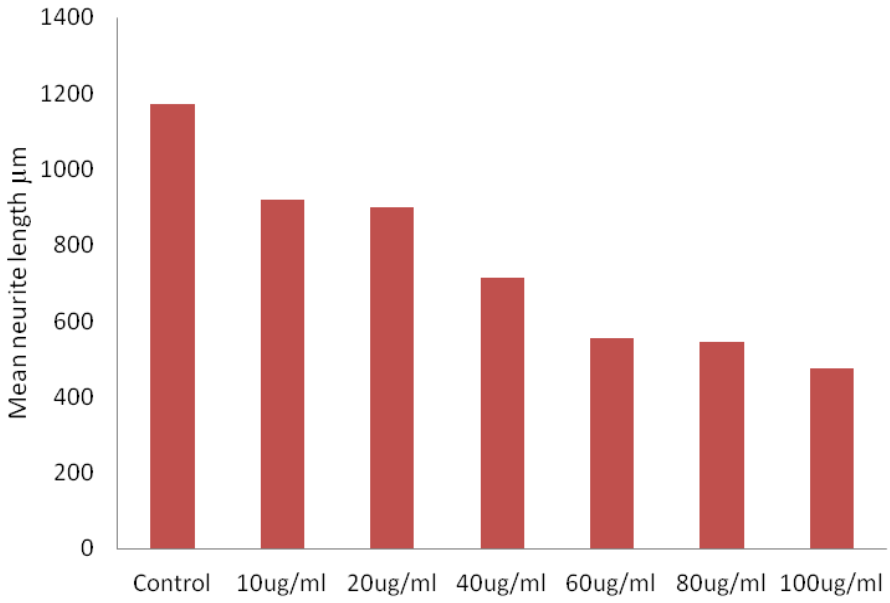


Figure 4.1: PolyI:C inhibits neurite outgrowth from neonatal mouse DRG neurons grown for 24 hrs on a poly lysine-laminin substrate. The inhibitory effects of Poly I:C are dose-dependant with least inhibition at at a conc of 10µg/ml and more than 50% inhibition at 100µg/ml. The concentration of Poly I:C that was chosen for further experiments was 100µg/ml.

When ErbB1 -/- neurons were cultured in the presence of Poly I:C, neurite outgrowth was markedly greater than that of wildtype or heterozygous neurons under the same conditions (Figure 4.2). This is compatible with the hypothesis that ErbB1 may also be involved in the signalling pathway from TLR3 leading to inhibition of neurite outgrowth.

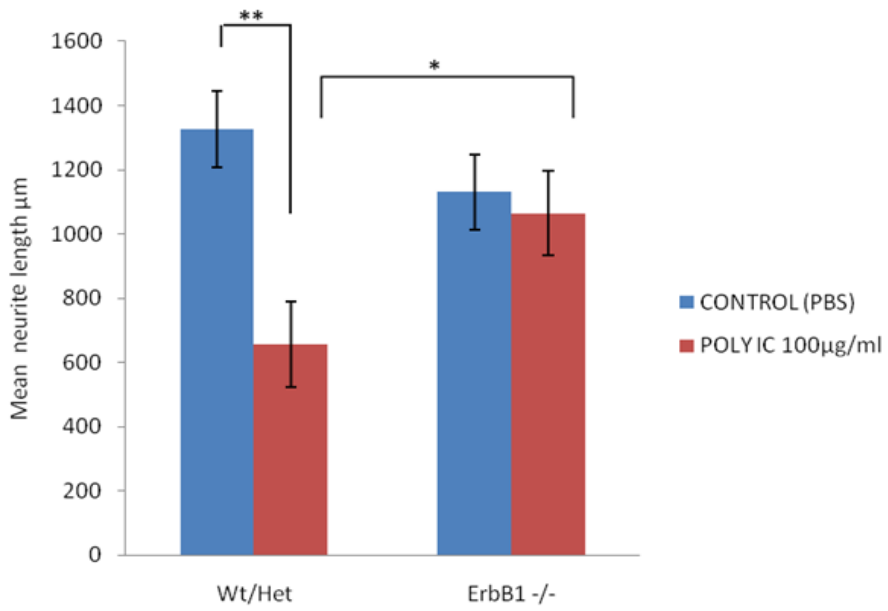


Figure 4.2: ErbB1 -/- neurons were less affected by Poly I:C. (**p<0.01 when compared to control;*p<0.05 when compared to ErbB1 -/- treated with Poly I:C, Student's t test, n=6). DRG neurons were cultured on Poyl-l-lysine and laminin for 24 hrs with or without 100µg/ml Poly I:C

PD168393 improves neurite outgrowth from wildtype but not ErbB1-/- DRG neurons in the presence of Poly I:C.

Neonatal DRG neurons from wildtype and ErbB1-/- mice were cultured in the presence of Poly I:C alone or Poly I:C and PD168393. The pharmacological inhibition of ErbB1 using PD168393 partially rescued wildtype DRG neurons from the inhibitory effects of Poly I:C (Figure 4.3). However, ErbB1 -/- neurons were unaffected by PD168393 (10nM and 100nM); once again this shows that the rescue from neurite inhibition by PD168393 was mediated through its effects on ErbB1 and was not a result of off-target effects. However, the experiment does not identify the cells on which PD168393 exerted its effects.

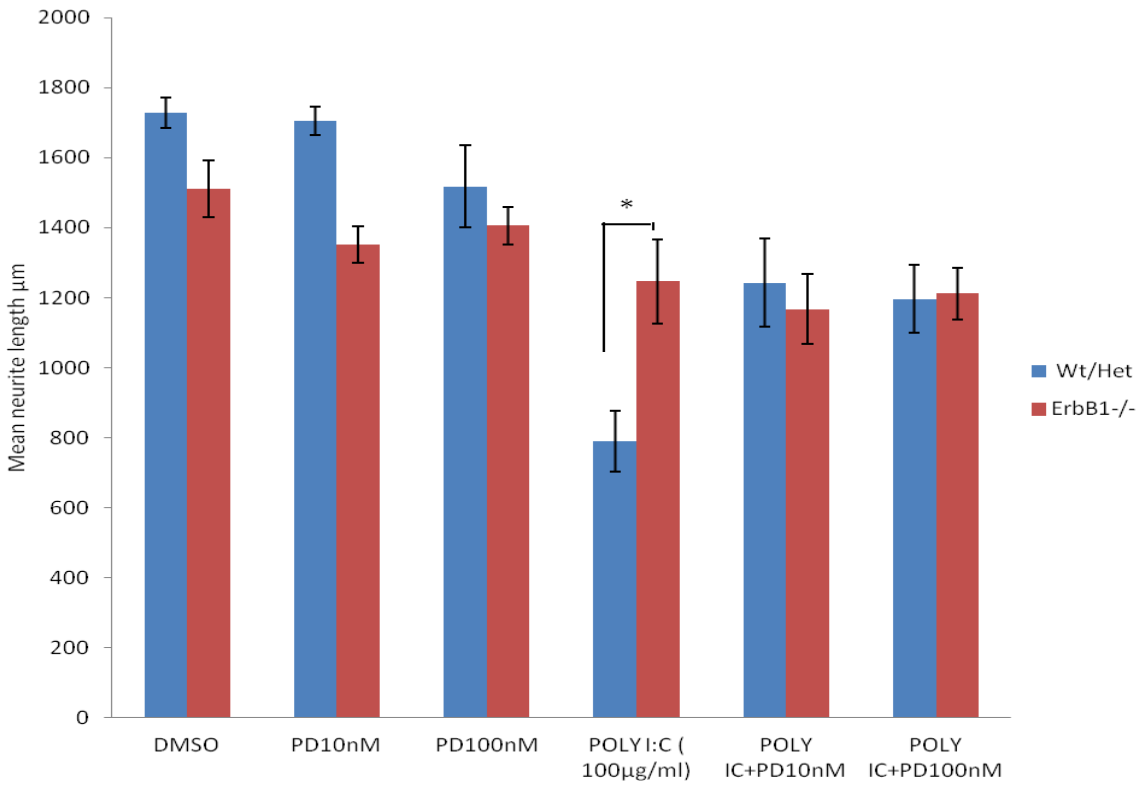


Figure 4.3: In the presence of Poly I:C (100µg/ml) PD168393 increased neurite outgrowth from wildtype neurons, but not from ErbB1 -/- neurons. DRG neurons were cultured for 24hrs. (*$p<0.01$ when compared to neurons treated with Poly I:C alone, Student's t test, n=5). b)

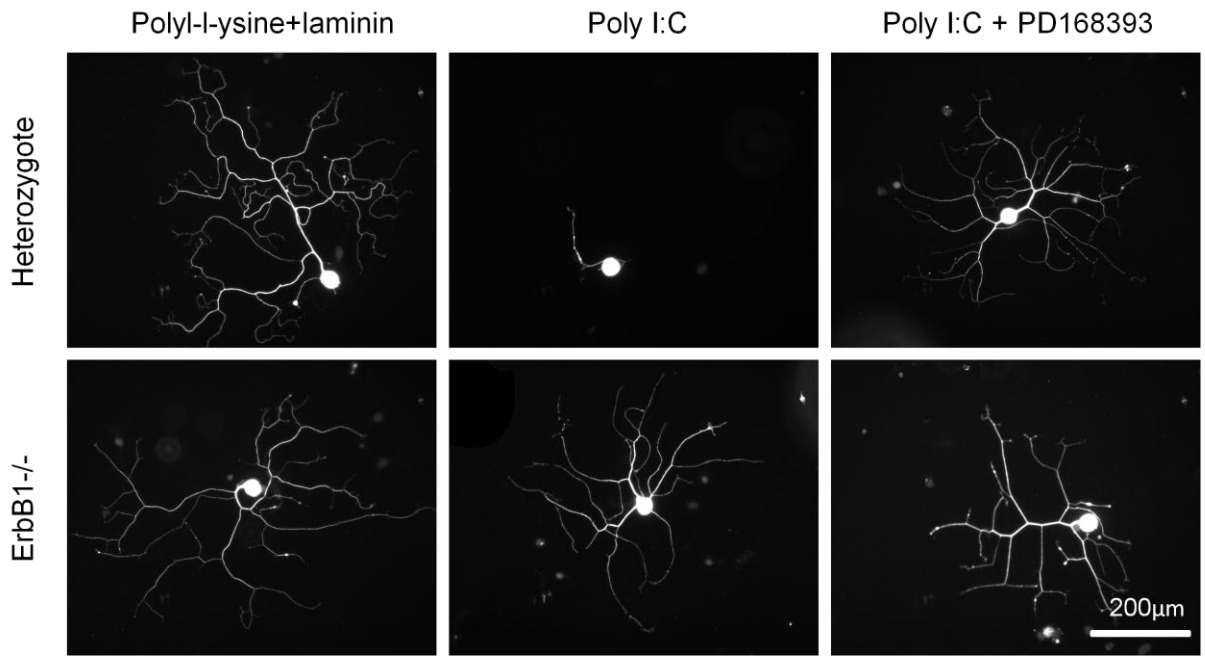


Figure 4.4: Photomicrographs of DRG neurons from heterozygous and ErbB1-/- mice showing the effects of various treatments (PolyI:C/ PolyI:C+PD168393, 100nM) on neurite outgrowth. PolyI:C inhibits neurite outgrowth from heterozygous neurons but not ErbB1-/- neurons. Addition of PD168393 (100nM) rescues neurite outgrowth from heterozygous neurons but has little effect on ErbB1-/- neurons.

Poly I:C also inhibits neurite outgrowth from cultured adult mouse DRG neurons and neonatal rat DRG neurons. Addition of PD168393 improves neurite outgrowth from both types of neurons in the presence of Poly I:C .

To test if Poly I:C has inhibitory effects on older neurons and neurons from different species, DRG neurons from adult mice and neonatal rats were cultured on poly-l-lysine and laminin in the presence of Poly I:C.

Neurite outgrowth from cultured DRG neurons from both adult mice and neonatal (P7-P8) rats was inhibited in the presence of Poly I:C (100µg/ml). However, the addition of PD168393 at 100nM improved neurite outgrowth from both types of neurons (Figures 4.5, 4.6). This data shows that the inhibitory effects of Poly I:C on neurite outgrowth as well as rescue effects seen with the addition of the ErbB1 kinase inhibitor are not age dependant. Moreover, these effects also apply to more than one species.

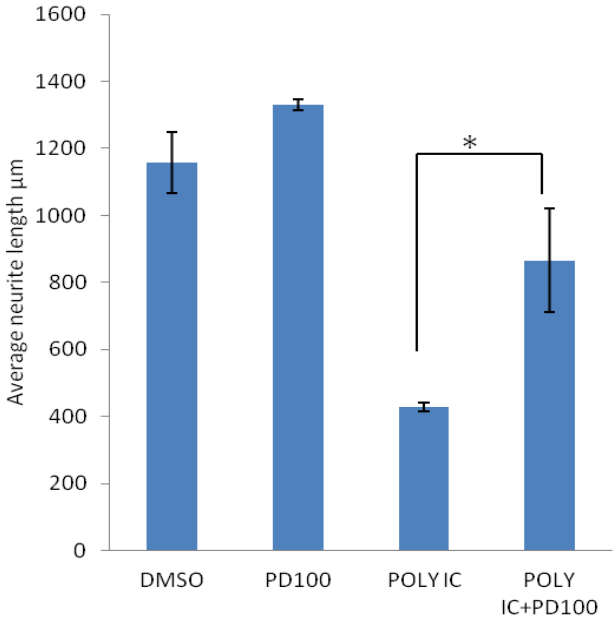


Figure 4.5: Poly I:C inhibits neurite outgrowth from cultured adult mouse DRGs. Addition of PD168393 improves neurite outgrowth. (*$p<0.01$ when compared to neurons treated with Poly I:C alone, Student's t test, n=3.

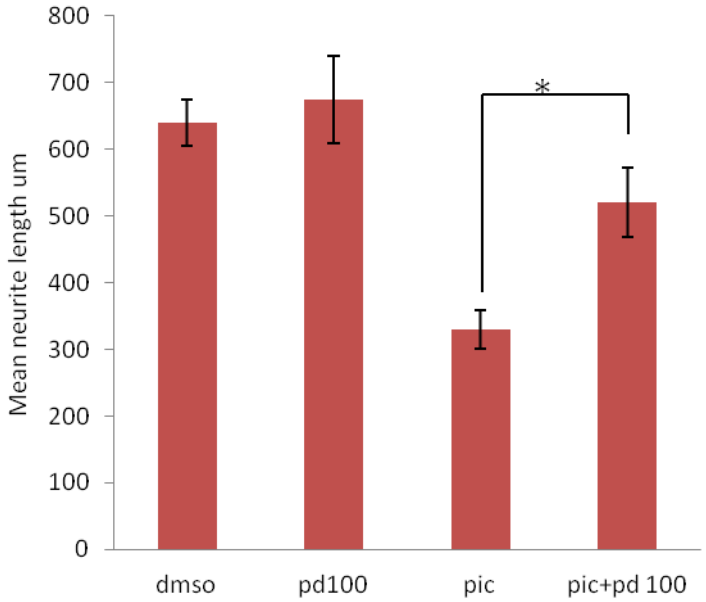


Figure 4.6: Poly I:C inhibits neurite outgrowth from cultured P7 rat DRGs. Addition of PD168393 improves neurite outgrowth. (*p<0.01 when compared to neurons treated with Poly I:C alone, Student's t test, n=3.

Poly I:C reduces the total number of neurons and also neurons bearing neurites on poly-l-lysine/laminin substrate. PD168393 does not affect adherence and neuritogenesis of DRG neurons treated with Poly I:C.

The total number of neurons (βIII tubulin+ve cells) that adhered to the substrate in each of the conditions was counted. The number of neurons adhering to the substrate was not affected by the addition PD168393 alone. However, in the presence of Poly I:C, counts revealed a reduction in the number of neurons on a poly-l-lysine laminin substrate when compared to a vehicle control (0.01M PBS). The addition of PD168393 does not increase the adherence of neurons treated with Poly I:C (Figure 4.7).

To investigate whether Poly I:C has an effect on neuritogenesis by DRG neurons, the number of neurons bearing neurites treated with PBS as well as in the presence of Poly I:C were counted. It was observed that under control conditions (PBS), approximately 62% of neurons produced neurites. In the presence of PD168393 (10nM) alone, the number of neurite-bearing neurons were similar to control neurons. However, when neurons were treated with Poly I:C, there was significant reduction in the number of neurons that produced neurites. The addition of

PD168393 did not improve this condition suggesting that ErbB1 does not play a role in neuritogenesis of DRG neurons inhibited by Poly I:C (Figure 4.7)

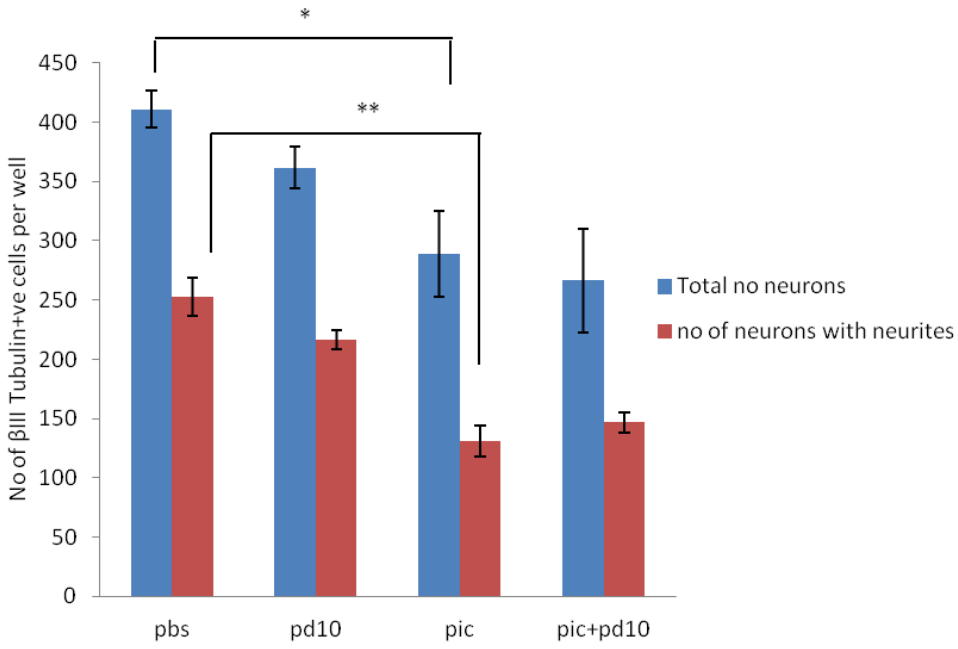


Figure 4.7: Poly I:C reduces number of neurons adhering to substrate and also the number of neurons bearing neurites. Total number of neurons and neurons with neurites adhering to the substrate were counted from one well per condition from each experiment. **p<0.005 when compared to total number of neurons treated with PBS; *p>0.5 when compared to neurons with neurites treated with PBS; Student's t test, n=3.

Addition of Poly I:C does not alter the proportion of S100 and NG2 positive non-neuronal cells in wildtype and ErbB1-/- DRG cultures.

Although many non-neuronal cells were removed in the preparation of partially-purified dorsal root ganglia neuronal cultures, such cultures none the less consisted of mixed populations of neurons and non-neuronal cells. One way in which Poly I:C could affect neurite outgrowth would be by altering the populations of non-neuronal cells. The addition of Poly I:C does not significantly alter the total number of cells when compared to control (PBS) in either wildtype or ErbB1-/- cultures (Figure4.8). To detect if there were differences in the proportion of non-

neuronal cells in the presence of Poly I:C and whether these effects varied with the absence of ErbB1, cultures were immunostained for NG2 and S100 (Figure 4.9) which detect partially overlapping sets of non-neuronal cells. Differential staining of non-neuronal cells in wildtype cultures revealed that 60-70% of the cell population were S100+ve and therefore comprised Schwann cells and satellite cells. 40% -60% of the cells were NG2+ve, marking them as fibroblasts and satellite cells. However, the addition of Poly I:C did not significantly alter the number of S100 or NG2+ve cells. In the case of ErbB1-/- DRG cultures, the proportions of NG2 and S100+ve cells were similar (65-75%) and the addition of Poly I:C did not significantly alter these proportions (Figure 4.9). Hence, these results show that addition of Poly I:C does not cause loss or stimulate proliferation of non- neuronal cells in DRG cultures and such activity is not altered in DRG cultures devoid of ErbB1.

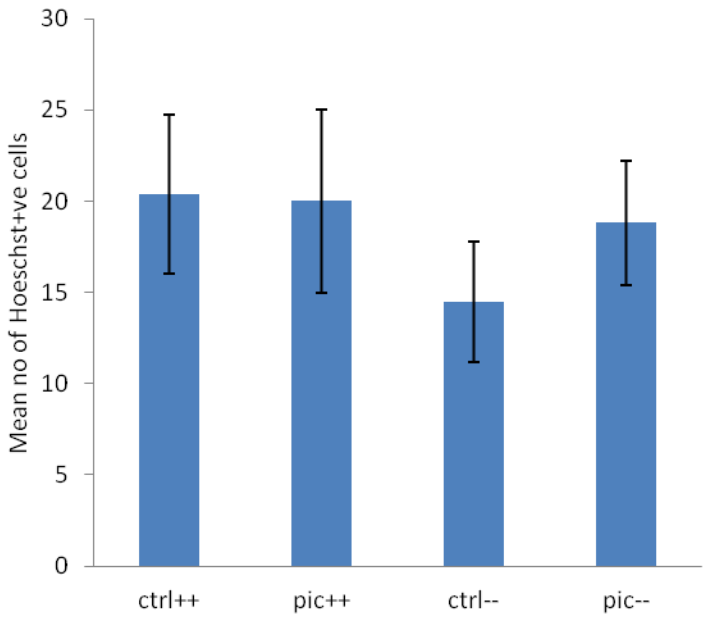


Figure 4.8: Addition of Poly I:C does not significantly alter the number of cells attached to poly laminin substrate in wildtype and ErbB1-/- cultures. Hoechst-positive cells were counted from 15 different fields of view and mean calculated from each experiment, N=6. Ctrl++ denotes wildtype DRG cultures treated with PBS, ctrl- - denotes ErbB1-/- DRG cultures treated with PBS, pic++ denotes wildtype DRG cultures treated with PolyI:C, pic--denotes ErbB1-/- DRG cultures treated with Poly I:C.

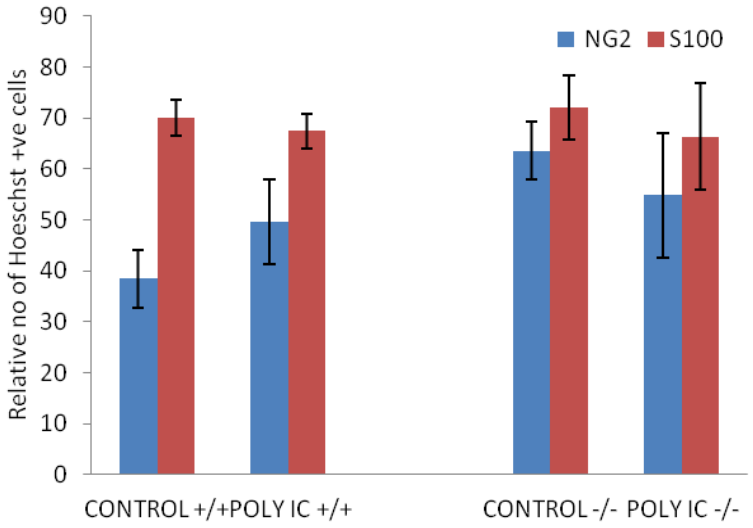


Figure 4.9: Differential staining of non-neuronal cells in wildtype and ErbB1-/-DRG cultures treated with Poly I:C . Total number of Hoechst and S100+ve or NG2+ve cells were counted from 10 different fields of view per condition from each experiment and the means of NG2+ve and S100 +ve cells relative to Hoechst +ve cells were calculated; Bars are expressed as percentage of NG2/S100+ve cells to the total no of Hoechst+ve cells. n=4.

PD168393 improves neurite outgrowth from CGRP+ve DRG neurons but not NF200+ve DRG neurons in the presence of Poly I:C

Neonatal DRG cultures were immunoreacted for CGRP, NF200 and IB4 to identify various subpopulations of DRG neurons. Characterisation of DRG subpopulations using these markers revealed that in control conditions, NF200+ve neurons produced significantly longer neurites than IB4+ve neurons (Figure 4.10). The addition of ErbB1 blocker, PD168393 at 100nM did not produce any effects on neurite lengths from neurons of the various identified subpopulations (Figure 4.10) in the absence of Poly I:C. In the presence of Poly I:C, neurite outgrowth from CGRP+ve and NF200+ve DRG neurons was strongly inhibited. However, the addition of PD168393 improved neurite outgrowth from CGRP+ve but not NF200+ve DRG neurons in the presence of Poly I:C (Figure 4.11). The effects of Poly I:C and possible rescue effects from the addition of ErbB1 blocker on neurite outgrowth from IB4+ve neurons were not quantified, as under such conditions the proportion of IB4+ve neurons were greatly reduced and in some experiments numbers were as low as 2-3 neurons. Moreover, the addition of PD168393 did not improve the number of IB4+ve neurons surviving in the cultures.

These results show that the ErbB1 blocker, PD168393 selectively rescues neurite outgrowth from CGRP+ve DRG neurons treated with Poly I:C .

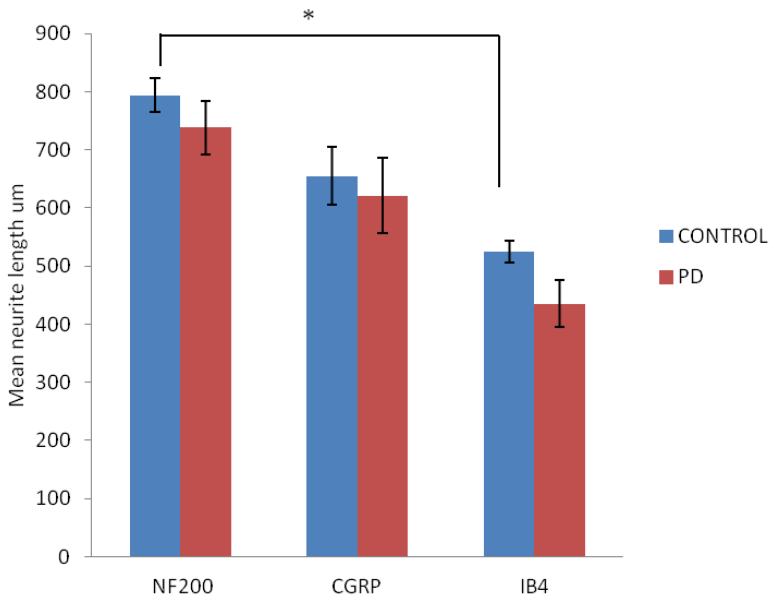


Figure 4.10: Characterization of neurite outgrowth from DRG sub-populations using markers NF200,CGRP and IB4. DRG neurons were cultured in the absence of PolyI:C. *$p<0.01$ when compared to NF200+ve neurons cultured under control conditions, Student's t test, n=3.

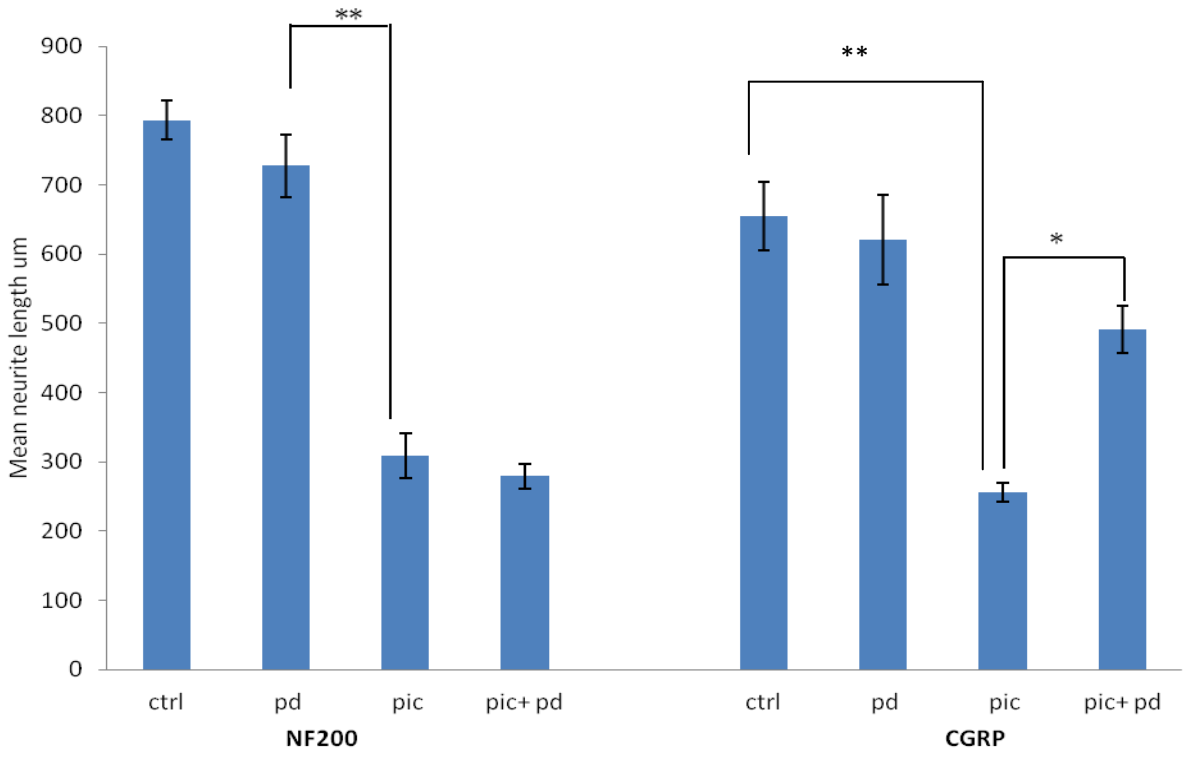


Figure 4.11: Poly I:C inhibits neurite outgrowth of CGRP+ve and NF200+ve DRG neurons. PD168393 improves neurite outgrowth of CGRP+ve but not NF200+ve neurons. **p<0.001 compared to control NF200+ve or control CGRP+ve neurons; *p<0.005 compared to CGRP+ve neurons treated with Poly I:C; Student's t test, n=3.

BAPTA improves neurite outgrowth from wildtype but not ErbB1-/- DRG neurons treated with Poly I:C.

Calcium signalling is believed to be involved in the responses of neurons to inhibitory cues such as CNS myelin and is upstream of ErbB1 activation by such cues (Koprivica et al., 2005a). To investigate if calcium signalling is involved in ErbB1 signalling in response to Poly I:C, cultures were treated with BAPTA or BCECF (acts as a control to BAPTA as it does not affect calcium homeostasis) at concentrations of 10nM and 100nM (Figure 4.12). BAPTA but not BCEFC improved neurite outgrowth from wildtype DRG cultures treated with Poly I:C. ErbB1 -/- neurons were unaffected by the presence of BAPTA suggesting that calcium signalling

is involved in TLR3 signalling that involves ErbB1 activation leading to inhibition of neurite outgrowth.

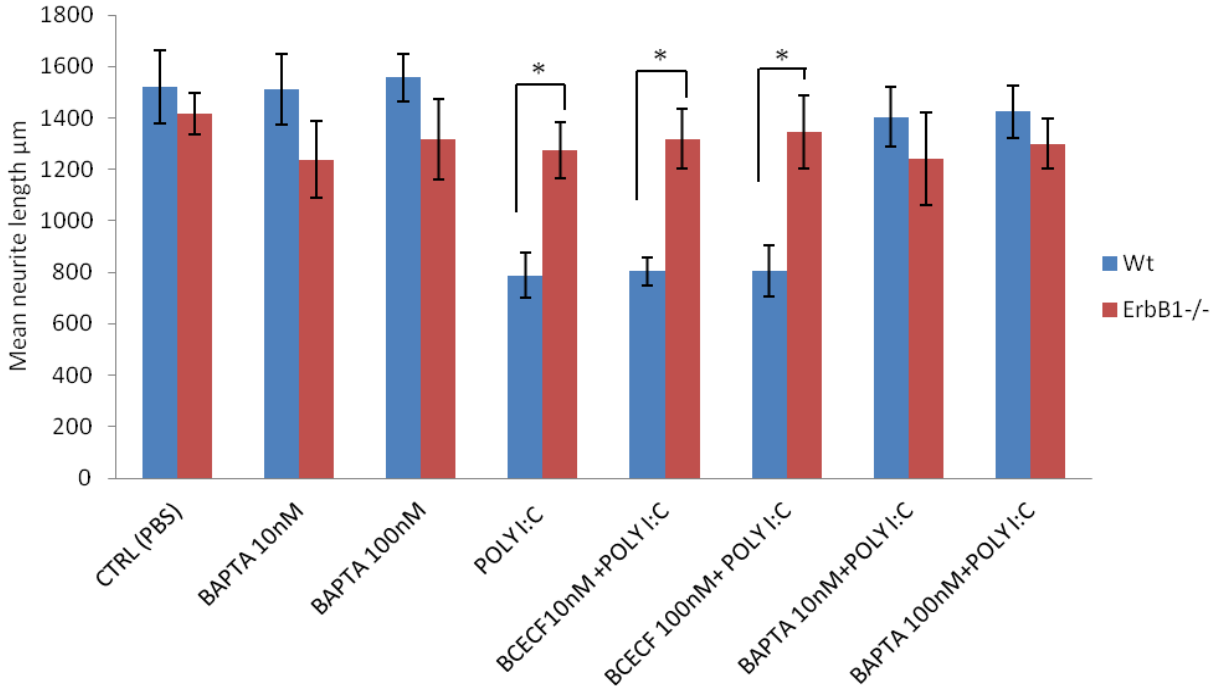


Figure 4.12: BAPTA improved neurite outgrowth from Poly I:C treated wildtype DRG neurons cultured for 24 hrs. No effects of BAPTA were observed on Poly I:C treated ErbB1 -/- neurons or on neurons that were not treated with Poly I:C. BCECF did not affect neurite outgrowth. (*p<0.01 when compared to neurons treated with Poly I:C alone or Poly I:C and BCECF, Student's t test, n=5).

Discussion

Poly I:C inhibition of neurite outgrowth is dependent on ErbB1 activation.

Recent studies on the effects of Poly I:C on neurons have shown that Poly I:C causes inhibition of neurite outgrowth as well as growth cone collapse. Most of this effect is attributable to Poly I:C binding to TLR3 (Cameron et al., 2007;Qi et al., 2012). Cameron et.al. (2007) also

showed that Poly (A) mRNA derived from brain, inhibited neurite outgrowth in a manner similar to Poly I:C . Their observation strengthens previous reports of similar effects produced by mRNA released from necrotic cells and subsequent activation of TLR3 leading to inhibition of neurite outgrowth (Kariko et al., 2005;Kariko et al., 2004). As mechanical insults to the spinal cord result in substantial cell necrosis, it is most likely that there is an abundance of such mRNA whose secondary structures are sufficient to activate TLR3. However, what remains unknown is the mechanism by which TLR3 signalling leads to inhibit neurite outgrowth.

Through a series of experiments, we have generated evidence that ErbB1 is on one of the signalling pathways from activated TLR3 that leads to inhibition of neurite outgrowth. To investigate the role of ErbB1, DRG neurons from ErBb1 -/- and wildtype mice were cultured in the presence of Poly I:C. Neurite outgrowth from ErbB1-/- neurons was less affected by Poly I:C than that from wildtype neurons. The effects of the irreversible pharmacological blocker of ErbB1, PD168393, were similar to that of genetically deleting ErbB1 on wildtype neurons, i.e. it reduced the effects of Poly I:C. However, PD168393 had no effect on ErbB1-/- neurons grown in the presence of Poly I:C, demonstrating once again that the drug does not act off-target. The neurite-inhibiting effects of Poly I:C and rescue effects seen with the addition of PD168393 were not limited to neonatal mouse DRG neurons but were also seen in neurons obtained from adult mouse DRGs as well as neonatal rat DRGs. This suggests that the effects of Poly I:C are not age dependant and that ErbB1 is a prominent signalling molecule through which activated TLR3 mediates its effects on neurite outgrowth from DRG neurons.

Effects of ErbB1 signalling from activated TLR3 in neuronal cultures are limited to reducing neurite outgrowth:

Poly I:C at 100μg/ml reduces the number of βIII tubulin-positive neurons extending neurites when compared to neurons treated with PBS. Treatment of DRG neurons with Poly I:C also reduces the total number of neurons adhering to a poly-l-lysine/laminin substrate when compared to neurons that were treated with PBS. However, no changes were observed in the total number of Hoechst positive cells treated with Poly I:C when compared to cells treated with vehicle control. This is compatible with results obtained by Cameron et.al., 2007 who showed

that the addition of PolyI:C at 20µg/ml neither altered the total number of Hoechst positive cells nor increased the proportion of tunnel positive cells when compared to control conditions.

The addition of PD168393 to DRG neurons does not alter the effects of Poly I:C in reducing the total number of neurons and number of neurons extending neurites. From these results, it can be interpreted that the effects of ErbB1 signalling resulting from activation of TLR3 is one that affects only neurite outgrowth without affecting neuritogenesis or mediating cell death.

The effects of Poly I:C on non-neuronal cells:

Since a reduction in the number of βIII tubulin-positive neurons was observed in the presence of Poly I:C, the next step was to investigate if Poly I:C significantly altered the proportion of other cells that may be present in DRG cultures. For the purpose of identifying non-neuronal cells, the markers S100 and NG2 were used to identify satellite cells, Schwann cells and other NG2-expressing non-neuronal cells. S100-positive cells were more numerous than NG2-expressing cells. However, it should be noted that the populations are not mutually exclusive. The addition of Poly I:C had no effect on the proportion of identified non-neuronal cell types. In the case of DRG cultures lacking ErbB1, the response of S100 and NG2+ve cells to Poly I:C remained unchanged. Although we were unable to identify macrophages in our cultures using IBA-1 immunohistochemistry, the possibility that Poly I:C may have an effect on other unidentified non-neuronal cells types cannot be ruled out.

Blocking ErbB1 signaling affects specific sub-populations of DRG neurons treated with Poly I:C.

Dorsal root ganglia cultures contain different sub-populations of DRG neurons which can be identified by various neurochemical markers, including NF200 (heavy neurofilament protein) for low threshold mechanoreceptors; calcitonin gene related peptide(CGRP) for peptidergic nociceptors and *Griffonia simplicifolia* IB4 isolectin for non-peptidergic nociceptors (reviewed by Priestly, 2009). Characterization of DRG cultures used in the present study, revealed expression of all three markers by different sub-populations of DRG neurons. However, relatively low number of neurons expressed IB4. In the absence of Poly I:C; NF200, CGRP and

IB4+ve neurons all extended neurites but it was observed that neurite lengths of IB4+ve neurons were significantly shorter than those of NF200 neurons. The addition of PD168393 did not alter neurite length from any of the subtypes of DRG neurons in the absence of inhibitors of neurite outgrowth. The addition of Poly I:C drastically reduced neurite outgrowth from NF200 and CGRP+ve neurons. Since the number of IB4+ve neurons was further diminished in the presence of Poly I:C, it proved difficult to obtain quantitative data on neurite length from these neurons. Surprisingly, the addition of PD168393 rescued neurite outgrowth from CGRP+ve neurons but had no effect on NF200+ve neurons thus showing that neurite enhancing effects achieved by blocking ErbB1 signaling is limited to certain subtypes of DRG neurons.

The differential effects of blocking ErbB1 in DRG neurons could be attributed to differences in the expression of the receptor in different sub-populations of DRG neurons. It has been previously reported that there is heterogeneity in immunoreactivity of ErbB1 in different subpopulations of human dorsal root ganglia neurons with some types of neurons completely lacking ErbB1 immunoreactivity (Werner et.al., 1988;Huerta et al., 1996;Gomez-Pinilla et al., 1988). Huerta et.al., (1996) reported that ErbB1 immunoreactivity is prominent in small and medium diameter neurons. If this were the case, it is most likely that a proportion of NF200+ve DRG neurons do not express ErbB1 explaining the existence of a different signalling pathway from activated TLR3 that does not involve ErbB1 in these neurons. However, more recently (Andres et al., 2010) reported uniform expression of ErbB1 in all subpopulations of DRG neurons. In our laboratory, we have also observed expression of ErbB1 in most DRG neurons by means of immunohistochemistry (Chapter-8). Although, we have evidence on the expression of ErbB1 mRNA in cultured DRG neurons and ErbB1 protein in DRG tissue lysates; immunocytochemical analysis of ErbB1 expression in cultured DRG neurons has proven extremely difficult. Nonetheless, the possibility of a different signalling pathway from TLR3 independent of ErbB1cannot be excluded.

Liu et al., 2012 reported that TL3 is expressed by CGRP+ve DRG neurons but not NF200+ve neurons. The lack of TLR3 to activate ErbB1 in NF200+ve neurons would explain why blocking ErbB1 in these neurons did not produce any significant improvement in neurite outgrowth. However, it is difficult to believe that TLR3 is absent from NF200+ve neurons as

neurite outgrowth from these neurons is profoundly inhibited by Poly I:C. The possible explanations would include Poly I:C acting on a different receptor or acting on TLR3-expressing non-neuronal cells which in turn may affect neurite outgrowth from NF200+ve neurons.. The effects of Poly I:C on cells has been shown to be specific to neurons when used at concentrations between 1.0-20 µg/ml (Cameron et.al.,2007) and is known to activate immune cells at above 25µg/ml (Alexopoulou et al., 2001). As optimal inhibition of neurite outgrowth in our experiments was achieved at 100µg/ml, it is most likely that non-neuronal TLR3 is also activated in our cultures. It could be possible that activation of non-neuronal TLR3 could lead to the secretion of factors that may affect neurite outgrowth from NF200+ve neurons which have been previously reported to be devoid of TLR3 expression. The secretion of factors induced by non-neuronal TLR3 is supported by a study that has reports that TLR3 activation in Schwann cells by Poly I:C (20µg/ml) leads to iNOS gene induction and subsequent production of NO that causes death of sensory neurons (Lee et al., 2007).

Apart from TLR3, Poly I:C has been reported to interact with the helicase protein, retinoic acid-inducible gene 1 (RIG-1) (Peltier et al., 2010;Kato et al., 2006). RIG-1 is expressed by neurons (Peltier et al., 2010). Although signalling through RIG-1 is associated with production of type 1 interferons, it not clear how such signalling may affect neurite outgrowth from DRG neurons and if at all an effect is seen, it is not known if these effects are confined to certain sub-populations of DRG neurons. Moreover, it is not known if ErbB1 is involved in this signalling pathway.

Calcium signaling is on the pathway from TLR3-induced activation of ErbB1 leading to inhibition of neurite outgrowth:

There is evidence that Nogo-66-induced activation of ErbB1 leading to inhibition of neurite outgrowth is not mediated by binding of ErbB1 ligands, such as EGF. Instead, in order to inhibit neurite outgrowth, ErbB1 is transactivated in a calcium-dependant manner. Such conclusions were based on results from experiments in which the addition of ErbB1 ligands such as EGF and HB EGF, although they induced ErbB1 phosphorylation, failed to inhibit neurite outgrowth. Moreover, buffering calcium failed to abrogate ErbB1 phosphorylation that was

induced by the binding of ligands such as EGF (Koprivica et al., 2005) but reduced the effects of Nogo-66.

In order to test if calcium signalling is involved in TLR3-mediated activation of ErbB1, wildtype as well as ErbB1 -/- neurons were treated with a calcium chelator, BAPTA-AM at concentrations of 10 and 100nM in the presence of Poly I:C. As AM-ester loading stresses the cell membrane, BCECF which is a pH indicator that does not affect calcium homeostasis was used as a control to BAPTA-AM. It was observed that the addition of BAPTA-AM to wildtype neurons significantly improved neurite outgrowth to a similar extent to that of blocking ErbB1 or using ErbB1 -/- neurons in DRG cultures. However, BAPTA-AM had no effect on neurite outgrowth from ErbB1-/- neurons treated with Poly I:C. No effects on neurite outgrowth were observed when the cells were treated with BCECF. The absence of neurite-enhancing effects when calcium was buffered in ErbB1-/- cells shows that ErbB1 plays an essential role in the pathway by which calcium signalling inhibits axonal elongation in the presence of activated TLR3.

Possible signalling mechanisms

Our experiments have confirmed previous reports by Cameron et.al. (2007) that Poly I:C inhibits neurite outgrowth. Although Poly I:C may bind to other receptors, most of the neurite-inhibiting effect was prevented in TLR3 knockout neurons (Cameron et.al., 2007) However, the mechanism through which activated TLR3 mediates inhibition of neurite outgrowth previously remained unknown. Koprovica (2005) reported that ErbB1 transactivation in the presence of CNS myelin or CSPGs occurs independently of metalloprotease activity or the binding of at least some of its ligands but depends on calcium signalling. The increased activation of ErbB1 seen after Poly I:C treatment of neuronal cultures is dependent on calcium signalling (Leinster et al., 20012). All three growth inhibitors- CNS myelin, CSPGs, and Poly I:C seem, therefore, to use calcium signalling to activate ErbB1 but the mechanisms of such signalling are unclear. However, it has been reported that the activation of TLR3 by its ligand requires binding of ErbB1 and Src which in turn phosphorylate two tyrosine residues in its cytoplasmic domain (Yamashita et al., 2012). Pharmacologically blocking ErbB1 activity leads to inactivation of TLR3. Presumably, a low level of tonic ErbB1 activation is present to allow

TLR3 signalling which somehow mobilises calcium to activate ErbB1. The possibility exists that TLR3 activation and ErbB1 activation could form a positive feedback loop leading to greater TLR3 and ErbB1 signalling.

Possible signalling pathways downstream of TLR3 are considered in the General Discussion (Chapter-10).

Conclusion

Our experiments have confirmed that Poly I:C, a potent ligand of TLR3, inhibits neurite outgrowth. The effects of activated TLR3 on neurite outgrowth can be ameliorated by pharmacologically inhibiting or genetically deleting ErbB1. Thus, we have shown that ErbB1 is actively involved in the signalling from TLR3 causing inhibition of neurite outgrowth from certain sub-populations of mouse DRG neurons *in vitro* and that signalling via ErbB1 is dependent on calcium signalling. Taken together, our results are compatible with the hypothesis that ErbB1 acts as a common signalling hub through which receptors for CNS myelin, CSPGs and Poly I:C signal leading to inhibition of neurite outgrowth.

Chapter-5 The role of non-neuronal cells in the responses of neurons to inhibitory molecules *in vitro.*

Introduction

In previous chapters it was shown that neurite outgrowth from neonatal mouse neurons was inhibited by CNS myelin and Poly I:C and that this inhibition was reduced by ErbB1 kinase inhibitors or by the genetic deletion of ErbB1. DRG cultures, even those enriched in neurons as used here, contain non-neuronal cells including satellite cells, Schwann cells, fibroblasts and macrophages. One might imagine that there are at least three possible explanations of the inhibitory effects of CNS myelin and Poly I:C on neurite outgrowth (in each case involving ErbB1 in some way) (See Figure 5.0).

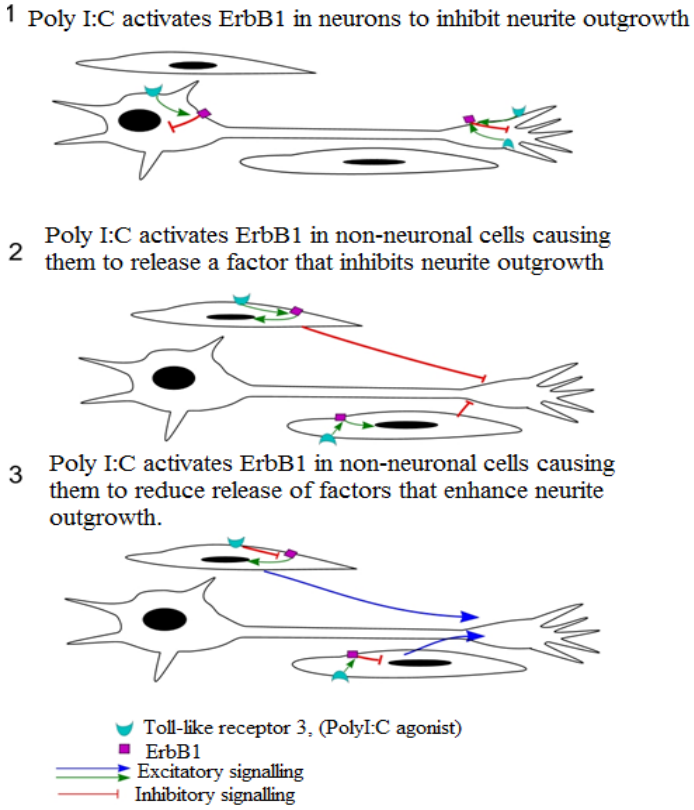


Figure 5.0: Some models for the regulation of axonal growth by factors derived from non-neuronal cells in dorsal root ganglia.

1. Direct inhibitory effects on neurons.

2. Causing non-neuronal cells to release a substance that inhibits neurite outgrowth.

3. Causing non-neuronal cells to stop releasing a substance that promotes neurite outgrowth.

Conditioned media obtained from cultures of many types of cells have been shown to enhance neurite outgrowth in culture, presumably because the cultured cells release neurite-promoting factors. Cells producing neurite outgrowth-promoting factors include embryonic heart cells that enhance neurite outgrowth from chick ciliary ganglion neurons (Collins, 1978) and Schwann cells that enhance neurite outgrowth from mouse DRG and chick sympathetic neurons (Varon et al., 1981). Cultured cells from most mammalian tissues produced conditioned media capable of enhancing neurite outgrowth from chick ciliary ganglion or mouse DRG neurons, although the effects on foetal CNS neurons were less obvious (Adler et al., 1981). Early studies showed that much of the neurite-promoting activity took the form of factors that bound to the substrate (Collins, 1978), although neurotrophic factors including NGF and CNTF were also soon implicated (Adler and Varon, 1980;Collins and Dawson, 1982). The substrate-bound neurite-promoting properties of conditioned media were found to be largely the result of laminin and heparin sulphate proteoglycans which adhered to substrates coated with poly-ornithine or poly-lysine (Davis et al., 1987;Lander et al., 1982;Lander et al., 1985). Subsequently, it was shown that a variety of neurotrophic factors and cytokines could be found in conditioned media, e.g. PDGF, LIF and BDNF in astrocyte-conditioned medium (Gard et al., 1995;Jean et al., 2008), IGF, NT3 and PDGF in neonatal Schwann cell conditioned medium (Meier et al., 1999), and Schwann cell-derived factors that do not require receptor kinases (Bampton and Taylor, 2005;Taylor and Bampton, 2004). At least some of the neurotrophic factors that have been found in conditioned media have been shown to disinhibit neurite outgrowth in the presence of CNS myelin (Ahmed et al., 2006;Logan et al., 1994;Logan et al., 2006). These authors have suggested that the means by which inhibitors of ErbB1 kinase disinhibit neurite outgrowth is primarily by stimulating the production of such factors.

We have shown that neurons from ErbB1-/- mice, grown in the presence of Poly I:C or CNS myelin are disinhibited and that inhibitors of ErbB1 have no effect on such cultures. The aims of this study were 1) to test the ability of ErbB1-/- cells to promote neurite outgrowth from ErbB1-expressing neurons and 2) to test the ability of medium conditioned by ErbB1-/- and wild type non-neuronal cells to promote or disinhibit neurite outgrowth.

Methods

For a detailed account of the methods please refer Chapter-2.

Experiments using the cell tracker, CFSE:

DRG cells were cultured from wildtype/heterozygous and ErbB1-/- animals aged between P7-P9. These experiments were designed such that neurite outgrowth could be measured from wildtype/heterozygous and ErbB1-/- neurons grown in the same well of an 8 well-chambered slide coated with CNS myelin. The aim was to test if neurite outgrowth from wildtype/heterozygous neurons were affected by the presence of ErbB1-/- neurons and non-neuronal cells and vice-versa. As βIII tubulin acts as a general marker for neurons, an ideal way to distinguish between ErbB1-/- and wildtype/ heterozygous neurons would be to treat one set of neurons (either ErbB1-/- or wildtype) with a cell tracker (CFSE- that stains neurons green). The diagram below explains the design of the experiment.

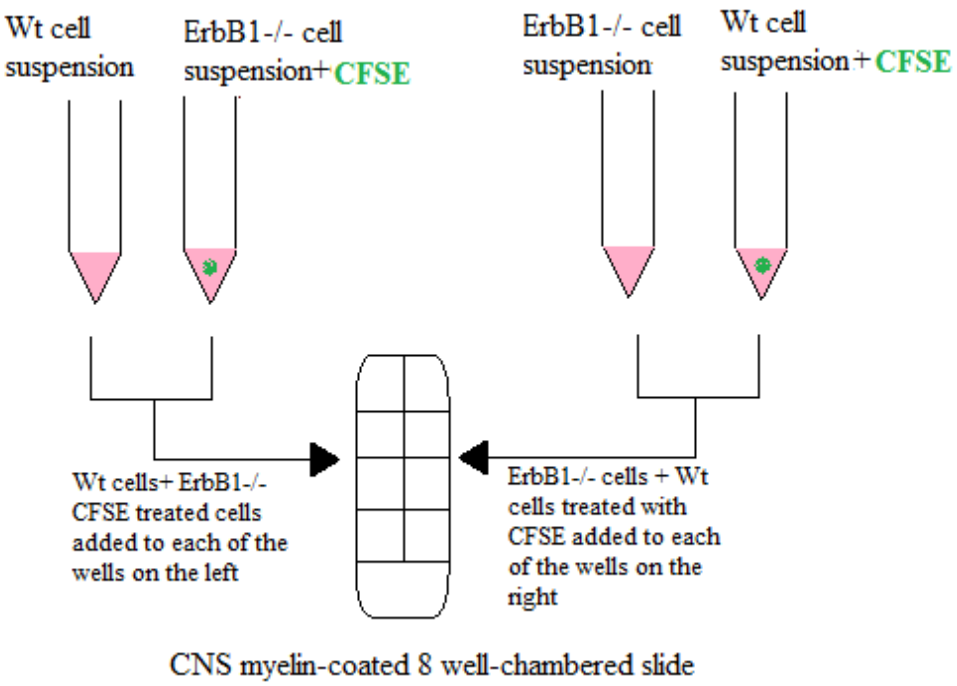


Figure 5.1: Diagram describing experiments with the cell tracker CFSE to test the effects of ErbB1-/- non-neuronal cells and neurons on neurite outgrowth from wildtype neurons on CNS myelin.

All neurons were immunostained with red βIII tubulin. Neurons that were CFSE and βIII tubulin-positive were photographed and neurite lengths were measured using Image J. As a control, wildtype neurons treated with CFSE were plated on wells containing wildtype neurons that were not treated with CFSE (not shown in figure 5.1). The same applied to ErbB1-/- cells. The reason for doing so was because comparisons on neurite outgrowth were made only between cells treated with CFSE as such treatment reduced neurite outgrowth compared to cells that were not treated with CFSE.

Experiments using conditioned media:

Conditioned media was collected from non-neuronal cells cultured in 24-well dishes for a period of 3 days as described in Chapter-2. Some of the wells containing non-neuronal cells from ErbB1-/- or wildtype/ heterozygous animals were treated Poly I:C. Conditioned media was added

to DRG neurons from ErbB1-/- and wildtype/heterozygous animals at a concentration of 1:10 at the time of plating neurons. The experimental design has been illustrated in figure 5.2.

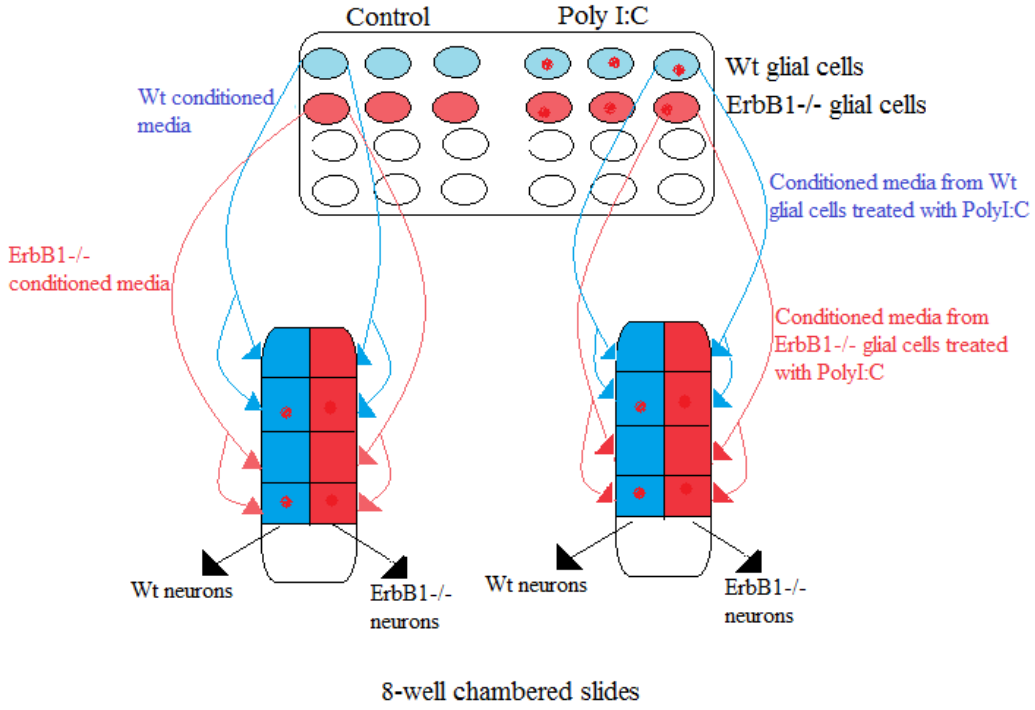


Figure 5.2: Illustration describing experiments with conditioned media. Red circles indicate wells treated with Poly I:C. Blue wells contain cells from wildtype (Wt) animals and red wells contain cells from ErbB1-/- animals.

Similarly, conditioned media was also collected from DRG neuronal cultures in which cells were plated at a density of 10^4 cells/ well and grown for a period of 3 days.

Neurons cultured in 8-well chambered slides were grown for a period of 24-hrs after which cells were fixed using 4% PFA and immunostained for βIII tubulin. Photomicrographs of βIII tubulin positive neurons were captured and neurite lengths measured using Image J.

Reverse Transcriptase (RT) PCR:

cDNA was obtained from cultured Schwann cells and DRG. The primers used to detect ErbB1 transcripts were:

- exon 1-CTCCCAGACAGACGACAGGT and exon 2 TGCCTTGGCAGACTTTCTTT.

- exon 1 GGGGCGTTGGAGGAAAAGAA and exon 7-ATGAGTGGTGGGCAGGTG.

The PCR reaction was as follows:

94°C - 2min
94°C - 1min.
58°C - 15sec. } X 32 cycles
72°C - 30sec.
72°C - 5min
4°C - ∞

Results

ErbB1-/- cells promote neurite outgrowth from wild type neurons.

First, the ability of ErbB1-/- cells to promote neurite outgrowth from ErbB1-expressing cells in the presence of CNS myelin was assessed. Neurite outgrowth from ErbB1-/- neurons and wildtype neurons was measured when they were grown in the presence of cells (neurons and non-neuronal) of the other genotype. βIII tubulin immunohistochemistry was used to identify all neurons in the cultures. Neurons of the genotype under investigation were labelled with a cell tracker -CFSE (green). The mixed cultures were grown on a myelin substrate for a period of 48hrs, fixed and stained and the lengths of neurites measured. The original plan was to measure both the green cell-tracker and the non-green neurons in the same cultures. However, treatment with the cell tracker (dissolved in DMSO) reduced neurite outgrowth from the treated cells, making direct comparison of neurite lengths in the same cultures of little value.

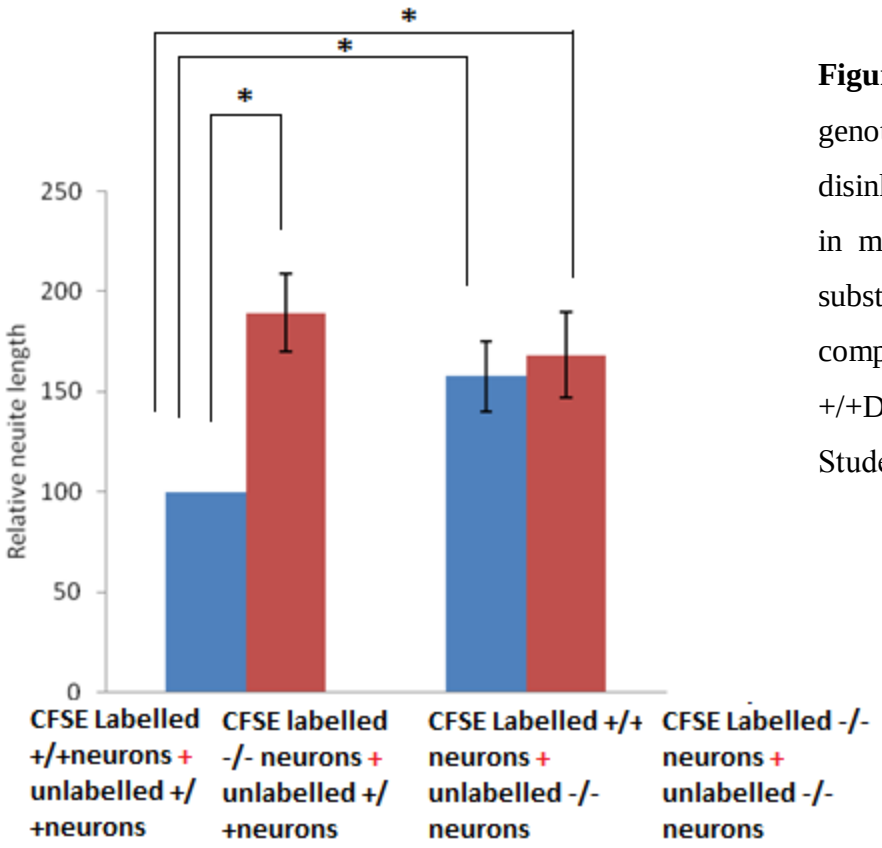


Figure 5.3: Effect of cell genotype on their ability to disinhibit neurite outgrowth in mixed cultures on myelin substrate. *p<0.001 when compared to +/+neurons in +/+DRG culture. n=5, Student's t test.

The presence of ErbB1-/- neurons and glia enhanced neurite outgrowth from wild type neurons in the presence of CNS myelin. Neurite outgrowth from ErbB1-/- neurons remained unaffected by the addition of ErbB1-/- or wildtype neurons and non-neuronal cells. These results do not show whether it was neurons or non-neuronal cells that exerted the growth-promoting effects, or whether secreted substances or cell contact was responsible.

Conditioned medium from neuron-enriched DRG cultures does not promote neurite outgrowth.

Conditioned medium was obtained from DRG cultures (10^4 neurons/well) grown for 3 days. Poly I:C, an inhibitor of neurite outgrowth which we have shown to require ErbB1 activity, was added to some wells. Conditioned media was collected and used immediately, being mixed with fresh culture medium at a concentration of 1:10 and its effects on neurite outgrowth from ErbB1 +/+ and ErbB1 -/- DRG neurons assessed in the presence and absence of Poly I:C.

Conditioned medium from none of the cultures enhanced neurite outgrowth with or without Poly I:C. It is therefore unlikely that the enhancement of neurite outgrowth on the co-culture experiments was due to factors released into the medium by neurons or their associated satellite cells.

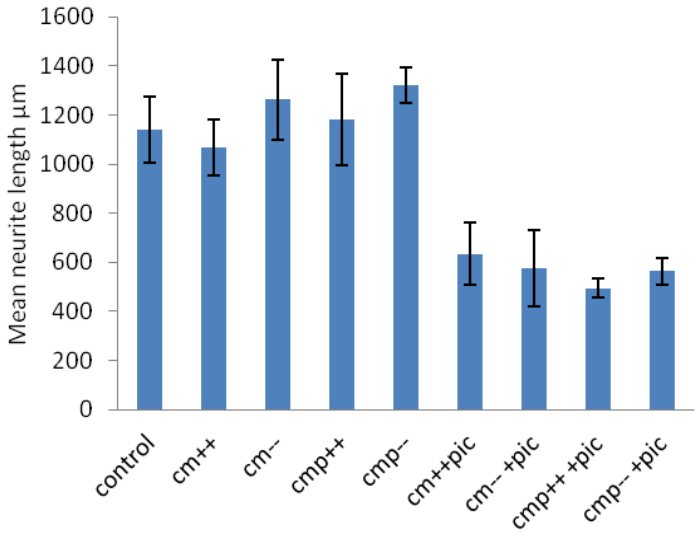


Figure 5.4: Effect of conditioned media from neuronal cultures. Conditioned media does not affect neurite outgrowth from DRG neurons.

CM++ = conditioned media from wildtype neuronal cultures;

CM-- = conditioned media from ErbB-/- cultures; pic denotes addition of Poly I:C. N=3.

CMP++ = conditioned media from wildtype cultures grown in the presence of Poly I:C;

CMP-- = conditioned media from ErbB1-/- cultures grown in the presence of Poly I:C;

Factors secreted into the medium by cultured non-neuronal cells promote neurite outgrowth from wildtype and ErbB1 -/- neurons.

Next, the ability of conditioned media from cultures of non-neuronal cells to enhance neurite outgrowth was assessed. During the normal purification of DRG neurons, non-neuronal cells are separated from the neurons by centrifugation with a cushion of bovine serum albumin (BSA). Non-neuronal cell cultures were prepared by taking cells from the BSA cushion during DRG dissociation. Non-neuronal cells were then seeded onto 24-well plates at a density of 40,000 cells per well. Cells in some of the wells were treated with Poly I:C, an inhibitor of neurite outgrowth which we have shown to require ErbB1 activity. The cells were allowed to grow for three days and conditioned media was collected and used immediately, being mixed with fresh culture medium at a concentration of 1:10 and its effects on neurite outgrowth assessed. Conditioned media from wildtype non-neuronal cells as well as ErbB1-/- non-neuronal

cells improved neurite outgrowth from wildtype neurons growing on a normal substrate, suggesting that neurite-promoting factors were secreted into the media by non-neuronal cells.

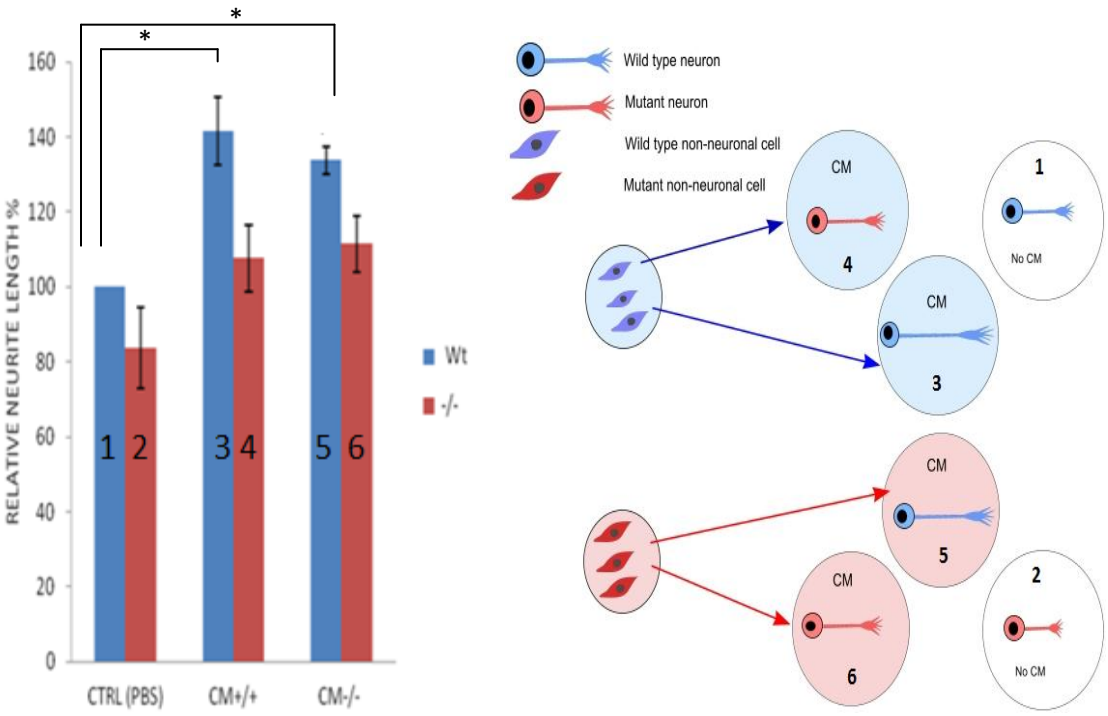


Figure 5.5: Effect of conditioned media from wildtype and ErbB1 KO non-neuronal cells on wildtype and KO DRG neurons. CM+/+: Neurons cultured with conditioned media from wildtype non-neuronal cells, CM-/- : Neurons cultured with conditioned media from ErbB1-/- non-neuronal cells.*$p<0.001$ when compared to wildtype neurons treated with PBS. n=4, Student's t test. The diagram on the right shows the plan of these experiments.

Conditioned media from Poly I:C treated wildtype or ErbB1-/- non-neuronal cells improves neurite outgrowth from both wildtype and ErbB1-/- neurons growing on a normal substrate .

Next, the ability of conditioned media from Poly I:C-treated non-neuronal cells to promote neurite outgrowth was tested. Conditioned media from cultures of wildtype as well as Erb1-/- non-neuronal cells improved neurite outgrowth from both wildtype and ErbB1-/- neurons grown on a normal substrate in the absence of Poly I:C. These results suggest that non-neuronal

cells of either phenotype, when treated with Poly I:C, continue to secrete substances that can enhance neurite outgrowth . Poly I:C does not inhibit neurite outgrowth by reducing secretion of such factors but seems to promote the secretion of neurite-promoting factors.

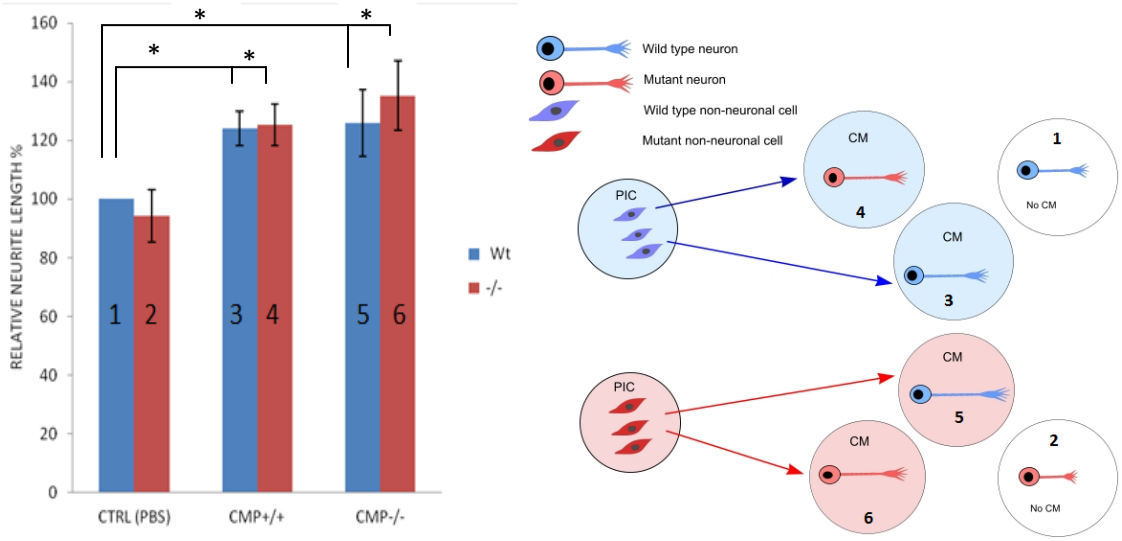


Figure 5.6: Effects of conditioned media from Poly I:C treated wildtype and ErbB1-/- glial cells on wildtype and ErbB1-/- DRG neurons . CMP+/+ = Conditioned media from poly I:C treated wildtype glial cells, CMP-/- = Conditioned media from poly I:C treated ErbB1-/- glial cells. *p<0.05 when compared to wildtype neurons treated with PBS. n=4, Student's t test.

Conditioned media from wildtype and ErbB1-/- non-neuronal cells disinhibits neurite outgrowth from wildtype DRG neurons treated with Poly I:C.

The ability of conditioned media to enhance neurite outgrowth from DRG neurons cultured in the presence of Poly I:C was then tested. In the presence of Poly I:C, neurite outgrowth from wildtype neurons was more strongly inhibited than that from ErB1-/- neurons. Addition of conditioned media from either wildtype non-neuronal cells or ErbB1-/- non-neuronal cells produced a modest enhancement (approx 150%) of neurite outgrowth from wildtype

neurons treated with Poly I:C . However, conditioned media from either type of non-neuronal cell failed to further enhance neurite outgrowth from Poly I:C-treated ErbB1-/- neurons.

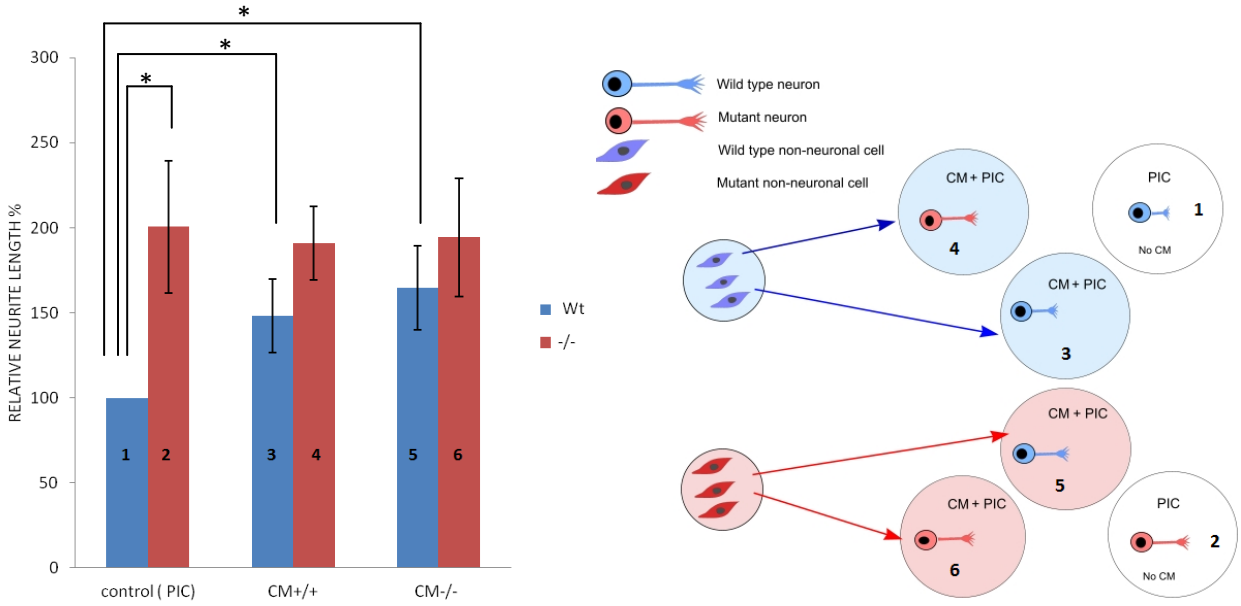


Figure 5.7: Effect of conditioned media from wildtype and ErbB1 KO glial cells on Poly I:C treated wildtype and KO DRG neurons. *p<0.05 when compared to wildtype neurons treated with Poly I:C; **p<0.05 when compared to wildtype neurons treated with Poly I:C n=4, Student's t test

Conditioned media from Poly I:C-treated wildtype non-neuronal cells strongly disinhibits neurite outgrowth from Poly I:C-treated wildtype neurons but conditioned medium from ErbB1-/- non-neuronal cells was less effective.

The ability of conditioned medium from Poly I:C- treated glia to enhance neurite outgrowth in the presence of Poly I:C was tested. Conditioned media from Poly I:C treated wildtype non-neuronal cells greatly enhanced (approx 250%) neurite outgrowth from Poly I:C treated wildtype neurons. This suggests that Poly I:C stimulates wildtype glia to secrete higher levels of growth-enhancing substances. In contrast, conditioned medium from ErBb1-/- non-neuronal cells only slightly improved neurite outgrowth of wildtype neurons treated with Poly I:C. Poly I:C treatment did not enhance the efficacy of conditioned media from ErbB1-/- non-neuronal cells on neurite outgrowth from Poly I:C- treated wildtype neurons, suggesting that

Poly I:C does not increase the secretion of neurite–promoting factors from the mutant glia. These results are compatible with the hypothesis that Poly I:C stimulates the secretion of neurite-promoting factors from glia via a pathway involving ErbB1. Neither type of conditioned medium had any effects on neurite outgrowth of ErbB1-/- neurons treated with Poly I:C.

Poly I:C probably acts through ErbB1 on both neurons and non-neuronal cells. Activation of ErbB1 on neurons probably reduces axon outgrowth. In contrast activation of ErbB1 on non-neuronal cells causes them to release a substance or substances that act on the neurons to reverse the inhibition otherwise caused by ErbB1 activation on the neuronal surface.

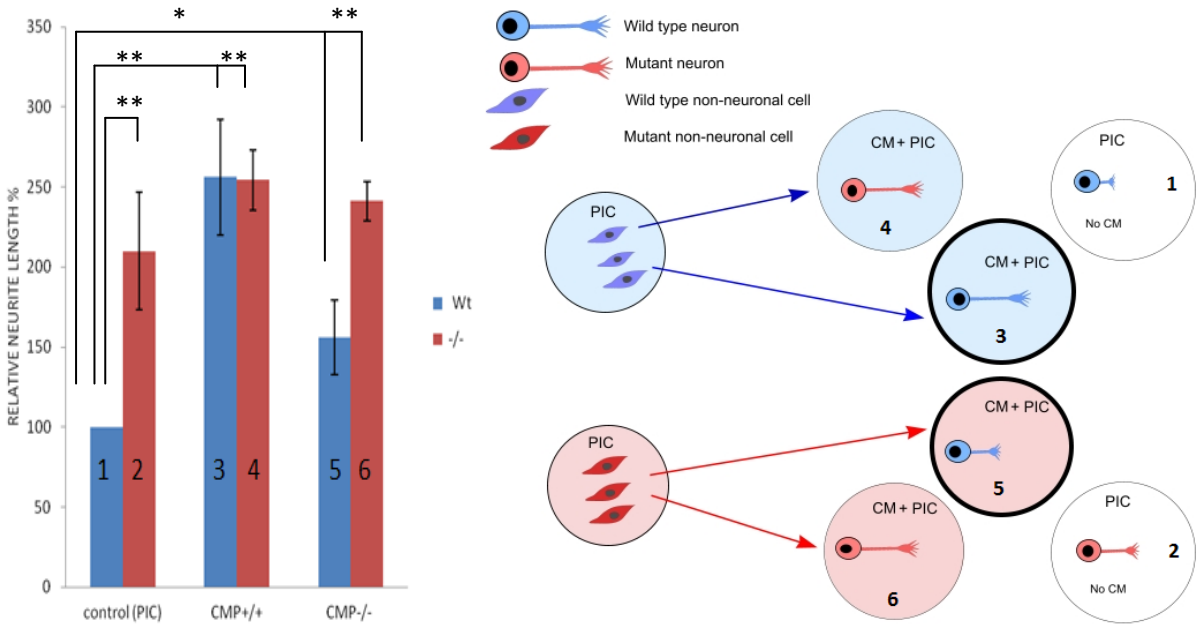


Figure 5.8: Effect of conditioned media from Poly I:C treated wildtype and ErBb1 KO glial cells on Poly I:C treated wildtype and Erb1-/-DRG neurons. **p<0.005 when compared to wildtype neurons treated with Poly I:C; *p<0.05 when compared to wildtype neurons treated with Poly I:C. n=4, Student's t test.

Cultured Schwann cells do not express ErbB1 mRNA.

The next step was to examine which cells might release molecules that disinhibit neurite outgrowth. For neurons or Schwann cells to be affected by the inhibition/deletion of ErbB1 they would have to express the receptor in the first place. There are several claims that neurons express ErbB1 (Huerta et al., 1996;Gomez-Pinilla et al., 1988;Werner et al., 1988). While it is generally assumed that the most important non-neuronal cells for the support of regenerating axon is the Schwann cell, most of the literature suggests that Schwann cells do not express ErbB1 and do not respond to EGF (Massa et al., 2006;DeClue et al., 2000). However, there are some claims to the contrary (Huerta et al., 1996c;Vega et al., 1994). It was therefore decided to investigate whether cells in DRG mixed cultures or Schwann cell cultures expressed ErbB1 mRNA.

The mutation used to create ErbB1-/- mice (EGFR Tm1 mag; Jackson's Laboratory) involved targeted disruption and replacement of 155bp surrounding the splice acceptor site in exon 2 with a Neo cassette . The mutation caused aberrant splicing around the targeted exon which led exon1 to join to exon3 or exon5. PCR primers in exon1 and 7, as previously published (Threadgill et al., 1995), gave rise to a single PCR product in wildtypes with no detectable protein product.

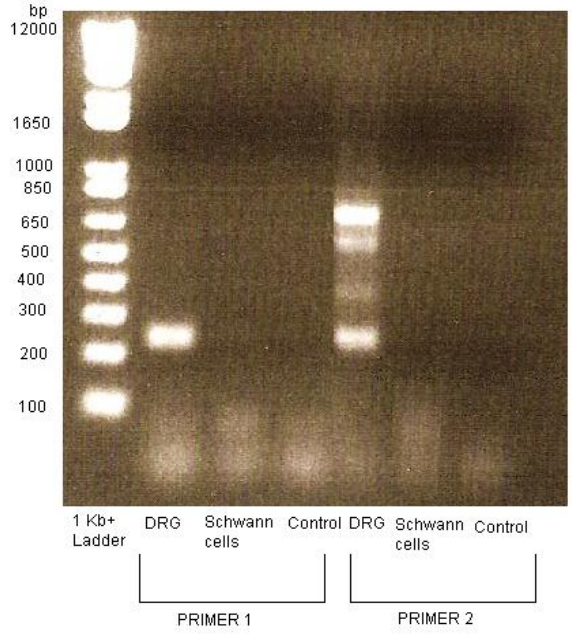


Figure 5.9: Gel showing the absence of ErbB1 mRNA from Schwann cell cultures but its presence in DRG cultures. RT PCR products from ErbB1 transcripts in DRG mixed cultures and Schwann cell cultures. Note the single band at 242bp generated from DRG cultures with the primer set 1 (exon 1-CTCCCAGACAGACGACAGGT and exon2-TGCCTTGGCA GACTTTCTTT) and the absence of a band in the Schwann cell lane. The second set of primers (exon1-GGGGCGTTGGAGGAA AAGAA and exon7-ATGAGTGGT GGG CAGGTG) demonstrates some splice variants also reported by Threadgill et al. 1995. The control lane is H20 to check for contamination.

Due to the difficulty in obtaining pure cultures of dorsal root ganglion neurons, it was necessary to investigate the presence of the receptor in other cell types that may be present in mixed cultures of dorsal root ganglia neurons. As data shown previously, approx 60-70% of non-neuronal cells in the DRG cultures are S100 positive, indicating that a large proportion of cells present in these cultures are Schwann cells and satellite cells. PCR revealed that ErbB1 was expressed in DRG cultures but was not expressed by Schwann cells. Hence, it is most likely that ErB1 is expressed by DRG neurons and non-neuronal cells other than Schwann cells (e.g. satellite cells, endothelial cells etc.).

Discussion

In previous chapters Poly I;C was shown to be a potent inhibitor of neurite outgrowth, whose effects were reduced by the inhibition or genetic deletion of ErbB1. In this chapter it has been shown that conditioned media from non-neuronal cells can improve neurite outgrowth from wild type neurons cultured in the presence of Poly I:C. However, the most potent conditioned medium was produced by wild type non-neuronal cells treated with Poly I:C. ErbB1 mRNA was shown to be absent from Schwann cell cultures but present in DRG cultures, which implies that it is expressed by neurons and/or satellite cells. Finally, neurite outgrowth from wildtype neurons cultured in the presence of CNS myelin extended longer neurites when they were co-cultured with ErBb1-/- DRG neurons and non-neuronal cells but not when they were co-cultured with additional wild type cells.

Molecules secreted by non-neuronal cells improves neurite outgrowth in the presence of Poly I:C but their activity does not explain the effects of inhibition or deletion of ErbB1.

Conditioned media from wildtype and ErbB1-/- non-neuronal cells promoted neurite outgrowth from wild-type DRG neurons to a similar extent. However, conditioned medium had no effect on neurite outgrowth from ErbB1 -/- neurons cultured in the presence of Poly I:C. It has been proposed that the neurite-promoting effects of ErbB1 kinase inhibitors is the result of increased production of such neurotrophic factors by glia (Ahmed et al., 2009;Ahmed et al., 2010). If the disinhibitory effects on neurite outgrowth of ErbB1 inactivation were mediated through a similar mechanism, the predicted outcome of the conditioned medium experiments

would be that media conditioned from ErbB1-/- non-neuronal cells grown in the presence of Poly I:C should stimulate better neurite outgrowth from wildtype neurons treated with Poly I:C. This was not the case; although medium conditioned by ErBb1-/- non-neuronal cells promoted neurite outgrowth in the presence of Poly I:C, the neurite-promoting effects of media conditioned by wildtype non-neuronal cells in the presence of Poly I:C was far greater. Hence non-neuronal cells can secrete neurite-promoting factors but their secretion is not enhanced by the absence of ErbB1 activity. Only a modified version of Model 1 of those suggested at the beginning of this chapter is compatible with the results of these experiments (see Figure 5.10). The secretion of non-neuronal-derived neurite-promoting factors does not explain the enhancement of neurite outgrowth in the absence of ErbB1 signalling.

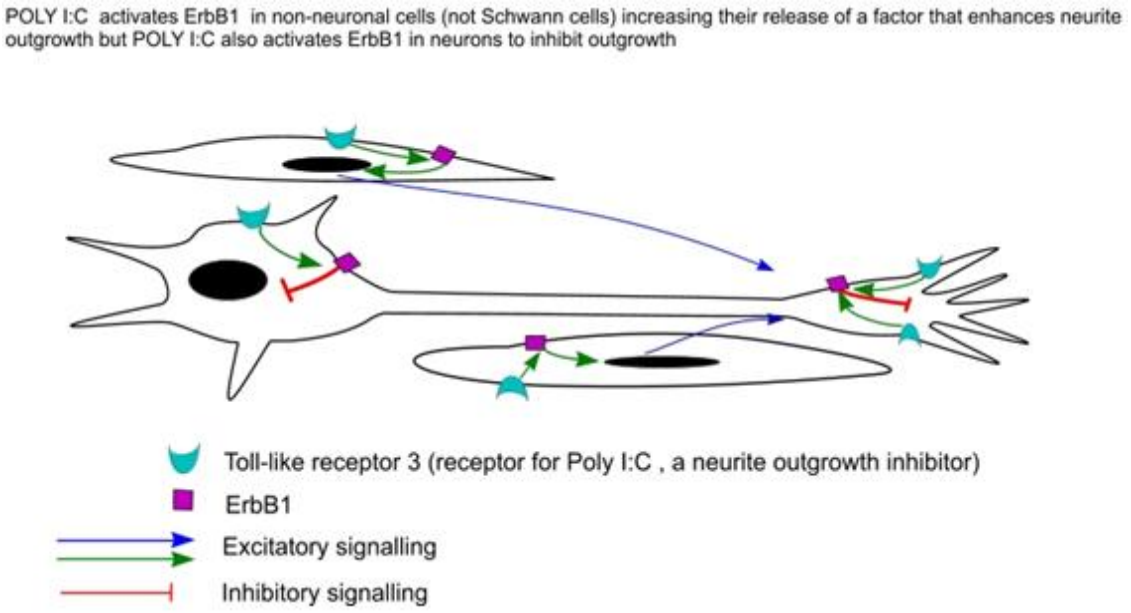


Figure 5.10: Model constructed on the basis of the results in this chapter, showing the involvement of ErbB1in the inhibition of neurite outgrowth by Poly I:C.

Differential effects on neurite outgrowth of conditioned media from knockout and wildtype non-neuronal cells were observed only in cases when the non-neuronal cells had been treated with Poly I:C . Poly I:C- treated wild type non-neuronal cells (i.e. cells with activated TLR3) secreted molecules that were very potent promoters of neurite outgrowth. Presumably

there are insufficient numbers of the appropriate non-neuronal cells in the DRG cultures to disinhibit the neurons in the presence of Poly I:C.

ErbB1 is absent from Schwann cell cultures but present in DRG cultures.

RT (reverse transcriptase) PCR of cDNA reverse transcribed from RNA isolated from wildtype/heterozygous DRG cultures and Schwann cell cultures showed that ErbB1 mRNA is detectable in DRG cultures but absent from Schwann cell cultures. The ErbB1-expressing cells in DRG cultures were, therefore, most probably neurons and/or satellite cells. Hence, it can be inferred that ErbB1-kinase inhibitors and the genetic deletion of ErbB1 should most likely affect neurons and satellite cells. This is compatible with the observation that medium conditioned by ErbB1-/- non-neuronal cells (lacking neurons and satellite cells) treated with Poly I:C was less effective at promoting neurite outgrowth in the presence of Poly I:C. However, the absence of ErbB1 mRNA from Schwann cells shows that ErbB1 inhibitors do not enhance neurite outgrowth through effects on Schwann cells.

A previous study (Ahmed et al., 2009) showed that conditioned medium from DRG cultures grown in the presence of CNS myelin and treated with an ErbB1 inhibitor, but not conditioned medium from DRG cultures grown with myelin in the absence of the ErbB1 inhibitor, contained elevated levels of NGF, BDNF and NT3. The neurotrophins were secreted at levels that were shown to be capable of partially disinhibiting neurite outgrowth. This led to their hypothesis that ErbB1 reduces secretion of neurotrophins from DRG neurons or satellite cells. Further experiments by Ahmed et al. (2009) showed that the enhancement of neurite outgrowth by AG1478-treatment was greatly attenuated if the proliferation of glia was inhibited. Although the experiments of Ahmed et al. (2009) are not directly comparable with the present study (see Table 5.1), they suggest a strikingly different mechanism for the disinhibition of neurite outgrowth by ErbB1 inhibitors, dependent mainly on enhanced non-neuronal secretion of neurotrophins. In the present study Poly I:C-treated ErbB1-expressing non-neuronal cells produced the most efficacious conditioned medium.

	The present study	**Ahmed et al. (2009)**
Inhibitor of neurite outgrowth	Poly I:C	CNS myelin
Mechanism of reduction of ErbB1 activity	Genetic deletion of ErbB1	Pharmacological treatment with AG1478 or PD168393
Cells used for assay of neurite outgrowth	Neonatal mouse DRG	6-8 week-old rat DRG
Effects of ErbB1 inhibition /deletion	Disinhibited neurite outgrowth (not more than cultures lacking Poly I:C)	Enhanced neurite outgrowth (more than cultures lacking CNS myelin)
Cultures used to generate conditioned medium	Mouse DRG non-neuronal cells	Rat DRG cultures (including neurons and satellite cells)
Nature of the neurite-promoting factors in conditioned medium	Unknown	Neurotrophins
Condition with maximum secretion of disinhibitors of neurite outgrowth	Poly I:C-treated non-neuronal cells expressing ErbB1	AG1478-/PD168393 treated DRG (ErbB1 inactivated)
Effects of inactivation of ErbB1 on neurite outgrowth in the absence of neurite outgrowth inhibitors	No enhancement of outgrowth	Not stated

Table 5.1: Some comparisons of the current study with that of Ahmed et al. (2009).

Our experiments show that ErbB1 inhibitors or the genetic deletion of ErbB1 does not promote neurite outgrowth under control conditions (e.g. cultures lacking CNS myelin or Poly I:C). If ErbB1 inhibitors enhanced neurotrophin secretion, they would be expected to enhance

neurite outgrowth even under control conditions. Such experiments were not reported in the experiments by Ahmed et al. (2009) although AG1478 produced greater neurite outgrowth on myelin than was obtained in the absence of myelin or AG1478. As discussed in earlier chapters, Ahmed et al. believed that ErbB1 kinase inhibitors enhanced neurite outgrowth though off-target effects, presumably on another kinase, but we have shown that their effects on neurite outgrowth are dependent on the presence of ErbB1.

ErbB1 -/- cells disinhibit neurite outgrowth from wild type DRG neurons in co-cultures.

The ability of ErbB1-/- cells to promote outgrowth from wildtype DRG neurons in co-cultures containing CNS myelin could in theory have been due to the effects of trophic factors released by the ErbB1 -/- cells. However, ErbB1 +/+ non-neuronal cells treated with poly I:C secreted more potent neurite-promoting factors that ErbB1-/- non-neuronal cells. Hence the disinhibition found in co-cultures may have been the result of direct cell/cell contact or trophic factors released by cells that were not present in the glial cultures i.e. neurons. One way in which the conditioned medium experiments can be made compatible with the co-culture experiments is by assuming that ErbB1 on neurons, or possibly on satellite cells attached to neurons, controls the release of neurite-promoting substances into the medium. Wild type neurons grown in the presence of ErbB1 knockout neurons (and consequently satellite cells) on a myelin substrate would then be disinhibited by factors released by the ErbB1-/- neurons and satellite cells. However, conditioned medium from cultures enriched in neurons did not disinhibit neurite outgrowth irrespective of the genotype, suggesting that neurons are not a potent source of factors capable of overcoming the effects of Poly I:C etc. Hence, the co-culture experiment allows the intriguing speculation that direct contact between neurons expressing ErbB1 and cells lacking ErbB1 may mediate disinhibition of neurite outgrowth.

Conclusion

In summary, we have shown that ErbB1-/- cells can disinhibit neurite outgrowth from ErbB1-expressing DRG neurons in co-cultures, that non-neuronal cells (glia) isolated from DRG secrete molecules capable of disinhibiting neurite outgrowth in the presence of PolyI:C and that ErbB1-expressing glia secrete more potent disinhibitory molecules than ErbB1-/- glia.

Chapter-6 Role of PTEN in ErbB1 mediated inhibition of neurite outgrowth

Introduction

PTEN –Phopshatase and Tensin homolog deleted on chromosome 10 was first discovered in 1997 (Li et al., 1997;Li and Sun, 1997) as a tumour suppressor gene which, when aberrantly expressed, causes malignant gliomas. Recent studies have shown that deleting PTEN induces robust regeneration of injured CNS axons allowing them to grow beyond the lesion site (Liu et al., 2010;Park et al., 2008;Sun et al., 2011). Using optic nerve crush as a CNS injury model, Park et al. (2008) demonstrated that deleting PTEN in retinal ganglion cells of adult mice greatly enhances regeneration, and that the principal signalling pathway leading to outgrowth is one that involves mTOR (Mammalian Target Of Rapamycin) (Park et al., 2008;Park et al., 2010). Apart from the effects of deleting PTEN in CNS axons, it has also been reported that PTEN knockdown using siRNA promotes regeneration of injured sciatic nerve in adult rats (Christie et al., 2010).

PTEN has been shown to be expressed by DRG neurons, particularly IB4+ve DRG cells (Christie et.al., 2010). The phosphorylated form of PTEN has been reported to be present in the nuclei of large and medium sized DRG neurons. However, post-injury there is a decrease in expression of nuclear PTEN, which was interpreted as showing that the relative concentration in the cytoplasm increased (Christie et al., 2010).

Deletion of PTEN normally results in tumorigenesis. Conditional knock out of PTEN in neural stem cells and glial progenitor cells leads to large and abnormal brains (Groszer et al., 2001) and deletion of PTEN in mature neuronal populations of the cerebral cortex and hippocampus leads to neuronal hypertrophy, macrocephaly, abnormal axon growth, synapse number and impaired response to sensory stimuli (Groszer et al., 2001;Kwon et al., 2006). In view of these and other reports that PTEN plays an important role in neuronal physiology, we wanted to test the effects of blocking PTEN using a pharmacological inhibitor in the systems in which we have studied the role of ErbB1. Hence the aims of this study were to:

- Investigate the effects of pharmacologically blocking PTEN using the drug VO-OHpic (Mak et al., 2010) on the outgrowth of neurites from dorsal root ganglia and cerebellar granule neurons cultured in the presence of Poly I:C.
- To investigate if PTEN is a part of the signalling pathway from activated TLR3.
- To look for interactions between PTEN and ErbB1 signalling pathways in neurite inhibition.
- To investigate if calcium signalling is involved in the signalling pathway comprising PTEN, ErbB1 and TLR3.

Methods

For a detailed account on methods, please refer Chapter-2.

***DRG cultures*:**

DRG neurons were cultured from mice aged between P7-P9. Briefly, DRGs were extracted and subjected to enzymatic dissociation using collagenase (2mg/ml) dispase (5mg/ml). Cells were plated at a density of 1,500 cells/well and grown for a period of 24 hrs at 37°C.

***CGC cultures*:**

Cerebellar granule neurons were cultured from mice aged between P5-P7. The cerebellum was dissected out and enzymatically digested using 0.05% trypsin. CGCs were plated at a density of 65,000 cells/ well and grown for a period of 16hrs at 37°C.

Treatment of cultures with drugs

PTEN inhibitor

A pharmacological inhibitor of PTEN namely VO-OHpic trihydrate (Sigma) was dissolved in sterile distilled water to make up a stock solution of 5mM and stored at -20°C. Desired concentrations of 50nM, 100nM, 1µM and 10 µM were made by diluting in appropriate volumes of growth media which were directly added to cells at the time of plating and allowed to grow for a period of 16hrs or 24hrs at 37°C.

Poly I:C

Poly I:C (Sigma) was dissolved in sterile 0.01M PBS as described in Chapter-2. Poly I:C at a concentration of 100µg/ml was made up by diluting stock in growth media and added to cultures at the time of plating cells.

BAPTA-AM

Stock solutions of BAPTA-AM (Invitrogen) were made using 2.5% pluronic F-127 and DMSO. A working concentration of 100nM was made by dissolving appropriate volumes of the stock in growth media and then added to cultures at the time of plating cells.

Immunocytochemistry

DRG neuronal cultures were grown for 24 hrs whereas CGCs were cultured for a duration of 16hrs after which cultures were fixed using 4%PFA. Cultures were then immunostained using a rabbit antibody to βIII tubulin (Sigma, 1:1000) as a neuronal marker. Nuclei of all cells were visualised using Hoechst stain.

***Data analysis*:**

Photomicrographs of cells expressing βIII tubulin were taken. Approximately 30-60 neurons from each condition of an experiment were photographed and total neurite length was analysed using the software ImageJ. Experiments were repeated from 5 different animals (n=5) and statistical significance was computed using Student's t test.

Results

PTEN inhibitor VO-OHpic improves neurite outgrowth from mouse DRG neurons treated with Poly I:C.

A series of different concentrations of PTEN inhibitor, VO-OHpic -50nM, 100nM, 1µM and 10µM were added to cultured mouse DRG neurons grown on a poly-l-lysine and laminin substrate. It was observed that inhibiting PTEN in DRG neurons grown on a normal substrate seemed to have little or no effect on neurite outgrowth when compared to DRG neurons grown in

the presence of vehicle control (water) (Figure 6.1). However, at the highest concentration of the drug i.e. 10µM, a small reduction in neurite outgrowth was observed, although it was not statistically significant when compared to neurite outgrowth from neurons treated with water.

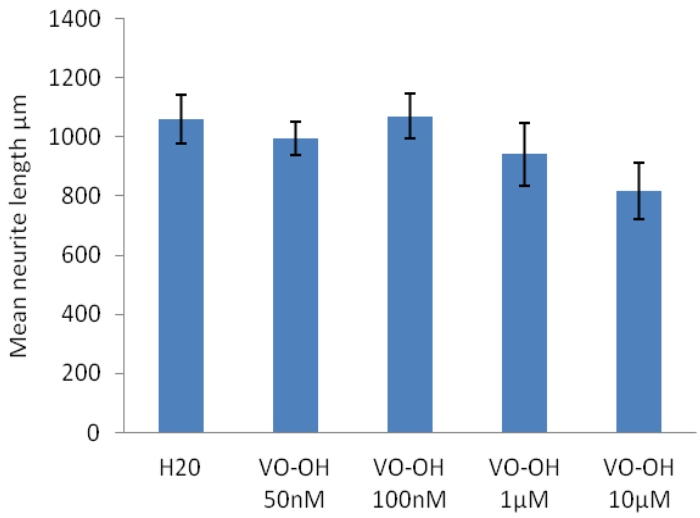


Figure 6.1: Effect of PTEN inhibitor VO-OHpic on neurite outgrowth from mouse DRG neurons grown on a poly-l-lysine laminin substrate for 24 hrs. N=5.

As shown previously, the addition of Poly I:C to DRG cultures caused inhibition of neurite outgrowth. In order to test if blocking PTEN activity improves neurite outgrowth from DRG neurons treated with Poly I:C, VO-OHpic was added at concentrations of 50nM, 100nM, 1µM and 10µM to these cultures. It was observed that the addition of all concentrations of VO-OHpic improved neurite outgrowth from neurons treated with Poly I:C (Figure6.2), even though they did not produce any significant effects on neurite outgtowth from neurons grown in the absence of Poly I:C. VO-OHpic used at concentrations of 50µM and 100µM proved most effective in enhancing neurite outgrowth and with a trend towards a decline in efficacy at the highest concentrations i.e. at 1 and 10µM.

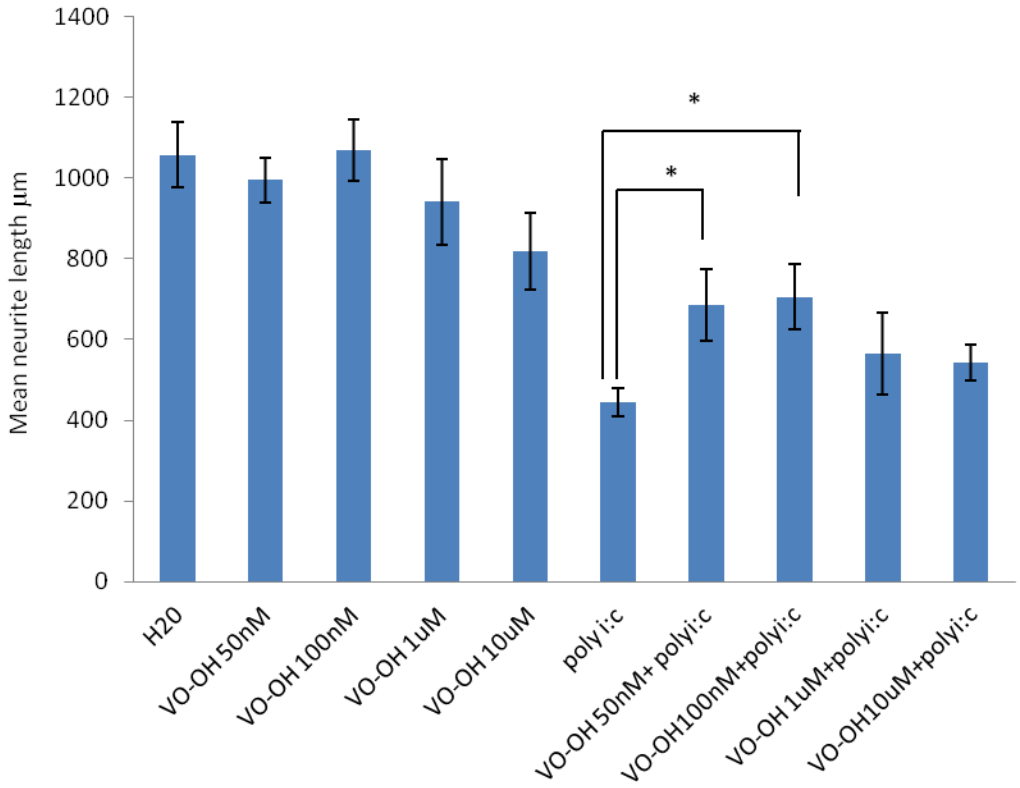


Figure 6.2: Effect of PTEN inhibitor VO-OHpic on neurite outgrowth from mouse DRG neurons treated with Poly I:C. Concentrations of VO-OHpic tested were 50nM, 100nm, 1µM and 10µM. *p<0.05 when compared to DRG neurons treated with PolyI:C; n=5, Student's t test.

Inhibiting both PTEN and ErbB1 promotes neurite outgrowth from mouse DRG neurons treated with Poly I: C but these effects are not additive:

Since blocking ErbB1 and PTEN individually promoted neurite outgrowth in the presence of Poly I:C, we then tested the effects of blocking both ErbB1 and PTEN on neurite outgrowth. Cultured DRG neurons were treated with pharmacological inhibitors to ErbB1 and PTEN, either in the presence or absence of Poly I:C. Neurons were treated with PD168393 (ErbB1 antagonist) at a concentration of 100nM or PD168393 (100nM) and VO-OHpic (PTEN antagonist) at concentrations of 50nM, 100nm, 1µM and 10µM .

It was observed that neurite outgrowth from mouse DRG neurons grown in the absence of Poly I:C was unaffected when both ErbB1 and PTEN were pharmacologically inhibited (Figure 6.3). However, in the presence of Poly I:C, the addition of PD168393 at 100nM and VO-OHpic at 50nM, 100nm and1µM significantly improved neurite outgrowth when compared to neurons treated with Poly I:C alone. No improvement in neurite outgrowth was observed at the highest concentration of VO-OHpic (10µM), as at this concentration, the drug proved detrimental to cells (they appeared to be dieing). However, there was no additive effect of using the two drugs together. It was observed that, in the presence of Poly I:C, the addition of both VOOHpic and PD168393 improved neurite outgrowth to a similar extent when compared to neurite outgrowth from neurons treated with PD68393 alone (Figure 6.3). One explanation of these results is that ErbB1 and PTEN converge on similar signalling pathways.

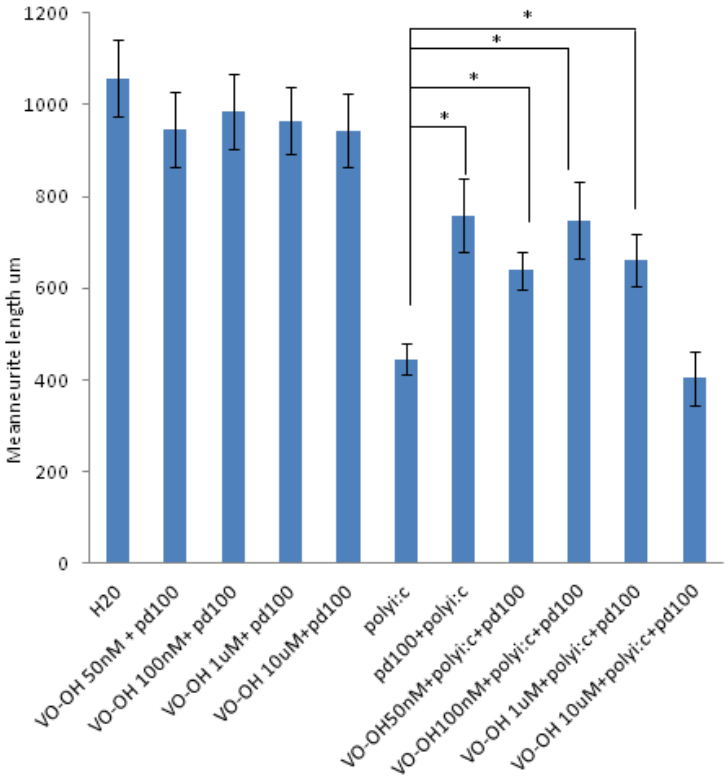


Figure 6.3: Effect of PTEN inhibitor VO-OHpic (50nM, 100nm, 1µM and 10µM) and ErbB1 inhibitor PD168393 (100nM) on neurite outgrowth from mouse DRG neurons treated with PolyI:C. *p<0.05 when compared to neurons treated with PolyI:C, n=5, Student's t test.

PTEN inhibitor VO-OHpic improves neurite outgrowth from mouse cerebellar granule neurons treated with Poly I:C.

To test the effects of blocking PTEN in neurons other than DRG neurons, mouse cerebellar granule cells (CGCs) were cultured and treated with VO-OHpic at concentrations of 50nM, 100nm, 1µM and 10µM. It was observed that at concentrations of 50nM, 100nM and 1µM, VO-OHpic had no effect on neurite outgrowth when compared to neurons treated with the vehicle control (water). However, at 10µM, a drastic reduction in neurite outgrowth was observed implying that the drug was toxic at this concentration (Figure 6.4).

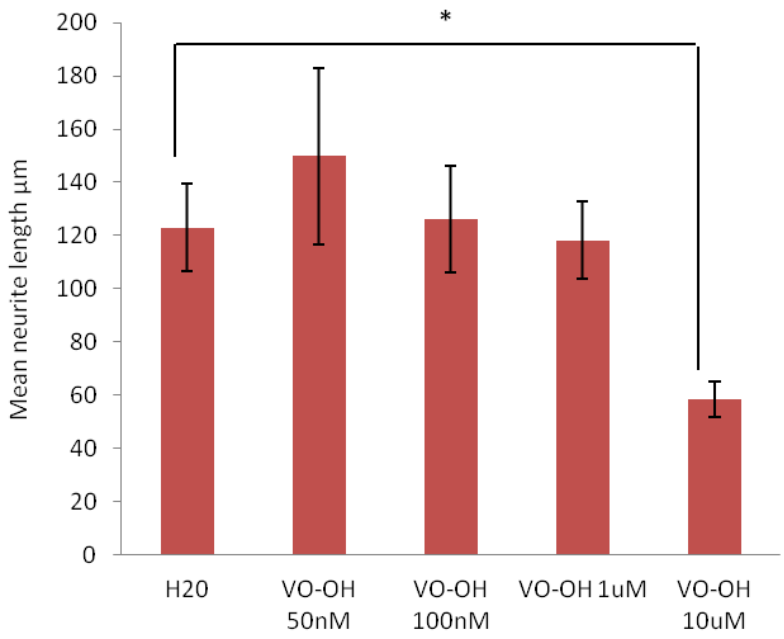


Figure 6.4: Effects of PTEN inhibitor VO-OHpic at concentrations of 50nM, 100nm, 1µM and 10µM on neurite outgrowth from cultured cerebellar granule neurons grown for a period of 16-20 hrs. *p<0.01 when compared to neurons treated with vehicle control- water; n=5, Student's t test.

As expected, when cerebellar granule neurons were treated with Poly I:C, a reduction in neurite outgrowth was seen when compared to neurons grown in the absence of Poly I:C (Figure 6.5). This shows that Poly I:C not only inhibits neurite outgrowth from DRG neurons but also cerebellar granule neurons. However, cerebellar granule neurons grown in the presence of Poly

I:C, when treated with PTEN inhibitor VO-OHpic at concentrations of 50nM, 100nM and 1µM showed an increase in neurite outgrowth when compared to neurons treated with Poly I:C alone. At the highest concentration of VO-OHpic (10µM) no improvement in neurite outgrowth was observed owing to the toxicity of the drug at such high concentrations. These results demonstrate that blocking PTEN improves neurite outgrowth from cerebellar granule neurons treated with Poly I:C.

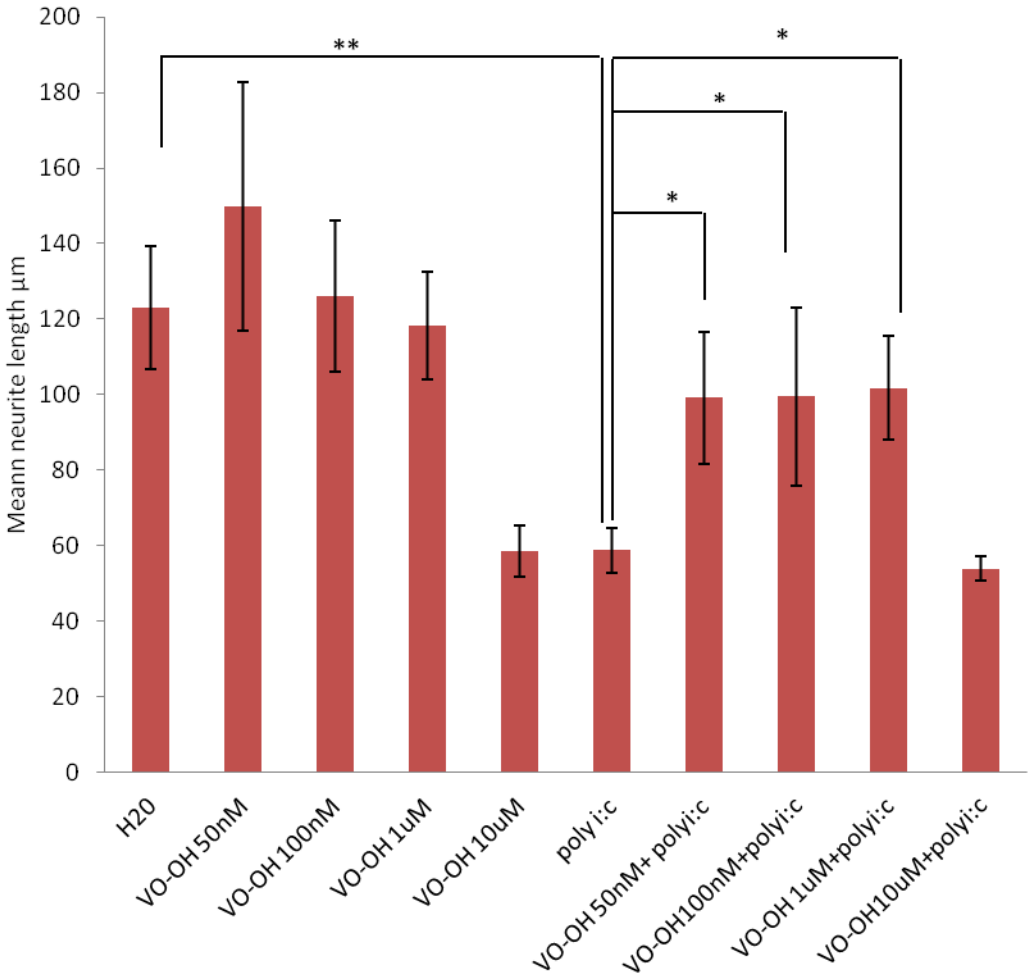


Figure 6.5: Effects of blocking PTEN using VO-OHpic at concentrations of 50nM, 100nm, 1µM and 10µM on neurite outgrowth from cerebellar granule neurons treated with PolyI:C. **p<0.01 when compared to neuron treated with water; *p<0.05 when compared to neurons treated with PolyI:C, n=5, Student's t test.

Inhibiting both PTEN and ErbB1 promotes neurite outgrowth from mouse cerebellar granule neurons treated with Poly I:C but these effects are not additive.

To test if blocking both PTEN and ErbB1 would produce additive effects on neurite outgrowth from mouse cerebellar granule neurons treated with Poly I:C, CGC cultures were treated with Poly I:C, PD168393 (100nM) and VO-OHpic (50nM, 100nm, 1µM and 10µM). In the absence of Poly I:C, the addition of PD168393 and VO-OHpic did not alter neurite outgrowth from cerebellar granule neurons irrespective of the concentration of VO-OHpic added when compared to neurons treated with vehicle control (water) (Figure 6.6).

In the presence of Poly I:C, as expected, it was observed that the addition of the ErbB1 antagonist improved neurite outgrowth from cerebellar granule neurons compared to neurons treated with Poly I:C alone. When both ErbB1 and PTEN antagonists were added to cultures treated with Poly I:C, an enhancement in neurite outgrowth was observed but only to a similar extent as seen in cultures treated with PD168393 alone (Figure 6.6). However, as was the case with DRG neurons, PTEN at a concentration of 10µM combined with PD168393, did not significantly enhance neurite outgrowth in the presence of Poly I:C. These results show that blocking both PTEN and ErbB1 in cerebellar granule neurons improves neurite outgrowth and similar to DRG neurons, these effects are not additive.

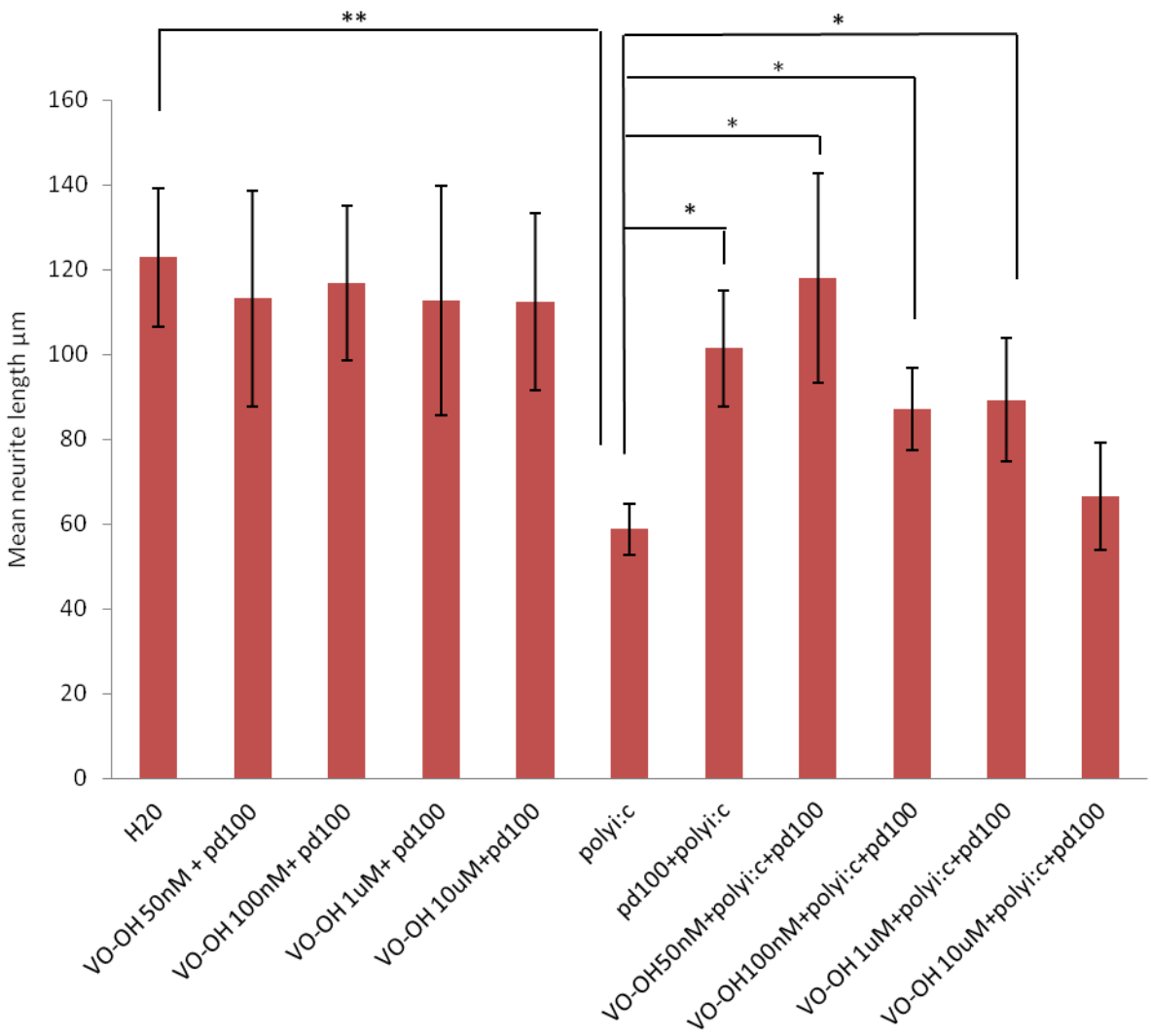


Figure 6.6: Effects of blocking PTEN using VO-OHpic (50nM, 100nM, 1µM and 10µM) and ErbB1 using PD168393 (100nM) on neurite outgrowth from mouse cerebellar granule neurons treated with Poly I:C grown for 16-20 hrs. **p<0.01 when compared to neuron treated with water; *p<0.05 when compared to neurons treated with Poly I:C, n=5, Student's t test.

Addition of BAPTA-AM , VO-OHpic or BAPTA-AM and VO-OHpic improves neurite outgrowth from DRG neurons in the presence of Poly I:C to similar extents.

As shown previously, buffering calcium using BAPTA-AM improves neurite outgrowth from DRG neurons treated with Poly I:C. In order to establish whether calcium signalling is

involved in the pathways by which PTEN reduces neurite outgrowth, calcium was buffered in VO-OHpic–treated DRG neurons. DRG cultures were treated with either BAPTA-AM (100nM), VO-OHpic (100nM) or BAPTA-AM and VO-OHpic. It was observed that in the absence of Poly I:C, the addition of BAPTA-AM and VO-OHpic did not significantly alter neurite outgrowth when compared to control (water). However, in the presence of Poly I:C, the addition of BAPTA-AM and PTEN inhibitor improved neurite outgrowth from mouse DRG neurons. It was also observed that the extent to which neurite outgrowth was enhanced was similar to improvements in outgrowth produced by the addition of either BAPTA-AM or VO-OHpic alone (Figure 6.7). Hence, the addition of both BAPTA-AM and VO-OHpic did not produce additive effects on neurite outgrowth in the presence of Poly I:C. These results show that calcium signalling is involved in the pathway that contains ErbB1, PTEN and TLR3 which leads to inhibition of neurite outgrowth.

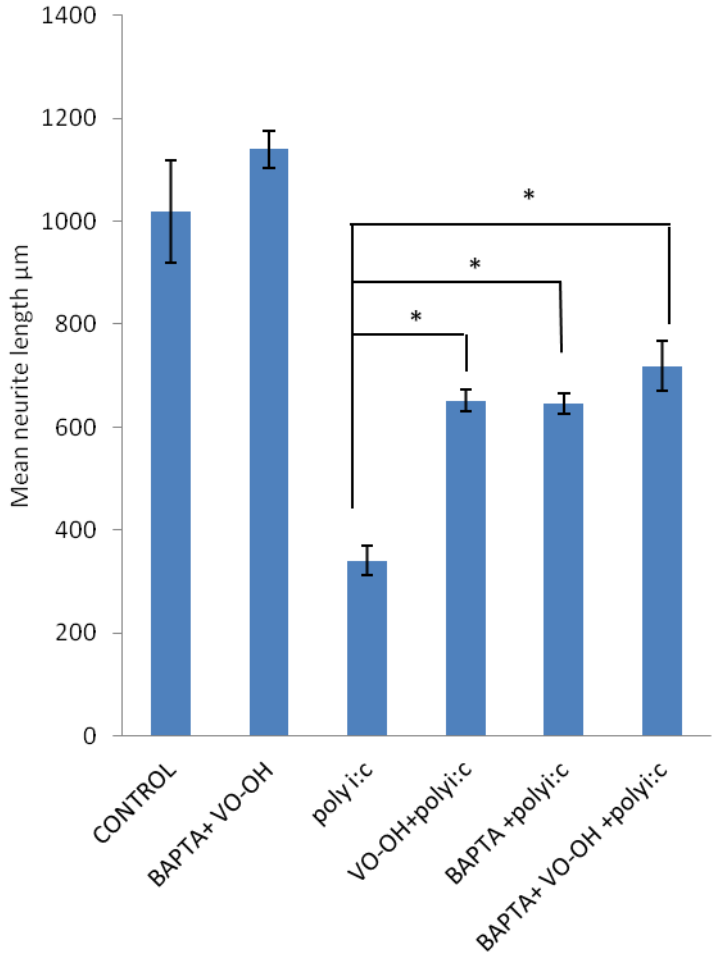


Figure 6.7: Effect of buffering calcium using BAPTA-AM (100nM) and PTEN antagonist VO-OHpic on neurite outgrowth from mouse DRG neurons in the presence of PolyI:C (100µg/ml) cultured for a period of 24hrs, *p<0.0001 when compared to neurons treated with PolyI:C, n=5, Student's t test.

Discussion

Recent studies have shown that PTEN plays a role in preventing regeneration of a variety of axons *in vivo* and *in vitro* incuding corticospinal axons (Liu et al., 2010), DRG axons (Christie et al., 2010) and retinal ganglion cell axons (Park et al., 2008; Sun et al., 2011). In the current study the interactions between PTEN, ErbB1 and calcium in the inhibition of neurite outgrowth *in vitro* by Poly I:C have been explored using a pharmacological blocker to PTEN namely VO-OHpic.

PTEN plays a role in TLR3 mediated inhibition of neurite outgrowth in vitro.

As shown in Chapter-4, the activation of TLR3 by its ligand Poly I:C leads to inhibition of neurite outgrowth. In order to investigate whether PTEN plays an active role in this signalling pathway, mouse DRG neurons as well cerebellar granule neurons were treated with a pharmacological blocker of PTEN, VO-OHpic. In the absence of Poly I:C, the PTEN antagonist had no effect on neurite outgrowth from DRG or cerebellar granule neurons, showing that it was not a general stimulator of neurite outgrowth. However, in the presence of Poly I:C, VO-OHpic at concentrations of 50nM, 100nM and 1µM greatly improved neurite outgrowth. VO-OHpic at 10µM did not increase neurite outgrowth but proved detrimental to cells. These results clearly indicate that blocking PTEN in DRG and cerebellar granule neurons disinhibits neurite outgrowth in the presence of Poly I:C and hence, PTEN is part of or interacts with the pathway by which TLR3 inhibits neurite outgrowth. This pathway also involves ErbB1.

PTEN is involved in the signalling pathway from ErbB1 and activated TLR3

We have previously shown that ErbB1 is an active component of the signalling pathway from TLR3 which is activated by Poly I:C. The inhibition of neurite outgrowth caused by the activation of this pathway can be greatly reduced by either pharmacologically blocking or genetically deleting ErbB1. Because blocking PTEN ameliorates the inhibition of neurite outgrowth caused by Poly I:C, it became necessary to investigate if PTEN plays a role in ErbB1 signalling triggered by activated TLR3. In order to so, DRG cultures as well as CGC cultures were treated with ErbB1 and PTEN antagonists. It was found that the extent to which neurite outgrowth was enhanced was similar in all three cases, i.e there was no additive effect when the two drugs were used together. It would be expected that if PTEN and ErbB1 were operating through separate signalling pathways to inhibit neurite outgrowth in the presence of Poly I:C, blocking both pathways would lead to a cumulative increase in neurite outgrowth. However, as no synergistic effects were observed when blocking both PTEN and ErbB1, it can be implied that ErbB1 and PTEN inhibit neurite outgrowth in the presence of Poly I:C via the same signalling pathway or pathways that converge on a common target.

Calcium signalling is integral to the signalling pathway from activated TLR3 containing PTEN and ErbB1

When calcium is buffered using BAPTA-AM, DRG neurons grown in the presence of Poly I:C are less inhibited by PolyI:C. However, buffering calcium in ErbB1-/- DRG neurons does not produce any effect on neurite outgrowth in the presence of Poly I:C (see Chapter-4). As calcium signalling is involved in activation of ErbB1 in response to CNS myelin etc. (Koprivica et al., 2005;Leinster et al., 2012), and PTEN may also be involved in the pathway from ErbB1, we tested the effects of buffering calcium in DRG neurons and cerebellar granule neurons treated with Poly I:C and the PTEN blocker. It was observed that buffering calcium in neurons in which PTEN activity was blocked using VO-OHpic produced no further promotion of neurite outgrowth. Taken these results together, it can be hypothesised that calcium signalling is involved in a pathway that involves TLR3, ErbB1 and PTEN. PTEN is probably a component of the pathway from TLR3 to neurite inhibition.

Signalling cascades from PTEN and ErbB1 that may cause inhibition of neurite outgrowth

There is emerging evidence that PTEN plays a role in the inhibition of neurite outgrowth in the presence of molecules which are believed to limit axonal regeneration in the CNS. However the exact signalling mechanism through which PTEN operates is not well understood.

It has recently been shown that knocking down PTEN using shRNA in mouse cortical neurons as well as cerebellar granule neurons rescues neurite outgrowth from inhibition by membrane-bound MAG (Perdigoto et al., 2011). Those authors showed that the effects of MAG on neurite outgrowth in these neurons were not mediated by the known receptors for MAG, but involved the PTEN-PI3-Akt pathway. Moreover, inhibiting ROCK-Rho kinase and PTEN produced additive effects on neurite outgrowth in presence of MAG, suggesting that PTEN and ROCK elicit their effects through separate signalling pathways (Perdigoto et al., 2011). In the case of DRG neurons, it has been reported that exposure to Sema3A suppresses PI3K signalling which activates GSK-3 in a manner dependant on PTEN activity and the accumulation of PTEN in the growth cone, causes it to collapse (Eickholt et al., 2002;Chadborn et al., 2006). Apart from studies suggesting that PTEN mediates its effects through suppressing the PI3/Akt pathway, it

has been reported that in retinal ganglion cells, PTEN mediates its effects predominantly by negative regulation of mTOR (Park et al., 2008). However, this may not be the case with DRG neurons as it has been shown that the activation of PI3K pathway in injured DRG neurons occurs independent of mTOR and that PTEN signalling may act on other signalling pathways (Christie et al., 2010).

Here, we have been able to show that PTEN is involved in the signalling pathway from activated TLR3 and that ErbB1 is a member of the same pathway which also involves calcium signalling. Other evidence shows that calcium signalling is upstream of ErbB1 activation in the pathway leading from CNS myelin etc. to cessation of neurite growth. We do not yet know if calcium signalling is upstream of PTEN activation. As signalling through ErbB1 is normally associated with growth and differentiation, it would be expected that suppression of PTEN would enhance ErbB1 activation. However, as shown through our experimental results, a strange correlation exists between ErbB1 and PTEN such that the two signalling molecules work together to inhibit neurite outgrowth.

Conclusion

We have shown that pharmacologically blocking PTEN using VO-OHpic rescues neurite outgrowth from mouse DRG neurons as well as cerebellar granule neurons treated with Poly I:C. Blocking both PTEN and ErbB1 improves neurite outgrowth but the effects are not additive. Buffering calcium using BAPTA-AM improves neurite outgrowth in the presence of Poly I:C and the addition of the PTEN blocker does not alter the levels to which an improvement in neurite outgrowth is produced. Hence it can be concluded that our findings show, that PTEN is part of a signalling pathway from ErbB1 transactivated by calcium in the presence of activated TLR3.

Chapter-7 Sulf1 and Sulf2- their role in axonal regeneration and expression in the nervous system.

Introduction

Heparan sulphate proteoglycans (Johnson et al., 2006) modulate extracellular signalling during development as well as after axotomy (Kantor et al., 2004;Minniti et al., 2004;Hu, 2001;Moon et al., 2002;Groves et al., 2005). HSPGs have been shown to mediate central and peripheral axon path finding and are required for specific directional cues (Irie et al., 2002). The predominant effects of HSPGs on neurons have been shown to be that of growth-promotion, unlike CSPGs that inhibit neurite outgrowth. However, more recently it has been reported that both HSPGs and CSPGs associate with the same receptor, PTPσ (Coles et al., 2011). The outcome of such association was dependant on the relative amounts of each type of proteoglycans that competitively bind to the receptor to control its oligomerisation (Coles et al., 2011). Moreover, the sulphation pattern of HSPGs has shown to be critical for determining the specificity and affinity of binding of growth factors and morphogens to its receptors (Esko and Selleck, 2002). The sulphation levels of HSPGs have been reported to be regulated by a novel class of sulfatases, Sulf1 and Sulf2 that remove 6-O-sulphate groups from HSPGs (Dhoot et al., 2001;Uchimura et al., 2006;Morimoto-Tomita et al., 2002). Sulfs can act on HSPGs that are either expressed on the cell surface or within Golgi bodies and alter binding of HSPGs to various growth factor receptors (Ai et al., 2003;Morimoto-Tomita et al., 2002). Active forms of Sulfs have also been detected in conditioned media, implying that active forms of the enzymes are also secreted (Ai et al., 2006). Recently, Sulfs have been reported to inhibit FGF signalling and promote BMP signalling in chondrocytes (Otsuki et al., 2010). Also Sulf1 and Sulf2 have been shown to reduce binding of GDNF to HSPGs. This allows GDNF to bind to the receptor RET expressed by GDNF-responsive neurons causing neuronal sprouting in the embryonic oesophagus (Ai et al., 2007). However, the role of Sulfs in axonal regeneration has not yet been characterised.

Considering the facts that both CSPGs and HSPGs mediate their functions through a common receptor and that Sulfs alter the binding of HSPGs to various growth factors or growth factor receptors which may include ErbB1(Narita et al., 2007) , we hypothesised that Sulfs may regulate responses of

neurons grown in the presence of CSPGs. Whether the effects of Sulfs were that of growth-promotion or inhibition of neurite outgrowth required investigation. The aims of this study were the following:

1) To test the effects of secreted Sulfs on neurite outgrowth from DRG neurons in the presence of CSPGs.
2) To test the effects of blocking Sulf1 or Sulf2, using function-blocking antibodies, on neurite outgrowth in the presence of CSPGs.
3) To investigate if ErbB1 plays a role in Sulf-mediated signalling.
4) To analyse the expression of Sulf1 and Sulf2 in the nervous system.

The chapter has been divided into two sections:

A) The role of Sulf1 and Sulf2 on CSPG-mediated inhibition of neurite outgrowth.
B) The expression of Sulf1 and Sulf2 in the nervous system.

7.1 The role of Sulf1 and Sulf2 on CSPG-mediated inhibition of neurite outgrowth.

Methods

DRG neurons from mice aged between P7-P9 were cultured as described in chapter 2. The conditions used to assess the effects of Sulf1 or Sulf2 on neurite outgrowth are listed in Table 7.1.

Condition	Treatment
Control	Neurons grown on a normal substrate in normal growth media.
Pre-immune serum	Neurons were treated with 1µl of pre-immune serum per well. Pre-immune serum was obtained from animals prior to immunisation to Sulf1 or Sulf2. The volume was the same as that of the experimental antibodies (Sulf1, Sulf2).

CSPGs	Neurons were treated with CSPGs at a concentration of 0.5µg/ml.
Pre-immune serum +CSPGs	Neurons were treated with 1µl of pre-immune serum and 0.5µg/ml of CSPGs.
Sulf1 Ab	Neurons treated with Sulf1 function-blocking antibody at a concentration of 200ng/ml
Sulf2 Ab	Neurons treated with Sulf2 function-blocking antibody at a concentration of 200ng/ml.
Sulf1 Ab + CSPG	Neurons treated with 200ng/ml of Sulf1 antibody and 0.5µg/ml of CSPGs.
Sulf2 Ab+CSPG	Neurons treated with 200ng/ml of Sulf2 antibody and 0.5µg/ml of CSPGs
Sulf 1Conditioned Media (Sulf1CM)	Conditioned media was collected from HEK cells transfected with SULF1 plasmids. Conditioned media was added to DRG cultures at a concentration of 1:10 ie 1 volume of conditioned media to 10 volumes normal DRG growth media.
Control conditioned media (Control CM)	Conditioned medium was collected from HEK cells that were not transfected with plasmids. Conditioned medium was added to DRG cultures at concentration of 1:10.
Sulf1 CM+CSPGs	Neurons were treated with 0.5µg/ml of CSPGs and Sulf1-conditioned medium (1:10).
Sulf1 Ab + PD100	Neurons were treated with 200ng/ml of Sulf1 antibody and PD168393 at 100nM.

Sulf2 Ab +PD100	Neurons were treated with 200ng/ml of Sulf2 antibody and PD168393 at 100nM.
CSPGs + PD100	Neurons were treated with 0.5µg/ml of CSPGs and ErbB1 antagonist PD168393 at a concentration of 100nM.
Sulf1Ab +PD100+CSPGS	Neurons were treated with 200ng/ml Sulf1 antibody,100nM PD168393 and 0.5µg/ml CSPGs.
Sulf2 Ab +PD100+CSPGs	Neurons were treated with 200ng/ml Sulf2 antibody,100nM PD168393 and 0.5µg/ml CSPGs.
Sulf -CM+PD100+CSPGs	Neurons were treated with Sulf–conditioned medium (1:10), 100nM PD168393 and 0.5µg/ml CSPGs.
Control-CM+PD100+CSPGs	Neurons were treated with control-conditioned medium (1:10),100nM PD168393 and 0.5µg/ml CSPGs.

Table 7.1: The different conditions under which DRG neurons were grown to assess the effects of Sulf1 or Sulf2 on neurite outgrowth.

Antibodies against full-length Sulf1 and Sulf2, pre-immune serum and conditioned media were kindly given to us by Dr. Gurtej Dhoot, Royal Veterinary College, London.

The above mentioned treatments were carried out at the time of plating cells. DRG neurons were cultured for 24 hours at 37°C after which they were fixed using 4% paraformaldehyde and immunostained for βIII tubulin. Photomicrographs of neurons were taken and neurite length was measured using the software, ImageJ.

Results

Addition of function-blocking antibody against Sulf1 improves neurite outgrowth from mouse DRG neurons in the presence of CSPGs.

There is ample evidence on the inhibitory effects of CSPGs on neurite outgrowth (see Chapter-3). These effects of CSPGs have been previously shown to be ameliorated by inhibiting or genetically deleting ErbB1 (Koprivica et al., 2005;Leinster et al., 2012). To confirm these reports before carrying out experiments using Sulf1 antibody, we first tested the effects of CSPGs on neurite outgrowth. It was observed that CSPGs, when added to DRG cultures at a concentration of 0.5µg/ml, significantly reduced neurite outgrowth by approximately 50% (Figure 7.1). The addition of the ErbB1 antagonist, PD168393, at 100nM promoted neurite outgrowth from neurons cultured in the presence of CSPGs but not of neurons grown in normal growth media (Figure 7.1).

To test the effects of blocking full-length Sulf1 on neurite outgrowth, DRG neurons were treated with a function-blocking antibody to Sulf1. The antibody was applied to cells either grown in normal growth media or in the presence of CSPGs. Sulf1 antibody did not affect neurite outgrowth from neurons grown in normal growth media (Figure 7.2). In the presence of CSPGs, there was a reduction in neurite outgrowth. However, the inhibitory effects of CSPGs were largely overcome when neurons were treated with the Sulf1 antibody (Figure7.2). Hence, blocking Sulf1, like PD168393 improved neurite outgrowth of DRG neurons grown in the presence of CSPGs.

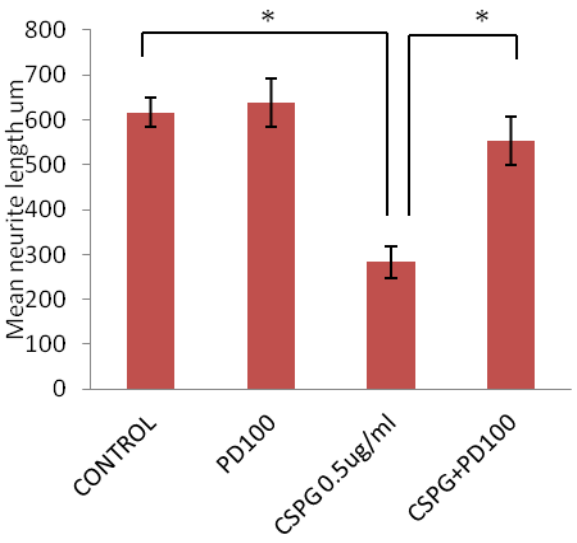


Figure 7.1: CSPGs (0.5µg/ml) inhibit neurite outgrowth from DRG neurons grown for 24hrs. The addition of ErbB1 antagonist, PD168393 (100nM) significantly improved neurite outgrowth in the presence of CSPGs.*p<0.005 when compared to neurite outgrowth from neurons treated with CSPGs; Student's t test; n=5.

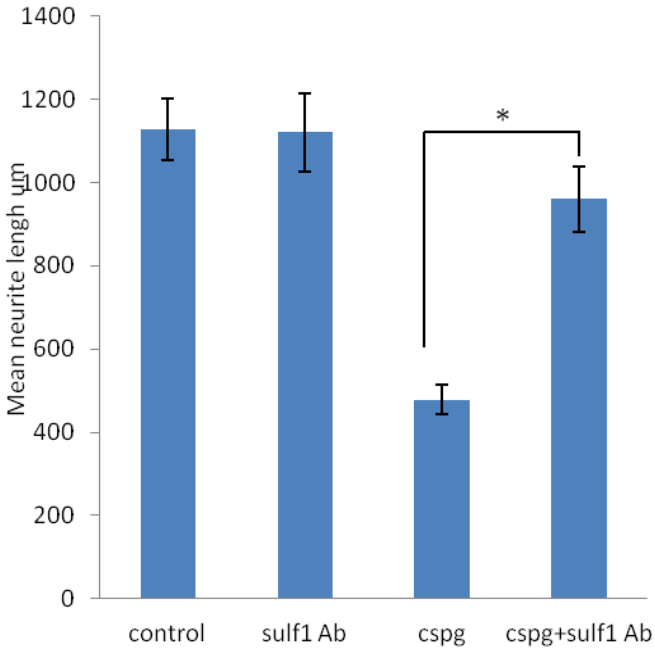


Figure 7.2: Blocking antibody to full-length Sulf1 (200ng/ml) improves neurite outgrowth from DRG neurons in the presence of CSPGs. Sulf1 antibody does not affect neurite outgrowth from neurons grown in the absence of CSPGs. When compared to neurons grown in the presence of CSPGs alone, the addition of Sulf1 antibody significantly enhances neurite outgrowth. *p<0.001 when compared to neurite outgrowth from neurons treated with CSPGs; Student's t test; n=5.

Blocking either Sulf1 or ErbB1 exerts similar effects on neurite outgrowth but these effects are not additive.

As shown previously, treatment with Sulf1 antibody does not alter neurite outgrowth from neurons grown in normal growth media but in the presence of CSPGs, Sulf1 antibody improves neurite outgrowth from DRG neurons. Moreover it seemed that the extent to which neurite outgrowth was

rescued was similar to that produced by PD168393. To test if ErbB1 and Sulf1 operate through similar signalling pathways, DRG cultures were treated with PD168393 (100nM) and Sulf1 antibody. In the absence of CSPGs, treatment with PD168393 and Sulf1 antibody slightly reduced neurite outgrowth when compared to neurons treated with Sulf1 antibody alone (not statistically significant) (Figure 7.3). In the presence of CSPGs, blocking both ErbB1 and Sulf1 improved neurite outgrowth when compared to neurons grown in the presence of CSPGs alone (Figure 7.3). These neurite enhancing effects were similar to the effects produced by blocking either ErbB1 or Sulf1 (Figure 7.3). Hence, it was observed that the addition of PD168393 and Sulf1 antibody improves neurite outgrowth in the presence of CSPGs, but these effects are not additive.

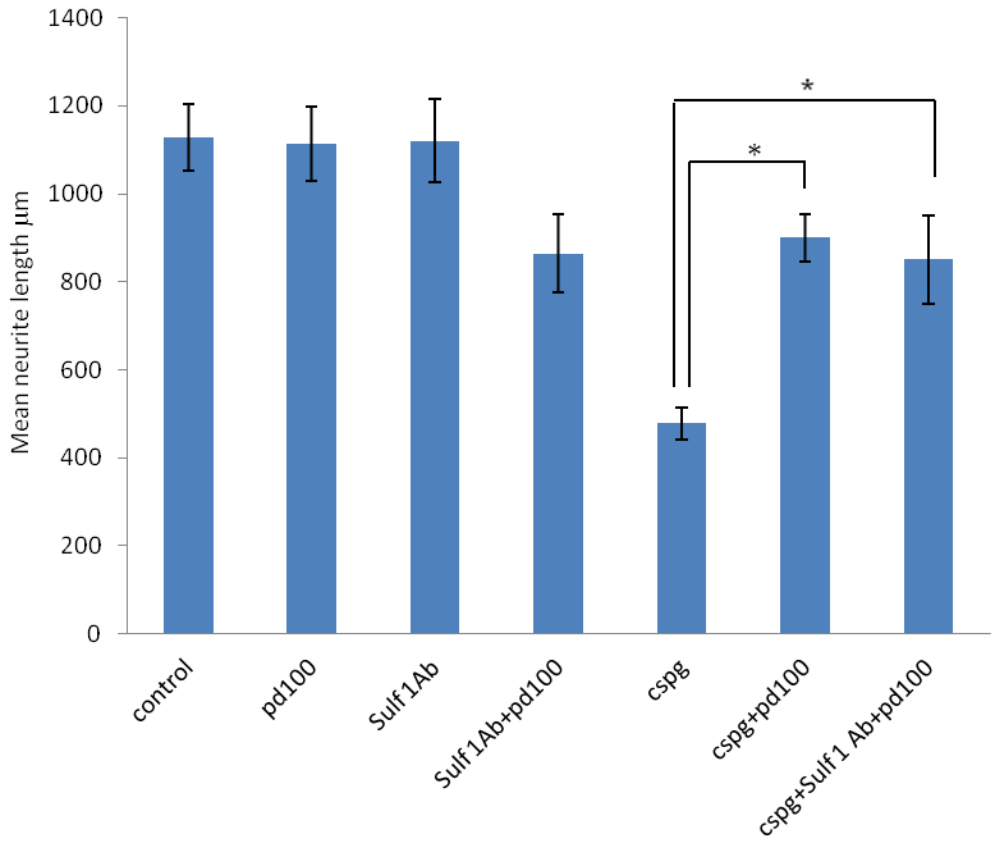


Figure 7.3: Effects of PD168393 (100nM) and full-length Sulf1 antibody (200ng/ml) on neurite outgrowth from DRG neurons. Blocking both ErbB1 and Sulf1 improves neurite outgrowth of DRG neurons compared to neurons grown in the presence of CSPGs alone, but these effects are not additive. There was no significant difference between neurite outgrowth in the presence of CSPGs with

PD168393 and that found in the presence of CSPGs with both PD168393 and antibody to Sulf1. *p<0.01when compared to neurite outgrowth from neurons treated with CSPGs; Student's t test, n=5.

Addition of function-blocking antibody against Sulf2 improves neurite outgrowth in the presence of CSPGs and also in conjunction with the ErbB1 blocker, but these effects are not additive.

The effects on neurite outgrowth of a different sulfatase enzyme, Sulf2 were tested by using a function-blocking antibody to full-length Sulf 2. The addition of Sulf2 antibody to neurons grown in normal media caused a slight reduction in neurite outgrowth (Figure 7.4). A further reduction in neurite outgrowth was seen when neurons were treated with both PD168393 (100nM) and Sulf2 antibody. In contrast, Sulf2 antibody improved neurite outgrowth in the presence of CSPGs when compared to neurons treated with CSPGs alone (Figure 7.4). Sulf2 antibody and PD168393 exerted similar neurite-enhancing effects on cultured DRG neurons. Moreover, as seen with the Sulf1antibody, the addition of Sulf2 and PD168393 produced non-synergistic effects on neurite outgrowth in the presence of CSPGs.

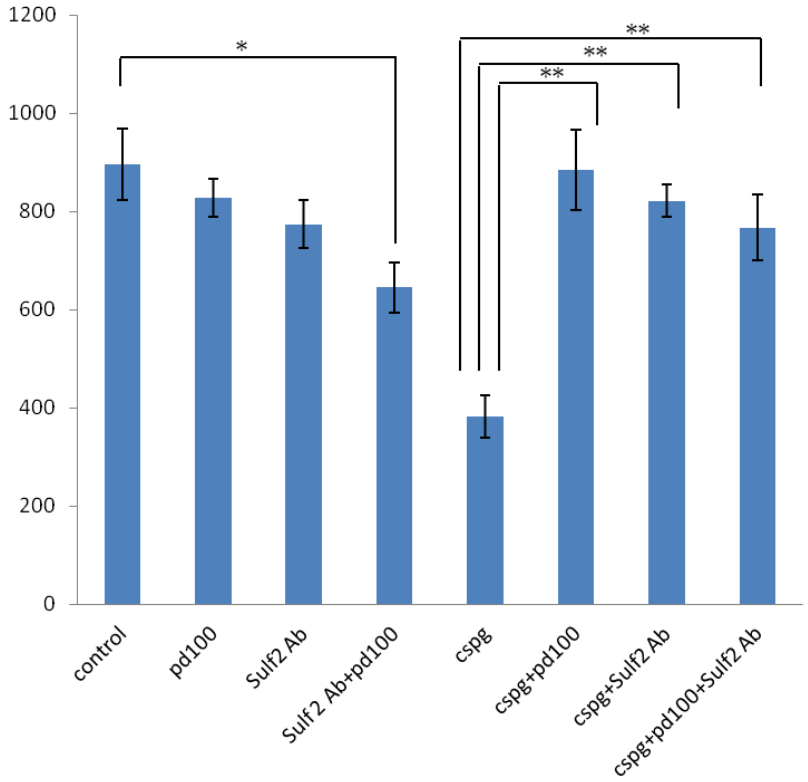


Figure 7.4: Effects of PD168393 (100nM) and full-length Sulf 2 (200ng/ml) antibody on neurite outgrowth from cultured DRG neurons. Addition of Sulf2 antibody improves neurite outgrowth from neurons in the presence of CSPGs. Blocking ErbB1 with PD 168393 improves neurite outgrowth in the presence of CSPGs to a similar extent but the effects of blocking both Sulf2 and ErbB1 are not additive. *p<0.05 when compared to neurite outgrowth from neurons grown under control conditions; **p<0.001 when compared to neurite outgrowth from neurons grown in the presence of CSPGs; Student's t test; n=5.

Pre-immune serum does not affect neurite outgrowth.

To test if the effects of blocking Sulf1 or Sulf2 on neurite outgrowth were a result of non-specific actions of the rabbit antiserum, DRG cultures were treated with pre-immune serum. Pre-immune serum was collected from animals prior to immunisation to full-length Sulf1 or full-length Sulf2. When pre-immune serum was added to DRG cultures, it was observed that there were no changes in neurite outgrowth from either neurons grown in normal growth media or neurons grown in the presence of CSPGs (Figure 7.5). Hence, it can be inferred that the actions on neurite outgrowth of antisera against Sulf1 or Sulf2 were dependent on the presence of antibodies to these proteins.

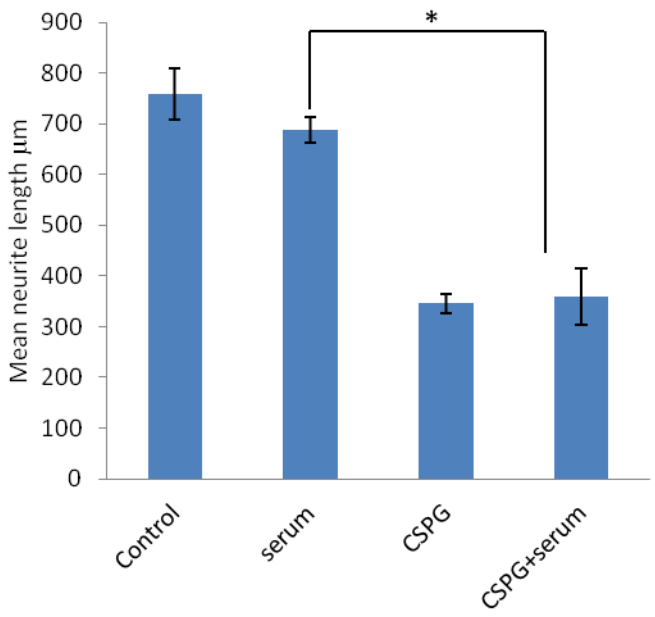


Figure 7.5: Effects of pre-immune serum on neurite outgrowth from DRG neurons. Pre-immune serum does not alter neurite outgrowth from neurons grown in normal growth media or in the presence of CSPGs.*p<0.01 when compared to neurons treated with pre-immune serum alone; Student's t test; n=3.

Conditioned media from Sulf1-secreting cells improves neurite outgrowth from DRG neurons treated with CSPGs.

DRG neurons were treated with two types of conditioned media; media conditioned by HEK cells genetically modified to secrete Sulf1 (Sulf1-conditioned medium) and media conditioned by non-genetically modified HEK cells (control conditioned medium). The two different types of conditioned medium were added to DRG cultures either grown in the presence or absence of CSPGs. Because blocking Sulf1 using a function-blocking antibody improves neurite outgrowth from neurons treated with CSPGs, it was to be expected that the addition of Sulf1-conditioned media would further reduce neurite outgrowth. However, to our surprise, treatment with Sulf1-conditioned media improved neurite outgrowth from neurons grown in the presence of CSPGs (Figure7.6). In the absence of CSPGs, Sulf1-conditioned media appeared to slightly reduce neurite outgrowth when compared to neurons grown in normal growth media (control) but the effect was not significant. Control-conditioned media did not alter neurite outgrowth (Figure 7.6).

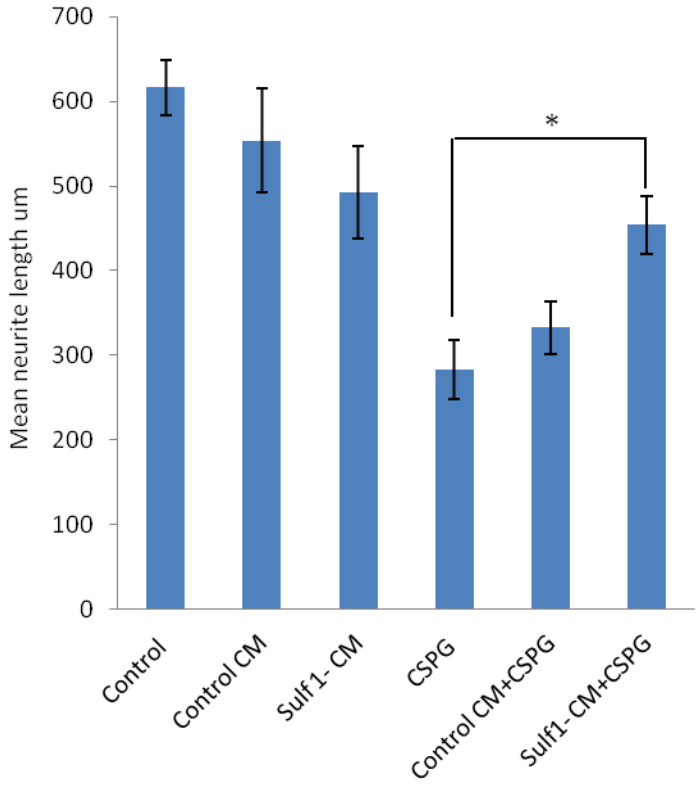


Figure 7.6: Effects of Sulf1-conditioned medium on neurite outgrowth from DRG neurons. Sulf1-conditioned medium improves neurite outgrowth from neurons treated with CSPGs.*$p<0.01$ when compared to neurons treated with CSPGs alone; Student's t test, n=5.

Pharmacological inhibition of ErbB1 blocker abolishes the neurite-enhancing effects of Sulf1-conditioned media.

To test if blocking ErbB1 would affect the neurite-enhancing properties of Sulf1-conditioned media, DRG neurons were treated with PD168393 (100nM). The addition of Sulf1-conditioned media and PD168393 does not alter neurite length of neurons grown in the absence of CSPGs (Figure 7.7). In the presence of CSPGs, as shown above, Sulf1-conditioned media improved neurite outgrowth. However, the addition of PD168393 cancelled the neurite-enhancing effects of Sulf1-conditioned media, although it improved neurite outgrowth from cells treated with control conditioned media (Figure7.7). Hence, addition of PD168393 or Sulf1-conditioned media but not both improves neurite outgrowth from neurons treated with CSPGs.

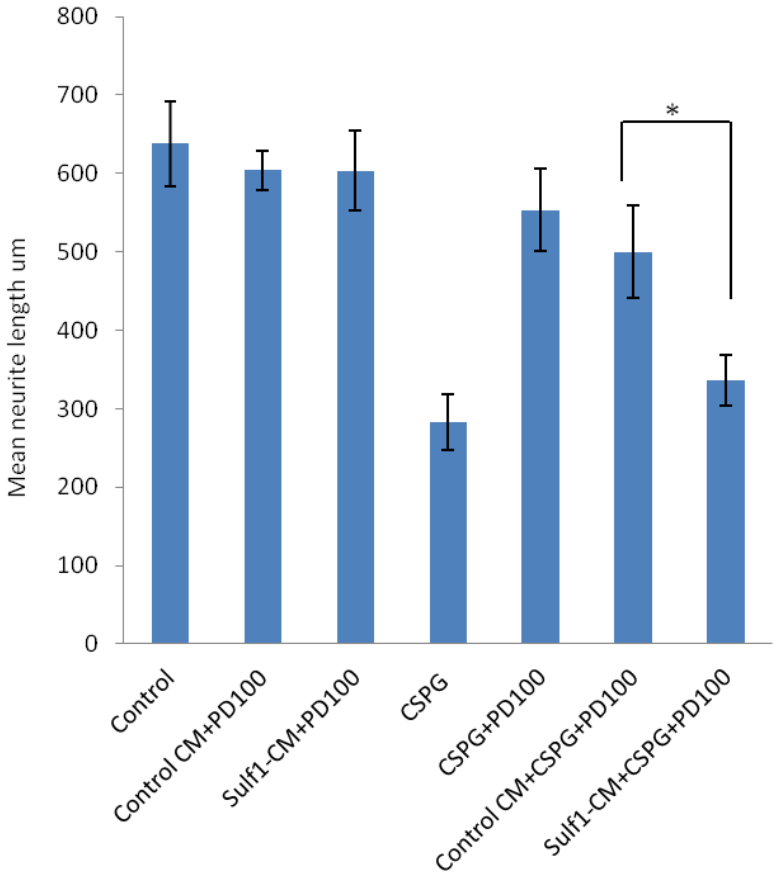


Figure 7.7: Effects of PD168393 on neurite outgrowth from DRG neurons treated with CSPGs and Sulf1-conditioned media. Addition of PD168393 to neurons treated with CSPGs improves

neurite outgrowth in the presence of control conditioned medium but not Sulf1-conditioned medium.*p<0.01when compared to neurons treated with CSPGs, control conditioned media and PD168393; Student's t test, n=5.

Discussion

The Sulfs are endosulfatases that cleave 6-O-sulfate groups of HSPGs (Dhoot et al., 2001;Morimoto-Tomita et al., 2002;Lum et al., 2007). HSPGs can provide binding sites for growth factors dependent on specific sulphation patterns, and Sulf1 and Sulf2 have been shown to regulate the activity of FGF, vascular endothelial growth factor (VEGF) and Wnt (Uchimura et al., 2006;Nawroth et al., 2007). Here, we have shown that Sulf1 and Sulf2 can influence neurite outgrowth from mouse DRG neurons grown in the presence of inhibitory CSPGs.

Blocking Sulf1 or Sulf2 improves neurite outgrowth in the presence of CSPGs.

We used function-blocking antibodies (Gill et al., 2010) to block the effects of Sulf1 and Sulf2 *in vitro*. When DRG neurons grown in normal growth media were treated with these antibodies, a slight reduction in neurite outgrowth was observed. However, in the presence of CSPGs, treatment with the antibodies resulted in an enhancement of neurite outgrowth when compared to neurons treated with CSPGs alone.

Functional studies on Sulf1 knockout and Sulf2 knockout mice have shown that genetic deletion of either enzyme causes impaired neurite outgrowth from hippocampal and cerebellar granule neurons *in vitro* (Kalus et al., 2009). That study implies that both Sulf1 and Sulf2 play a role in the developing nervous system, although an overt phenotype was not observed in mice in which Sulf1 was deleted. Our observations of a trend towards reduced neurite outgrowth from DRG neurons grown on a normal substrate when treated with Sulf2 antibody can be explained by proposing that Sulf2-mediated signalling is involved in the growth of neurites *in vitro*. However, these effects were small when compared to the effects of blocking Sulf activity in DRG cultures treated with CSPGs. The addition of Sulf1 or Sulf2 antibodies greatly disinhibited neurite outgrowth in the presence of CSPGs. Although Sulf1 and Sulf2 have shown to exert opposing

effects on different signalling pathways involved in cancer (Yang et al., 2011), our results have shown that Sulf1 and Sulf2 exert similar disinhibiting effects on neurite outgrowth from DRG neurons *in vitro*.

ErbB1 signalling interacts with Sulf1 and Sulf2 to inhibit neurite outgrowth.

We have previously shown that pharmacological inhibitors to ErbB1 act as broad-spectrum disinhibitors of neurite outgrowth in the presence of CNS myelin, Poly I:C and CSPGs. As blocking Sulf1 or Sulf2 ameliorates the inhibitory effects of CSPGs, we tested the effects of blocking ErbB1in cultures treated with Sulf1 or Sulf2 antibody. In the absence of CSPGs, it was observed that blocking both Sulfs and ErbB1 slightly reduced neurite outgrowth suggesting that growth factor signalling mediated via ErbB1 which involved Sulfs may promote neurite extension. However, signalling via ErbB1 involving Sulfs also acted to inhibit neurite growth in the presence of CSPGs. It was noted that the effects of blocking both Sulf1/2 and ErbB1 on neurite outgrowth were not additive. These results imply that Sulf1 and Sulf2 probably affect ErbB1signalling leading to inhibition of neurite outgrowth.

Conditioned medum from Sulf1-secreting cells and Sulf1/2 function-blocking antibodies both disinhibit neurite outgrowth.

Experiments with blocking antibodies to Sulf1 or Sulf2 in the presence of CSPGs have allowed to us characterise Sulfs as enzymes which take part in a signalling pathway which is also activated by ErbB1. The outcome of such signalling led to inhibition of neurite outgrowth. However, contrary to our expectations, the addition of conditioned medium collected from Sulf1-secreting cells promoted neurite outgrowth in the presence of CSPGs. Thus, on one hand blocking Sulf1 activity disinhibits neurite outgrowth and on the other hand adding Sulf1 in solution also disinhibits outgrowth. Another unexpected finding was that when the ErbB1 blocker was added, the growth-promoting effects of conditioned medium were abolished, suggesting that the factors in the conditioned medium, presumably including secreted Sulf 1, promote outgrowth via ErbB1.

These anomalous neurite outgrowth-promoting effects obtained with Sulf1-conditioned medium may have been brought about by components of the medium other than Sulf1. However,

medium conditioned by cells not expressing Sulf1 was ineffective. Sulf1 inhibits autocrine activation of ErbB1 by amphiregulin in cancer cells (Narita et al., 2007).

HSPGs may affect different signalling pathways and these effects may be dependant on the level of sulphation. For example, it has been reported that Schwann cells express lower levels of Sulfs than olfactory ensheathing cells, which results in their secreting HSPGs with highly sulphated GAGs, whereas OECs express higher levels of Sulfs resulting in the secretion of HSPGs with less sulphated GAGs. The different forms of HSPGs have been suggested to have opposing effects on FGF signalling (Higginson et al., 2012). It could also be the case that conditioned medium from Sulf1- secreting cells may also contain its isoforms such as Sulf1A and Sulf1B which have been previously reported to have functions opposing that of Sulf1 (Sahota and Dhoot, 2009). Sulf1A inhibits angiogenesis and promotes Wnt signalling whereas Sulf1B promotes angiogenesis and inhibits Wnt signalling (Sahota and Dhoot, 2009). An obvious way of testing some of these ideas would be to pre-absorb the conditioned medium with antibodies to Sulf 1, to determine whether the effects were mediated directly by the secreted enzyme.

Differences in the form in which Sulfs are expressed i.e. membrane-bound or secreted may alter the function of Sulf1 because that could act on different molecules. The antibody could have had its effects by binding cell-surface Sulf1. None the less, it remains surprising that Sulf1 conditioned medium promoted neurite outgrowth on CSPGs by a mechanism involving ErbB1. An obvious way to elucidate this would be to identify the neurite-promoting molecules in Sulf1 conditioned medium.

Signalling mechanisms

The results imply that Sulf1 and Sulf2 act as molecules that are involved in signalling pathways resulting in inhibition of neurite outgrowth. The exact mechanisms by which Sulfs exert their effects on neurite outgrowth are unknown but we have been able to demonstrate that this signalling pathway involves ErbB1.

Although, it might be tempting to think that Sulfs directly act on CSPGs in a manner that contributes to its inhibitory effects, this may not be the case. The endosulfatase activity of Sulfs

has been reported to be highly specific to HSPGs and these enzymes do not alter sulphation patterns of CSPGs (Nagamine et al., 2012). Hence, their mode of activity would be by modulating HSPG-dependant signalling rather than acting directly on CSPGs. A previous study reported that certain HSPGs may act as receptors for neurocan and such binding facilitates outgrowth of neurites from cerebellar granule neurons (Akita et al., 2004). More recent evidence suggests that both HSPGs and CSPGs interact through the same receptor, PTPσ (Coles et al., 2011).Hence, it can be hypothesised that regulating sulfation activity of HSPGs may regulate responses of neurons towards CSPGs.

We have shown that Sulfs mediate inhibition of neurite outgrowth by CSPGs through a signalling pathway that involves ErbB1. Studies in Drosophila have shown that Sulf1 fine-tunes ErbB1 signalling extracellularly by negatively regulating interactions between the ligand and the receptor (Wojcinski et al., 2011;Butchar et al., 2012). In contrast, it has been proposed that ErbB1 inhibits neurite outgrowth through a mechanism that is independent of ligand-binding in mouse cerebellar granule neurons cultured in the presence of CNS myelin (Koprivica et al., 2005). Taken these results together, there lies a possibility that on one hand ErbB1 signalling mediated by ligand-binding resulting in growth promotion is inhibited by Sulfs.; on the other hand, transactivation of ErbB1in neurons results in growth inhibition and this signalling maybe enhanced by Sulfs.

7.2 Expression of Sulfs in the nervous system

Methods

Reverse transcriptase PCR:

RNA was isolated using Qiagen's RNAeasy kit as described in chapter 2. cDNA was generated using Superscript reverse transcriptase (Invitrogen). The primer combinations for mSulf1 were –forward 5'- AGAG GGA TCC ATG AAG TAT TCC CTC TGG GCT CTG; reverse-5'-CAA TGT GGT AGC CGT GGT CC. For mSulf2, the primer combinations used were –forward 5'- AGAG GGA TCC ATG GCA CCC CCT GGC CTG CCA CTA T and reverse-5'-CAT AGA CTT GCC CTT CAC CAG CCC. The conditions for denaturing, annealing and extension of the template cDNA were as follows: 94°C for 30 sec, 52°C for 45 sec and 72°C for 1 min for 40 cycles. PCR products were separated by electrophoresis on 1.8% agarose gels and visualized with ethidium bromide.

Western blotting:

Protein lysates were prepared from cortex, cerebellum, spinal cord, DRGs and sciatic nerve from adult rats using RIPA lysis buffer (Sigma). Equal quantities of samples (10µg) were resolved on 10% Tris-acrylamide gels. Proteins were transferred onto a nitrocellulose membrane (Amersham), blocked in 5% milk and immunostained with antibodies against Sulf1 (1:3000) and Sulf2 (1:3000). Following washes in 1X PBS, the membranes were treated with secondary antibody (Anti-rabbit IgG HRP; 1:2000) followed by treatment with ECL. Bands were detected by chemiluminesence captured on X-ray sheets using XO-graph.

Immunohistochemistry:

Animals including those that underwent surgical intervention were transcardially perfused with 4% paraformaldehyde. Cryostat sections of width 12µm or free floating sections (40µm) were blocked in blocking solution containing 5% goat serum. The sections were immunostained using the following antibodies:

Primary antibody	Working concentration
Sulf1antibody produced in rabbit (recognises exon6 and detects full length Sulf1)	1:200
Sulf2antibody produced in rabbit (recognises exon6 and detects full length Sulf2)	1:100
Anti- Sulf1isoform produced in rabbit(recognises exon 20/21 and detects all sulf1 isoforms)	1:200
Anti- Sulf2 isoform produced in rabbit (recognises exon 23 and detects all sulf2 isoforms)	1:100
Polyclonal anti-GFAP produced in rabbit (isolated from bovine spinal cord and recognises rat, mouse, sheep, cow and dog GFAP, purchased from DAKO).	1:500
Monoclonal anti-neurofilament produced in mouse (recognises medium and heavy neurofilament -molecular weight:160kDa and 200KDa of mouse, rat and human, purchased from Sigma).	1:1000
Monoclonal anti-Nogo A produced in mouse (generously provided by RK Prinjha, GSK)	1:500

Table 7.2: List of primary antibodies used to detect Sulfs and their isoforms.

Sections were incubated in primary antibodies overnight. Following washes in 0.1M PBS, the sections were treated secondary antibodies (Alexa flour 488, anti-rabbit-1:400 ; Atto 594, anti-mouse-1:400) and nuclear stain, DAPI (1:1000) for two hours. This was followed by washes in 0.1M PBS and the sections were mounted using mounting medium (DABCO, glycerol).

Immunocytochemistry

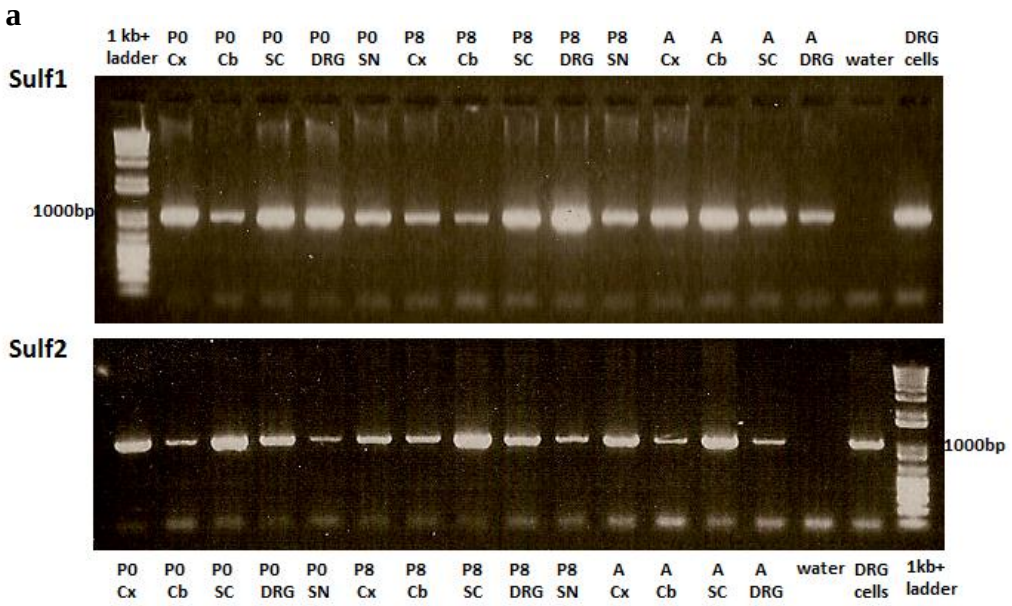
DRG neurons were cultured from neonatal rats or mice aged between P7-P8. Cultures were fixed using 4% paraformaldehyde and immunostained using antibodies against Sulfs at concentrations mentioned in Table7.2.

Microscopy

Images were acquired using a Zeiss Axiophot microscope, Hamamatsu C4742 camera and Improvision software.

Results

Sulf1 and Sulf2 transcripts are expressed in nervous tissue of mice from different age groups:



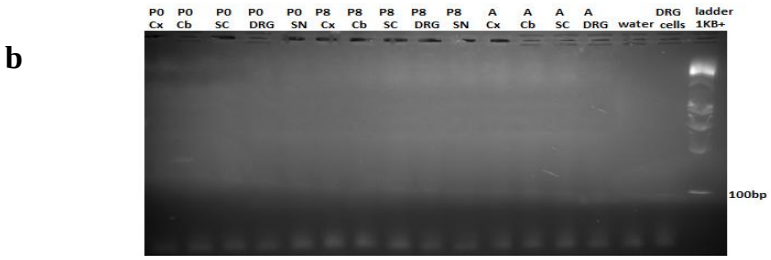


Figure 7.8: Expression of Sulf1 and Sulf2 transcripts in nervous tissue from P0, P8 and adult mice. Transcripts for Sulf1 and Sulf2 were detected in all tissue samples. a) Gel images show transcripts of Sulf1 and Sulf2 (bottom half) detected by bands of size 1000bp from the cerebral cortex (Cx), cerebellum (Cb), spinal cord (SC), DRGs, sciatic nerve (SN) from P0,P8 and adult mice and also from cultured DRG cells. Water was used as control to check for contamination of reagents. b) Gel picture showing samples generated by PCR reactions in the absence of reverse transcriptase enzyme to check for non-specific amplification as a result of contamination from

genomic DNA. The absence of bands confirms specific amplification of target sequence shown in (a).

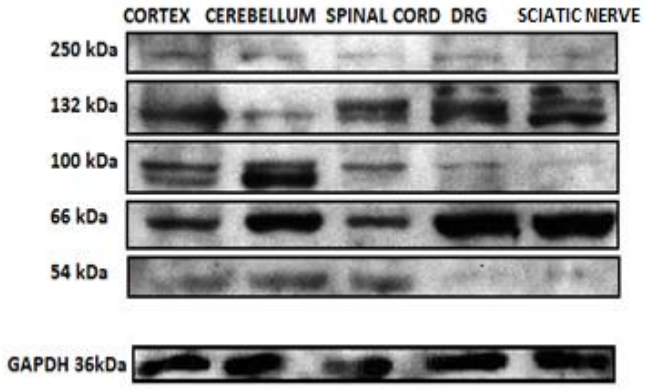
To investigate if Sulf1 and Sulf2 are expressed in CNS and PNS tissue from mice of different age groups, RNA was isolated and reverse transcribed. PCR reactions using primer combinations described in methods yield bands of sizes approximately 1000 bp (expected size-956bp) (Figure 7.9). It is to be noted that these primers anneal to exons present in all forms which include full-length and short variants. RT PCR reveals that both Sulf1 and Sulf2 transcripts are expressed by cortex, cerebellum, spinal cord, DRG and sciatic nerve from P0 and P8 mice. In adult mice, Sulf1 and Sulf2 transcripts were detected in the cortex, cerebellum, spinal cord and DRGs. Moreover, DRG neurons cultured from P7-P8 mice also expressed both Sulf1 and Sulf2. Samples contained equal volumes of cDNA and differences in the band intensities may indicate different levels of expression of Sulf1 and Sulf2 transcripts in different tissues from varying age groups. Specific amplification of target sequences were confirmed by the absence of bands in samples generated in the absence of reverse transcriptase (Figure 7.9b). Moreover, use of water in place of cDNA served as an additional control measure to check for contamination from reagents used to generate PCR reactions.

Sulf1 and Sulf2 proteins are expressed in CNS and PNS tissue from adult rats.

Sulf1 and Sulf2 proteins were detected in lysates from the cortex, cerebellum, spinal cord, DRGs and sciatic nerve from adult rats (figure 7.11). The presence of multiple bands indicates the presence of different sub-domains of Sulf1 and Sulf2 (Tang and Rosen, 2009).The calculated mass of Sulf1 and Sulf2 is 100kDa and the remaining mass (32kDa) is attributed to N-glycosylation (Morimoto-Tomita et al., 2002). Apart from bands corresponding to a size of 132 kDa, other bands were visible at sizes 250kDa, 66kDa and 54kDa. Bands at 250kDa indicate oligomers of higher order whereas bands at 66kDa and 54 kDa may correspond to the two different sub-domains of Sulf1 and Sulf2 (Tang and Rosen, 2009). Immunoblots revealed that all samples expressed full-length Sulf1 (132 kDa) (figure 7.11a) but not Sulf2. While samples from the cortex, cerebellum and spinal cord expressed full-length Sulf2, DRGs and sciatic nerve expressed a 54 kDa fragment which may correspond to one of the sub-domains of Sulf2 (figure 7.11b). Moreover, not all samples expressed the 66kDa and 55kDa fragments. In the case of

Sulf1, while all samples expressed the 66 kDa fragment, the 54 kDa fragment was not expressed by the DRGs and sciatic nerve. In the case of Sulf2, the native protein (100kDa) was not detected and the 132 kDa glycosylated full-length protein was detected in samples from the cortex, cerebellum and spinal cord but not from the DRGs and sciatic nerve. The 66 kDa fragment was expressed by the cortex and the cerebellum and all samples strongly expressed the 54kDa fragment. Also the intensity of the bands corresponding to different sizes varied among samples.

a) Sulf1



b) Sulf2

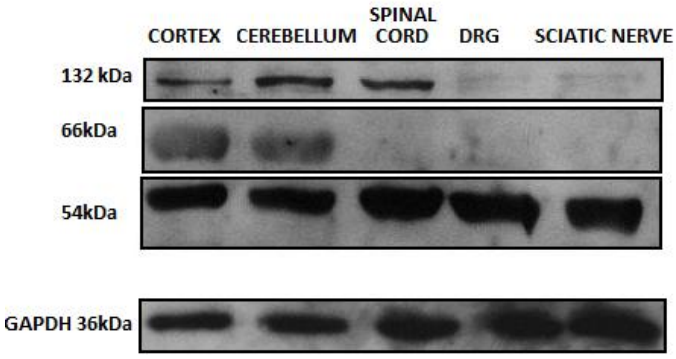


Figure 7.9: Immunoblots from protein lysates (10µg) extracted from cerebral cortex, cerebellum, spinal cord, DRG and Sciatic nerve of adult rats showing expression of a) Sulf1 and b) Sulf2. Bands corresponding to 250 kDa indicate oligomers of higher order. Full-length protein detected at 100kDa and glycosylated forms at 132 kDa. Bands at 66kDa and 54kDa may correspond to different sub-domains of Sulf1 and Sulf2. Immunostaining with GAPDH confirms even loading of samples.

Sulf1 and Sulf2 are expressed by many neurons in the nervous system

Using immunohistochemistry, we were able to detect expression of Sulf1 and Sulf2 in neurons of the cerebral cortex, cerebellum, spinal cord and DRGs as well as in cultured DRG

neurons. Different antibodies were used to detect Sulf1 and Sulf2. Antibodies to regions of the antigen encoded by exon 6 that confers functional activity to Sulf1 and Sulf2 were used to detect the full-length form of the Sulfs. As the expression of various isoforms for Sulf1 and Sulf2 have been reported (Gill et.al., 2011; Sahota and Dhoot, 2009) , we looked for their expression in the nervous system. As the shorter isoforms lack exon 6, antibodies to regions in the antigen encoded by exon 20/21 in the case of Sulf1 and exon 23 in the case of Sulf2 were used as these exons are generally not spliced out and hence present in all isoforms (which also include full-length forms). Therefore the presence of additional staining that was absent when antibodies to exon 6 were used indicates the expression of the isoforms of Sulf1 and Sulf2 in these regions.

Although the expression of Sulf1 and Sulf2 was observed in various neurons and their axons in the cerebral cortex, cerebellum, spinal cord and DRGs, the expression of its shorter isoforms was absent except in the case of the cerebellar cortex. These inferences were made based on the lack of differences in the pattern of staining when antibodies to the full-length and isoforms were used. Images have been shown below.

Figure 7.10: Expression of Sulf1 and Sulf2 (all isoforms) in DRG cells cultured for 5 days. The non-neuronal cells have proliferated during the longer period of culture compared with Figure 7.13. a) Neuronal and non-neuronal cells express all isoforms of Sulf1 (green). Neurons and some neurites can be identified by immunostaining for neurofilament (red). Arrowhead points to a non-neuronal cells that express Sulf1 isoform in the nucleus. Sulf1 (all isoforms) is weakly expressed in the cytoplasm of non-neuronal cells. b) Nuclear expression was confirmed by counterstaining with DAPI. Arrow points to a neurofilament-positive neuron that express Sulf1 (all isoforms) in the cytoplasm and nucleus. c) Sulf2 (all isoforms) is expressed by cell bodies and neurites of DRG cells. Neuronal localisation was confirmed by neurofilament (red) expression. d) DAPI stains all nuclei. Arrow point to a neurofilament-positive neuron that express Sulf2 (all isoform) in the cytoplasm and the nucleus.

Sulf1 and Sulf2 (all isoforms) are expressed by cultured DRG cells

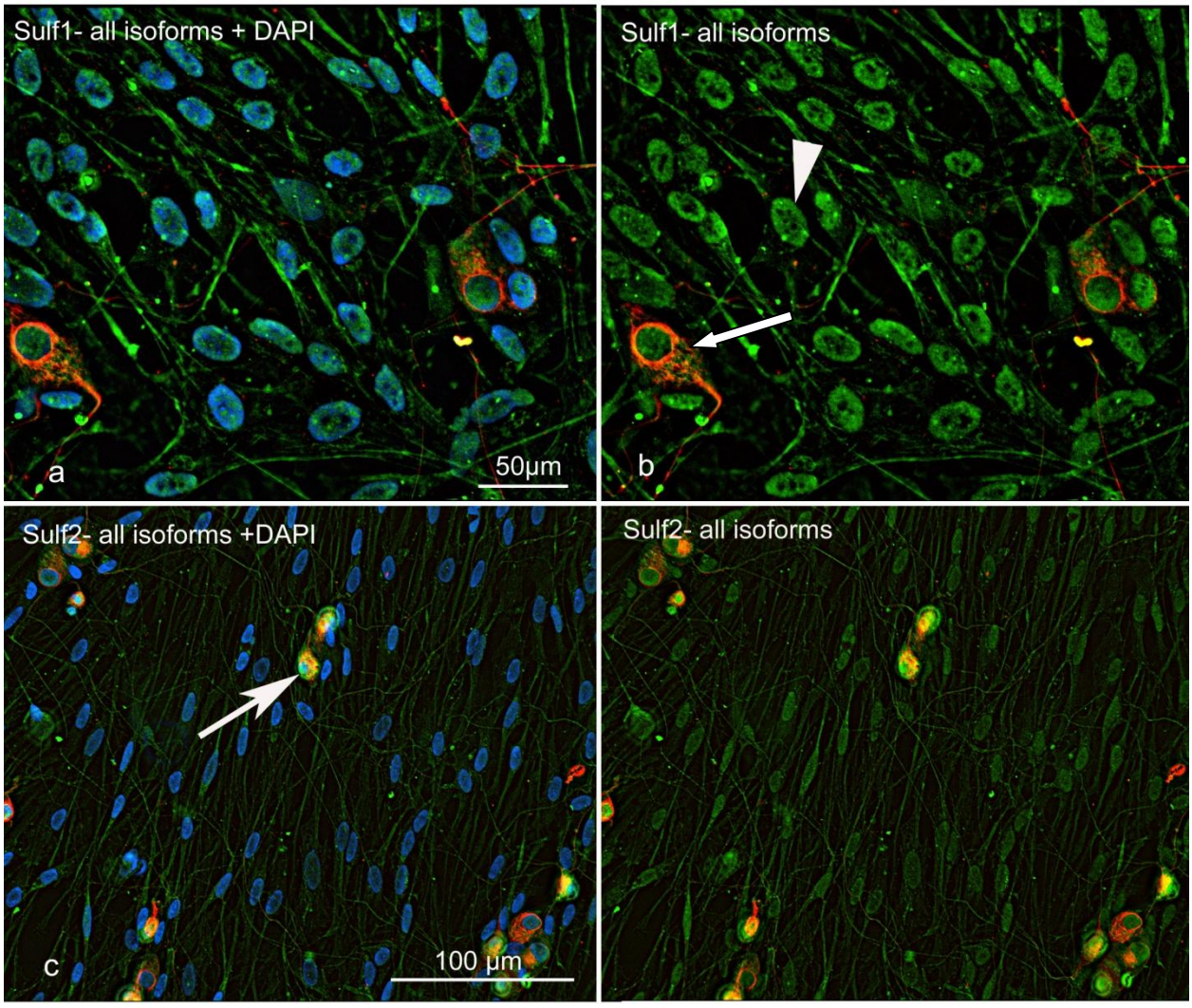


Figure 7.11: Immunoreactivity for full-length Sulf1 (a,b) and Sulf2 (c,d) (green) in DRG cells cultured for 48 hrs. a) Sulf1 (full-length) expression in a DRG neuron. Both cell body and neurites are Sulf1-positive. b) Arrows indicate cytoplasmic localisation of Sulf1 in non-neuronal cells. Presence of non-neuronal cells was confirmed by the nuclear stain, DAPI. c) A DRG neuron that expresses Sulf2 (full-length). Pattern of expression is similar to that of Sulf1. d) DRG cells counterstained with DAPI confirming the presence of non-neuronal cells that show cytoplasmic localisation of Sulf2.

Sulf1 and Sulf2 (full-length) are expressed by cultured DRG cells

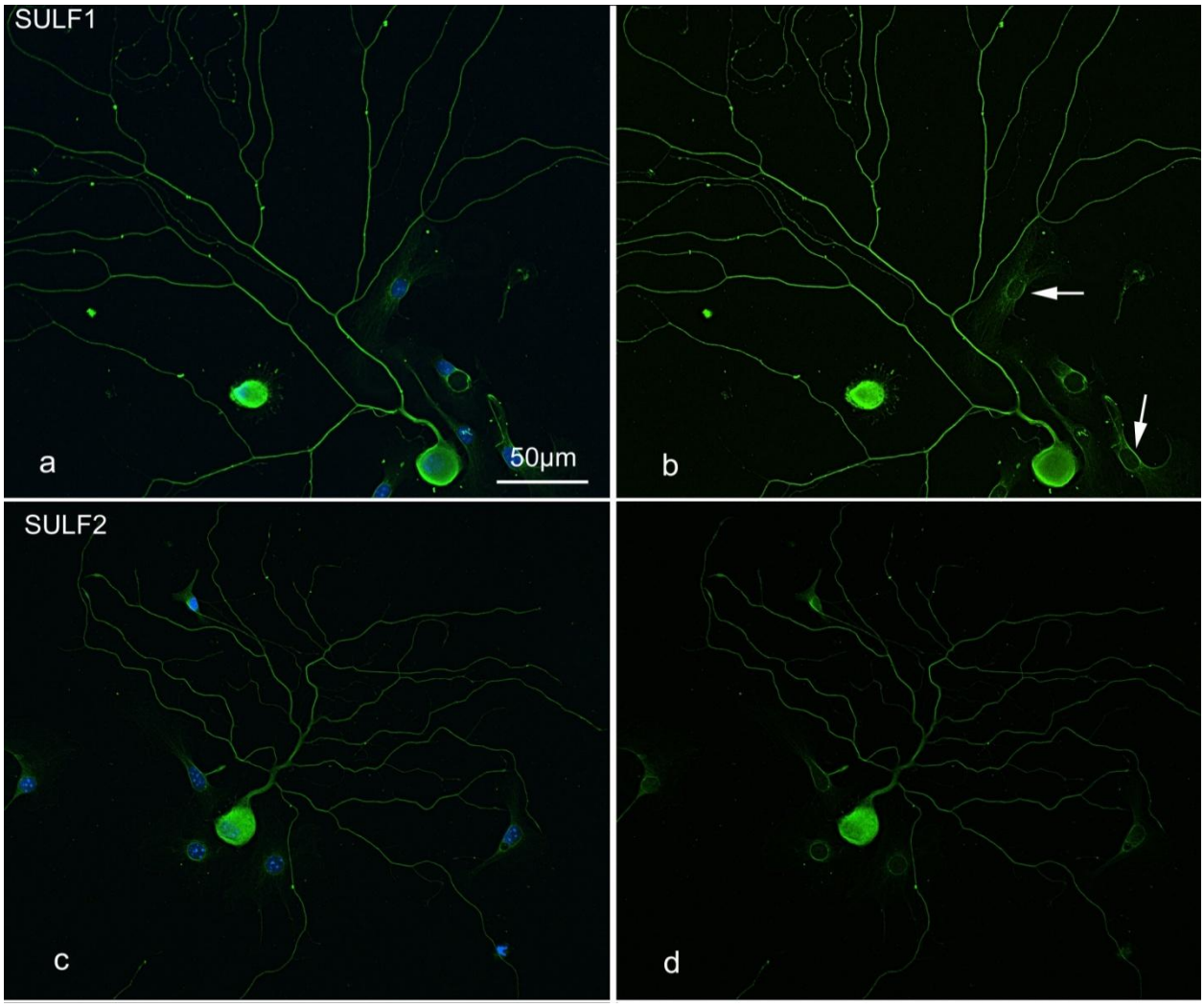


Figure 7.12: Expression of Sulf1 (all isoforms) in the motor cortex of adult rat. a) Most layer 5 (*) pyramidal neurons and many other cells express Sulf1 (all isoforms). b) Pyramidal neurons express NogoA. c) Co-localisation of Sulf1 (all isoform) and NogoA confirms the expression of Sulf1 (all isoforms) in neurons. d) Nuclear stain, DAPI allows the identification of layer 5 of motor cortex in which pyramidal neurons express NogoA and Sulf1 (all isoforms). e) Localisation of Sulf1 (all isoforms) is predominantly nuclear (e.g. arrow) and weakly cytoplasmic. f) Pyramidal neurons that express NogoA in the cytoplasm and Sulf1(all isoforms) in the nucleus (e.g. arrow).

Expression of Sulf1 (all isoforms) in the motor cortex of adult rat

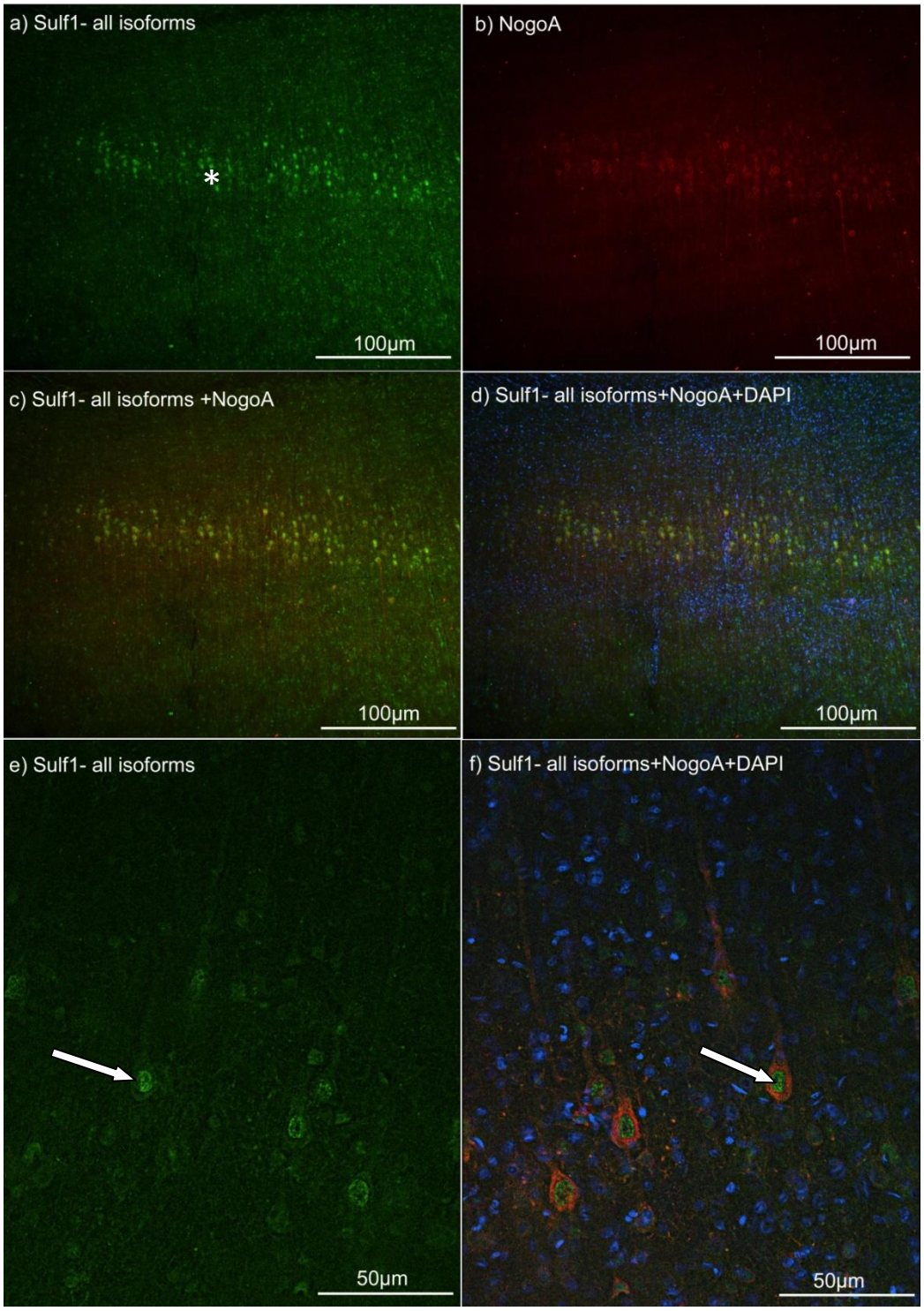


Figure 7.13: Full-length Sulf1 immunoreactivity in the motor cortex of adult rat. a) The nucleus, cytoplasm and some apical dendrites (arrows) of pyramidal neurons are positive for full-length Sulf1. b) Neurofilament stains pyramidal neurons in layer 5 of the motor cortex. c) The same image as shown in (a) and (b) shows co-localisation of Sulf1 (full-length) with neurofilament in the cytoplasm of pyramidal neurons. Sulf1 (full-length) can also be seen expressed in the nucleus of neurons. c) Nuclear stain, DAPI reveals the presence of various cells that may be of neuronal or non-neuronal origin in the field of view. d) The same cells as shown in (c) captured under higher magnification showing nuclear staining of Sulf1 (full-length) in pyramidal neurons detected using neurofilament.

Full-length Sulf1 is expressed in the motor cortex of adult rat

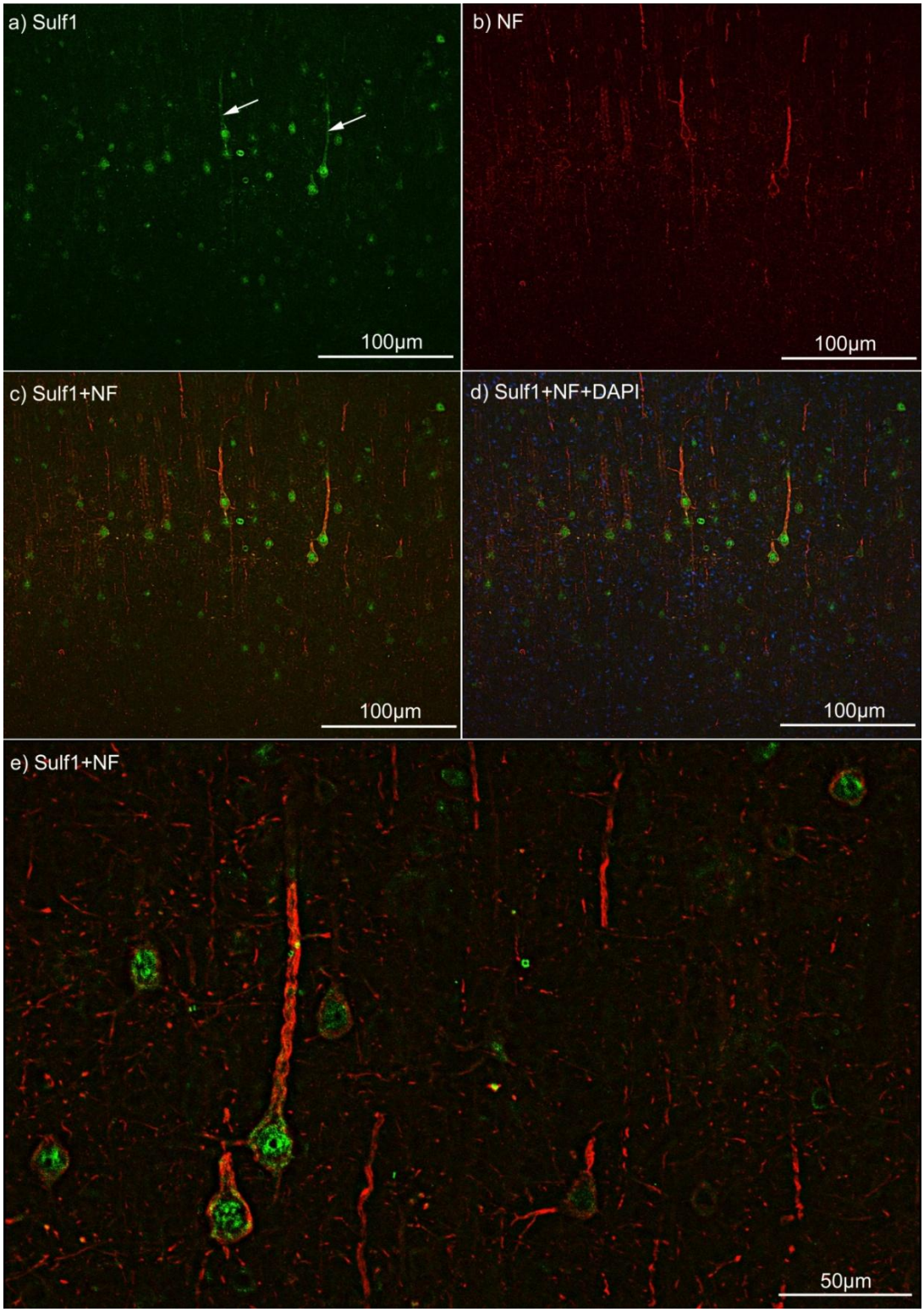


Figure 7:14: Immunoreactivity of Sulf2 (all isoforms) in the motor cortex of adult rat. a) Glial cells in the sub-cortical white matter (SWM), pyramidal neurons in layer 5 (*) and cells in other layers stain positive for Sulf2 (all isoforms). b) DAPI staining helps to indicate various layers in the motor cortex. c) Image captured under higher magnification shows strong Sulf2 (all isoforms) expression in the nucleus of pyramidal neurons (seen in c and d; e.g. arrow). NogoA stains the cytoplasm of pyramidal neurons (c).

Expression of Sulf2 (all isoforms) in the motor cortex of adult rat

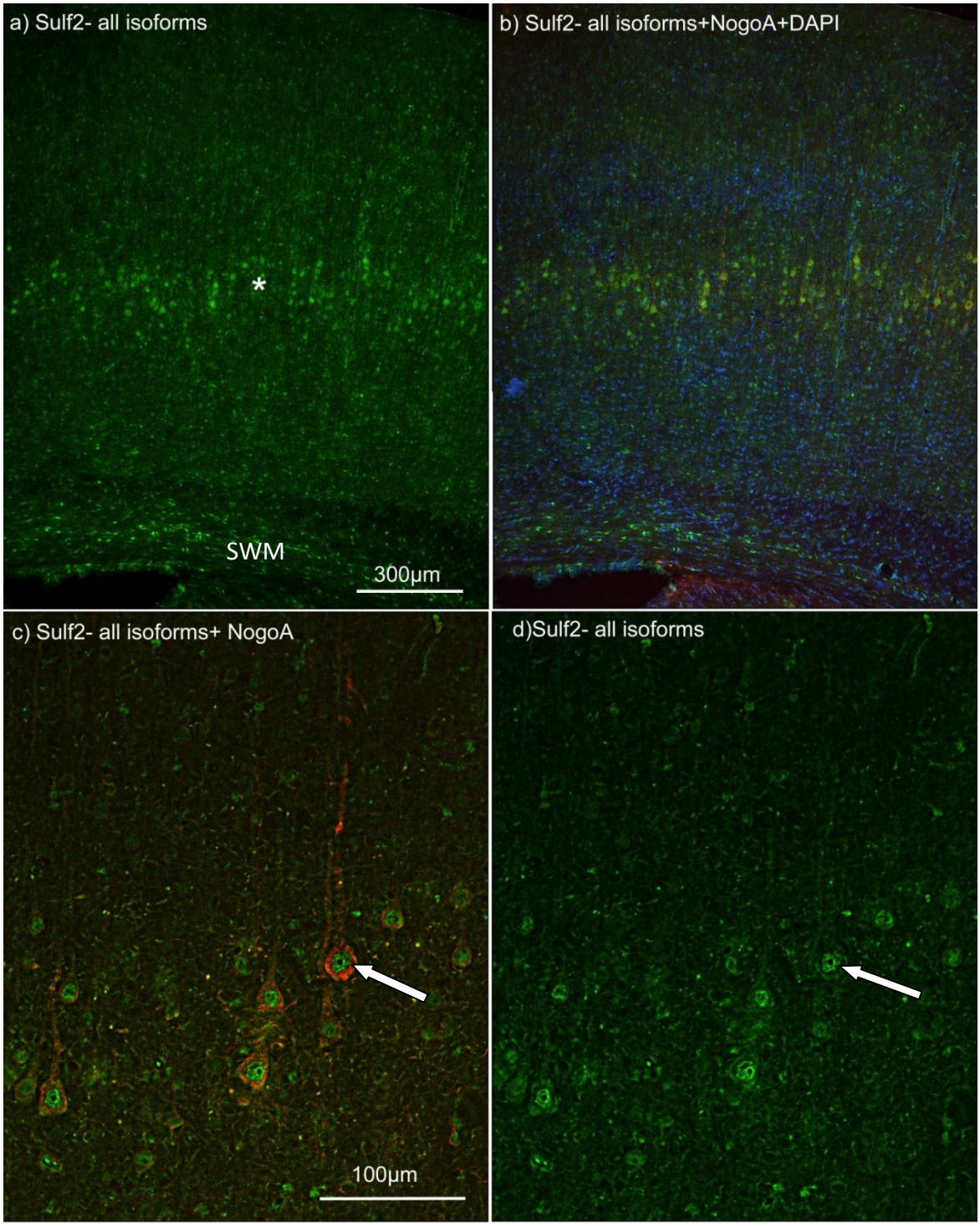


Figure 7:15: Immunoreactivity of full-length Sulf2 in the motor cortex of adult rat. a) Sulf2 is expressed by pyramidal neurons in layer 5 (*) of the motor cortex and cells in other layers. b) Sulf2 (full-length) is strongly expressed in neuronal nuclei with weaker expression observed in the cytoplasm and apical dendrites (e.g. arrow). c) Positive staining for neurofilament confirms neuronal localisation of full-length Sulf2. DAPI stains nuclei and helps to determine the layers in the motor cortex. d) Image shows pyramidal neurons that express neurofilament in the cytoplasm and Sulf2 (full-length) in the nucleus. e,f) The same field of view as shown in d, captured under higher magnification showing nuclear localisation of Sulf2 in many neurofilament-positive neurons as well as in the apical dendrites and basal dendrites (e.g. arrow in e).

Full-length Sulf2 is expressed in the motor cortex of the adult rat.

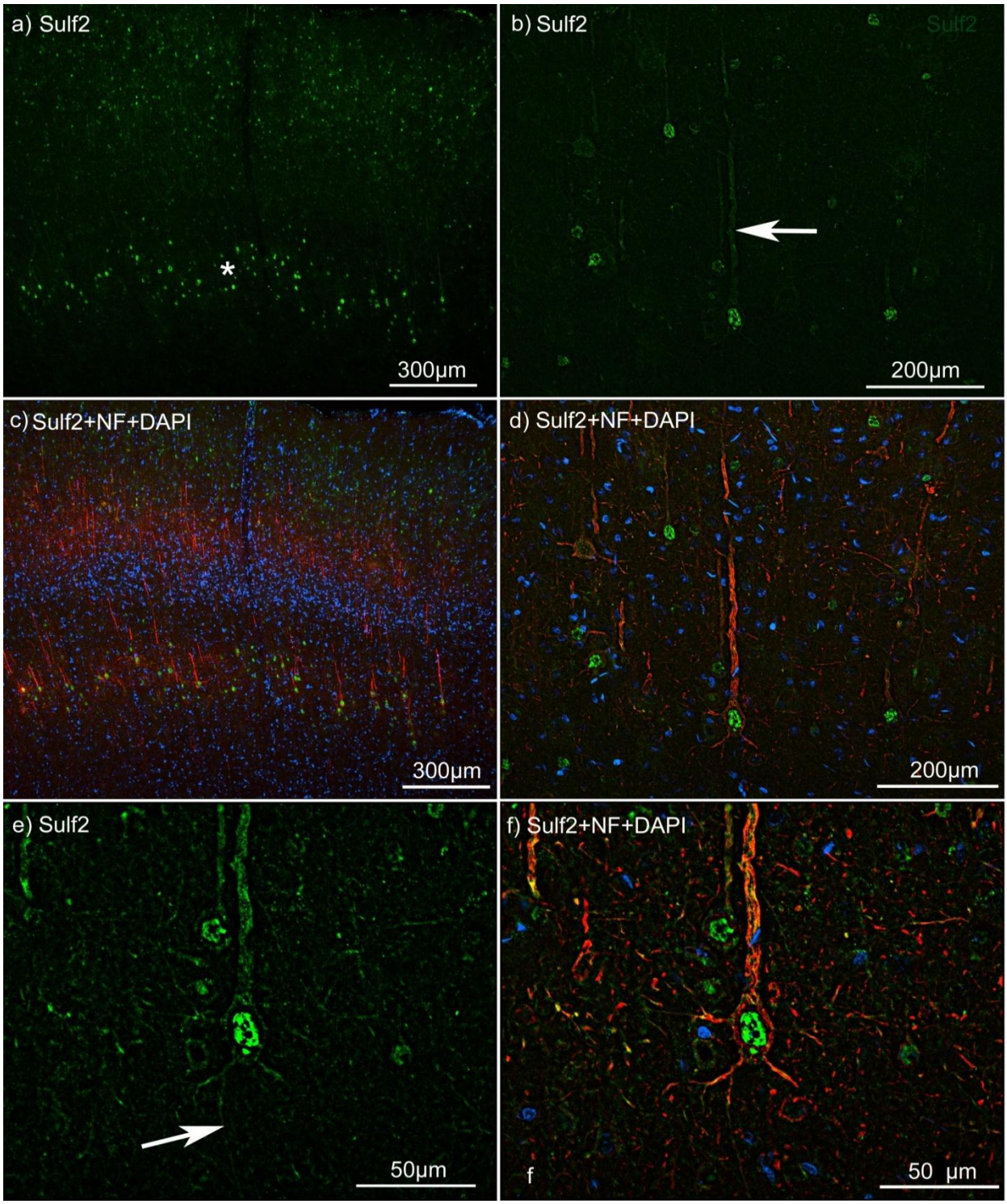


Figure 7.16: Full-length Sulf1 and full-length Sulf2 immunoreactivity in the hippocampal formation of adult rat. a) Sulf1 is expressed in the granule cells (*) of the dentate gyrus. b,d) Dapi stains nuclei in the hippocampal formation. c) Sulf2 is expressed in granule cells in the dentate gyrus and also in neurons present in the hilus of the dentate gyrus (*).

Full-length Sulf1 and full-length Sulf2 are expressed in the hippocampal formation

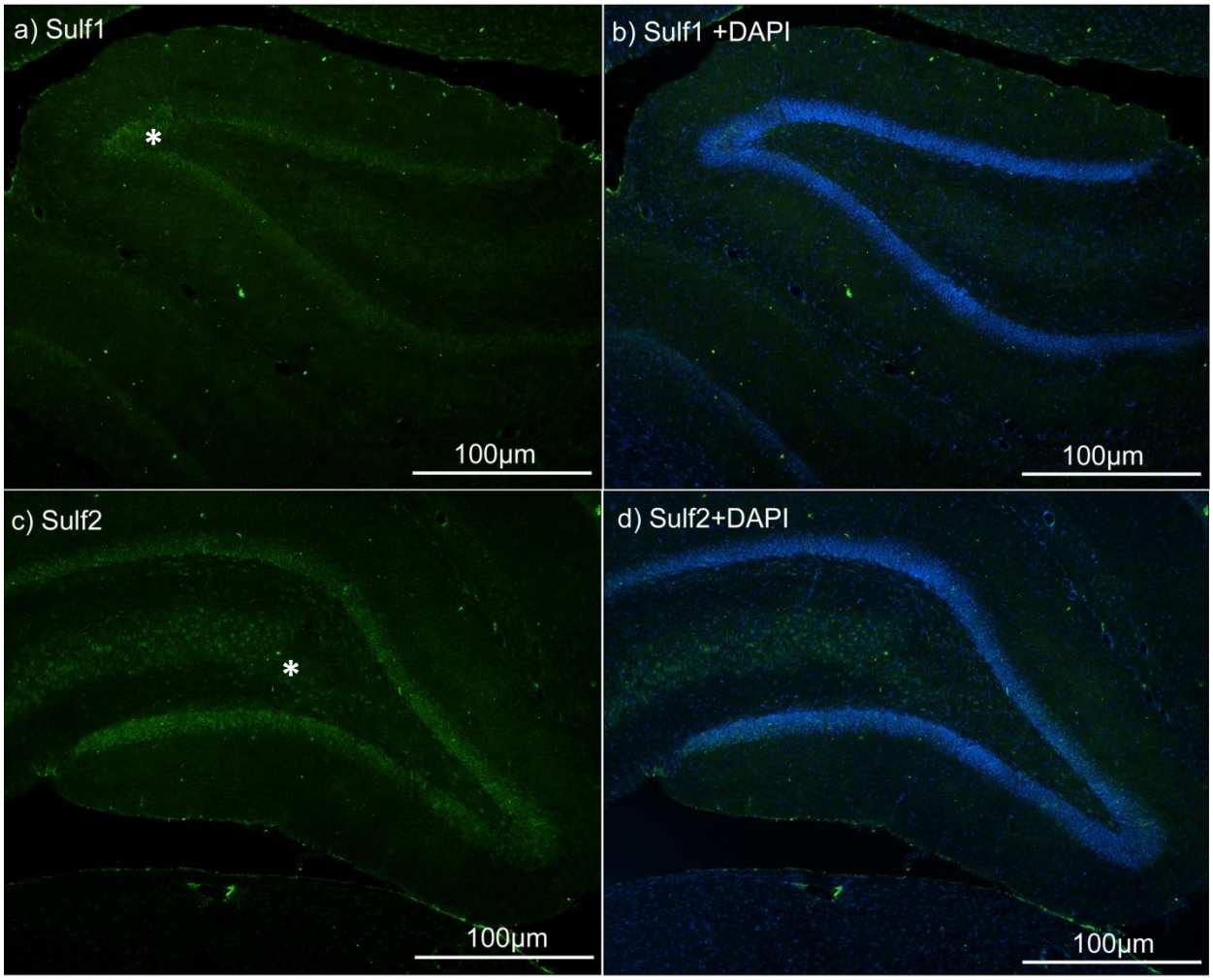


Figure 7.17: Full-length Sulf1 and full-length Sulf2 immunoreactivity in the thalamic reticular nucleus. Neurons in the thalamic reticular nucleus (arrows) and in the corpus striatum (St) stain positive for full-length Sulf1 (a) and full-length Sulf2 (b). DAPI stains nuclei in the fimbria/fornix (*), corpus striatum (St) and the thalamic reticular nuclei.

Full- length Sulf1 and Sulf2 are expressed in the thalamic reticular nuclei of adult rat

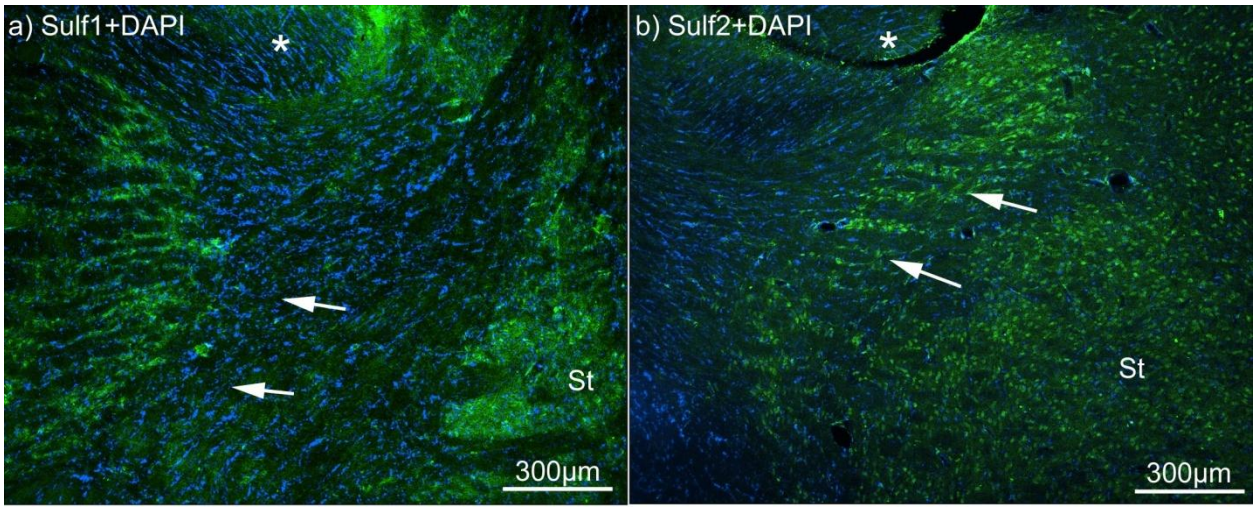


Figure 7.18: Expression of Sulf1 (all isoforms) in cerebellar cortex of adult rat. a) Sulf1 (all isoforms) is expressed by the cell bodies of Purkinje cells, and what appear to be Bergmann glia (on the basis of their size, number and position) (arrow) and their processes (arrowheads). There may be expression of Sulf1 isoforms in the parallel fibres of that project into the molecular layer. b) DAPI stains nuclei in the granule layer and molecular layer. Arrows point to cerebellar granule neurons that express Sulf1 isoforms. Arrowheads indicate processes, probably axons, in the granule cell layer, that stain positive for Sulf1 isoforms.

Expression of Sulf1 (all isoforms) in the cerebellum

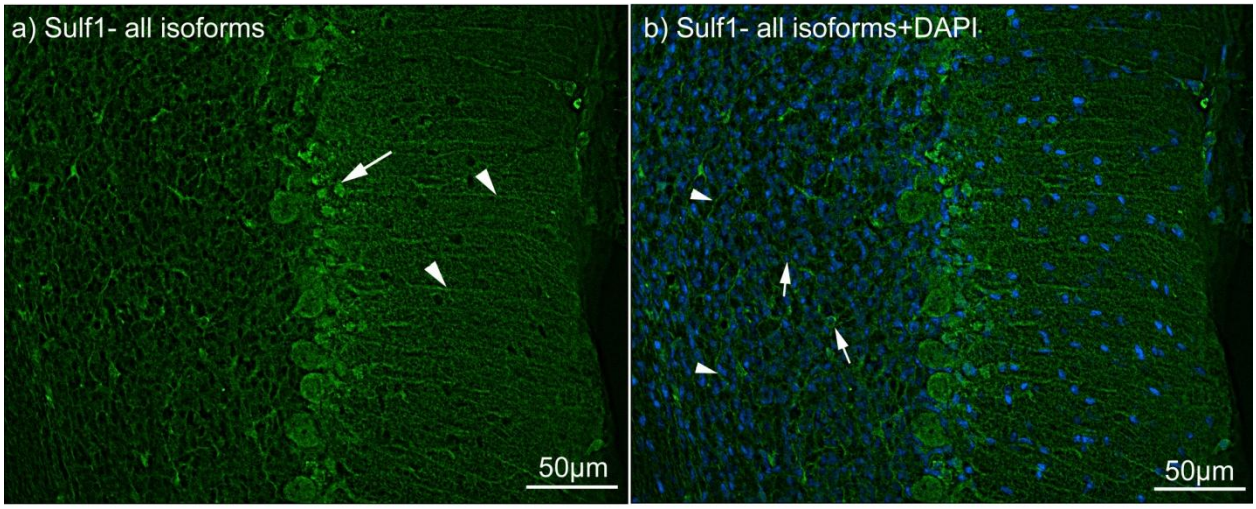


Figure 7.19: Immunoreactivity of full-length Sulf1 in the cerebellum of adult rat. a) Sulf1 is expressed in Purkinje cells and neurons in the deep nuclei (*). Low levels of Sulf1 may be expressed in the sub-cortical white matter. b) The same field of view captured under higher magnification showing numerous dendrites from Purkinje cells that stain positive for Sulf1. c) Axons from Purkinje cells show no obvious sign of Sulf1. d) Sulf1 is expressed by dendrites of Purkinje cells and parallel fibres present in the molecular layer. e,f) The same field of view immunostained for Sulf1(e) and NogoA(f). Sulf1 is expressed in parallel fibres, cell bodies and dendrites of Purkinje cells whereas Nogo A stains Purkinje cell bodies and their dendrites.

Sulf1 is expressed in the cerebellum of adult rat

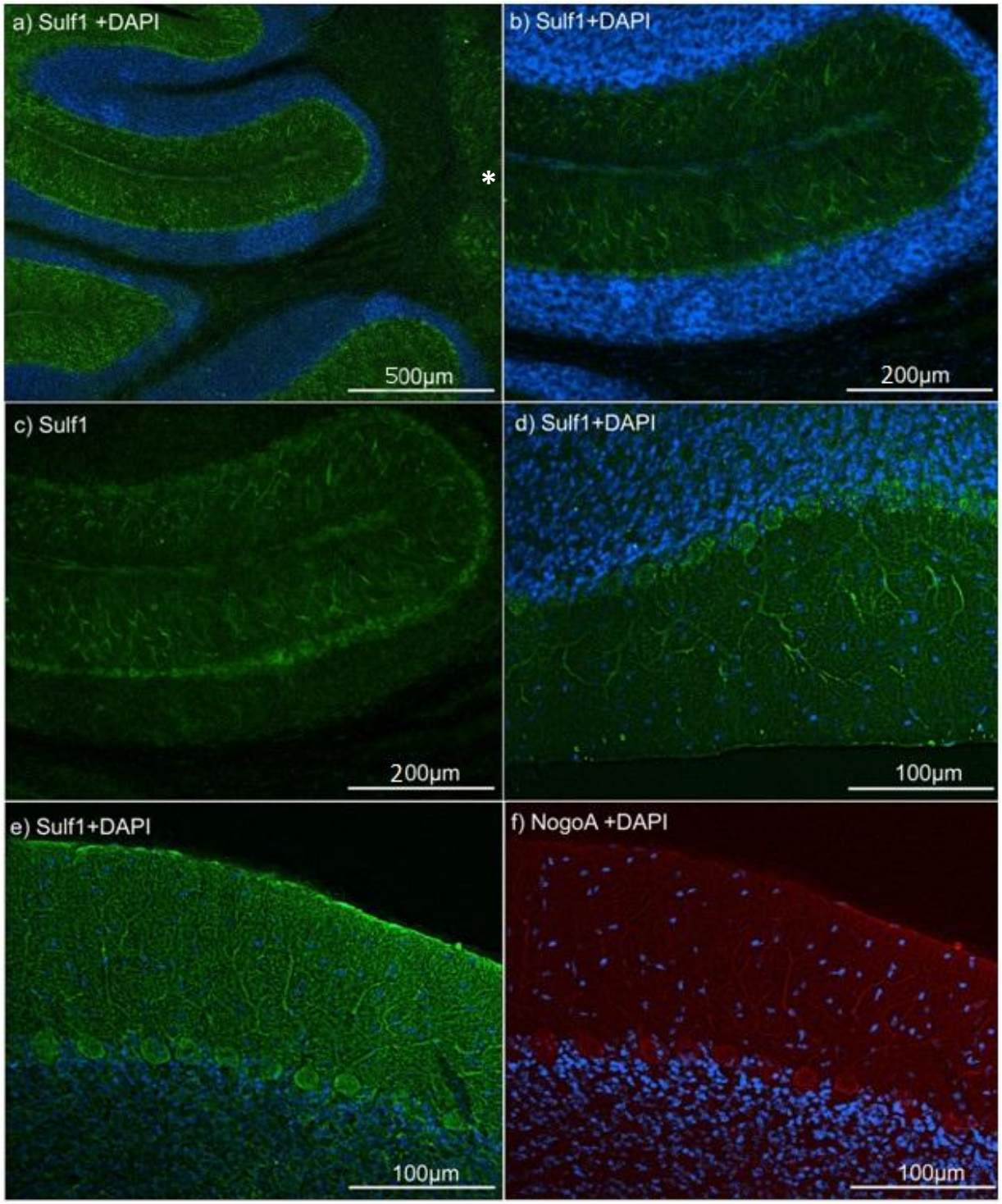


Figure 7.20: Immunoreactivity of full-length Sulf2 in the cerebellum of adult rat. a) Sulf2 (full-length) is expressed in and around the nucleus of Purkinje cells (arrow) and in Purkinje cell dendrites extending into the molecular layer. b) Cell bodies, dendrites and axons of Purkinje cells stain positive for neurofilament. c) Co-localisation of neurofilament and full-length Sulf2 confirms neuronal localisation of Sulf2. There was no obvious sign of full-length Sulf 2 on the axons of Purkinje cells (a and d).

Full-length Sulf2 is expressed in the cerebellum of adult rat

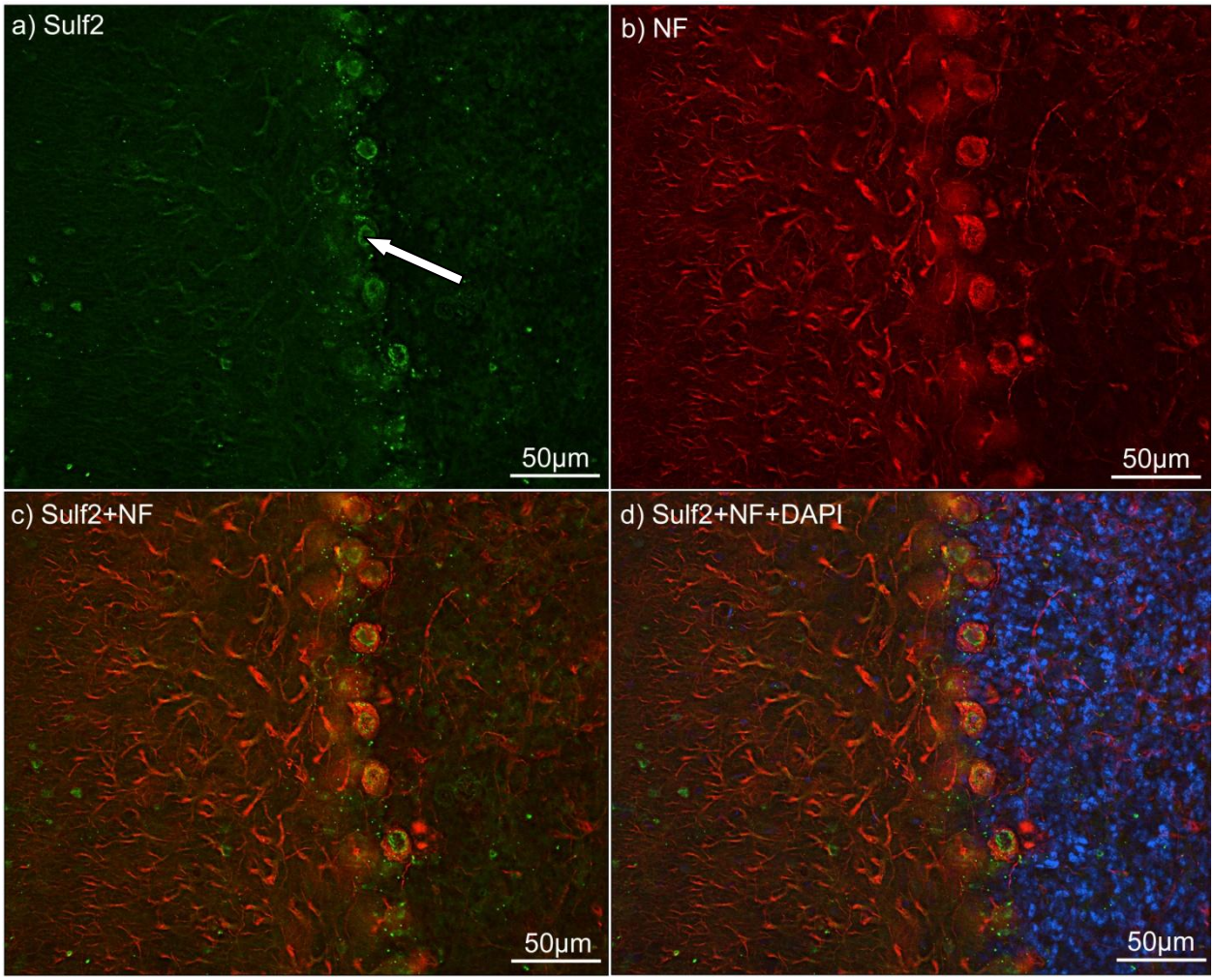


Figure 7.21: Sulf1 (all isoforms) is expressed in neurons and axons the spinal cord of adult rat. a) Sulf1 (all isoforms) is expressed in the neurons of the grey matter. b) Image captured under higher magnification shows immunoreactitivity of Sulf1 (all isoforms) in ependymal cells of the central canal. There is little co-localisation of GFAP (red) and Sulf1 isoforms. c,d) Same field of view showing neurons in the ventral horn that stain positive for (c) NogoA and Sulf1 (all isoforms) (d). Arrows indicate the same neuron that expresses NogoA as well Sulf1 (all isoforms).Sulf1 (all isoforms) is strongly expressed in the cytoplasm of neurons. In the case of NogoA (c), expression is confined to neuronal cytoplasm and axons and is absent in the nucleus. e,f) Sulf1 isoforms are expressed in axons in the ventral columns (e) and dorsal columns (DC) (f). In the ventral columns (e), some GFAP positive processes may also express Sulf1 (all isoforms).

Expression of Sulf1 (all isoforms) in the spinal cord of adult rat

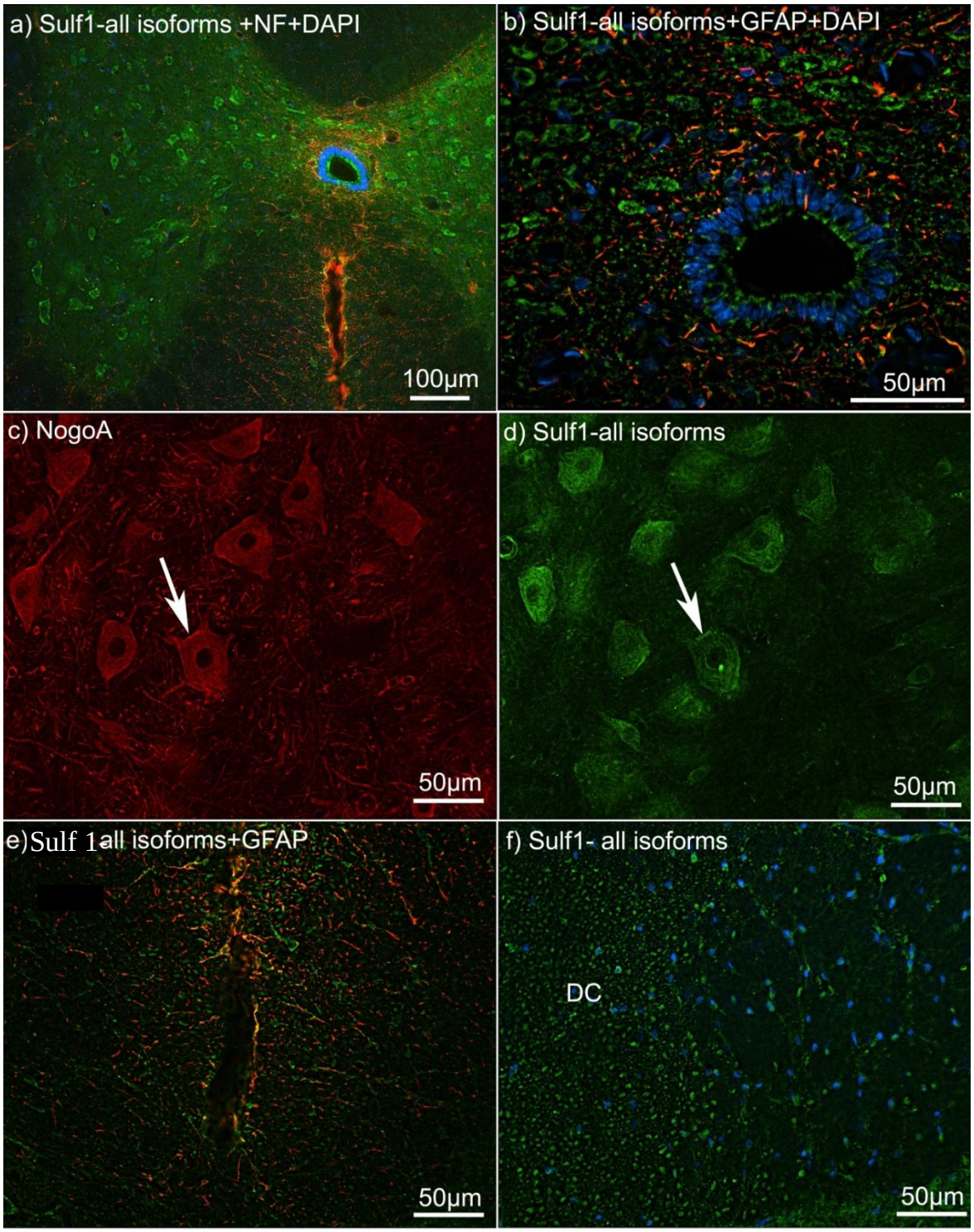


Figure 7.22: Immunoreactivity of full-length Sulf1 is found in neurons, glial cells and axons in the lumbar spinal cord of adult rat. a) Full-length Sulf1 is expressed in the grey matter and by non-neuronal cell processes, probably astrocytes, in the white matter. b) The luminal side of ependymal cells of the central canal stain positive for full-length Sulf1 (green). A NogoA positive neuron (arrow) shows weak expression of full-length Sulf1. c) Motor neurons in the ventral horn express full-length Sulf1. d) Motor neurons express NogoA. Arrows indicate same cell positive for NogoA and full-length Sulf1. e) Axons in the ventral columns are positive for full-length Sulf1. Presumptive glia may also be positive for Sulf1. f) Neurofilament identifies axons in the ventral horn. Arrow heads indicate examples of axons that are both neurofilament and Sulf1 positive.

Full-length Sulf1 is expressed in the spinal cord of adult rat

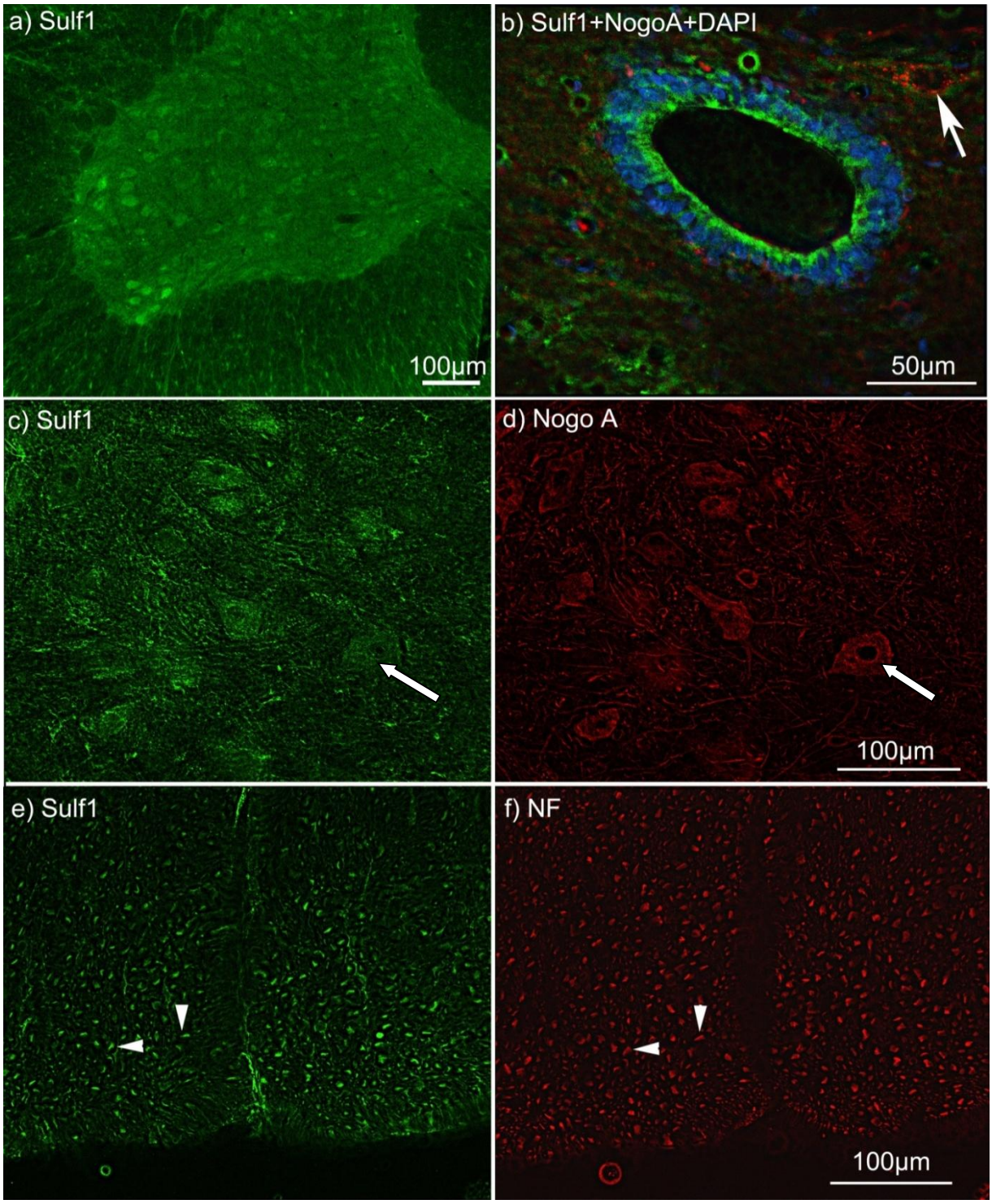


Figure 7.23: Expression of Sulf2 (all isoforms) in neurons and axons the spinal cord of adult rat. a,b) Sulf2 (all isoforms) is expressed by neurons in the ventral horn and glial processes in the white matter (a) as well as by neurons in the superficial dorsal horn (b,*). c) Axons in the ventral columns express Sulf2 (all isoforms) (e.g. arrow).d) A neuron in the grey matter (arrow) that expresses Sulf2 (all isoforms) in the cytoplasm with stronger expression in the nucleus but not nucleolus. NogoA is expressed in the cytoplasm of the neuron and can be seen concentrated in the perinuclear region. e) Sulf2 isoform stains various glial processes in the white matter, presumably those of astrocytes (e.g. arrow) and oligodendrocytes whereas NogoA immonoreactivity is seen only in oligodendrocytes. f) Same field of view as shown in (e) captured under higher magnification that shows oligodendrocytes that are immunoreactive for both NogoA and Sulf2 (all isoforms) (e.g. arrow).

Expression of Sulf2 (all isoforms) in the spinal cord of adult rat

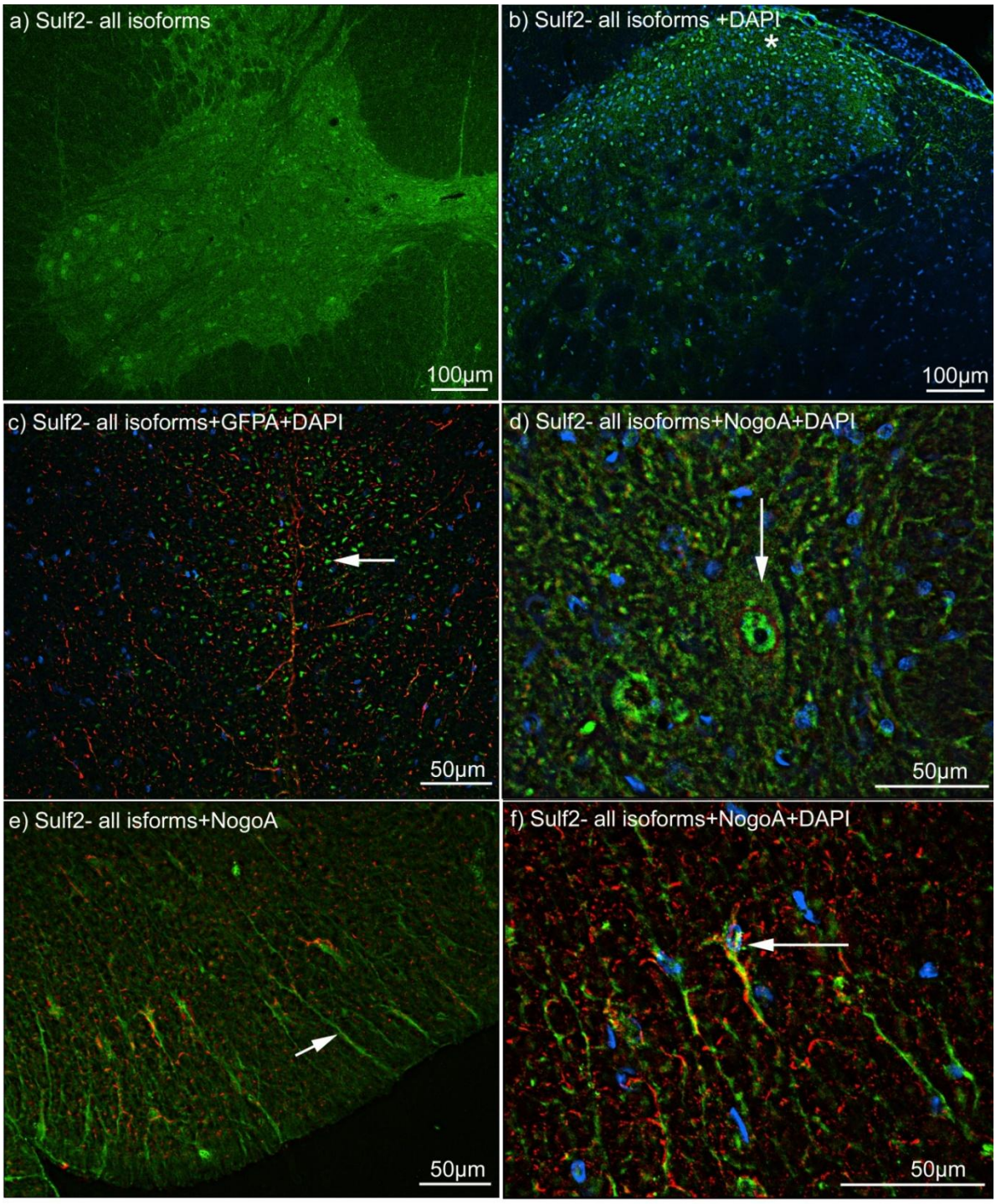


Figure 7.24: Full-length Sulf2 is expressed in the nuclei of neurons and in some axons in the spinal cord of adult rat. a) Neurons in the ventral horn express full-length Sulf2. b) Image captured under lower magnification shows Sulf2 immunoreactivity in neurons in the grey matter and axons in the ventral columns. Weak immunoreactivity may be seen in glial processes in the white matter and also on the luminal side of ependymal cells around central canal. GFAP (red) stains astrocytes. c,f) Cellular localisation of full-length Sulf2 in the grey matter. Immunostaining with GFAP(red) in (c) shows hardly any overlap with Sulf2 immunoreactivity whereas immunostaining with neurofilament (f) clearly identifies motor neurons in the nuclei in which Sulf2 can be seen .However, Sulf2 maybe absent in the nucleolus (arrow). d) Image captured under higher magnification shows axons in the ventral columns expressing full-length Sulf2 (e.g. arrows). However, full-length Sulf2 may be absent in astrocytes detected by immunostaining for GFAP (red). (g) Presumptive oligodendrocytes in the white matter that express NogoA and Sulf2 (e.g. arrow).

Full-length Sulf2 is expressed in the spinal cord of adult rat

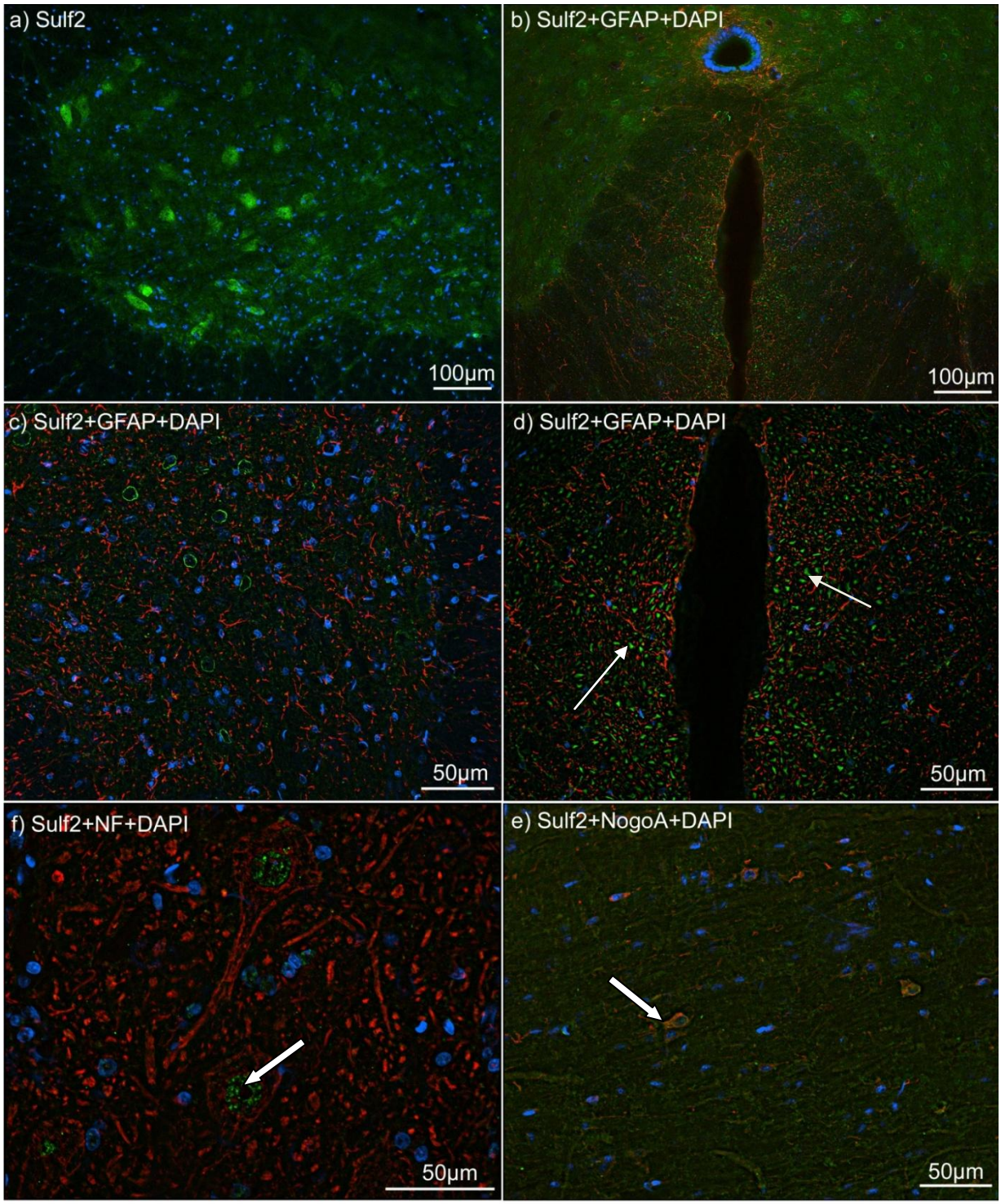
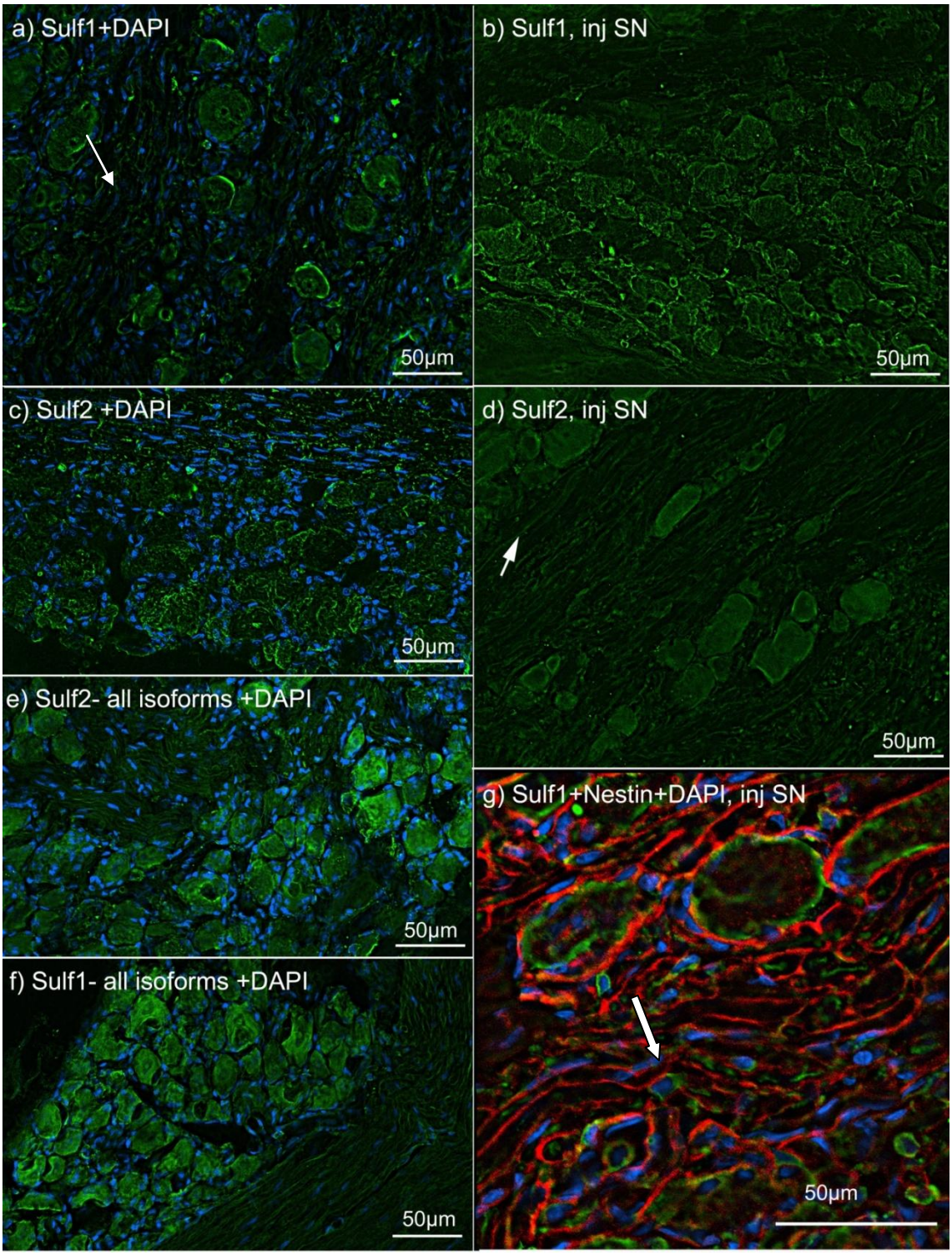


Figure 7.25: Expression of Sulfs in the intact and injured lumbar DRG of adult rats. a) Full-length Sulf1 expressed in neuronal cell bodies and in the neuropil, possibly on axons (e.g. arrow). b) Full-length Sulf1 is still expressed in and around the periphery of neuronal cell bodies in DRGs of animals that underwent sciatic nerve transection. c) Full-length Sulf2 is expressed in the cell bodies of DRG neurons and in the neuropil. d) Full-length Sulf2 is expressed on the cell bodies and some axons (e.g. arrow) of DRG neurons from animals that underwent sciatic nerve transection. e) Sulf2 (all isoforms) is expressed in the cell bodies of DRG neurons. Sulf2 (all isoforms) is localised to the nucleus and nucleus of some cells and probably present on some axons. f) Expression of Sulf1 (all isoforms) in the cell bodies of DRG neurons and the neuropil, possibly axons. g) Injured DRG neurons captured under higher magnification showing full-length Sulf1 immunoreactivity in and around the periphery of DRG cell bodies. Schwann cells and satellite cells were detected by staining for Nestin (red). Arrow points to a cell that express nestin on the periphery and full-length Sulf1 in the axon surrounded by the cell. DAPI has been used to stain nuclei.

Sulf1 and Sulf2 are expressed in intact and injured DRGs of adult rat



Discussion

Expression of Sulf1 and Sulf2 in the nervous system

Using RT-PCR, western blotting and immunohistochemistry we have been able to identify the expression of sulfatase enzymes Sulf1 and Sulf2 in the nervous system. The expression of Sulfs in various tissues of the nervous system as detected by immunohistochemistry has been summarised in Table 7.3. In general the antibodies to all isoforms of the Sulfs stained the same structures as the antibodies to the full-length isoforms. Hence we have little data on the distribution of the shorter variants. An exception was found in the cerebellum where antibodies to all isoforms of Sulf1 stained small cells in the Purkinje cell layer much more strongly than did antibody to full-length Sulf1 (Figure 7.18). This indicates that such cells, by their position and size are likely to be Bergmann glia, express one or more short variants of Sulf1. Full-length Sulf1 and Sulf2 are expressed by a wide majority of neurons but there are differences in the pattern of expression. Full-length Sulf1 immunoreactivity was mainly localised to the cytoplasm of most neurons (Figures 7.19, 7.21, 7.22) whereas Sulf2, including full-length Sulf2, was expressed most strongly in the nucleus, but not in the nucleolus, with comparatively weaker expression in the cytoplasm (Figures 7.14, 7.23, 7.24). In the motor cortex Sulf1 was also strongly expressed in the nuclei of layer 5 pyramidal neurons (Figures 7.12, 7.13), similar to Sulf2. Full-length Sulf1 and Sulf2 were expressed in axons in the spinal cord and would therefore be in a position to influence axonal regeneration following cord injury.

Although, expression of Sulf1 and Sulf2 proteins in the nervous system of adult rat has not been previously characterised using immunohistochemistry, there have been a few reports on the expression of its transcripts in embryonic chick, adult mouse and rat nervous tissue (Nagamine et al., 2005;Garcia-Lopez et al., 2009;Braquart-Varnier et al., 2004;Kalus et al., 2009) (summarised in table7.4). A previous study using in-situ hybridisation has reported punctate expression of Sulf2 transcripts in the grey matter with less abundant signals in the white matter of the spinal cord (Nagamine et al., 2005). That study also reported expression of Sulf2 in a small proportion of DRG neurons. In the brain, strong expression of Sulf2 mRNA in layer 5 of the motor cortex as well as in the CA_3 region of the hippocampus was reported (Nagamine et al., 2005). In comparison, we have shown strong immunoreactivity for both Sulf1 and Sulf2 in axons

within the white matter of the spinal cord and motor neurons of the grey matter. Since both the antibodies to full-length Sulf 1 and 2 and the antibodies to all isoforms of those enzymes stained the axons, immunohistochemistry tells us little about the distribution of the shorter splice variants in axons.

Full-length Sulf2 was prominently expressed by NogoA-positive oligodendrocytes (Figure 7.23, 7.24). Expression of Sulf1 by oligodendrocyte precursors in the embryonic chick spinal cord has been previously reported (Braquart-Varnier et al., 2004). Moreover, it has been proposed that Sulf1 acts as a major component of a pathway that controls the fate of neural progenitor cells inducing them to differentiate into oligodendrocyte precursors as opposed to motor neurons in the ventral spinal cord of the chick embryo (Touahri et al., 2012). In the case of DRG neurons, unlike previously reported (Nagamine et al., 2005), we were able to detect Sulf1, Sulf2 including the full-legth isoforms in most DRG neurons particularly cultured neurons. Moreover, expression of Sulfs remained detectable in DRGs following a sciatic nerve lesion.

In the current study, we analysed the expression of Sulf1 and Sulf2 in the nervous system. Using RT PCR, we have shown the expression of Sulf1 and Sulf2 transcripts in the cerebral cortex, cerebellum, spinal cord, DRGs and sciatic nerve of mice from different age groups- P0, P8 and adult. The expression of its transcripts in all age groups suggests that Sulf1 and Sulf2 may not only be involved in developmental processes but may also play a role in other aspects of cell physiology, even in the mature animal. Our studies using western blotting have shown that full-length Sulf1 (132kDa/100kDa) is expressed in adult cerebral cortex, cerebellum, spinal cord, DRG and sciatic nerve. In contrast, full-length Sulf2 (132kDa/100kDa) was absent in tissue lysates from DRGs and sciatic nerve. Instead, a 54kDa fragment which has been suggested to be a sub-domain of Sulf2 (Tang and Rosen, 2009) was detected in these tissues in addition to its detection in lysates from the cortex, spinal cord and cerebellum. Nonetheless, we were able to detect Sulf1 and Sulf2 in neurons particularly their axons using the same antibodies to full-length forms using immunohistochemistry. As our data from PCR, western blotting and immunohistochemistry serves as strong evidence for the expression of Sulfs in the nervous system, it remains intriguing as to why the full-length forms of Sulf2 (132kDa/100kDa) were undetectable in lysates from the adult sciatic nerve and DRGs. It can be speculated that the

detected forms of Sulf2 in DRGs using PCR and immunohistochemistry may correspond to the 54kDa sub-unit and that the full-length protein corresponding to a size of 132kDa would be synthesised under varied circumstances such as after injury. In addition, we also detected expression of Sulf1 and Sulf2 in DRGs following a lesion to the sciatic nerve. It would be of interest to see if the 132 kDa full-length protein was expressed under such conditions.

Functional significance of the expression of Sulf1 and Sulf2 in the nervous system

Immunohistochemical evidence for the expression of shorter isoforms of Sulfs was only detectable in the cerebellar cortex where full-length Sulf1 was barely detectable in presumptive Bergmann glia but the antibody to all isoforms of Sulf1 showed strong staining. This suggests that these cells express an enzymatically inactive Sulf1. The antibody to full-length Sulf1 detects a region which is essential for enzymatic activity but is spliced out in the shorter isoforms. Cysteine 89 on exon 6 has been reported to be critical for the enzymatic activity of Sulfs (Sahota and Dhoot, 2009). Isoforms that lack this residue are enzymatically inactive i.e. incapable of desulfating HSPGs. However, exon 20/21 of Sulf1 and exon23 of Sulf2 are not spliced out and hence present in all isoforms. It has been shown that a short variant of the full-length Sulf1 has opposing effects to full-length Sulf1 with regard to modulating wnt and VEGF/FGF signalling (Sahota and Dhoot, 2009). A possible mechanism is that short isoforms would compete with full-length Sulf1 and Sulf2 and bind to HSPGs thereby protecting HSPGs from desulfation and hence promoting signalling.

The presence of HSPGs in the nucleus of neurons in the spinal cord has been previously reported suggesting their participation in nuclear processes (Liang et al., 1997). One study has reported that nuclear heparan sulphate inhibits the activity of DNA topoisomerase I, a nuclear enzyme translocated at active sites of transcription (Kovalszky et al., 1998). Also, other enzymes that act on HSPG substrates such as heparanase have been shown to be translocated to the nucleus where it acts on nuclear heparan sulphate chains (Schubert et al., 2004). In the light of these studies, it can be suggested that the expression of Sulfs in the nucleus as demonstrated by immunohistochemistry may affect transcription by modulating HSPG activity. However, the exact mechanism by which Sulfs regulate transcription requires further investigation. If HSPGs inhibit DNA transcription by inhibition of topoisomerse activity, it would of interest to know if

this activity was dependant on the presence of 6-O sulphate groups on HSPGs. Also, HSPGs have been reported to modulate the internalisation and nuclear localisation of FGF-1 and FGF-2 (Sperinde and Nugent, 1998;Sperinde and Nugent, 2000) and that FGF interferes with HSPG-mediated inhibition of topoisomerase (Kovalszky et al., 1998). As there are numerous reports on nuclear localisation of ErbB1(Wang et al., 2010;Lo, 2010), it would also be of interest to investigate if nuclear uptake of ErbB1 is modulated by HSPGs, if so, this would attribute Sulfs with the role of regulating neuronal ErbB1 activity. Most importantly, it would be interesting to investigate changes in the pattern of expression of Sulf1 and Sulf2 in the spinal cord following injury.

Conclusion

We have shown that Sulf1 and Sulf2 transcripts are expressed in various types of nervous tissues which include cerebral cortex, cerebellum, spinal cord, and DRGs from neonatal and adult mice. Also Sulf1 and Sulf2 proteins were detected in tissue lysates from cerebral cortex, cerebellum, spinal cord, DRG and sciatic nerve from adult rats. Sulfs, detected using immunohistochemistry have shown to be expressed by a wide majority of neurons and also by non-neuronal cells. While Sulf1 is expressed in the cytoplasm of many neurons, Sulf2 is predominantly expressed in the nucleus. The exact role of Sulfs in the nucleus would be an interesting area for further investigation.

Nervous tissue	Sulf1	Sulf2
Motor cortex, layer 5		
Pyramidal neurons	+++	+++
Hippocampal formation		
CA_4	++	+++
Dentate gyrus	+	++
Hilus of dentate gyrus	++	+++
Thalamic reticular nucleus	+++	+++
Corpus Striatum	+++	+++
Cerebellum		
Purkinje cells	+++	++
Granule layer	+	+
Parallel fibres in the molecular layer	++	+/-
Deep nuclei	+++	ne
Sub-cortical white matter	+	ne
Spinal cord		
Ventral horn neurons	+++	+++
Ependymal cells	+++	+
Dorsal horn neurons	+++	+++
Ventral column axons	+++	+++
Dorsal column axons	ne	ne
Astrocytes	++	+/-
Oligodendrocytes	+?	++
DRG	++	++
Cultured DRGs	+++	+++

Table7.3: Expression of Sulfs in the nervous system as detected by immunohistochemistry. +++ denotes strong expression, ++ medium expression, + weak expression, +/- may or may not be expressed, ne- not investigated.

	Sulf1	Sulf2
Cortex		
Layer1	- (Klaus	+
Layer2	et.al.,2009)	+
Layer3	-	+
Layer4	-	+
Layer5	-	++(Nagamine
Layer6	+ +?(Ohto	et.al.,2005)
	et.al,2002)	++
Hippocampus	-	
CA1		
CA2		
CA3	-	+
Dentate gyrus	-	+
Olfactory tubercle	-	++
Hypothalamus	-	+
	+	NE
Cerebellum	+	+
Molecular layer		
Purkinje layer		
Granule layer	++	++
Choroid Plexus	-	++
Spinal cord	-	-+
Dorsal horn	+ +	+
Ventral horn		
White matter		
DRG	+	NE
	+	NE
	+	NE
	+	NE

Table7.4: Expression of Sulf transcripts that have been previously reported. +++ denotes strong expression, ++ medium expression, + weak expression, +/- may or may not be expressed, NE- not investigated. + or- in black font corresponds to data reported by Kalus et.al., 2009. Likewise, blue font corresponds to data reported by Ohto et.al, 2002 and red font corresponds to data reported by Nagamine et.al., 2005.

Chapter 8- Expression of ErbB1in the nervous system

Introduction

The expression of ErbB1 in neurons starts during brain development and peaks before birth in rodents, suggesting a role for ErbB1 in the regulation of neurogenesis (Yamada et al., 1997). After birth, the expression of ErbB1 declines and basal levels of expression are maintained throughout adulthood (Yamada et al., 1997;Liu and Neufeld, 2007). The expression of ErbB1 before birth has been predominantly localized to the subventricular regions. Post-natal expression of ErbB1mRNA has been observed in the cerebellar external granular layer, with continued weak expression in the dentate gyrus, and cerebral periventricular zone, regions where neurogenesis is a prominent feature even in the adult (Cameron et al., 1998;Seroogy et al., 1995). The expression of ErbB1 protein in developing and adult human dorsal root ganglia neurons have been reported but the quality of such immunohistochemical evidence is poor (Huerta et al., 1996;Werner et al., 1988).

In the injured nervous system, there is strong evidence for increased expression of ErbB1 in astrocytes, although such evidence comes mainly from one laboratory (Liu and Neufeld, 2007;Liu and Neufeld, 2004;Zhang and Neufeld, 2005). Although there are numerous reports of increased expression of the phosphorylated form of ErbB1 in non-neuronal cells (Erschbamer et al., 2007;White et al., 2011;Liu et al., 2006), it remains unclear if phosphorylated ErbB1 is upregulated in neurons in the injured nervous system. While one report suggests expression of ErbB1 in neurons following spinal cord injury (Schachtrup et al., 2007), there are also reports suggesting that phosphorylated ErbB1 is expressed by non-neuronal cells associated with DRG neurons and not by neurons themselves (Ahmed et al., 2009;Ahmed et al., 2010).

As our functional data suggests that ErbB1 signalling is associated with the failure of regeneration, we wanted to confirm if ErbB1 is expressed in neurons, particularly by DRG neurons. It was also of interest to investigate if there were changes in the pattern and strength of expression of ErbB1 following injury.

Methods

For a detailed account of the methods, please refer Chapter-2.

Surgery

Briefly, the sciatic nerve of adult Sprague Dawley rats (n=2) were transected and 3mm of nerve segment was removed. The skin was sutured and animals were killed 7 days post-surgery. Control group involved animals that did not receive any surgical intervention.

Immunohistochemistry

Animals were perfused transcardially with 2% paraformaldehyde. The sciatic nerve and roots leading to the dorsal root ganglia, brain and spinal cord were dissected and immersed in 2% paraformaldehyde for 2 hrs followed by overnight incubation in 30% buffered sucrose at 4°C. The next day, DRG tissue was embedded in tissue-tek (Sakura). 12µm thick sections were taken using the cryostat. The brain and spinal cord were not embedded, instead freezing microtome sections of 40µm thickness were taken and reacted free-floating.

Sections were blocked in blocking solution contain goat serum. Sections were then immunostained using the following primary antibodies:

Primary antibody	**Working concentration**
Polyclonal anti-EGFR produced in rabbit (recognises C-terminus of EGFR of human origin, purchased from Santa Cruz)	1:10
Monoclonl anti- phospho EGFR produced in rabbit (recognises EGFR when phosphorylated at Tyr 1173, purchased from Cell Signalling)	1:100
Monoclonal anti-neurofilament produced in mouse (recognises medium and heavy neurofilament - molecular weight:160kDa and 200KDa of mouse, rat and human, purchased from Sigma).	1:400
Monoclonal anti-nestin produced in mouse (purchased from Sigma)	1:500

Table 8.1: List of primary antibodies used to immunostain sections of brain, spinal cord, DRG and sciatic nerve.

Sections were incubated in primary antibody overnight at 4°C. Free floating sections were incubated with primary antibody at room temperature for 2 hours with agitation followed by overnight incubation at 4°C. After several washes in 0.1M PBS, sections were treated with secondary antibodies which included Anti-rabbit Alexa Fluor-488 (Invitrogen) and anti-mouse Atto-594 (Sigma) used at a working concentration of 1:600. Nuclei were visualized using DAPI stain.

Anatomical evaluation of DRGs

DRGs were dissected from wildtype and ErbB1-/- mice aged between P0-P7. The ganglia were placed in 2% paraformaldehyde for 2 hours and then immersed in fixative solution containing 2% glyceraldehyde and 2% paraformaldehyde. Tissue was stored at 4°C. Cryostat sections were taken.

Western blotting

Protein was extracted from cortex, cerebellum, spinal cord, DRG and sciatic nerve from adult rats using lysis buffer (RIPA or 5M Urea buffer). 10µg of protein was loaded into each well. Protein was then subject to electrophoresis and transferred into a nitrocellulose membrane (Hybond ECL, Amersham). The membranes were blocked for half an hour with gentle agitation using 5% skimmed milk followed by overnight incubation with primary antibody (EGFR-Rabbit, Santa Cruz) at a concentration of 1:100. After several washes in 1X PBS, the membranes were treated with secondary antibody (Anti-IgG HRP, Promega ; 1:2000) for 1hr with gentle agitation. Membranes were visualized using chemiluminescence by treatment with ECL for 2 min followed by visualization of bands on an X-ray sheet developed using the XO-graph. Membranes were then stripped using 1X re-blotting buffer (Pierce) and immunostained with primary antibody to GAPDH (Mouse, Abcam; 1:2000) which acted as the loading control. This was detected with anti-mouse IgG-HRP(Promega, 1:2000).

PCR

RNA was extracted from cerebral cortex, spinal cord, DRGs and sciatic nerve from neonatal and adult mice and also from DRG neurons cultured from mice pups aged P8. RNA was reverse transcribed and used for either quantitative or reverse transcriptase PCR reactions. The details of the primers and reactions have been described in Chapter-2.

Results

ErbB1 transcripts are expressed in DRG cultures.

To confirm the expression of ErbB1 in DRG cells, RNA was isolated from ErbB1+/- and ErbB1-/- neuronal cultures. Two separate PCR reactions were carried out using different sets of primers. PCR products shown in Figure 8.1a were obtained using previously published primers- 5'-GGG GC GTTGGAGGAAAAGAA which anneals to regions in exon 1 and 5'-ATGAGTG GT GGG CAGGTG which anneals to regions in exon7 (Threadgill et al., 1995). As the mutation in ErbB1-/- animals involved targeted disruption in exon2, this resulted in aberrant splicing where exon 1 joined to either exon 3 or exon 5. In wildtypes, exon 1 would join to exon 2 and a single PCR product would be produced with a size of 700bp (not shown). In the case ErbB1-/- cells, the two splice variants which included joining of exon 1 to exon 3(300bp) or exon 1 to exon 5 (600bp) were amplified, but the product corresponding to joining of exon1 to exon2 as seen in wildtypes was absent. In the case of heterozygotes, all three forms were amplified.

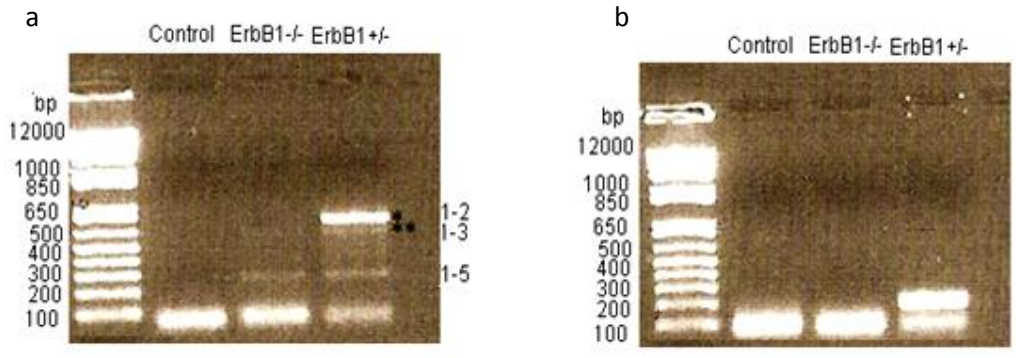


Figure 8.1: Expression of ErbB1 transcripts in DRG cultures. RNA was isolated from ErbB1-/- and ErbB1+/- DRG cultures and reverse transcribed . Control denotes reaction mixtures in which cDNA was replaced with water a) PCR products from ErbB1 transcripts using primers 5'-GGGGCGTTGGAGGAAAAGAA which anneals to regions in exon 1and 5'-ATGAGTG GTGGGCAGGTG which anneals to regions in exon7. Band sizes are indicated on the left and splice variants on the right. Expected bands correspond to sizes 300bp, 600bp and 736bp. **denotes splice variant of size 600bp and * denotes full-length PCR product b) PCR products from transcripts using primers 5'TGCCAAGGCACAAGTAACAG and 5'GTTGA GGCAATGAGGACAT; both primers anneal to regions between exon2 and exon3. Band size is 191 bp.

We also used a different set of primers which amplified regions between exon2 and exon3 (Figure 8.1b). In ErbB1-/- cells, as the target sequence is lacking, no PCR products were formed whereas in heterozygotes, a single band corresponding to a size of 191bp was obtained. In each of the cases, a control reaction was set up where cDNA was replaced with water. Bands seen below a size of 100bp denotes primer dimmers or unused primers which has a decreased band intensity in the case of heterozygote as part of the primers were used up for the reaction. These results show that DRG cultures express ErbB1transcripts.

ErbB1 protein is expressed in CNS and PNS tissue.

To investigate if ErbB1 is expressed in different types of nervous tissue, protein lysates were prepared from adult rat cerebral cortex, cerebellum, spinal cord, DRGs and sciatic nerve. Total ErbB1 that corresponds to a band size of 150kDA was detected in all types of samples tested (Figure 8.2). While the greatest quantity of ErbB1 was detected in adult cortex (Figure 8.2a), least amounts were seen in the sciatic nerve (Figure 8.2b). It was worth noting that DRGs appeared to have more ErbB1 protein than the spinal cord and the sciatic nerve. Also the cortex and cerebellum expressed higher amounts of ErbB1 when compared to other tissue samples. These inferences are made based on band intensities from equal amounts of protein lysates that were loaded onto the gel. Even loading was confirmed by immunostaining for GAPDH.

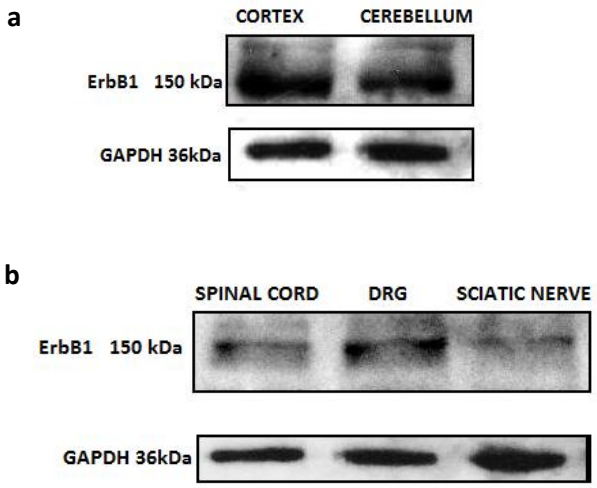


Figure 8.2: Immunoblots from protein lysates showing expression of total ErbB1 in different types of nervous tissue. 10µg of protein was loaded into each well. a) Immunoblots from adult rat cerebral cortex and cerebellum lysed used RIPA buffer. b) Immunoblots from spinal cord, DRG and sciatic nerve of adult rats lysed used 5M urea buffer. Membranes were immunostained with GAPDH to confirm even loading of samples.

ErbB1 is expressed in different types of nervous tissue in neonatal and adult rats.

Quantitative PCR analysis shows that ErbB1 transcripts are expressed by adult and neonatal rats. Although, there are no drastic differences in the expression of ErbB1 transcripts with age in the different types of tissue, the general trend is that ErbB1 expression declines with age. The levels of ErbB1 expression is highest in the P0 cortex when compared to P8 and adult cortex, and also when compared to ErbB1 levels in spinal cord, DRG and sciatic nerve from all age groups (Figure 8.3). However, the expression of ErbB1 is slightly higher in the adult cortex when compared to P8 cortex. In the spinal cord, DRG and sciatic nerve, ErbB1 expression is highest in P0 animals and slowly declines with age. This decline in ErbB1 expression with age was particularly obvious in DRGs, and the differences are subtle in the spinal cord. When comparing expression of ErbB1 in different age groups, it can be said that at P0 ErbB1 expression is highest in cortex; at P8, ErbB1 expression is highest in the spinal cord and in the adult, once again ErbB1 expression is highest in the cortex.

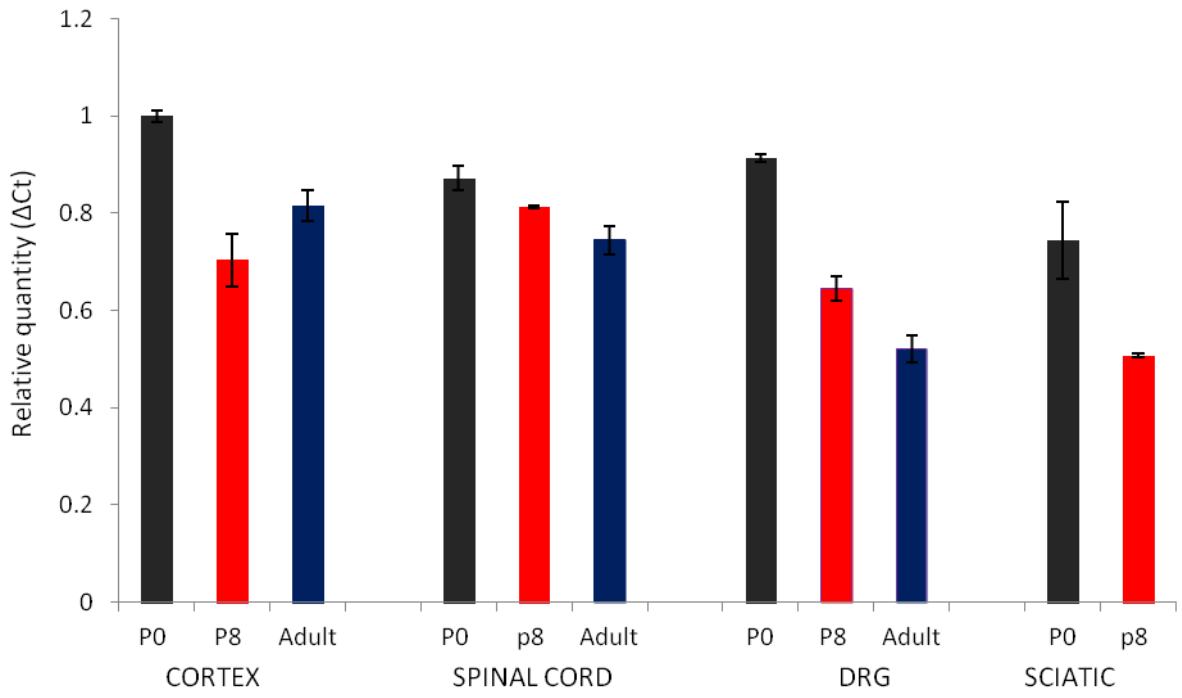


Figure 8.3: Expression levels ErbB1 detected by qPCR in different types of nervous tissue from animals of different age groups. ErbB1 expression has been standardized against the internal reference gene RPL3. The experiments were done in triplicates.

ErbB1 is expressed by various neurons in the nervous system of adult rats.

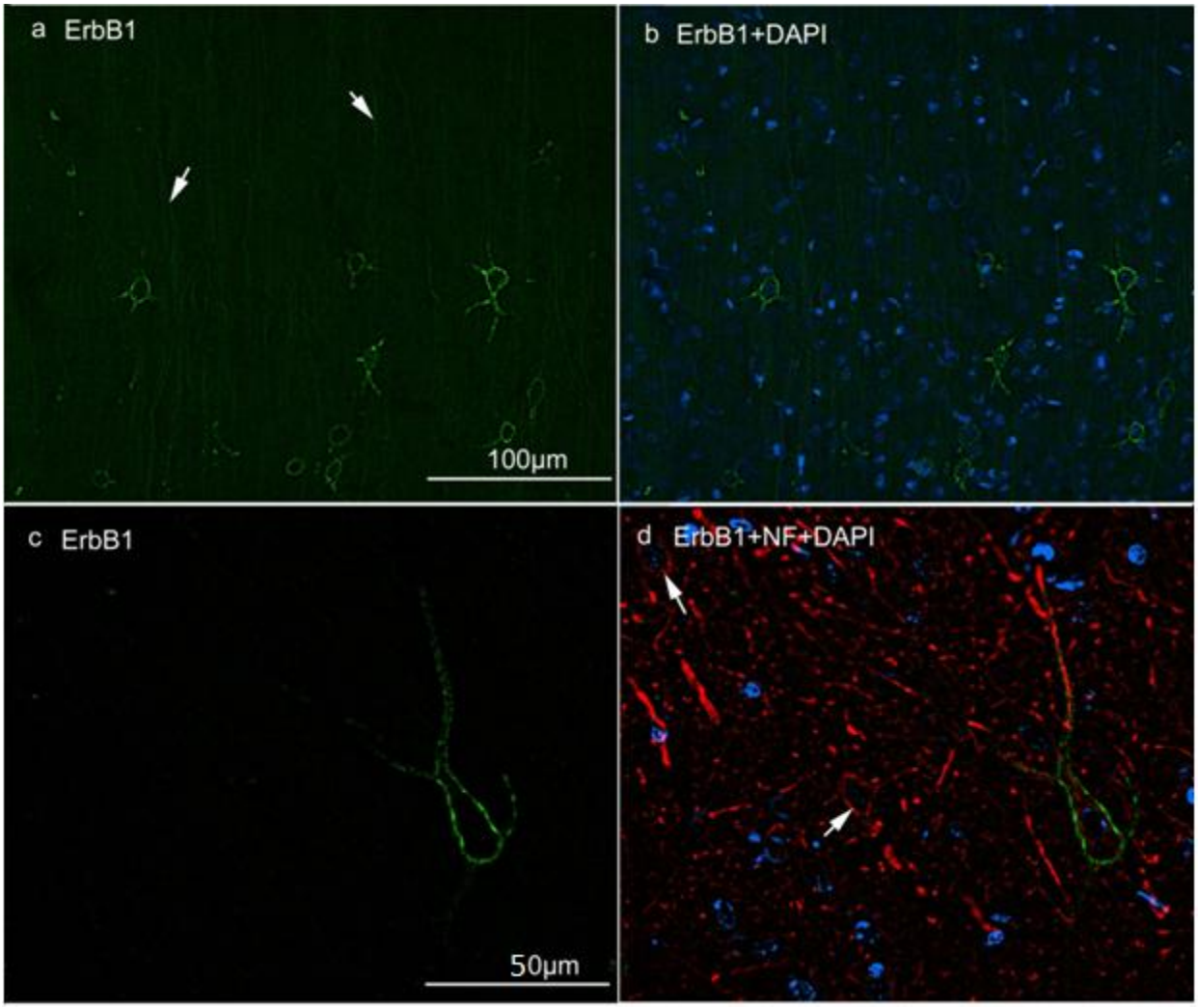
Using immunohistochemistry we were able to detect expression of ErbB1 in various neurons in the nervous system particularly their axons. In the cerebral cortex, ErbB1 was detected in some pyaramidal neurons and their apical dendrites in layer 5 of the motor cortex (Figure 8.4, Figure 8.5). In the cerebellum, ErbB1 expression was prominent in the axons of the sub-cortical white matter (Figure 8.6). A comparatively weaker signal for ErbB1 was detected in deep nuclei neurons (Figure 8.6). Also structures similar to parallel fibres in the molecular layer stained positive for ErbB1 (Figure 8.7). In the spinal cord, ErbB1 is expressed in dorsal column axons and some staining was observed in the superficial dorsal horn (Figure 8.8). A phosphorylated form of ErbB1 (phosphorylated at tyrosine residue 1173) was detected in neurons and nerve fibres in DRGs (Figure 8.9). Moreover, the expression of the phosphorylated

form of ErbB1 (pErbB1) was investigated in the intact and transected sciatic nerve (Fiure 8.11). pErbB1 is expressed in the nerve fibres in the sciatic nerve. It was noted that pErbB1 expression was absent in Schwann cells that stained positive for nestin. Following transection to the sciatic nerve, pErbB1 expression was stronger in the proximal segment when compared to the injured nerve. There was very little expression of pErbB1 in the distal segment. Moreover, pErbB1 expression was detected in DRGs following transection to the sciatic nerve (Figure 8.10).

Apart from immunohistochemical detection of ErbB1, we looked for anatomical diffrences between DRGs from ErbB1-/- and wildtype animals. It was observed that ErbB1-/- DRGs had a smaller proportion of large diametre neurons and a larger proportion of small diameter neurons when compared to wildtype DRGs (Figure 8.12).

Figure 8.4: Expression of ErbB1 in the motor cortex of adult rat. a) ErbB1 (green) is expressed by some neuronal perikarya and by many apical dendrites; arrows point to apical dendrites expressing ErbB1.b) ErbB1 positive neurons counterstained with nuclear stain, DAPI c) An ErbB1 positive neuronal cell body captured under higher magnification shows the antigen located at the cell surface. d) Co-localisation of ErbB1-positive cell body with neurofilament (red) and DAPI stain; arrow points to a neuronal cell body positive for neurofilament but not ErbB1.

ErbB1 is expressed in the adult rat cortex



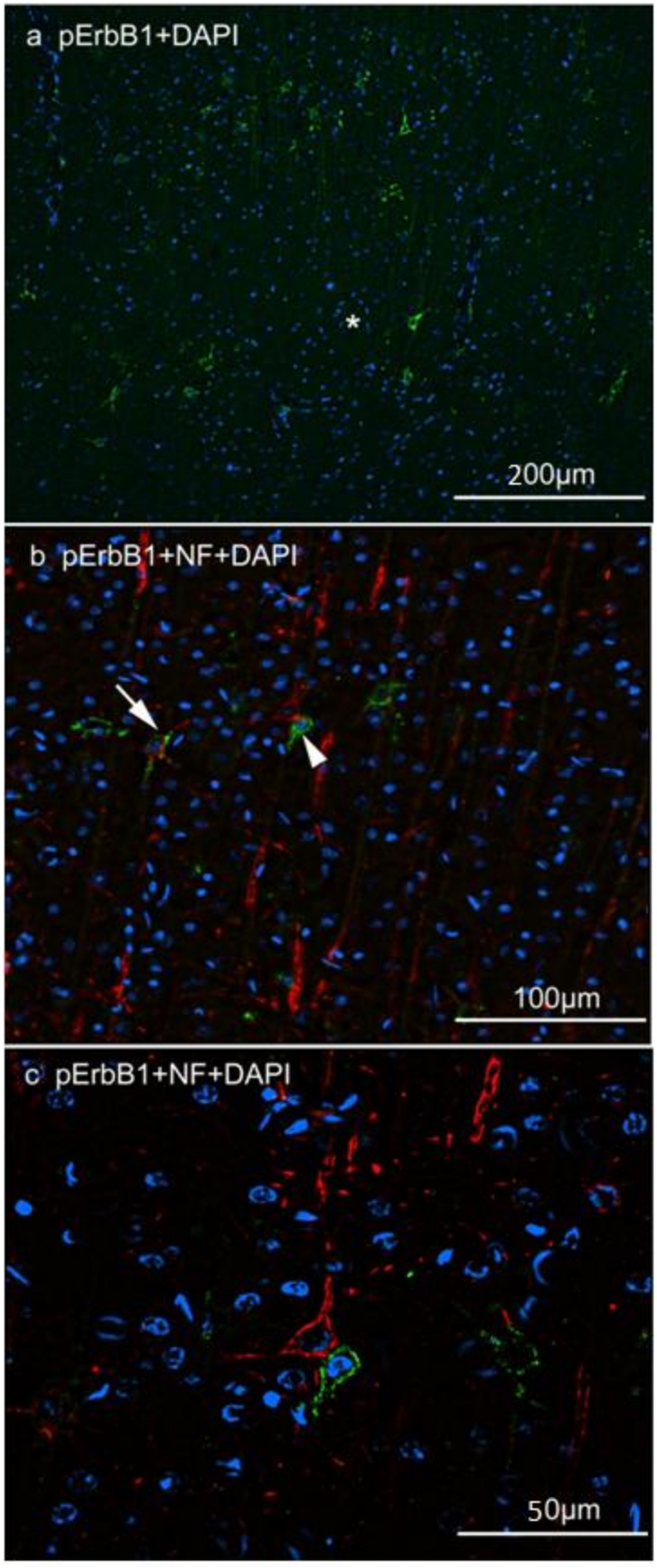

a pErbB1+DAPI
*
200μm
b pErbB1+NF+DAPI
100μm
c pErbB1+NF+DAPI
50μm

Figure 8.6: Expression of ErbB1 in axons and deep cerebellar nucleus neurons in the adult rat cerebellum. a) ErbB1 (green) is weakly expressed by neuronal cell bodies and some neurites in the deep nuclei of the cerebellum; arrowheads point to neurons expressing ErbB1 in the cytoplasm. b) Image showing ErbB1 (green) in neurons, neurofilament positive axons (red) and DAPI positive nuclei. c) ErbB1 positive axons in the sub-cortical white matter (*). d) The same image but with Purkinje cell bodies (PCL) (top left corner) and axons stained for neurofilament protein (red) and the granule layer (GCL) containing densely-packed DAPI positive nuclei. e) ErbB1 positive axons shown in (c) captured under higher magnification. f) image shown in (d) captured under higher magnification, arrowheads point to some axons that are both neurofilament and ErbB1 positive.

ErbB1 is expressed in the adult rat cerebellum

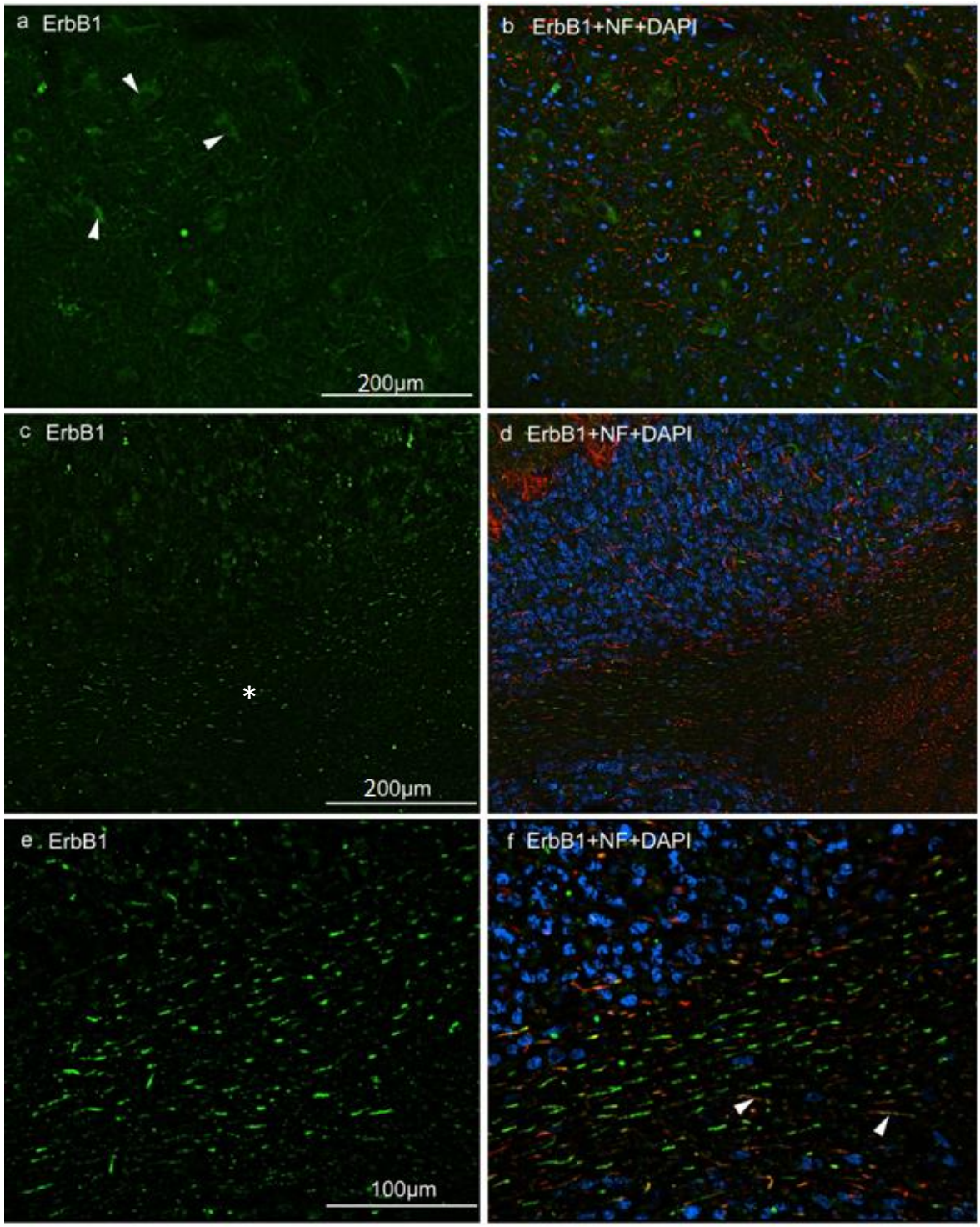


Figure 8.7: Expression of ErbB1 in the molecular layer of the cerebellum. a) Transverse sections through the cerebellum showing ErbB1 expression in structures with the same orientation as parallel fibres. The arrow points to the edge of a blood vessel around which ErbB1 is expressed. b) ErbB1 is absent in dendrites from Purkinjee cells stained for neurofilament (red), blue indicates nuclei stained with DAPI.

ErbB1 is expressed in the molecular layer of the cerebellum

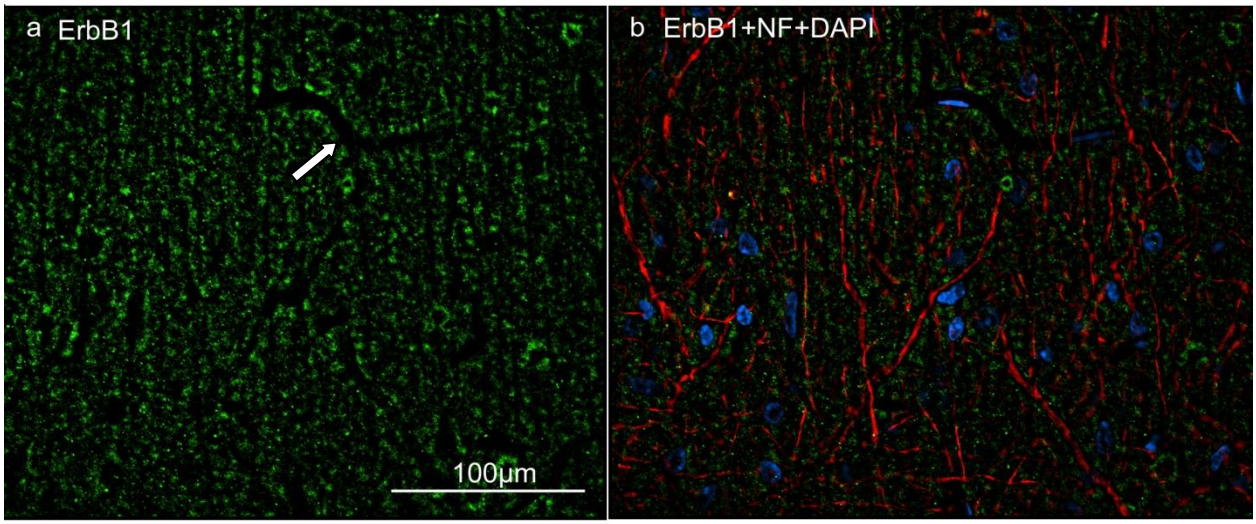


Figure 8.8: Expression of ErbB1 in the adult rat spinal cord. a) ErbB1 (green) is expressed in the superficial dorsal horn (indicated by *) and by axons in the dorsal column (DC). b) Neurofilament staining (red) of axons in the dorsal column, ErbB1co-localises with neurofilament. c,d) Images from the same field shown in a) and b) captured under higher magnification. Arrows show co-localisation of ErbB1 and neurofilament in large diameter axons. Nuclei stained with DAPI .

ErB1 is expressed in the adult rat spinal cord

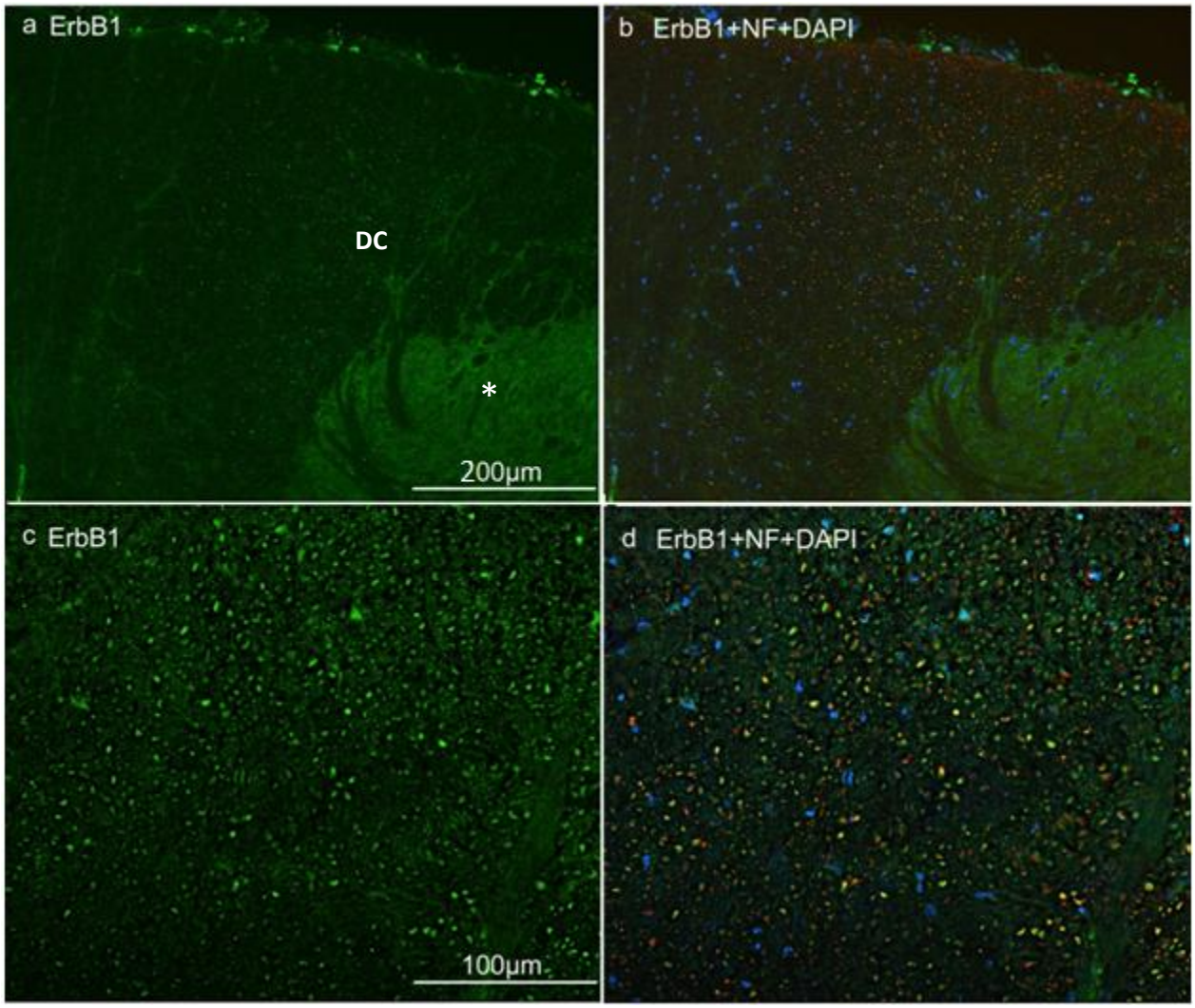


Figure 8.9: Expression of pErbB1 by adult rat DRGs. a) and c) are images of sections treated with secondary antibody but not primary antibody. a) DAPI stains nuclei of DRGs as wells as Schwann cells and satellite cells around DRG neurons. b) deconvolved image showing pErbB1 (green) expressed by neuronal cell bodies and nerve fibres in DRGs. c) shows background staining from treatment with secondary antibody. d) Unmanipulated image showing pErbB1 staining in DRG neurons. e) DRG neurons captured under higher magnification. Satellite and Schwann cells are stained for nestin (red). pErbB1 is expressed in neuronal cell bodies. Arrows indicate staining of pErbB1 in axons. Nestin and pErbB1 does not co-localise, suggesting that pErbB1 is not found in Schwann or satellite cells.

Phosphorylated ErbB1 is expressed by adult rat DRGs

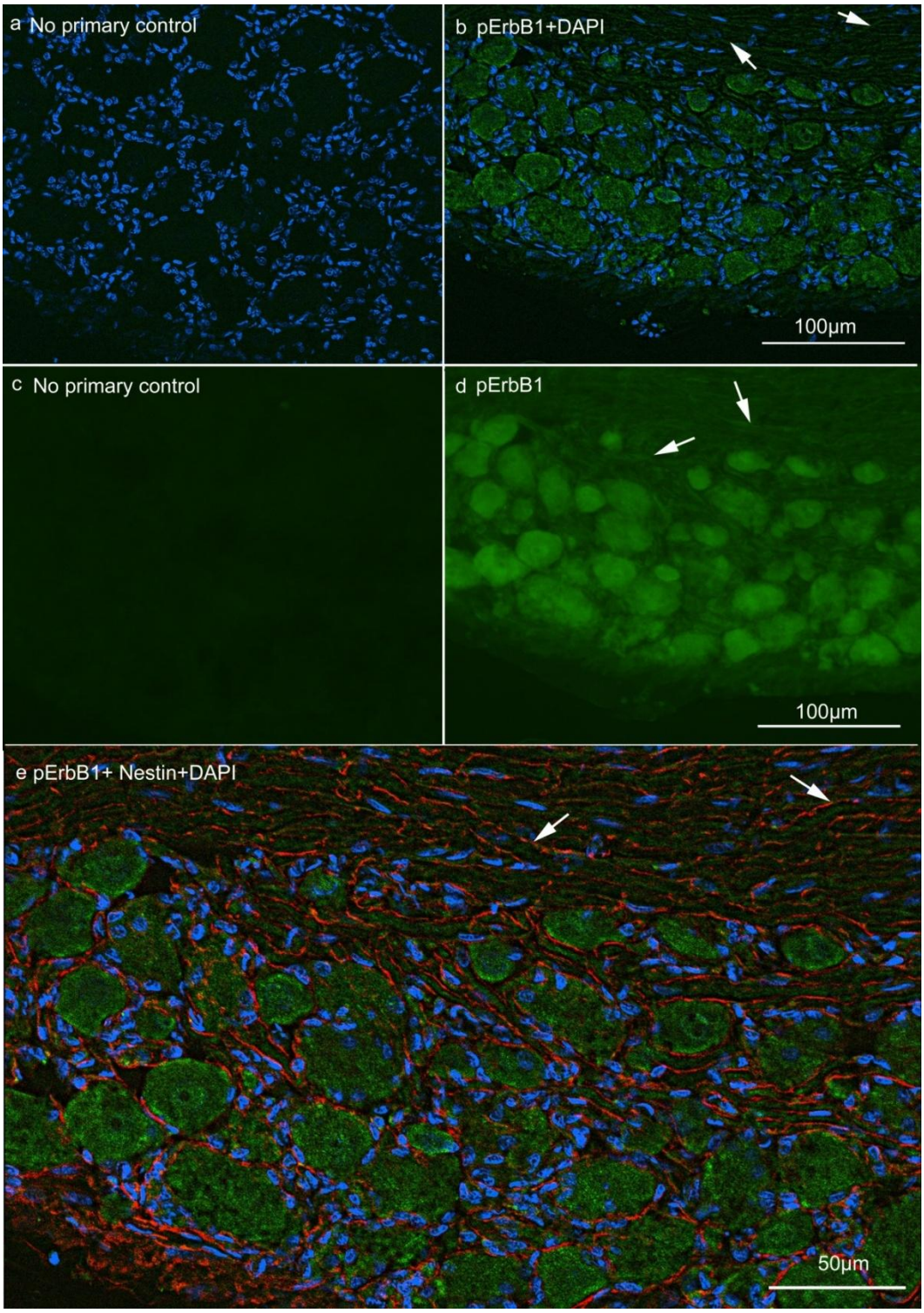


Figure 8.10: Expression of pErbB1 in L4 DRG of adult rats following sciatic nerve transection. a) pErbB1(green) is expressed in the cell bodies of DRG neurons. Axons are weakly stained with pErbB1 as indicated by arrows. b) pErbB1 immunoreactivity does not co-localise with nestin (red). c) Image taken from same field of view as shown in (a) under higher magnification. Some axons are weakly stained for pErbB1 but pErbB1 expression is mainly localized to the cytoplasm of DRG cell bodies. DAPI stains nuclei.

pErbB1 is expressed in DRGs following transection to the sciatic nerve

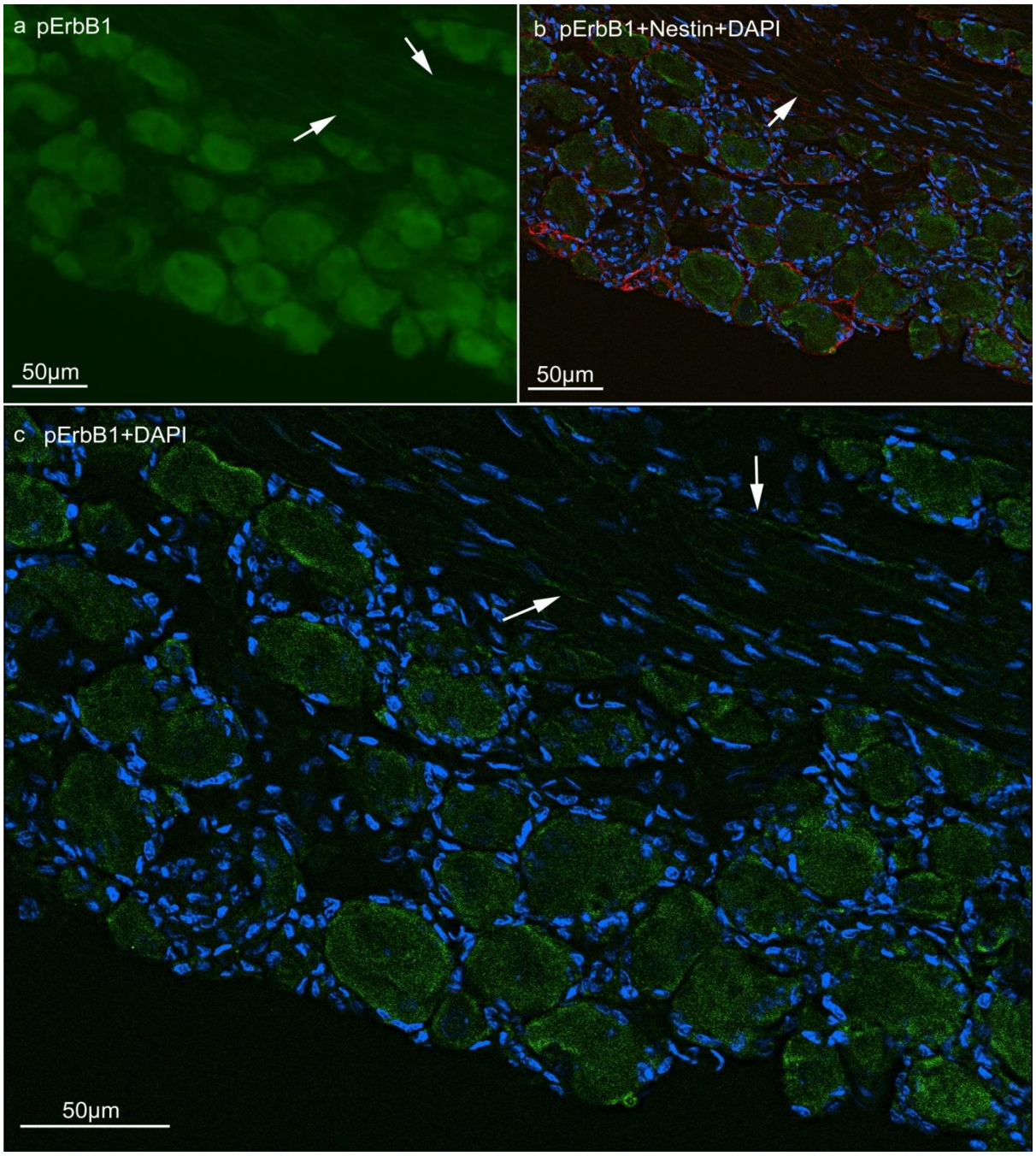


Figure 8.11: Expression of pErbB1 in the uninjured and injured sciatic nerve of adult rat. a,b and c are non-deconvolved images taken at the same settings and in the same session of microscope use. b,d and f are deconvolved images designed to show any co-localization. a) and b) pErbB1 (green) is weakly expressed in the uninjured sciatic nerve where Schwann cells stain positive for nestin (red) but not pErbB1. Arrows indicate pErbB1 immunoreactivity in nerve fibres. c) and d) The distal stump of the injured sciatic nerve shows background levels of flourescence and does not co-localise with nestin-positive Schwann cells. e and f) show increased expression of pErbB1 in the proximal stump of the transected sciatic nerve where axons appear to express ErbB1(arrows) at levels greater that the uninjured nerve (shown in a). Nestin positive cells do not express ErbB1.

pErbB1 is expressed in sciatic nerve following transection to the sciatic nerve

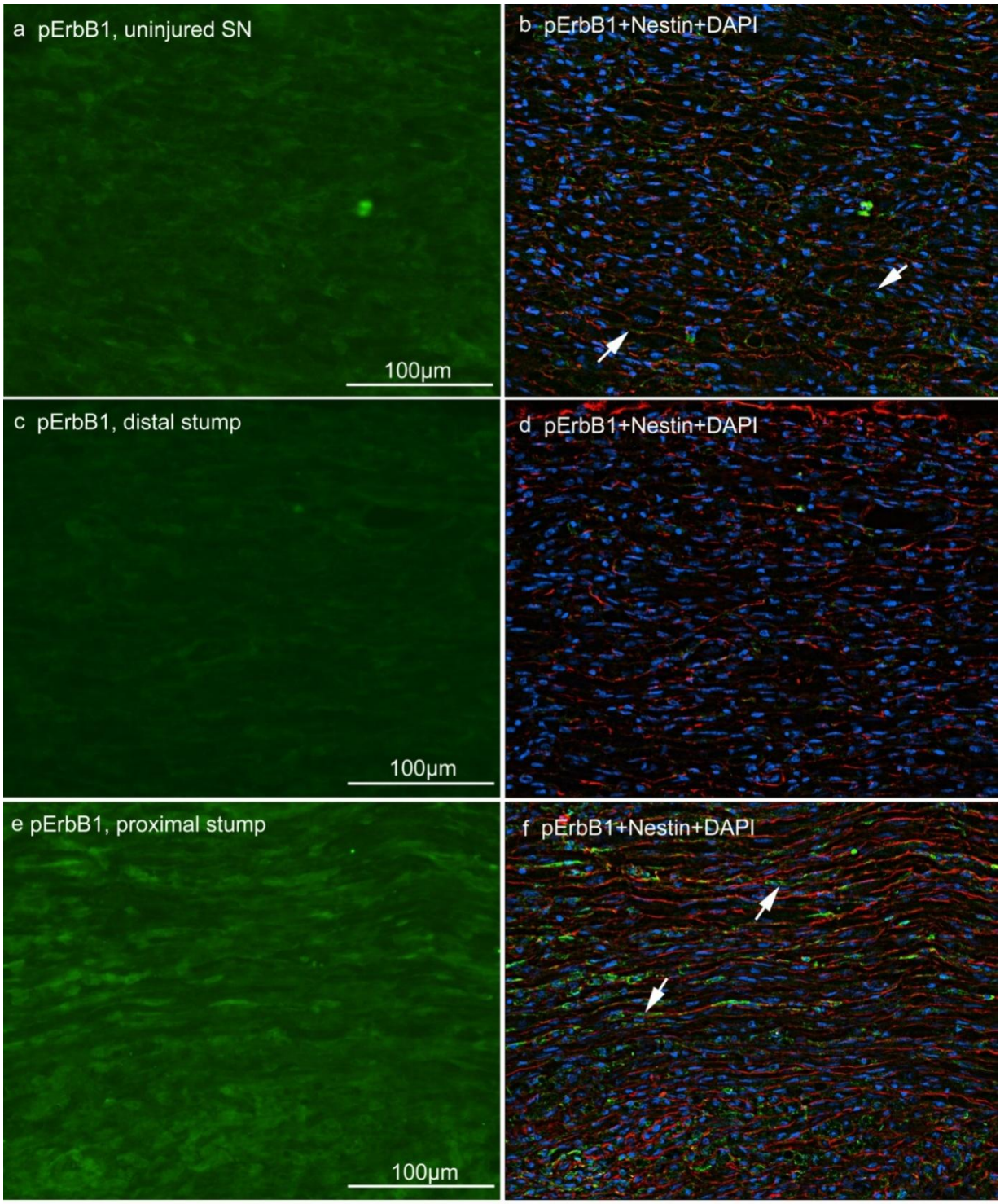
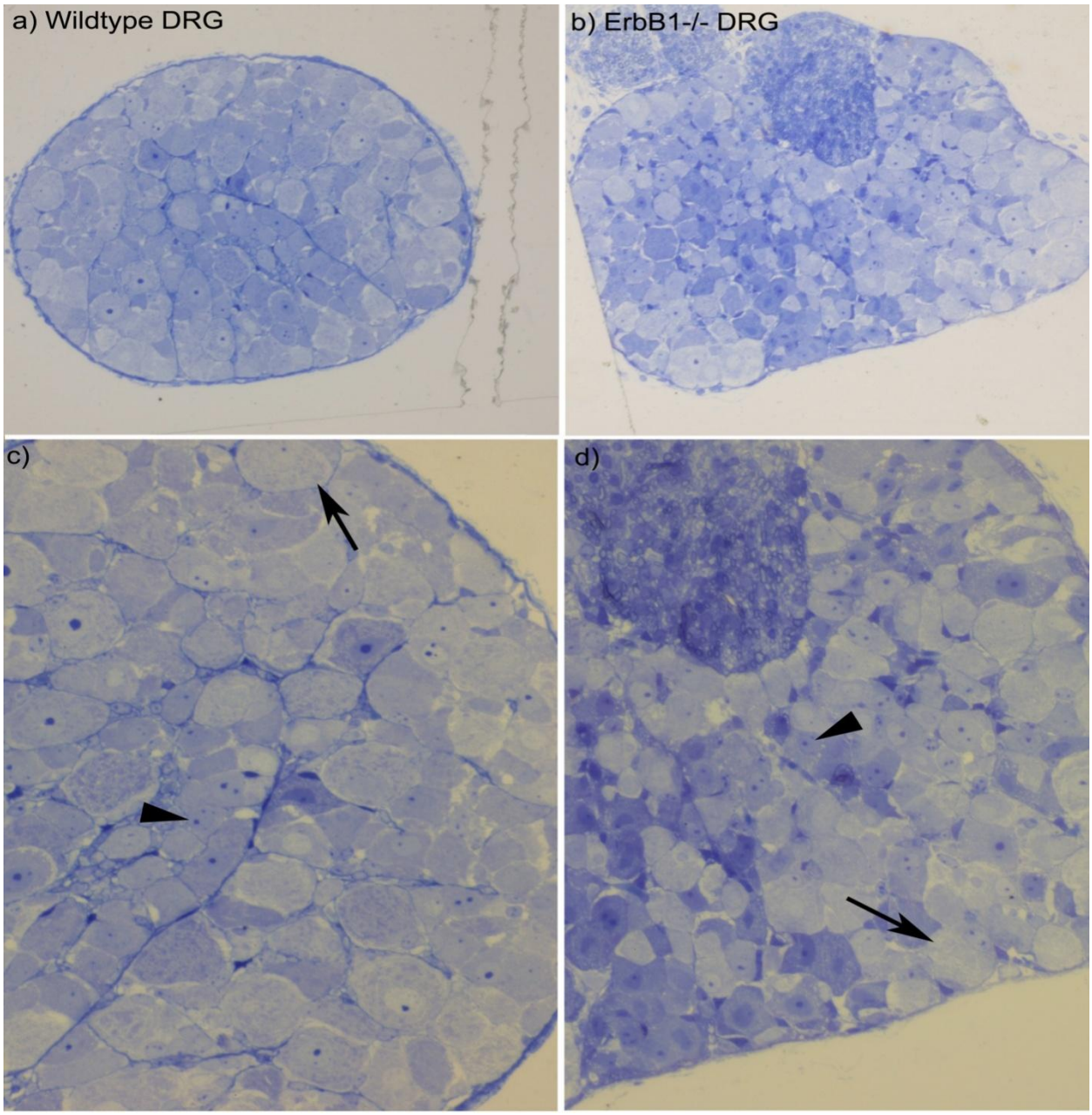


Figure 8.12: Anatomical differences between wildtype and ErbB1-/- DRGs. ErbB1 deletion in mice (Meng et al., 2012) results in abnormal phenotype in DRGs. a) DRG from wildtype mouse. b) DRG from ErbB1-/- mouse showing that both small dark and large light neurons are present but the smaller neurons are more numerous than in the control DRG. c,d) Images captured under higher magnification showing differences between ErbB1-/- and wildtype DRGs. ErbB1-/- DRG (d) contain a larger proportion of small diameter neurons (e.g. arrowheads) and a smaller proportion of large diameter neurons (e.g. arrow) when compared to the wildtype DRG (c).

ErbB1 deletion causes abnormal phenotype in neonatal mouse DRGs



Discussion

There is substantial literature on the expression of ErbB1 in the developing brain particularly in regions associated with active neurogenesis (Yamada et al., 1997;Liu and Neufeld, 2007). By using immunohistochemical techniques, western blotting and PCR, we have identified ErbB1 expression in various types of nervous tissue in animals from different age groups.

ErbB1 transcripts are expressed in CNS tissue, PNS tissue and cultured DRG cells

Using quantitative and RT PCR, we have shown that ErbB1 transcripts are present in the cortex, spinal cord, DRGs and sciatic nerve of P0, P8 and adult mice. As signalling via ErbB1 is associated with growth and differentiation, it was not surprising to observe highest expression of ErbB1 in tissue from P0 animals. Moreover, among the different types of tissue tested for ErbB1 expression, it was observed that the sciatic nerve expressed comparatively lower levels of ErbB1. Our PCR data (Chapter-5) shows that cultured Schwann cells do not express ErbB1 mRNA; presumably the cells which express the transcripts *in vivo* are lost from the cultures. Hence it can be inferred that ErbB1 levels detected in the sciatic nerve is a result of its expression in structures other than Schwann cells, mainly axons or by cells eliminated during preparation of the cultures such as fibroblasts. Moreover, our general observation was that ErbB1 expression declines with age.

As our functional data shows that signalling via ErbB1 mediates inhibition of neurite outgrowth in the presence of CNS myelin, CSPGs and Poly I:C , it was important to determine which cultured DRG cells express ErbB1. As our attempts to detect ErbB1 immunocytochemically in cultured cells has proven extremely difficult, we resorted to techniques that would aid in the detection of ErbB1 transcripts in cultured cells. Using RT PCR, we have clearly shown that ErbB1 transcripts are expressed by DRG cultures from wildtype/ ErbB1+/- animals and absent in DRG cultures from ErbB1-/- animals (Figure 8.1) and cultured Scwhann cells (Chapter-5). We used two different sets of primers to confirm our PCR data. PCR products obtained by using previously published primers were compatible with the results obtained by Threadgill (1995).

Detection of ErbB1 protein in the nervous system

We have shown using immunohistochemistry and western blotting that ErbB1 is expressed by cells in the CNS and PNS. Data from western blotting has shown that ErbB1 is expressed in the cortex, cerebellum, spinal cord, DRGs and sciatic nerve in adult rats. These results are in compliance with our results from qPCR that show expression of its transcripts in adult tissue (discussed above). Based on our data from immunoblots, it can be said that total ErbB1 levels are highest in the brain (cortex and cerebellum) when compared to spinal cord, DRG and sciatic nerve. Even as our results from qPCR suggest that ErbB1 levels are higher in the adult spinal cord compared to the levels in the adult DRGs, immunoblotting has shown that DRGs express higher amounts of ErbB1 when compared to the spinal cord and sciatic nerve. This can be explained by the fact that the levels of mRNA are not always a direct indication of the amount of protein expressed. It can be speculated that ErbB1 is expressed in the central and peripheral branches from DRGs which extend to the spinal cord as well as the sciatic nerve and hence may account for its expression in these tissues.

In order to investigate the types of cells in which ErbB1 is expressed, immunohistochemistry was performed on tissue section from adult rats. We have been able to detect ErbB1 expression in pyramidal neurons and their apical dendrites and non-pyramidal neurons in the motor cortex; deep nuclei, axons in the sub-cortical white matter and parallel fibres in the molecular layer in the cerebellum; superficial ventral horn and dorsal column axons in the spinal cord. The phosphorylated form of ErbB1 has been detected in the cell bodies and axons of DRGs and nerve fibers in the sciatic nerve. In general, ErbB1 was found to be cytoplasmic, with strong axonal expression, and only in cortical neurons was the expression predominantly at the cell surface. Cytoplasmic expression of ErbB1 has been reported in most previous studies (e.g. Chen et al., 2007; Ahmed et al., 2010). Based on immunohistochemical data, it was observed that ErbB1 was predominantly expressed by neuronal cells particularly in their axons. In the adult CNS, we were not able to detect ErbB1 expression in non-neuronal cells and in the PNS, we observed expression of pErbB1 mainly on neurons and their axons. Double immunostaining for pErbB1 and nestin has clearly shown that pErbB1 expression is absent in

Schwann cells, which is supported by data that shows the absence of ErbB1 transcripts in cultured Schwann cells.

In the current study, due to limitations on time and resources, we were unable to further determine the specifity of immunohistochemical staining for ErbB1 using a blocking peptide or by performing similar reactions on tissue from ErbB1-/- mice. However, the results obtained are in compliance with other studies that have shown ErbB1 expression in certain neurons of the CNS (Werner et al., 1988;Gomez-Pinilla et al., 1988;Chen et al., 2007;Ferrer et al., 1996). Moreover, our results are in agreement with previous studies that report ErbB1 expression in DRG neurons (Huerta et al., 1996;Andres et al., 2010) and are contrary to observations made by Ahmed et.al. (2010) on the confinement of ErbB1 expression to non-neuronal cells rather than neurons in DRGs. The absence of ErbB1 expression in non-neuronal cells such Schwann cells and satellite cells in the PNS and prominent expression in neurons of the CNS strongly indicate that ErbB1 mediates distinct signalling pathways in neurons. These observations add further weight to our hypothesis that ErbB1 activation in neurons causes inhibition of axonal outgrowth in the presence of known inhibitors of axonal regeneration.

Changes in ErbB1 expression following injury to the PNS

We analysed changes in pErbB1 expression following transection to the sciatic nerve. 7 days after injury, increased expression of ErbB1in nerve fibers of the proximal stump was observed. As expected, ErbB1 expression was absent in the distal stump. However, subtle changes were observed in the DRGs which showed reduction in pErbB1 expression and expression was mainly localized to the cytoplasm and certain axonal fibers.

There have been previous reports on changes in the expression of ErbB1 in spinal cord and optic nerve following injury (Erschbamer et al., 2007;Liu and Neufeld, 2004). These studies have shown that changes in ErbB1 expression were predominantly localized to astrocytes. However, there have not been many reports on changes in ErbB1 expression following transection to the sciatic nerve. There are numerous studies on the expression of other members of the ErbB family in the sciatic nerve (Pearson, Jr. and Carroll, 2004;Atanasoski et al., 2006;Carroll et al., 1997), but clear-cut evidence on the expression of ErbB1 in the sciatic nerve

is lacking. Although, some studies report expression of ErbB1 in Schwann cells (Toma et al., 1992;Ahmed et al., 2010), there are other studies that report absence of ErbB1 expression by Schwann cells (Gonzalez-Martinez et al., 2007;Ling et al., 2005). Our studies have confirmed that ErbB1 is expressed by nerve fibers in the sciatic nerve but not by Schwann cells. The increased expression of pErbB1 in axons of the transected sciatic nerve clearly indicates the importance of the receptor in regulating responses of axons following injury. However, it is not entirely certain if increased expression is associated with eliciting or inhibiting regenerative responses. One study has reported that there is declined expression of ErbB1 mRNA under regenerating conditions in the transected sciatic nerve repaired using end-to-end or end-to-side neurorrhaphy (Audisio et al., 2008). Also, PTPσ, a receptor for CSPGs which has been implicated in ErbB1 signalling, when knocked out, improves the rate of nerve regeneration in the injured sciatic nerve (McLean et al., 2002). Based on these reports that show decline in ErbB1 mRNA expression in the injured sciatic under conditions induced to regenerate and also improved rate of regeneration in the absence of PTPσ , it can be speculated that upregulation of pErbB1 is associated with delayed regenerative responses in the transected sciatic nerve. One way of testing this would be to assess the rate of regeneration following a sciatic nerve injury in wildtype and ErbB1 conditional knock out mice.

ErbB1-/- and wildtype DRGs exhibit anatomical diffrences

The importance of ErbB1 signalling in cells of the nervous system has been studied using knockout mouse models in which genetic deletion of the receptor has shown to result in severe neurodegenertaion in the frontal cortex, olfactory bulb and thalamus (Sibilia et al., 1998). In our experience, most ErbB1-/- mice die after the first week of life. Anatomical evaluation of DRGs from wildtype and ErbB1-/- animals (aged P6) has revealed differences in the proportion of large and small diameter neurons in DRGs from the the different animals. ErbB1-/- DRGs had a smaller proportion of large diameter neurons and a larger proportion of small diameter neurons when compared to wildtypes. These results are not in compliance with the observations made by (Maklad et al., 2009) as they report normal phenotype in knockout and wildtype DRGs. However, neurons from wildtype and ErbB1-/- animals did not differ in their ability to extend neurites (Chapters-3) when grown on a normal substrate in the absence of growth inhibitors.

Nonetheless, ,the anatomical diffrences between wildtype and ErbB1-/- DRGs can be attributed to the fact that ErbB1 signalling plays a critical role in the developing nervous system as shown by numerous other studies (Threadgill et al., 1995;Sibilia et al., 1998;Kornblum et al., 1998).

Conclusion

We have shown that ErbB1 is expressed by certain neurons of the peripheral and central nervous system. Expression of ErbB1 transcripts is prominent in the cortex, spinal cord, DRGs and sciatic nerve in neonatal and adult mice. Comparison between expression levels in different age groups have shown that ErbB1expression declines with age. Moreover, we have shown that ErbB1 is present in protein lysates from the cortex, cerebellum, spinal cord, DRG and sciatic nerve of adult rats. Immunohistochemical data suggests that ErbB1 expression is more pronounced in axonal fibers and various types of neurons stain positive for ErbB1. Schwann cells and satellite cells do not express ErbB1. Following injury to the sciatic nerve, expression of pErbB1 is upregulated in nerve fibers of the proximal stump and expression is absent in the distal stump. Based on the expression pattern of ErbB1, it can be concluded that ErbB1 may regulate distinct signalling processes in neurons.

Chapter -9 Role of ErbB1 in axonal regeneration *in vivo*

Introduction

The failure of axonal regeneration following injury to the CNS can be attributed to regenerative incompetence of mature CNS axons as well as the extracellular environment to which these axons are exposed. *In vitro* studies as described in this thesis and from other labs suggest that ErbB1 activation in response to CSPGs, CNS myelin and fibrinogen mediates signalling that causes growth cone collapse and inhibition of neurite outgrowth (Leinster et al., 2012;Koprivica et al., 2005;Schachtrup et al., 2007). Based on these studies it can be inferred that ErbB1 activation is a common signalling event in response to neural injury and may act as a target for neural repair.

To assess the effects of blocking ErbB1 signalling *in vivo*, we aimed to use two different injury models- optic nerve crush and a dorsolateral section of the spinal cord. The optic nerve contains axons from retinal ganglion cells and is a pure CNS axonal tract and is a single projection that innervates a few target sites. The glial environment of the optic nerve appears to be identical to that of other parts of the CNS. Injury to the optic nerve produces an astrocytic scar around the site of lesion through which few axons regenerate (Berry et al., 2008;Benowitz and Yin, 2008). Due to its simplicity, this model is one of the best for investigating the fundamental mechanisms that underlie regeneration failure. Moreover, the completeness of the lesion can be easily assessed by the complete absence of regenerating axons in the distal parts of the optic nerve and optic tract. However, injury to the optic nerve does not fully replicate the more complicated situation found in the injured spinal cord. The spinal cord contains both grey and white matter and is characterised by complex circuitry which is probably maintained by a variety of signalling cues, e.g those which limit projections across the midline. Injury to the spinal cord results in more extensive hemorrhage, inflammation, necrosis, secondary cell death and cavitation than injury to the optic nerve. Whereas limited regeneration of a few axons occurs following complete lesions of the optic nerve even without experimental interventions (Campbell et al., 1999) no such regeneration occurs after complete spinal lesions. In view of my previous *in vitro* observations, it was of interest to assess the effects of blocking ErbB1 firstly, in the injured

optic nerve and then in the injured spinal cord, and to assess the effect of blocking PTEN on optic axon regeneration in vivo. These experiments are essentially a preliminary study that was limited by the time available at the end of my studies.

The goals of the present study are summarized below:

- To assess the effects of blocking ErbB1in the crushed optic nerve of adult rats by using PD168393 delivered by soaking the drug in gel foam and also by means of intravitreal injections.
- To compare any regeneration observed in the optic nerve treated with PD168393 with that produced by a PTEN anatagonist
- To assess the effects on rubrospinal axon regeneration of injecting a lentivirus carrying dominant-negative ErbB1 into the red nucleus of adult rats which have received a dorsolateral section to their spinal cords.

Methods

Optic nerve crush

The optic nerve of Sprague-Dawley adult rats were crushed intradurally using a fine pair of forceps. The experimental group (n=2) received treatment with PD168393 (10µM) or PTEN antagonist, VO-OHpic (1µM). The drug was delivered in a gelfoam pad placed in the region of the crush and, at the same time, 6µl of PD168939 or VO-OHpic was injected into the vitreous body eyeball using a Hamilton syringe. The animals received a second intravitreal dose of PD168393 or VO-OHpic 7 days post operation. The control group received similar treatments with 0.1%DMSO. Regeneration was assessed 14 days following injury. Treatment strategy which has also been outlined in Chapter-2 is given below.

DAY	EXPERIMENTAL PROCEDURE	ANIMAL	
		EXPERIMENT (n=2)	CONTROL (n=2)
DAY 1	OPTIC NERVE CRUSH	1) Gel foam soaked in PD168393 (10µM) or VO-OHpic (1µM) placed at crush site. 2) 6µl of PD168393 or VO-OHpic injected into eyeball.	1) Gel foam soaked in 0.1% DMSO or water placed at crush site. 2) 6µl of 0.1%DMSO or water injected into eyeball.
DAY 7	EYE BALL INJECTIONS	6µl of PD168393 or VO-OHpic injected into eyeball.	6µl of 0.1%DMSO or water injected into eyeball.
DAY 14	PERFUSION	Animals sacrificed.	

Table 9.1: Treatment paradigm of adult rats that received optic nerve injuries.

Spinal cord injury

Adult Sprague-Dawley rats received an injection of 4µl of a 50% mixture of the lentivirus and the axonal tracer BDA (Molecular Probes, Oregon, USA; 10% solution in dissolved in 0.1 M PBS) into the right red nucleus. The lentivirus vectors carried with dominant-negative ErbB1-IRES-EGFP or EGFP. The lamina of the C3 vertebra was removed and the left dorsolateral part of the spinal cord, including part of the left dorsal column, the left dorsal horn and the dorsal part of the left lateral white column was cut with microsurgical scissors.

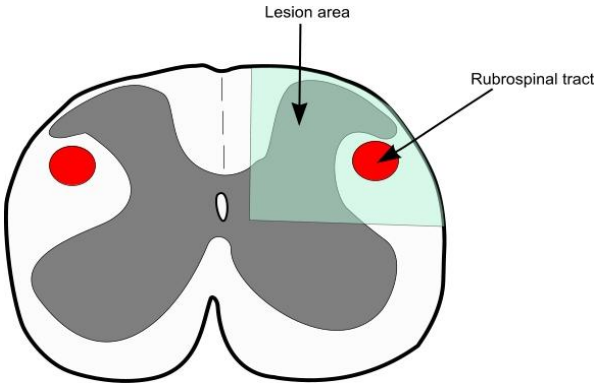


Figure 9.0: Model of spinal cord injury used in this study.

After three weeks the animals were perfused with 2% paraformaldehyde and the brain and spinal cord prepared for immunohistochemistry.

Immunohistochemistry

Cryostat sections (12µm thick) of the optic nerve were cut and immunostained for rabbit βIII tubulin (1:1000) and mouse GFAP (1:500). The secondary antibodies used include goat anti-rabbit Alexa flour 488 (1:400) and goat anti-mouse Atto 594 (1:400).

For the spinal cord and red nucleus, 40µm freezing microtome sections were cut, immunoreacted for GFAP and reacted with streptavidin-Alexaflor 594.

Data analysis

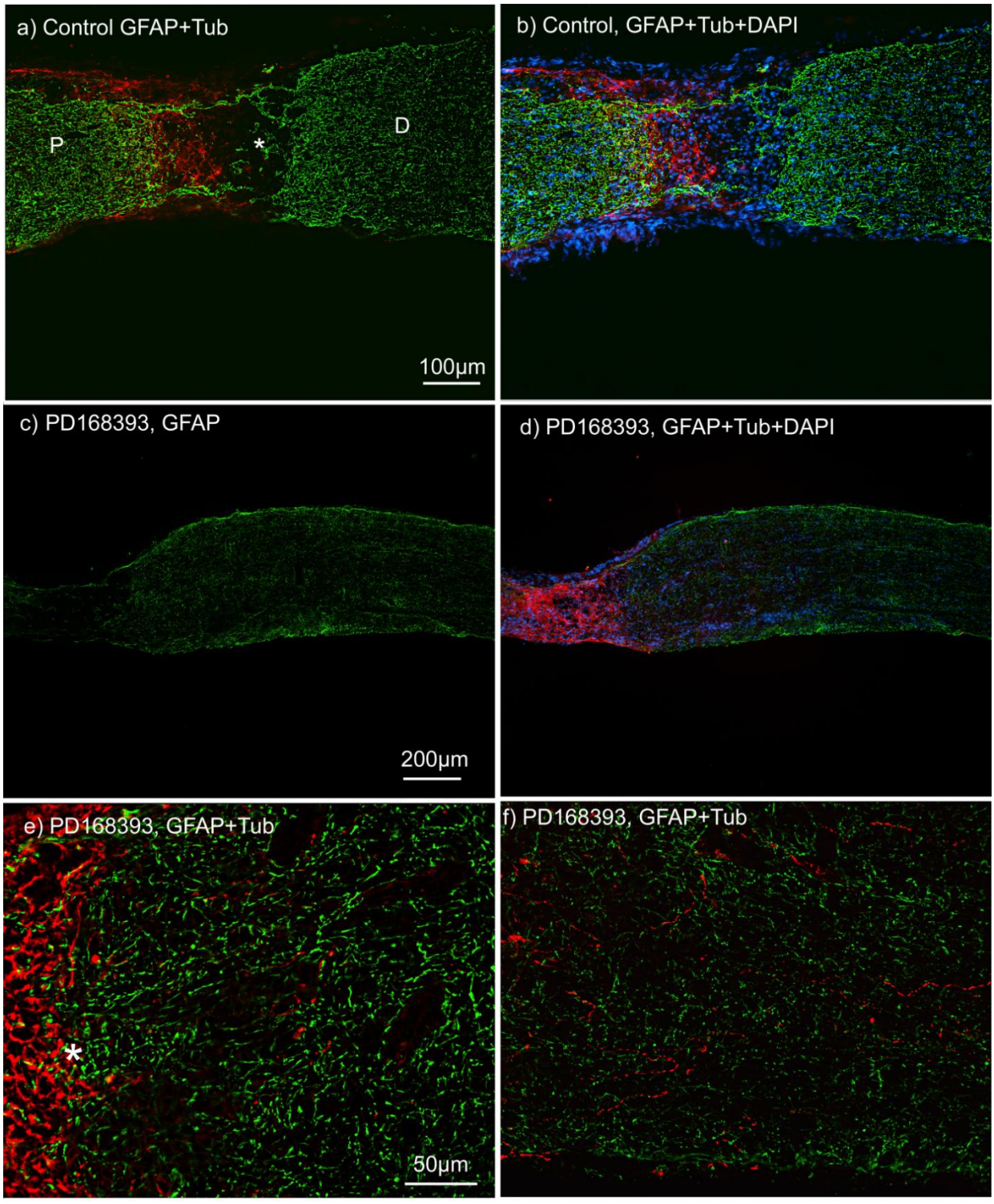
Images were captured using Hamamatsu camera and Improvision software. In images of the optic nerve, the number of axonal fibers that were βIII tubulin (green) positive and 0.1mm, 0.5mm and 1mm from the lesion site were counted. Counts were taken from the experimental and the control group and the mean number of fibers that regenerated a distance of 0.1.0.5 and 1mm were calculated.

Results

PD168393 promotes limited axonal regeneration in the injured optic nerve.

To assess the effects of blocking ErbB1 on axon regeneration *in vivo*, the optic nerve of adult rats were crushed. The ErbB1 antagonist, PD168393 (10µM) was delivered by means of soaking a gel foam with the drug placed at the crush site and also by means of intravitreal injections. Axons in the crushed optic nerve were detected by means of βIII tubulin staining and the site of injury was determined by immunostaining for GFAP. Regeneration was assessed 14 days after injury. In animals that underwent treatment with 0.1% DMSO, it was observed that axons sprouted into but not beyond the site of lesion (Figure 9.1a,b). However, animals that were treated with PD168393 regenerated their axons beyond the site of lesion into the distal segment of the injured optic nerve (Figure 9.1d,e). Regenerating axons could be seen as far as 1.25mm from the site of injury (Figure 9.1f).

Figure 9.1: PD168393 promotes axonal regeneration in the injured optic nerve. a) A longitudinal section through the crushed optic nerve from animals that received control treatments. Immunostaining for GFAP (green) helps to determine the region of the lesion. Lesion site denoted by *. Axons stained for βIII tubulin (Tub, red) seen in proximal segment (P) sprout into the site of lesion but no axons are seen regenerating beyond the lesion site into the distal segment (D). b) Staining of nuclei with DAPI shows the presence of cells in the lesion site but these are not GFAP positive. c) Longitudinal section through the optic nerve from adult rats that received treatments with PD168393 (10μM). GFAP (green) stains the optic nerve but not the lesion site. d) βIII tubulin (red) axons seen regenerating into the lesion site and many axons regenerated into the distal segment. Regenerated axons in the distal segment are not clearly visible. e) Image captured under higher magnification showing regenerating axons in the distal segment. * indicates the boundary of the lesion. f) axons (red) regenerating into the distal stump at a distance of 1.25mm from the site of lesion.

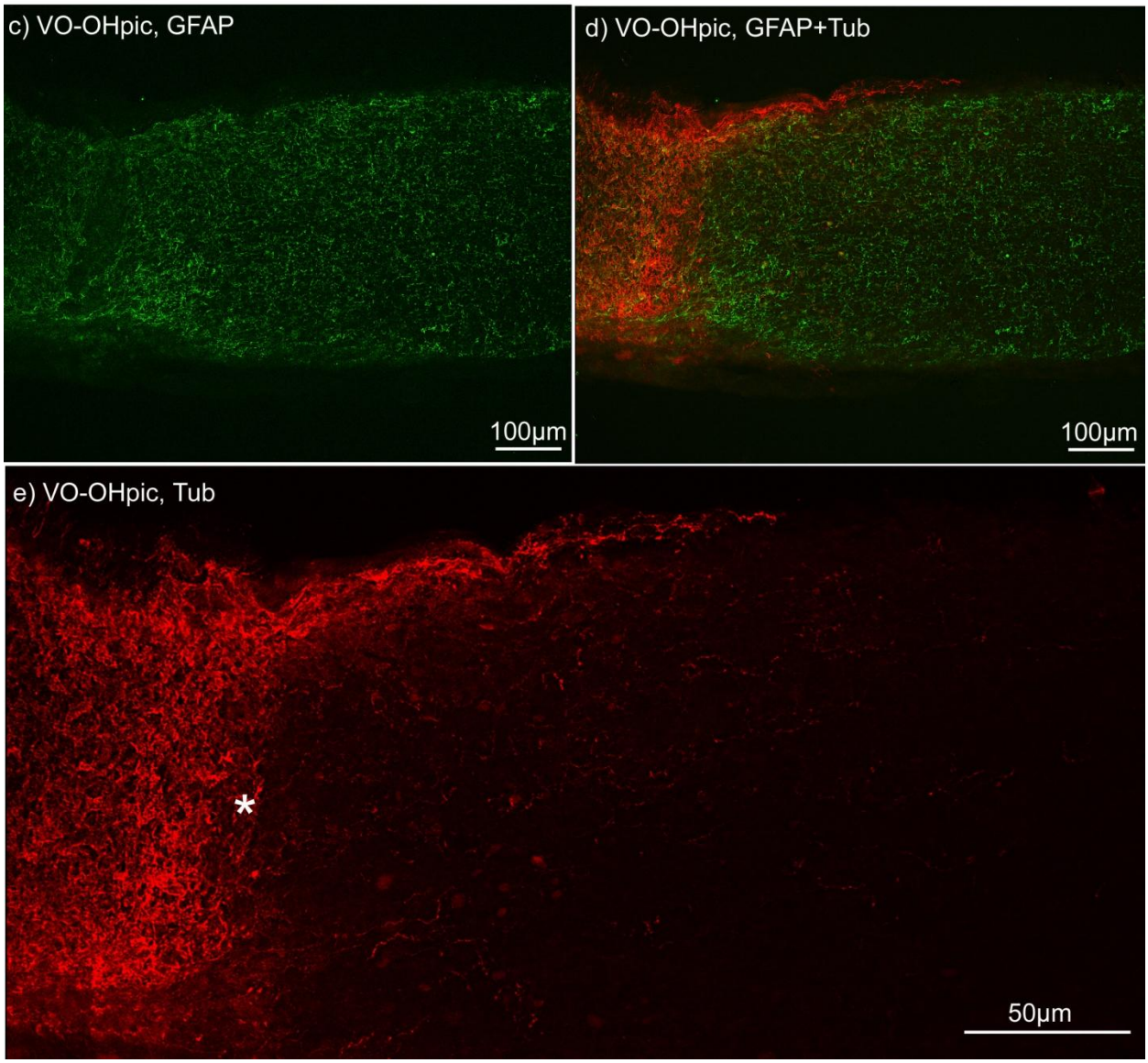

a) Control GFAP+Tub
P
*
D
100µm
b) Control, GFAP+Tub+DAPI
c) PD168393, GFAP
200µm
d) PD168393, GFAP+Tub+DAPI
e) PD168393, GFAP+Tub
*
50µm
f) PD168393, GFAP+Tub

PTEN antagonist, VO-OHpic promotes limited axonal regeneration in the injured optic nerve.

To test if blocking PTEN promotes axonal regeneration, the optic nerve of adult rats were crushed and treated with VO-OH pic (1µM) (Figure 9.2). The treatment strategy was the same as that of the ErbB1 antagonist described above. 14 days after injury, axons could be seen regenerating into and beyond the site of lesion. Modest numbers of regenerating fibers were present up to a distance of 0.5mm into the distal segment of the optic nerve (Figure 9.b, c).

Axons that regenerated into the distal segment were quantified (Figure 9.3). The numbers of regenerating fibers in the distal segment of the injured optic nerve from animals treated with VO-OHpic were comparable to those animals treated with PD168393. Although modest amounts of regeneration was observed in both types of experimental groups, axons from animals treated with PD168393 extended axons to longer distances.

Figure 9.2: VO-OHpic promotes axonal regeneration in the injured optic nerve. a) Longitudianl section through the optic nerve from adult rats treated with VO-OHpic (1µM). GFAP (green) stains astrocytes in the optic nerve are absent in the site of lesion. b) βIII tubulin positive axons seen regenerating into and beyond the lesion site. c) Image captured under higher magnification showing numerous axons that regenerate into the distal segment. * denotes the boundary of the lesion.


c) VO-OHpic, GFAP
100µm
d) VO-OHpic, GFAP+Tub
100µm
e) VO-OHpic, Tub
*
50µm

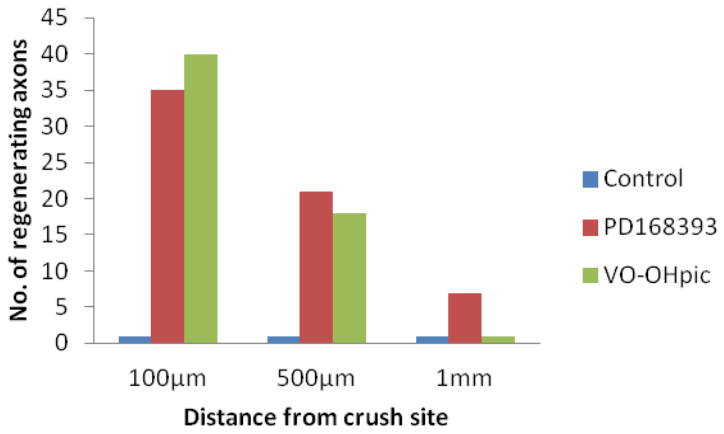
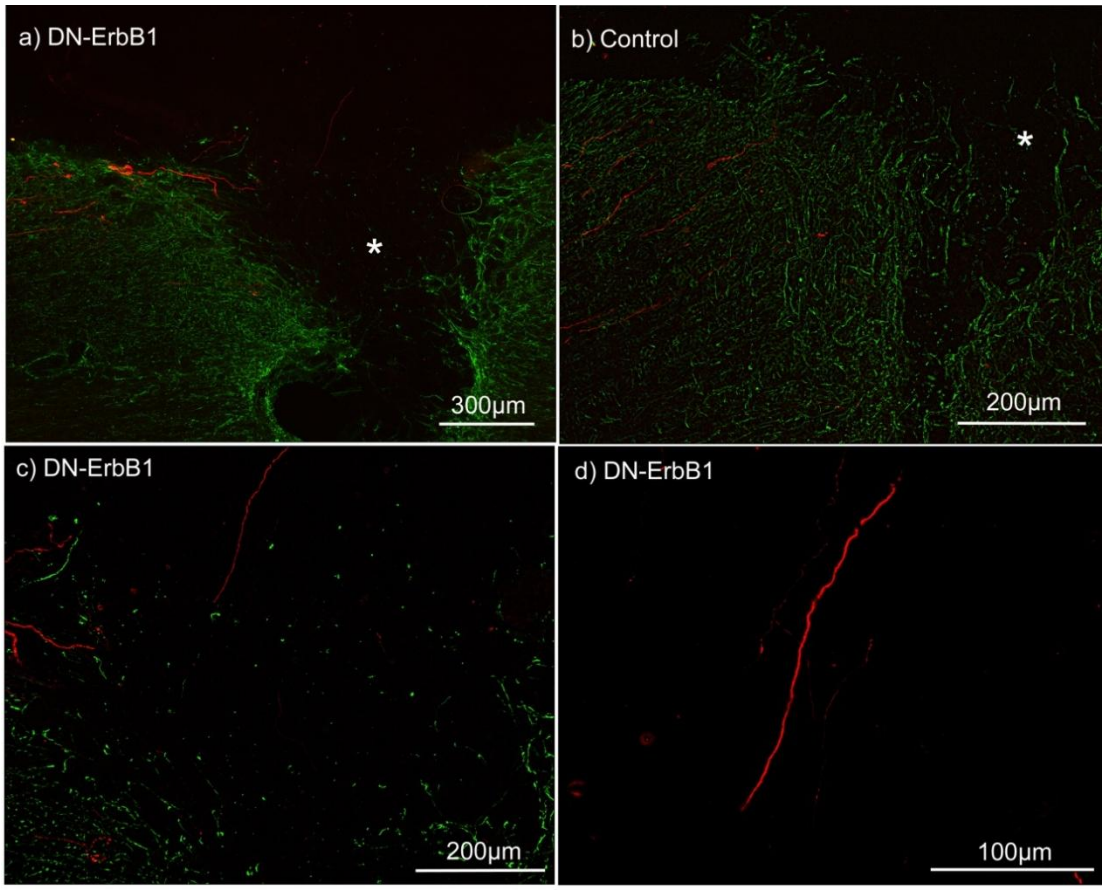


Figure 9.3: Quantification of the number of axons that regenerated into the distal segment of the crushed optic nerve. βIII tubulin positive axons were counted from 3 sections through the optic nerve from each animal. Experiments were repeated from 2 animals from each group ie, control, animals treated with PD16839 and animals treated with VO-OH-pic.

Preliminary results suggest that dominant negative (DN) ErbB1 promotes axonal regeneration in the injured spinal cord.

A DN-ErbB1-IRES-EGFP lentivirus was constructed (described in Chapter-2). The left dorsolateral part of the spinal cord of adult rats was sectioned at the C3 level and DN-ErbB1-IRES-EGFP or EGFP lentivirus was injected into the right red nucleus. Regeneration was assessed 3 weeks after injury. In control animals which received treatment with the EGFP virus, rubrospinal axons were seen regenerating towards the site of lesion but no axons were seen regenerating into the lesion site (Figure 9.4b). However, in the animals that received an injection with the DN-ErbB1 lentivirus, a small number of rubrospinal axons were seen regenerating into the site of lesion (Figure 9.4 a,c,d).

Figure 9.4: Lentiviral delivery of DN-ErbB1 improves axonal regeneration in the injured spinal cord. a) Treatment with DN-ErbB1 causes rubrospinal axons (red) in the dorsolateral columns of the injured spinal cord to sprout into the site of lesion (* denoted lesion site). b) Section through the injured spinal cord of adult rat that received injections with EGFP virus shows rubrospinal axons near the site of lesion but failed to grow into the lesion site. c,d) Images captured under higher magnification showing axons that regenerated into the lesion site.


a) DN-ErbB1
*
300µm
b) Control
*
200µm
c) DN-ErbB1
200µm
d) DN-ErbB1
100µm

Discussion

The effects of blocking ErbB1 on axonal regeneration *in vivo* were investigated using a pharmacological inhibitor as well as by lentiviral delivery of DN-ErbB1. Also, the effects of blocking PTEN on axonal regeneration were assessed using a pharmacological blocker to PTEN. Although these results were obtained from preliminary experiments and further experimentation would be required to confirm such observations, these results are in compliance with data obtained from *in vitro* studies on cultured neurons. Also these observations are in accordance with published data from other laboratories (Koprivica et al., 2005;Erschbamer et al., 2007).

The original study that shows ErbB1 antagonists improve axonal regeneration in the crushed optic nerve reported regeneration of axons to a distance of 2mm from the crush site (Koprivica et al., 2005). This is comparable with our observations of regenerating axons seen at a distance of 1.25mm from the site of injury, but the regenerative effects of the ErbB1 and PTEN antagonists were less that those of lens injury in our laboratory (data not shown). Although long distance axonal regeneration was observed in both studies, only a limited number of fibers were seen regenerating in the distal segment. This can be explained by previous observations that only a small fraction of postnatal RGCs retain their ability to regrow axons (Goldberg et al., 2002). Moreover, following optic nerve injury, a large proportion of retinal ganglion cells undergo apoptosis and hence any regeneration observed would be from the few retinal ganglion cells that have escaped apoptotic cell death. Also, Koprivica (2005) reported that the ErbB1 antagonists had no effect on cell survival. Although repeated delivery of PD168393 was shown to improve axonal regeneration, more recently it has been reported that a single administration of AG1478 delivered using nanospheres also improves regeneration in the injured optic nerve (Robinson et al., 2011). However, if anything, less regeneration was observed by Robinson et al. than were seen with PD168393 in our study. Also, out of the two antagonists tested, Koprivica (2005) reported greater regeneration in animals treated with the irreversible ErbB1 antagonist, PD168393 when compared to the reversible blocker, AG1478.

Apart from the effects of PD168393, we have also shown that the PTEN antagonist, VO-OHpic improves regeneration in the crushed optic nerve. Recent reports have shown that conditional deletion of PTEN induces robust regeneration of axons *in vivo* (Leibinger et al.,

2012;Liu et al., 2010;Sun et al., 2011;Christie et al., 2010). Treatment with the pharmacological blocker did not induce the robust regeneration seen following conditional deletion of PTEN; based on our preliminary results we report modest amounts of regeneration. The regenerative capabilities of the ErbB1 and PTEN antagonists were similar and these observations strengthen *in vitro* data that suggests that PTEN and ErbB1 converge on similar signalling pathways (Chapter-6). Also the association between the two signalling molecules is supported by similar expression patterns of ErbB1(Chapter-7) and PTEN (Christie et al., 2010) in DRGs and sciatic nerve following axotomy i.e increased expression in nerve fibers of the proximal stump and cytoplasmic localization in DRGs following transection to the sciatic nerve.

Another aim of this study was to assess the effects of blocking ErbB1 on regeneration in the injured spinal cord of animals. Due to lack of time, we have only prelininary observations that lentiviral delivery of DN-ErbB1 may promote regeneration of rubrospinal axons following dorsolateral section to the spinal cord. None the less, the result of considerable experience in our laboratory is that rubrospinal axons do not normally regenerate into lesion sites as they did in the present experiments. Lesion sites contain little myelin but large amounts of CSPGs (Davies et al., 1997) and probably RNA fragments (Chapter-4) whose inhibitory action can be at least partially overcome by blocking ErbB1. However, there have been previous studies that have shown that blocking ErbB1 activation facilitates axonal regeneration in the injured spinal cord. One study shows that intrathecal delivery of PD168393 into adult rats with contused spinal cords improves axonal sparing, increases myelin quantity in the injury zone and improves functional recovery (Erschbamer et al., 2007). Although these observation were recently questioned (Sharp et al., 2012), differences in experimental outcome can be based on technique rather than the subject under investigation (Erschbamer et al., 2012). Also, it has been reported that blocking ErbB1 activation reduces neuroinflammation-associated secondary damage and decreases cavitation in the injured spinal cord (Qu et al., 2012).

In contrast, it has been reported that ErbB1 activation in astrocytes in the injured spinal cord causes its transformation to a growth supportive phenotype and that ErbB1 activation is associated with neuroprotection (White et al., 2011). However, preliminary results from experiments in our laboratory and other laboratories as discussed above have shown that

blocking ErbB1 activation improves axonal regeneration *in vivo*. Moreover, *in vitro* studies in our laboratory have also shown that ErbB1 activation causes inhibition of neurite outgrowth in the presence of activated TLR3 (Leinster et al., 2012).

Conclusion

Our results from preliminary experiments suggest that blocking ErbB1 activation by treatment with PD168393 improves axonal regeneration in the crushed optic nerve. Moreover, blocking PTEN also improves optic nerve regeneration. PTEN and ErbB1 antagonists exert similar effects on axonal regeneration in the injured optic nerve. Also, lentiviral delivery of DN-ErbB1 improves regeneration of rubrospinal axons into lesion sites in the injured spinal cord.

Chapter 10- Discussion

I have investigated the role of ErbB1 in limiting axonal regeneration. The reason for this line of investigation was the work carried out by two laboratories with differing views on the mechanisms by which pharmacological inhibitors of Erbb1 promote neurite outgrowth *in vitro* (Koprivica et al., 2005; Ahmed et al., 2009; Ahmed et al., 2010). Numerous receptors have been implicated in signalling from molecules present in and around the lesion site formed after injury to the CNS. These include receptors for Nogo (NgR1, PirB), receptors for MAG (NgR1, NgR2, PirB) and receptors for CSPGs (PTPσ, NgR1, NgR3) (Fournier et al., 2001; Atwal et al., 2008; Shen et al., 2009; Dickendesher et al., 2012). Koprivica and colleagues (2005) provided evidence that ErbB1 acts as a signalling hub through which these receptors elicit their effects. In contrast, Ahmed et el. (2010) proposed that neuronal ErbB1 activation was not associated with regeneration failure. These differences in opinion are largely based on interpretations of the effects of pharmacological antagonists (including PD168393) used to block the activity of ErbB1 *in vitro*. Thus, although studies from both laboratories reported that ErbB1 antagonists enhanced neurite outgrowth in the presence of CNS myelin, Ahmed et.al. (2009) claimed that PD168393 acted off-target to ErbB1, presumably on another kinase. These claims were based on studies that used siRNA to knock down ErbB1activation. The failure to observe improved neurite outgrowth under such conditions led to the hypothesis that neurite outgrowth resulting from the addition of the pharmacological blocker were down to off-target effects of the drug. Moreover, they (Ahmed et.al. 2009; Douglas et al., 2009) suggested that ErbB1 antagonists induce non-neuronal cells to produce neurotrophins that in turn disinhibit neurite outgrowth in the presence of CNS myelin.

It was difficult to understand why multiple well-characterized pharmacological ErbB1 kinase inhibitors, widely used in cancer studies, would act on a different receptor when used at a concentration within their range of its specificity and produce similar effects on neurite outgrowth. The western blots published by Douglas et al. (2009) clearly indicate that the ErbB1 antagonists were having their expected effect on ErbB1 phosporylation (see Fig. 2A from (Douglas et al., 2009), but it remained possible that residual ErbB1 activity that had escaped knockdown by siRNA was responsible for eliciting inhibition of neurite outgrowth. To clarify this situation we studied the effects of CNS myelin on neurons from ErbB1 knockout animals.

As genetic deletion results in the absence of the protein, any effects observed on neurite outgrowth would be due to the lack of receptor. In Chapter-3, we have shown that ErbB1 knockout neurons are less inhibited by CNS myelin. Also, and of critical importance, the addition of PD168393 failed to further disinhibit neurite outgrowth from ErbB1 knockout neurons, confirming that ErbB1 antagonists act "on-target" for this function. It appears that the "off target" hypothesis for the effects of PD168393 on neurite outgrowth is incorrect.

Apart from CNS myelin, we were also able to assess the effects of Poly I:C on neurite outgrowth (Chapter-4). Poly I:C is a synthetic double-stranded RNA and a viral mimetic (Town et al., 2006; Matsumoto and Seya, 2008) that binds to a receptor, TLR3, which is present in the nervous system (Farina et al., 2005; Town et al., 2006). Activation of TLR3 following binding of its ligand, Poly I:C, has been reported to inhibit neurite outgrowth from DRG neurons (Cameron et al., 2007). A repetition of this work allowed us to confirm the inhibitory effects of Poly I:C on neurite outgrowth. However, the signalling pathways through which these inhibitory effects are mediated were unknown. I have shown that ErbB1 is an active component of this signalling pathway and that ErbB1 knockout neurons are less affected by Poly I:C. Although there is no direct evidence on expression of activated TLR3 following spinal cord injury, there is indirect evidence that suggests TLR3 may be activated following injury. It has been reported that secondary structures in mRNA released from necrotic cells are capable of activating TLR3 (Kariko et al., 2004). As necrosis is a widespread phenomena associated with spinal cord injury, it could be hypothesized that TLR3 is activated in the event of an injury and that activated TLR3 is associated with regeneration failure.

After having shown that ErbB1 mediates signalling that leads to inhibition of neurite outgrowth in the presence of CNS myelin and Poly I:C, we next investigated if it was signalling via neuronal or non-neuronal ErbB1 that mediated such responses (Chapter-5). As immunocytochemical detection of ErbB1 proved difficult, we used conditioned media from non-neuronal cells to test if genetic deletion of ErbB1 increased neurotrophin production leading to increased neurite outgrowth. The hypothesis that ErbB1 antagonists increase neurotrophin secretion by non-neuronal cells, leading to disinhibition of neurite outgrowth (Ahmed et.al., 2009), predicts that there should be increased neurite outgrowth when neurons were treated with

media conditioned by ErbB1-/- but not wildtype non-neuronal cells. Our results are not compatible with this hypothesis. Media conditioned by non-neuronal cells does disinhibit neurite outgrowth but we have shown that absence of ErbB1 signalling in non-neuronal cell cultures reduces the efficacy of their conditioned medium. We propose a model in which ErbB1 signalling may have dual effects based on the cell type and also the conditions to which cells are exposed i.e. treatment with either Poly I:C, CNS myelin or CSPGs. While ErbB1 signalling may induce non-neuronal cells to produce growth promoting factors, neuronal ErbB1 activation mediates responses that results in growth inhibition. However, it is to be noted that the effects of conditioned media were tested on cells treated with Poly I:C and the possibility that the effects of conditioned media may vary in the presence of CNS myelin or CSPGs cannot be excluded.

The identity of the secreted factors present in these conditioned media that are capable of disinhibiting neurite outgrowth remains unknown. Although we would expect Schwann cell-derived factors to be found in conditioned media to include NGF, BDNF and LIF, IGFs, PDGF, NT-3 (Meier et al., 1999), it could be the case that Poly I:C may stimulate the secretion of additional factors which may affect neurite outgrowth. Poly I:C-treated non-neuronal cells produced a medium which disinhibited neurite outgrowth in the presence of Poly I:C to a greater extent than medium produced by control non-neuronal cells. However, the nature of any factors produced by Schwann cells in response to Poly I:C is unknown. It has been reported that Poly I:C stimulates the activation of inducible nitric oxide synthase gene in Schwann cells (Lee et al., 2007) but the effects of NO production in such cultures are unpredictable. Poly I:C may affect other cells in the cultures. Its best known role is that of an activator of the innate immune system. It has been shown to stimulate microglial activation in the spinal cord following intraperitoneal injections and activation of macrophages in the sciatic nerve following intraneural injections (Zhang et al., 2005; Lee et al., 2007). It is known that inflammatory responses are associated with production of cytokines, some of which may prove beneficial to neurite outgrowth. However, the lack of macrophages in our cultures rules out the possibility that ErbB1 signalling modulates the secretion of inflammatory cytokines from macrophages. Moreover, the lack of differential upregulation of S100- and NG2- positive cells from the two types of cultures treated with Poly I:C; and the lack of expression of ErbB1 transcripts in Schwann cells (Chapter-4)

added further weight to our hypothesis that neuronal ErbB1 signaling causes inhibition of neurite outgrowth.

Transactivation of ErbB1

While experiments in our laboratory have clearly shown that ErbB1 signalling is associated with inhibition of neurite outgrowth *in vitro*, the signalling mechanisms by which ErbB1 is activated by neurite outgrowth inhibitors and those by which ErbB1 activation reduces neurite outgrowth are unclear. It was initially suggested that in the presence of neurite outgrowth inhibitors, ErbB1 is transactivated by a calcium-dependent process (Koprivica et al., 2005). Certainly the effects of multiple inhibitors of neurite outgrowth are calcium-dependent (Bandtlow et al., 1993; the present study). That calcium signalling is upstream of ErbB1 activation is supported by experiments in our laboratory that showed that ErbB1 activation by Poly I:C was blocked by an intracellular calcium buffer, BAPTA-AM (Leinster et al., 2012). The present experiments show that the effects of inhibitors of neurite outgrowth are attenuated when calcium is buffered intracellularly in ErbB1-expressing neurons but not in ErbB1 knockout neurons (Chapter4). Much transactivation of ErbB1 occurs via the metalloprotease-mediated release of ErbB1 ligands and calcium signalling has been implicated in such release (Eguchi et al., 1998; Saito et al., 2002) (Figure 10.1). However, Koprivica et al. (2005) could not demonstrate the involvement of metalloproteases or ligand binding in ErbB1-mediated neurite growth inhibition, so calcium may also be involved in intracellular transactivation of ErbB1. There is some evidence that calcium can cause Pyk2 and Src to transactivate ErbB1 (Montiel et al., 2007).

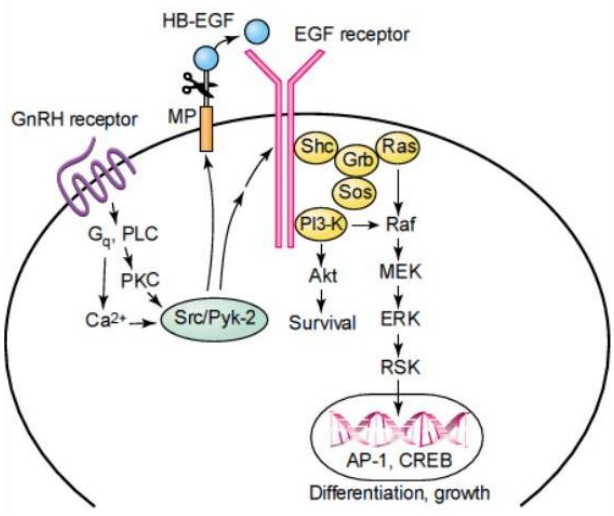


Figure 10.1: Transactivation of ErbB1. Depending on the agonist and the cell type, GPCR stimulation can cause activation of metalloproteases (MP) [e.g. disintegrin and metalloproteases (ADAMs)] that cause ectodomain shedding of soluble ligands, such as heparin-binding EGF(HB-EGF) and transforming growth factor α (TGF-α), that bind to ErbB1. Several intermediate signaling molecules such as PKC, Ca2+ and Src/Pyk2 contribute to GPCR-mediated transactivation of ErbB1. This triggers phosphorylation of the MAPK cascade through recruitment of adaptor proteins such as the Shc–Grb–Sos complex that increase Ras GTP activity and cause activation of the Raf–MEK–ERK–RSK cascade. Stimulation of ErbB1 in this way can also activate phosphatidylinositol 3-kinase (PtdIns3-K, or PI3-K) and Akt to promote. This causes phosphorylation and activation of several nuclear proteins, including c-Myc, Elk-1, c-Fos, c-Jun, AP-1 and cAMP response-element-binding protein (CREB), leading to differentiation and growth (Shah and Catt, 2004).

ErbB1 signalling and the inhibition of neurite outgrowth

A central problem with the idea that ErbB1 activation is on the pathway from the Nogo receptors etc. to inhibition of neurite outgrowth is the fact that most of the pathways downstream of ErbB1 activation are similar to those employed by neurotrophin receptors and would be expected to enhance neurite outgrowth. Neurotrophins bind to Trks to activate Ras, PI3-kinase, and phospholipase C-γ and the MAP kinases (Reichardt, 2006). These promote neurite outgrowth under control conditions and disinhibit neurite outgrowth in the presence of CNS myelin etc (Logan et al., 2006). MAP kinase, phospholipase C-γ and PI3-kinase signalling are also prominent features of ErbB1 activation (Fuller et al., 2008) although signalling from ErbB1 is particularly complex and very much still under investigation. The consequences for neurite

outgrowth of some other ErbB1 signalling pathways such as nuclear translocation are quite unclear. One very interesting finding that may explain the contrasting effects of ErbB1 and Trk activation is provided by the observation that activated ErbB1 directly associates with TLR3 (Yamashita et al., 2012)(Figure 10.2) and such binding is necessary for TLR3 activation. TLR3 activation inhibits neurite outgrowth (Cameron et al., 2007). In the model proposed by Yamashita et al. this interaction is dependent on ligand stimulation of TLR3. Binding of double-stranded RNA induces conformational changes in TLR3 that allow interactions with activated ErbB1. It may be speculated, however, that activated ErbB1 may bind to any TLR3 that is in an appropriate conformational state and induce downstream signalling. The ErbB1/TLR3 complex then binds Src, another tyrosine kinase, and together ErbB1 and Src phosphorylate the appropriate tyrosine residues of TLR3. The tyrosine kinase activity of ErbB1 is required for phosphorylation of the Tyr^{858} residue on TLR3 whereas tyrosine kinase activity of Src is required for phosphorylation of the Tyr^{759} residue. Phosphorylation of both residues on TLR3 results in the recruitment of TRIF (Figure 10.2).

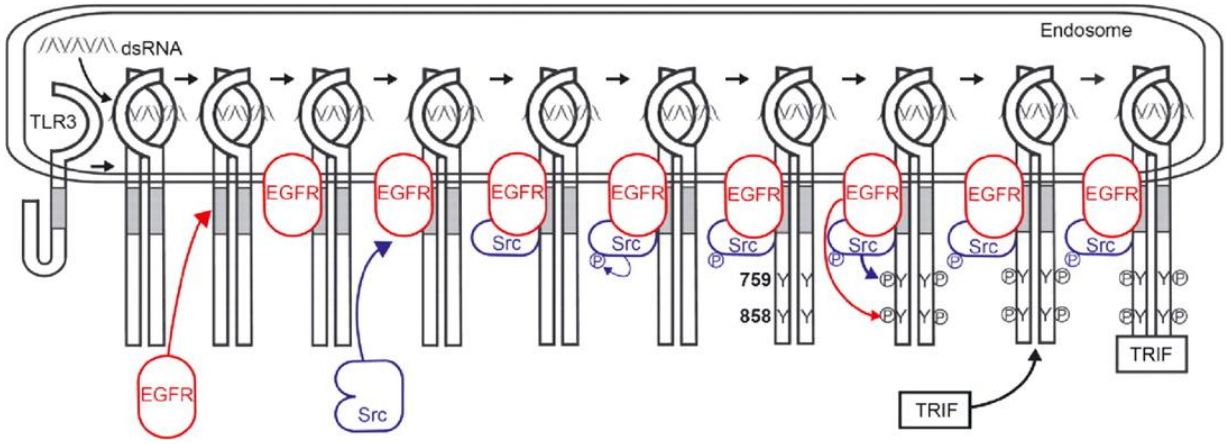


Figure 10.2: Model depicting early events in TLR3 signalling. From (Yamashita et al., 2012)

The interactions of TRIF (TIR domain-containing adaptor inducing interferon-β) with other proteins that result in inhibition of neurite outgrowth are not clearly understood. TRIF has been shown to interact with TBK1 which in turn phosphorylates IRF-3 (interferon regulatory factor-3) (Sarkar et al., 2004). IRF-3 exists as a monomer. Phosphorylation induces its dimerisation, but although dimerised IRF3 can translocate to the nucleus it cannot activate gene

transcription. The activation of the dimer requires additional phosphorylation by the PI3K pathway (Sarkar et al., 2004). PTEN is known to negatively regulate the PI3K pathway. Others have shown that blocking PTEN improves neurite outgrowth from neurons cultured in the presence of CNS myelin etc. (Christie et al., 2010; Perdigoto et al., 2011; Zhang et al., 2012). We have added Poly I:C to the list of molecules whose effects are attenuated by blocking PTEN and have shown that combined blockade of PTEN and ErbB1 produced non-additive effects on neurite outgrowth (Chapter-6). This could be explained by the activation of the dimer, IRF-3 by PI3K signalling being impeded by PTEN (Figure 10.3).

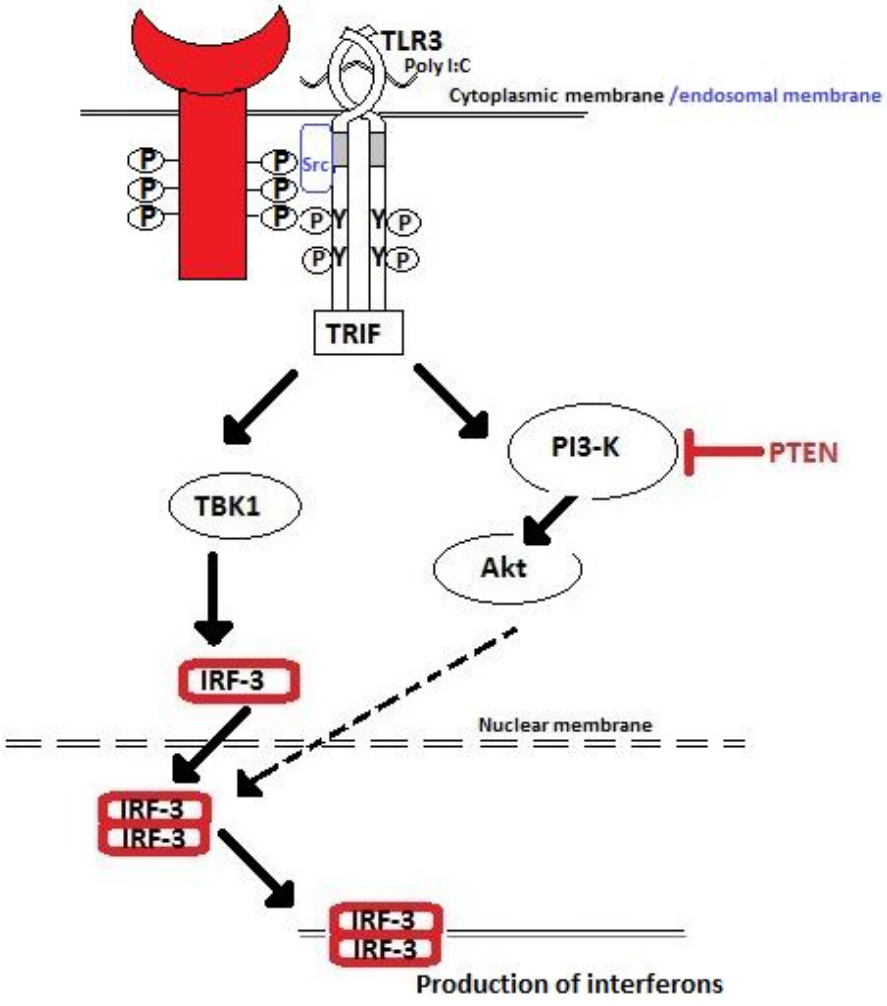


Figure 10.3: Model for downstream signalling targets following phosphorylation of TLR3 by ErbB1. TRIF, an essential adaptor protein, is recruited to phosphorylated TLR3. TBK1 recruitment through TRIF leads to phosphorylation of the IRF-3 monomer causing the formation IRF-3 dimer, the IRF-3 inactive dimer, which can translocate to the nucleus but cannot activate gene transcription. Additional phosphorylation by the PI3K pathway results in activation of IRF-3 dimer IRF-3 transcriptional activation is associated with the production of interferons. PTEN negatively regulates PI3-K/Akt signalling and does not allow activation of the IRF-3 dimer. The dotted line denotes multiple steps between Akt and IRF-3 phosphorylation. Adapted from Sarkar et.al., 2004.

Although, it is known that IRF-3 would aid in the transcription of interferons (Takeuchi et al., 2004; Sen and Sarkar, 2005), it is not entirely certain if the outcome of such transcription would cause inhibition of neurite outgrowth. Another pathway that can be speculated is the NF-κB signalling pathway. Activation of TLR3 has shown to be associated with NF-κB signalling (Siednienko et al., 2011; Meyer et al., 2011). The components of NF-κB signalling pathway have been implicated in regulating various responses in neuronal and non-neuronal cells (Brambilla et al., 2005; Gutierrez et al., 2008; Sitcheran et al., 2008; Sanchez-Ponce et al., 2008) but it has been suggested that activation of TLR3 in neurons does not result in nuclear localization of NF-κB (Cameron et al., 2007).

Nuclear factor-kappa B (NF-κB) is a ubiquitously expressed transcription factor system that consists of homodimers or heterodimers. The p65/p50 heterodimer is the most abundant and widely expressed form of NF-κB . IκBα is the predominantly expressed inhibitor that holds NF-κB in an inactive form in the cytosol. In the classical NF-κB signalling pathway, NF-κB is activated by phosphorylation of IκBα by the IκB kinase-β (IKKβ) catalytic subunit of an IκB kinase (IKK) complex. This complex consists of IKKβ together with another catalytic subunit IKKα and a regulatory subunit IKKγ. This leads to dissociation of IκBα and translocation of the liberated NF-κB to the nucleus where it binds to κB elements in the promoter and enhancer regions of responsive genes, resulting in gene induction or gene repression (Perkins, 2007).

NF-κB signalling plays a role in regulating the expression of genes involved in inflammatory responses, cell survival and cell proliferation (Liang et al., 2004). In the nervous

system, NF-κB is activated by neurotrophic factors, cytokines and neurotransmitters (Sole et al., 2004; Gutierrez et al., 2005; Memet, 2006; Gallagher et al., 2007).

Glutamate, a critical neurotransmitter of the CNS, when bound to glutamate receptors activates signalling pathways that lead to the activation of NF-κB in primary astrocytes (Sitcheran et al., 2008). It has been reported that the ability of glutamate to activate NF-κB is dependant on cross coupling with ErbB1(Sitcheran et al., 2008).The interaction of ErbB1 with the glutamate receptor (mGluR5) is enhanced in the presence of glutamate but not EGF. Moreover, in response to glutamate signalling in glial cells, ErbB1 induces calcium release and activates NF-κB through IKKα/β (figure10.4).

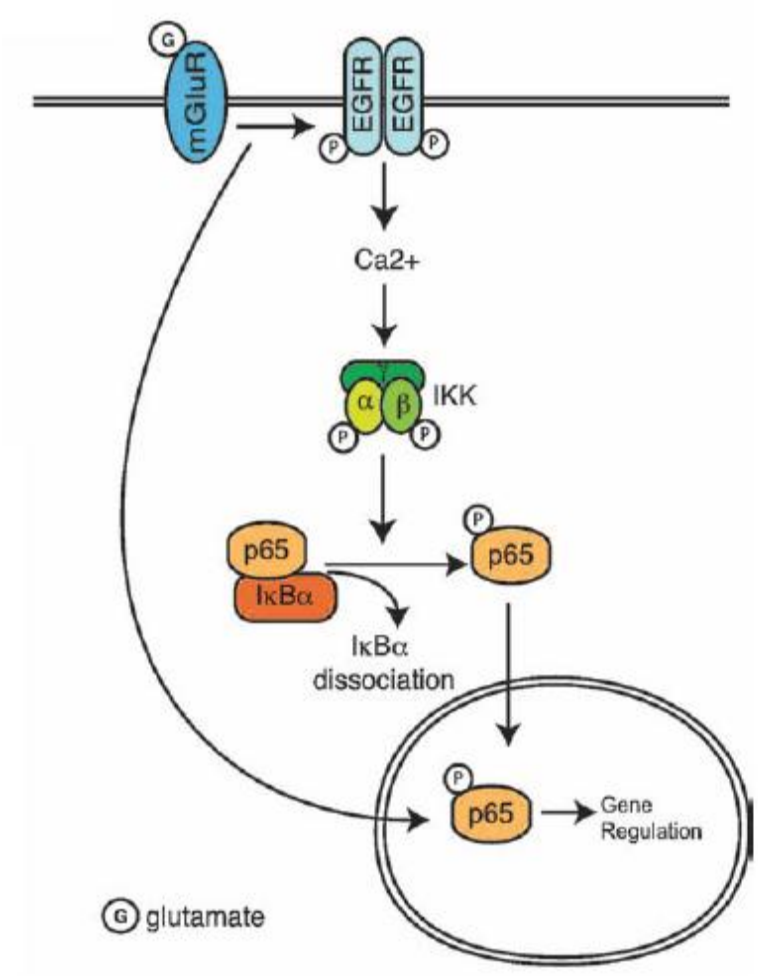


Figure 10.4: Model for glutamate signalling to NF-κB. (Sitcheran et al., 2008). Binding of glutamate to glutamate receptor, mGluR causes transactivation of ErbB1 (EGFR) causing calcium mobilization, calcium dependant phosphonrylatio of IKK α/β and dissociation of IκBα p65 complex. Dissociated p65 is phosphorylated and translocated to the nucleus.

Evidence for NF-κB signalling *in vivo* has been supported by a separate study showing that NF-κB plays a role in injury-mediated responses following spinal cord injury (Brambilla et al., 2005). It was shown that expression of p65 sub-unit of NF-κB was upregulated on day 1 and day 3 following injury. Inactivation of NF-κB in astrocytes by means of a GFAP driven promoter improves functional recovery following contusive spinal cord injury. Moreover, the study showed that deletion of astroglial NF-κB improved white matter sparing and reduced expression of CSPGs such as neurocan and phosphacan. As phenotypic changes in astrocytes are induced by the expression of ErbB1 and ErbB1 signalling may regulate the secretory activity of astrocytes, it is possible that ErbB1 may signal to NF-κB by the signalling pathway demonstrated by Sitcheran et al., 2008.

Although, NF-κB signalling has been associated with survival and growth (Sanchez-Ponce et al., 2008), more recently it has been shown that NF-κB can mediate opposing responses in the same cell depending on the phosphorylation status of p65. NF-κB signalling has been shown to inhibit and promote neurite outgrowth from sensory neurons and the outcome of such signalling was dependant on the phosphorylation of a single serine residue on the p65 sub-unit of NF-κB (Gutierrez et al., 2008). Overexpression of IKKβ induced phosphorylation of p65 at the serine residue 536 which resulted in inhibition of neurite outgrowth.

NF-κB is also known to interact with heterologous transcription factors either through direct binding or occupying adjacent sites on DNA. Such associations profoundly influence transcriptional responses. The p65 sub-unit of NF-κB and IRF-3 has been reported to form stable complexes which can be recruited through an interferon response element (IRE) or a κB site. The complex then recruits other transcription factors which act as co-factors to activate transcription. NF-κB , IRF-3 and transcriptions factors c-Jun, ATF2 have been reported to form complexes following viral infection (Panne et al., 2004; Apostolou and Thanos, 2008).

Taken these results together we propose a signalling pathway as shown in figure 10.5. Stimulation of TLR3 by Poly I:C causes conformational changes that allows binding of ErbB1to TLR3. This is followed by binding of Src to TLR3. Binding of ErbB1 and Src to TLR3 phosphorylates TLR3. Phosphorylated TLR3 recruits the adaptor protein TRIF which in turn activates TBK1. TBK1 activates IRF-3 causing its dimerisation. Inactive IRF-3 dimer is

phosphorylated by PI-3K/ Akt pathway. Activated IRF-3 dimer traslocates to the nucleus and can then form a stable complex with NF-κB. The stable complex then recruits other transcription factors such as c-JUN and ATF-2. This heterologous complex translates interferons. ErbB1 in the presence of elevated calcium activates the IKK complex which catalyses the dissociation of IκBα p65. The liberated p65 is phosphorylated by IKKβ. Phosphorylated p65 translocates to the nucleus and regulates transcription of genes that results in inhibition of neurite outgrowth. The phosphotyrosine residues in the cytoplasmic domain of TLR3 recruits PI-3K which in turn activates the PI-3K –Akt pathway. Akt phosphorylates a wide spectrum of substrates and regulates cellular functions such as cell survival, cell proliferation and growth (Song et al., 2005). Substrates phosphorylated by Akt include GSK3, NF-κB and TSC (Tuberous sclerosis). The inactivation of TSC complex leads to the activation of mTOR which in turn activates the ribosomal protein S6 (Park et al., 2010). The mTOR pathway is associated with protein synthesis and cell growth. The PI3-K pathway is negatively regulated by PTEN.

This signalling cascade shows that PTEN may act downstream to ErbB1 by regulating the PI-3K pathway. Hence, the active pathway from ErbB1 would be the one that leads to phosphorylation of p65 which in turn mediates inhibitory responses towards neurite outgrowth in the presence of PolyI:C. Moreover, as we have shown that buffering calcium abolishes the inhibitory effects of ErbB1-PTEN signalling on neurite outgrowth (Chapter-6), it can be suggested that calcium may also act downstream to ErbB1 and may be required for PTEN to inhibit PI-3K.Also it has been reported that treatment of DRG cultures with Poly I:C raises TRPV1-mediated calcium flux in neurons (Qi et al., 2011).Activated TLR3 could also mediate signalling via IRF-3 which results in production of interferons.

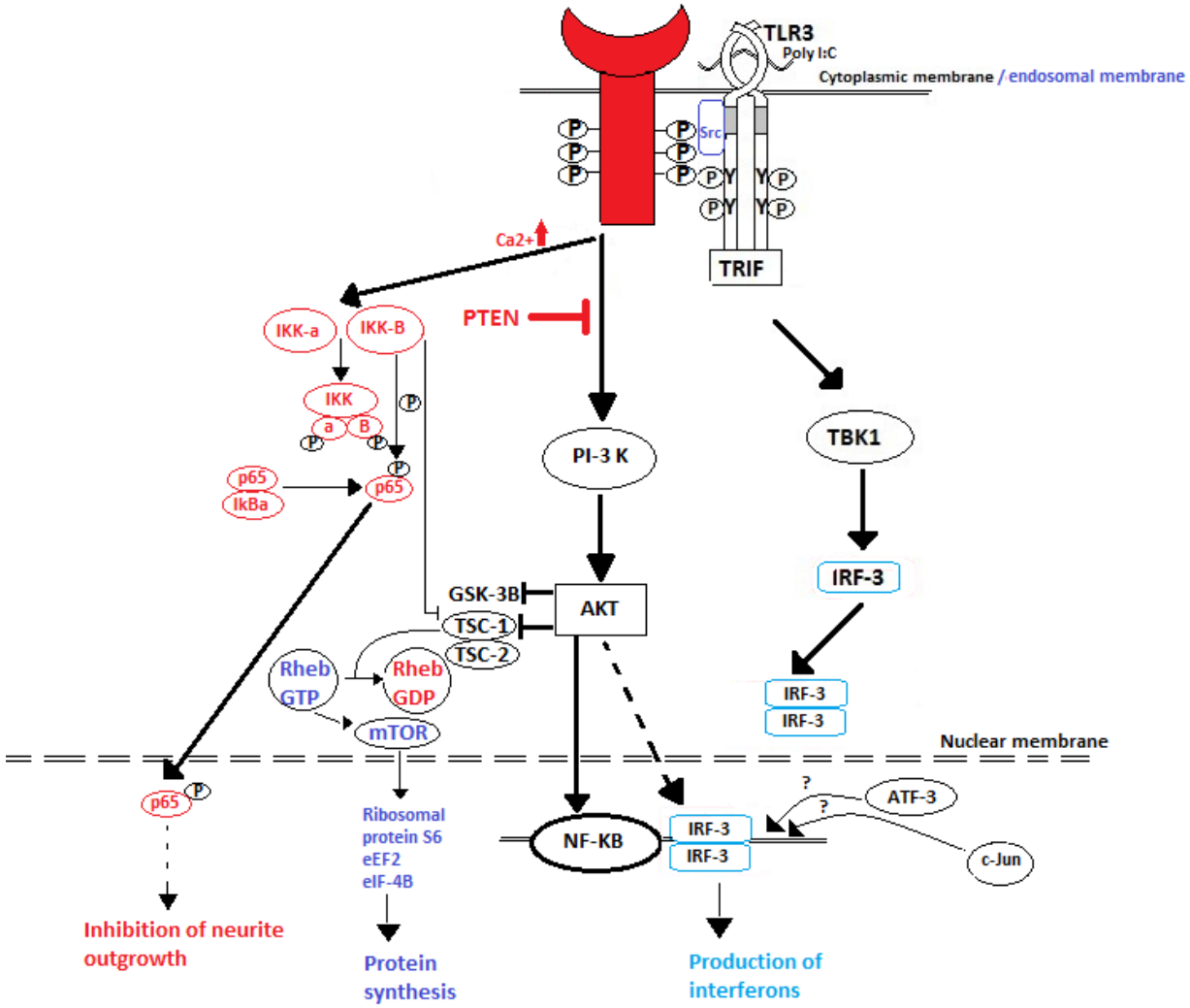


Figure 10.5: Model depicting the signalling cascade from activated TLR3**.** Dotted arrows indicate multiple steps that are unknown. Adapted from Sarkar et.al, 2004; Sitcheran et.al, 2008; Park et.al, 2010; Yamashita et.al, 2012.

The expression pattern of ErbB1 in DRG tissue suggests that the phosphorylated form of the receptor is expressed in the cytoplasm and nucleus of neurons (Chapter-7). Following injury to the sciatic nerve, expression was predominant in the cytoplasm of the neuron. These observations fit in with previous reports on intracellular expression of ErbB1 particularly in endosomes (Stahl and Barbieri, 2002; Sorkin and Goh, 2008; Hanafusa et al., 2011). Moreover, TLR3 has been reported to be expressed in endosomes (Cameron et al., 2007; Peltier et al., 2010). Similar patterns of localization of both ErbB1 and TLR3 formed the basis for

investigating whether ErbB1 directly associates with TLR3 (Yamashita et al., 2012). The results from our experiments support the hypothesis that ErbB1 is required for the effects of TLR3 activation on neurite outgrowth and that calcium is either required for this association (probably because calcium is required for ErbB1 activation) and/or may be required for the activation of downstream targets from ErbB1.

Apart from investigating the effects of TLR3 on neurite outgrowth, we were given an opportunity to study the role of endosulfatases, Sulf1 and Sulf2 on neurite outgrowth (Chapter-8). As the effects of Sulfs on neurite outgrowth from DRG neurons were unknown, we tested their activity by either using blocking antibodies or conditioned media containing Sulf1. Our results have demonstrated a rather intriguing role for Sulfs in regulating neurite outgrowth. While blocking the function of either Sulf1 or Sulf2 improves outgrowth of neurites in the presence of CSPGs, addition of Sulf1 conditioned media also improved neurite outgrowth. Although we are not able to explain the effects of conditioned media (discussed in Chapter-7), experiments using function-blocking antibodies provides preliminary evidence that Sulfs are enzymes that mediate inhibition of neurite outgrowth.

Sulfatases alter the activity of HSPGs, which play an important role in regulating neuronal responses in a CSPG-contained environment (Coles et al., 2011). The role of HS-GAG chains in modulating FGF signalling has also been widely studied. The sulfate group at the 6-O position of glucosamine has been shown to be essential for the association of FGF with FGFR (Pellegrini, 2001). Moreover, the contact of GAG chains with FGF and FGFR brings the signalling complex together and stabilizes it (Schlessinger et al., 2000). The presence of the 6-0 sulfate group has been reported to be crucial for the mitogenic activity of FGF (Lundin et al., 2000). Hence, it is expected that Sulfs can affect FGF signalling. The predominant effects of Sulfs on FGF signalling are that of inhibiting the signalling pathway by cleaving 6-O sulfate groups from HSPGs (Otsuki et al., 2010b). FGF signalling has been reported to mediate cell growth, proliferation and survival (Guillemot and Zimmer, 2011). However, this pathway is tightly regulated by an autoregulatory feedback loop. Proteins that negatively regulate signalling via FGFR include Sprouty and Spred {Sprouty-related EVH1 [Ena/VASP (vasodilator-stimulated phosphoprotein) homology 1] domain}(Yu et al., 2011; Quintanar-Audelo et al.,

2011). The MAP kinase pathway which is downstream of FGFR is required for transcription of Sprouty (Hanafusa et al., 2002). Sprouty in turn inhibits different components of FGF signalling. There is evidence on the inhibitory roles of Sprouty on neurite outgrowth *in vitro*. In adult DRG cultures, down-regulation of Sprouty2 promotes neurite outgrowth (Hausott et al., 2009). Sprouty 2 and 4 have been implicated in regulating neurite outgrowth from hippocampal neurons (Hausott et al., 2012).

Sprouty has also been reported to regulate ErbB1 signalling. While it downregulates FGF signalling, Sprouty acts to prolong the duration of signalling from ErbB1 (Egan et al., 2002; Wong et al., 2002; Rubin et al., 2003). This is predominantly achieved by direct association with the ubiquitin ligase, Cbl. Sprouty competes with ErbB1 to bind to Cbl resulting in targeted lysosomal degradation of Sprouty. Apart from the effects of sprouty on prolonging ErbB1 signalling, Spred has recently been shown to associate with ErbB1 (Meng et al., 2012). Spred2 is constitutively associated with unstimulated ErbB1. Ligand stimulation of ErbB1, results in posphorylation of Spred2 which then dissociates from ErbB1 and binds to p85. The spred2-p85 complex mediates its inhibitory effects on cell growth by inhibiting the Ras/Erk pathway (Meng et al., 2012).

CSPGs can bind to various receptors which include PTPσ, LAR, NgR1 and NgR3(Shen et al., 2009; Fisher et al., 2011; Dickendesher et al., 2012). HSPGs can also bind to PTPσ and NgR1 (Aricescu et al., 2002; Lee et al., 2008). Binding of HSPGs to PTPσ induces clustering of the receptor whereas binding of CSPGs does not induce receptor oligomerisation (Coles et al., 2011). Clustering of receptors can alter signalling pathways. It has been suggested that protein tyrosine phosphatases (PTPs) are inactive when dimerised (Weiss and Schlessinger, 1998; Ostman and Bohmer, 2001). Dimerisation of phosphatases can be easily induced when they are clustered. Even if the catalytic domain of the receptor may be rendered inactive, the ectodomain may take part in signalling. Although the phosphatase activity of PTPσ is associated with inhibition of neurite outgrowth, the ectodomain has been shown to promote neurite outgrowth from retinal ganglion cells (Sajnani et al., 2005). These authors suggested a model where ligand binding to PTPσ was followed by shedding of the ectodomain. The shed ectodomain would then act as a ligand for a different receptor that would mediate signalling resulting in neuritogenesis.

It is quite possible that binding of HSPGs to clustered PTPσ with inactive catalytic domains could be followed by the shedding of ectodomains which would in turn act as ligands for membrane bound HSPGs (reverse signalling as in the case of Ephrins). Nonetheless, it can be concluded that clustering of PTPσ inhibits its activity in regions lacking oligomerisation and the receptor's phosphatase activity is inhibited by oligomerisation. This in turn facilitates signalling through receptors such as FGF receptor resulting in promotion of neurite outgrowth. As it is most likely that sulfate groups in HSPGs influence tight binding and clustering of the receptors, the activity of Sulf1 and Sulf2 would disrupt such binding. CSPGs do not mediate oligomerisation of the receptors. CSPG bound to PTPσ monomers with an active catalytic domain that is more evenly distributed on the membrane mediates inhibitory responses. The PTPσ CSPG complex can dephosphorylate various substrates such which may include receptors for Trks(Faux et al., 2007) and possibly PTEN. While dephosphorylation of Trk proteins may result in abrogation of neutrophin mediated neurite outgrowth, dephosphorylation of PTEN causes it activation and subsequent downregulation of PI-3K and mTOR.

Taken these observations together, we propose a model to explain the signalling events in the presence of two different proteoglycan-enriched environments as shown in figure 10.6. In the presence of HSPGs, PTPσ is oligomerised. The GAG chains of HSPGs bring the receptors together. This facilitates dimerisation of PTPσ causing the inactivation of its catalytic domain. The ectodomain to which HSPGs are bound are shed and this complex in turn acts as ligands for membrane-bound HSPGs. Reverse signalling follows which results in promotion of neurite outgrowth. The dotted arrow indicates multiple steps that are unknown which leads to transcription of growth-associated factors.

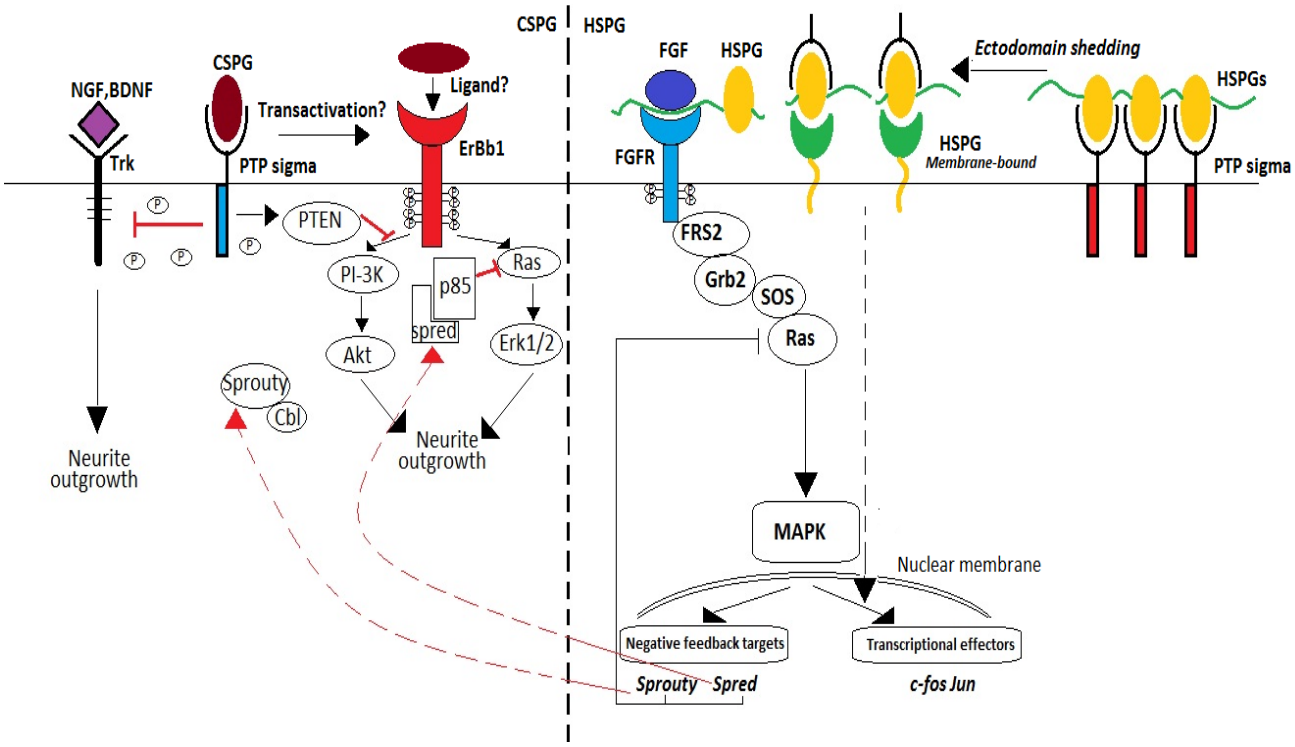


Figure 10.6: Suggested model for FGFR/ErbB1 signalling in the presence of HSPGs/CSPGs.

HSPGs also bind and stabilize FGF-FGFR interactions. The formation of the ternary complex activates FGFR. Activated FGFR recruits FRSα. Downstream signalling molecules from FRSα include Grb2, SOS, Ras and MAPK. The activation of MAPK results in the activation of transcription factors which either act as transcriptional regulators or effectors. Transcriptional regulators activated by MAPK include Sprouty and Spred. In the presence of CSPGs, clustering of PTPσ is abolished. The PTPσ monomers with active catalytic domain associates with CSPGs. This association may trigger auto phosphosphorylation of ErbB1or may be activated by ligand binding. Spred associates with inactive ErbB1. Activation of ErbB1 causes dissociation of Spred from ErbB1 which then binds to p85. Spred-p85 complex negatively regulates neurite outgrowth by inhibiting the the Ras- Erk pathway. ErbB1 is rescued from lysosomal degradation by Sprouty which competes with ErbB1 to bind to Cbl. Binding of Sprouty to Cbl results in its degradation. The CSPG-bound active PTPσ dephosphorylates various substrates such as Trk proteins which mediate NGF or BDNF signalling. The complex also dephosphorylates PTEN thereby activating it. PTEN then negatively regulates the PI-3/Akt

pathway from acitivated ErbB1. As a result of the phosphatase activity of PTPσ, neurite outgrowth is inhibited by blocking PI-3/Akt and Trk signalling pathways.

Based on our observations of increased cell surface expression of Sulfs in DRG neurons following sciatic nerve lesion (chapter8), it can be suggested that Sulf expression is upregulated in an altered extracellular environment or when the cell senses injury. Increased expression of Sulfs in turn cleaves the sulfate groups which are necessary for binding of HSPGs to either PTPσ or FGF attenuating signaling from these receptors. Clusters of PTPσ which are no longer tightly bound by HSPG chains de-oligomerise facilitating scattered expression of PTPσ monomers with active phosphatase activity. Hence the shift from growth promoting to growth inhibition when the extracellular environment is altered is regulated by Sulf activity.

Expression studies have revealed the presence of Sulfs, particularly Sulf2 in neuronal nuclei (Chapter-8). This introduces the possibility that Sulfs influence gene transcription. However, further investigation is required. As we have shown that blocking ErbB1 has positive influences on axonal regeneration (Chapter-9), it would be of interest to investigate the role of blocking Sulfs on axonal regeneration in *vivo*.

Chapter-11 Future directions of this research

The work carried out so far has been useful in understanding the role of ErbB1 in limiting neurite outgrowth *in vitro*. A clear priority for further work would be to extend the in vivo studies on which I have preliminary data. Unfortunately, time prevented the performance of more extensive *in vivo* studies for inclusion in this thesis. As there has been only one previous study showing that pharmacological blocking of ErbB1 improves axonal regeneration *in vivo,* it would be interest to be able to confirm our initial observations on the ability of the dominant-negative ErbB1 lentivirus to promote axonal regeneration in the injured spinal cord. More experiments over a greater number of time-points would be required to see just how far rubrospinal axons expressing DN-ErbB1 regenerate (the current data only show regeneration into the scar tissue at the lesion site) and into what tissue they extend, e.g. into the meninges or into the caudal spinal cord. It would also be exciting to extend the study to other tracts including the corticospinal tracts, ascending dorsal columns and optic nerves. These tracts arise from very different types of neurons with different capacities for axonal regeneration (Anderson et al., 1998). Retinal ganglion cells and DRG neurons can be stimulated to mount a more profound regenerative response by a number of techniques, including lens injury and the induction of perineuronal inflammation. However, a different viral vector would be required to transfect DRG or retinal ganglion cells, which are refractory to treatment with lentiviral vectors. Adeno-associated viral vectors would seem the obvious candidates for such experiments.

The exact mechanism through which ErbB1 elicits its effects are unknown. It can be envisaged that ErbB1is downstream on the signalling pathway from receptors such as Nogo receptors and receptors for CSPGs, and the activation of these receptors leads to the activation of ErbB1 in a manner dependant on calcium. In the case of TLR3, as discussed in Chapter-10, there is evidence that shows activated ErbB1 is not downstream of TLR3 but is essential for the activation of TLR3. It is possible that all the inhibitory effects on neurite outgrowth produced by ErbB1 activation involve signalling through TLR3 and this hypothesis could be tested using neurons from TLR3 knockout mice.

However not all receptors for inhibitors of neurite outgrowth signal via ErbB1. In the case of CNS myelin, treatment of cultured DRG neurons with PD168393 improves neurite outgrowth but does not completely disinhibit the neurons. This can be explained by the presence of additional receptors such as receptors for Ephrin B3 and Sema 3a that have been previously reported to elicit responses independent of ErbB1 signalling (Koprivica et al., 2005). Similarly, in the case of CSPGs, blocking ErbB1 does not lead to complete disinhibition of neurite outgrowth. As several receptors for CSPGs have been identified, including PTPσ, NgR1 and NgR3 (Atwal et al., 2008; Dickendesher et al., 2012) and that deletion of all three receptors produces additive effects on axonal regeneration in the injured optic nerve(Dickendesher et al., 2012), it would be worth investigating which of these receptors signal via ErbB1. This could be done by using PTPσ and ErbB1 double knockout mice or ErbB1, NgR1, NgR3 tripple knockouts mice to test if such deletions produced additive effects in promoting neurite outgrowth from cultured neurons grown in the presence of CSPGs.

With regard to the activation of ErbB1, we and others have shown that calcium is critical for the activation of the receptor but it is unclear how calcium transactivates ErbB1. One known pathway for such transactivatiion involves calcium activating a metalloprotease with releases an ErbB1 ligand at the cell surface, which in turn activates ErbB1. Although ligand-independent activation of ErbB1 has been suggested, this requires further clarification. Koprivica et.al. (2005) have shown that ErbB1 activation in response to CNS myelin is independent of metalloproteases and at least some of its ligands such as EGF and HB-EGF. Experiments with neurons expressing mutant ErbB1 with impaired ligand-binding might help clarify this situation.

White et.al. (2011) have shown that ErbB1 activation by TGFα promotes axonal regeneration *in vivo,* predominantly by converting astrocytes to a supportive phenotype. It would be of interest to test if ErbB1 activation by TGFα promotes neurite outgrowth from cultured cerebellar neurons in the presence of CNS myelin or CSPGs. However, observations made by White et.al. (2011) are intriguing as they relate to the fact that axonal regeneration in vivo takes place in the presence of a complex mixture of cell types, including both neurons and glia several of which may express ErbB1. It could be that activation in astrocytes promotes regeneration while activation in neurons inhibits that process. It can be suggested that activation of ErbB1 may elicit different effects on

regeneration depending on the cell-type in which it is activated. The best way to study this would be to use conditional knockouts for ErbB1 in which ErbB1 is either selectively deleted in astrocytes or in neurons. Breeding a mouse carrying floxed ErbB1 with animals carrying Cre under the control of the GFAP promoter or the synapsin promoter would clarify this point. Assessment of regeneration following CNS lesions in both types of knockouts would give us a better understanding into the role of ErbB1 signalling in neuronal and non-neuronal cells cultured in the presence of inhibitory molecules *in vitro* or following injury *in vivo*.

In our efforts to investigate the downstream signalling targets of ErbB1, we analysed the role of PTEN in signalling from ErbB1. Although it can be said that PTEN is associated with signalling from ErbB1, it is unclear whether PTEN is a direct downstream target or if signalling pathways through ErbB1 and PTEN converge at the same signalling point. In light of our functional data on the association between ErbB1 and PTEN, it would be of interest to analyse the activation of ErbB1 in neurons treated with a PTEN inhibitor. The specific phosphotyrosine residues on ErbB1 responsible for eliciting inhibitory responses to neurite outgrowth require investigation but could be investigated using mutant forms of ErbB1. Targeting potential downstream molecules using pharmacological blockers would aid in elucidating the pathway from ErbB1 activated in the presence of CNS myelin and CSPGs. Proteins such as canonical signalling molecules from ErbB1 which include MAPK, JAK/STAT and other signalling molecules such as calmodulin, Pyk2 and GSK3β could act as potential downstream targets.

With regard to endosulfatases Sulf1 and Sulf2, it is not known if there are changes in the expression pattern of these enzymes following injury to the spinal cord or optic nerve. Based on data from *in vitro* experiments using function-blocking antibodies to Sulfs, we have shown that Sulfs and ErbB1 share similar signalling pathways. It would be of interest to look into the activation of ErbB1 in cultured neurons treated with antibodies that block the activity of Sulfs. Finally, the effect of blocking Sulf1 and Sulf2 on axonal regeneration in the injured spinal cord is a subject of study that is open to investigation.

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