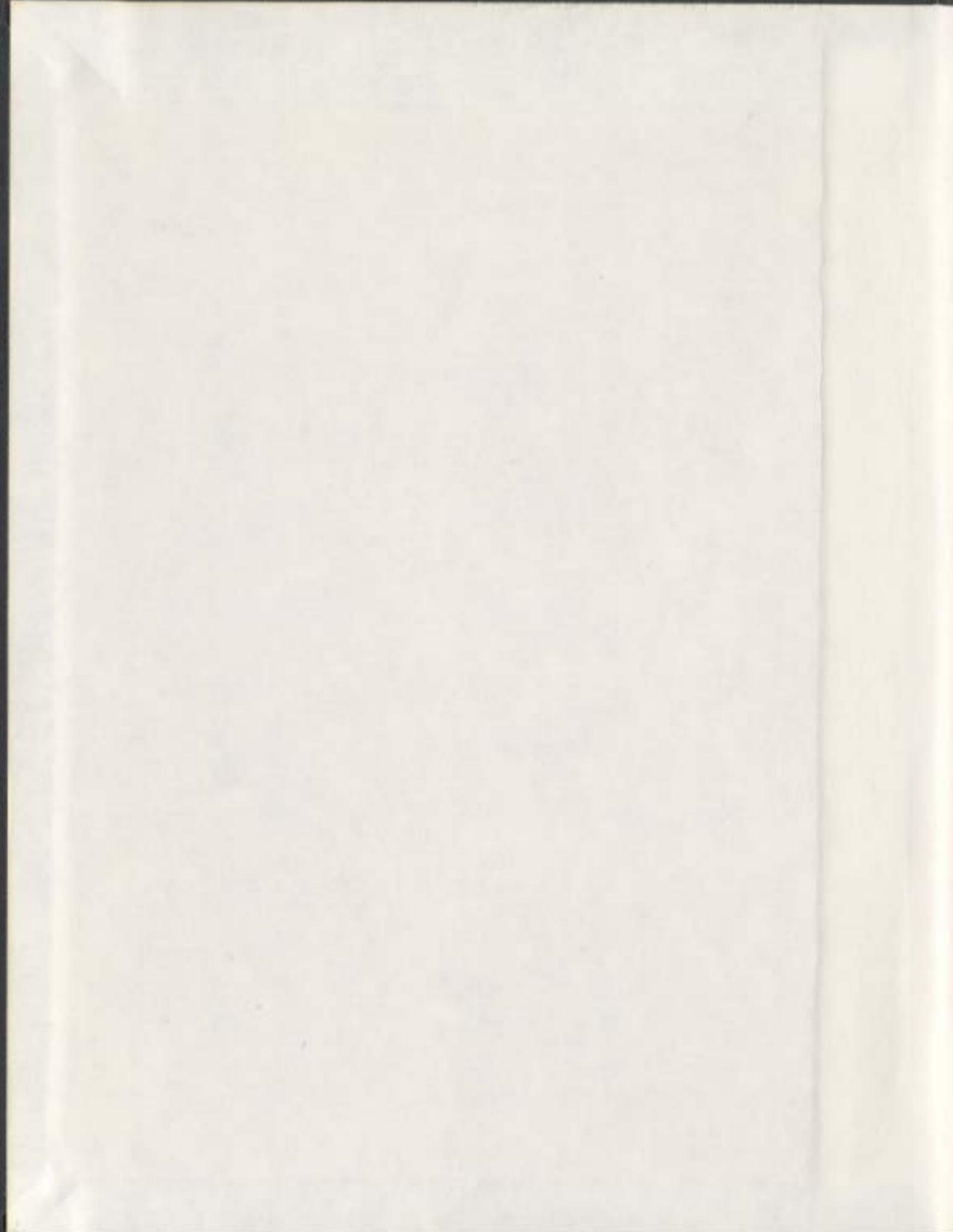


COOPERATION BETWEEN INTEGRIN AND GROWTH
FACTOR RECEPTOR SIGNALLING IN THE REGULATION
OF NEURITE GROWTH FROM RAT SENSORY NEURONS

BUDD A. TUCKER



Cooperation between integrin and growth factor receptor signalling in the regulation of
neurite growth from rat sensory neurons

By

© Budd A. Tucker

A thesis submitted to the School of Graduate Studies in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

Division of Basic Medical Sciences
Faculty of Medicine
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Abstract

Like growth and guidance of developing axons, regeneration after axonal injury is predominantly influenced by factors located in a neuron's extracellular environment. These factors include neurotrophins, such as Nerve Growth Factor (NGF), and adhesion molecules, such as laminin. However, how these factors interact to stimulate optimal levels of neurite growth is largely unknown. In this thesis, I initially investigated whether attachment to permissive culture substrates is sufficient to promote neurite outgrowth from DRG neurons, and whether this interaction is able to enhance the response of neurons to added neurotrophic factors, such as NGF. Adult Dorsal Root Ganglion (DRG) neurons plated on surfaces coated with a thin film of laminin exhibited increased neurite outgrowth, this growth was correlated with increased expression of laminin binding integrin subunits. Laminin-induced neurite growth was integrin-dependent as it was attenuated by treatment with either a cyclic Arg-Gly-Asp (RGD) peptide or a $\beta 1$ integrin-blocking antibody ($\beta 1i$). The addition of NGF to cells plated on laminin resulted in a significant and synergistic increase in the integrin-dependent outgrowth.

In performing these experiments it was observed that not all DRG neurons were responding to laminin and NGF with increased neurite growth. As neurons in the adult rat DRG can be classified into at least three separate subpopulations based on morphologic and phenotypic differences, a cell selection approach was utilized to show that laminin-induced neurite growth occurs in the absence of added trophic factors only in heavy chain neurofilament positive and calcitonin gene related peptide positive DRG neurons (NGF-responsive population). In contrast, laminin alone is not sufficient to stimulate significant neurite growth from lectin *Griffonia Simplicifolia* IB4 positive

neurons (IB4+ve), although it is still required to elicit a growth response from these cells in the presence of Glial Cell Line Derived Neurotrophic Factor (GDNF) (eg., neurite growth occurred only when cells were plated on laminin in the presence of GDNF). By using chemical inhibitors we demonstrated that only the PI 3-K/Akt pathway was required for neurite growth from the NGF-responsive cell population. However, both the PI 3-K/Akt and MEK/MAPK signalling pathways were required for neurite growth from the IB4+ve cell population. Further analysis indicated that Src was potentially a key point of collaboration between NGF and laminin induced neurite growth in NGF-responsive adult DRG neurons. Src appeared to be located upstream of the PI 3-K/Akt signalling pathway and was responsible for increased neurite growth induced by the addition of laminin and NGF together.

In this series of studies we have identified specific signalling events and environmental requirements associated with neurite growth for different subpopulations of adult DRG neurons, pointing to potential therapeutic targets while identifying why any one treatment alone may be insufficient to totally repair peripheral nerve damage.

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List of Abbreviations

ANOVA	analysis of variance
Akt/PKB	protein kinase B
Akti	Akt inhibitor
ARAC	cytosine arabinoside
ART	artemin
β 1i	Beta 1 integrin inhibitor
BDNF	brain derived neurotrophic factor
C3 enzyme	clostridium limosum exoenzyme C3
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CGRP+ve	calcitonin gene-related peptide positive DRG neurons
CNS	central nervous system
Col	collagen
CSPG	chondroitin sulphate proteoglycan
DAB	diaminobenzidine
DAG	diacylglycerol
DCC	deleted in colorectal cancer
DRG	dorsal root ganglion
ECM	extracellular matrix
ERK	extracellular signal related kinase
FAK	focal adhesion kinase
FB	fibronectin
FCS	fetal calf serum
GAP-43	growth associated protein 43
GDNF	glial cell line-derived neurotrophic factor
GFR α	growth factor receptor alpha
GPI	glycosylphosphatidyl inositol
HBSS	Hanks balanced salt solution
HRP	horseradish peroxidase
IB4+ve	<i>griffonia simplicifolia</i> isolectin B4 binding DRG neurons
ICC	immunocytochemistry
IGF	insulin-like growth factor
ILK	integrin-linked kinase
K252a	tyrosine kinase inhibitor
LIMK	Lin-11 Islet 1 and Mec 3 kinase
LN	laminin
LNN	laminin plus NGF
LY294002	PI 3-K inhibitor
MAG	myelin associated glycoprotein
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MG	matrigel
MODNB	modified neurobasal media

NANs	non-surface activated nanofibers
NB	neurobasal media
NCAM	neural cell adhesion molecule
NF200	heavy chain neurofilament
NGF	nerve growth factor
NgR	nogo receptor
NT	neurotrophin
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NTN	neurturin
Omgp	oligodendrocyte myelin glycoprotein
P75NTR	p75 neurotrophin receptor
PAK	p21 activated kinase
PBS	phosphate-buffered saline
PC12	pheochromocytoma cell line
PGP 9.5	protein gene product 9.5
PI 3-K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKC	protein kinase C
PL	poly-D-lysine
PLC	phospholipase C
PMSF	phenylmethanesulphonyl fluoride
PNS	peripheral nervous system
PP2	SFK inhibitor
PSP	persephin
RET	transmembrane tyrosine kinase receptor
RGD	Arg-Gly-Asp
ROBO	roundabout
ROCK	rho associated coiled-coil kinase
SANs	surface activated nanofibers
SDS	sodium dodecyl sulfate
SFK	Src family kinase
SH-SY5Y	neuroblastoma cell line
TBS	tris-buffered saline
TBST	tris-buffered saline plus tween-20
TESK1	testicular protein kinase 1
Trk	tropomyosin-related kinase
TrkA	tropomyosin-related kinase A
TrkB	tropomyosin-related kinase B
TrkC	tropomyosin-related kinase C
U0126	MEK inhibitor
Y27632	ROCK inhibitor

CHAPTER 1

INTRODUCTION

1.1 Neuronal regeneration of the adult nervous system

1.1.1 The regenerative response

In order for a nerve cell to undergo axonal regeneration and stimulate functional recovery, it must first survive the initial insult. Once this is achieved, the cell has to mount a regenerative response in which the severed axon must grow from its remaining proximal segment, across the injury site, and back to the appropriate target, where it must reform its original synaptic connections. In order for this regenerative response to occur, the cell body must receive signals from its injured axon telling it to switch from the normal operating mode, whereby information in the form of action potentials is sent along the length of its axon, to a mode where axonal growth becomes its main priority (Fu and Gordon, 1997). Thus, axonal injury causes neurons to revert back to a state similar to that seen early in development, where attempts to form the appropriate terminal connections are made.

During nervous system development, axons are required to travel great distances with accuracy in order to make the required synaptic connections. For instance, a pseudounipolar DRG neuron extends a single axon from its cell body, which bifurcates sending one process centrally, to the spinal cord, while the other process is sent peripherally to the skin, muscle, or visceral organs. For axon growth and accurate target innervation to occur, a number of excitatory and inhibitory growth and guidance cues must be utilized.

The growth cone is a specialized structure located at the leading end of an axon and acts as the major sensory-motor component for the detection of growth and guidance cues in the extracellular environment. The growth cone is made up of 2 major domains consisting of a central domain surrounded by a peripheral domain (Dent and Gertler, 2003). The central domain has an abundance of microtubules and membranous organelles, whereas the peripheral domain is made up of two actin rich structures. These structures include the lamellipodium, which is made up of a dense meshwork of actin filaments and directly surrounds the central domain, and the filopodia, which are longer finger-like projections of actin that extend from the outer edge of the lamellopodium (Dent and Gertler, 2003; Forscher and Smith, 1988; Krause et al., 2003; Lebrand et al., 2004; Letourneau et al., 1986; Letourneau et al., 1987). Axon elongation has been shown to occur in three stages and is critically dependent on the presence of these filament structures. The first stage of axon growth is protrusion where the growth cone quickly extends lamellopodia and filopodia, which begin to probe their environment. The second stage is engorgement where the organelles and cytoplasm move forward into the central domain of the growth cone. The third and final step is consolidation where a new section of axon is formed by the collapse of the proximal portion of the newly elongated growth cone (Dent and Gertler, 2003; Krause et al., 2003; Lebrand et al., 2004).

Thus, both lamellopodia and filopodia extending from the growth cone act as sensors which undergo continuous cycles of extension and collapse while probing their environment (Burmeister and Goldberg, 1988; Goldberg and Burmeister, 1986; Goldberg and Burmeister, 1988; Goldberg and Burmeister, 1989). Filopodia, for example, act as fingers that probe the environment, transducing signals from both excitatory and

inhibitory cues. These cues are considered to be short or long range, stimulating specific cell surface receptors and inducing advancement, retraction, turning, or collapse of the growth cone. The short-range cues include those that are attached to either cellular membranes or the extracellular matrix and typically exert precise control over growth cone movement. The long-range cues, however, are typically secreted, soluble factors that act on growth cones a distance from the source of production. These factors typically exert less precise control over growth cone movement.

Like the processes involved in nervous system development, growth cone interaction with the extracellular environment is essential to the regenerative response following axonal injury. Being interested in these processes the fundamental goal of my research was to identify the key environmental conditions and subsequent cellular signalling components required to stimulate optimal levels of neurite growth from adult sensory neurons. Knowing that both short- and long-range growth and guidance cues are important developmentally, these factors became my main focus. Initially I was concerned with the role of neurotrophins, specifically NGF, in axon growth. However, during early collaborative studies between David Jones (a former MSc student in the lab) and myself, the observation was made that adult DRG neurons plated on the extracellular matrix molecule laminin were extending neurites in the absence of added trophic factors. This led us to suspect that the extracellular matrix on which the neurons were plated was having a positive effect on neurite outgrowth. Thus, my key interest became the interactions between laminin activated signalling pathways and NGF activated signalling pathways in stimulating axonal regeneration in adult DRG neurons. The reasons for

focusing on the extracellular matrix molecule laminin and the neurotrophin NGF are numerous and will be discussed in great detail in the upcoming sections.

1.1.1.1 Signalling at the growth cone

As suggested in Section 1.1.1, both lamellopodia and filopodia extending from the growth cone are responsible for sensing and transducing cues from a cell's environment that are required for growth and elongation of axons. The ability of an axon to respond to external growth and guidance cues is largely the function of a group of proteins belonging to the Rho family of small GTPases (Luo, 2002; Ng *et al.*, 2002). These proteins have been shown to directly regulate actin filament dynamics, thus regulating growth cone motility (Luo, 2002; Ng *et al.*, 2002). Members of this family of proteins (including RhoA, Rock, Rac, Pak, and Cdc42) are important in the regulation of growth cone extension and retraction, and are also important in the promotion or inhibition of axon growth. As shown in Fig 1.1 Rho GTPase activity is regulated by two distinct classes of molecules, the Rho GAPs, or GTPase activating proteins, and the Rho GEFs, or guanine nucleotide exchange factors (Huber *et al.*, 2003). The Rho GAPs function to inhibit Rho GTPase activity, while the Rho GEFs function to activate Rho GTPase activity (Huber *et al.*, 2003). In general, activation of RhoA stimulates the activation of ROCK, which induces the retrograde flow of actin (Huber *et al.*, 2003). This stimulates growth cone retraction and inhibition of axon extension. Activation of Rac, and Cdc42 act in opposition to RhoA and inhibit the retrograde flow of actin and stimulate actin polymerization and branching (Huber *et al.*, 2003). This stimulates growth

Figure 1.1 Schematic illustration indicating how the family of small Rho GTPases are utilized at the growth cone for axon extension and guidance. GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein. Adapted from Huber et al., 2003.

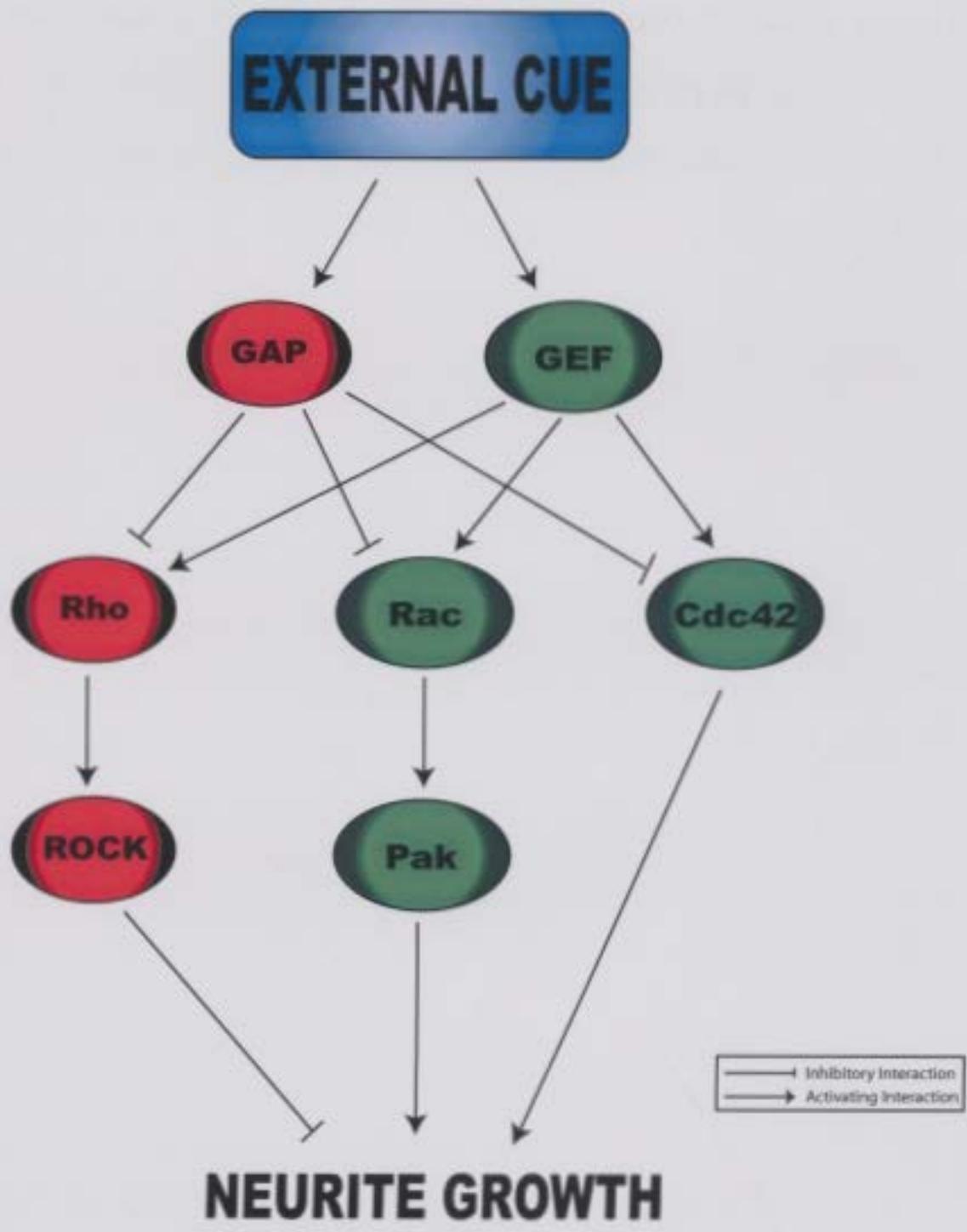


Fig 1.1

cone protrusion and axon extension. Thus, external cues can have direct effects on GAPs and GEFs that act to either stimulate or inhibit axonal regeneration.

1.1.2 Regeneration in the CNS vs. PNS

Following axotomy, the distal segment of the severed axon undergoes Wallerian degeneration. This is the process by which the distal part of the severed axon, which no longer has contact with its cell body, undergoes degeneration and subsequent removal (Stoll *et al.*, 2002). During this process, there is an immune response that stimulates the infiltration of leukocytes and macrophages, which act to remove the degenerating myelin sheet via phagocytosis (Stoll *et al.*, 2002).

Although the presence and rate of Wallerian degeneration is roughly the same in both the CNS and PNS, differences exist in this process that are very important to axonal regeneration. In the CNS, myelin removal is slow and inefficient, and myelin debris, which contains a variety of inhibitory proteins, remains in the injury site for extended periods of time (George and Griffin, 1994). The injury site in the CNS is also plagued by the formation of a glial scar, which has also been shown to contain growth inhibitory molecules (Klapka *et al.*, 2005; Ribotta *et al.*, 2004). In contrast, axotomy of PNS neurons stimulates the complete removal of the myelin sheet, and Schwann cells change from their normal state of myelination, begin to proliferate, and form bands of Bungner, which act as basal lamina tubes providing guidance tracts for regenerating axons (Fu and Gordon, 1997). The Schwann cells of the distal nerve stump begin to release a variety of cell adhesion, extracellular matrix, and neurotrophic molecules in an attempt to provide a permissive environment for axonal regeneration (Fu and Gordon, 1997; Stoll *et al.*,

2002). In the CNS during Wallerian degeneration, oligodendrocytes do not change from a myelinating to a proliferating state and the formation of structures like the bands of Bungner does not occur. Unlike the PNS, there is a lack of permissive molecules such as NGF and laminin that are secreted after CNS injury in the adult (Fenrich and Gordon, 2004; Stoll et al., 2002).

It has long been known that CNS neurons, unlike PNS neurons, lack the ability to achieve significant amounts of axonal regeneration following injury (Aguayo et al., 1981; Aguayo et al., 1991; David and Aguayo, 1981; Filbin, 2003). However, when provided with the appropriate environmental conditions, CNS neurons have been shown to regenerate (Azanchi et al., 2004; David and Aguayo, 1981; Fairless and Barnett, 2005; Klapka and Muller, 2006; Kuffler, 2000). These findings suggest that the marked difference in regenerative capacity between CNS and PNS neurons is largely due to different environmental factors. Specifically, the adult CNS is faced with an inhibitory environment, which is created by the presence of inhibitory molecules associated with the myelin sheath and glial scar, and a lack of permissive molecules such as laminin and NGF associated with the Schwann cells of the PNS (Azanchi et al., 2004; Fouad et al., 2005; Kuffler, 2000).

1.1.2.1 The CNS environment

As noted in Section 1.1.2, it is postulated that the extracellular environment of the CNS is unfavourable for axonal regeneration, making functional recovery after CNS injury limited. The major inhibitory components of the CNS environment are derived from either the myelin sheath or the glial scar. I will first discuss the inhibitory

molecules Nogo, Myelin Associated Glycoprotein (MAG), and Oligodendrocyte Myelin Glycoprotein (OMgp) that are found in myelin and act as potent inhibitors to approaching axons. I will then discuss how both the physical properties of the glial scar and its molecular components act to inhibit neurite growth.

The inhibitory Nogo molecule was first identified as an antigen for the IN-1 antibody, which was shown by Schwab and colleagues to block the inhibitory action of myelin on the growth of corticospinal tract axons in young adult rats (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Schnell and Schwab, 1990). Three major isoforms of the Nogo molecule have been reported, including Nogo-A, Nogo-B, and Nogo-C (Schweigreiter and Bandtlow, 2006). While Nogo-B and Nogo-C are expressed outside the nervous system, Nogo-A is specific to the mammalian CNS and is located on both the surface of oligodendrocytes and in the lumen of the endoplasmic reticulum (Filbin, 2000; Hunt *et al.*, 2002). Nogo-A has been shown to contain two functional components, an internal amino-Nogo, and the external Nogo66. The Nogo66 region has been identified on all Nogo isoforms and acts as the major inhibitory component of axonal regeneration (Fournier et al., 2001; GrandPre et al., 2000). Strittmatter and colleagues identified a GPI linked receptor that is predominantly expressed on neuronal surfaces, which they termed the Nogo receptor (NgR). Inhibition of this receptor via GPI protein cleavage blocked Nogo66 binding and subsequent growth cone collapse and stimulated axon growth from CNS spinal explants (Fournier *et al.*, 2001).

MAG, a member of the Ig superfamily of molecules, was the first of the myelin-derived proteins to be identified as an inhibitor of axon growth (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). Early in development, like other members of the Ig

super family of proteins, MAG functions to promote axon growth and guidance. The switch to axonal inhibition occurs at different times in different neurons; for instance, retinal ganglion cells have been shown to switch from MAG induced attraction to inhibition embryonically (Salzer *et al.*, 1990). This switch to inhibition of axon growth by MAG was later shown to be dependent on cellular expression of cAMP and activation of its downstream effector PKA; the embryonic switch to MAG inhibition in retinal ganglion cells occurs between E18 and P5, which coincides perfectly with a decrease in cAMP expression (Cai *et al.*, 2001). Activation of the cAMP/PKA pathway before MAG stimulation, via neurotrophin pre-treatment, blocks MAG induced growth inhibition (Cai *et al.*, 1999). Similarly, inhibiting cAMP embryonically, prior to MAG induced inhibition, causes growth cone collapse (Cai *et al.*, 2001).

Of the three myelin-derived inhibitory proteins, OMgp is the most recently identified. This protein was initially called arretin and was identified from chromatographic fractionation of MAG (Kottis *et al.*, 2002). Like NogoA and MAG, OMgp also acts to inhibit neurite growth from regenerating neurons. The addition of OMgp inhibited neurite growth from both cerebellar granule and hippocampal neurons *in vitro* (Kottis *et al.*, 2002).

Like Nogo, MAG and OMgp have also been found to bind to the NgR. NgR transfection into neurons that do not normally express the receptor causes OMgp to stimulate growth cone collapse, and similarly, dominant negative expression of NgR blocks MAG induced growth inhibition (Domeniconi *et al.*, 2002; Wang *et al.*, 2002). However, as indicated, NgR is a GPI linked receptor, meaning that it does not extend into the cytoplasm and, thus, cannot exert a direct influence on internal signalling cascades.

Rather, NgR was found to function as a co-receptor with the low affinity neurotrophin receptor p75 and lingo-1 (Bandtlow and Dechant, 2004; Ceni and Barker, 2005; Mi et al., 2004; Yamashita et al., 2002). Neurons that lack the p75 receptor or were transfected with a dominant negative lingo-1 protein do not undergo MAG-induced growth cone inhibition (Mi et al., 2004; Yamashita et al., 2002). Together, this signalling complex functions as a regulator of the Rho family of GTPases. Upon binding, myelin activates the NgR complex and stimulates RhoA activation which in turn inhibits neurite growth from a variety of different cell types in the adult CNS (Bandtlow, 2003; Domeniconi *et al.*, 2005). Conversely inhibition of RhoA or its downstream signalling intermediates with the C3 exoenzyme or a dominant negative form of the RhoA protein has been shown to stimulate axon growth and regeneration (Ellezam et al., 2002; Lehmann et al., 1999; Winton et al., 2002; Yamashita et al., 2002). Thus, negative regulation of Rho and its downstream effectors has a positive effect on neurite growth in a variety of CNS cell types including, retinal ganglion, cerebellar, spinal, and cortical neurons (Bandtlow, 2003; Domeniconi et al., 2005; Ellezam et al., 2002; Hunt et al., 2002; Lehmann et al., 1999; Logan et al., 2006; Mi et al., 2004; Winton et al., 2002; Yamashita et al., 2002).

The formation of the glial scar is a relatively late occurrence after CNS injury and if myelin inhibition can be overcome, allowing the regenerating axon to traverse the injury site before the scar forms, then this event becomes less concerning. The glial scar is formed in a process called reactive gliosis, whereby the infiltration of reactive astrocytes into the injury site causes the formation of a physical barrier along with the accumulation of chondroitin sulfate proteoglycans (McKeon et al., 1999; McKeon et al., 1991). These molecules, which include neurocan, brevican, phosphocan, NG2, tenascin

and chondroitin sulfate, are known to inhibit axonal regeneration. For instance, NG2 has been shown to be responsible for inhibiting sensory neuron regeneration into the dorsal root entry zone (Zhang *et al.*, 2001), while neurocan and phosphocan inhibit the regeneration of adult cortical and retinal ganglion neurons (McKeon *et al.*, 1999; Monnier *et al.*, 2003) (see Fig 1.1). Like the myelin-associated inhibitors, chondroitin sulfates exert their inhibitory effects on neurite growth via activation of RhoA and its downstream effector Rho Kinase ROCK (Monnier *et al.*, 2003). Inhibition of this signalling pathway via the C3 exoenzyme, or the use of the chemical inhibitor specific to ROCK, Y-27632, allows the axon to overcome chondroitin sulfate induced inhibitory effects (Dergham *et al.*, 2002; Monnier *et al.*, 2003). Many other attempts to overcome axonal inhibition by chondroitin sulfates have also been explored. For instance, the use of Chondroitinase ABC, an enzyme that removes the glycosaminoglycan side chains of chondroitin sulfates rendering them inactive, has seen some success in stimulating regeneration in both corticospinal tract and dorsal column neurons (Bradbury *et al.*, 2002). To date, specific receptors for this class of molecules are elusive, and thus, experimental attempts to stimulate regeneration via receptor inhibition have not been possible.

1.1.2.2 The PNS environment

As suggested in Section 1.1.2, the PNS environment is much more favourable for axonal regrowth than the CNS environment. However, this is not because the PNS does not express the inhibitory myelin proteins or the receptors required for binding to these molecules. Many studies have shown that myelin-associated inhibitors have the ability to

inhibit axonal regeneration from PNS neurons. For example, Stritmatter's group found that Nogo stimulates growth cone collapse from sensory neurons (Fournier *et al.*, 2001). Thus, peripheral neurons express the receptors required for binding to inhibitory myelin molecules and react to contact in the same way as CNS neurons. Therefore, the permissive growth environment of the PNS may be due to the efficient removal of myelin and its associated inhibitory molecules by proliferating Schwann cells and macrophages during Wallerian degeneration (Fenrich and Gordon, 2004; Stoll *et al.*, 2002). However, the removal of these inhibitory substances is only a part of the reason why the PNS environment is more favourable for axon growth than the CNS environment. For instance, unlike the CNS, axotomy in the PNS stimulates the increased expression of a variety of permissive growth and guidance molecules (Fenrich and Gordon, 2004).

Schwann cells are the prominent producers of the permissive PNS growth environment; these cells normally form the myelin sheath and have a one to one relationship with peripheral axons (Bunge, 1994a; Bunge and Bunge, 1986; Bunge, 1987; Bunge, 1993; Bunge, 1994b; Bunge and Wood, 1987; Dezawa, 2002; Fenrich and Gordon, 2004). As previously noted, axotomy stimulates Schwann cell proliferation, which in turn, induces the expression of a wide range of cell adhesion molecules, neurotrophic factors, and extracellular matrix molecules, all of which have been shown to promote growth during nervous system development. For example, following axotomy, Schwann cells upregulate their expression of cell adhesion molecules, such as Neural Cell Adhesion Molecule (NCAM) and L1 (Jessen *et al.*, 1987; Martini and Schachner, 1988), they deposit extracellular matrix molecules, such as laminin, collagen, and fibronectin, which make up the basement membrane (Chernousov *et al.*, 1999;

Kuecherer-Ehret et al., 1990; Lefcort et al., 1992), and they produce growth factors, such as Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), and Glial Cell Line Derived Neurotrophic Factor (GDNF) (Acheson et al., 1991; Bandtlow et al., 1987; Matheson et al., 1997; Meyer et al., 1992; Saika et al., 1991a; Saika et al., 1991b). The process of Schwann cell proliferation and the presence of these factors act to stimulate axonal regeneration and functional recovery; inhibition of this process by depletion of Schwann cells after nerve injury impaired axonal elongation (Fugleholm *et al.*, 1994).

1.1.3 Anatomy of the peripheral nerve

The PNS encompasses all nerves that lie peripheral to the pial covering of the CNS and include the craniospinal and autonomic nerves. Peripheral nerves are made up of both afferent and efferent nerve fibers. Afferent nerve fibers connect external receptors to the central nervous system, bringing information in from the periphery, and include, the peripheral process of the sensory DRG neurons projecting from the skin, muscle and visceral organs to the central process which projects to the dorsal aspect of the spinal cord. Efferent nerve fibers, include the primary motor neurons which have their cell bodies located in the grey matter of the spinal cord, and send information through their axons to muscle fibers in the periphery. In higher vertebrates, these afferent and efferent fibers join just outside the spinal cord to make up peripheral spinal nerves (Fig 1.2). Peripheral spinal nerves are covered by the epineurium, which is a tough

Figure 1.2. Location and structure of the DRG. Ag stained cross section of a rat spinal Cord with Dorsal Root Ganglion attached, this image has been adapted from

http://biology.clc.uc.edu/fankhauser/Labs/Anatomy_&_Physiology/A&P202/CNS_Histology/Spinal_Cord/Spinal_Cord_Histology.htm.

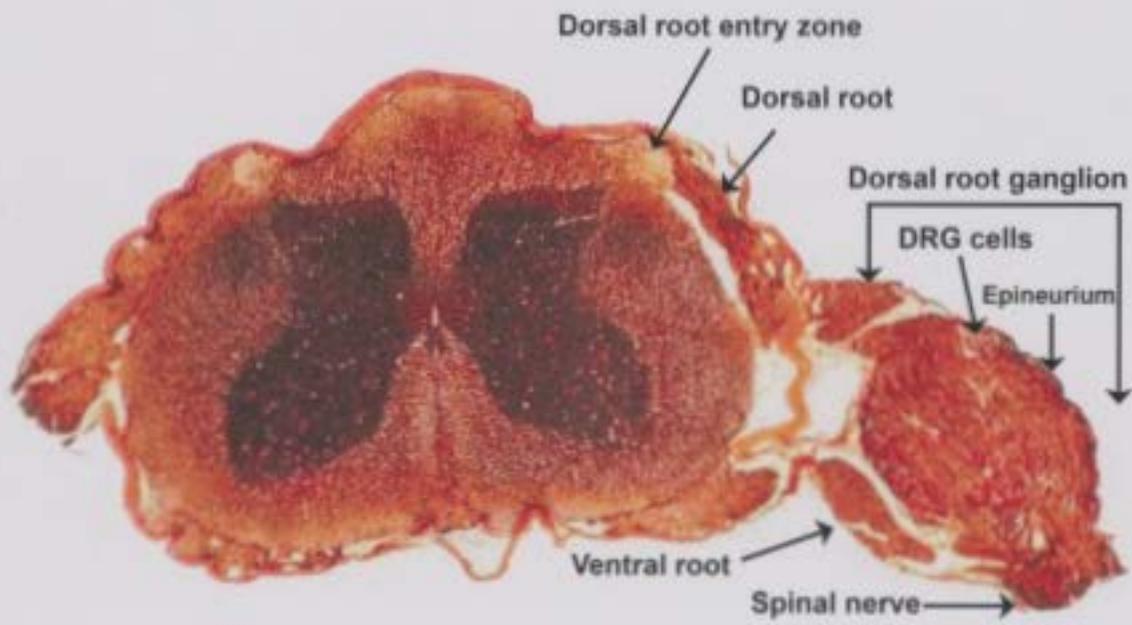


Fig 1.2

connective tissue layer derived from the mesoderm. Inside the epineurium, another layer called the perineurium covers bundles of individual fibers called fasciculi, which are filled with an intrafascicular connective tissue called the endoneurium. The epineurium provides the major structural support for the peripheral nerve and is made up of fibroblasts, collagen and fat. The perineurial layer is made up of alternating layers of flattened polygonal cells derived from fibroblast and collagen; between these layers is the basal lamina that contains important growth promoting molecules that will be discussed in later sections of this study. The endoneurium is made of mostly type 1 collagen fibers and is found adjacent to individual Schwann cell-axon units; between these units and the endoneurium lies another basal lamina layer (Fig 1.3).

1.1.3.1 Anatomy of the dorsal root ganglion

Although spinal nerves contain both motor and sensory fibers, my studies have focused on the sensory portion of the PNS, in order to study the processes involved in axonal regeneration. I will now describe the anatomy of the sensory dorsal root ganglion neuron, on which all of my studies have been performed.

Sensory neurons that convey information from peripheral sensory organs to the dorsal aspect of the spinal cord are housed in connective tissue similar to the perineurium, known as the dorsal root ganglion (DRG) (Fig 1.2) (Gray *et al.*, 1995). DRG neurons are pseudounipolar with spherical cell bodies that vary in diameter and are surrounded by small round satellite cells (Devor, 1999; Yoshida and Matsuda, 1979). A single axon that arises from these neurons exits the cell body and bifurcates, sending one

Figure 1.3. Anatomy of the peripheral nerve. **A:** Illustration of the typical structure of a peripheral nerve. **B:** Histological section of a sciatic nerve. The peripheral nerve is made up of three connective tissue layers, the outermost epineurium, the middle perineurium, and the innermost endoneurium. The endoneurium surrounds individual nerve bundles that house axons, Schwann cells, and the basal lamina. Adapted from <http://science.nhmccd.edu/biol/nervous/nervous.htm> and <http://www.apsu.edu/thompsonj/Anatomy%20&%20Physiology/2010/2010%20Exam%20Reviews/Exam%204%20Review/CH%2013%20Peripheral%20Nerve%20Histology.htm>.

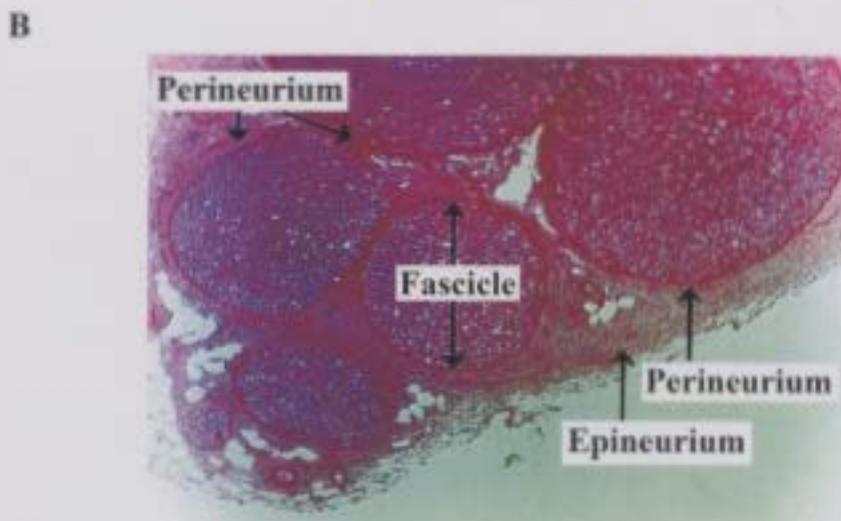
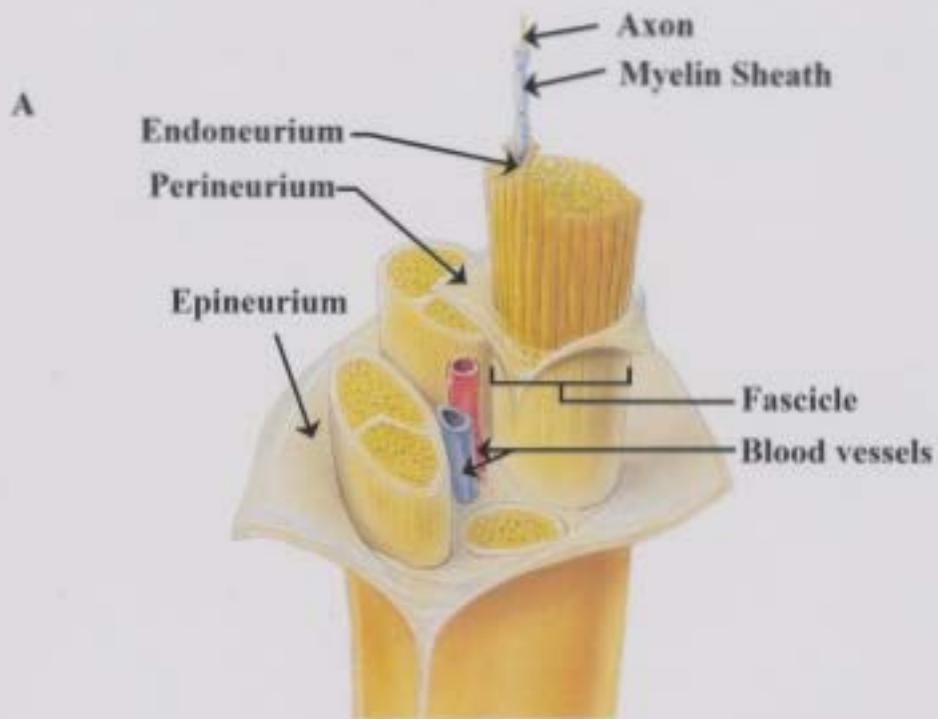


Fig 1.3

process centrally to form a synapse in the dorsal aspect of the spinal cord via the dorsal root entry zone, while the other process goes peripherally to the sensory receptors located in the skin, muscle, and visceral organs (Devor, 1999). Thus, the sensory message is carried from the periphery to the CNS via an extremely long axon that essentially bypasses the cell body, which makes up less than 1% of the cell's cytoplasm, and acts to supply protein and energy to the enormous length of axon (Devor, 1999).

The adult rat DRG is made up of a heterogeneous population of cells subdivided on the basis of neurochemistry, morphology, trophic requirements, and sensory modalities (Averill et al., 1995; Gavazzi et al., 1999; Ishikawa et al., 2005; Petruska et al., 2000; Priestley et al., 2002a). Three major cell groups have been identified with potential for further subclassification. To aid in the explanation of the different cellular subpopulations within the DRG, a schematic illustration adapted from Priestley and colleagues has been included (Fig 1.4). As shown in Fig 1.4, the first group (denoted in red) contains roughly 30-40% of the cells within the DRG. This population comprises the large and medium diameter neurons, and can be identified based on their expression of the heavy chain neurofilament (NF200+ve). These neurons typically have large myelinated axons, and function as mechanoreceptors and proprioceptors that have the ability to rapidly conduct action potentials. With regards to receptor expression, the NF200+ve neurons express the p75 neurotrophin receptor and TrkC, although TrkA and TrkB receptors are also reported (Averill et al., 1995; Ishikawa et al., 2005; Priestley et al., 2002a). Therefore, these neurons are able to respond to NT-3 (binds TrkC), NGF (binds TrkA), and BDNF (binds TrkB). The next two major groups consist of mainly

Figure 1.4 Pie chart summarising the three major populations of neurons that are found in the DRG. Large diameter myelinated Proprioceptive and Mechanoreceptive neurons are NF200+ve; small and media diameter nociceptive peptidergic neurons are CGRP+ve; small diameter non-peptidergic nociceptive neurons bind the lectin *Griffonia Simplicifolia* IB4. This figure has been adapted from Priestley et. al. 2002.

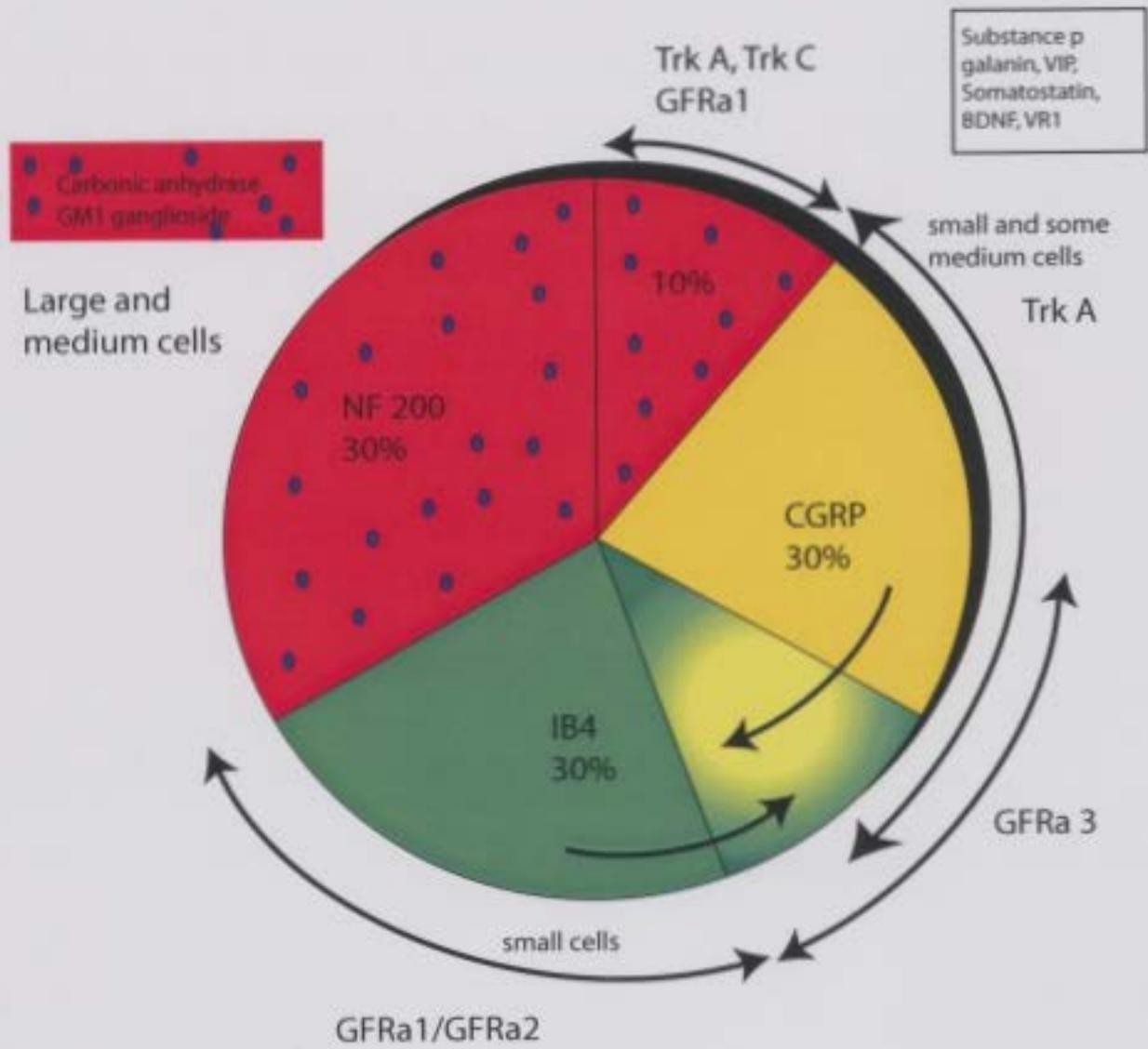


Fig 1.4

small diameter neurons and are considered to be either peptidergic, expressing calcitonin gene related peptide (CGRP+ve, denoted in yellow), or non-peptidergic, binding the lectin *Griffonia Simplicifolia* IB4 (denoted in green) (Averill et al., 1995; Ishikawa et al., 2005; Priestley et al., 2002a). These groups are considered to be unmyelinated or thinly myelinated nociceptive afferents. The CGRP+ve population contains roughly 30% of the cells within the DRG and also expresses a high level of both the p75 neurotrophin receptor and TrkA, thus these neurons preferentially respond to NGF (Averill et al., 1995; Ishikawa et al., 2005; Priestley et al., 2002a). The IB4-binding population also contains roughly 30% of the cells within the DRG. These neurons do not express either the p75 nor Trk receptors, but instead express the receptor tyrosine kinase RET and the GFR α 1 subunit, which makes these cells responsive to GDNF and unresponsive to the neurotrophins NGF, NT3, and BDNF (Bennett et al., 1998; Kashiba et al., 2001; Molliver et al., 1997).

The internal complexity of the DRG coupled with the fact that each neuron extends a single axon a great distance to its periphery gives the DRG the unique morphological properties that make it a great candidate for the study of axonal regeneration.

Another important property of adult DRG neurons that make them ideal for studying axonal regeneration is that, unlike neonatal DRG neurons that show a dependence on neurotrophic factors for their survival, postnatal DRG neurons are neurotrophin independent and are able to survive both *in vitro* and *in vivo* in the absence of exogenous trophic factors (Dodge *et al.*, 2002; Dodge *et al.*, 2006). This property allows neurotrophin-induced growth effects to be clearly distinguished from neurotrophin

induced survival effects. Studies that focus on regeneration in embryonic or neonatal neurons are always plagued by the fact that neurotrophins have to be present. Thus, the interaction between extracellular matrix molecules and neurotrophins on axon growth can be difficult to determine.

1.1.4 Classification of nerve injury

Functional recovery after axonal injury is limited, and in many cases, reliant on the type and severity of nerve injury. Seddon identified three major categories for the classification of peripheral nerve injury, which include neuropraxia, axonotmesis, and neurotmesis, brief descriptions of each of these injuries are as follows (Seddon, 1975).

Neuropraxia is the least severe type of nerve injury and is the result of a mild insult, such as local ischemia, which induces a block in impulse conduction. Although this type of injury can result in both motor and sensory loss, axonal continuity remains intact, and, Wallerian degeneration does not occur. Such pathology is usually the result of a myelin sheath injury and patients undergo full functional recovery within a relatively short period of time (Seddon, 1975).

Axonotmesis is a more severe condition in which axonal continuity is lost while the connective tissue, including the Schwann tubes, endoneurial, and perineurial structures, remain intact. This condition is commonly seen in crush/compression, stretch and percussion injuries, and results in a less favourable prognosis. Unlike neuropraxia where the axon remains intact, axonotmesis results in the proximal portion of the severed axon dying back to the node of Ranvier adjacent to the injury site, while the distal portion, due to the loss of cell body contact, undergoes Wallerian degeneration. For regeneration to occur, the axon has to leave the proximal stump and grow a substantial distance across

the site of injury back to the original target. In this situation, the remaining endoneurial tube provides a path for regenerating axons, and thus, functional recovery is possible and depends on the degree of internal disorganization of the nerve, severity of damage to the cell body, and the distance required for target innervation (Seddon, 1975).

Neurotmesis is the most severe type of nerve injury, resulting in the poorest prognosis. This type of injury occurs when there is a complete loss of continuity of the entire nerve and is usually the result of a laceration or severe nerve crush, whereby all axons and connective layers, including the perineurium and endoneurium, are severed. In this situation, spontaneous functional recovery is poor; not only does the nerve have to grow large distances to reach its target, but it has to do so without any guidance from the original connective structures (Seddon, 1975). Although functional recovery is rare after this type of insult, clinical attempts are still made to repair sites of injury and stimulate axonal regeneration. For instance, a variety of surgical techniques, including coaptation by epineurial suture, fascicular suture, and glue, have been used, to essentially bring the distal and proximal nerve stumps together while trying to reduce the extent of scar formation and provide a path for nerve growth (Chen, 2002). In many cases, the amount of damage inflicted by the injury is so severe that sections of the nerve are lost or have to be removed. In such a situation, coaptation without stretching and inflicting further damage to the proximal and distal aspects of the remaining nerve becomes impossible. Intervention in this case requires that a bridge be formed between the two nerve stumps. A variety of materials have been used to bridge the gap, including both natural biologically active materials, such as skeletal muscle, blood vessels, and autogenous nerve, and non-biological materials, such as silicone, nylon fiber, and polyurethanes

(Chen, 2002; Hall, 1997; Hall and Kent, 1996; Meek and Coert, 2002; Strauch, 2000). Both types of materials have their own benefits and shortcomings. For instance, the biological materials have good compatibility, and thus, a low rejection rate, but they tend to collapse and promote scar tissue proliferation, resulting in poor regeneration. The non-biological materials provide much better support and can be laden with growth promoting molecules like growth factors and extracellular matrix proteins, but, they are foreign objects and can be subject to rejection.

1.1.5 Models of injury and disease affecting the PNS

Peripheral neuropathy refers to an injury or degeneration of peripheral axon fibers. In general, peripheral neuropathies can be mononeuropathies, or a polyneuropathies (Marrs and Newton, 2003). Mononeuropathies affect a single nerve root or peripheral nerve fiber and are usually caused by a direct injury or insult to the axon. Polyneuropathies affect more than one peripheral nerve and are usually the result of a disease. Both of these types of nerve injury result in substantial disability and loss of function; and spontaneous full functional recovery is limited.

1.1.5.1 Physical nerve insults

Traumatic peripheral nerve injury is a relatively common occurrence, seen in upwards of 5% of the people admitted to trauma centers. Currently, one of the most common causes of peripheral nerve injury are motor vehicle accidents, although, many of the early clinical cases occurred during the first and second world wars (Noble et al., 1998; Woodhall, 1951). In many occurrences, peripheral nerve damage is the result of

fractures to adjacent bones; for instance, upper extremity nerve injuries such as radial, ulnar, and median nerve damage may be due to broken radial and/or ulnar bones, and similarly, femoral nerve injuries commonly result from fractures of the femur (Noble *et al.*, 1998). Although mononeuropathies of this nature result in substantial functional impairment, these types of physical injuries are asymmetric, affecting one body area only. For instance, a severed right radial nerve would result in a partial loss of function of the right hand. As suggested previously, regeneration and functional recovery after physical nerve injury is dependent on the severity of the nerve injury. For example, in severe crush/transection injuries, where no real organized distal stump remains, the connective tissue and immature nerve fibers form a neuroma, which is a disorganized bundle of fibers that prevents any further regeneration (Young, 1948).

1.1.5.2 Diabetes

Diabetes is a disease that results from either a deficiency of insulin or an insulin receptor malfunction. In either case, glucose accumulates in the extracellular fluid and exerts negative effects on a number of sensitive cell populations. Alterations in glucose levels induce diabetic polyneuropathies, which are the most common complication, and in many cases, the first indicator, associated with diabetes (Feldman *et al.*, 1997; Polydefkis *et al.*, 2004). Unlike physical nerve injury, diabetic polyneuropathies are symmetric and readily affect the sensory nerves of the DRG. DRG neurons are especially susceptible to glycemic regulation because the cell body and long peripheral axons are located outside of the protective blood nerve barrier (Arvidson, 1979). Hyperglycemia associated with diabetes induces a variety of functional and structural

abnormalities in these cells, including decreased axonal caliber and conduction velocity, dying back and dwindling of axons, disruption of the cell membrane, and possible cell death. Many of these abnormalities are associated with alterations in structural proteins such as tubulin and neurofilament (Pierson et al., 2002; Scott et al., 1999; Yagihashi et al., 1990). These changes may lead to a loss of sensation for pressure, temperature, and vibration. Eventually reflexes carried by large sensory neurons are diminished, and the patient can experience pain or numbness (Feldman *et al.*, 1999). A major complication associated with this disease has been termed the diabetic foot. The diabetic foot is a condition where the loss of pain sensation, through disruption of distal sensory contacts, predisposes diabetics to foot ulcerations. Foot ulcers go undetected for extended periods of time, and failing to heal, they become gangrenous and result in foot amputation, or potentially patient death (Le Quesne and Fowler, 1986).

Although peripheral neuropathies are a major problem associated with diabetes, little effort has gone into promoting regeneration of injured axons (McHugh and McHugh, 2004). This is a concern as many environmental alterations associated with diabetes have been reported. As suggested in section 1.1.2, in the normal PNS, injury results in rapid macrophage infiltration and phagocytosis of the inhibitory myelin sheath. Schwann cells take on a proliferative nature forming guidance tubes for regenerating axons and increasing their expression of permissive extracellular matrix molecules, which help to produce a new basement membrane. In diabetic nerves, these regeneration promoting processes are delayed or interrupted, producing an environment that more closely resembles that of the CNS (Cheng and Zochodne, 2002; Kennedy and Zochodne, 2000; Levy et al., 2001). For example, delayed macrophage infiltration results in the

prolonged presence of inhibitory myelin proteins (Kennedy and Zochodne, 2000; Levy et al., 2001), and the failure of Schwann cells to migrate out of the distal stump results in poor basement membrane production and the absence of bands of Bungner (Cheng and Zochodne, 2002). These events make the normal permissive environment of the PNS inhibitory, resulting in poor axonal regeneration and prolonged degeneration.

In addition to the altered immune and Schwann cell responses, diabetes also produces alterations in the expression of both growth factor molecules and their corresponding cell surface receptors. For instance, decreased NGF production, as well as reduced Trk A expression and NGF retrograde signalling have been found in DRG neurons and their targets in diabetic models (Brewster et al., 1994; Hellweg and Hartung, 1990; Jakobsen et al., 1981; Pierson et al., 2003). An increase in p75 expression, which has been reported to stimulate sensory neuron cell death, has been found in diabetic peripheral nerves (Jiang and Jakobsen, 2004; Scarpini et al., 1996). Alterations in neurotrophin signalling and expression may contribute to the impaired nerve function associated with diabetes, as it has been shown that NGF treatment has the potential to reverse diabetes-related nerve alterations (Unger *et al.*, 1998).

These findings indicate that the extracellular environment is critically important in peripheral nerve regeneration regardless of whether the loss of function comes from direct physical insult or disease.

1.1.6 Models for the study of axonal regeneration in the PNS

A variety of different models, both *in vivo* and *in vitro*, have been utilized to study the processes involved in axonal regeneration. Each of these model systems has its

advantages and disadvantages. For my studies, I have chosen to use an *in vitro* model to study aspects related to sensory nerve regeneration. I will briefly discuss the widely used nerve crush model for the study of axonal regeneration and explain why I have chosen to use dissociated DRG neurons instead.

1.1.6.1 *In vivo* vs. *in vitro*

Nerve crush is one of the most commonly used *in vivo* models for the study of axonal regeneration. In the use of this model, animals are anaesthetized, the nerve of interest is exposed, and a mechanical crush is performed. The crush induces axotomy and stimulates Wallerian degeneration and die back of the distal and proximal axons respectively. This allows the researcher to study a number of different experimental questions. For instance, early studies by Ramon Cajal that expanded on Waller's original work utilized nerve crush to identify fine details associated with Wallerian degeneration (Stoll et al., 2002). Years later, this technique is still being used to study the effects of axotomy on gene expression in both neurons and support cells, in an attempt to understand regenerative conditions. For example, axotomy has recently been shown to stimulate the upregulation of the cell surface adhesion receptors, or integrins, in the proximal axon (Wallquist *et al.*, 2004). So why have I chosen to use an *in vitro* cell culture model to study the aspects of peripheral nerve regeneration?

The research that I have conducted has focused on the effects of environmental factors on sensory nerve regeneration. Thus, it made more sense to be able to study individual cells in isolation without confounding extraneous variables found in the surrounding peripheral nerve. By removing the non-neuronal cells and all of the support

structures found in the dorsal root ganglion, I was able to exert precise experimental control by adding the factors of interest to the neurons one at a time while looking for potential interactions. This method of study also allowed me to investigate fine details at the cellular level associated with sensory axon regeneration.

I realize that *in vitro* models such as this are not the same as the nerve crush models of real physiological conditions, as the peripheral environment is made up of more than just one or two components, in a three dimensional space. However, valuable data that would be difficult to collect using *in vivo* methods can be obtained, and applied to *in vivo* models of regeneration using *in vitro* models.

1.1.7 Growth factors and their role in neurite growth

Trophic factors are well known mediators of differentiation, survival, and growth of both CNS and PNS neurons during development and regeneration (Airaksinen and Saarma, 2002; Allen and Dawbarn, 2006; Chao et al., 2006; Kaplan and Miller, 2000; Lu et al., 2005; Markus et al., 2002a; Snider et al., 2002). Of the various trophic factors available, I will discuss members of two distinct families that are critically important to DRG neurons. The first group consists of the classical neurotrophins NGF, BDNF, Neurotrophin-4/5 (NT4/5), and Neurotrophin-3 (NT3), while the second group is closely related to members of the transforming growth factor β superfamily and includes GDNF, neurturin (NTN), persephin (PSP) and artemin (ART). Both groups of molecules are secreted, soluble factors that have a noncovalent homodimer structure consisting of two pairs of non-parallel β subunits (McDonald and Blundell, 1991; McDonald and Chao, 1995; McDonald et al., 1990; McDonald et al., 1991; Yano and Chao, 2000). Thus,

trophic factors are very similar in structure and differ mainly by amino acid sequences found in three distinct loops that join the β strands (McDonald and Chao, 1995). Of these trophic factors, I will first discuss the neurotrophin NGF, its receptors, and subsequent signalling mechanisms, followed by GDNF, its receptors and subsequent signalling mechanisms (the populations of DRG neurons that do not respond to NGF, express the receptors for and respond to GDNF).

1.1.7.1 NGF and its receptors

NGF was the first member of the neurotrophin family to be identified and has become the prototypical member of the neurotrophins. This molecule was first discovered by Rita Levi-Montalcini and Viktor Hamburger in the early 1950s as a stimulant of survival and differentiation in embryonic sensory and sympathetic neurons (Aloe, 2004; Cowan, 2001; Hamburger, 1993; Levi-Montalcini et al., 1995). As suggested previously, NGF exerts its effects by binding to two separate classes of receptors, the low affinity p75 neurotrophin receptor (p75NTR) and the high affinity tropomyosin receptor kinase A (TrkA). The p75NTR receptor is a member of the tumor necrosis factor family and has the ability to bind all neurotrophins with the same affinity (Yano and Chao, 2000). As noted above, p75NTR functions as a component of the Nogo, Lingo receptor complex and aids in the activation of RhoA and subsequent inhibition of neurite growth following growth cone binding to inhibitory myelin molecules. Aside from this function, p75NTR also acts as a co-receptor for Trk, altering the binding kinetics of NGF (Hempstead et al., 1991; Mahadeo et al., 1994; Yano and Chao, 2000). For instance, the use of I^{125} -NGF indicates that binding to, and release of

NGF from TrkA happens at a much slower rate when p75NTR is absent than when p75NTR is present (Mahadeo *et al.*, 1994). Similarly, removal of the p75NTR receptor decreases the sensitivity of sensory neurons to NGF (Lee *et al.*, 1994). The signalling components downstream of p75NTR following NGF binding are essentially the same as those found following myelin binding. However, instead of stimulating the activation of RhoA and inhibiting neurite growth, NGF binding inhibits RhoA activation, which acts to enhance neurite growth (Blochl *et al.*, 2004; Gallo and Letourneau, 2004). Thus, p75NTR can act as both a positive and negative regulator of neuronal regeneration.

Unlike the p75 receptor that binds all neurotrophins with the same low affinity, Trk receptors act by binding with a high affinity to specific members of the neurotrophin family. For instance, TrkA preferentially binds NGF, TrkB preferentially binds BDNF and NT4/5, and TrkC preferentially binds NT3. As shown in Fig 1.5 three major signalling pathways stimulated by NGF binding to TrkA have been identified, these pathways consist of the PI 3-K/Akt, Ras/MAPK, and PLC- γ signalling intermediates. Upon NGF binding, TrkA dimerizes and autophosphorylates its cytoplasmic tyrosine residues Y490 and Y785. The phosphorylation of these residues stimulates the recruitment and binding of adaptor proteins, which in turn, activate downstream

Figure 1.5. Schematic illustration summarizing proposed signalling intermediates involved in NGF induce neurite growth of adult DRG neurons.

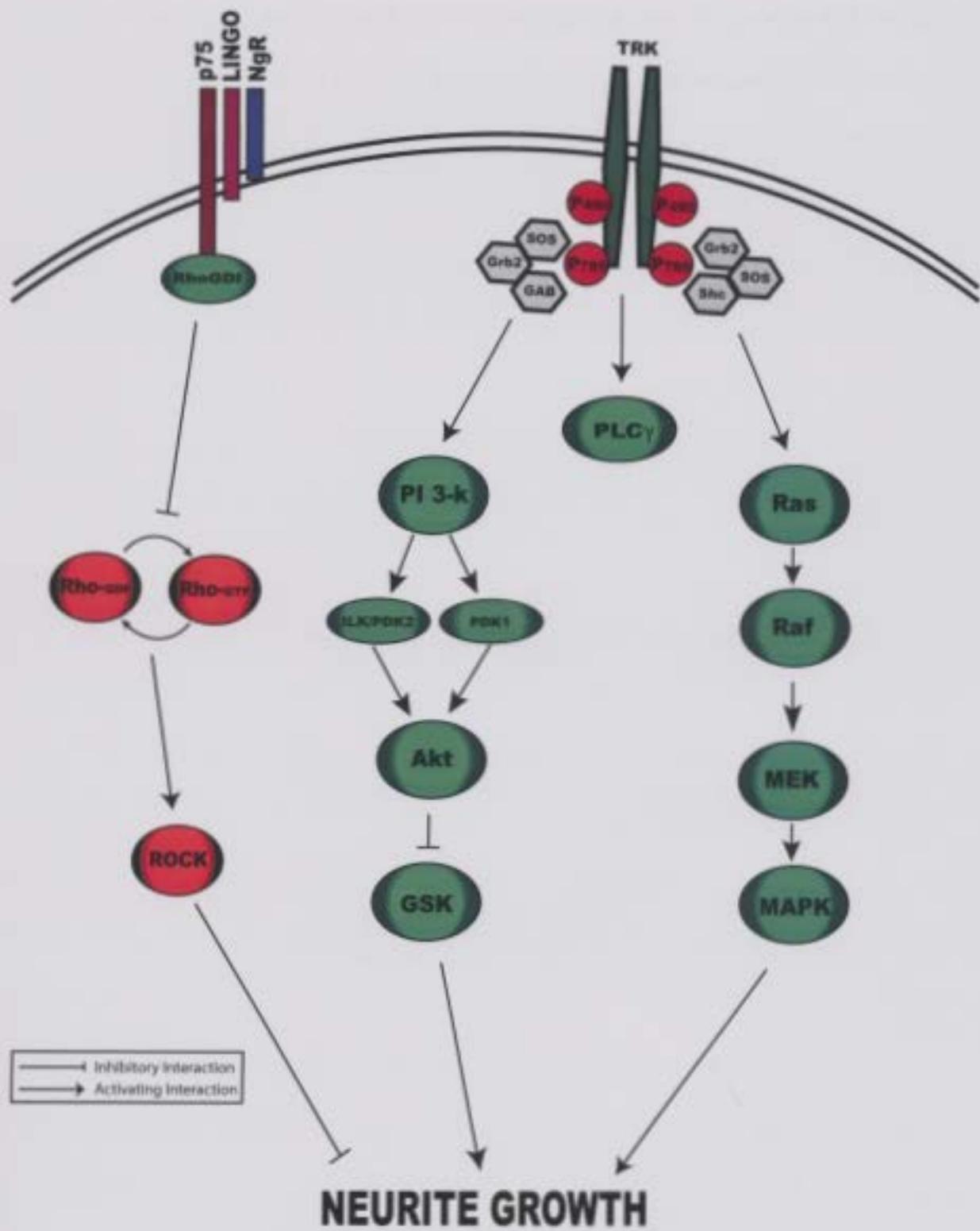


Fig 1.5

signalling intermediates (Cunningham and Greene, 1998; Kaplan and Miller, 1997; Kaplan and Miller, 2000; Markus et al., 2002a; Snider et al., 2002; Yano and Chao, 2000). Phosphorylation of Y490 stimulates the activation of both Ras/MAPK and PI 3-K/Akt signalling pathways, whereas phosphorylation of Y785 activates PLC- γ , which in turn, stimulates PKC activation (Kaplan and Miller, 1997; Kaplan and Miller, 2000). Stimulation of these pathways has been shown to play significant roles in cellular differentiation, survival and neurite outgrowth in a variety of different cell types. For example, inhibition of the Ras/MAPK pathway blocks NGF-induced differentiation in PC12 cells, while inhibition of the PI 3-K/Akt pathway has a negative impact on cell survival (Kaplan and Miller, 2000; Kim et al., 2004; Klesse et al., 1999). Conversely, activation of the PI 3-K/Akt signalling pathway in these cells has been shown to inhibit neurite branch formation and aid in neurite elongation (Higuchi *et al.*, 2003). Similarly, neurite growth stimulated by the addition of NGF is dependent on the PI 3-K/Akt signalling pathway in both DRG and sympathetic neurons, and the inhibition of either of PI 3-K or Akt has a negative effect on axonal regeneration (Atwal et al., 2000; Edstrom and Ekstrom, 2003; Jones et al., 2003; Kimpinski and Mearow, 2001; Markus et al., 2002b; Zhou et al., 2004).

Although the above signalling intermediates have been identified as important components for axonal regeneration, how these pathways actually regulate axon growth is not well understood.

1.1.7.2 GDNF and its receptors

GDNF was the first member of the GDNF family of trophic factors to be discovered and has been identified as a molecule that could promote survival in a variety of cell types including PC12 cells, mesencephalic dopaminergic neurons, motor neurons, sympathetic neurons, locus coeruleus noradrenergic neurons, and subpopulations of sensory neurons (Airaksinen and Saarma, 2002; Arenas et al., 1995; Couplier and Ibanez, 2004; Henderson et al., 1994; Lin et al., 1993; Oppenheim et al., 1995; Park et al., 2005). Shortly after the discovery of GDNF, the other family members NTN, PSP and ART were identified. Like NGF, the GDNF family of trophic factors exerts its actions by binding to two separate cell surface receptors. The first receptor GFR- α , like Trk, is the high affinity growth factor receptor responsible for ligand specificity (Airaksinen and Saarma, 2002). For instance, although some overlap exists, GFR- α 1 preferentially binds GDNF, GFR- α 2 preferentially binds NTN, GFR- α 3 preferentially binds PSP, and GFR- α 4 preferentially binds ART. Although binding to the GFR- α receptor alone has been shown to stimulate internal signalling cascades, it is commonly accepted that this GPI linked receptor signals via activation of the second GDNF receptor, RET (Durbec *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996). Like Trk, RET is a transmembrane tyrosine kinase receptor, which upon activation, has the ability to stimulate internal signalling cascades (Sariola and Saarma, 2003). Signalling pathways are activated by initial GDNF binding to the GFR- α 1 receptor, which stimulates the recruitment and dimerization of two RET receptor subunits, the combination of which stimulates transphosphorylation of specific tyrosine residues on the receptors' cytoplasmic tails

(Airaksinen and Saarma, 2002). GDNF induced phosphorylation of the cytoplasmic tyrosine residues on RET stimulates the recruitment of specific adaptor proteins, which in turn, activate specific signalling cascades. As shown in Fig 1.6, Like NGF, RET phosphorylation has the ability to activate the MEK/MAPK, PI 3-K/Akt and PLC- γ pathways (Sariola and Saarma, 2003). For instance, phosphorylation of Y1015 stimulates the activation of PLC- γ (Borrello *et al.*, 1996; Trupp *et al.*, 1999), phosphorylation of Y1062 stimulates the activation of MEK/MAPK (Trupp *et al.*, 1999), and phosphorylation of Y1096 stimulates the activation of PI 3-K/AKT (Soler *et al.*, 1999; Trupp *et al.*, 1999). Aside from stimulating survival in various cell types, activation of these signalling intermediates via GDNF can also stimulate neurite growth. For instance, GDNF-induced activation of PI 3-K, but not MAPK, is required for lamellopodia formation and process extension in both neuroepithelioma SK-N-MC cells and enteric neuroblasts (Maeda *et al.*, 2004; Srinivasan *et al.*, 2005; van Weering and Bos, 1997). GDNF-induced activation of MAPK, but not PI 3-K, stimulates differentiation and neurite outgrowth in PC12 cells (Creedon *et al.*, 1997; Park *et al.*, 2005; Xing *et al.*, 1998). However, in sympathetic neurons GDNF has been shown to stimulate neurite growth by activating both PI 3-K and MAPK signalling pathways (Coulpier and Ibanez, 2004). These results not only show the importance of the different signalling intermediates in neurite growth and cellular survival but they also illustrate that many of the results are cell type specific, and what is important in one cell type may not be important in another.

To date most of the studies on trophic factor-induced neurite growth have been

Figure 1.6. Schematic illustration summarizing proposed signalling intermediates involved in GDNF induced neurite growth of adult DRG neurons.

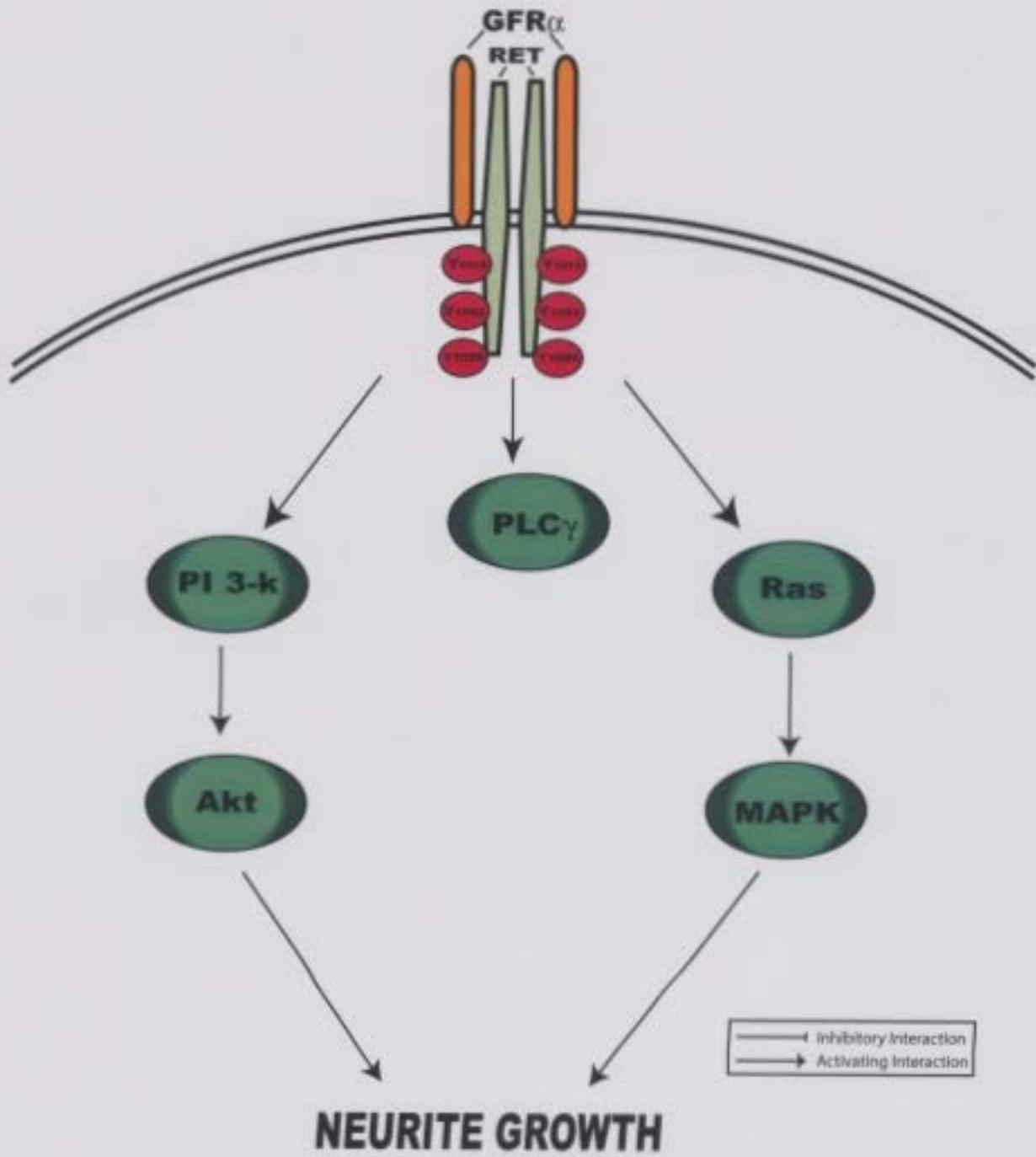


Fig 1.6

carried out using different cell types with cells cultured in the presence of extracellular matrix molecules, with the result that the influence of these molecules are often overlooked. Therefore, the complex interplay between neurotrophin and extracellular matrix receptors that lead to neurite growth are not well understood.

1.1.8 Extracellular matrix molecules and their role in neurite growth

The ability of a neuron to extend a process over a biological substrate requires there to be a direct interaction with an insoluble network of proteins called the extracellular matrix, or basement membrane. Depending on the different cellular locations, this matrix consists of a number of different molecules; for example, laminin, collagen, and fibronectin have all been identified in the PNS (Guo and Giancotti, 2004). Extracellular matrix molecules (ECM) exert their effect by binding to a class of cell surface adhesion receptors called integrins (Aplin et al., 1999; Giancotti and Ruoslahti, 1999; Guo and Giancotti, 2004). Integrin receptors are abundant on the leading edge of growth cones peripheral domains, and on the leading edge of migrating cells. Here these receptors act as sensors for ECM molecules and stimulate internal signalling cascades upon binding. Of the extracellular matrix molecules, we are most interested in the effects of laminin and the corresponding integrin receptors on neurite growth. In early studies on basal lamina composition, it was found that laminin is the most highly expressed ECM molecule in the basement membrane (Martin and Timpl, 1987). This molecule is detected earlier than any other ECM molecule during development and persists into adulthood (Martin and Timpl, 1987). More recent studies have confirmed these results, for example, studies by Matesz et al. on the role extracellular matrix molecules in

nervous system regeneration, have found that laminin is highly expressed in the PNS of the common water frog *Rana Esculenta* and is upregulated after PNS injury (Matesz *et al.*, 2005). Although other ECM molecules are present at lower levels in the PNS, laminin has been shown to be a stronger promoter of neurite growth from DRG neurons than collagen or fibronectin (Gundersen, 1988).

As suggested previously, the lack of ECM proteins in the adult CNS provides a partial reason why little or no axonal regeneration occurs from CNS neurons after injury (Matesz *et al.*, 2005). The presence of these molecules has been shown to be able to overcome the inhibitory influences of CNS myelin and CSPGs on neurite growth (Condic *et al.*, 1999; David *et al.*, 1995). For example, recent studies by Laforest *et al.*, show that PC12 cells plated on MAG normally have little neurite growth, poor cellular adhesion, and little cell spreading, but, when laminin was added to these cultures the inhibitory MAG induced effects were overcome. By using specific integrin inhibitors, the authors found that these effects were integrin dependent and that a laminin specific integrin was involved (Laforest *et al.*, 2005).

The following section will provide a brief discussion of laminins, their structure, and corresponding function; I will then discuss the laminin-binding integrin receptors and known downstream signalling cascades.

1.1.8.1 Laminins

The ECM molecule laminin was initially isolated from basement membranes produced by mouse tumor cells called Engelbreth-Holm-Swarm or EHS cells (Timpl *et al.*, 1979). Individual laminin molecules are large trimeric proteins with α , β , and γ

subunits that predominantly bind to cell surface adhesion receptors called integrins (Luckenbill-Edds, 1997; Malinda and Kleinman, 1996). The three separate laminin subunits bind together to form a cross-like structure and each domain has been suggested to serve specific cellular functions, such as attachment, migration, and neurite growth and elongation (Luckenbill-Edds, 1997). At least 10 different laminin isoforms have been identified, which are expressed in various regions of the mammalian body and by different cell types. These isoforms vary only by their expression of α , β , and γ subunits, and have been termed laminin-1 to laminin-15 (Colognato et al., 2005; Luckenbill-Edds, 1997).

In the PNS, laminin is a molecule that is synthesized and secreted by a variety of cell types. Schwann cells, being a major producer of laminin in the peripheral nerve, deposit these molecules within the basal lamina. This basal lamina and subsequently laminins have been shown to be involved in many different processes, and have been shown to function in different stages of development. For example, their function is required for proper neural crest cell migration (Previtali et al., 2001; Tarone et al., 2000) and in the attraction and stimulation of neurite growth (Luckenbill-Edds, 1997). Laminins have also been shown to play important roles in Schwann cell-induced myelination of PNS axons. In normal developmental and regenerative processes, myelinating Schwann cells are surrounded by a basal lamina rich in laminin-2, and laminin-8 which have both been shown to be required for normal myelin formation; disruption of Schwann cell production of either of these laminin isoforms results in defects in synthesis of myelin proteins and an abundance of unsorted, unmyelinated axons (Chen and Strickland, 2003; Oguievetskaia et al., 2005; Yang et al., 2005).

As suggested previously, laminin production is increased after PNS injury and is prominent during bands of Bungner formation, which are important for forming growth and guidance tubes for regenerating axons. Studies indicated that these molecules could play a positive role in neurite growth. The addition of an anti-laminin antibody prevents axon growth into peripheral basal lamina tubes of rat sciatic nerves (Wang *et al.*, 1992), while laminin availability is able to override the inhibitory influences of myelin on both CNS and PNS neurons (David *et al.*, 1995; Matesz *et al.*, 2005). As suggested above, the major laminin receptors are the integrins, and although these receptors have been shown to bind different laminin isoforms, laminin-1 is a predominant mediator of neurite outgrowth in the PNS (Luckenbill-Edds, 1997). Therefore, in my studies this was the main laminin isoform that was used.

1.1.8.2 Integrin receptors

Growth cone attachment to laminin and subsequent activation of internal signalling cascades, are mediated through integrins. Integrins are a class of heterodimeric transmembrane cell surface adhesion receptors that are composed of α and β subunits, the cytoplasmic tails of which are short and have no intrinsic enzymatic activity (Giancotti, 2003). To date, 18 α and 8 β mammalian subunits have been identified, and different combinations of these subunits allow for the creation of at least 24 different integrin receptors (Guo and Giancotti, 2004). Each of these receptors have been shown to bind different ECM molecules with partial overlap between ligands, and most integrins recognize more than one ECM molecule (Giancotti, 2003; Giancotti and Ruoslahti, 1999; Hynes, 2002). For instance, $\alpha 1\beta 1$ binds both laminin and collagen; however, laminin

also acts as a ligand for $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7\beta 1$ (Archelos et al., 1999; Hynes, 1992; Previtali et al., 2001). Much of the work performed on integrin receptors has focused on their role in cell migration, angiogenesis, wound healing, and metastasis (Caswell and Norman, 2006; Eble and Haier, 2006; Lenz, 2005; Sato, 2001; Serini et al., 2006). However, the importance of these receptors in growth and repair of PNS neurons has also been identified (Previtali *et al.*, 2001). As with laminin, integrin receptors have been shown to be upregulated after injury to PNS neurons. For example, upon injury of the peripheral processes of the DRG, the laminin-associated integrin receptors $\alpha 6\beta 1$ and $\alpha 7\beta 1$ are upregulated, and this has been suggested to play an important role in regeneration after PNS injury (Wallquist *et al.*, 2004). Similarly it has been shown that DRG neurons receiving a preconditioning lesion prior to severe injury have significantly better regeneration in the presence of laminin than neurons that did not receive a preconditioning lesion. This effect was suggested to be due to an upregulation of the $\alpha 7\beta 1$ integrin receptor after the initial injury, and inhibition of this receptor was shown to inhibit the precondition growth effect (Ekstrom *et al.*, 2003).

Although integrins have short cytoplasmic tails with no intrinsic enzymatic activity, they have been shown to function by signalling across the membrane in two distinct directions: via “inside-out” signalling, molecular intermediates within the cell act to alter receptor affinity and activation state by altering the conformation of its cytoplasmic tails. In “outside-in” signalling, ligand binding activates the integrin receptor which stimulates a conformational change within the extracellular component of the receptor, which in turn mobilizes complexes of molecules that transmit a variety of intracellular signals (Aplin et al., 1999; Clark and Brugge, 1995; Clark and Hynes, 1997;

Giancotti, 2003; Giancotti and Ruoslahti, 1999; Ginsberg et al., 2005; Guo and Giancotti, 2004; Schwartz et al., 1995; Travis et al., 2003).

As I am most interested in the effects of laminin binding on axonal regeneration, I will focus my discussion on the “outside-in” signalling processes involved in integrin activation. The mechanisms involved in “outside-in” signalling have been most widely studied in non-neuronal cells and are associated with proliferation, survival, and migration of different cell types. Integrin binding to the ECM has been shown to stimulate a conformational change in the cytoplasmic tail of the receptor, such that the tails straighten and stimulate the recruitment of adaptor proteins such as talin and vinculin. These proteins then act to link integrins to the actin cytoskeleton and stimulate the recruitment of a number of different cytoplasmic protein kinases (Giancotti, 2003; Giancotti and Ruoslahti, 1999). Coupled with ligand-induced integrin clustering these events stimulate the formation of aggregates at the cell surface called focal adhesions (Giancotti, 2003; Giancotti and Ruoslahti, 1999). The kinases associated with these focal adhesions include focal adhesion kinase (FAK), integrin-linked kinase (ILK), and the Src-family kinases (such as Src, Fyn, and Yes) (Chen et al., 1996; Guo and Giancotti, 2004; Hannigan et al., 1996; Howe et al., 1998; Kornberg et al., 1992; Schaller and Parsons, 1994; Schlaepfer and Hunter, 1998; Wary et al., 1996; Wary et al., 1998). Activation of these early integrin-linked intermediates has also been suggested to activate the downstream signalling components Ras, MAPK, and PI 3-K, all of which have been shown to be activated by growth factor receptors in different cell types (Giancotti and Ruoslahti, 1999; Guo and Giancotti, 2004)

The processes associated with focal adhesion formation and actin dynamics are regulated by the Rho family of small GTPases. As suggested earlier this family of proteins plays an essential role in growth cone remodeling and axonal extension, and thus these intermediates are potentially very important in axon growth during regeneration (Brunton *et al.*, 2004; Wiesner *et al.*, 2005).

For both neurotrophin and integrin signalling, I have suggested that the Rho family of small GTPases are important for axon growth, but the complex interactions between the potential activators and effectors within the cell are poorly understood. For example, it has been suggested that activation of the PI 3-K/Akt pathway is important for NGF- induced neurite growth in adult DRG neurons (Jones *et al.*, 2003; Kimpinski *et al.*, 1997; Kimpinski and Mearow, 2001), yet how this is linked to growth cone remodeling and axon extension is poorly understood. Similarly the connection between integrin-activated signalling events and axon growth are even more elusive. As noted previously, much of the work investigating integrin-mediated signalling has been performed in non-neuronal cells. This is important because many of the above mentioned signalling intermediates have been suggested to be cell type and behavior dependent (Brunton *et al.*, 2004; French-Constant and Colognato, 2004). The potential role of integrins in regeneration of sensory neuron axons and the complex interactions between integrin- and growth factor-activated signalling pathways responsible for axonal regeneration in adult sensory neurons are not well understood.

To summarize the signalling cascades that were suggested to be downstream of integrin activation at the time that I started these studies, I have included a schematic diagram as an illustration of the possible chain of events (Fig 1.7).

Figure 1.7. Schematic illustration summarizing proposed signalling intermediates involved in integrin induced neurite growth of adult DRG neurons.

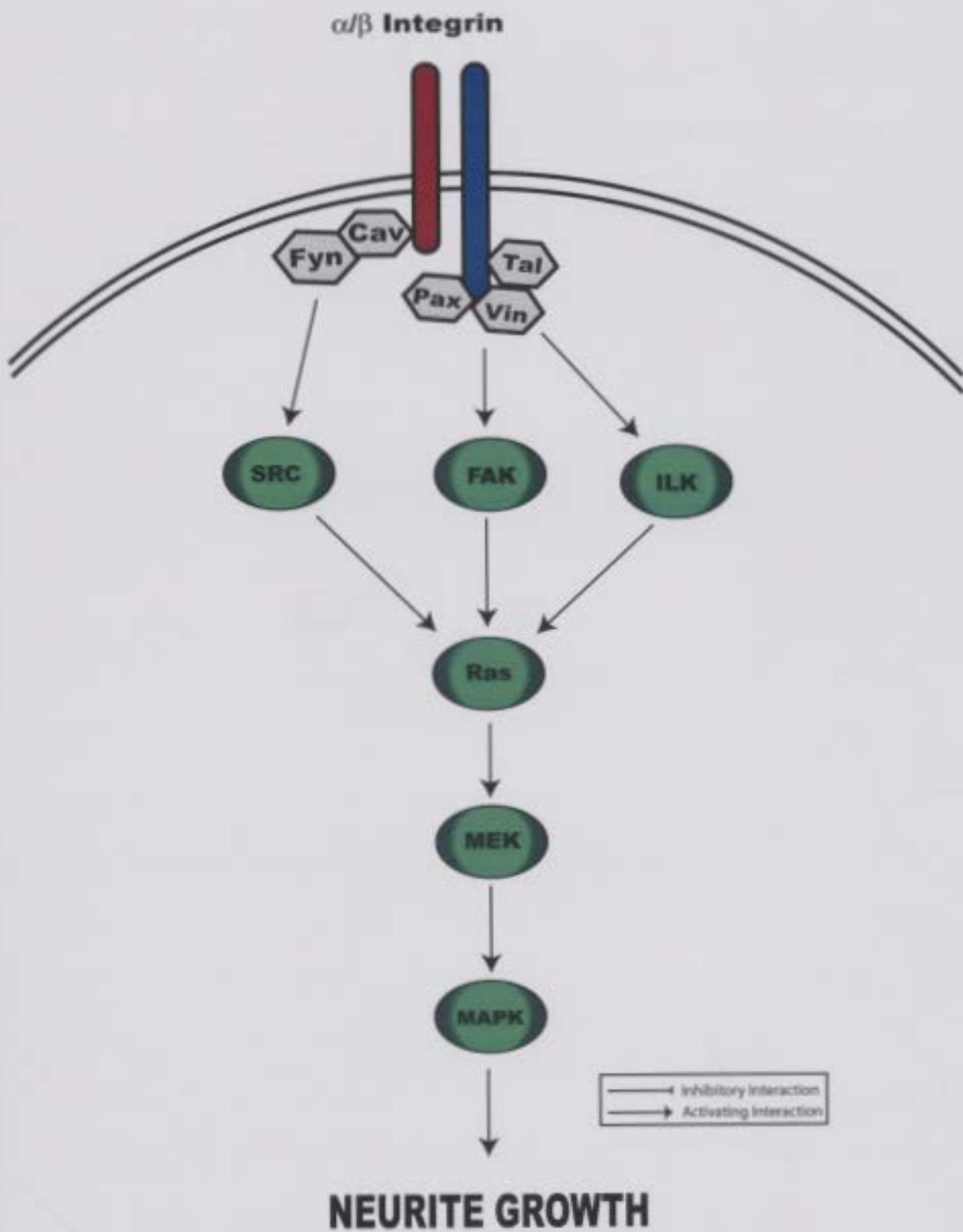


Fig 1.7

1.2 Hypothesis and objectives

The main objective of my thesis was to investigate the mechanisms involved in the regulation of neurite growth of adult sensory DRG neurons. My focus was on the effects of environmental factors, specifically laminin and NGF, on axonal regeneration. Although it is becoming clear that laminin and subsequent integrin binding are important processes for axon growth and regeneration, when I began my experiments very little information with regards to integrin signalling in neurons was available. At the time, it had been suggested that various intermediates in the NGF-induced Trk activated signalling pathways were important for neurite growth; however, most of the *in vitro* studies had been performed with cells plated on various extracellular matrix molecules such as laminin, and thus the potential interactions and dependence on integrin-induced intermediates had been overlooked. Therefore, I became interested in the interactions between laminin and NGF signalling intermediates responsible for axonal regeneration within the peripheral sensory nervous system. In this study, it was hypothesized that the activation of laminin-induced signalling intermediates was sufficient to promote significant neurite growth from adult rat DRG neurons in the absence of exogenous NGF, and that the addition of NGF acted to potentiate laminin-induced signalling, stimulating a level of neurite growth not achievable by the presence of either factor alone. Thus, integrin-activated signalling pathways could synergize with NGF-activated signalling pathways to stimulate optimal levels of axonal regeneration in peripheral sensory DRG neurons.

To study this hypothesis, three specific objectives were developed.

Objective 1: Test whether integrin activation via binding the ECM molecule laminin could stimulate significant neurite growth from adult rat DRG neurons in the absence of added neurotrophins. In initial experiments, dissociated adult DRG neurons were cultured on substrates coated with either poly-D-lysine (a neutral non-integrin binding molecule) or laminin (an ECM molecule that binds and activates integrin receptors), in the presence or absence of added NGF. Using a camera lucida imaging unit and corresponding software, the quantity and pattern of neurite growth were assessed. To ensure that growth results were due to the engagement of the integrins, these experiments were performed in the presence and absence of integrin blocking peptides and antibodies (Chapter 2).

Objective 2: To characterize the signalling pathways stimulated by laminin-induced integrin activation and NGF-induced Trk activation responsible for neurite growth in adult rat DRG neurons. To study the signalling events responsible for neurite growth, dissociated DRG neurons were plated on either poly-D-lysine, or laminin \pm added NGF, \pm pathway specific pharmacological inhibitors. At 24 hr, cells were harvested, lysed, and Western blot analyses were performed. These experiments were initially performed in a heterogeneous population of cells (Chapter 2). However, differential growth responses were observed in different neurons, so a similar series of experiments was performed on specific cell populations (Chapter 4, and 5). To carry out these experiments, I developed a cell isolation technique that utilized magnetic beads to select, release, and plate homogeneous populations of neurons (Chapter 3).

Objective 3: To identify key points of collaboration between laminin-induced signalling pathways and NGF-induced signalling pathways responsible for optimal

levels of neurite growth in adult rat DRG neurons. To identify potential points of collaboration between integrin and Trk induced signalling pathways responsible for neurite growth, homogeneous populations of dissociated DRG neurons were plated on poly-D-lysine \pm pathway specific pharmacological inhibitors for 2 h prior to treatment with media containing NGF, laminin, or laminin plus NGF. Neurons were subsequently harvested and lysed at 10 min, 1 hr, or 6 hrs after treatment, and Western blot analyses were performed (Chapter 5).

Co-authorship statement

I, Budd Tucker, am the principle author for all manuscripts that are contained within this thesis (chapters 2-5). However, each of these chapters has been co-authored by my supervisor Dr. K. M. Mearow, and her research assistant Mrs. M. Rahimtula. The contributions from both Dr. Mearow and Mrs. Rahimtula have been invaluable in the completion of this work. The specific contribution of each author to each manuscript is described below. Chapters 2, 3, and 4 are published manuscripts, while the manuscript in chapter 5 is in preparation.

Chapter 2, “Integrin activation and neurotrophin signalling cooperate to enhance neurite outgrowth in sensory neurons”. As the principle author, I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Mrs. Rahimtula provided extensive amounts of technical help including animal dissection and preparation of chemical compounds. Dr. Mearow provided extensive amounts of help with the experimental design and writing of the first to final drafts of this manuscript (Tucker et al., 2005a).

Chapter 3, “A procedure for selecting and culturing sub-populations of neurons from rat dorsal root ganglia using magnetic beads”. As the principle author I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Mrs. Rahimtula provided extensive amounts of technical help including animal dissection and preparation of chemical compounds. Dr. Mearow

participated in the experimental design, trouble-shooting, correction and improvement of this manuscript (Tucker et al., 2005b).

Chapter 4, “Laminin and growth factor receptor activation stimulates differential growth responses in sub-populations of adult DRG neurons”. As the principle author, I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Mrs. Rahimtula provided extensive amounts of technical help including animal dissection and preparation of chemical compounds. Dr. Mearow participated in the experimental design, correction and improvement of this manuscript (Tucker, 2006, in press).

Chapter 5, “Identification of Src as the key intermediate between integrin and Trk activated signalling pathways in the stimulation of neurite growth in NGF-Responsive DRG neurons”. As the principle author I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Mrs. Rahimtula provided extensive amounts of technical help including animal dissection and preparation of chemical compounds. Dr. Mearow participated in the experimental design, correction and improvement of this manuscript.

CHAPTER 2

INTEGRIN ACTIVATION AND NEUROTROPHIN SIGNALLING COOPERATE TO ENHANCE NEURITE OUTGROWTH IN SENSORY NEURONS

(Published in the Journal of Comparative Neurology, 2005, 486(3):267-80)

2.1 Introduction

We know that various elements can influence and promote regeneration of peripheral axons. In addition to soluble factors (neurotrophins, cytokines and other growth factors), the extracellular environment in which growth occurs is critically important. In the CNS, axonal regeneration does not occur to any great extent, and while this is due to a number of factors, the most prominent is a non-permissive growth environment as well as an unavailability of appropriate growth-promoting factors. In the PNS, on the other hand, peripheral axons (both motor and sensory) generally regenerate quite well relative to CNS axons. We have recently characterized signalling pathways activated by NGF and IGF-1 in promoting neuronal survival and neurite growth from adult DRG sensory neurons *in vitro* (Dodge et al., 2002; Jones et al., 2003; Kimpinski and Mearow, 2001). While our results show that the provision of both NGF and IGF supports the most extensive growth, this enhancement is dependent upon the availability of a permissive culture substrate.

In the PNS, components of the basal lamina, such as laminin, collagen IV and entactin are well known to promote neurite growth both *in vivo* and *in vitro*. In this regard, the integrins play a key role, as they are the cell surface receptors for ECM

molecules such as laminin. The integrin receptors consist of heterodimers of α and β subunits, the cytoplasmic tails of which are short and have no intrinsic enzymatic activity (Giancotti and Ruoslahti, 1999; Howe et al., 1998). Different combinations of these receptors bind to different substrate molecules; for instance, integrin $\alpha1\beta1$ binds to specific domains of the laminin molecule and has been shown to mediate the binding of neurons and neuronal cell lines to laminin (Tomaselli *et al.*, 1993). Embryonic and neonatal chick and rat DRG neurons are reported to express high levels of $\alpha1\beta1$ and $\alpha3\beta1$, and lower levels of $\alpha5\beta1$ and $\alpha6\beta1$ (Condic, 2001; Condic and Letourneau, 1997; Lefcort et al., 1992; Tomaselli et al., 1993; Toyota et al., 1990; Yanagida et al., 1999). Similarly, adult DRG neurons and their axons express integrins that allow for interaction with ECM and basal lamina molecules like laminin, collagen and fibronectin, although the level of expression is reported to be much lower than in developing neurons (Condic, 2001; Condic and Letourneau, 1997).

Integrin signalling mechanisms have been most widely studied in non-neuronal cells, where it has been shown that integrin-mediated events activate a number of cytoplasmic protein kinases, in particular FAK (focal adhesion kinase), ILK (integrin linked kinase), and the Src-family kinases (Aplin et al., 1998; Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). These intermediates are recruited to the sites of integrin-mediated cellular attachment along with other structural proteins such as paxillin, talin and vinculin to form focal adhesions, leading to morphological changes contributing to events such as cell spreading, survival and migration (Attwell et al., 2003; Ivankovic-Dikic et al., 2000; Park et al., 2000).

Our objective was to investigate how integrin-activated signalling pathways interact with growth factor-activated pathways, and to determine whether integrin signalling would be sufficient in the absence of NGF to promote neurite growth.

2.2 Materials and methods

2.2.1 Neuronal cultures

Dorsal root ganglia (DRG) from young adult (4-6 wk) Sprague-Dawley rats (Memorial University of Newfoundland Vivarium and Charles River Canada, Montreal, QC) were dissected and dissociated. In accordance with University Animal Care guidelines, animals were live decapitated, ganglia from all spinal levels were removed, and the roots were trimmed. To isolate individual DRG neurons, both enzymatic and mechanical dissociation was used. Enzymatic dissociation was carried out first and performed by incubating the ganglia in 0.25% collagenase for 45 min, and 0.25% trypsin for 40 min (Invitrogen/ Gibco BRL, Burlington, Ont). Mechanical dissociation was carried out next and performed by repeated cycles of aspiration and expulsion of the DRG fragments through a flame narrowed pasture pipette. Upon completion, dissociated DRG neurons were suspended in serum-free Neurobasal medium (NB, Invitrogen) supplemented with 100U penicillin/streptomycin, B27 supplement (Invitrogen), and 20mM cytosine arabinoside (modified NB). This suspension was then layered on top of a 30% Percoll solution (Amersham Bioscience, Baie d'Urfe, QC) in 15ml conical tubes and centrifuged at 400g for 20 min at room temperature. Pellets were then carefully extracted with a sterile pasture pipette, placed in a fresh 15ml tube, washed with the previous suspension media, and centrifuged to remove any remaining Percoll. Neurons were

plated in either Lab-Tek 16-well chamber slides (Nunc International, Naperville, NC) for neurite growth assessment or 12-well plates for Western blot analysis, and incubated at 37°C with 95% O₂ and 5% CO₂ for 24 and 48 hrs. Slides and culture plates were coated twice with poly-lysine (PL, 1 mg/ml, BD Bioscience, Bedford, MA) laminin, (LN, 50 mg/ml, Invitrogen) or Matrigel (MG, BD Bioscience; 1:100 dilution in ice-cold NB). Surface coating for MG was similar to that used for laminin, and not at all like the MG gels used for ganglionic explants (Tonge *et al.*, 1997). Coating with these extracellular matrix molecules resulted in a thin film of LN or MG on the plating surfaces. The neurons were cultured in modified serum-free NB alone or supplemented with NGF (25 ng/ml, Cedarlane Labs, Hornby, ON).

2.2.2 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde (pH 7-7.4) in PBS for 15 minutes, permeabilized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS. For neuronal and neuritic identification, TRITC-labeled Phalloidin (1:100, Sigma), and antibodies directed against peripherin were employed (1:250, Chemicon, Temucula, CA). For analyses of integrin signalling components, antibodies directed against β 1 (Chemicon AB 1952) and α 1 (Chemicon AB 1934) integrin subunits; pFAK (pY 397, Biosource Inc), ILK (Upstate Biotech Inc, Lake Placid, NY), and pAkt (Cell Signalling Technology, Beverly, MA) were employed. Cells were incubated with the primary antibodies at 4°C for 16-20 hrs, followed by Cy2- or Cy5-tagged secondary antibodies (Jackson Immunoresearch Labs, West Grove, PA). These slides were cover slipped with glycerol and imaged with confocal laser scanning microscopy. For neuronal tracing

analyses, HRP-tagged secondary antibodies were used followed by visualization with the ABC system and di amino-benzidine (DAB) as the developing agent (Vector Laboratories, Burlingame, CA).

2.2.3 Measurements of Neurite Growth

Neurite initiation (neuritogenesis) was first quantified by determining the percentage of total phase bright neurons with neurites twice the length of their cell bodies. Growth was assessed at both 24 and 48 hrs. Individual neuron tracings of DAB-stained neurons were carried out using the NeuroLucida® (MicroBrightField, VT) tracing program. Only neurons for which we were able to identify unambiguously the associated neurites or neuritic networks were chosen for tracing analysis. Data analyses were carried out with the NeuroExplorer® software package; here Scholl analysis (intersection point and length) and total neurite length data were collected. Scholl analysis measures both the number of intersection points of axons crossing 20 μ m concentric circles radiating from the cell body and the length of the neurites within each concentric circle, thus giving a measure of both elaboration and total growth of the neurites. Total neurite length is a measure of the cumulative length of neurites produced. Data were imported into a graphing and statistical analysis software program (Prism 3, GraphPad Corp) for further analyses. For each condition examined (control (PL) +NGF or -NGF, LM +NGF or -NGF, and MG + NGF or -NGF) 50 neurons were completely traced, with cells pooled from approximately 3-6 different plating experiments.

2.2.4 Integrin Blocking

Integrin activation was blocked by the use of a synthetic cyclic RGD peptide (Arg-Gly-Asp, 0.1 mg/ml, Chemicon), which had been added to culture media two hours after plating and replenished every 24 hrs where applicable. In some experiments a β 1-integrin blocking antibody (β 1i) (Chemicon) was used; results were similar to those obtained with the RGD peptide.

2.2.5 Immunoblotting

For Western analyses, neurons were plated in 12-well plates that had been coated with PL, LN (25 μ g/ml, Invitrogen) or MG in the presence or absence of NGF (25 ng/ml, Cedarlane Labs). Neurons were subsequently processed at 24 hrs after stimulation by removing the original plating media, adding 300 μ l of PBS containing 0.5 mM Orthovanadate to each well, and scraping the cells off the plating surface using a beveled tip rubber scrapper. Collected samples were briefly centrifuged to pellet the cells, supernatant was removed, and the cells were lysed by gentle rotation overnight using 50 μ l of lysis buffer containing TBS with 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate. Following cell lysis and centrifugation (10,000 rpm, 5 min), the supernatants were used to determine protein concentrations using a BCA protein assay (Pierce Chemicals, Rockford, IL.). Equivalent amounts of protein (50 μ g) were subjected to SDS-PAGE (8%-10% acrylamide). Following transfer to nitrocellulose, the blots were stained with Ponceau Red, photographed, assessed for the equivalency of protein loading, and cut to allow the top and bottom portions of the blot to be probed simultaneously.

Ponceau Red was subsequently removed using Tris-Buffered Saline and the blots were blocked with 3% milk in Tris-Buffered Saline with 0.2% Tween-20, and subsequently incubated with the following antibodies (1:1,000) overnight at 4°C: phospho-p44/42 MAPK, (NEB/Cell Signalling Technology); FAK, phosphotyrosine 4G10 (Upstate Biotech Inc); pFAK^{Y397} (Biosource Inc); α 1 integrin (Chemicon); ERK (MAPK), β 1 integrin (Santa Cruz Biotech, SC8978); pAkt, Akt (NEB/Cell Signalling Technology); Src (Santa Cruz Biotech); pSrc PY²¹⁶ (Biosource Inc). The next day, blots were washed, incubated in the appropriate horseradish peroxidase conjugated secondary antibody (anti-rabbit or anti-mouse IgG- HRP; Boehringer Mannheim, 1:5,000), and visualized using chemiluminescence reagents (ECL, NEN, Boston, MA) and exposure to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC). Developed films were subsequently digitized and densitometrically analyzed using the Image J software (NIH image downloadable software). Phosphorylated substrates were normalized against their non-phosphorylated forms, for non-phosphorylated substrates, actin, or MAPK were used. Digital images of the blots were used to make composite figures with Adobe Photoshop graphics software (Adobe Corp, MountainView CA).

2.2.6 Src inhibition

PP2 (25 μ M, Chemicon), a potent and selective inhibitor of the Src family of tyrosine kinases (SFK) was used to inhibit Src intermediates in the integrin-signalling pathway. The inhibitor was added to the culture media 2 hrs after plating, and 24 hrs later cells were harvested and lysed for use in Immunoblotting.

2.2.7 Adhesion assay

Cells were plated on 12 well plates and allowed to sit for 2 hrs before initial counts were performed. Prior to counting, cells were washed with Modified Neural Basal media to simulate normal feedings. To ensure that the same population of cells was counted each time, an X was drawn on the bottom of each well separating the area into four equal quadrants. A quadrant was initially chosen at random and cells in this area were counted at 2, 24, 48, and 72 hrs after plating. Once completed the percent of cells that remained attached was calculated and plotted in a bar graph, whereby initial count at 2 hrs after plating was considered to be 100 percent adhesion.

2.2.8 Statistical Analysis

All reported data analyses are based on a minimum of 3 separate experiments for each manipulation. Data are plotted as mean \pm SEM and significance is noted only if $p < 0.05$ as determined by ANOVA using Tukey testing for post hoc comparisons for multiple group analyses.

2.3 Results

2.3.1 Effects of polylysine, laminin and matrigel extracellular matrix on neurite growth and signalling

In these experiments, we were interested in how neurite growth could be promoted by extracellular matrix (ECM) molecules in the absence of NGF, and whether this growth, if present, would be potentiated by the presence of NGF. However, we first compared the adherence of neurons plated on poly-lysine (PL) or a thin film of growth

factor-reduced MG to determine whether differences in adhesion would confound effects on neurite outgrowth. Adhesion assays were carried out as noted in the Methods section. Cells were plated on either PL or MG coated 12-well dishes, an 'X' was inscribed on the outside bottom of the wells, and the number of cells adhering to the substrate within the chosen quadrant of this 'X' were counted at 2 hrs after plating; this number was taken as $t=0$. Subsequently, the medium in the cultures was exchanged every 24 hrs and the number of neurons still adherent in the quadrants was counted at 24, 48 and 72 hrs postplating. The data were expressed as a percentage of the counts at $t=0$ and are presented in Fig 2.1 for neurons plated on PL (Fig 2.1 A, B) or MG (Fig 2.1 C, D). There was no significant difference within the groups or between the groups plated on PL or MG, nor did NGF appear to have any influence on adherence. Although we did not directly test adhesion to LN in this experiment, our observations indicate that neuronal adherence to LN and MG, and PL are similar.

In our initial series of experiments we examined several combinations of ECM, including collagen, laminin (LN) and, growth factor reduced Matrigel (MG). In these experiments, all substrates were capable of eliciting neurite growth in the absence of added growth factors. The first series of growth quantitation results were carried out with MG, but we also tested whether the effects we observed with MG were comparable to equivalent concentrations of LN. We observed little difference between the LN- and MG-coated substrates in both percentage of neurite growth and expression of down

Figure 2.1. Neuron adherence is similar on polylysine and Matrigel-coated substrates. Neurons were plated on PL (A, B) or MG (C, D) coated slides, and the number of adhering neurons counted over 72 hrs according to the methods outlined in the Results section. There is no significant difference between the substrates in terms of adherent neurons.

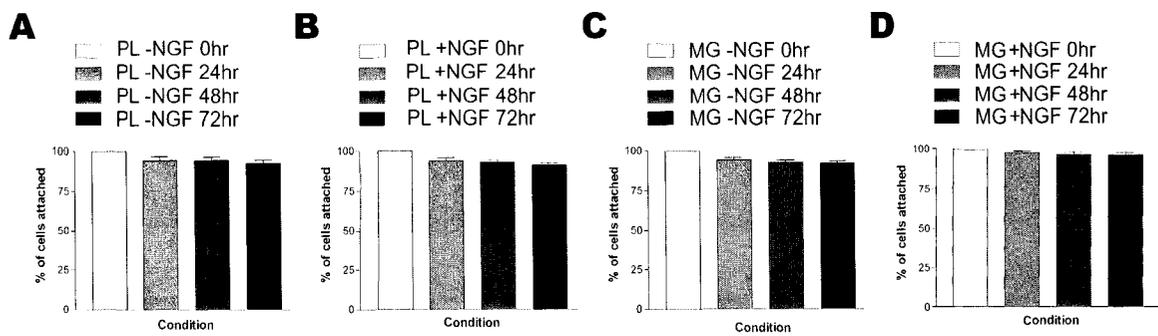


Fig 2.1

stream signalling intermediates (data not shown). For instance, Fig 2.2A shows that, although the addition of NGF produced a significant increase in the percentage of cells with neurites in both conditions, there was no significant difference between LN and MG conditions (Fig 2.2 A, $p > 0.05$). However, cells plated on LN or MG \pm NGF showed a significant increase in the percentage of cells with neurites compared to cells plated on PL (Fig 2.2 A, $p < 0.001$). While it is possible that the other components of the MG (such as collagen IV) may have a role in the growth responses we have observed, our preliminary studies and previous reports indicate that laminin is the major growth effector in MG as previously reported by Tonge and colleagues (Tonge *et al.*, 1997).

To determine the influence of ECM molecules on growth from young adult DRG sensory neurons, we plated dissociated DRG neurons on plastic or glass surfaces coated with one of two substrates, either PL (lacking any sequences required to produce integrin activation) or a thin film of growth factor-reduced MG (which will activate integrins). Fig 2.2 B-E presents representative micrographs of neurite growth 24 hrs after plating on either PL or MG in the presence or absence of NGF. Neurons plated on PL showed little or no neurite outgrowth in the absence of NGF (Fig 2.2 B), although addition of NGF resulted in the appearance of some short neurites (Fig 2.2 D). In contrast, neurons plated on the MG coating displayed substantial neurite outgrowth in the absence of NGF (Fig 2.2 C), which was further enhanced by the addition of NGF (Fig 2.2 E).

2.3.2 Integrin activation enhances neurite growth

Differences in neurite growth as observed in the representative images in Fig 2.2

Figure 2.2. Comparison of the effects of Polylysine, laminin and Matrigel on neuronal growth. **A:** Cells were plated on polylysine (PL), laminin (LN) or growth factor reduced matrigel (MG), and the number of cells with neurites were counted 24 hrs after plating. There was no significant difference in the percentage of neurite-bearing cells on LN or MG \pm NGF. However, the addition of NGF in both substrate conditions produced a significant increase in the percentage of cells with neurite growth. ** p<0.001. **B-E:** Neurons were plated on PL (B,D) or MG (C,E) coated culture plates. **B,** neurons cultured on PL in absence of added NGF; **D,** neurons cultured on PL in the presence of added NGF, 25 ng/ml. **C,** neurons were cultured on MG in the absence of added NGF; **E,** neurons cultured on MG in the presence of added NGF, 25 ng/ml. Neurite growth on MG is greater than on PL even in absence of added NGF. Addition of NGF enhances growth on both PL and MG. Scale bar – 50 μ m (applies to A-D).

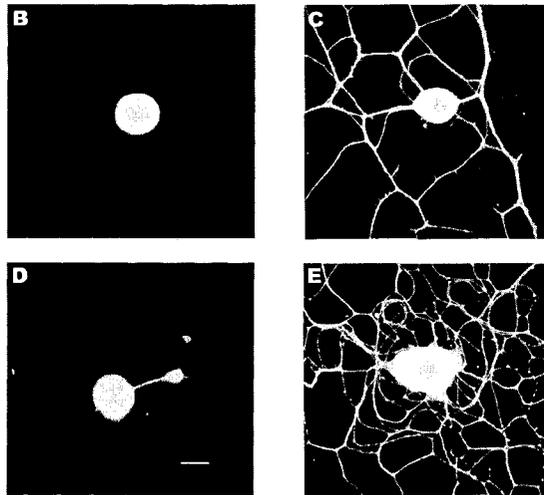
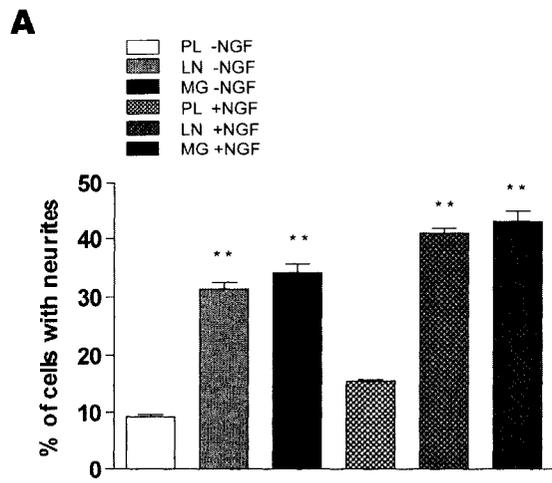


Fig 2.2

were quantified as noted below. In order to evaluate the contribution of integrin activation to growth, some cultures were treated with a synthetic cyclic RGD peptide. This sequence (Arginine-Glycine-Aspartate, RGD) is recognized by the majority of known integrins, and plays a key role in integrin-mediated adhesion; extracellular matrix molecules such as laminin contain one or more RGD sites (Aplin et al., 1998; Ruoslahti, 1996). The RGD peptides can block integrin-dependent interactions with the substrate and cell-surface receptors. Neurite growth was assessed following fixation and immunostaining with peripherin. Individual neurons were traced at 24 hr and 48 hrs after plating, using image analysis computer software (NeuroLucida® tracing program). Data were extracted and analyzed using the NeuroExplorer® component of this software as outlined in the Methods and as previously reported (Jones et al., 2003).

The results of these analyses are presented in Fig 2.3; the panels on the left side of the figure present total neurite growth, while the panels on the right side of the figure present the numbers of intersections using Scholl analysis. It is obvious that neurons plated on MG have much more extensive neurite growth than cells plated on PL, regardless of whether NGF is present or not (panels A, C vs. E, G). On PL, addition of NGF resulted in approximately a 45% increase in growth by 24 hrs, which was not significantly altered by the RGD peptide (panel A). On MG, the addition of NGF resulted in about a 35% increase in growth by 24 hrs; the RGD peptide decreased the growth on MG by about 35% regardless of whether NGF was present or not (panel E). By 48 hrs, growth on PL in the presence of NGF remained similar to the 24 hr results; again the RGD peptide did not have a significant effect on growth (panel C). In contrast, neurites continued to

Figure 2.3. Integrin activation enhances neurite growth - quantitation of neurite growth and branching. Neurons were plated on PL or MG coated dishes in the presence or absence of NGF. Panel A, B- PL, 24 hrs; Panel C, D – PL, 48 hrs; Panels E, F – MG, 24 hrs; Panel G, H – MG, 48 hrs. In addition, some cultures were treated with the cyclic peptide RGD (0.1 μ g/ml) two hours after plating. Cultures were fixed and stained with antibodies to peripherin followed by HRP-DAB detection. Quantitation of growth and branching was assessed using the NeuroLucida® tracing program, and as described in the Methods. Left hand panels: total neurite length (in μ m); data are presented as mean \pm SEM. Right panels: the number of intersections (an indication of neurite branching and complexity); data are presented as the mean of the number of intersection points in 20 μ m radial increments from the cell body (see Methods for details). Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

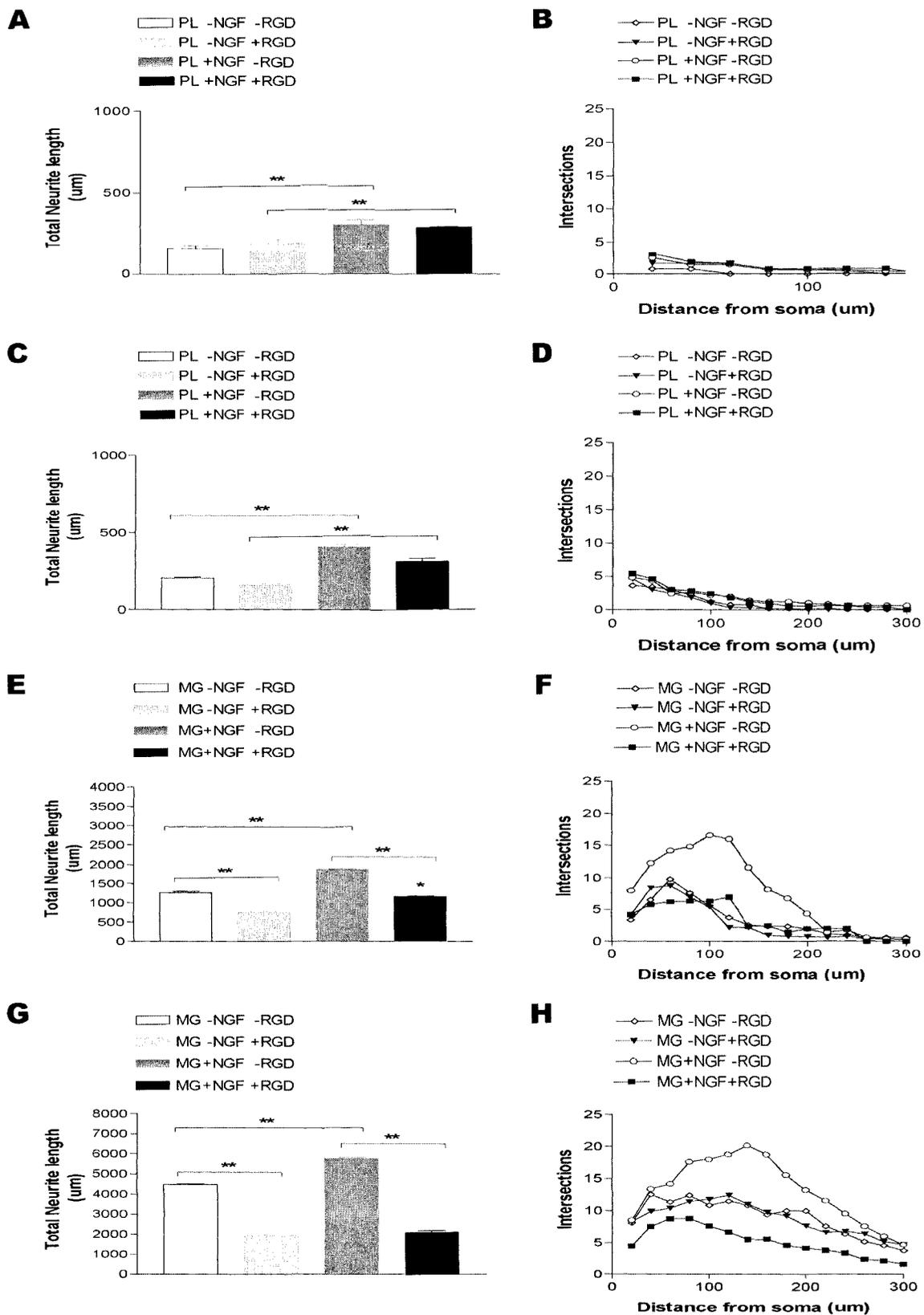


Fig 2.3

neurites continued to extend on the MG increasing the amount of growth by almost 3-fold at 48 hrs (panel G). At 48 hrs, the apparent NGF-dependent growth was somewhat less than that observed at 24 hrs, with the addition of NGF resulting in about a 25% increase in growth compared to MG alone (panel G). In the presence of the RGD peptide, NGF effect was limited to an increase of only 10%.

The results of the Sholl analyses indicate that the effect of integrin and NGF signalling on total growth seems to be not necessarily a result of increased extension but rather an increase in the complexity of the growth patterns, eg., an increased number of branches (Fig 2.3 F, H). This effect appears to be synergistic in that the number of intersections in the MG+NGF conditions (at both 24 and 48 hrs) is greater than an additive effect of either condition alone. *In vivo* these neurons have rather extensive peripheral fields of innervation and the ability to respond to both ECM signals and growth factor signal may provide for optimal re-establishment of reinnervation patterns after nerve injury. The ability of these neurons to respond to substrate and growth factor in the manner shown may provide a basis for the observations of increased collateral sprouting that occurs in the intact peripheral processes of NGF-responsive neurons after denervation of adjacent receptive fields (Diamond *et al.*, 1992; Mearow, 1998).

These data support the notion that integrin engagement by ECM molecules and subsequent activation are sufficient for neurite growth from DRG neurons in the absence of added neurotrophins like NGF, and that this growth can be further enhanced by the addition of trophic factors such as NGF. However, the observation that the RGD peptide appears to almost completely block the effect of NGF by 48 hours was not expected and points to a potentially more complex interaction between NGF-dependent and the

integrin-dependent signalling pathways. Indeed, one interpretation is that integrin activation may be required to respond to growth factors, although numerous studies indicate that cultured neurons are still able to respond to growth factors when plated on synthetic substrates. Since integrin signalling can be influenced by outside-in and inside-out signalling events (Giancotti, 2003), it is possible that in addition to the outside-in signalling evoked by integrin-LN interactions, there may also be important inside-out events evoked by NGF-dependent signalling that could contribute to the results. Our data also support the interpretation that while growth factors alone can elicit a certain amount of neurite growth, interactions with permissive ECM substrates can clearly enhance this response.

2.3.3 Enhancement of growth of adult DRG neurons correlates with increased expression of integrin subunits

Although growth patterns and signalling at 24 hours between cells plated on LN and MG were identical, to avoid problems with potential confounding components found in MG, LN (see Methods) was used to further study the signalling mechanisms involved in integrin activation. Neurons were plated on PL or LN in the presence or absence of NGF, and 24 hrs later either cell lysates were prepared and processed for Western blotting analyses, or cells were fixed and immunostained for confocal microscopy, (as described in Methods section).

As shown by Western blot analysis (Fig 2.4 A) a significant increase in $\beta 1$ integrin expression was seen when cells were plated on LN as compared to cells plated

Figure 2.4. Expression of $\beta 1$ integrin in adult DRG neurons in the presence or absence of a β -1 blocking antibody ($\beta 1i$). Adult DRG neurons were plated on either PL or LN coated dishes for 24 hrs \pm NGF \pm $\beta 1i$, followed by Western blotting of cell lysates (A) or immunocytochemistry (B-I); when applicable $\beta 1i$ was added two hours after plating. **A:** Expression of $\beta 1$ integrin subunit was increased in adult neurons plated on LN, but not significantly influenced by the addition of NGF. This increase was abolished when media was supplemented with a $\beta 1$ inhibitor. MAPK was used as a loading control, and the values for $\beta 1$ are expressed relative to MAPK expression. **B-I:** Cells were fixed and immunostained with anti- $\beta 1$ antibody, followed by Cy2-tagged secondary antibodies. Confocal laser scanning microscopy was used to visualize the stained neurons. Cells plated on LN (**D:** LN-NGF, **E:** LN +NGF) show increased staining intensity for $\beta 1$ when compared to cells plated on PL (**B:** PL -NGF, **C:** PL +NGF), where expression was unaffected by the presence of NGF. Cells plated on LN in the presence of a $\beta 1$ inhibitor (**H:** LN -NGF + $\beta 1i$, **I:** LN +NGF + $\beta 1i$) show decreased growth, with no significant effect of NGF above control levels. Cells plated on PL in the presence of the $\beta 1$ inhibitor (**F:** PL -NGF + $\beta 1i$, **G:** PL +NGF + $\beta 1i$) were not significantly affected. Scale bar – 20 μ m. Graphs represent the mean \pm SEM of relative densitometric data of 3 blots from 3 experiments; data are expressed as percentages with the control value (PL) for each experiment taken as 100 %. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

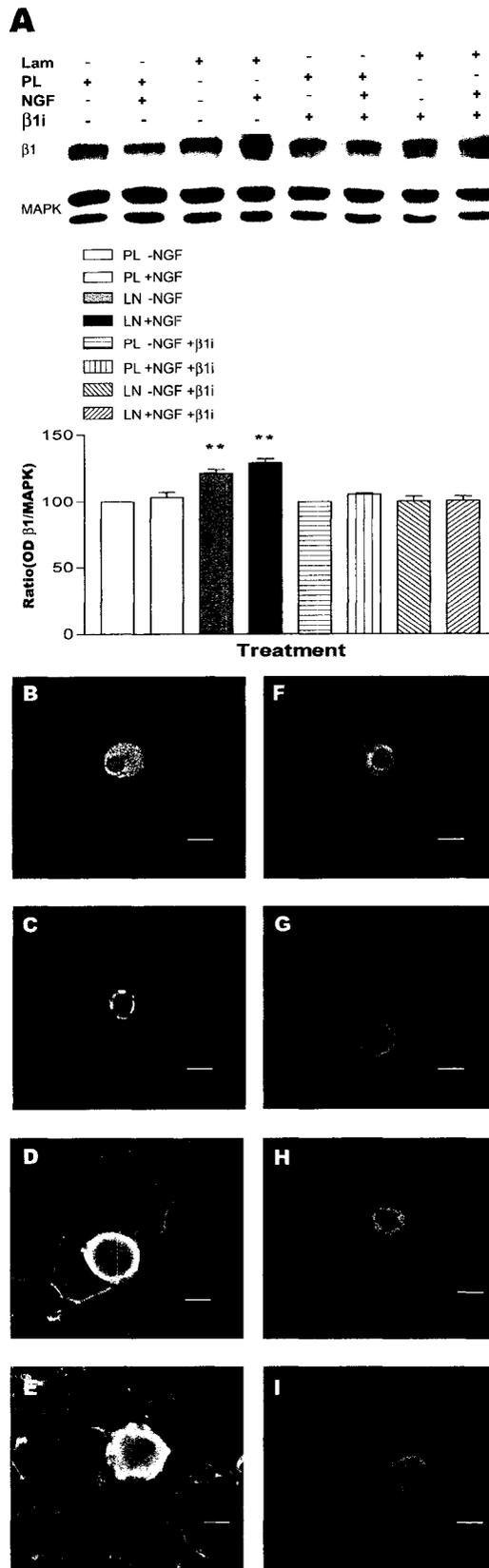


Fig 2.4

on PL (** $p < 0.001$). This effect was abolished with the addition of a functional blocking $\beta 1$ integrin antibody ($\beta 1i$). Examining expression at the cellular level, (Fig 2.4 B-E) a modest increase in $\beta 1$ was observed for cells plated on LN (D, E) when compared to cells plated on PL (B, C). Patterns of staining for $\beta 1$ were different between substrate conditions with LN substrates punctuate staining of the cell body, neurites and growth cones. Fig 2.4 F-I shows that when $\beta 1i$ was added to the culture media, the LN-induced increase in $\beta 1$ (H, I) was reduced to levels detected on PL (B, C). More importantly the addition of $\beta 1i$ reduced neurite growth seen in integrin-activated LN conditions to control non-integrin activated PL conditions.

As shown by Western blot analysis (Fig 2.5 A) a significant increase in $\alpha 1$ integrin was also observed when cells were plated on LN as compared to cells plated on PL (* $p < 0.05$, ** $p < 0.001$). As with $\beta 1$, this effect was abolished with the addition of a functional blocking $\beta 1$ integrin antibody ($\beta 1i$). At the cellular level (Fig 2.5 B-E) an increase in $\alpha 1$ was seen for cells plated on LN (D, E) compared to cells plated on PL (B, C). Patterns of staining for $\alpha 1$ were also different between substrate conditions with LN substrates showing staining at points of bifurcation, growth cones, and throughout the cell body, whereas cells plated on PL displayed lower $\alpha 1$ expression apparently concentrated at the edges of the cell membrane (B, C, F, G). Fig 2.5 F-I shows that when $\beta 1i$ was added to the culture media there was no increase in $\alpha 1$. As noted above, the addition of $\beta 1i$ reduced neurite growth on LN, supporting the interpretation that this is integrin-mediated growth. These data support the role of $\alpha 1\beta 1$ integrin as a laminin receptor. We

Figure 2.5. Expression of $\alpha 1$ integrin in adult DRG neurons in the presence and absence of a $\beta 1$ blocking antibody ($\beta 1i$). Adult DRG neurons were plated on either PL or LN coated dishes for 24 hrs \pm NGF \pm $\beta 1i$ followed by Western blotting of cell lysates (A) and immunocytochemistry (B-I). **A:** Expression of $\alpha 1$ integrin subunit was increased in adult neurons plated on LN, but not significantly influenced by the addition of NGF. This increase was abolished when media was supplemented with the $\beta 1$ antibody. MAPK was used as a loading control, and $\alpha 1$ relative expression. **B-I:** Cells were fixed and immunostained with anti- $\alpha 1$ antibody followed by Cy2-tagged secondary antibodies. Confocal laser scanning microscopy was used to visualize the stained neurons. Cells plated on LN (**D:** LN-NGF, **E:** LN +NGF) show an increase in staining intensity for $\alpha 1$ when compared to cells plated on PL (**B:** PL -NGF, **C:** PL +NGF) whereas expression was unaffected by the presence of NGF. Cells plated on LN in the presence of a $\beta 1$ inhibitor (**H:** LN -NGF + $\beta 1i$, **I:** LN +NGF + $\beta 1i$) show decreased growth and staining intensity for $\alpha 1$, with no significant effect of NGF above control levels. Cells plated on PL in the presence of the $\beta 1$ inhibitor (**F:** PL -NGF + $\beta 1i$, **G:** PL +NGF + $\beta 1i$) were not significantly affected. Scale bar – 20 μ m. Graphs represent the mean \pm SEM of relative densitometric data from 3 blots from 3 experiments; data are expressed as percentages with the control value (PL) for each experiment taken as 100 %. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

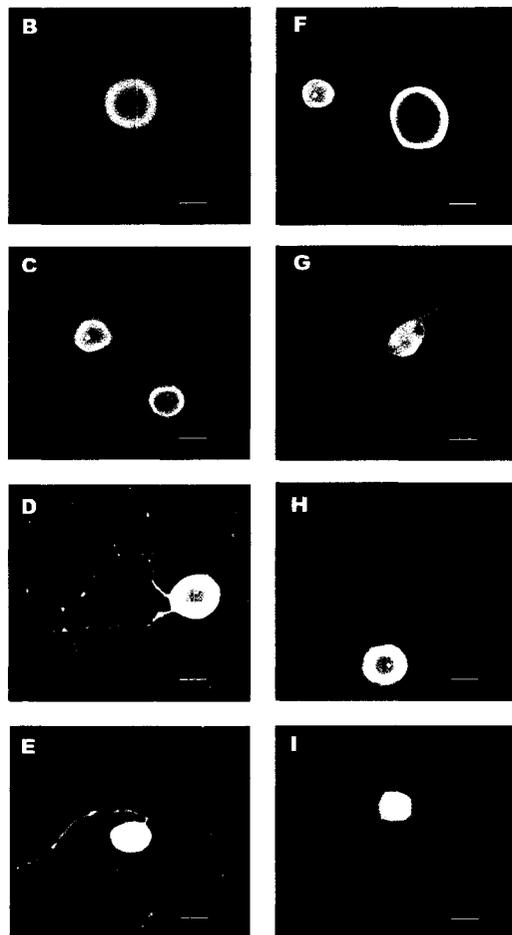
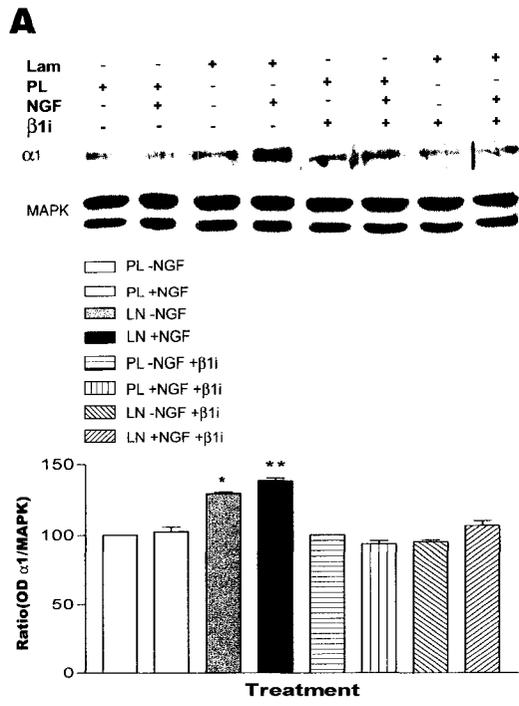


Fig 2.5

also attempted analysis of $\alpha 3$ and $\alpha 6$ integrin subunits, however, commercially available antibodies did not provide consistent results for either Western or immunocytochemical analysis.

2.3.4 Cellular expression of downstream signalling intermediates in the integrin and NGF signalling pathways

Expression of pFak, ILK, and pAkt, was examined using immunocytochemistry and confocal microscopy on adult neurons plated on PL or LN. Representative results are presented in Fig 2.6. As shown in Fig 2.6 A-D, Fak phosphorylation was increased in cells plated on LN (C, D) when compared to cells plated on PL (A, B). There was also a trend toward a further increase in pFak levels with the addition of NGF. Similarly, ILK expression (Fig 2.6 E-H) was increased when cells were plated on LN (A, B) as compared to those plated on PL (E, F). Notable was the large increase in membrane and neurite localization with very bright staining at branching points. As our growth observations noted a significant increase in the complexity of growth (Fig 2.3) for cells plated on MG in the presence and absence of NGF, it is tempting to speculate that the local accumulation of both Fak and ILK (along with apparent accumulation of the integrins (Fig 2.4, D, E, & Fig 2.5 D, E) at branching points may play a role in pathway decision-making. We were unable to obtain a phospho-specific ILK antibody for these studies. In Fig 2.6 I-J it is apparent that Akt phosphorylation is increased with the addition of NGF and that pAkt expression was higher in LN conditions than in PL conditions.

Figure 2.6. Neuronal expression of signalling intermediates in the integrin and NGF pathways. Adult DRG neurons were plated on either PL or LN, in the presence or absence of NGF. Cells were fixed and immunostained with anti-pFak (A: PL-NGF, B: PL +NGF, C: LN -NGF, D: LN +NGF), anti-ILK (E: PL -NGF, F: PL +NGF, G: LN -NGF, H: LN +NGF), and anti-pAkt (I: PL-NGF, J: PL +NGF, K: LN -NGF, L: LN +NGF) followed by Cy2-tagged secondary antibodies. Confocal laser scanning microscopy was used to visualize the stained neurons. Note increased growth of neurons plated on LN with a further increase when plated in the presence of NGF. A-D: Cells plated on laminin show increased expression of pFak with no obvious effect of NGF. E-H: Cells plated on laminin show increased expression of ILK with no obvious effect of NGF. I-L: Cells in the presence of NGF show increased phosphorylation of Akt when plated on either substrate condition. Slight increases in pAkt are seen in cells plated on laminin in the absence of NGF compared to controls. Scale bar – 20 μ m.

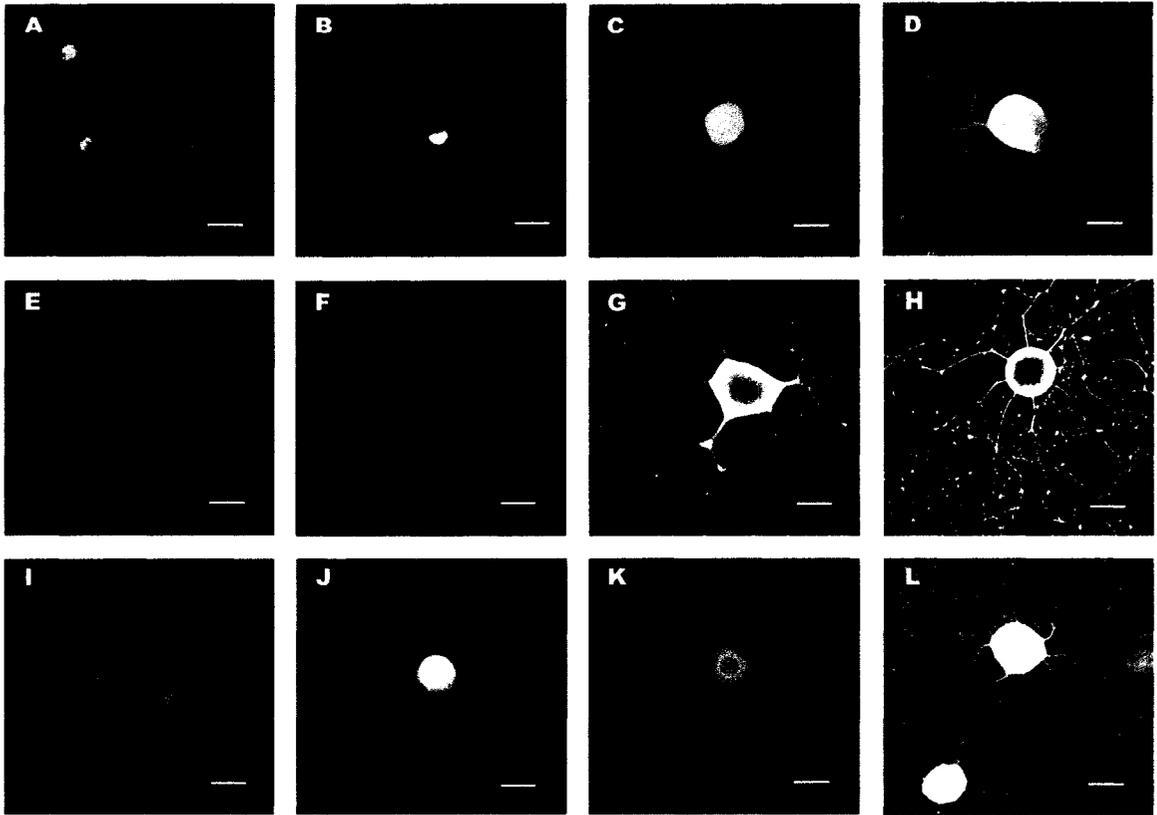


Fig 2.6

2.3.5 Analysis of downstream signalling intermediates common to both integrin and NGF signalling pathways.

To quantitatively analyse activation and expression of downstream signalling components, we employed Western blotting of cell lysates as described in Methods. To further examine the relationship between integrin and growth factor signalling, a selective inhibitor of the Src family of tyrosine kinases, PP2 (Hanke *et al.*, 1996), and a functional blocking β -1 integrin antibody, β 1i, was used. Neurons were plated on PL or LN in the presence or absence of NGF for 2 hours, then media was aspirated and supplemented with new media of the same composition (Fig 2.7, control condition), media with PP2 (Fig 2.8), or with β 1i (Fig 2.9). Cells were then incubated for a further 24 hrs at 37°C, after which time lysates were processed for Western blotting analyses.

As shown in Fig 2.7 A, a significant increase in Src activation (as assessed by phospho-Src antibody) was observed in cells plated on LN compared to those plated on PL (** $p < 0.001$), and this was further enhanced by the addition of NGF (** $p < 0.001$). LN-induced increases in Src activity were abolished with the addition of PP2 (Fig 2.8 A) and β 1i (Fig 2.9 A). In the case of Akt, LN alone had little effect on pAkt, although the combination of LN and NGF resulted in increased activation. The addition of PP2 abolished both the NGF- and LN-induced increases in active Akt (Fig 2.8 B), although little or no effect was seen with the addition of β 1i (Fig 2.9 B). Activation of FAK (Fig 2.7 C) was observed primarily in the LN conditions with no further increase with the addition of NGF, (** $p < 0.001$). As with Src activation, FAK activation was abolished with the addition of both PP2 (Fig 2.8 C) and β 1i (Fig 2.9 C). We also examined the

Figure 2.7. Western blot analysis of downstream signalling intermediates in the integrin and NGF signalling pathways. Adult DRG neurons were plated on either PL or LN coated dishes for 24 hrs \pm NGF, and Western blotting of cell lysates was subsequently carried out. **A:** Activation of Src (as assessed by p-Src) was significantly increased in cells plated on laminin in both the presence and absence of NGF (Fig 8A, $p < 0.001$), with a further significant increase when NGF is present (Fig 8A, $p < 0.001$). **B:** Phosphorylation of Akt was significantly increased with the addition of NGF (Fig 8B, $p < 0.001$), with a slight but non-significant influence from the ECM. **C:** Activation of Fak was significantly increased in cells plated on laminin in both the presence and absence of NGF, with no further increase when NGF is present (Fig 8C, $p < 0.001$). **D:** Phosphorylation of MAPK was significantly increased with the addition of NGF (Fig 8D, $p < 0.001$), but not significantly influenced by the ECM. Graphs represent the mean \pm SEM of densitometric data from at least 3 blots from at least 3 experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. ** $p < 0.001$.

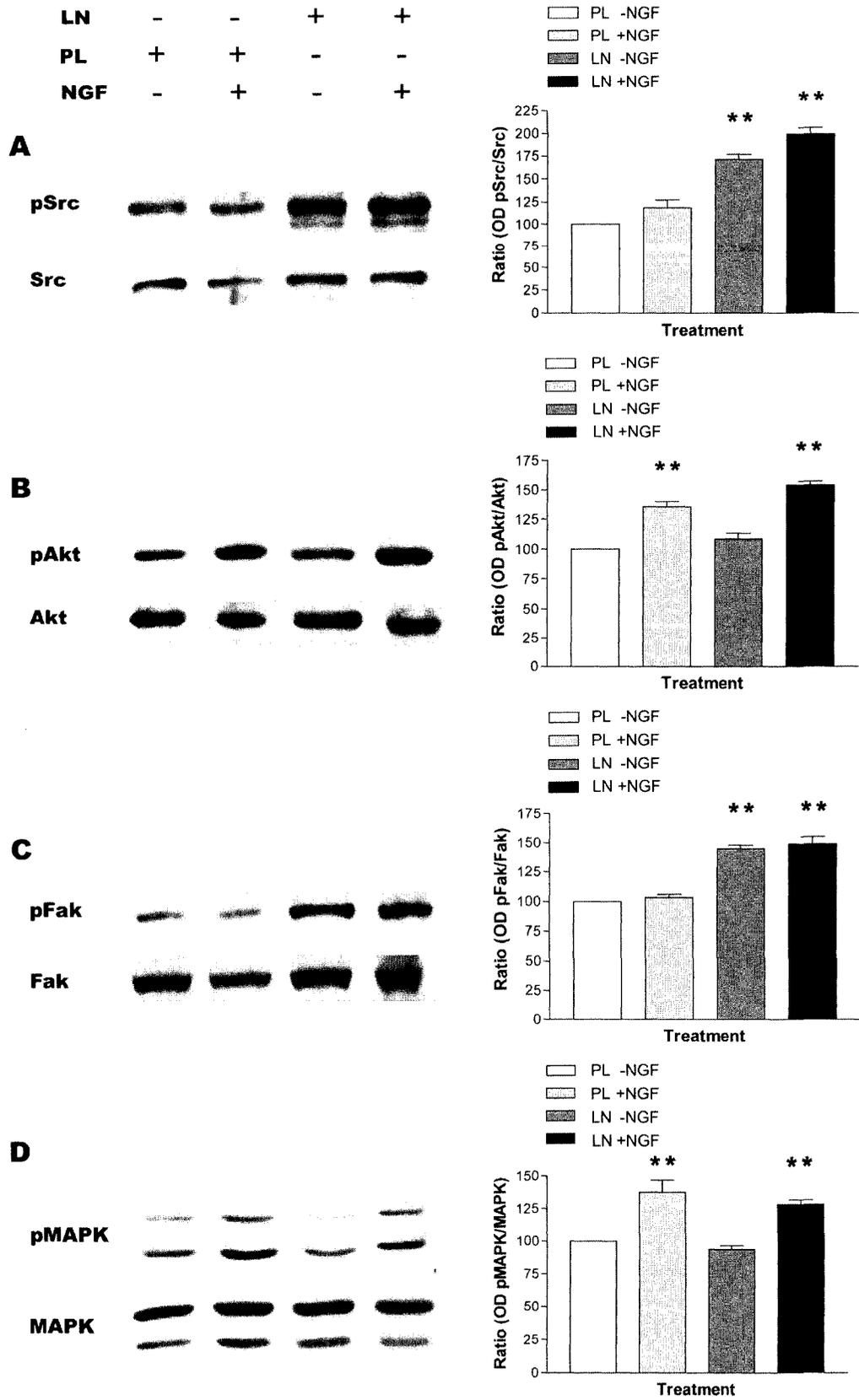


Fig 2.7

Figure 2.8. Western blot analysis of downstream signalling intermediates in the integrin and NGF signalling pathways in the presence of a specific Src family kinase (SFK) inhibitor (PP2). Adult DRG neurons were plated on either PL or LN coated dishes for 24 hrs \pm NGF +PP2 and Western blotting of cell lysates was subsequently carried out. **A:** Increased activation of Src by LN and NGF was reduced to control levels in the presence of SFK inhibitor. **B:** Increased phosphorylation of Akt with the addition of NGF was reduced to control levels in the presence of the SFK inhibitor. **C:** Increased activation of Fak in the LN conditions in both the presence and absence of NGF, was reduced to control levels in the presence of the SFK inhibitor. **D:** Phosphorylation of MAPK was not influenced by the addition of the SFK inhibitor. Graphs represent the mean \pm SEM of densitometric data from at least 3 blots from at least 3 experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

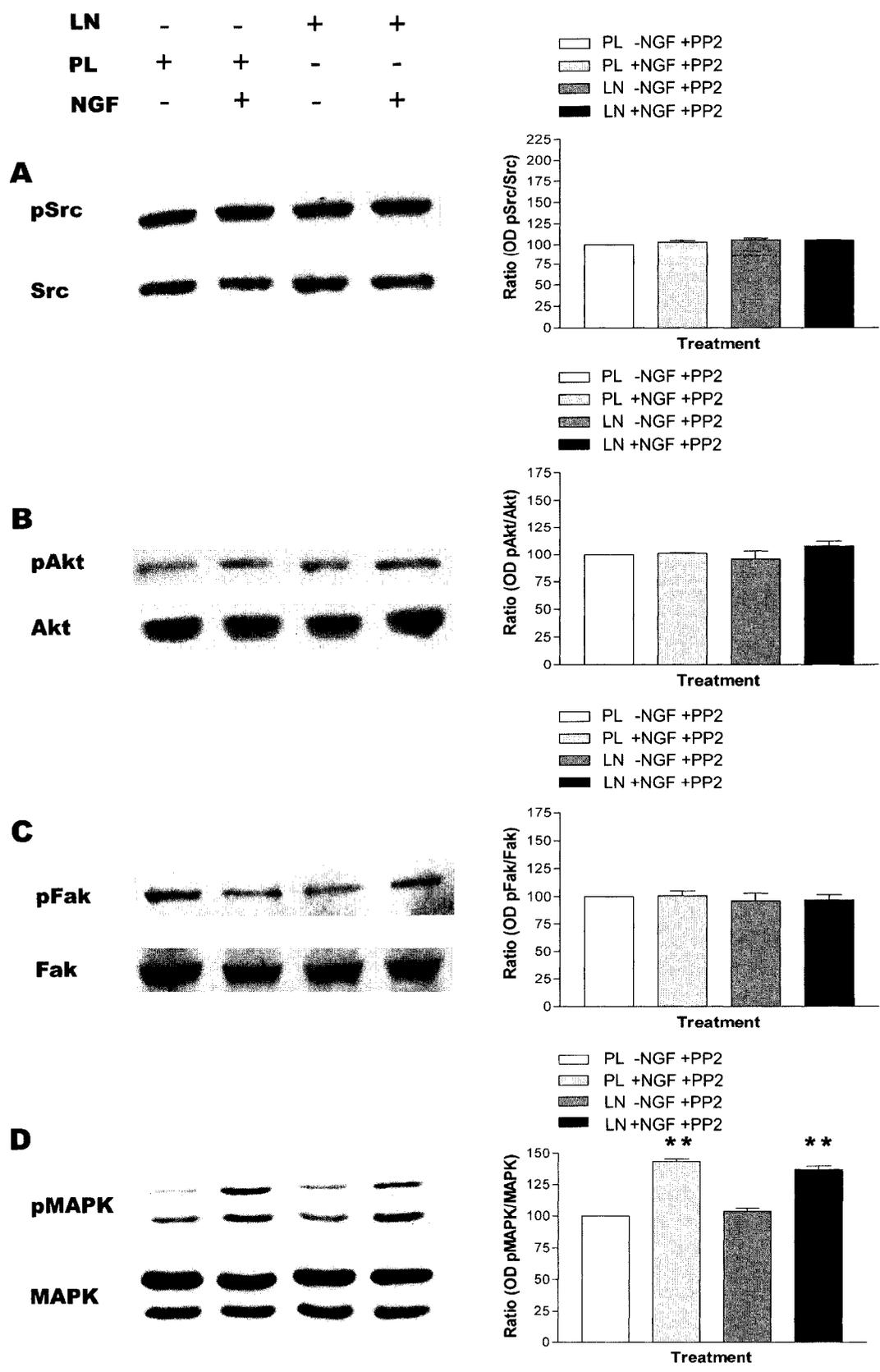


Fig 2.8

Figure 2.9. Western blot analysis of downstream signalling intermediates in the integrin and NGF signalling pathways in the presence of a $\beta 1$ blocking antibody ($\beta 1i$). Adult DRG neurons were plated on either PL or LN coated dishes for 24 hrs \pm NGF + $\beta 1i$ and Western blotting of cell lysates were subsequently carried out. **A:** Increased activation of Src by LN and NGF was reduced to control levels in the presence of the $\beta 1$ inhibitor. **B:** Increased phosphorylation of Akt with the addition of NGF was unaffected by the presence of the $\beta 1$ inhibitor. **C:** Increased activation of Fak in the LN conditions in both the presence and absence of NGF was reduced to control levels in the presence of the $\beta 1$ inhibitor. **D:** Phosphorylation of MAPK was not influenced by the addition of the $\beta 1$ inhibitor. Graphs represent the mean \pm SEM of densitometric data from at least 3 blots from at least 3 experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

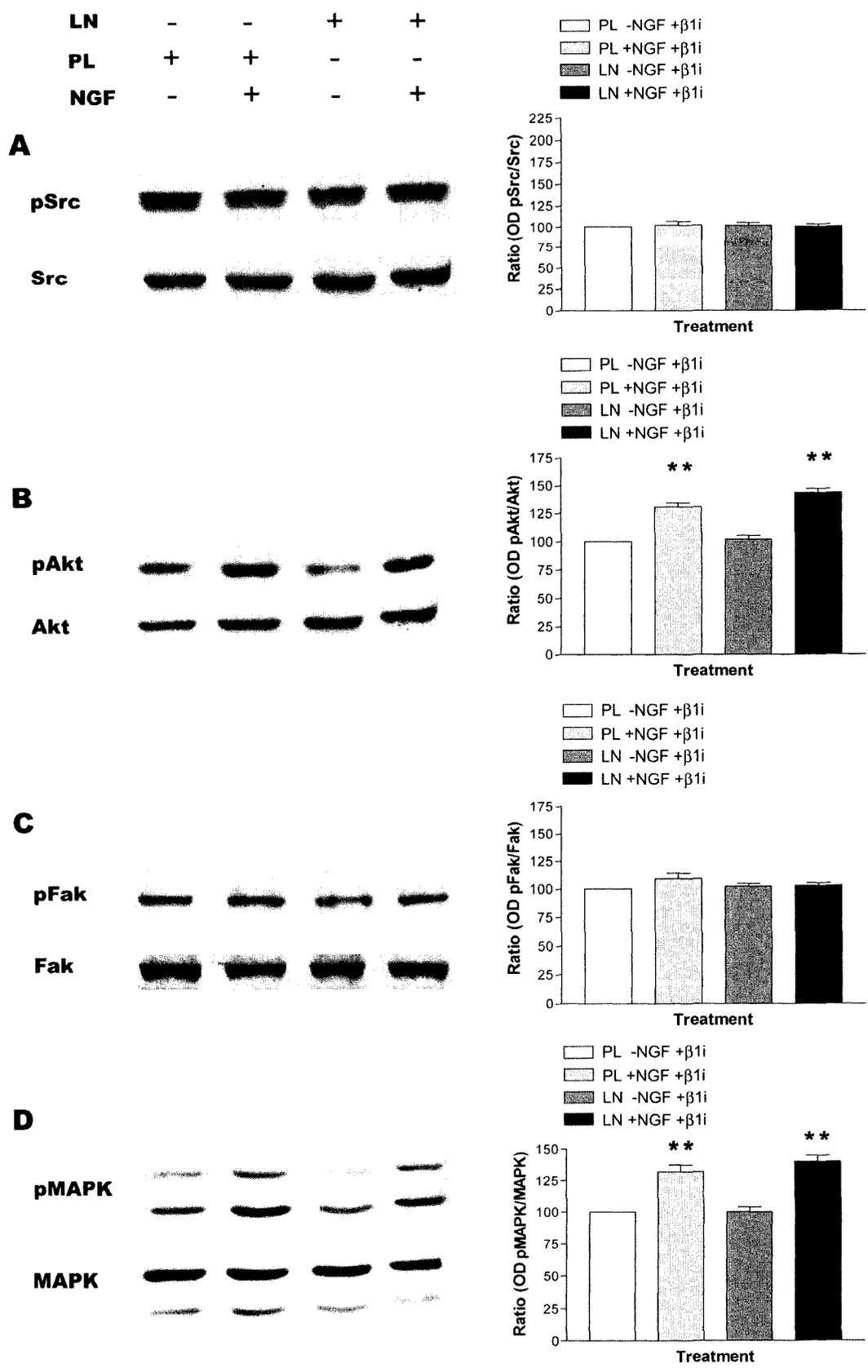


Fig 2.9

activation of MAPK, which has been reported to be activated by integrin engagement in other cell types. In these neurons LN had no obvious effect on MAPK activation measured at 24hrs, although it was obvious that NGF treatment resulted in MAPK activation. Neither PP2 (Fig 2.8 D) nor β 1i (Fig 2.9 D) had any effect on MAPK activation. These results suggest that any integrin-growth factor convergence is not occurring through the Ras-MAPK pathway in these neurons, but rather through a Src-mediated pathway, with subsequent downstream activation of PI3K.

2.4 Discussion

We are interested in understanding how integrin-activated signalling pathways interact with those activated by growth factors like NGF to result in optimal axonal regeneration. In the present study we have investigated how ECM molecules such as LN can act to enhance neurite growth from adult DRG neurons compared to an adhesive, but non-integrin activating, substrate (PL). Our results demonstrate that neurons plated on ECM exhibit significantly more outgrowth than those plated on PL, and that this growth can be further enhanced by NGF, suggesting convergence of these signalling pathways.

We also investigated activation of signalling intermediates in NGF- and integrin-dependent growth and our findings are summarized in Table 2.1. NGF-dependent signalling in the absence of integrin activation (eg., PL+N) resulted in the sustained activation of Akt and MAPK above baseline, but not FAK or Src. With integrin activation alone (eg., LN), pFAK and pSrc were elevated above baseline, and pAkt also increased, although as noted previously this increase did not reach significance;

Table 2.1. Summary of signalling intermediates in integrin and growth factor signalling pathways

Substrate condition	Signalling component						
	FAK	Src	Akt	MAPK	$\beta 1$	$\alpha 1$	Neurite Growth
PL	-	-	-	-	-	-	-
PL+N	-	-	+	+	-	-	+
LN	+	+	+	-	+	+	++
LN+N	+	++	++	+	+	+	+++
PL+N+PP2	-	-	-	+	ND	ND	-
LN+PP2	-	-	-	-	ND	ND	-
LN+N+PP2	-	-	-	+	ND	ND	-
PL+N+ $\beta 1i$	-	-	+	+	-	-	-
LN+ $\beta 1i$	-	-	-	-	-	-	-
LN+N+ $\beta 1i$	-	-	+	+	-	-	+

- Base line, + increase over baseline, ++ increase over N or L alone, ND not determined

activation of MAPK was not observed. We also examined ILK expression and observed an increase in cellular expression and localization in the LN condition. However, due to the lack of phospho-specific antibodies we were unable to determine the activation state of ILK. In both these experimental conditions, our data indicated that these signalling events correlated with increased neurite growth.

In the conditions where both integrin- and NGF-evoked signalling pathways come into play, pFAK, pSrc, pAkt and pMAPK were again elevated above baseline. However, only the Src activation appeared to be significantly enhanced above either the NGF- or LN-elicited activation. This observation suggests that Src may be the point of convergence of the two pathways.

Use of the β 1-integrin blocking antibody and the SFK selective inhibitor PP2 allowed us to further dissect the signalling events. β 1i-treatment resulted in the attenuation of FAK and Src activation, but had no detectable influence on pAkt or pMAPK, indicating that the latter two events were NGF-dependent. β 1i also blocked the LN-dependent increase in expression of α 1 and β 1 integrin subunits. Similarly, PP2 treatment resulted in the inhibition of FAK and Src activation, but in contrast to β 1i, PP2 also blocked the NGF-dependent Akt activation. PP2 had no effect on NGF-dependent MAPK activation. Both these inhibitors also blocked neurite outgrowth, with PP2 being more effective than β 1i in inhibiting growth in the presence of both NGF and LN.

Our data suggest that the enhanced growth on the ECM was at least partially due to modestly increased integrin expression, as well as increased intracellular signalling via integrin engagement. We have not examined expression of other integrins in our culture system, but it is also possible that other α integrins such as α 6 or α 7 may be involved in

the regulation of neurite outgrowth (Belkin and Stepp, 2000; Previtali et al., 2001). For example, the $\alpha 4 \beta 1$ integrin is upregulated during peripheral nerve regeneration and has been shown to enhance DRG neurite growth on fibronectin (Vogelezang *et al.*, 2001).

How growth factor and integrin activated signalling actually regulate neuritogenesis is not clear. Data from the integrin-signalling field indicate that complex interactions between many of these co-ordinately activated pathways modulate both cellular adhesion and the reorganization of cytoskeletal elements required for cellular motility (and by analogy, neuritic extension and growth). Our results point to a convergence of NGF and integrin activated signalling pathways along with the potential requirement for integrin signalling to elicit a response to specific neurotrophins. For instance, neurons that have been plated on MG in the presence of the RGD peptide for 48 hours show not only inhibition of the integrin-dependent neurite outgrowth, but inhibition of NGF dependent outgrowth as well. How this NGF-induced outgrowth inhibition takes place is unclear. But, because both integrin and NGF contribute to activation of common signalling components such as Src it is possible that when inhibited (eg. Fig 2.8 and Fig 2.9), added NGF can no longer stimulate the required level of activation resulting in lower amounts of growth. Thus, not only are there convergent interactions between integrin and NGF signalling pathways, but it is also possible that activation of components downstream of integrin signalling may be necessary to achieve an effective cellular responses to neurotrophins such as NGF.

Integrin engagement can trigger a number of different intracellular pathways that would appear to use the same intermediates as the neurotrophin-trk cascades. Some of these intermediates are primarily activated by integrins, such as FAK, while others like

Ras-MAPK appear to be more reliant on growth factor receptor signalling (Giancotti and Tarone, 2003). For example, FAK is activated by integrin engagement, and can interact either directly or via adapter proteins talin or paxillin with the cytoplasmic tail of integrin β subunits (de Curtis and Malanchini, 1997; Giancotti and Ruoslahti, 1999). FAK has been suggested to be the central coordinator of signalling downstream from integrins, since it plays a key role in cell motility, migration and survival (Schwartz and Ginsberg, 2002). A number of proteins can bind phosphorylated FAK, including paxillin, Src, CAS, Grb2 and PI 3-kinase, pointing to roles not only in cellular motility but also in promotion of survival (Schwartz and Ginsberg, 2002; Vuori et al., 1996). We have shown that there was a significant increase in FAK activation when adult neurons were plated on LN, although this did not appear to increase further with the addition of NGF. Src activation has also been reported to be integrin-dependent, although whether this is upstream or downstream of FAK activation seems to depend upon the experimental system (Giancotti and Tarone, 2003; Jones et al., 2000). Our data suggest that Src activation is upstream of FAK and potentially PI 3-K signalling, since PP2 treatment blocked both Fak and Akt activation. NGF-dependent TrkA signalling also results in Src activation in PC12 and neuroblastoma cells (Gatti, 2003; Tsuruda et al., 2004; Wooten et al., 2001; Altun-Gultekin, 1996; Rankin and Mearow, unpublished observations). Transactivation of TrkA via G-protein coupled receptors and src activation has also been observed (Lee *et al.*, 2002). In ongoing experiments, we have observed that NGF-mediated Src phosphorylation can be blocked by K252a and appears to require the Y490 site in TrkA; however, we have found no evidence that PP2 inhibits NGF-TrkA

signalling or at least via traditional PI 3-K→Akt, Ras→MAPK pathways (Rankin and Mearow, unpublished observations).

We have previously reported that the PI 3-K→Akt, rather than Ras→MAPK, signalling pathway plays a primary role in NGF-dependent growth from adult DRG neurons (Jones et al., 2003; Kimpinski and Mearow, 2001). Use of the PI 3-K inhibitor, LY200928 resulted in inhibition of neurite growth, while the MEK inhibitor, U0126, actually appear to enhance growth somewhat (Jones et al., 2003). The fact that NGF, but not LN, resulted in sustained MAPK activation is consistent with our previous observations in these sensory neurons, where even in short-term stimulation experiments we have not observed significant LN-dependent phosphorylation of MAPK. These data also point to differences between cellular systems. For example, in our experiments with PC12 cells, both LN- and NGF-evoked activation of MAPK are detectable within minutes but are not sustained and disappear within 30 minutes of treatment (unpublished observations).

Both the extracellular environment through which axons grow and the availability and action of neurotrophic factors play key roles in regulating axonal growth and regeneration. Our data suggest that potentiation of similar signalling pathways or convergence of distinctly-activated pathways could underlie the observed enhancing effects of laminin or other ECM molecules on growth factor-dependent cellular responses.

CHAPTER 3

A PROCEDURE FOR SELECTING AND CULTURING SUB-POPULATIONS OF NEURONS FROM RAT DORSAL ROOT GANGLIA USING MAGNETIC BEADS

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3.1 Type of research

Sensory neurons of the DRG consist of a heterogeneous population of cells that can be classified on the basis of neurochemistry, morphology, trophic requirements, and sensory modalities (Averill et al., 1995; Gavazzi et al., 1999; Petruska et al., 2000; Priestley et al., 2002a). Three major cell groups have been identified. These include large and medium diameter neurons identified based on their expression of phosphorylated heavy chain neurofilament NF200, p75 Neurotrophin Receptor (p75NTR), and tyrosine kinase receptors Trk A, B, or C, and typically have myelinated axons and function as peripheral mechanoreceptors (Priestley et al., 2002a). The next group consists of mainly small diameter neurons, express calcitonin gene related peptide (CGRP), p75NTR and TrkA, and are thought to have unmyelinated or thinly myelinated axons and function as peripheral nociceptive neurons (Molliver et al., 1997; Priestley et al., 2002a). The final group are also mainly small diameter neurons, bind the lectin *Griffonia Simplicifolia* IB4, express the receptor tyrosine kinase RET and at least one of the Glial Cell Line-derived neurotrophic factor family receptor subunits (GFR α), are considered to have unmyelinated or thinly myelinated axons and function as peripheral nociceptive neurons (Averill et al., 1995; Bennett et al., 1998; Kashiba et al., 2001;

Molliver et al., 1997; Snider and McMahon, 1998). These differences have been shown to be important when considering diseases such as diabetes that affect the peripheral nervous system. For instance, it has been shown that the small diameter nociceptive nerve fibres of the IB4 and CGRP positive populations (IB4+ve, CGRP+ve) are the first affected and most susceptible neurons to diabetic environments (Le Quesne and Fowler, 1986; Polydefkis et al., 2003). Thus, it is important that we be able to study different treatments on specific subpopulations of DRG neurons in isolation. Here we describe a procedure that uses magnetic beads for preparing viable homogenous cultures of DRG neurons based on their expression of previously mentioned cell surface markers. Specifically, here we have used the lectin IB4 to select out the population of non-peptide nociceptive neurons, leaving the CGRP and NF200 expressing populations behind (NF200+ve). However, this protocol can easily be adapted to select cells that express any cell surface marker with the appropriate antibody by the use of monoclonal or polyclonal IgG-coated magnetic beads.

3.2 Time required

Step 1. Dissection of 4-6 week old Sprague-Dawley rat DRGs (2-3 rats): 30 min.

Step 2. Enzymatic and mechanical dissociation: 1 hr and 20 min.

Step 3. Removal of non-neuronal debris via 30% Percol gradient: 30 min.

Step 4. Magnetic bead selection and separation: 30 min.

Step 5. Plate selected cells: 5 min.

Step 6. Growth of DRG cultures: 24 hr.

Step 7. Immunocytochemistry: 24 hr.

Step 8. Confocal microscopy: 4 hr.

Total: 54 – 60 hr

3.3 Materials

3.3.1 Animals

Male Sprague-Dawley rats 4-6 weeks old (Memorial University of Newfoundland

Animal Care Facility) were used.

3.3.2 Dissection and Special equipment

- Small animal guillotine
- Large sharp tip scissors
- Large notch tip forceps
- 45 degree curved fine-tipped jeweler's forceps
- 45 degree curved fine-tipped micro-surgical scissors
- Autoclaved 9" Pasteur pipets
- 15 ml conical tubes
- 1.5 ml eppendorf tubes
- 37° C water bath
- Variable speed swinging bucket centrifuge
- Adams Nutator single speed orbital mixer (Fisher, Ottawa, ON)
- Stereo dissection microscope

- Poly-D-Lysine (BD Bioscience, Canada), and Laminin (Gibco/Invitrogen, Grand Island, NY) coated Lab-Tec 16 well chamber slides (Nalge NUNC international, VWR, Mississauga, ON)
- 22x74mm glass cover-slip (Nalge NUNC international)
- Dynal Magnetic Separator (Dynal, Product # 120.20)

3.3.3 Chemicals and reagents for preparing viable selected DRG cultures.

- Neurobasal (NB) media containing 100 units/ml penicillin, 100mg/ml streptomycin
- Modified Neurobasal media (ModNB) containing 100 units/ml penicillin, 100mg/ml streptomycin, 29.2mg/ml L-glutamine (Gibco/Invitrogen), supplemented with 500 μ l/50ml of 50x B27, (Gibco/Invitrogen), and 20mM cytosine arabinofuranoside, (AraC) (Gibco/Invitrogen)
- 70% ethanol
- 1x Phosphate buffered saline (PBS; Gibco/Invitrogen)
- 1x Hanks buffered saline solution without phenol red (HBSS; Gibco/Invitrogen)
- 1x Hanks buffered saline solution without calcium chloride, magnesium chloride, magnesium sulfate, and phenol red (HBSS-M; Gibco/Invitrogen)
- DNase free Dispase dissociation media with 5mg/ml Protease (Sigma-Aldrich, Oakvill, ON), and 2mg/ml Collagenase type II (Gibco/Invitrogen)
- Fetal calf serum (FCS, Gibco/Invitrogen)
- Percol/Redigrad (Amersham Biosciences/ GE Healthcare, Baie D'Urfé, QC)

- 4.5mm diameter streptavidin-coated magnetic beads (Dyna beads; Dynal Biotech LLC, Brown Deer, WI, Product # 115.21)
- DNase 15,000 to 20,000 units (Gibco/Invitrogen)

3.3.4 Chemicals and reagents for immunocytochemistry

- Lectin binding buffer containing 0.1mM MnCl₂, 0.1 mM MgCl₂, and 0.1mM CaCl₂ (Gibco/Invitrogen)
- 1xPBS (Gibco/Invitrogen)
- Fluorescein-conjugated avidin (Vector Labs, Burlington, CA; 1:50, in 1xPBS, stock solution 1mg/ml)
- Biotinylated IB4 (Sigma-Aldrich; 1:250, in 1xPBS, stock solution 1mg/ml)
- Glycerol 50% (Fisher, Nepean, ON) in 1xPBS
- Paraformaldehyde, 4% (Fisher) in 1xPBS (Gibco/Invitrogen)
- Triton X-100, 0.1% (Sigma-Aldrich)

3.4 Detailed procedure

Animal procedures were approved by the Animal Care Committee at Memorial University of Newfoundland. Male Sprague-Dawley rats 4-6 weeks of age were housed 4 animals per cage in a temperature- and humidity-controlled animal care facility with a 12-h light/dark cycle. Animals were given a constant supply of laboratory chow and tap water.

3.4.1 Dyna bead coating and preparation (see Fig 3. 1)

Figure 3.1. Procedural summary of cellular-selection. A schematic diagram summarizing the steps required in preparing magnetic beads for use, capture and release of the desired cells, and plating of both selected and non-selected cell populations.

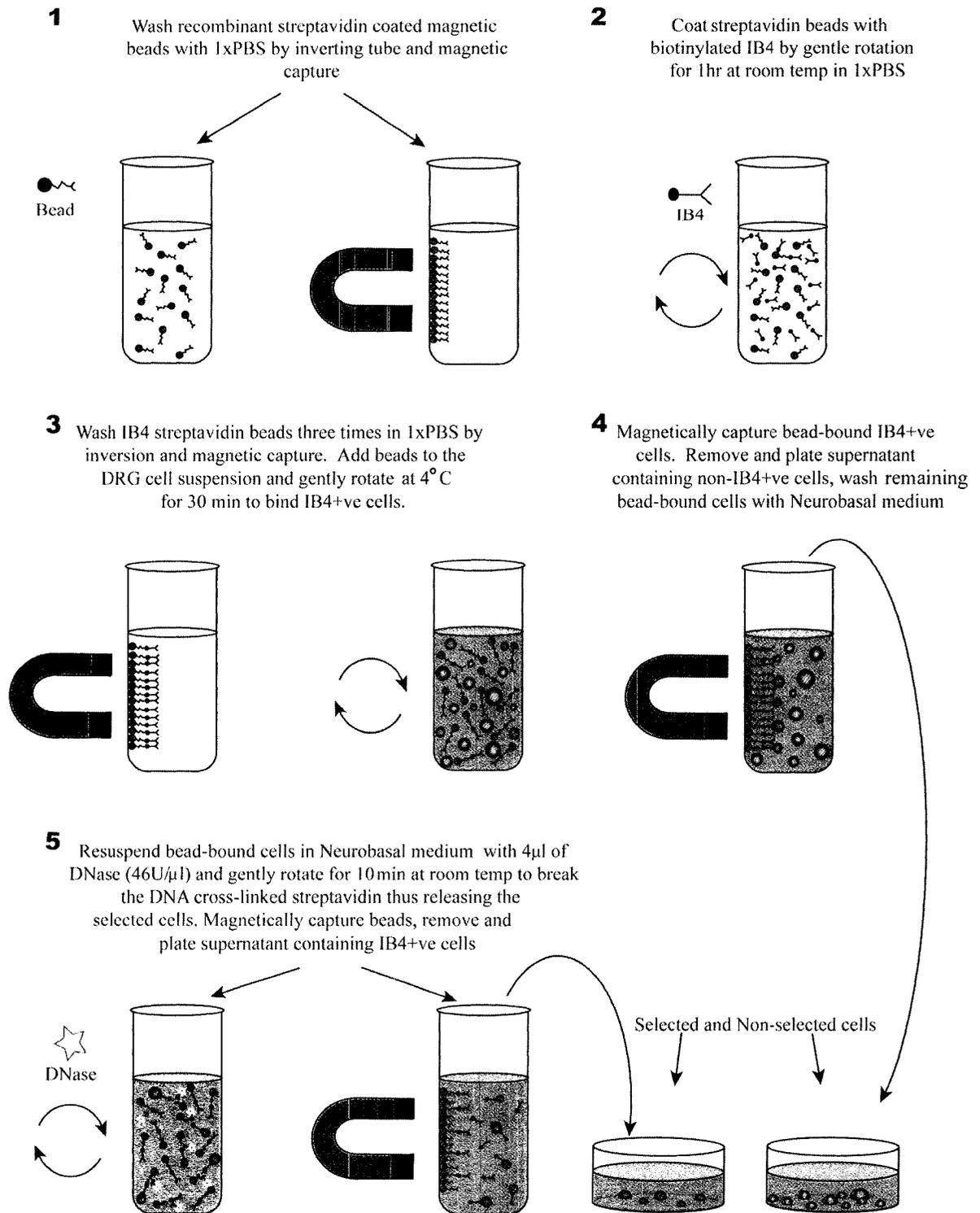


Fig 3.1

- (i) Transfer 100ml of streptavidin-coated beads (4.5mm, Product # 115.21) to a 1.5ml eppendorf tube and place tube in the Dynal Magnetic Separator for a minimum of 1 min to allow adequate bead capture.
- (ii) Aspirate and discard supernatant, remove tube from the magnetic separator and resuspend the beads in 1ml of 1xPBS; invert several times to ensure that the beads are mixed completely and that no beads have settled.
- (iii) Place beads back in the Dynal Magnetic separator for a minimum of 1 min to ensure that all beads are captured and that solution is free of suspended beads. Repeat steps (ii) and (iii) three times.
- (iv) On the third wash aspirate all remaining supernatant and resuspend coated beads in 500ml of 1xPBS with 40mg of biotinylated-IB4. Mix the bead / IB4 suspension (on the Nutator) at room temperature for 1 hr or until ready to use; to ensure that the beads do not settle during this step flip the tube every 10-15 mins.
- (v) Just prior to use, remove the tube with the coated beads from the Nutator, place in the magnetic separator and allow a minimum of 1 min to ensure that all beads have been captured, then aspirate and discard the supernatant. Wash three times as in steps (ii) and (iii); on the third wash leave the beads in 100ml of 1xPBS. Coated beads are now ready to be added to the cell suspension.

3.4.2 DRG dissociation, selection, and culture

3.4.2.1 DRG Dissociation

- (i) Sacrifice rats by live decapitation ($n = 3$) and remove spinal column using large sharp tip scissors and notch-tip forceps, cut a v-shaped opening in the back of the spinal column, expose and remove the spinal cord.
- (ii) Remove DRGs from all spinal levels using a stereomicroscope with 45-degree fine-tip jewelers forceps and 45-degree fine-tip micro-surgical scissors (all equipment has been sterilized with 70% ethanol). Place the DRGs in a 35mm Petri dish of ice-cold NB (Gibco). Generally, 48-52 ganglia can be isolated from one spinal column.
- (iii) Transfer DRGs to a 15ml conical tube using a cracked Pasteur pipette that was previously rinsed with HBSS to prevent the DRGs from sticking (by cracking the tip off the Pasteur pipette an opening large enough to aspirate the DRGs is created).
- (iv) Wash DRGs by filling the 15ml conical tube to the 10ml mark with HBSS, cap and invert tube 3 times. Allow the DRGs to settle, aspirate the media, and repeat 1 time.
- (v) Aspirate all remaining supernatant and resuspend DRGs in 1ml of disperse dissociation media and place in a 37° C water bath for 50 min. Tap the tube every 5 min to dislodge the DRGs ensuring adequate exposure.
- (vi) Add 1ml of FCS and 8ml of HBSS-M and centrifuge at room temperature for 5 min at 200xg.
- (vii) Aspirate supernatant and resuspend pellet in 2ml of ModNB.
- (viii) Triturate DRGs by using a series of polished Pasteur pipets each with a smaller diameter opening. Triturate for 10 times and allow large DRG

fragments to settle. Remove the supernatant with dissociated cells and place in a new 15ml tube. Suspend DRG fragments in 2ml of ModNB and continue trituration for another 10 cycles. Again, the media containing any DRG fragments is allowed to settle and the supernatant is removed and added to the tube containing the previously dissociated cells. This process is repeated using smaller diameter pipets each time until no DRG fragments remain.

- (ix) Centrifuge the tube of dissociated DRG neurons at 200xg for 5 mins at room temperature, remove and discard the supernatant and resuspend the cells in 3ml of fresh ModNB (more or less media is required when not using 3 rats, generally 1ml per rat is sufficient).
- (x) Transfer the cell suspension with a Pasteur pipette onto a previously made continuous 30% Percol gradient (Amersham Biosciences) in 15ml conical tubes. This gradient is produced by mixing 3ml of 100% Percol solution with 7ml of 0.15% NaCl. A maximum of 1ml of the cell suspension is used per 10ml of Percol gradient.
- (xi) Centrifuge at 500xg for 20 mins at room temperature. Use a Pasteur pipette to carefully aspirate the DRG enriched pellet that has migrated through the 30% Percol gradient. Be very cautious not to take an excessive amount of the surrounding Percol solution nor to expel any air that could disturb the pellet.
- (xii) Transfer the cells to a new 15ml tube filled with 12ml of Mod-NB and centrifuge at 200xg for 5 mins at room temp. Remove and discard supernatant and resuspend the cells in 1ml of Mod-NB.

3.4.3 Selection and culture (see Fig 3. 1)

- (i) Take previously coated and washed Dyna beads and place in the magnetic separator to allow adequate bead capture and remove any remaining supernatant. Add the cell suspension to the coated Dyna beads in a 1.5ml eppendorf tube and gently rotate at 4° C for 30 mins on the Nutator; flip the tube every 5 min to ensure that the beads do not settle.
- (ii) Remove tube with cell/bead suspension from the Nutator, place in the Dynal Magnetic Separator and allow to sit for a minimum of 1 min to ensure that all beads have been captured. Carefully transfer the supernatant to a new tube marked NS and place it back in the magnetic separator until ready for plating (NS contains the non-selected cell population that does not have any beads attached). Carefully resuspend the beads in 1ml of Mod-NB and gently invert to wash away any remaining non-selected cells. Place bead-bound cells back in the Dynal Magnetic Separator and allow to sit for a minimum of 1 min, and then remove and discard the supernatant.
- (iii) Resuspend the beads in 1ml of Mod-NB with 4ml of DNase and rotate on the Nutator at room temperature for 10 mins. Streptavidin is bound to the beads via a DNA cross linker, thus DNase treatment breaks the DNA cross-linked-Streptavidin from the beads, releasing the selected cells into the media.
- (iv) Remove tube from Nutator, gently pipet the cell/bead suspension up and down 5 times and place tube in the Dynal Magnetic Separator, allow the suspension to sit for a minimum of 1 min to ensure adequate bead capture. Because Streptavidin has been released from the beads via the DNase treatment, the

captured beads now will not be bound to any cells. Transfer the supernatant to a new tube labeled S and place it in the magnetic separator. This tube now contains the selected cell population with the beads removed.

- (v) To ensure that all selected cells have been released, add 500ml of Mod-NB to the original tube with beads and using a polished Pasteur pipet gently aspirate and expel the beads. Place them back into the magnetic separator, allow 1 min for adequate capture and transfer the supernatant to tube S; the supernatant will contain any remaining cells that have been released from the beads.
- (vi) Plate the selected cells from tube S and non-selected from tube NS (in separate wells) at a concentration of 100-150 cells per well in pre-coated 16 well chamber slides. In these experiments slides have been coated with 2x PL followed by 2x LN. Cultures were maintained for 24 hrs after plating for analysis of neurite growth.

3.4.4 Immunocytochemistry

- (i) Wash cells plated on 16 well chamber slides two times with 1xPBS for 5 mins and subsequently fix with 4% paraformaldehyde in 1xPBS for 15 mins.
- (ii) Three 5 mins washes in 1xPBS are subsequently followed by permeabilization with 0.1% Triton X-100 in PBS for 10 mins.
- (iii) For IB4 detection, wash 1 time for 5 mins with 1xPBS and subsequently replace PBS with the lectin binding buffer for 1hr to block non-specific

binding. When immunostaining for NF200, CGRP, Trk, p75, or other neuronal markers proceed to step (vi).

- (iv) Incubate with biotinylated IB4 (40mg/ml) overnight at 4° C for detection of IB4 +ve cell populations.
- (v) Perform three 5 min washes with 1xPBS, incubate for 1hr with Fluorescein-conjugated avidin to localize biotinylated-IB4. If not immunostaining, proceed to step (ix).
- (vi) For co-immunostaining, incubate with 10% NGS in 1xPBS for 1hr.
- (vii) Incubate cells with desired antibody (1:250-1:1000, eg. pan Trk 1:250, Santa Cruz Biotech, Santa Cruz, CA) overnight at 4° C.
- (viii) Perform three 5 min washes with 1xPBS, incubate with Cy2 or Cy5 labeled secondary antibodies for 1hr at room temperature to localize primary marker. Note – for double labeling, use of Cy2 and Cy3 is not recommended due to overlap in the spectra.
- (ix) Perform three 5 min washes with 1xPBS, and subsequently mount a coverslip on the slide with 50% glycerol in 1xPBS and seal with clear nail polish.
- (x) Stained cells were viewed and images captured using an Olympus laser-scanning microscope at 20x magnification equipped with the appropriate filters to view Fluorescein-conjugated avidin, Cy2, and Cy5.

3.5 Results

The protocol is summarized in Fig 3. 1. With the use of IB4-coated magnetic beads we were able to obtain a pure culture of IB4+ve DRG neurons. Fig 3.2A shows

Figure 3.2. IB4-selected and non-selected cell populations plated on LN. The IB4+ve cells that were selected using magnetic beads and the remaining non-selected IB4-ve cells were plated on 50µg/ml LN. Live cell phase microscopy was performed immediately after selection before bead release (A) or 24 hrs after plating (B, C). **A:** IB4+ve cell during selection before bead release with Dyna beads attached to its surface. Scale bar – 100 µm. **B-C:** Selected IB4+ve and non-selected IB4-ve cells plated on laminin for 24 hrs. Scale bar – 50 µm.

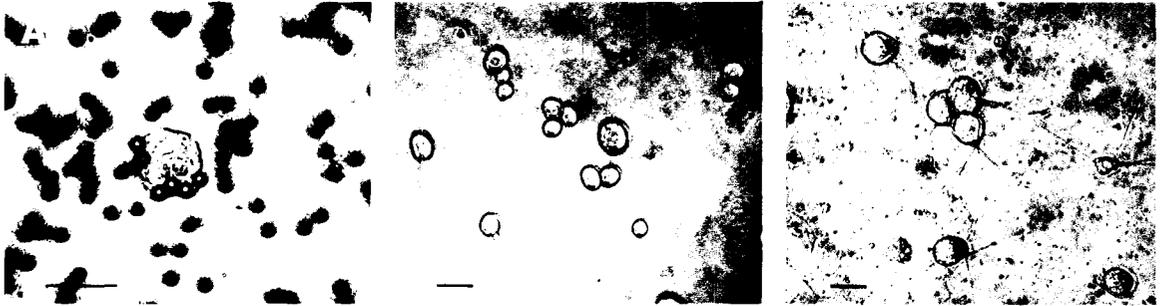


Fig 3.2

the typical IB4+ve cell that had IB4-coated beads attached to its surface. It is apparent that the IB4-coated beads are attached to the cell as they are in focus at the upper field of view whereas other unattached beads sitting on the surface of the plate are out of focus. After selection, both selected IB4+ve and non-selected IB4 negative (IB4-ve) cells were plated on LN. These cultures were grown at 37 ° C for 24 hrs and later imaged using phase microscopy. The IB4-selected population contained mainly small, with few large, diameter neurons (Fig 3.2 B) while the non-selected cells ranged in size, with some small but mainly medium and large diameter neurons (Fig 3.2C). These observations are in accordance with previous reports which suggest that the IB4+ve population is mainly small with a limited number of large diameter neurons (Bennett et al., 2000; Priestley et al., 2002a). Also the non-selected population is made up of small and medium CGRP+ve neurons as well as medium and large NF200+ve neurons (Averill et al., 2001; Gavazzi et al., 1999; Priestley et al., 2002a). To validate if cultures of selected and non-selected cells were made up of homogenous populations, the cells were co-labeled with IB4 and a pan Trk (directed against Trk A, B, & C) antibody and imaged using confocal microscopy (Fig 3.3). As shown in Fig 3.3, the IB4-selected cells only show positive labeling for IB4 and do not show Trk staining (Fig 3.3, A-C). Conversely cells in the non-selected condition only show Trk positive staining and do not show positive IB4 labeling (Fig 3.3, D-F). Thus, we are confident that the selection protocol is functioning exactly as expected. The IB4-selected population consists of IB4+ve cells with no detectable contamination of Trk+ve (Fig 3.3) nor P75+ve neurons (data not shown). The

Figure 3.3. IB4-selected and non-selected cell populations plated on LN. The IB4+ve cells that were selected using magnetic beads and the remaining non-selected IB4-ve cells were plated on 50 $\mu\text{g/ml}$ LN. **A-C:** Selected IB4+ve cells plated on LN, Co-labeled with IB4 and a panTrk antibody 24 hrs after plating. **A:** Positive IB4 labeling. **B:** Trk immunostaining shows no co-labeled cells. **C:** Merged confocal image of IB4 and Trk labeling together. These three panels show that selected IB4+ve cells do not show significant Trk staining, although they are positively labeled with IB4. **D-F:** Non-selected IB4-ve cells plated on LN and co-labeled with IB4 a panTrk antibody 24 hrs after plating. **D:** IB4 labeling shows no labeled cells. **E:** Positive Trk immunostaining. **F:** Merged confocal images of both Trk and IB4 labeling together. These three panels show that non-selected IB4-ve cells show significant Trk staining but display no IB4 labeling. Scale bar – 50 μm .

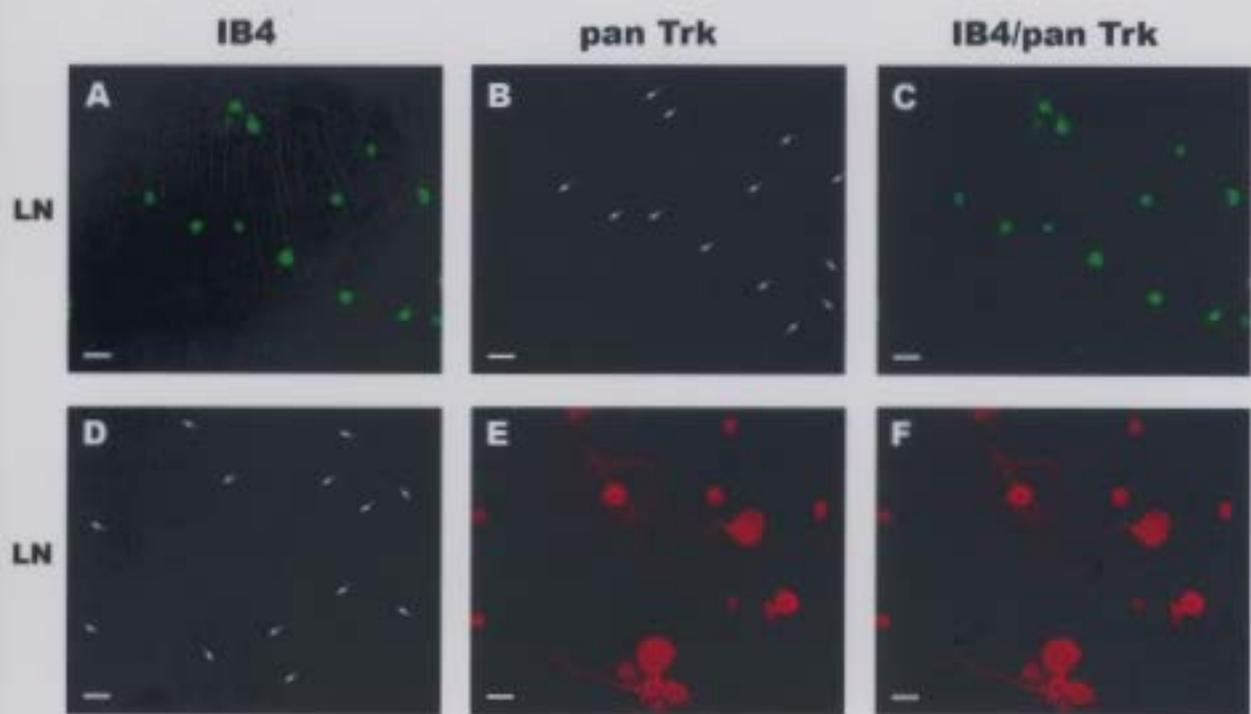


Fig 3.3

non-selected population shows little or no residual IB4+ve cells. As previously reported, IB4+ve neurons do not generally express the Trk nor the p75NTR family of receptors (Averill et al., 1995; Priestley et al., 2002a).

To show that these selected IB4+ve neurons are alive and responsive, IB4-selected neurons were plated on LN in the presence or absence of NGF or GDNF. With the absence of Trk and p75NTR these cells should not respond to NGF. However, these cells do express GFR α 1 and RET and are therefore responsive to GDNF (Averill et al., 1995; Priestley et al., 2002a). As shown in Fig 3.4 IB4+ve cells plated in the absence of added growth factors (Fig 3.4 A) or in the presence of NGF (Fig 3.4 B) had no significant neurite outgrowth. However, when GDNF was added IB4+ve cells responded with a robust increase in neurite outgrowth, indicating that these are viable and healthy neurons (Fig 3.4 C).

Figure 3.4. IB4-selected cell population plated on LN in the presence or absence of added growth factors and labeled for IB4. IB4+ve cells were plated on 50 $\mu\text{g/ml}$ of LN in the presence or absence of added growth factors (NGF or GDNF). Cells were fixed and labeled with IB4. IB4+ve cells do not show significant neurite outgrowth when plated on LN (A) in the absence of added growth factors, nor is there any stimulation of growth with NGF (B). However, GDNF does elicit significant growth from these IB4+ve cells when plated on LN (K). Scale bar – 50 μm .

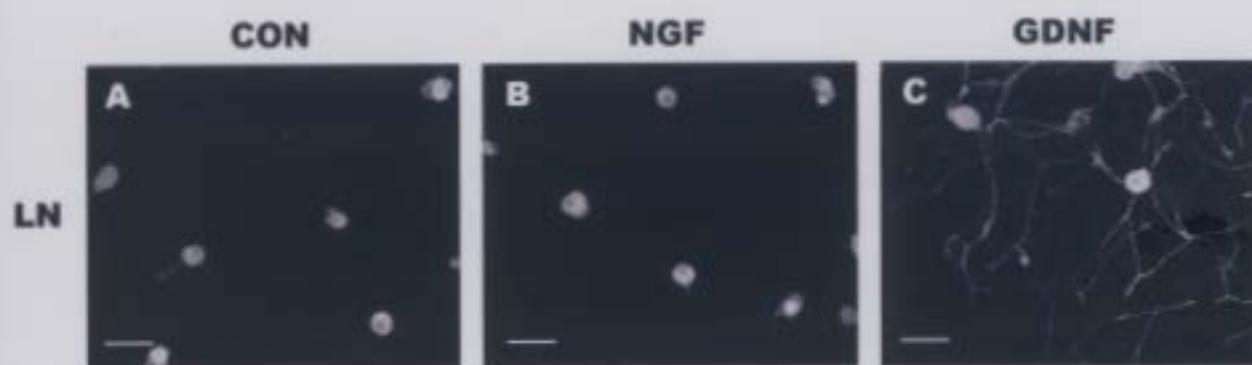


Fig 3.4

3.6 Discussion

3.6.1 Overall assessment of protocol

Normal cultures of DRG neurons are typically generated by whole ganglion dissociation resulting in the presence of each of the subpopulations of DRG neurons. Our results illustrate that pure subpopulations of viable DRG neuron cultures can be reproducibly generated by the use of magnetic beads from Dynal. This technique offers new possibilities in the study of survival and regeneration of sensory neurons. As different environmental conditions, such as availability of growth factors and extracellular matrix molecules, elicit different responses from different DRG subpopulations it is important to be able to study each population separately (Averill et al., 1995; Gavazzi et al., 1999; Priestley et al., 2002b). Although we have detailed methods for selection of IB4+ve cells, we have also used this method for selecting p75NTR+ve cells (using anti mouse-IgG coated Dyna beads) and Trk+ve cells (using biotinylated anti rabbit antibodies and streptavidin coated Dyna beads). The only difference in these selections is that either streptavidin or anti mouse-IgG coated beads are used.

3.6.2 Trouble-shooting

A key factor for successful DRG selection is that the rotation and washing steps are done gently. If these steps are not done carefully, then the cells will be released from the beads resulting in a low yield of selected neurons. At the same time care must also be taken to do the procedure as quickly as possible, for if long periods of time are taken the viability of the cells drop dramatically. For this reason it is not recommended that any more than 4 rat dissections be carried out at once. Also care has to be taken when

removing the DRGs to ensure that the roots are carefully trimmed away, as contaminating non-neuronal cells will interfere with the selection process (i.e. they may express the same cell surface marker as the cells you are trying to capture). It is also important to keep all dissection media ice cold. When performing the enzymatic dissociation in section 3.2, step (V), it is important that DNase is not used or does not come in contact with beads or cells to be selected. DNase will release cross-linked avidin from the beads releasing the biotinylated IB4 and preventing cellular selection. Finally, during the selection process when the coated beads are being incubated with the cell suspension, it is important that the beads are not allowed to settle in the bottom or on the side of the tube (the beads are relatively heavy and tend to settle very quickly). Thus, for the 30 min rotation period carefully turn the tube over every 5 mins gently dislodging any beads that may have settled.

3.6.3 Alternative and support protocols

The enzymatic dissociation and purification procedure in section 3.2 (iv-xi) can be replaced with previously described procedures (Mearow and Kril, 1995).

- Enzymatic dissociation using 1ml of dissociation media containing collagenase and place in a 37° C water bath for 40 mins, wash 3 times with 1xHBSS, resuspend in 500ml Trypsin 500ml HBSS and place in a 37° C water bath for 20 mins, tap the tube every 5 mins in each step to ensure that DRGs receive adequate exposure (iv).
- Filter the cell suspension through a 70mm nylon filter (Falcon, BD Biosciences) to remove debris, allowing the suspension to be used for bead selection (xi).

Cultures prepared in this way have less chance for cell loss because more vigorous enzymatic dissociation has been used and the percol step has been eliminated. However, these cultures will contain more non-neuronal cells, which may pose a problem when selecting.

3.7 Quick procedure

3.7.1 Dyna bead coating and preparation

- (i) Wash 100 μ l of streptavidin coated beads with 1xPBS in a 1.5ml eppendorf tube by using a Dynal Magnetic Separator.
- (ii) Resuspend washed beads in 500ml of 1xPBS with 40mg of biotinylated anti-IB4. Rotate at room temperature for a minimum of 1 hr.
- (iii) Just prior to use aspirate and discard the supernatant, wash three times resuspend in 100ml of 1xPBS. Beads are now ready to be added to the cell suspension of interest.

3.7.2 DRG dissociation, selection, and culture

- (i) Remove DRGs place in a 35mm Petri dish of ice-cold neurobasal media supplemented with 1% penicillin/streptomycin.
- (ii) Wash DRGs with 10ml 1xHBSS.
- (iii) Resuspend DRGs in 1ml of dissociation media containing dispase and place in a 37° C water bath for 50 mins.
- (iv) Add 1ml FCS and 8ml of HBSS-M and centrifuge at room temperature for 5 mins at 200xg.

- (v) Resuspend pellet in 2ml of ModNB.
- (vi) Triturate DRGs 10 times and allow remaining DRG fragments to settle, move supernatant to a new tube and repeat until all remaining DRG fragments are dissociated.
- (vii) Centrifuge and resuspend the pellet in fresh ModNB.
- (viii) Transfer the cell suspension to a 30% Percol gradient in 15ml conical tubes and centrifuge. Aspirate enriched DRG fraction taking extreme caution not to take an excessive amount of the surrounding Percol solution or to disturb the pellet.
- (ix) Wash pellet in fresh ModNB, centrifuge and resuspend in 1ml of ModNB with previously coated Dyna beads and gently rotate at 4° C for 30 mins.
- (x) Capture selected cells using the magnetic separator, remove and plate the non-selected cells. Wash selected cells, add DNase, rotate at room temperature and remove and plate selected cells.

Fig 3.1 is a summary diagram of bead coating, cellular selection, capture, release, and plating of both selected and non-selected cell populations.

3.7.3 Immunocytochemistry

- (xi) Cells plated on glass slides are washed and fixed.
- (xii) After washing, cells are permeabilized with 0.1% Triton X-100 and blocked with a lectin binding buffer for IB4 labeling, or with 10% NGS in PBS for immunostaining.

- (xiii) Incubate at 4° C overnight with the biotinylated IB4 or with appropriate primary antibodies (i.e. Fig 3.2, pan Trk, 1:250, Santa Cruz).
- (xiv) After washing localize biotinylated IB4 with fluorescein-conjugated avidin or primary antibodies with appropriate Cy2 or Cy5 secondary markers (1 hr at room temperature).
- (xv) Wash slides with 1xPBS, and mount coverslips with 50% glycerol, seal with clear nail polish.
- (i) Visualize using a laser-scanning confocal microscope equipped with the appropriate filters.

CHAPTER 4

LAMININ AND GROWTH FACTOR INDUCED NEURITE GROWTH IS DIFFERENTIALLY REGULATED IN SUBPOPULATIONS OF ADULT DRG NEURONS VIA THE PI 3-K/AKT AND MEK/MAPK SIGNALLING PATHWAYS

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4.1 Introduction

The adult DRG is made up of a heterogeneous population of cells subdivided on the basis of neurochemistry, morphology, trophic requirements, and sensory modalities (Averill et al., 1995; Gavazzi et al., 1999; Petruska et al., 2000; Priestley et al., 2002a). Three major cell groups have been identified with potential for further subclassification. The first group comprises the large and medium diameter neurons, identified based on expression of heavy chain neurofilament (NF200+ve) and typically have myelinated axons. These neurons express the p75 neurotrophin receptor and TrkC, although TrkA and TrkB receptors are also reported (Averill et al., 1995; Priestley et al., 2002a). Thus, these cells are able to respond to NT-3, NGF, and BDNF. The next two major groups consist of mainly small diameter neurons and are considered to be either peptidergic, expressing calcitonin gene related peptide (CGRP+ve), or non-peptidergic, binding the lectin *Griffonia Simplicifolia* IB4 (Averill et al., 1995; Priestley et al., 2002a). These groups are considered to be unmyelinated or thinly myelinated nociceptive afferents. The CGRP+ve population expresses a high level of both p75 neurotrophin receptor and TrkA and preferentially respond to NGF. The IB4-binding population has neither p75 nor Trk

receptors, but instead expresses the receptor tyrosine kinase RET and the GFR α 1 subunit, making these cells responsive to GDNF and unresponsive to the neurotrophins NGF, NT3, and BDNF (Bennett et al., 1998; Kashiba et al., 2001; Molliver et al., 1997).

In the current study we demonstrate that these cellular differences are important when attempting to stimulate axonal regeneration from these neurons. We have previously shown that both NGF and laminin (a major component of the peripheral extracellular matrix) are strong promoters of neuronal regeneration in vitro (Jones et al., 2003; Tucker et al., 2005a). These factors can work in conjunction with each other to stimulate neurite growth not achievable by either factor alone, an effect which was shown to be in part due to crosstalk between integrin and growth factor signalling pathways (Tucker et al., 2005a). However, laminin-induced integrin activation (in both the presence and absence of added NGF) was not sufficient to promote neurite growth from all DRG neurons, despite the fact that laminin-binding integrins are expressed in the majority of these cells regardless of subpopulation (Condic and Letourneau, 1997; Tomaselli et al., 1993). Thus, it was hypothesized that laminin induced neurite growth is population specific, and in part due to the differential requirement for specific signalling pathways by the different neuronal subpopulations.

To test this hypothesis we have focused on the growth response of selected subpopulations of adult DRG neurons with respect to laminin and growth factor receptor activation, using a magnetic bead-based selection protocol (Tucker et al., 2005b). Because adult DRG neurons survive in the absence of added trophic factors they make an ideal model for studying the effects of neurotrophins and extracellular matrix molecules on peripheral nervous system regeneration (Dodge et al., 2002; Tucker et al., 2005a).

Our results indicate that laminin-induced neurite growth initially occurs in the NF200+ve cell population, followed by a delayed growth response from the CGRP+ve cells; because both NF200+ve and CGRP+ve populations are known to be responsive to NGF to avoid further confusion these cells are identified as the NGF-responsive population (Chattopadhyay *et al.*, 2003). In contrast, laminin alone is insufficient in stimulating significant neurite growth in the IB4+ve cell population, although this substrate is still required to elicit a growth response from these cells in the presence of the appropriate growth factor. In analysing potential downstream signalling pathways we demonstrate a requirement of only the PI 3-K/Akt pathway for neurite growth from the NGF-responsive cell population, while neurite growth in the IB4+ve cell population required both PI 3-K/Akt and MEK/MAPK signalling. While we have previously published data showing a role for laminin and the PI 3-K signalling in DRG neurite growth, for the first time (to our knowledge) we are now able to show very distinct responses from selected populations of sensory neurons, responses that are masked by the usual culture models of these cells. Of particular significance is the fact that the small nociceptive GDNF responsive cells do not appear to be able to respond with neurite growth to any of the extracellular matrix molecules usually found in the peripheral nerve, which is in contrast to the situation observed with both NGF-responsive nociceptive and proprioceptive neurons. It is also important to note that this is the first time that neuronal subpopulations within the adult DRG have been identified as having different signalling requirements for axonal regeneration. Therefore, these results are important for the development of potential therapies for nerve regeneration after injury or in peripheral neuropathies such as those associated with diabetes.

4.2 Materials and methods

4.2.1 Neuronal culture

Dorsal root ganglia (DRG) from young adult (4-6 wk) Sprague-Dawley rats (Memorial University of Newfoundland Vivarium and Charles River Canada, Montreal, QC) were dissected and dissociated. In accordance with University Animal Care guidelines, animals were live decapitated, ganglia from all spinal levels were removed, and the roots were trimmed. To isolate individual DRG neurons, both enzymatic and mechanical dissociation was used. Enzymatic dissociation was carried out first and performed by incubating the ganglia in 0.25% collagenase for 45 min, and 0.25% trypsin for 40 min (Invitrogen/ Gibco BRL, Burlington, Ont). Mechanical dissociation was carried out next and performed by repeated cycles of aspiration and expulsion of the DRG fragments through a flame narrowed pasture pipette. Upon completion, dissociated DRG neurons were suspended in serum-free Neurobasal medium (NB, Invitrogen) supplemented with 100U penicillin/streptomycin, B27 supplement (Invitrogen), and 20mM cytosine arabinoside (modified NB). This suspension was then layered on top of a 30% Percoll solution (Amersham Bioscience, Baie d'Urfe, QC) in 15ml conical tubes and centrifuged at 400g for 20 min at room temperature. Pellets were then carefully extracted with a sterile pasture pipette, placed in a fresh 15ml tube, washed with the previous suspension media, and centrifuged to remove any remaining Percoll. Neurons were plated in either Lab-Tek 16-well chamber slides (Nunc International, Naperville, NC) for neurite growth assessment or 12-well plates for Western blot analysis, and incubated at 37°C with 95% O₂ and 5% CO₂ for 24 and 48 hrs. Slides and culture plates were coated

twice with poly-D-lysine (PL, 1 µg/ml, BD Bioscience, Bedford, MA), laminin, (LN, 1 µg/ml, 10 µg/ml, 25 µg/ml or 50 µg/ml, Invitrogen), collagen (COL, 50 µg/ml, Sigma), or fibronectin (FB, 50 µg/ml, Gibco). The neurons were cultured in modified serum-free NB alone or supplemented with NGF (25 ng/ml, Cedarlane Labs, Hornby, ON), or GDNF (25 ng/ml, Cedarlane Labs).

4.2.2 Cell Selection

Specific cell populations were selected as discussed in chapter 3; Briefly streptavidin-coated beads were incubated with biotinylated *Griffonia Simplicifolia* IB4 lectin (Sigma) for 2 hrs at room temp by gentle rotation. Beads were then washed removing any excess lectin and added to the cell suspension. This suspension of cells and Dynal beads was incubated at room temp for 30 min followed by bead capture with a Dynal magnetic separator. The supernatant was removed and plated as the non-selected NGF-responsive cell population, which included both NF200+ve and CGRP+ve cells. Next the beads and selected cells were washed and further incubated with DNase for 15 mins to release the selected cells from the beads. These cells were then plated as a homogenous selected IB4+ve cell population (purity \geq 95%, with $<$ 2% non-neuronal cells).

4.2.3 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde (pH 7-7.4) in PBS for 15 mins, permeablized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS. For neuronal and neuritic identification, antibodies directed against peripherin (1:250),

PGP 9.5 (1:250, Chemicon, Temucua, CA), and tubulin (1:1000, Sigma) were employed. To identify cellular subpopulations antibodies directed against heavy chain neurofilament NF200 (1:1500, RT97, University of Iowa Developmental Biology Hybridoma Bank), calcitonin gene related peptide (1:1500, CGRP, Sigma), panTrk (1:250, Santa Cruz, Santa Cruz, CA) and a biotin-conjugated lectin IB4 (1:250, Sigma) were used. Cells were incubated with the primary antibodies at 4°C for 16-20 hrs, followed by Cy2- or Cy5-tagged secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA) or HRP-DAB detection; in the case of IB4-detection FITC-Streptavidin was used. These slides were cover slipped with glycerol and imaged with light or confocal laser scanning microscopy.

4.2.4 Enzyme Inhibition

Signalling pathways were inhibited by the use of specific pharmacological inhibitors. Inhibitors targeting PI 3-K (LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one, Calbiochem), Akt (Akti, 1L-6-hydroxymethyl-chiro-inositol 2-[(R)-2-0-methyl-3-0-octadecylcarbonate, Calbiochem), and MEK (U0126, 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene, Calbiochem) were added to the culture media at the time of plating, thus the cells were treated for 24 hrs. The cultures were fixed and labelled for the appropriate markers.

4.2.5 Measurements of Neurite Growth

Neurite initiation (neuritogenesis) was first quantified by determining the percentage of total tubulin stained neurons with neurites twice the length of their cell

body diameter. Growth was assessed at 24 hrs after plating. Individual neuron tracings of DAB-stained neurons were carried out using the NeuroLucida® (MicroBrightField, VT) tracing program. Only neurons for which we were able to identify unambiguously the associated neurites or neuritic networks were chosen for tracing analysis. Data analyses were carried out with the NeuroExplorer® software package; here Sholl analysis (intersection point and length) and total neurite length data were collected. Sholl analysis measures both the number of intersection points of axons crossing 20 μm concentric circles radiating from the cell body and the length of the neurites within each concentric circle, thus giving a measure of both elaboration and total growth of the neurites. Total neurite length is a measure of the cumulative length of neurites produced. Data were imported into a graphing and statistical analysis software program (Prism 4, GraphPad Corp) for further analyses. For each condition examined (control-PL +NGF or -NGF, LN +NGF or -NGF) 50 neurons were completely traced, with cells pooled from approximately 3-6 different plating experiments. A minimum of 800 cells was analyzed for the entirety of this study.

4.2.6 Immunoblotting

For Western analyses, neurons were plated in 12-well plates that had been coated with PL or LN (25 $\mu\text{g}/\text{ml}$, Invitrogen) in the presence or absence of NGF (25 ng/ml , Cedarlane Labs), or GDNF (25 ng/ml , Cedarlane Labs). Neurons were subsequently processed at 24 hrs after plating by removing the original plating media, adding 300 μl of PBS containing 0.5 mM Orthovanadate to each well, and scraping the cells off the plating surface using a beveled tip rubber scrapper. Collected samples were briefly centrifuged

to pellet the cells, supernatant was removed, and the cells were lysed by gentle rotation overnight using 50 μ l of lysis buffer containing TBS with 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate. Following cell lysis and centrifugation (10,000 rpm, 5 min), the supernatants were used to determine protein concentrations using a BCA protein assay (Pierce Chemicals, Rockford, IL.). Equivalent amounts of protein (50 μ g) were subjected to SDS-PAGE (8%-10% acrylamide). Following transfer to nitrocellulose, the blots were stained with Ponceau Red, photographed, assessed for the equivalency of protein loading, and cut to allow the top and bottom portions of the blot to be probed simultaneously. Ponceau Red was subsequently removed using Tris-Buffered Saline and the blots were blocked with 3% milk in Tris-Buffered Saline with 0.2% Tween-20, and subsequently incubated with the following antibodies (1:1,000) overnight at 4°C: with β 1 integrin (1:1000, Santa Cruz, Santa Cruz CA), and Actin (1:1000, Sigma). The next day, blots were washed, incubated in the appropriate horseradish peroxidase conjugated secondary antibody (anti-rabbit or anti-mouse IgG- HRP; Boehringer Mannheim, 1:5,000), and visualized using chemiluminescence reagents (ECL, NEN, Boston, MA) and exposure to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC). Developed films were subsequently digitized and densitometrically analyzed using the Image J software (NIH image downloadable software). β 1 integrin was normalized against actin, and digital images of the blots were used to make composite figures with Adobe Photoshop graphics software (Adobe Corp, Mountain View CA).

4.2.7 Statistical Analysis

All reported data analyses are based on a minimum of 3 separate experiments for each manipulation. Data are plotted as mean \pm SEM and significance is noted only if $p < 0.05$ as determined by ANOVA using Tukey testing for post hoc comparisons for multiple group analyses.

4.3 Results

4.3.1 DRG neurons plated on increasing concentrations of laminin show increased neurite growth and increased expression of $\beta 1$ integrin

In these experiments, we were interested in the effects of both the extracellular matrix molecule laminin (LN) and the growth factor NGF on neurite growth. Briefly DRG neurons were plated on either poly-D-lysine (PL, lacking any sequence required to produce integrin activation) or increasing concentrations of LN (which will activate integrins) in the presence or absence of NGF. To allow for neuronal visualization cells were fixed and immunostained 24 hrs after plating for tubulin and PGP 9.5. When cells were plated on increasing concentrations of LN, from 0 (PL) to 50 $\mu\text{g/ml}$, greater amounts of neurite growth were observed (Fig 4.1 A-E). Further increases in neurite growth were achieved by plating the DRG cells in the presence of added NGF (Fig 4.1 F-J). Optimal growth conditions existed when cells were plated on 50 $\mu\text{g/ml}$ of LN in the presence of 25 ng/ml of NGF (Fig 4.1 J).

We also examined $\beta 1$ integrin subunit expression, and observed a significant

Figure 4.1. Increasing concentrations of laminin stimulates increased neurite growth and $\beta 1$ integrin expression in adult DRG neurons. A-J: Neurons were plated on poly-D-lysine (PL, A, F), or laminin (LN) at concentrations of 1 $\mu\text{g/ml}$ (B, G), 10 $\mu\text{g/ml}$ (C, H), 25 $\mu\text{g/ml}$ (D, I), and 50 $\mu\text{g/ml}$ (E, J). They were subsequently fixed and immunostained for anti-peripherin as outlined in the methods. Confocal laser scanning microscopy was used to visualize stained neurons 24 hrs after plating. Increased neurite growth was seen with increasing concentrations of LN with maximal growth observed at 25-50 $\mu\text{g/ml}$. Scale bar – 20 μm . K-L: Western blots of neurons plated on PL or previously stated LN concentrations in the absence (K) or presence (L) of NGF. Blots were probed for $\beta 1$ integrin and actin. Significant increases in $\beta 1$ expression were observed with increasing LN concentrations. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

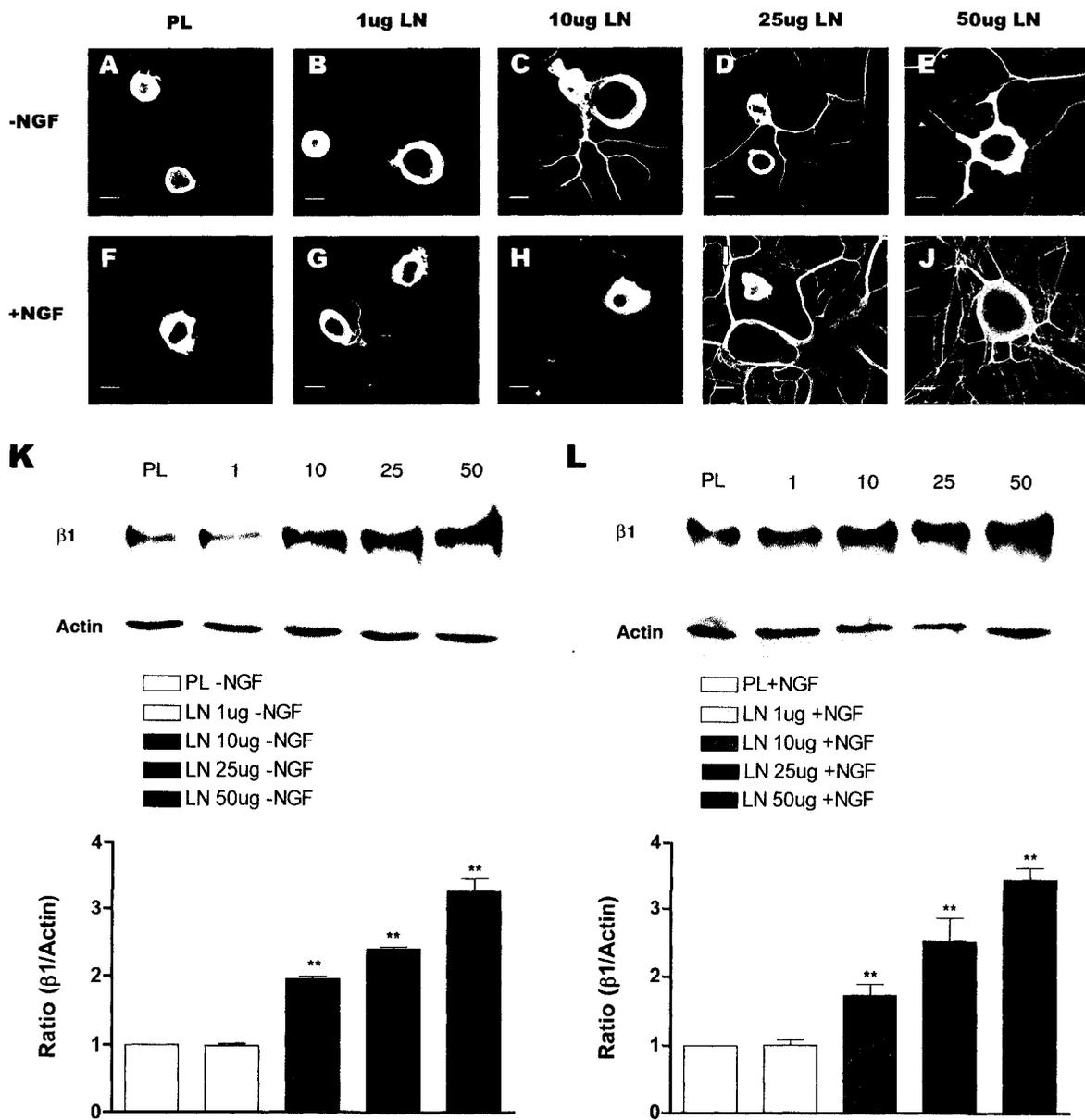


Fig 4.1

increase in $\beta 1$ integrin expression in cells in response to increasing LN concentrations in both the presence and absence of NGF (Fig 4.1 K-L, * $p < 0.05$, ** $p < 0.001$). The first significant increase in integrin expression was seen at 10 $\mu\text{g/ml}$ with a further increase at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$. Similar results have been reported for the effect of LN and NGF on embryonic DRG neurons (Condic and Letourneau, 1997); it has also been noted that increasing integrin expression in adult DRG neurons can enhance neurite growth (Condic, 2001).

4.3.2 Laminin-induced neurite growth at 24 hrs after plating is most extensive in NF200+ve DRG cells

We had noticed both here and in previous studies that not all neurons plated on LN, or those that underwent soluble LN stimulation, responded with significant neurite growth. As shown in Fig 4.2, in this mixed population of cells there are some neurons that put out neurites on LN in the absence of NGF (Fig 4.2C, arrows), and when NGF is added, there is an enhancement of growth in some neurons (Fig 4.2D arrows), although others fail to respond (Fig 4.2D, arrowheads). To further investigate this, we first sought to identify the responsive cells initially using immunocytochemistry for markers reported to be relatively specific for different subcategories of DRG neurons. Cells were plated on LN or PL in the presence or absence of NGF, and fixed and immunostained for NF200 (RT97) which tends to label the medium and large myelinated neurons (Priestley et al., 2002a) and CGRP, which is selective for smaller nociceptive myelinated and unmyelinated neurons (Priestley et al., 2002a). As shown in Fig 4.3, at 24 hrs after

Figure 4.2. Not all DRG neurons respond to laminin with increased neurite growth.

Neurons were plated on either PL (A, B), or LN, 50 $\mu\text{g/ml}$ (C, D) in the presence (B, D) or absence (A, C) of NGF. They were fixed, immunostained for PGP 9.5, and confocal laser scanning microscopy was used to visualize neurons 24 hrs after plating. A subpopulation of DRG neurons responds to LN-induced integrin stimulation with increased neurite growth (C, arrow) while other cells remain unaffected (C, D, arrowheads). The growth response can be further enhanced with the addition of NGF (D, arrow). Scale bar – 50 μm .

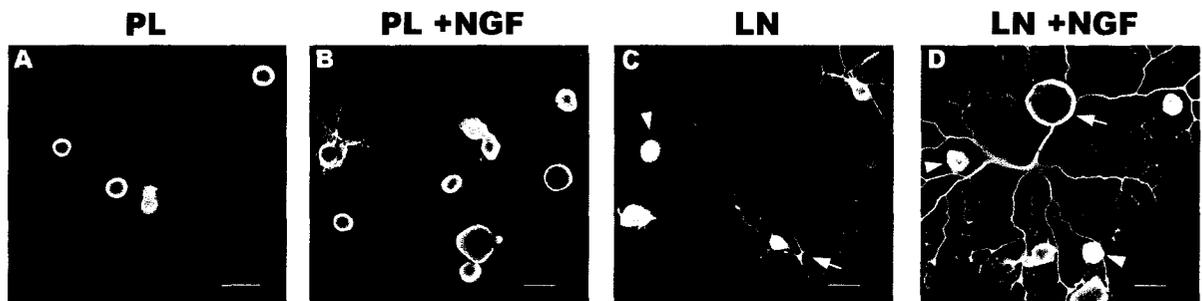


Fig 4.2

Figure 4.3. Laminin-induced neurite growth is most extensive in the NF200+ve DRG neurons. Neurons were plated on PL or LN (50 $\mu\text{g/ml}$) in the presence or absence of NGF. At 24 hrs after plating cells were fixed and immunostained for anti-CGRP (green) and anti-NF200 (red). **A-D:** NF200+ve (red) neurons plated on LN show a robust increase in neurite growth (B, arrowhead), which was potentiated by the addition of NGF (D, red cell). CGRP+ve neurons (green) show only small amounts of neurite growth when plated on LN alone, however plating them in the presence of NGF did stimulate increased neurite growth (D, green cell and arrowheads) that was not observed when plated on PL plus NGF. Scale bar – 50 μm .

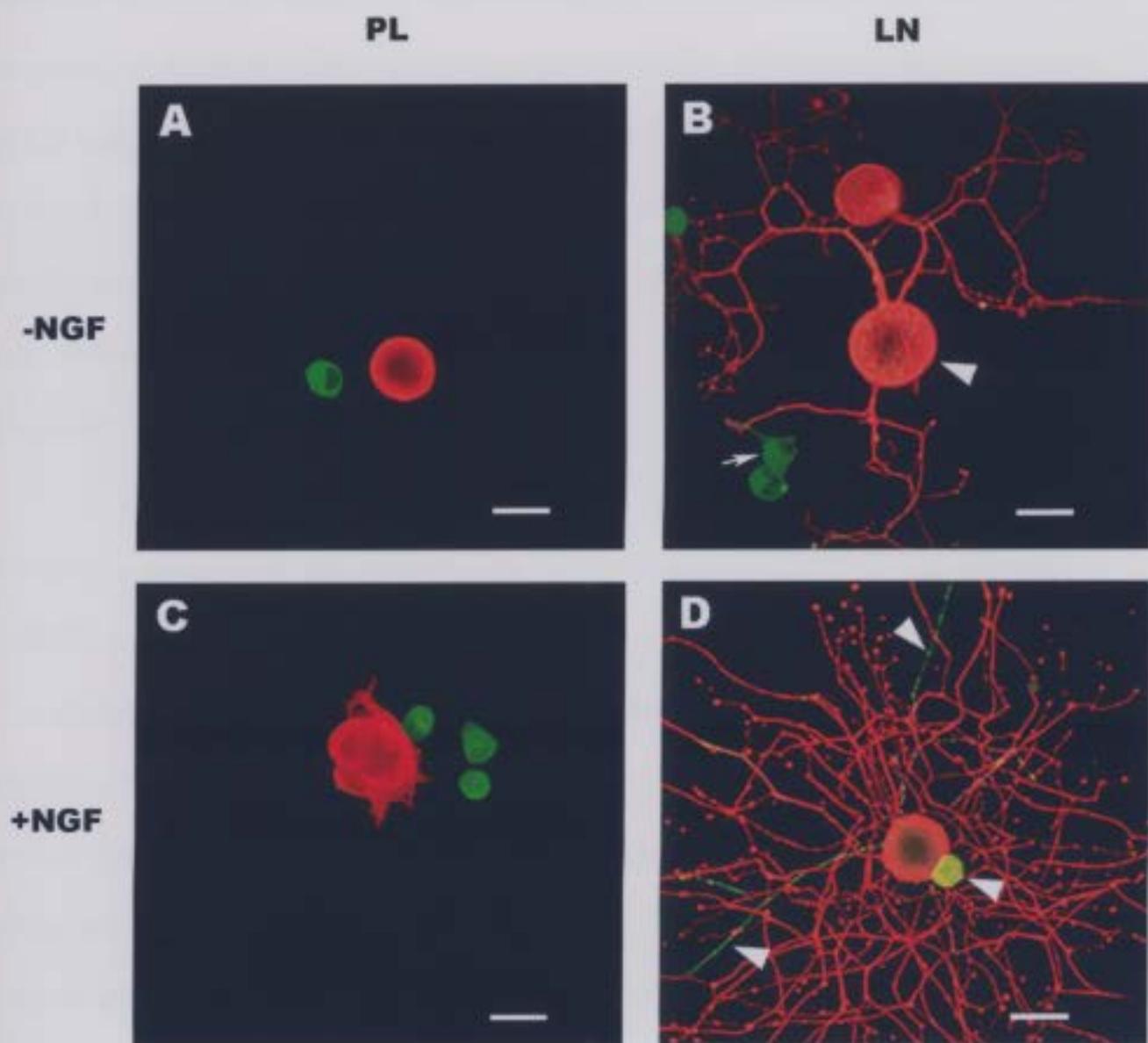


Fig 4.3

plating NF200+ve neurons (red) exhibit extensive neurite growth on LN (Fig 4.3 B), which was further increased by the addition of NGF (Fig 4.3 D). In contrast, the small CGRP+ve cells (green) show only small amounts of neurite growth on LN alone at this time point (Fig 4.3 B, note arrow pointing to green cell with some small neurites; also see Fig 4.6 below for 48 hr growth on LN), but did show extensive neurite growth in response to NGF (Fig 4.3 D, note green neuron and associated neurites, arrowheads). They did not, however, appear to respond to NGF with neurite growth at this time point when plated on PL (Fig 4.3 C) (further analysis of neurite growth for these cell populations can be found in Fig 4.10).

4.3.3 IB4+ve neurons do not respond with increased neurite growth in the absence of GDNF

Because the DRG neuronal population is heterogeneous, with cells selectively responding to at least LN and various trophic factors, we decided to undertake a cellular selection protocol in order to further examine factors contributing to growth in the specific populations. In order to do this we utilized Dynal magnetic beads coated with the ligand of interest (Tucker et al., 2005b). Briefly, streptavidin-coated magnetic beads were washed and coated with biotinylated lectin IB4. The beads were then incubated with the dissociated neuronal suspension in order to capture IB4+ve cells. Bead-bound cells were then isolated by being placed in a magnetic separator and the supernatant was removed. This allowed us to culture the selected IB4+ve population separately from the remaining non-selected population, which were termed the NGF-responsive cell population and consisted of the NF200+ve and CGRP+ve neurons. We opted for the

selection of the IB4+ve cells for a number of reasons, not the least of which was the lack of other appropriate cell surface markers compatible with the available Dynal beads. At the time we carried out these experiments, only anti-mouse IgG-coated beads (and not anti-rabbit IgG) were available, and although we could select p75+ve neurons using a monoclonal antibody, because of the lack of monoclonal antibodies against the external portion of the Trk receptors we were unable to select out specific Trk-expressing populations.

Selected IB4+ve and non-selected NGF-responsive cells (NF200+ve and CGRP+ve cells remaining in supernatant after magnetic bead capture) were then plated on PL or LN in the presence or absence of NGF or GDNF for 24 hrs. Cells were subsequently fixed and labeled with IB4 and immunostained using a pan-Trk antibody. As shown in Fig 4.4, IB4+ve cells (green) show little or no neurite growth when plated on PL (Fig 4.4 A, PL-S) or LN (Fig 4.4 C, LN-S). Furthermore, as might be expected, the IB4+ve cells did not respond to NGF on either PL (Fig 4.4 E) or LN (Fig 4.4 H). Since IB4+ve neurons are reported to be GDNF-responsive (Bennett *et al.*, 1998; Paveliev *et al.*, 2004), we treated neurons on either PL (Fig 4.4 I) or LN (Fig 4.4 K) with GDNF and observed that GDNF elicited neurite growth only from neurons plated on LN (Fig 4.4 K). Because of the apparent lack of response of the IB4+ve neurons to the LN substrate alone, we asked whether other ECM molecules that are known to be present within the PNS were better able to promote neurite growth from this population of cells. We thus plated the selected IB4+ve population of cells on fibronectin (FB) or collagen-IV (Col) coated slides and assessed the amount of growth in the presence or absence of

Figure 4.4. IB4+ve and NGF-responsive cell populations labelled for IB4 and immunostained with a pan Trk antibody. The IB4+ve cells that were selected using magnetic beads (as outlined in Methods) and the remaining non-selected NGF-responsive cells were plated on PL or 50 μ g/ml LN in the presence or absence of added growth factors (NGF or GDNF). Cells were fixed and labelled with biotinylated IB4 lectin (green) or immunostained using a pan Trk antibody (red). IB4+ve cells do not show significant neurite growth on PL (A) or LN (C), nor is there any stimulation of growth with NGF (E, G). However, GDNF does elicit significant growth from these IB4+ve cells when plated on LN (K). NGF-responsive cells show significant neurite growth when plated on LN (D), which was potentiated by the addition of NGF (H). However, these cells were not responsive to GDNF (L). Scale bar – 50 μ m.

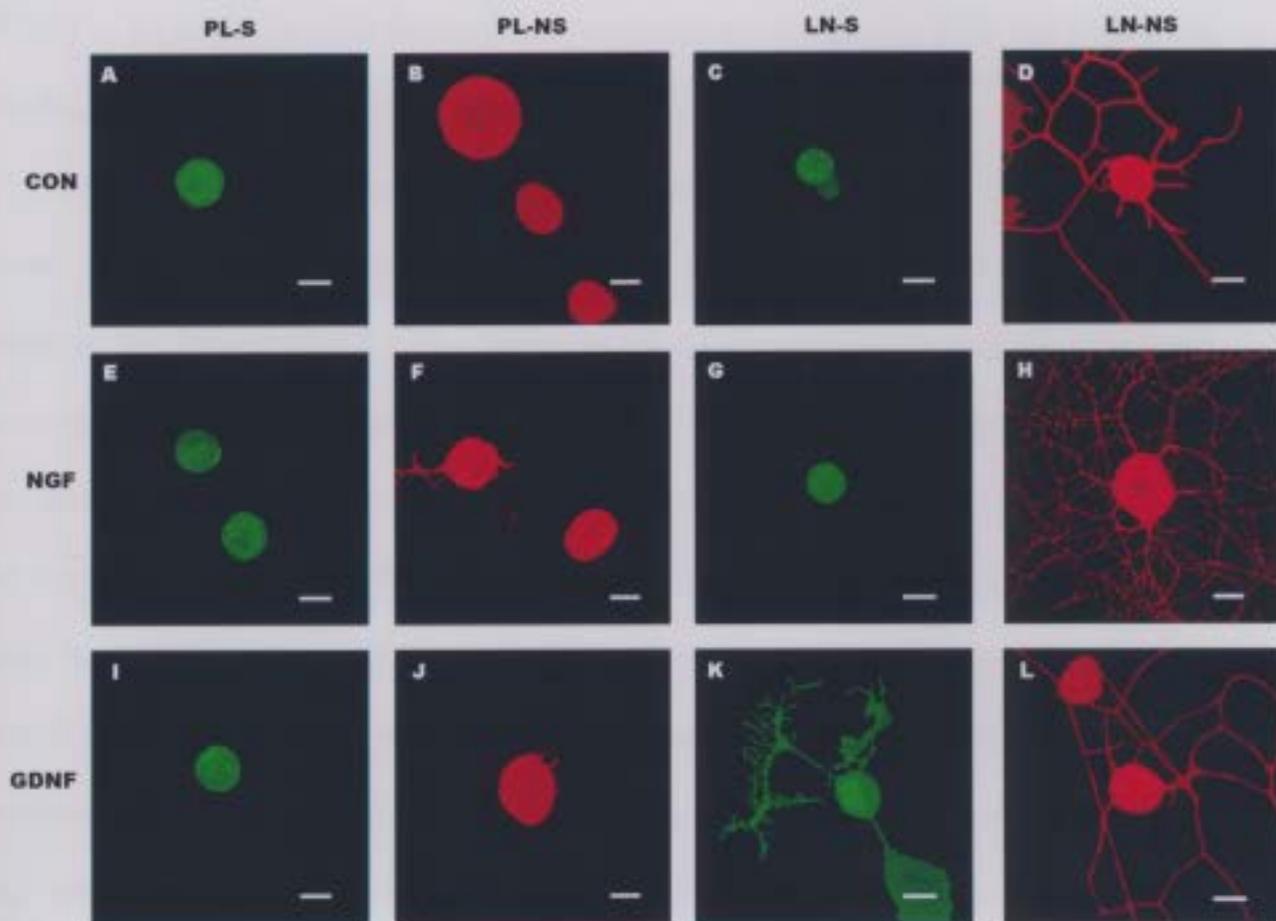


Fig 4.4

added NGF or GDNF at 24 hrs after plating. IB4+ve neurons did not exhibit any significant amount of growth on either Col or FB alone, and the addition of NGF or GDNF had no further influence (data not shown). In addition to these integrin ligands, NCAM has been reported to be a ligand for GDNF receptor components (Liu et al., 2002; Paratcha et al., 2003; Thelen et al., 2002). It is possible that cell adhesion molecules like NCAM or L1 might be more important in promoting neurite growth from this population of cells, although we have not investigated this.

Cells of the NGF-responsive Trk+ve population (red) displayed significant neurite growth when plated on LN alone (Fig 4.4 D, LN-NS), and the addition of NGF further enhanced this growth (Fig 4.4 H). Note that the addition of GDNF (Fig 4.4 L) did not result in further increased growth in the Trk+ve cells on LN (compare Figs 4.4 D and 4.4 L). These results indicate that the selected IB4+ve neurons are unresponsive to LN alone and require both LN and GDNF to elicit significant neurite growth. Unlike the IB4+ve cells, the NGF-responsive Trk+ve neurons respond with robust increases in neurite growth when plated on LN which was further increased by the addition of NGF and unaffected by GDNF. In our cultures, the majority of the NF200+ve cells appear to be NGF-responsive. Prior work has also noted that large proprioceptive neurons do express TrkA and respond to NGF (Chattopadhyay *et al.*, 2003), and we have previously reported that only a small proportion of cells in our cultures express TrkC (<10%) (Jones et al., 2003; Kimpinski et al., 1997).

Fig 4.5 provides quantitation of the results noted above. The number of cells with

Figure 4.5. Quantitative analysis of neurite growth from IB4+ve and NGF-responsive cell populations in the presence or absence of NGF and GDNF. **A, D:** Percentage of IB4+ve (**A**) and NGF-responsive (**D**) cells with neurites when plated on PL or LN +/- added growth factors (NGF, GDNF). **B, E:** Total neurite length of IB4+ve (**B**) and NGF-responsive (**E**) cells plated on PL or LN +/- added growth factors (NGF, GDNF). **C, F:** Number of intersections of IB4+ve (**C**) and NGF-responsive (**F**) cells plated on PL or LN +/- added growth factors (NGF, GDNF). Panels, A-C, clearly show that IB4+ve cells plated on LN or PL show little growth in the absence of GDNF. The addition of GDNF to LN-plated cells results in a significant increase in all parameters. NGF had no significant effect on this population of cells. In contrast, panels D-F show that a significant increase in the percentage of cells with neurites, total neurite length, and neuritic elaboration was observed when NGF-responsive cells were plated on LN, with all parameters further enhanced by the addition of NGF. NGF also stimulated a significant increase in the percentage of cells with neurites and total neurite length of NGF-responsive cells when plated on PL. However, the presence of GDNF had no significant effect on this cell population. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$ ** $p < 0.001$.

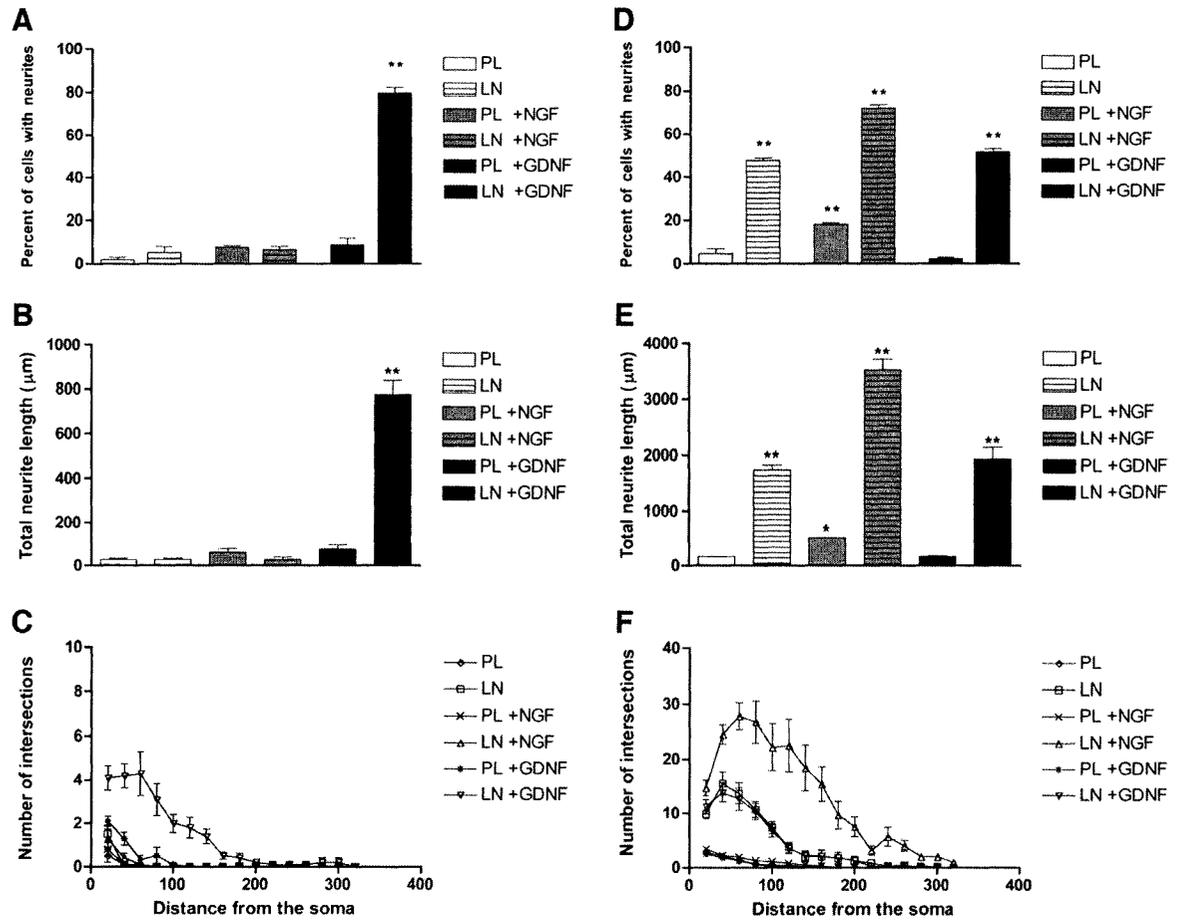


Fig 4.5

neurites, neuritic length and degree of neuritic elaboration for the IB4+ve and NGF-responsive cell populations were calculated. In order to quantitate the amount and pattern of growth (here and in subsequent experiments), neurons were traced and analyzed for total neurite length and complexity or patterns of neurite growth (Sholl analysis) using the image analysis software NeuroLucida® and NeuroExplorer® as per the Methods section and as previously reported (Jones et al., 2003; Tucker et al., 2005a). As shown in Fig 4.5 A, the IB4+ve population did not show a significant number of neurite bearing cells when plated on PL or LN in the absence of added GDNF; similarly, no significant increase in total neurite length (B) or complexity of neurite growth (C) were observed. However, a striking increase in the percentage of neurons with neurites was observed when plated on LN in the presence of GDNF, with greater than 80% of the cells showing growth. Likewise a significant increase in total neurite growth (total neurite length/neuron) (B) and neurite complexity (C) was observed with the addition of GDNF. The addition of NGF has a small, although non-significant, effect on increasing neurite growth; this is likely related to the small percentage of neurons that have been reported to be both IB4+ve and express TrkA receptors (Averill *et al.*, 1995; Kashiba *et al.*, 2003).

Unlike the IB4+ve cell population, LN alone stimulates a significant increase in neurite growth in the NGF-responsive cell population. Approximately 50% of these neurons exhibit neurite growth in response to LN alone, and this percentage is increased to almost 80% when NGF is present. This percent increase correlates with observed significant increases in total neurite length (Fig 4.5 E) and neurite complexity (Fig 4.5 F), wherein growth (total neurite length/neuron) was increased from approximately 300 μm on PL to approximately 2000 μm on LN. A further significant increase in total neurite

growth (Fig 4.5 E) and neurite complexity (Fig 4.5 F) was observed with the addition of NGF, with mean total neurite growth reaching 3800 $\mu\text{m}/\text{neuron}$. As noted above, the addition of GDNF does not influence neurite growth from this population of cells. Note that NGF treatment of cells plated on PL also results in about 20% of the cells expressing small neurites (Fig 4.5 D) with a small but significant increase in total neurite length (Fig 4.5 E).

These results again show that the NGF-responsive population is responsive to LN in the absence of added growth factors and that this response is potentiated by the addition of NGF but unaffected by GDNF. Conversely, the IB4+ve cell population seems to be unresponsive to LN alone but exhibits a robust response when GDNF is present.

4.3.4 The lack of LN induced neurite growth of the IB4+ve neurons is not due to a delayed growth response

To test whether or not the lack of neurite growth observed in the IB4+ve cell population was due to a delay in neurite growth, IB4+ve and NGF-responsive cells were plated on LN for 48 hrs and subsequently fixed and labelled with IB4, or immunostained for CGRP and NF200. At 48 hrs after plating selected IB4+ve cells still did not express any significant amount of neurite growth in the absence of added GDNF, with less than 10% of the cells having neurites (Fig 4.6 A, D). However, the NGF-responsive cell

Figure 4.6. Neurite growth from IB4+ve and NGF-responsive cell populations plated on Laminin (LN) in the absence of added growth factors for 48 hrs. **A:** Selected IB4+ve cells were plated on LN in the absence of added growth factors and labeled for IB4 (LN-S). **B-C:** NGF-responsive cells were plated on LN in the absence of added growth factors and immunostained for CGRP (LN-NS-CGRP) and NF200 (LN-NS-CGRP). **D:** Percent of IB4+ve and NGF-responsive cells with neurites plated on LN in the absence of added growth factors. Confocal laser scanning microscopy was used to visualize stained neurons 48 hrs after plating. IB4+ve cells do not show any significant amount of neurite growth at this time point when plated on LN in the absence of added growth factors (A, D). Similar to the situation at 24 hrs, a significant number of NGF-responsive cells extended neurites (B, C, D) when plated on LN in the absence of added growth factors. However, neurite growth from the CGRP+ve cell population was much more extensive at this time point than that observed at 24 hrs after plating (B). Significance was tested using one-way ANOVA with Tukey post-hoc testing. ** $p < 0.001$.

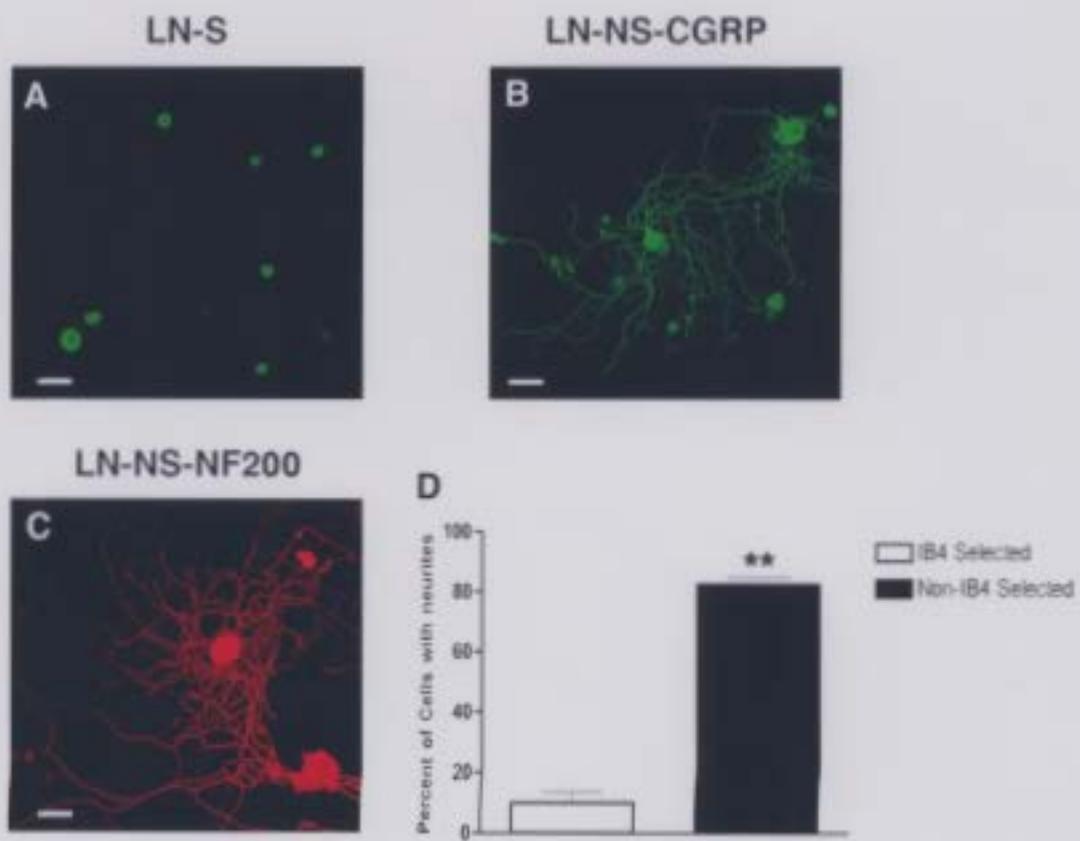


Fig 4.6

population again showed a significant amount of neurite growth, with approximately 80% of the cells extending neurites (Fig 4.6 B, C, D). Most notably, extensive neurite growth (which was much smaller at the earlier time point) was observed in the CGRP+ve cell population (Fig 4.6 B). This suggests that the CGRP+ve cells are indeed responsive to LN in the absence of added growth factors, but require an extended period of time to elicit a similar response to that seen in the NF200+ve cell population. These results suggest that lack of LN induced neurite growth of the IB4+ve neurons is not due to a delayed growth response and that integrin activation alone is insufficient in eliciting neurite growth from this population of cells.

4.3.5 Signalling pathways important in neurite growth for the IB4+ve cell population

To elucidate the signalling pathways employed by neurons that responded to LN and GDNF together (i.e., the selected IB4+ve cells), a series of experiments using pharmacological inhibitors of phosphoinositide 3-kinase (PI3-K, LY290034), protein kinaseB/Akt (Akti) and MAP kinase kinase (MEK, U0126) were carried out. IB4+ve cells were plated on LN in the presence or absence of GDNF for 24 hrs, subsequently fixed, immunostained with peripherin, and processed for HRP-DAB histochemistry followed by light microscopy (Fig 4.7). As noted above, cells plated on LN in the absence of added growth factors have little or no neurite growth (Fig 4.7 E), while neurons plated on LN in the presence of GDNF display extensive neurite growth (Fig 4.7 A). To elucidate the signalling components important in the LN+GDNF-induced neurite

Figure 4.7. Inhibition of PI-3K, Akt and MAPK attenuates growth of IB4+ve cells plated on LN. A-H: Cells were plated on LN +/- GDNF and immunostained for peripherin, followed by DAB secondary labeling. Light microscopy was used to visualize stained neurons 24 hrs after plating. IB4+ve cells plated on LN in the presence of GDNF show an increase in neurite growth (A). Increased neurite growth observed when cells were plated on LN in the presence of GDNF was attenuated with the addition of LY294002 (B), Akti (C), and U0126 (D). No increase in neurite growth was observed when IB4+ve cells were plated on LN in the absence of GDNF (E), therefore, no effect on neurite growth was observed with the addition of LY294002 (F), Akti (G), or U0126 (H). Scale bar – 25 μ m.

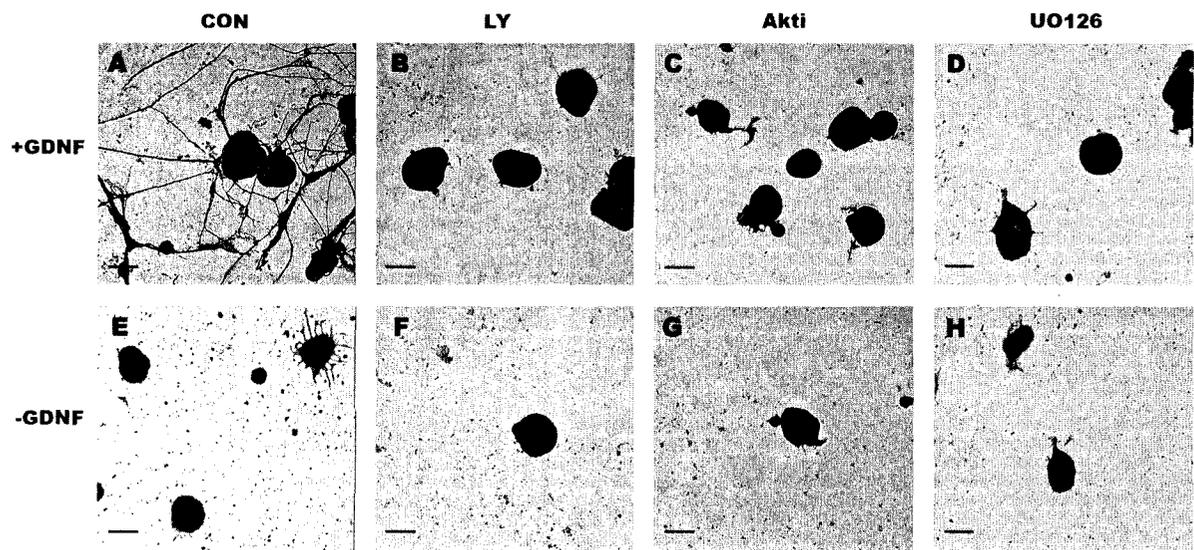


Fig 4.7

growth, pharmacological inhibitors of PI3-K (LY, 10 mM), Akt (Akti, 10 mM) and MEK (U0126, 10 mM) were added to the culture medium. Each inhibitor blocked neurite growth on LN in the presence of GDNF (Fig 4.7 B -LY, Fig 4.7 C-Akti, Fig 4.7 D-U0126); because there was little or no growth in the absence of GDNF, the inhibitors had no apparent effect (Fig 4.7 F-LY, Fig 4.7 G-Akti, Fig 4.7 H-U0126).

Quantitative analyses of the number of cells with neurites and neuritic length and elaboration are presented in Fig 4.8. First, the total number of neurons plated was counted and then the number exhibiting neurites greater than 2 soma diameters was recorded. Second, each neuron that exhibited neurite growth was traced providing data for complex and total neurite length. The data are presented in Fig 4.8 with cells plated in the absence (Fig 4.8 A, C, and E) or presence (Fig 4.8 B, D, and F) of GDNF.

As shown in Fig 4.8, less than 10% of the IB4+ve cells extend neurites when plated on LN alone (Fig 4.8 A, first column), while the addition of GDNF increases this to about 80% (Fig 4.8 B, first column). However, the addition of LY (Fig 4.8 B, second column), Akti (Fig 4.8 B, third column) or U0126 (Fig 4.8 B, fourth column) significantly inhibits the number of cells with neurites. In order to further quantitate the amount and pattern of growth, neurons were traced and analyzed for total neurite length and complexity or patterns of neurite growth (Sholl analysis) using the image analysis software NeuroLucida® and NeuroExplorer® as per the Methods section and as previously reported (Jones et al., 2003; Tucker et al., 2005a). Results of these analyses are presented in Fig 4.8 C-F, where panels C and D present total neurite growth of IB4+ve neurons plated on LN in either the absence (Fig 4.8C) or presence (Fig 4.8D) of GDNF; and panels E and F present the data from the Sholl analyses as the number of intersection

Figure 4.8. Quantitative analysis of the effect of LY294002, Akti, or U0126 on neurite growth from IB4+ve cells 24 hrs after plating. **A:** Percent of cells with neurites on LN minus GDNF. **B:** Percent of cells with neurites on LN plus GDNF. **C:** Total neurite length on LN minus GDNF. **D:** Total neurite length on LN plus GDNF. **E:** Number of intersections on LN minus GDNF. **F:** Number of intersections on LN plus GDNF. The percent of cells with neurites was decreased with the addition of LY294002, Akti, or U0126 in the presence of GDNF (B), as was total neurite length (D). The amount of neuritic elaboration (indicated by the number of intersections that neurites make with concentric 20 μ m rings) was attenuated with the addition of LY294002, Akti or U0126 in the presence of GDNF (F). Significance was tested using one-way ANOVA with Tukey post-hoc testing. ** $p < 0.001$.

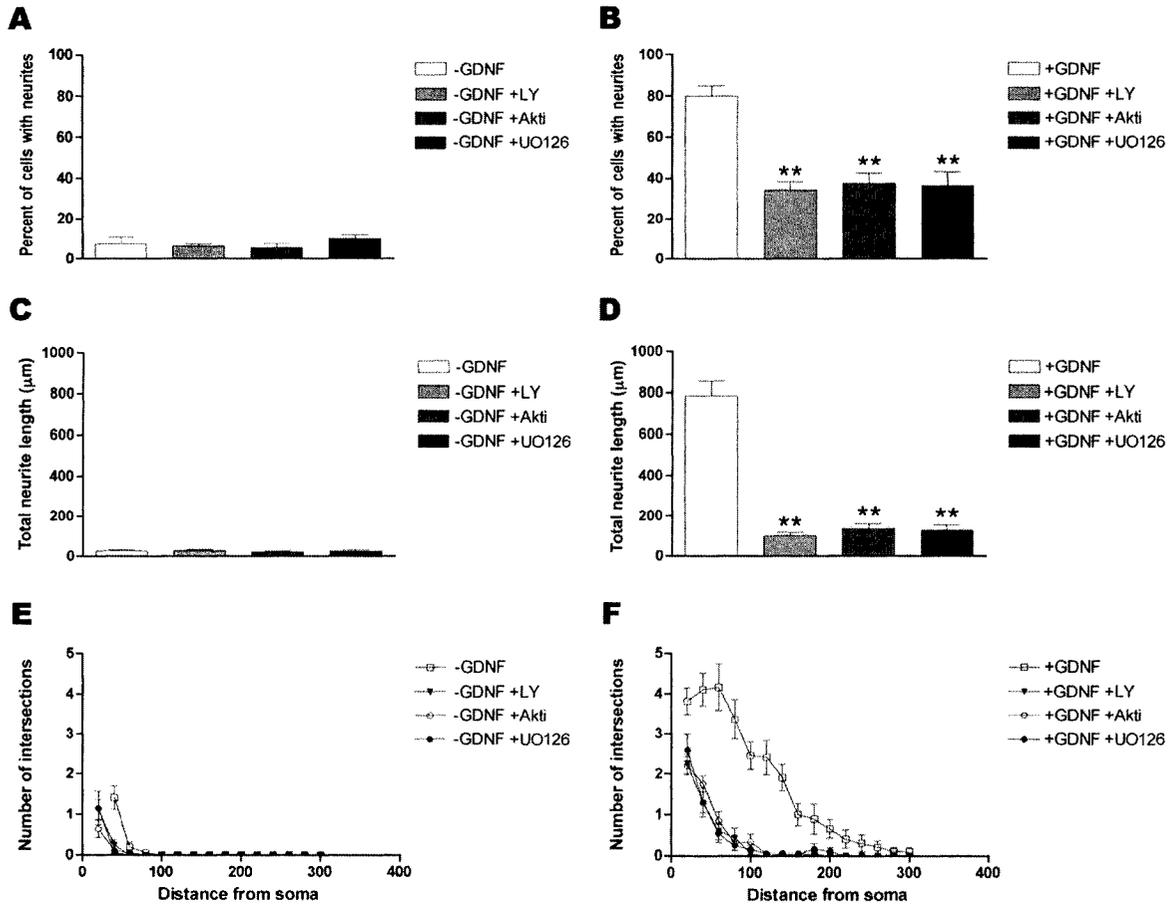


Fig 4.8

points for the IB4+ve neurons plated on LN in either the absence (Fig 4.8E) or presence (Fig 4.8F) of GDNF.

The data in Fig 4.8 D show more clearly the effect of inhibiting either the PI 3-K, Akt, or MAPK pathways on total neurite growth of IB4+ve cells plated plus GDNF. Growth was attenuated by approximately 75%, with total neurite length dropping from 800 μm to less than 200 μm with the addition of inhibitors (Fig 4.8 D-LY column 2, Fig 4.8 D-Akti column 3, Fig 4.8 D-U0126 column 4). Because total neurite growth for IB4+ve cells plated in the absence of GDNF was negligible, the inhibitors had no significant effect.

The Sholl analyses provide an estimation of the complexity of growth or the amount of branching, by quantifying neurite intersection points as described in the Methods section. As with the amount of neurite growth, neurite complexity (equated by the number of intersections across 20 μm concentric rings) of IB4+ve neurons plated in the presence of GDNF was significantly inhibited by the addition of LY, Akti, or U0126 (Fig 4.8 F).

Results from these experiments indicate that both the PI3-K/Akt and MEK/MAPK pathways are important in stimulating neurite growth in adult IB4+ve DRG neurons since inhibition of either of these pathways clearly resulted in a significant decrease in growth compared to the control situation.

4.3.6 Signalling pathways important in neurite growth for the NGF-responsive cell population

Because we were also interested in the signalling pathways employed by neurons that responded to LN and NGF (i.e., the NF200+ve and CGRP+ve cell populations), we carried out a series of experiments with the NGF-responsive population using the same pharmacological inhibitors noted above.

NGF-responsive cells were plated on PL or LN in the presence or absence of NGF for 24 hrs, subsequently fixed and immunostained for tubulin followed by confocal analysis (Fig 4.9). As noted above, cells plated on PL have little or no growth (Fig 4.9 A-B), while neurons plated on LN display extensive neurite growth (Fig 4.9 C), which is further enhanced by NGF (Fig 4.9 D). To elucidate the signalling components important in the LN-induced neurite growth, pharmacological inhibitors of PI3-K (LY, 10 mM), Akt (Akti, 10 mM) and MEK (U0126, 10 mM) were added to the culture medium. Both LY and Akti blocked neurite growth on LN in the absence of NGF (Fig 4.9 G-LY, Fig 4.9 K-Akti), although there was still some remaining growth in the LN+NGF condition (Fig 4.9 H, L; see below). As expected there was no effect on the cells plated on PL in the presence (Fig 4.9 F) or absence (Fig 4.9 E) of NGF; the PL conditions were included as controls. In contrast to the effects of LY and Akti, inhibition of MEK, and thus MAPK, did not inhibit growth on LN alone (Fig 4.9 O) nor in the presence (Fig 4.9 P) of NGF. In fact, the addition of U0126 appeared to have increased neurite growth (Fig 4.9 P). Again little effect was observed for cells plated on PL in the presence of U0126 (Fig 4.9 M, N).

Figure 4.9. Inhibition of PI-3K or Akt, but not MAPK, blocks neurite growth of NGF-responsive cells plated on LN. A-P: Cells were plated on PL, or LN, +/- NGF and immunostained for peripherin. Confocal laser scanning microscopy was used to visualize stained neurons 24 hrs after plating. NGF-responsive cells plated on LN show substantial neurite growth (C), and the addition of NGF stimulates an increase in neurite growth on both PL (B) and LN (D). Increases in neurite growth observed on PL+NGF, LN-NGF, and LN+NGF were attenuated with the addition of LY294002 (F, G, and H respectively) and Akti (J, K, and L respectively). Neurite growth was unaffected or potentially enhanced by the addition of U0126 (N, O, and P respectively). Scale bar – 50 μ m.

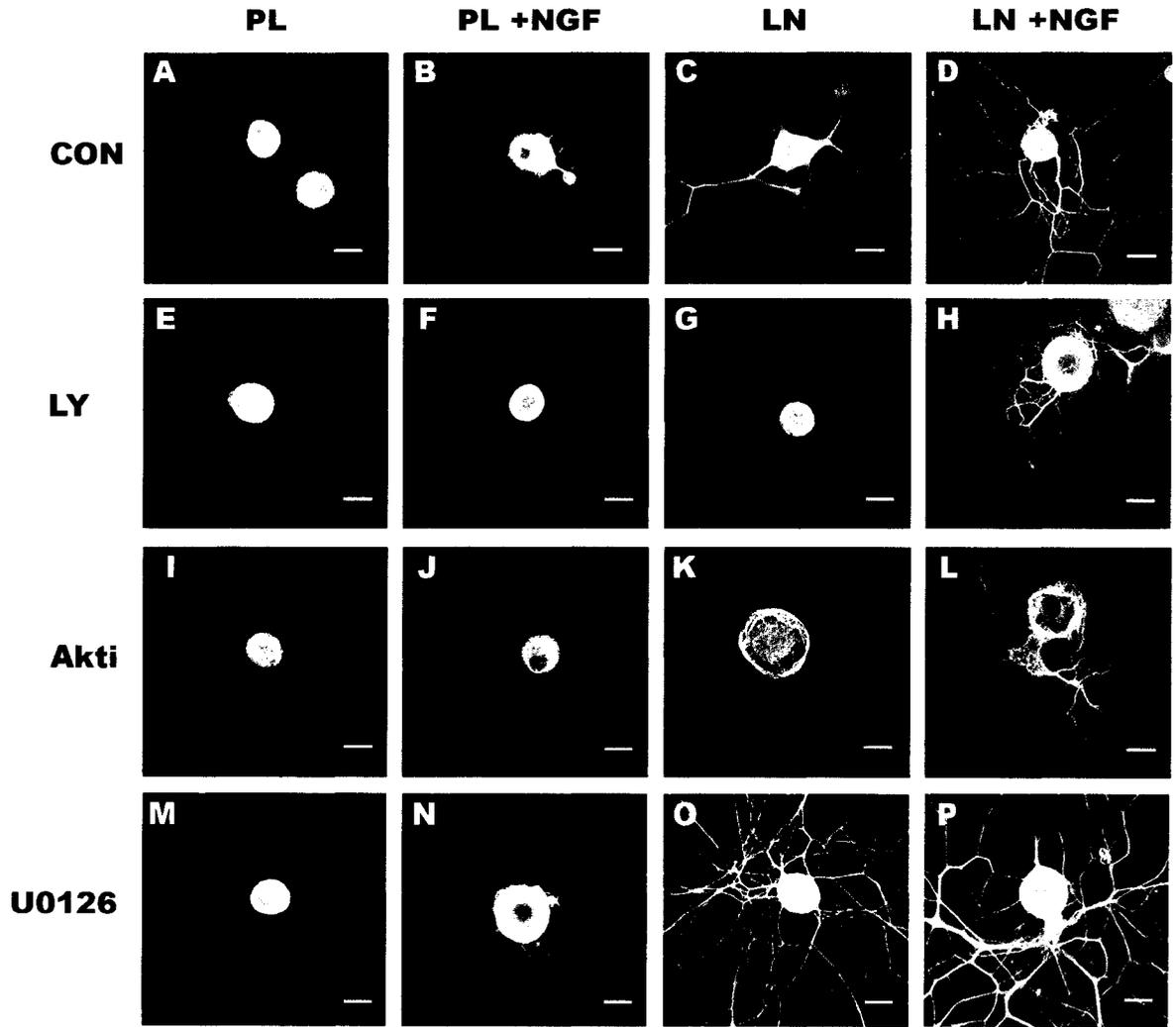


Fig 4.9

In subsequent experiments, cells were plated on LN and treated with NGF or the inhibitors as noted above, but were immunostained and processed for HRP-DAB histochemistry, to facilitate quantitative analyses of the number of cells with neurites, neuritic length and degree of neuritic elaboration. Total numbers of neurons plated were counted and the number of neurons exhibiting neurites greater than 2 soma diameters was analyzed. The data are presented in Fig 4.10 for the cells plated on LN minus NGF (Fig 4.10 A, C, E) or plus NGF (Fig 4.10 B, D, F).

As shown in Fig 4.10, approximately 50% of the cells extend neurites when plated on LN alone (Fig 4.10 A, first column), while NGF increases this to about 75% (Fig 4.10 B, first column). LY inhibits the percentage of neurons with neurite growth by approximately 20% in the absence of NGF (Fig 4.10 A, second column) and by about 35% in the presence of NGF (Fig 4.10 B, second column). Akti further inhibits the number of neurons with growth by about 30-35% in the absence of NGF (Fig 4.10 A, third column) and by about 40% in presence of NGF (Fig 4.10 B, third column). However, unlike LY or Akti, U0126 had no significant effect on the percentage of cells with neurites (Fig 4.10 A, B – last column).

In order to further quantitate the amount and pattern of growth, neurons were analyzed by tracing neurites, as noted in Section 4.3.5. Results of these analyses are also presented in Fig 4.10, where panels C-D present total neurite growth for cells plated on LN alone (Fig 4.10 C) or plus NGF (Fig 4.10 D). Panels E-F present the data from the Scholl analyses as the number of intersection points for the same set of cells again in the absence (Fig 4.10 E) or presence (Fig 4.10 F) of NGF. The data in Fig 4.10 C-D show more clearly the effect of inhibiting the PI 3-K/Akt and MEK/MAPK pathways on total

Figure 4.10. Quantitative analysis of the effect of LY294002, Akti, or U0126 on neurite growth from NGF-responsive cells at 24 hrs after plating. A: Percent of cells with neurites on LN minus NGF. **B:** Percent of cells with neurites on LN plus NGF. **C:** Total neurite length on LN minus NGF. **D:** Total neurite length on LN plus NGF. **E:** Number of intersections on LN minus NGF. **F:** Number of intersections on LN plus NGF. The percent of cells with neurites was attenuated with the addition of LY294002 and Akti in both the presence (B) and absence (A) of NGF. However the addition of U0126 had no significant effect on the percent of cells with neurites (A, B). Total neurite length was also attenuated with the addition of LY294002 and Akti in both the presence (D) and absence (C) of NGF. Although the addition of U0126 had no significant effect on total neurite length when plated in the absence (C) of NGF, a significant increase in total neurite length was observed in the presence (D) of NGF. The amount of neuritic elaboration was attenuated with the addition of LY294002 and Akti in both the presence (F) and absence (E) of NGF. However the addition of U0126 had no significant effect on neuritic elaboration in either the presence (F) or absence (E) of NGF. Significance was tested using one-way ANOVA with Tukey post-hoc testing. ** $p < 0.001$.

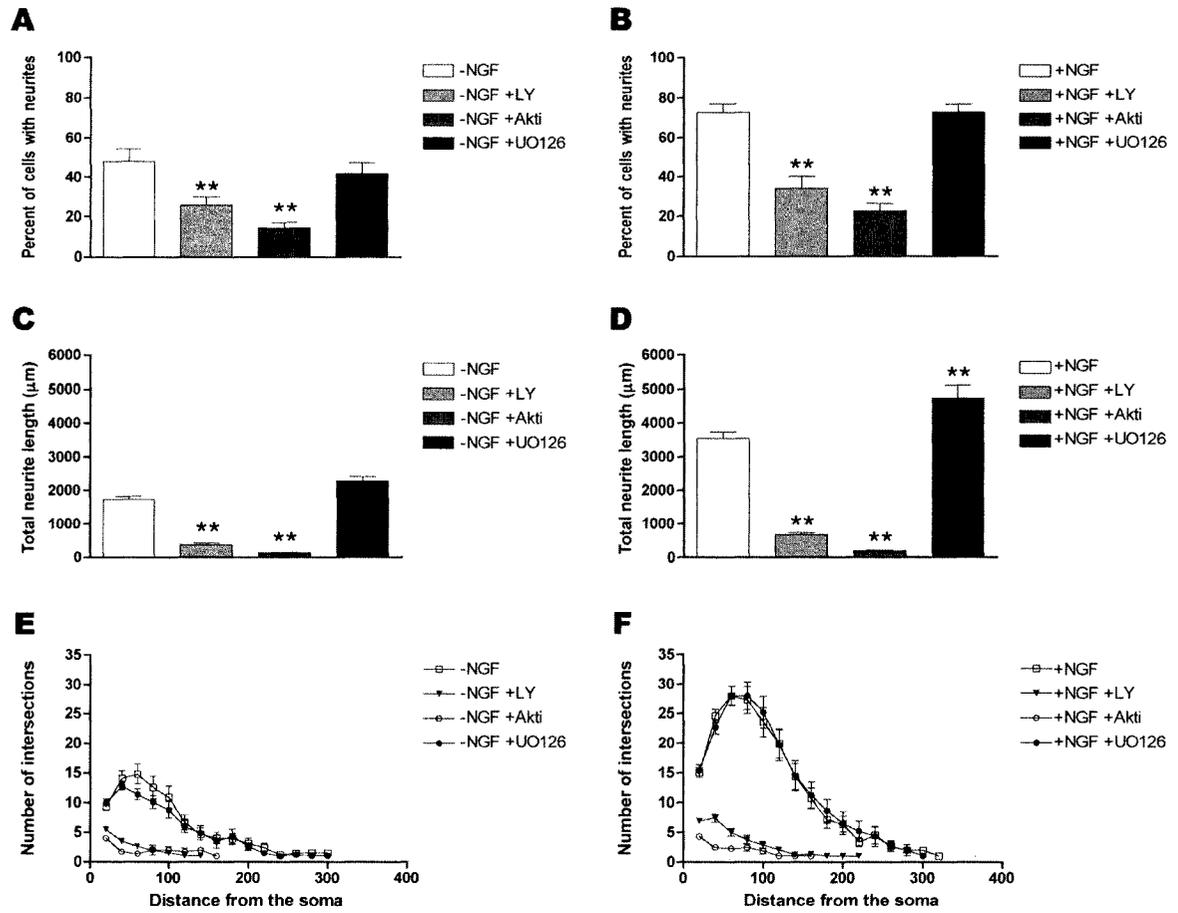


Fig 4.10

neurite growth of NGF-responsive cells. In the absence of NGF (Fig 4.10 C), growth was attenuated by approximately 75% with the addition of LY (Fig 4.10 C, second column, decreased from about 1800 μm to $< 400 \mu\text{m}$), and by 80% with the addition of Akti (Fig 4.10 C, third column, total neurite length decreased from 1800 μm to $< 200 \mu\text{m}$). A slight, but non-significant, increase in neurite growth was seen with the addition of U0126 in the absence of NGF (Fig 4.10 C, column 4). In the presence of NGF (Fig 4.10 D) growth was decreased by about 80% with the addition of LY (Fig 4.10 D, second column, 3800 μm to $< 800 \mu\text{m}$), and by about 90% with the addition of Akti (Fig 4.10 D, third column, 3800 μm to $< 300 \mu\text{m}$). In contrast, a significant increase in neurite growth of about 20% was observed with the addition of U0126 in the presence of NGF (Fig 4.10 D, column 4, total neurite length increased from 3800 μm to 5700 μm).

As with the amount of neurite growth, neurite complexity of the NGF-responsive neurons in the absence (Fig 4.10 E) or presence (Fig 4.10 F) of NGF was also significantly inhibited by the addition of both LY, and Akti, but was unaffected by U0126.

Results from these sets of experiments indicate that the PI 3-K/Akt pathway is important in stimulating LN-induced neurite growth in NGF-responsive DRG neurons, since inhibition of this pathway clearly results in a significant decrease in growth compared to the control situation. These results also illustrate that, although selected IB4+ve cells extend neurites on LN in the presence of GDNF, this growth is much less extensive than that observed from the NGF-responsive cells. For instance, in selected IB4+ve cells with LN plus GDNF, total neurite growth reached a maximum of 800 μm (Fig 4.8 D, column one), while the corresponding amount for the NGF-responsive

neurons is approximately 3500 μm (Fig 4.10 D, column one). The Sholl analyses in these studies also illustrate that the NGF-responsive neurons clearly display more complex patterns of branching; compare a maximum of 3 intersection points at 100 μm away from the cell body for IB4+ve cells (Fig 4.8 F), with 12 intersections (minus NGF, Fig 4.10 E) increasing to 25 intersections with NGF present (Fig 4.10 F).

4.4 Discussion

In the present study we have investigated the role of growth factor and extracellular matrix molecule (ECM) stimulation on neurite growth and regeneration of selected sub-populations of DRG neurons. We have utilized a magnetic bead selection technique to study growth responses of two major DRG neuron subpopulations in isolation. Our results demonstrate that the NGF-responsive neurons show neurite growth when plated on LN, even in the absence of added neurotrophins, and this can be further enhanced by the provision of NGF, while neither BDNF nor NT3 were able to duplicate the effects of NGF (data not shown). These effects appeared to be selective to LN, as these cells did not respond with neurite growth when plated on FB or Col (data not shown). In contrast, the IB4+ve neurons did not exhibit any detectable neurite growth when plated on LN or any of the other ECM molecules tested (FB, Col) in the absence of GDNF. However, these cells did respond with a robust increase in neurite growth when plated on LN and treated with GDNF (GDNF did not elicit neurite growth in these cells when plated on FB or Col).

The potential signalling mechanisms were studied for each of the different subpopulations of DRG neurons. We investigated the dependence of the NGF-responsive

neurons on the PI3-K/Akt and MEK/MAPK signalling pathways. These pathways have been shown to play a role in axonal growth from DRG explants and dissociated neurons (Edstrom and Ekstrom, 2003; Jones et al., 2003; Jones et al., 2003; Kimpinski and Mearow, 2001; Liu and Snider, 2001; Markus et al., 2002b; Tucker et al., 2005a; Wiklund et al., 1996; Zhou et al., 2004). We have also noted that integrin-dependent activation of components of these pathways are important for neurite growth in these cells (Tucker et al., 2005a). Inhibition of the PI3-K/Akt pathway blocked neurite growth in all situations, while there was no obvious requirement for the MEK/MAPK pathway, as inhibition with U0126 had no negative effect. In fact inhibition of MEK/MAPK tended to have a positive effect on growth, which may be due to increased activity of Akt (Jones et al., 2003; Lad et al., 2003).

Dependence on these same signalling pathways was also examined in the IB4+ve cell population. Inhibition of the PI3-K/Akt pathway blocked neurite growth from IB4+ve cells, while, unlike the NGF-responsive cell population, inhibition of the MEK/MAPK pathway also attenuated neurite growth in these cells. GDNF can activate several intracellular signalling pathways to promote cell survival and neurite growth in a variety of cell types (Airaksinen and Saarma, 2002). However, there have been relatively few studies examining the contribution of GDNF-activated signalling to neurite growth in DRG neurons (Leclere *et al.*, 1998; Paveliev *et al.*, 2004). A recent study notes the importance of Src family kinases (SFKs), and suggests that, while the signalling pathways mediating the effects of SFKs on DRG neurite growth are unknown, PI 3-K could play a role (Paveliev *et al.*, 2004). The link between SFK and GDNF-induced

growth in that study also supports our data indicating a requirement for integrin-mediated component to the GDNF-induced growth in DRG neurons.

The influence of ECM, particularly laminin, on axonal growth and regeneration is well established (Grimpe and Silver, 2002; Luckenbill-Edds, 1997; McKerracher et al., 1996). Studies dealing specifically with DRG explants and neurons have also reported the positive effects of ECM and neurotrophic factors (Edstrom et al., 1996; Hari et al., 2004; Liu et al., 2002; Tonge et al., 1997) but few, if any, have noted any differences in the responsiveness of particular classes of neurons to ECM substrates or have considered the importance of the extracellular matrix as a requirement for many of these processes. Our results indicate clear distinctions in the response of neuronal subpopulations to integrin activation by one ECM molecule, LN, as well as the requirement for specific signalling pathways.

4.4.1 What is the reason for the different responses? Are there intrinsic differences in ECM receptors expressed by the different neuron populations?

Our data show that IB4+ve cells differ from the NGF-responsive neurons in their ability to regenerate processes after dissociation. The obvious question is whether/how these cells differ in either their intrinsic growth capacity or their ability to respond to ECM molecules such as LN, FB or Col.

In terms of intrinsic growth ability, GAP-43 is associated with axonal growth and regeneration (Bomze et al., 2001; Skene, 1989). While GAP-43 is reported to be primarily expressed by small-medium sized DRG neurons in vivo, there are no reported differences in the expression or upregulation of GAP-43 in these different neuronal

subpopulations after injury or culturing that would account for our observations here (Mearow et al., 1994; Verge et al., 1990; Woolf et al., 1990). Another possibility relates to observations that the initial arborizing growth displayed by some DRG neurons is transcription-independent and does not appear to require neurotrophins (Smith and Skene, 1997); the larger neurons may express higher levels of proteins required to initiate neurite growth in the absence of added neurotrophic factors compared to the smaller cells, which could explain why the CGRP+ve cell populations exhibit a delayed growth response. However, the lack of growth on LN in the absence of GDNF in the IB4+ve cells cannot be explained simply by a lag in growth. We have followed cultures for up to 72 hrs and the IB4+ve cells still do not respond in the absence of GDNF, although when stimulated with GDNF they are able to put out processes (data not shown).

Are there differences in their ability to respond to ECM molecules? Ability to respond to ECM essentially depends upon the expression of the appropriate ligands for these molecules, eg., the integrins. The majority of DRG neurons express $\beta 1$ integrin and a combination of the various α subunits, (Condic and Letourneau, 1997; Guan et al., 2003; Tomaselli et al., 1993; Wallquist et al., 2004). The integrins $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 7\beta 1$ are able to act as laminin receptors (Malinda and Kleinman, 1996). Although LN alone was not sufficient to elicit a significant growth response from the IB4+ve cell population, these cells express LN binding integrins (Guan et al., 2003; Wallquist et al., 2004) and this substrate was still required in order to achieve GDNF-induced neurite growth. During embryonic development the different subpopulations of chick DRG neurons have been reported to express different α chains, with presumptive proprioceptive afferents having higher expression of $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ and

extending neurites on both LN and FB; cutaneous afferents had a stronger preference for LN and higher expression of $\alpha7\beta1$ (Guan et al., 2003). Recent data have suggested that $\alpha7$ may play a role in the regeneration of the large proprioceptive sensory axons and motor axons in mice (Ekstrom et al., 2003; Gardiner et al., 2005; Werner et al., 2000). These studies reported that $\alpha7$ is expressed by only the large cells, although it was noted that $\alpha7$ was upregulated in both small and large neurons after injury (Gardiner et al., 2005; Werner et al., 2000). In contrast, Wallquist and colleagues reported that almost all DRG neurons expressed $\alpha7$ and that all cells upregulated it after injury (Wallquist *et al.*, 2004). Our preliminary data (Mearow, Tucker and Rahimtula, in preparation) also suggest that there is no apparent difference in the expression of $\alpha7$, or in αV or $\alpha6$ expression between the selected and non-selected neurons. We have previously reported expression of both $\alpha3$ and $\alpha1$ in cultured DRG neurons (Tucker et al., 2005a), although we have not yet completed our studies of potential difference among the subpopulations.

Thus, there do not appear to be any obvious differences amongst the subpopulations in terms of the integrins expressed. Since they do appear to express a similar repertoire of LN receptors, another possibility is that the relative numbers of receptors, and thus the effectiveness of activating signalling cascades, may differ. The NGF-responsive cells might have more receptors and thus be able to respond more efficiently to limiting amounts of the ECM in the absence of growth factors such as NGF. The IB4+ve neurons might have fewer receptors and require contribution from both integrin-activated and growth factor activated components to reach a threshold of signalling that could result in growth. We are currently examining this possibility, which would seem to be supported by the work of Condic and colleagues (Condic, 2001;

Condic and Letourneau, 1997; Lemons et al., 2005). Overexpression/upregulation of expression of integrin subunits in adult DRG neurons enhances their regenerative ability (Condic, 2001), and upregulation of select $\alpha 1$, $\alpha 3$ and $\alpha 6$ subunits in embryonic neurons provides them with the ability to overcome the inhibitory effects of CSPGs (Condic *et al.*, 1999; Lemons *et al.*, 2005).

4.4.2 Relevance to repair of peripheral neuropathies.

In peripheral neuropathy, such as that associated with diabetes, the most susceptible axons tend to be the small diameter nociceptive nerve fiber populations, while the larger diameter fibers tend to be less vulnerable and affected later in the disease (Polydefkis et al., 2003; Yasuda et al., 2003; Zochodne, 1999; Zochodne et al., 2001). Much of the research associated with sensory polyneuropathies has focused on prevention through treatment, although there is more emphasis now being placed on attempts to stimulate regeneration. As we have noted in this study, in order to achieve an effective regenerative response, the cell requires support from both trophic factors and components of the ECM. In experimental models of diabetes, trophic support is altered (Leininger et al., 2004; Tomlinson et al., 1997), attachment of DRG neurons to the ECM is impaired (Sango *et al.*, 1995), the axonal cytoskeleton and transport is compromised (Ferryhough and Schmidt, 2002; McLean, 1997; Scott et al., 1999), and the regeneration of peripheral nerves is impaired (Kennedy and Zochodne, 2005). Our study points to potential specific environmental requirements and signalling events that could provide therapeutic targets for the treatment and repair of neuropathies such as those associated with diabetes. However, future studies are required to further characterize the points of

collaboration between integrin and growth factor activated pathways important in optimizing regeneration.

CHAPTER 5

IDENTIFICATION OF SRC AS THE KEY INTERMEDIATE BETWEEN INTEGRIN AND TRK ACTIVATED SIGNALLING PATHWAYS IN THE STIMULATION OF NEURITE GROWTH IN NGF-RESPONSIVE DRG NEURONS

(Manuscript in preparation)

5.1 Introduction

It has long been known that axonal regeneration is much more extensive in the PNS than in the CNS. The poor regenerative ability of the CNS is in part due to the presence of inhibitory extracellular matrix substrates such as MAG, Nogo, and OMgp, and the paucity of essential neurotrophins such as NGF, and BDNF (Cai et al., 1999; Hunt et al., 2002; Jacobs and Fehlings, 2003). Inhibitory conditions such as these are not present in the PNS; however, like the CNS, the extracellular environment in which growth occurs is still critically important. We have recently characterized signalling pathways activated by NGF and IGF-1, which promote neuronal survival and neurite growth from adult DRG sensory neurons *in vitro* (Kimpinski, 2001; Dodge, 2002; Jones, 2003; Tucker, 2005; Tucker et al, 2006 in press). Our results illustrate that the provision of such growth factors supports an increase in extensive neurite growth, which is dependent upon the availability of a permissive culture substrate (i.e. laminin).

Unlike the inhibitory substrates found in the CNS, components of the ECM found in the PNS, such as laminin, entactin, and fibronectin, are essential for axonal regeneration.

These permissive molecules have an effect by signalling via integrin receptors, which consist of heterodimers of α and β subunits, the cytoplasmic tails of which are short and have no intrinsic enzymatic activity. Upon activation, integrin receptors cluster, form focal adhesions, and stimulate the recruitment of specific adapter proteins, which activate a series of downstream signalling molecules (Giancotti and Ruoslahti, 1999; Guo and Giancotti, 2004; Howe et al., 1998). Different combinations of these receptors bind to different substrate molecules; for instance, integrins $\alpha1\beta1$, $\alpha3\beta1$, and $\alpha7\beta1$ act as laminin receptors and have been shown to mediate the binding of DRG neurons and neuronal cell lines to laminin (Condic, 2001; Condic and Letourneau, 1997; Gardiner et al., 2005; Guo and Giancotti, 2004; Lefcort et al., 1992; Tomaselli et al., 1993; Tucker et al., 2005a).

We have previously shown that both NGF and laminin are strong promoters of adult DRG neurite growth (Jones et al., 2003; Tucker et al., 2005a). These factors are able to work in conjunction with each other to stimulate increased neurite growth not achievable by either factor alone, which is in part due to crosstalk between integrin and growth factor signalling pathways (Tucker et al., 2005a).

The adult DRG is made up of a heterogeneous population of cells subdivided on the basis of neurochemistry, morphology, trophic requirements, and sensory modalities (Averill et al., 1995; Gavazzi et al., 1999; Petruska et al., 2000; Priestley et al., 2002a). At least three major cell groups have been identified with the potential for further subclassification. The first is comprised of large and medium diameter neurons, identified based on their expression of heavy chain neurofilament (NF200), and typically have myelinated axons. These neurons express the p75 neurotrophin receptor as well as

TrkA, TrkB, and TrkC, making them responsive to NGF, BDNF, NT3, and NT4/5 (Averill et al., 1995; Priestley et al., 2002a). The next two groups consist mainly of small diameter neurons, have unmyelinated or thinly myelinated nociceptive afferents, and are either peptidergic, expressing calcitonin gene related peptide (CGRP), or non-peptidergic, binding the lectin *Griffonia Simplicifolia* IB4 (Averill et al., 1995; Priestley et al., 2002a). The CGRP+ve population expresses a high level of both p75 neurotrophin receptor and TrkA, and responds to NGF. However, the IB4-binding population has neither p75 nor Trk receptors, but instead expresses the receptor tyrosine kinase RET and the GFR α 1 subunit, making these cells responsive to GDNF and unresponsive to NGF (Bennett et al., 1998; Kashiba et al., 2001; Molliver et al., 1997).

We have previously shown that the NGF-responsive cell population (NF200+ve and CGRP+ve) responds to laminin with increased neurite growth in the absence of added trophic factors, and that neurite growth is potentiated by the addition of NGF (Tucker et al., 2006, in press). This growth response has been shown to be critically dependent on the PI 3-K/Akt pathway; however, the upstream point of collaboration between LN and NGF stimulation remains to be elucidated. The signalling intermediates, Src, Fyn, ILK, and Fak, are common components to various cell adhesion and growth factor receptors. For instance, Src has been shown to be activated by integrins, the netrin receptor DCC, Eph receptors, IGF receptors, Trk receptors, and the GDNF receptor Ret in various cell types (Altun-Gultekin and Wagner, 1996; Encinas et al., 2001; Helmke et al., 1998; Iwasaki et al., 1998; Li et al., 2004; Sato et al., 1998; Tucker et al., 2005a). Therefore, the possibility exists that one of these common components could provide the essential link between NGF and laminin induced axonal regeneration in adult DRG neurons.

Our previous findings have identified Src as a possible collaborative component upstream of PI 3-K/Akt signalling, activated by both NGF and LN, which leads to stimulation of optimal levels of neurite growth from adult NGF-responsive DRG neurons. These studies examined NGF and LN induced neurite growth 24 hr after plating; however, in the course of these experiments it became evident that extensive neurite growth was occurring at much earlier time points. Thus, to determine the critical points of collaboration between NGF and LN induced signalling, a time course of early signalling intermediate activation was conducted. If Src is the critical point of collaboration between NGF and integrin activated signalling in these cells, inhibition of this component should lead to the interruption of neurite growth via alteration of downstream targets. To test this hypothesis, pharmacological inhibition of specific signalling components was performed, and early neurite growth and signalling analysis was carried out. Adult NGF-responsive DRG neurons were treated with NGF, laminin (LN), and laminin plus NGF (LN+N) for 10 min, 1 hr, or 6 hrs and subsequently analysed. Adult DRG neurons make an ideal model for studying the effects of neurotrophins and extracellular matrix molecules on peripheral nervous system regeneration because they survive in the absence of added trophic factors, as well as in the presence of the added inhibitors (Dodge et al., 2002; Tucker et al., 2005a).

Short-term stimulation with NGF, LN, or LN+N for 6 hrs induces increased neurite growth, which can be blocked by inhibition of PI 3-K, and subsequent decreased activation of Akt. Inhibition of Src at the tested time points also inhibits neurite growth following NGF, LN, or LN+N stimulation, due to the subsequent inhibition of downstream signalling intermediates Fak and Akt. Src inhibition also results in

decreased activation of MAPK, indicating that Src is upstream of this signalling component. Inhibition of PI 3-K via the pharmacological inhibitor LY294002 does not affect MAPK activation, indicating that this intermediate is not upstream of MAPK, and is subsequently located downstream of Src. However, inhibition of PI 3-K does affect the activity of Src and Fak, potentially due to the presence of a negative feedback system, giving this pathway ultimate control over neurite growth. To our knowledge, this is the first time that Src has been shown to be the key point of collaboration between NGF and laminin induced signalling, and subsequent neurite growth from adult NGF-responsive DRG neurons. These findings have identified a key target in the attempt to stimulate optimal levels of neuronal regeneration in both PNS and CNS neurons.

5.2 Materials and Methods

5.2.1 Neuronal cultures

Dorsal root ganglia (DRG) from young adult (4-6 wk) Sprague-Dawley rats (Memorial University of Newfoundland Vivarium and Charles River Canada, Montreal, QC) were dissected and dissociated. In accordance with University Animal Care guidelines, animals were live decapitated, ganglia from all spinal levels were removed, and the roots were trimmed. To isolate individual DRG neurons, both enzymatic and mechanical dissociation was used. Enzymatic dissociation was carried out first and performed by incubating the ganglia in 0.25% collagenase for 45 min, and 0.25% trypsin for 40 min (Invitrogen/ Gibco BRL, Burlington, Ont). Mechanical dissociation was carried out next and performed by repeated cycles of aspiration and expulsion of the DRG fragments through a flame narrowed pasture pipette. Upon completion, dissociated DRG neurons were suspended in serum-free Neurobasal medium (NB, Invitrogen)

supplemented with 100U penicillin/streptomycin, B27 supplement (Invitrogen), and 20mM cytosine arabinoside (modified NB). This suspension was then layered on top of a 30% Percoll solution (Amersham Bioscience, Baie d'Urfe, QC) in 15ml conical tubes and centrifuged at 400g for 20 min at room temperature. Pellets were then carefully extracted with a sterile pasture pipette, placed in a fresh 15ml tube, washed with the previous suspension media, and centrifuged to remove any remaining Percoll. Neurons were plated in either Lab-Tek 16-well chamber slides (Nunc International, Naperville, NC) for neurite growth assessment or 12-well plates for Western blot analysis, (plates and slides were coated twice with Poly-D-lysine, PL, 1 µg/ml, BD Bioscience, Bedford, MA) and incubated at 37°C with 95% O₂ and 5% CO₂ for 2 hrs in the presence or absence of the pharmacological inhibitors LY294002, U0126 or PP2. After the initial 2 hr incubation with inhibitors, NGF (25 ng/ml, Cedarlane Labs, Hornby, ON), laminin (LN, 25 µg/ml, Invitrogen) or laminin +NGF (LNN, 25 ng/ml of NGF Cedarlane Labs, and 25 µg/ml of LN Invitrogen) were added to the cells where appropriate and cultures were then incubated for a further 10 min, 1 hr, or 6 hrs. For the Western blot analysis, 4 animals were used to provide enough neurons for one 12-well culture plate. Each 12-well plate provided a single time point for the control (no treatment), NGF, LN, and LN+N treatment conditions. Therefore, three, 12-well plates were required to complete a single experiment for each of the 10 min, 1 hr, and 6 hr time points studied. This procedure was performed for both the control (no inhibitor condition), and pathway specific inhibitor conditions, and was repeated a minimum of three times. Thus, to complete the entire experiment, thirty-six 12-well plates, and a minimum of 144 animals were required.

5.2.2 Cell Selection

Specific cell populations were selected as discussed in chapter 3; briefly streptavidin-coated beads were incubated with biotinylated *Griffonia Simplicifolia* IB4 lectin (Sigma) for 2 hrs at room temp by gentle rotation. Beads were then washed removing any excess lectin and added to the cell suspension. This suspension of cells and Dynal beads was incubated at room temp for 30 min followed by bead capture with a Dynal magnetic separator. The supernatant was removed and plated as the non-selected NGF-responsive cell population, which included both NF200+ve and CGRP+ve cells. In this series of experiments we were primarily interested in the interaction between LN and NGF in the NGF-responsive cell population, thus, the beads and selected IB4+ve cells were discarded.

5.2.3 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde (pH 7-7.4) in PBS for 15 mins, permeablized with 0.1% Triton-X-100 and blocked with 10% normal goat serum in PBS. For neuronal and neurite identification, antibodies directed against total tubulin (1:1000, Sigma) were employed. Cells were incubated with the primary antibodies at 4°C for 16-20 hrs, followed by Cy2-tagged secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) detection. These slides were cover slipped with glycerol and imaged with confocal laser scanning microscopy.

5.2.4 Enzyme Inhibition

Signalling pathways were inhibited by the use of specific pharmacological inhibitors. Inhibitors targeting PI 3-K (LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one, Calbiochem), MEK (U0126, 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene, Calbiochem), and the Src Family Kinases (SFKs) (PP2, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, Calbiochem) were added to the culture media at the time of plating (2 hrs prior to NGF and/or LN treatment). Cultures were either fixed and labelled for the appropriate markers, or collected for Western blot analysis, at 10 min, 1 hr, or 6 hrs after treatment.

5.2.5 Neurite Growth Analysis

Neurite growth was quantified by determining the percentage of total tubulin stained neurons that extended processes. Growth was assessed at 10 min, 1 hr, and 6 hrs after stimulation. For each condition examined (control, NGF, LN, or LN+N), a minimum of 200 neurons was counted, and each experiment was repeated 3 times. Thus, a minimum of 600 cells per condition was analyzed, giving a total of 2400 cells for the entire experiment.

5.2.6 Immunoblotting

For Western analyses, neurons were cultured in 12-well plates containing modified serum-free NB, with or without LY294002, U0126 or PP2 for 2 h, and subsequently treated with either normal media, or media containing NGF (25 ng/ml, Cedarlane Labs), LN (25 µg/ml, Invitrogen) or LN+N (25 ng/ml of NGF Cedarlane Labs,

and 25 µg/ml of LN Invitrogen). Neurons were subsequently processed at 10 min, 1 hr, or 6 hrs after stimulation by removing the original plating media, adding 300 µl of PBS containing 0.5 mM Orthovanadate to each well, and scraping the cells off the plating surface using a beveled tip rubber scrapper. Collected samples were briefly centrifuged to pellet the cells, supernatant was removed, and the cells were lysed by gentle rotation overnight using 50 µl of lysis buffer containing TBS with 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate. Following cell lysis and centrifugation (10,000 rpm, 5 min), the supernatants were used to determine protein concentrations using a BCA protein assay (Pierce Chemicals, Rockford, IL.). Equivalent amounts of protein (50 µg) were subjected to SDS-PAGE (8%-10% acrylamide). Following transfer to nitrocellulose, the blots were stained with Ponceau Red, photographed, assessed for the equivalency of protein loading, and cut to allow the top and bottom portions of the blot to be probed simultaneously. Ponceau Red was subsequently removed using Tris-Buffered Saline and the blots were blocked with 3% milk in Tris-Buffered Saline with 0.2% Tween-20, and subsequently incubated with the following antibodies (1:1,000) overnight at 4°C: phospho-Src, MAPK (1:1000, Santa Cruz, Santa Cruz CA), phospho-Akt, Akt, phospho-p44/42 MAPK (1:1000, NEB/Cell Signaling, Beverley, MA), phospho-Fak^{Y397}, and Fak (1:1000, Biosource Inc, Camerillo, CA). The next day, blots were washed, incubated in the appropriate horseradish peroxidase conjugated secondary antibody (anti-rabbit or anti-mouse IgG- HRP; Boehringer Mannheim, 1:5,000), and visualized using chemiluminescence reagents (ECL, NEN, Boston, MA) and exposure to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC). Developed films were subsequently

digitized and densitometrically analyzed using the Image J software (NIH image downloadable software). Phosphorylated substrates were normalized against their non-phosphorylated forms, however, as we did not have a functioning Src antibody, phospho-Src was normalized against MAPK. Digital images of the blots were used to make composite figures with Adobe Photoshop graphics software (Adobe Corp, Mountain View CA).

5.2.7 Statistical Analysis

All reported data analyses are based on a minimum of 3 separate experiments for each manipulation. Data are plotted as mean \pm SEM and significance is noted only if $p < 0.05$ as determined by ANOVA using Tukey testing for post hoc comparisons for multiple group analyses.

5.3 Results

5.3.1 Inhibition of Src or PI 3-K blocks NGF- and LN-induced axon growth

In our previous studies, we examined the requirement for both the PI 3-K/Akt and MEK/MAPK signalling pathways on LN- and NGF-induced neurite growth at 24 hrs. However, because neurite growth is detectable as early as 1-2 hrs after plating, we know that the intracellular signalling events required for neuritogenesis must be initiated almost immediately after ligand binding (Williams et al., 2005). Thus, by looking at the 24 hr time point previously, we were not able to identify the potential signalling intermediate relationships and temporal signalling events required for neurite growth.

Thus, in order to establish the relationship between the signalling intermediates required for neurite growth and the potential temporal differences in their activation we opted to perform a series of early time point experiments. We first analysed the effects of the inhibitors on the morphology of early neurite growth and then assessed the time and degree of intermediate activation by Western blot analysis.

To assess the requirement for PI 3-K, MEK, and Src on neurite growth, adult NGF-responsive DRG neurons were plated on PL for 2 hrs in the presence (Fig 5.1, LY, D-F; UO, G-I; PP2, J-L) or absence (Fig 5.1, Con, A-C) of enzyme specific pharmacological inhibitors prior to NGF, LN, or LN+N treatment. After addition of NGF, LN or LN+N, neurons were further incubated for 10 min, 1 hr, or 6 hrs at 37°C, and subsequently fixed and immunostained with antibodies directed against total tubulin to allow for neuritic identification. As shown in Fig 5.1, 6 hr treatment with NGF (A), LN (B), or LN+N (C) results in neurite outgrowth in the absence of added inhibitors (since 6 hr treatment elicited the most robust growth response, data for these time point are shown). Although growth was minimal with the addition of NGF alone (Fig 5.1 A), extensive growth was observed when the neurons were treated with LN (Fig 5.1 B), which was further increased with LN+N treatment (Fig 5.1 C).

When cultured in the presence of the PI 3-K inhibitor, LY294002, growth induced by NGF (Fig 5.1 D), LN (Fig 5.1 E), and LN+N (Fig 5.1 F) was inhibited. Similar results were observed with the addition of the Src family kinase inhibitor, PP2 (Fig 5.1 J-L). However, the addition of the MEK inhibitor, U0126, did not appear to have any affect on neurite growth at the tested time points (Fig 5.1 G-I).

These data suggest that NGF, LN, and LN+N-induced neurite growth from adult

Figure 5.1. Inhibition of either PI-3K, or Src, but not MAPK, blocks neuritogenesis of NGF-responsive cells 6 hrs after stimulation. A-L: Cells were plated on PL and immunostained for total tubulin. Confocal laser scanning microscopy was used to visualize stained neurons 6 hrs after plating. NGF-responsive cells plated on PL show increased neurite growth when treated with NGF (A), LN (B), or LN+N (C). Observed increases in neurite growth after NGF, LN, or LN+N stimulation were attenuated with the addition of LY294002 (D, E, and F respectively), or PP2 (J, K, and L respectively). Neurite growth was unaffected by the addition of U0126 (G, H, and I respectively). Scale bar – 50 μ m.

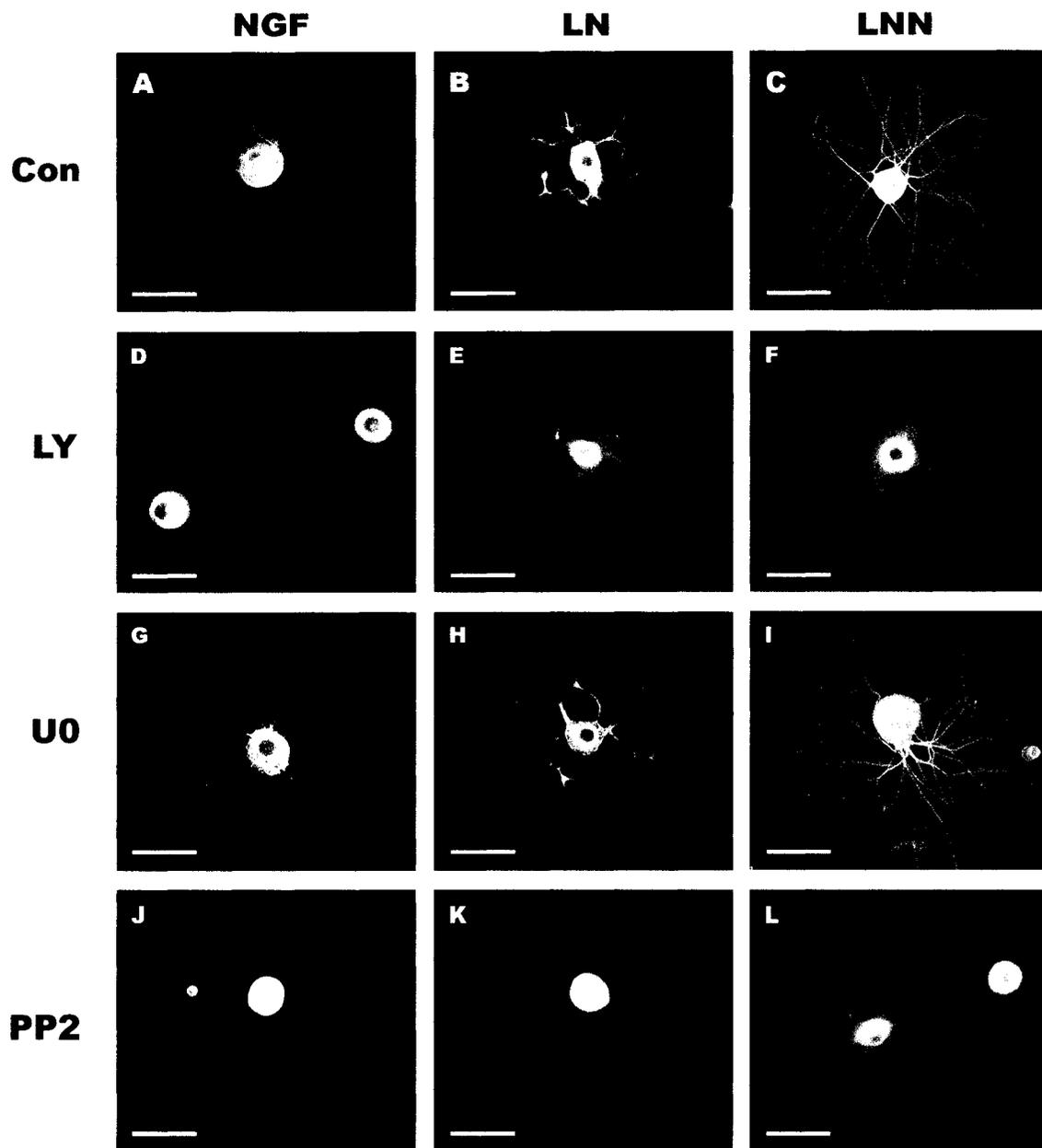


Fig 5.1

NGF-responsive DRG neurons can be detected by 6 hrs after treatment and is dependent on Src and PI 3-K signalling, but is not dependent on MEK/MAPK signalling. This confirms that neurite growth is initiated at very early stages after treatment, and that signalling initiated for this growth must occur almost immediately after ligand binding.

Quantitation for conditions represented in Fig 5.1 is presented in Fig 5.2 as the percent of cells with neurites. Varying amounts and patterns of neurite growth were detected by 6 hrs after treatment with NGF, LN, and LN+N as noted in Fig 5.1. In order to quantify these results, we categorized cells with elaborate neurite growth as having +++ growth. Cells with 1-2 neurites that were at least 2 cell body diameters in length, but with little branching were categorized as ++, and cells that had little growth, but possessed large lamellopodia were categorized by + (see Fig 5.2A for representative examples). Quantification of these results are presented in Fig 5.2 B-E.

The most neurite growth was observed after 6 hr LN+N treatment (Fig 5.2B), in this condition 30-35% of the cells had elaborate neurite growth, 10% of the cells had smaller, less branched neurites, and 10-15% of the cells displayed large lamellopodia. Approximately 20% of the cells extended elaborate neurite growth after LN treatment, while 8-10% of the cells had smaller, less branched neurites, and roughly 5% of the cells displayed large lamellopodia. Although 6 hr treatment with NGF induced significantly more neurite growth than the un-treated control condition, there were no cells with elaborate neurite growth, and only 10-12% with smaller neurites, and 5% with large lamellopodia. These data show that the co-treatment with LN+N appears to accelerate the growth that is observed with either LN or NGF alone at this early time point.

Figure 5.2. Analysis of the effect of LY294002, U0126, and PP2 on neurite growth from NGF-responsive cells 6 hrs after stimulation. **A:** Legend indicating growth patterns for subsequent analysis (+: little neurite growth with large lamellopodia; ++: few neurites extending at least two cell body diameters with little branching; +++: extensive neurite growth with extensive branching). **B-E:** Percent of cells with neurites on PL in the absence of added inhibitors or in the presence of LY294002, U0126, and PP2, before and after NGF (A, C), LN (A, D), or LN+N (A, E) stimulation. The percent of cells with neurites was attenuated with the addition of LY294002, and PP2, after NGF (C), LN (D), and LN+N (E) stimulation, however U0126 had no significant effect on the percent of cells with neurites. The control condition in C, D, and E, correspond to the circled area in B of the same color. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.005$, ** $p < 0.001$.

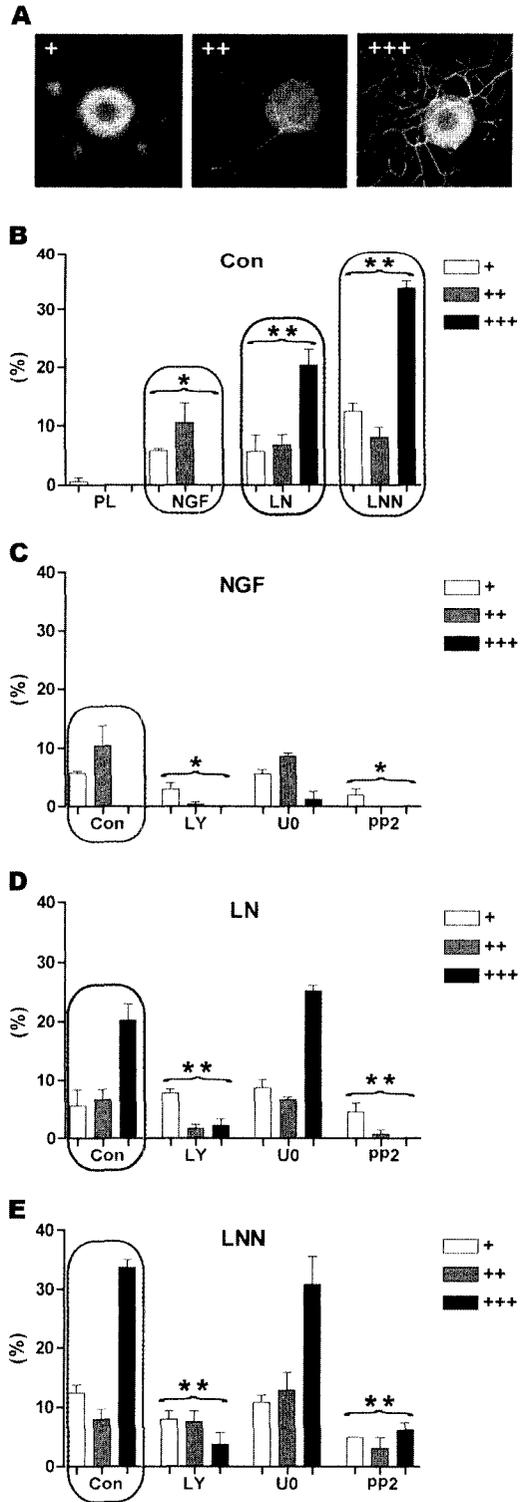


Fig 5.2

To analyse the contribution of PI 3-K, MEK, and Src to neurite growth, the neurons were plated in the presence of the pharmacological inhibitors LY294002 (PI 3-K inhibitor), U0126 (MEK inhibitor), and PP2 (Src family kinase inhibitor). As indicated in Fig 5.2 C, inhibition of either PI 3-K or Src, significantly inhibited NGF-induced neurite initiation and growth. Inhibition of PI 3-K with LY294002, and Src with PP2, blocked NGF (Fig 5.2 C), LN (Fig 5.2 D), and LN+N-induced (Fig 5.2 E) neurite growth; however, MEK inhibition had no effect. Interestingly while neuritogenesis was inhibited, neither LY294002 nor PP2 appeared to influence the number of cells with lamellopodia, suggesting perhaps that the effects of these pathway specific inhibitors are at the stage of neuritogenesis (Williams *et al.*, 2005).

5.3.2 Timeline of activation of the signalling intermediates Src, Fak, Akt, and MAPK after short-term stimulation with NGF, LN, and LN+N.

We have previously shown that 24 hrs after treatment, LN+N stimulates a level of neurite growth from adult NGF-responsive DRG neurons that is not achievable with either LN or NGF treatment alone. We have also noted that this neurite growth is critically dependent on the activation of the PI 3-K/Akt pathway. However, it remains to be determined how the early activation of these signalling intermediates is responsible for the increased neurite growth induced by LN+N. Therefore, in order to investigate the early events activated by NGF, LN, or LN+N experiments similar to those noted above were carried out. Again neurons were plated on PL, and incubated for 2 hrs with or without pathway specific inhibitors prior to treatment with NGF, LN, or LN+N for 10min, 1 hr, or 6 hrs. We hypothesized that NGF or LN activates its signalling cascades

with different temporal characteristics. The combination of the two might then act to either result in stronger or more sustained signalling that results in enhanced neurite growth.

In Fig 5.3, representative Western blots and corresponding densitometric analyses plotted as a function of treatment time are shown. Each graph is designed such that it represents a single signalling intermediate, and each line on the graph represents the timeline of activation for the corresponding intermediate after a specific treatment. The PL line is the control (no treatment) condition; since no detectable phosphorylation was observed for any of the studied intermediates except MAPK, this lane was not included on the representative Western blot. However, it is important to note that there is a basal level of constitutive MAPK phosphorylation in these cells, and thus, the treatment effect appears to be much smaller than that observed for the other signalling intermediates. It is also important to note that for each time point and condition studied triplicate platings were analysed and data represents the mean \pm SEM; thus, even though some of the error bars are masked by the symbols in Fig 5.3- Fig 5.7 they are present. To simplify the results, the effects of each treatment (NGF, LN, or LN+N) on the activation of Src, Fak, Akt and MAPK respectively, are grouped together and described separately below.

Effect of NGF treatment on the activity of Src, Fak, Akt, and MAPK

As shown in Fig 5.3 A, NGF treatment stimulated a significant increase in the phosphorylation of Src at the earliest point analyzed, 10 min relative to control, this increase was sustained over the tested time course, with maximal levels noted at 6 hrs. In contrast, NGF resulted in early and transient Fak phosphorylation; maximal levels were

Figure 5.3. Western blot analysis indicating the timeline of Src, Fak, Akt, and MAPK activation, essential for neurite growth after short-term stimulation with NGF, LN, and LN+N. Adult DRG neurons were plated on PL coated dishes, and incubated for 2 h at 37°C. Neurons were subsequently treated with NGF, LN, or LN+N for 10min, 1 hr, or 6 hrs, after which time Western blotting of cell lysates was carried out. **A-D:** Western blot and corresponding timeline of activation of early signalling intermediates: Src (A), Fak (B), Akt (C), and MAPK (D), in response to short term (10min, 1 hr, and 6 hrs) NGF and LN stimulation. n = 3-4 separate experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * p< 0.05, ** p<0.001.

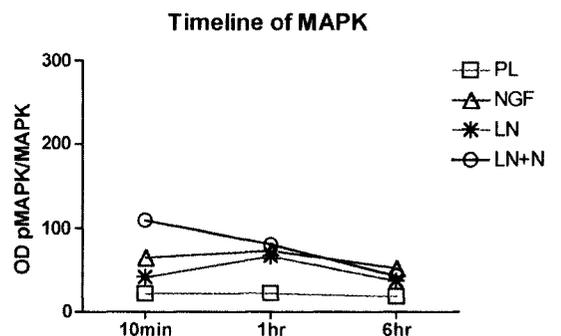
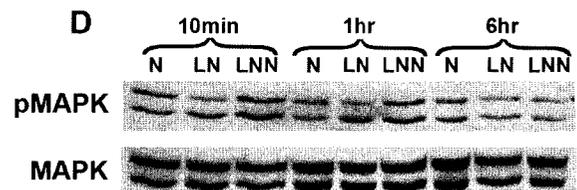
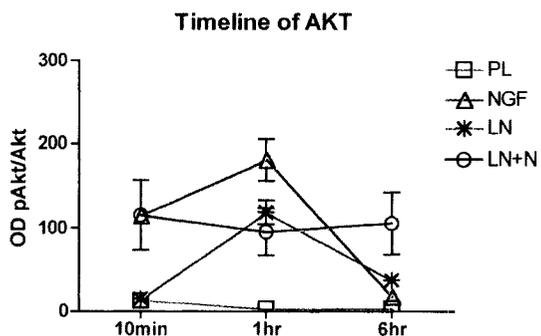
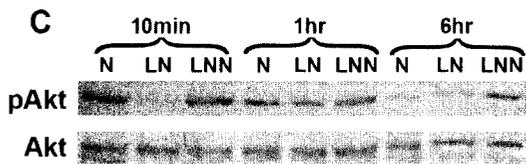
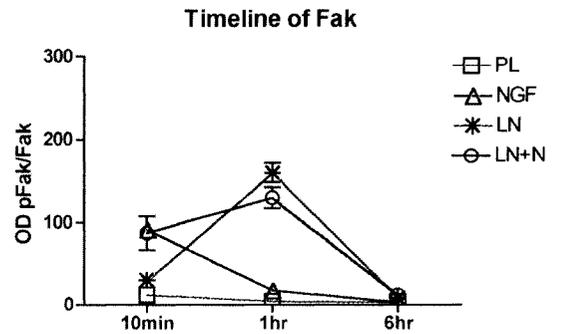
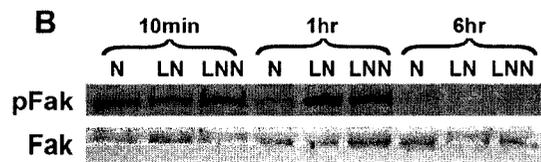
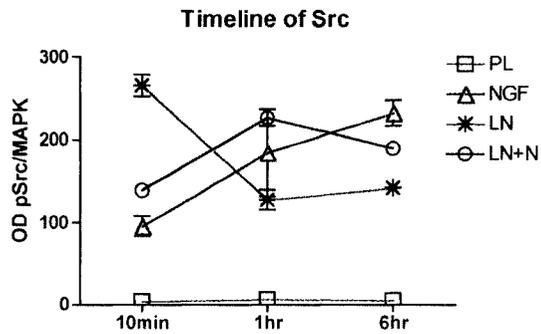
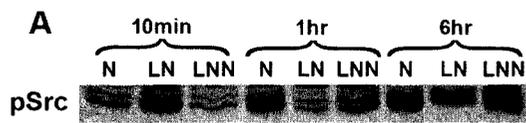


Fig 5.3

observed at 10 min, but levels dropped back to baseline by 1 hr (Fig 5.3 B). NGF also induced a significant level of phosphorylated Akt at 10 min relative to control, which further increased, peaking at 1 hr, before declining to baseline by the 6 hr time point (Fig 5.3 C). Finally, NGF treatment induced a significant increase in MAPK phosphorylation at 10 min relative to control, and the increase was sustained across all time points (Fig 5.3 D).

Effect of LN treatment on the activity of Src, Fak, Akt, and MAPK

As shown in Fig 5.3 A, LN treatment resulted in a significant increase in the phosphorylation of Src at 10 min relative to control, but levels declined significantly by 1 hr where it remained up to 6 hrs. An examination of the effects of LN treatment on Fak, Akt, and MAPK phosphorylation revealed a delayed activation with a significant increase in phosphorylated Fak, Akt, and MAPK being detected by 1 hr after LN treatment, and subsequently declining to baseline by 6 hrs (Fig 5.3 B, C, D).

Effect of LN+N treatment on the activity of Src, Fak, Akt, and MAPK

As shown in Fig 5.3 A, LN+N treatment resulted in a significant increase in the phosphorylation of Src at 10 min relative to control. This was less than that observed with LN alone and greater than that with NGF alone. The phosphorylation increased to a peak at 1 hr which was similar to that observed with NGF alone. Fak phosphorylation was increased by 10 min relative to control, and unlike NGF alone, was sustained for 1 hr, before declining to baseline by 6 hrs (Fig 5.3 B). LN+N also induced a significant level of phosphorylated Akt at 10 min relative to control, but pAkt levels were sustained

across all time points (Fig 5.3 C). Finally, LN+N treatment induced a significant increase in MAPK phosphorylation at 10 min relative to control; the phosphorylation subsequently declined to baseline levels over the tested time course (Fig 5.3 D).

Summary of the timeline of activation data

In summary, the time at which each of the tested signalling intermediates is activated is highly dependent on the treatment performed. Notably the pattern and time at which each of the intermediates is activated varies greatly.

LN+N appears to sustain Src phosphorylation over the course of the experiment, keeping phosphorylated Src levels up in contrast to the relative transient effect of LN treatment alone: the LN+N this treatment also induces a level of Src phosphorylation by 10 min that is higher than that seen with NGF alone. Similarly LN+N gives a higher level of Src phosphorylation at 1 hr than either NGF or LN alone.

LN+N treatment results in early Fak phosphorylation (similar to that with NGF alone) which is sustained for 1 hr (to give levels similar with LN alone). In contrast, phosphorylation of Fak by NGF drops to base line by 1 hr, while phosphorylation of Fak by LN does not occur until 1 hr. Thus, it appears that LN+N takes the early phosphorylation of Fak that NGF induces and couples it with the later phosphorylation of Fak that LN induces.

LN+N acts to maintain the activation of Akt over the course of the experiment, such that an early increase in Akt phosphorylation by 10 min is similar to that after NGF treatment alone, and the level of Akt phosphorylation by 1 hr is similar to that after LN

treatment alone. In contrast, LN+N keeps the phosphorylated levels of Akt up by 6 hrs, which is lost by either LN or NGF treatment alone.

These results indicate that the enhanced growth induced by LN+N treatment is not merely due to an additive response in signalling intermediate phosphorylation. For example, it was possible that increased neurite growth induced by LN+N was simply due to a level of signalling intermediate phosphorylation across all time points that was equal to that induced by LN and NGF added together. Thus, it is important to note that this was not the case. For example, as suggested above unlike treatment with LN or NGF alone, LN+N induces a sustained activation of Akt across all time points, which potentially explains why LN+N rather than either treatment alone gives enhanced neurite growth.

5.3.3 Addition of PP2, LY294002, and U0126 identify Src as a key signalling intermediate located upstream of both PI 3-K/Akt and MEK/MAPK signalling pathways.

Although we have identified the time at which each of the different signalling intermediates is activated after NGF, LN, and LN+N treatment, we also wished to identify the hierarchical location of each of these signalling intermediates. Thus, to try and determine where each of the studied intermediates are located with relation to each other, the neurons were treated with the PI 3-K inhibitor LY294002 (Fig 5.4), the MEK inhibitor U0126 (Fig 5.5), or the Src family kinase inhibitor PP2 (Fig 5.6), and incubated for 2 hrs prior to treatment with NGF, LN, or LN+N for 10 min, 1 hr, or 6 hrs. Cells were lysed and Western analysis was carried out as outlined in Section 5.2.6.

As in the previous experiment, representative Western blots and corresponding densitometric analysis plotted as a function of treatment time are shown. Again each graph is designed such that it represents a single signalling intermediate, and each line on the graph represents the timeline of activation for the corresponding intermediate after a specific treatment. The only difference is that specific pharmacological inhibitors are added to the culture media at the time of plating. Again the PL line is the control (no treatment) condition, and as no identifiable phosphorylation was seen for any of the studied intermediates except MAPK, this lane was again excluded from the representative Western blots. It is important to note that in all conditions, including the PL (no treatment) group, the pharmacological inhibitors were present before and during treatment.

Effect of LY294002 treatment on the activity of Src, Fak, Akt, and MAPK

NGF induced phosphorylation of Src, Fak, and Akt are significantly attenuated across all time points in the presence of the PI 3-K inhibitor, LY294002 (Fig 5.4 A, B, C), relative to control (Fig 5.3 A, B, C, respectively). In contrast, the pattern of MAPK phosphorylation in response to NGF treatment was unaffected by the inhibition of PI 3-K (Fig 5.4 D). Phosphorylation of Src, Fak, and Akt by LN and LN+N treatment was also inhibited by LY294002 (Fig 5.4 A, B, C), relative to control (Fig 5.3 A, B, C), while MAPK phosphorylation was unaffected (Fig 5.4 D). These results indicate that PI 3-K may influence the activity of Src, Fak, and Akt, but is not upstream of MAPK.

Figure 5.4. Inhibition of PI 3-K results in attenuation of NGF, LN and LN+N signalling. Adult DRG neurons were plated on PL coated dishes, and incubated for 2 h at 37°C. Neurons were subsequently treated with NGF, LN, or LN+N for 10min, 1 hr, or 6 hrs, after which time Western blotting of cell lysates was carried out. **A-D:** Western blot and corresponding timeline of activation of early signalling intermediates: Src (A), Fak (B), Akt (C), and MAPK (D), in response to short term (10min, 1 hr, and 6 hrs) NGF and LN stimulation. n = 3-4 separate experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * p< 0.05, ** p<0.001.

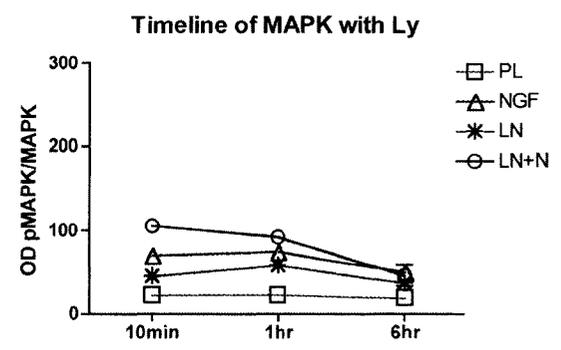
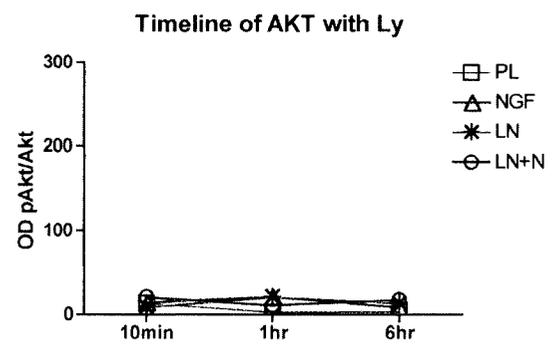
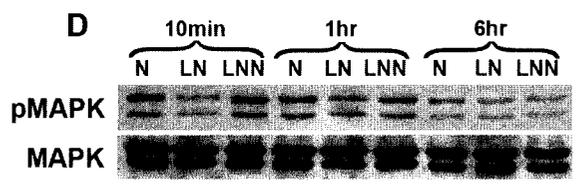
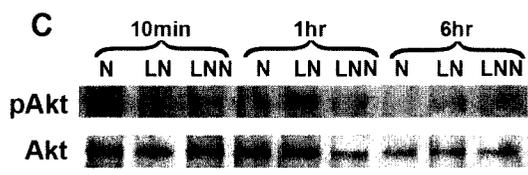
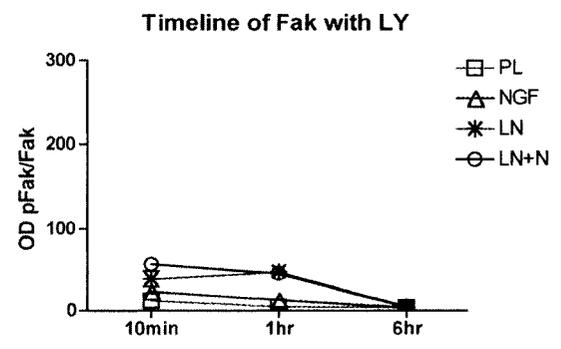
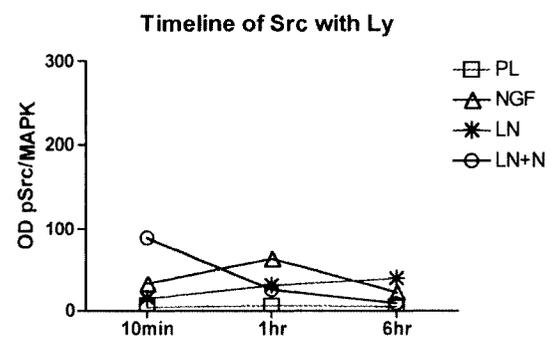
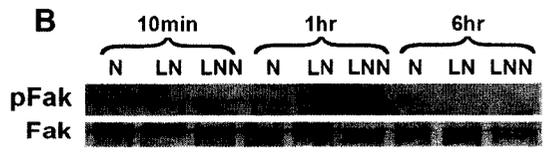
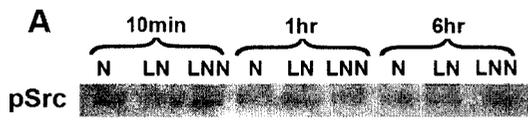


Fig 5.4

Effect of U0126 on the activity of Src, Fak, Akt, and MAPK

NGF induced phosphorylation of Src, Fak, and Akt were not significantly affected at any of the tested time points in the presence of the MEK inhibitor, U0126 (Fig 5.5 A, B, C), relative to control (Fig 5.3 A, B, C respectively). In contrast, the pattern of MAPK phosphorylation in response to NGF treatment was significantly attenuated by the inhibition of MEK (Fig 5.5 D). Cellular responses to LN and LN+N treatment yielded a similar attenuation MAPK phosphorylation (Fig 5.5 D), relative to control (Fig 5.3 D), while Src, Fak, and Akt phosphorylation were unaffected (Fig 5.5 A, B, C). These results indicate that MEK is located upstream of MAPK and does not exert any apparent influence over the activity of Akt, Src, or Fak.

Effect of PP2 on the activity of Src, Fak, Akt, and MAPK

PP2 inhibits the activation of Src, Fak, Akt, and MAPK by all the treatments (Fig 5.6 A, B, C, D), relative to control (Fig 5.3 A, B, C, D respectively). These results indicate that Src is likely located upstream of Fak, PI 3-K, Akt, and MAPK, and acts as the major point of collaboration between NGF and LN induced signalling pathways and subsequent neurite growth.

Figure 5.5. MEK inhibition does not negatively influence the activation of Src, Fak, or Akt by NGF, LN, or LN+N. Adult DRG neurons were plated on PL coated dishes, and incubated for 2 h at 37°C. Neurons were subsequently treated with NGF, LN, or LN+N for 10min, 1 hr, or 6 hrs, after which time Western blotting of cell lysates was carried out. **A-D:** Western blot and corresponding timeline of activation of early signalling intermediates: Src (A), Fak (B), Akt (C), and MAPK (D), in response to short term (10 min, 1 hr, and 6 hrs) NGF and LN stimulation. n = 3-4 separate experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * p< 0.05, ** p<0.001.

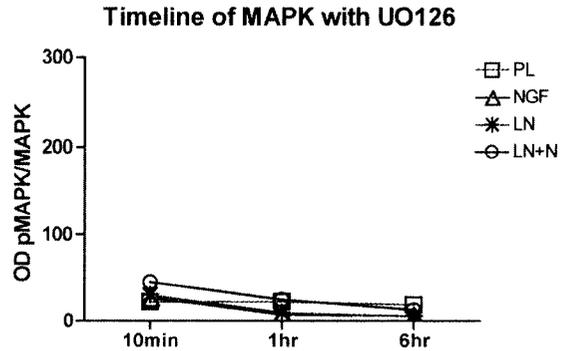
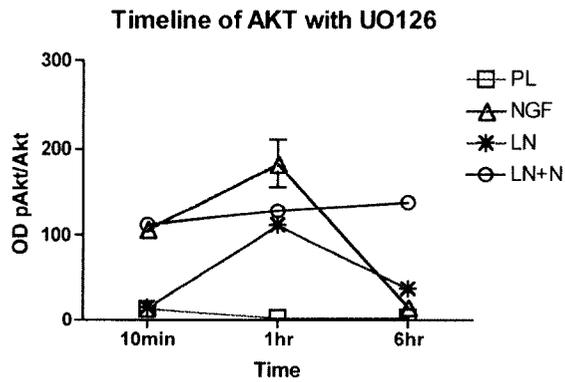
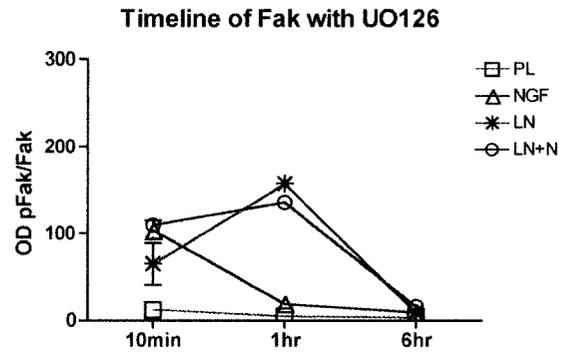
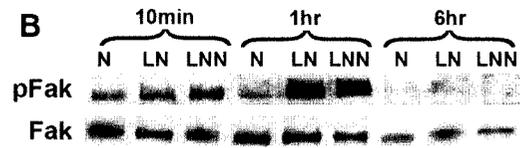
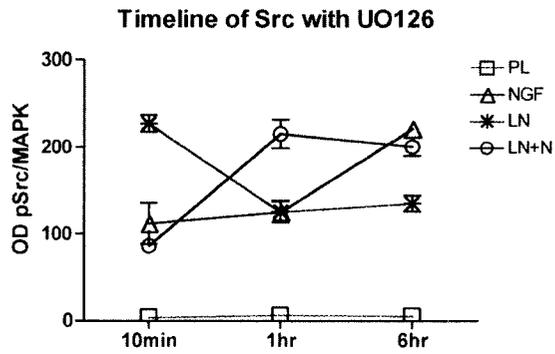
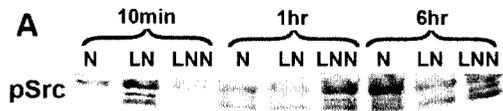


Fig 5.5

Figure 5.6. Inhibition of Src attenuates NGF, LN and LN+N signalling.

Adult DRG neurons were plated on PL coated dishes, and incubated for 2 hrs at 37°C. Neurons were subsequently treated with NGF, LN, or LN+N for 10 min, 1 hr, or 6 hrs, after which time Western blotting of cell lysates was carried out. **A-D:** Western blot and corresponding timeline of activation of early signalling intermediates: Src (A), Fak (B), Akt (C), and MAPK (D), in response to short term (10 min, 1 hr, and 6 hr) NGF and LN stimulation. n = 3-4 separate experiments. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * p< 0.05, ** p<0.001.

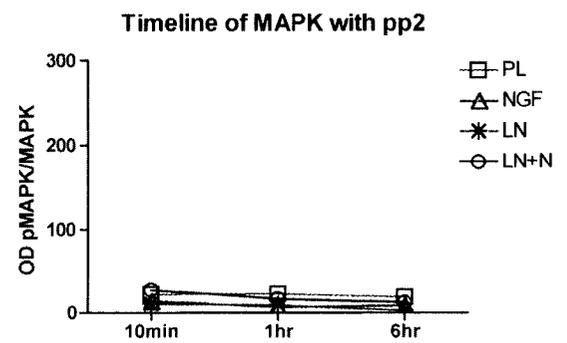
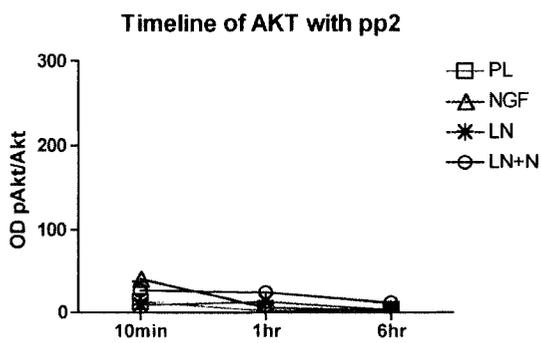
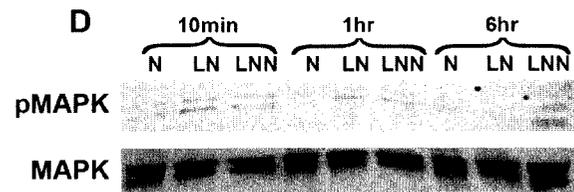
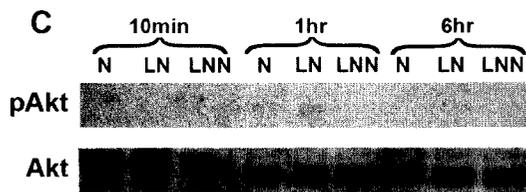
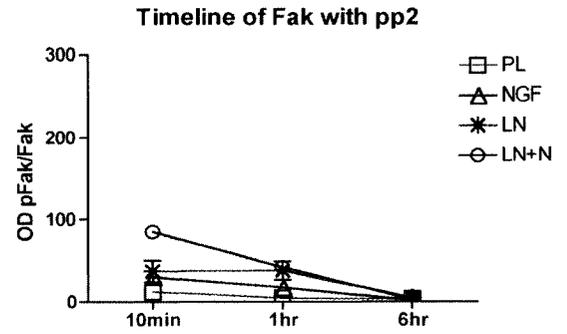
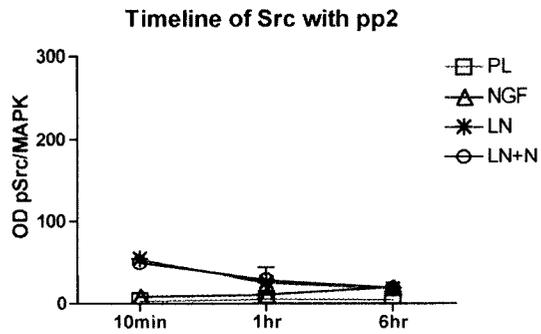
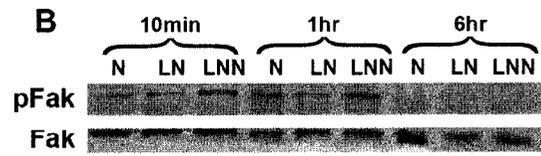
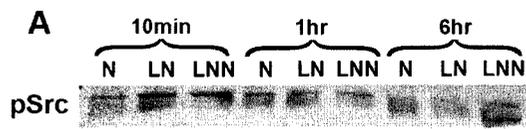


Fig 5.6

5.4 Discussion

The ability of the PI 3-K/Akt pathway to promote neurite growth has been well documented in a variety of different cell types (Higuchi et al., 2003; Jones et al., 2003; Kaplan and Miller, 2000; Mills et al., 2003; Tucker et al., 2005a). Activation of this pathway, and subsequent neurite growth, can be achieved by signalling through both integrin receptors, via laminin binding (Sarner et al., 2000; Tucker et al., 2005a), and growth factor receptors, via NGF binding (Jones et al., 2003; Kaplan and Miller, 2000). However, the process by which cross talk occurs between integrin and growth factor receptor activation, leading to the stimulation of PI 3-K/Akt and optimal neurite growth is poorly understood. Although we have previously identified Src as a possible collaborative component upstream of PI 3-K/Akt signalling, these studies were carried out at 24 hrs after plating, and following further investigation, we found that extensive neurite growth was occurring between 1-6 hrs after treatment. Thus one would expect to see activation of the relevant signalling intermediates within this early time frame. It is clear that initial ligand binding can activate signalling cascades over relatively short periods of time, so in order to determine the patterns of activation induced by NGF, LN, and LN+N, we undertook a temporal analysis of signalling intermediate activation between 10 min and 6 hrs.

LN stimulation resulted in the early activation of Akt and MAPK, not just Src and Fak, indicating that the appropriate time points for LN and NGF induced activation is very early and may act to prime neurite growth. Our results suggest that the optimal neurite growth that occurs with LN+N treatment is due to crosstalk between LN and NGF induced signalling intermediates located upstream of PI 3-K. It is important to note that the addition of both NGF and LN together did not result in an additive level of Src phosphorylation. For instance, rather than LN+N resulting in maximal Src activation at

all time points, it actually acted to stabilize the level of active Src, resulting in a level that was midway between either LN or NGF treatment alone. Shortly after the 10 min LN stimulation, Src reached its highest level of phosphorylation; however it quickly dropped and stabilized by 1 hr. NGF stimulation on the other hand, started out with the lowest level of Src activation but continued to climb, resulting in maximum Src activation by 6 hrs. LN+N stimulation induced the highest levels of Src activation, which was between NGF and LN at both 10 min and 6 hrs. As with Src phosphorylation, LN+N treatment induced a sustained level of Akt phosphorylation that was maintained across all time points. This response occurred despite the fact that phosphorylated Akt had returned to baseline by 6 hrs with either NGF or LN treatment alone.

An important determinant of our conclusion that Src is a potential upstream point of convergence between LN and NGF induced signalling pathways is that inhibition of Src, via PP2, resulted in inhibition of Akt, Fak, and MAPK. This finding, paired with the fact that Akt and MAPK are known intermediates in both NGF-induced Trk signalling (Delcroix et al., 2003; Higuchi et al., 2003; Jones et al., 2003; Markus et al., 2002b; Mills et al., 2003; Tucker et al., 2005a; Zhou et al., 2004) and LN induced integrin signalling (Dey et al., 2005; Gary and Mattson, 2001; Koul et al., 2005; Mills et al., 2003; Tucker et al., 2005a), suggests that these intermediates are located downstream of Src. It is also important to note that inhibition of MEK in our experiments had no detectable effect on early Akt activation, and inhibition of PI 3-K has no effect on the activation of MAPK. This suggests that these intermediates are not in the same signalling pathway and do not influence one another's activity. Thus, Src activation is playing a dual role in the

influence of two distinct signalling pathways, again suggesting that it acts as an upstream collaborative intermediate.

Although we are confident that Src is a key point of collaboration between integrin and neurotrophin signalling in the studied population of cells, we are well aware that this is not the only possible point of integrin and growth factor cross talk. For instance, in a series of elegant experiments performed by Mills and colleagues (2003), integrin-linked kinase (ILK) was identified as being a necessary component of NGF induced neurite outgrowth in embryonic chick DRG neurons. In these experiments, they showed that Akt is directly phosphorylated by ILK, and the presence of a dominant negative ILK construct attenuated Akt induced neurite growth. Their study also pointed out that inhibition of ILK, although directly affecting Akt phosphorylation, does not affect the activation of the MEK/MAPK signalling pathway, suggesting that ILK is an important component of the PI 3-K/Akt signalling pathway, and potentially located downstream of Src. Thus, inhibition of Src may also lead to inhibition of ILK. However, because antibodies towards activated ILK were not commercially available, we were unable to explore this possibility further.

As indicated previously, phosphorylation of Fak may be induced by both integrin and growth factor receptor activation; for instance, signalling induced by EGFR and integrin activation in PC12 and SH-SY5Y cells both stimulate the phosphorylation of Fak (Ivankovic-Dikic et al., 2000; Tucker et al., 2005a). Fak is known to bind components of the Src family kinases, specifically c-Src, following which this complex is translocated to sites of focal adhesion (Dikic et al., 1996; Schlaepfer and Hunter, 1998). These events then activate a series of downstream signalling molecules such as PI 3-K and MAPK,

which stimulate cell survival, migration, and gene expression (Clark and Brugge, 1995; Dikic et al., 1996; Schlaepfer and Hunter, 1998). Our results show that inhibition of Src, via PP2, inhibits Fak activation by NGF, LN, and LN+N treatment in DRG neurons. This, coupled with the fact that activation of Src occurs prior to Fak activation, indicates that Fak is probably not the key upstream point of collaboration between NGF and LN signalling in our system. However, due to a lack of available pharmacological Fak inhibitors, and poor transfection efficiency of dominant negative cDNA constructs into DRG neurons, the function of this intermediate could not be directly examined in these studies. The possibility still exists that activation of Src may stimulate recruitment and binding of Fak, leading to the activation of the PI 3-K/Akt and MEK/MAPK signalling pathways. As indicated by Ivankovic-Dikic and colleagues (2000), expression of Fak^{Y397F}, which is unable to bind Src in SH-SY5Y cells, leads to an inhibition of neurite growth, although similar results were not found in PC12 cells. It is important to note that even though we find that PI 3-K is located downstream of Src, this molecule still has the ability to influence both Src and Fak signalling, potentially by the presence of a negative feedback system. For instance, inhibition of Src, via PP2, could inhibit PI 3-K activation, and inhibition of PI 3-K, via LY294002, could attenuate Src activity.

We have summarized our data in a model that illustrates the hierarchy of signalling intermediates required for LN+N induced optimal neurite growth in NGF-responsive adult DRG neurons (Fig 5.7). To our knowledge, this is the first

Figure 5.7. Schematic illustration of the hierarchy of intermediates essential to our model of Neurotrophin and Integrin mediated signalling cascades for the stimulation of neurite growth. LY294002, PI 3-K inhibitor; PP2, Src family kinase inhibitor; U0126, MEK inhibitor.

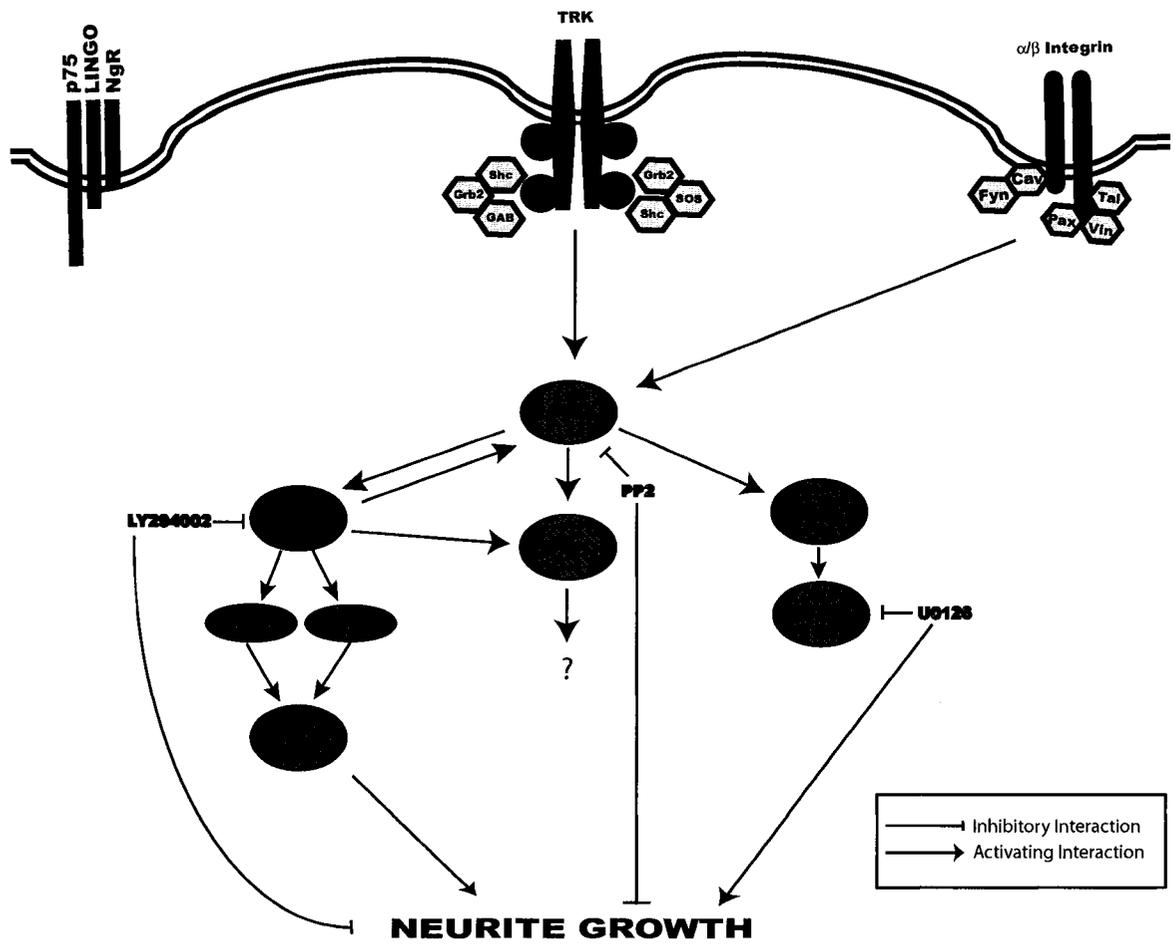


Fig 5.7

study identifying Src as a key point of collaboration between NGF and laminin induced signalling in adult NGF-responsive DRG neurons. As we have indicated, enhanced neurite growth elicited by LN+N stimulation appears to be due to the sustained activation of Src, which results in the sustained activation of the downstream signalling intermediate Akt. We have also shown that Src is required for NGF and LN induced activation of the MEK/MAPK signalling pathway; however, this pathway is not required for neurite extension in the tested NGF-responsive cell population. These findings are important as they provide new insights into the process by which NGF and LN induced signalling pathways collaborate to stimulate optimal levels of neurite growth. We have also identified that Src is a key signalling molecule that can be targeted to potentially stimulate maximal axonal regeneration after injury to either PNS or CNS neurons.

CHAPTER 6

DISCUSSION AND SUMMARY

6.1 Research outcomes

The fundamental goal of my research was to identify the key environmental conditions and subsequent cellular signalling components required to stimulate optimal levels of neurite growth from adult sensory neurons. As previously suggested, this work stemmed from work in our lab, which looked at the effects of NGF and IGF-1 on neurite growth in adult rat DRG neurons. At the time, we were concerned with the signalling mechanisms related to trophic factor stimulation, specifically interactions between NGF and IGF-1, in relation to neurite growth. We had noticed that neurons plated on laminin were extending processes in the absence of added growth factors. From these observations it was hypothesized that the ECM, laminin (which we happened to be using at the time), was sufficient to stimulate significant amounts of neurite growth in the absence of added growth factors. Although the neurotrophin field was well advanced by the time these experiments began, there was little information available with respect to the interaction between the extracellular matrix and neurotrophins. At the time, most *in vitro* neurotrophin experiments, ours included, were performed with neurons plated on laminin, and thus, the importance of this ECM was overlooked. This led us to formulate the second part of the hypothesis, which suggested that laminin-induced neurite growth could be potentiated by neurotrophins, especially NGF, and that this potentiation was due to crosstalk between extracellular matrix and neurotrophin induced signalling intermediates.

As obvious from previous chapters, a variety of different experiments were performed in order to test these hypotheses. To summarize my results, I have placed my findings in three separate categories: (1) neuronal response to laminin; (2) the effect of added trophic factors; (3) signalling required for neurite growth.

6.1.1 Neuronal response to laminin

DRG neurons plated on the ECM laminin in the absence of added growth factors elaborate significantly more neurite growth than cells plated on poly-D-lysine plus or minus NGF. Unlike laminin, poly-D-lysine is a synthetic molecule used to charge plating surfaces. Although this aids in cellular attachment, it does not have the ability to activate integrin receptors, which suggests that binding and activation of integrins are key for stimulating growth in DRG neurons. However, it was possible that the major difference in neurite growth was simply due to a difference in cell/substrate adherence. These concerns were satisfied by the fact that adherence was essentially identical for neurons plated on either surface. To determine whether increased neurite growth was integrin dependent we used two different integrin inhibitors, the RGD peptide and the anti- β 1 integrin antibody. From these experiments, we were able to show that integrin inhibition resulted in an attenuation of neurite growth.

Although these results suggested that integrin activation alone was sufficient to stimulate significant neurite growth in the absence of added growth factors, we observed that not all neurons were responding in this way. As noted above, the DRG is made up of a heterogeneous population of cells that can be classified crudely based on cell body diameter, and more specifically, upon their ability to bind the lectin IB4 (predominantly

small diameter neurons), express the peptide CGRP (small and medium diameter neurons) or express the heavy chain neurofilament NF200 (predominantly medium and large diameter neurons) (Averill et al., 1995; Priestley et al., 2002a). Initial observations indicated that the small diameter DRG neurons were unresponsive to laminin, and only medium and large diameter neurons were extending neurites. However, by culturing cells for 48 hrs we found that this was not entirely the case; in fact, some of the small diameter neurons responded with extensive neurite growth while some large diameter neurons remained unresponsive. By using a magnetic bead-based cell selection technique, further analyses indicated that, unlike the CGRP and NF200+ve cell populations, the IB4+ve cell population was not responsive to laminin in the absence of added growth factors. These findings corresponded nicely with our initial observations; this population consisted of predominantly small diameter neurons and just a few large diameter neurons, which is exactly the ratio of unresponsive cells that we had observed.

6.1.2 Effect of added trophic factors

In initial experiments, we found that laminin-induced neurite growth was potentiated by the addition of NGF. However, as with the previously mentioned findings, NGF exerts its effects on specific cell populations. As indicated previously, the IB4+ve cell population does not express the NGF receptors Trk or p75, and thus these cells are not NGF-responsive. However, this population of cells does express the GDNF receptor components GFR α and Ret. When these cells were plated on laminin in the presence of GDNF, they responded with increased neurite growth; this result was not seen when these cells were plated on poly-D-lysine. These findings suggest that the

IB4+ve cell population is responsive to laminin; but, they require GDNF in order to extend neurites. On the other hand, NGF could potentiate laminin induced neurite growth in both CGRP and NF200+ve cell populations. As our interests lay in the interaction between NGF and laminin induced signalling in the stimulation of neurite growth, these two cell populations (later collectively termed the NGF-responsive cells) became our main focus, in chapter 5.

6.1.3 Signalling required for neurite growth

Although we were mainly concerned with the signalling events required to elicit neurite growth in the NGF-responsive cell population, the signalling pathways utilized by the IB4+ve cell population were also identified (chapter 4). Initially through pharmacological inhibition, we found that laminin induced neurite growth in sensory DRG neurons was critically dependent on signalling through the PI-3k/Akt pathway. However, when we examined the GDNF- and NGF- responsive populations separately we identified population specific signalling. The NGF-responsive cell population required signalling through the PI 3-K/Akt pathway for neurite growth but did not require MEK/MAPK signalling. In contrast, the IB4+ve cell population required both the PI 3-K/Akt and MEK/MAPK signalling pathways.

As I was interested in the population of neurons that responded to both laminin and NGF, my studies in chapter 5 focused on the NGF-responsive cells only. As indicated above, in order for these cells to extend neurites, signalling through the PI 3-K/Akt pathway is required. The next step then was to identify potential upstream points of convergence between laminin and NGF induced receptor activation, leading to the

stimulation of key downstream signalling components. By performing a series of timed stimulation experiments in the presence or absence of enzyme specific pharmacological inhibitors, we have identified Src as a potential upstream signalling component responsible for activation of the PI 3-K/Akt pathway by LN and/or NGF stimulation. Inhibition of Src attenuated the activation of all downstream signalling components, including those located in the MEK/MAPK signalling pathway. However, neither the PI 3-K/Akt nor the MEK/MAPK pathways were able to exert any influence over the other; inhibition of PI 3-K did not effect the activation of MAPK and inhibition of MEK did not affect the activation of Akt.

In these experiments, we also found that LN, NGF, or LN plus NGF could exert differences in the time and duration of signalling intermediate activation. Most interesting was the fact that laminin plus NGF stimulation induced a sustained activation of both Src and Akt that was not seen by the addition of either laminin or NGF alone. This finding could potentially explain how laminin plus NGF stimulates optimal levels of neurite growth.

Figure 6.1 presents a summary of signalling events thought to be involved in integrin and/or growth factor activated signalling that could be attributed to neurite growth. Although we have identified the importance of the intermediates highlighted in yellow, many of the other intermediates have been suggested in the literature to be potential effectors in a variety of different cell types.

Figure 6.1. Proposed model for the hierarchy of signalling intermediates that could be involved in regulating neurotrophin and integrin induced neurite growth. This is a summary diagram from the literature that points to potential signalling intermediates that could possibly act as downstream effectors for the intermediates that we have identified. Our findings are summarized in the yellow highlighted section. Akti: AKT inhibitor; Echistatin, anti-b1, RGD: integrin inhibitors; K252a: TrkA inhibitor; LY294002: PI 3-K inhibitor; PP2: Src family kinase inhibitor; Rex: p75 inhibitor; Y27632: ROCK inhibitor. LIMK: Lin-II, Islet I and Mec 3 kinase. SSH1: slingshot. TESK 1: Testicular protein kinase 1.

6.2 Relevance to the field of axonal regeneration

These studies are among the first to identify the importance of an interaction between ECM and trophic factors in stimulating neurite growth from adult rat sensory DRG neurons. At the time I began these studies very little was known with respect to integrin mediated signalling responsible for growth in neurons, similarly even less was known about how these pathways synergise with growth factor pathways in stimulating neurite growth. Importantly, we have identified both the key environmental conditions and signalling components required for neurite growth *in vitro* and potentially for nerve regeneration *in vivo*. In turn, these results can provide potential therapeutic targets for the treatment and repair of both peripheral and central nerves following injury. To briefly discuss the global significance of these findings, I will begin by focusing on the two types of peripheral nerve insult mentioned in the introduction, physical nerve injury and diabetes. This discussion will be followed by a brief mention of the relevance of these findings to CNS repair.

6.2.1 Physical nerve injury

To alleviate the loss of function and disability associated with physical injury to the PNS, axonal regeneration is required. However, as suggested in the Introduction, although PNS regeneration is possible, it is often poor and full functional recovery rarely occurs (Abrams and Widenfalk, 2005; Fu and Gordon, 1995a; Fu and Gordon, 1995b). Many different factors contribute to insufficient functional recovery after PNS damage, including prolonged time for or inability to reestablish synaptic connections. When the time between nerve injury and reinnervation is prolonged, target tissue often degenerates,

making functional recovery impossible (Fu and Gordon, 1995a; Fu and Gordon, 1995b). A major component of delayed target reinnervation is the formation of peripheral nerve gaps. Nerve gaps are formed when a segment of the peripheral nerve is lost and the proximal and distal nerve segments cannot simply be reconnected (Abrams and Widenfalk, 2005). When nerve gaps occur, surgical intervention is often necessary, and in many cases, one or more grafting procedures are performed (Newton and Nunamaker, 1985). A major problem associated with nerve grafting is that it is difficult to recreate the healthy peripheral nerve environment. However, a variety of grafting procedures have been used with limited success. Grafts range from biological materials such as nerve, vein, and muscle, which can be isolated from either the same (autografts) or different (heterografts) species, to synthetic nonbiological materials such as silicone and polyester (Meek and Coert, 2002; Muller and Stoll, 1998; Newton and Nunamaker, 1985). For the most part, use of these types of materials has been plagued with problems such as conduit collapse, enhanced scar production, and immunological rejection. The most commonly used grafting material still appears to be the autologous nerve; however, this graft is still quite poor in stimulating nerve regeneration, and in many cases, secondary problems as severe as the initial nerve injury may be created (Meek and Coert, 2002; Weber and Mackinnon, 2005). More recently, bio-absorbable conduits have become the focus for stimulating and guiding regenerating peripheral axons. For instance, polyglycolic acid, L-lactide and epsilon-caprolactone have all recently been used and have met with some success when attempting to stimulate peripheral nerve regeneration (Giardino et al., 1999; Giardino et al., 1995; Koshimune et al., 2003; Nakamura et al., 2004; Navissano et al., 2005; Weber et al., 2000). These conduits have

many promising characteristics in that they are non-toxic, non-immunogenic, resistant to collapse, and absorbable. Thus, scar formation using these materials is kept to a minimal and secondary surgeries are not required for their removal (Bini et al., 2004; Weber and Mackinnon, 2005). One of the main advantages of using this type of conduit to bridge peripheral nerve gaps is that they can be filled with permissive growth materials to aid in axonal regeneration. My current findings provide strong support for the use of the extracellular matrix molecule laminin in conjunction with NGF and GDNF in these bioabsorbable conduits in order to stimulate optimal levels of axon growth from all populations of regenerating sensory neurons. As I have shown, the provision of these factors together would stimulate a level of growth from sensory neurons that is not achievable by the provision of either factor alone. Treatments that only focus on one permissive molecule may be inadequate in helping axons to cross the injury site and get back to their original targets before their ability to stimulate functional recovery is lost.

Another major problem associated with peripheral nerve damage, especially when nerve gaps are formed, is the creation of pain causing neuromas (Lewin-Kowalik et al., 2006; Sakai et al., 2005; Vernadakis et al., 2003). These neuromas are formed when growing axons, especially sensory C fibers become tangled and form an aggregate of axon fibers at the beginning of the injury site. It has been suggested that the provision of NGF alone at sites of injury may actually act to induce neuroma formation, potentially because the regenerating fibers are getting cues to grow but have nowhere to travel because there is no remaining ECM outside of the proximal nerve stump (Lewin-Kowalik et al., 2006). Filling conduits with laminin, NGF, and GDNF could possibly provide

regenerating sensory axons with the support they need to traverse the nerve gap, thus preventing neuroma formation and neuropathic pain.

6.2.2 Diabetes

Metabolic diseases such as diabetes inflict damage on the peripheral sensory nervous system, characterized by demyelination, dying-back of axons, and subsequent failure of axonal regeneration (Kennedy and Zochodne, 2005). These neuropathies occur due to reduced neurotrophic support, decreased axonal flow and disruption of the ECM (Fernyhough and Schmidt, 2002; McLean, 1997; Scott et al., 1999), and often lead to a loss of sensations including pain, pressure, temperature, and vibration. Eventually reflexes mediated by sensory and motor fibers are also lost (McHugh and McHugh, 2004). Sensory neurons of the DRG are among the most vulnerable to this type of metabolic injury (McHugh and McHugh, 2004; Zochodne, 1996). This increased vulnerability is in part due to the fact that DRG neurons and their peripheral axons lay outside of the blood nerve barrier, as well as the fact that the neurons have a unique morphological property in that 99.8% of their cytoplasm is axonal (Devor, 1999; McHugh and McHugh, 2004). Of the different sub-classifications of DRG neurons, the most susceptible and first affected neurons in diabetic environments tend to be the small diameter nociceptive nerve fibers of the IB4 and CGRP populations (Le Quesne and Fowler, 1986; Polydefkis et al., 2003; Yasuda et al., 2003; Zochodne, 1999; Zochodne et al., 2001). The medium and large diameter NF200 positive neurons tend to be less susceptible and are affected later in the disease (Lacomis, 2002; Leininger et al., 2004; Sumner et al., 2003). Disruption of these neurons ultimately leads to the loss of sensation

for painful or noxious stimuli, as well as tactile and vibratory sensation, putting tissue at increased risk for neuropathic damage (Ali *et al.*, 1989; Dellon, 2002; Leininger *et al.*, 2004). The most common and debilitating damage occurs on the plantar surface of the foot; because the loss of sensation often goes undetected and untreated, this can result in non-healing foot ulcers, severe infection, and gangrene, the leading causes of amputation and morbidity in diabetic patients (Leininger *et al.*, 2004).

To date, much of the research associated with sensory polyneuropathies produced by metabolic diseases have focused on prevention through treatments such as glucose regulation (McHugh and McHugh, 2004). However, due to the unique structure and location of the DRG, even slight undetected alterations in the extracellular environment can produce detrimental disruptions in sensory nerves, resulting in problems such as the diabetic foot (Ali *et al.*, 1989; Le Quesne and Fowler, 1986; McHugh and McHugh, 2004; Sumner *et al.*, 2003; Zochodne, 1996). Thus, the focus on treatment of sensory polyneuropathies has shifted toward attempts to stimulate regeneration. As we have noted in this study, in order to achieve an effective regenerative response, the cell requires support from both trophic factors and components of the extracellular matrix, specifically laminin, which are both disrupted in diabetes. In identifying the environmental requirements, as well as specific signalling events associated with unique laminin-induced growth responses for each of the sub-populations of DRG neurons, we have identified potential therapeutic targets for the treatment and repair of peripheral sensory nerve damage. This illustrates the inability of any one treatment alone to repair the damage inflicted by diseases such as diabetes on the different subpopulations of DRG neurons. For instance, NGF treatments would do little in the way of stimulating

regeneration of IB4+ve neurons, which require both LN and GDNF for neurite outgrowth. Similarly, if, as suggested, the extracellular matrix is disrupted and LN is not present, then NGF treatment will be ineffective in stimulating regeneration of CGRP+ve neurons. As with NGF treatment, GDNF treatment would be ineffective in stimulating regeneration in CGRP and NF200+ve neurons.

6.2.3 Overcoming influences from inhibitory CNS molecules

It has long been suggested that axonal regeneration in the CNS is poor. A variety of reasons for the poor regenerative capacity of CNS neurons have been suggested, two of which are the presence of inhibitory ECM molecules and the lack of an adequate neurotrophin supply (Hunt *et al.*, 2002). The inhibitory molecules of the CNS come from two main sources, the myelin sheath and the glial scar (Sandvig *et al.*, 2004). As suggested in the Introduction, the major inhibitory molecules found in the myelin sheath, including Nogo, MAG, and OMgp, are left behind after brain and spinal cord injury, while the inhibitory molecules of the glial scar are deposited by infiltration of reactive astrocytes (Hunt *et al.*, 2002; Sandvig *et al.*, 2004). Until recently, much of the CNS regeneration research had focused on how to remove these inhibitory molecules. However, more recently the focus has shifted to the provision of permissive molecules especially those found in the PNS that are known to be lacking in the CNS. For instance, laminin purified from the PNS has been shown to have the ability to override the inhibitory actions of myelin and glial scar proteins on CNS neurons (Condic *et al.*, 1999; David *et al.*, 1995; Laforest *et al.*, 2005; Snow *et al.*, 1996). Similarly, priming of CNS neurons with NGF allows them to overcome myelin induced growth inhibition (Cai *et al.*,

1999; Gao *et al.*, 2003). Thus, one can see how the balance could be potentially shifted toward promoting regeneration of CNS neurons by providing enough positive cues to overwhelm the inhibitory ones. Therefore, by providing both laminin and the appropriate neurotrophin to cells of the CNS, one may be able to overcome the influence of inhibitory CNS molecules. The ability of LN to overcome environmental inhibition in the CNS has been suggested to occur through binding of integrin receptors and subsequent activation of integrin dependent signalling components, such as Rac (Laforest *et al.*, 2005). Similarly NGF has also been shown to activate the Rac pathway in CNS neurons (Gao *et al.*, 2003). As I have suggested, in our system the presence of laminin and NGF together may work by altering the kinetics of specific signalling intermediates that are important for neurite growth. Thus, the addition of both NGF and laminin in the CNS may act to change the kinetics of Rac signalling, thereby stimulating enhanced axonal regeneration after CNS injury. For example, as I have indicated in Fig 6.1 it is possible that LN and NGF together activate both PI 3-K, which could in turn activate Rac, and Src which may activate Ras which in turn could activate Rac. In either situation these signalling events may work to enhance neurite growth.

Another important finding that could potentially relate to CNS regeneration is the fact that we have identified Src as a critical signalling intermediate for the regeneration of peripheral sensory axons. It is quite possible that the laminin- and NGF-induced increases in Src seen in the PNS could be directly paralleled in the CNS. Therefore, these findings identify another signalling target with potential therapeutic significance for CNS neurons. For instance, much of the research in CNS repair has focused on inhibition of the Rho pathway by the use of inhibitors such as the C3 enzyme to inhibit RhoA or the

pharmacological inhibitor Y-27632 to inhibit Rho kinase (Dergham *et al.*, 2002; Yamashita *et al.*, 2002). In conjunction with these inhibitors, it may be beneficial to activate the intermediates such as Src, which could potentially shift the balance even further toward the positive end of the spectrum (see Fig 6.1).

6.3 Future directions

Although I have identified many key environmental factors and intracellular signalling intermediates that are involved in neuritogenesis of adult sensory DRG neurons, many questions with regards to axonal regeneration remain. I will briefly discuss four lines of work that I see as being important to further advance the knowledge base in this field.

1) We have identified the upstream and intermediate signalling components required to stimulate neurite growth in adult DRG neurons. However, the requirement or contributions of downstream effectors have not yet been identified. It has been previously shown that both the Rho family of small GTPases (Rho, Rac, Cdc42, PAK1) and the actin binding proteins (Cofilin, LIMK, TESK1) are key regulators of growth cone motility and axonal advancement (Gallo and Letourneau, 2004; Korey and Van Vactor, 2000; Meyer and Feldman, 2002) (see Fig 6.1). Therefore, the role of these proteins in laminin and NGF induced neurite growth need to be identified. From the lower highlighted portion of Fig 6.2, it becomes evident that the required signalling intermediates that we have identified could possibly interact with potential downstream

Figure 6.2. Proposed schematic illustration of how the Rho family of small GTPases (highlighted in blue) could be regulated by the known signalling intermediates (highlighted in yellow) that have been identified as being required for neurite growth from adult sensory DRG neurons. The area highlighted in yellow represents the signalling intermediates that we have identified as being important for LN and NGF induced neurite growth. The area highlighted in blue represents the Rho family of small GTPases that have been identified in the literature that could possibly play a role in axonal regeneration. Akti: AKT inhibitor; Echistatin, anti- β 1, RGD: integrin inhibitors; K252a: TrkA inhibitor; LY294002: PI 3-K inhibitor; PP2: Src family kinase inhibitor; Rex: p75 inhibitor; Y27632: ROCK inhibitor. LIMK: Lin-II, Islet I and Mec 3 kinase. SSH1: slingshot. TESK 1: Testicular protein kinase 1.

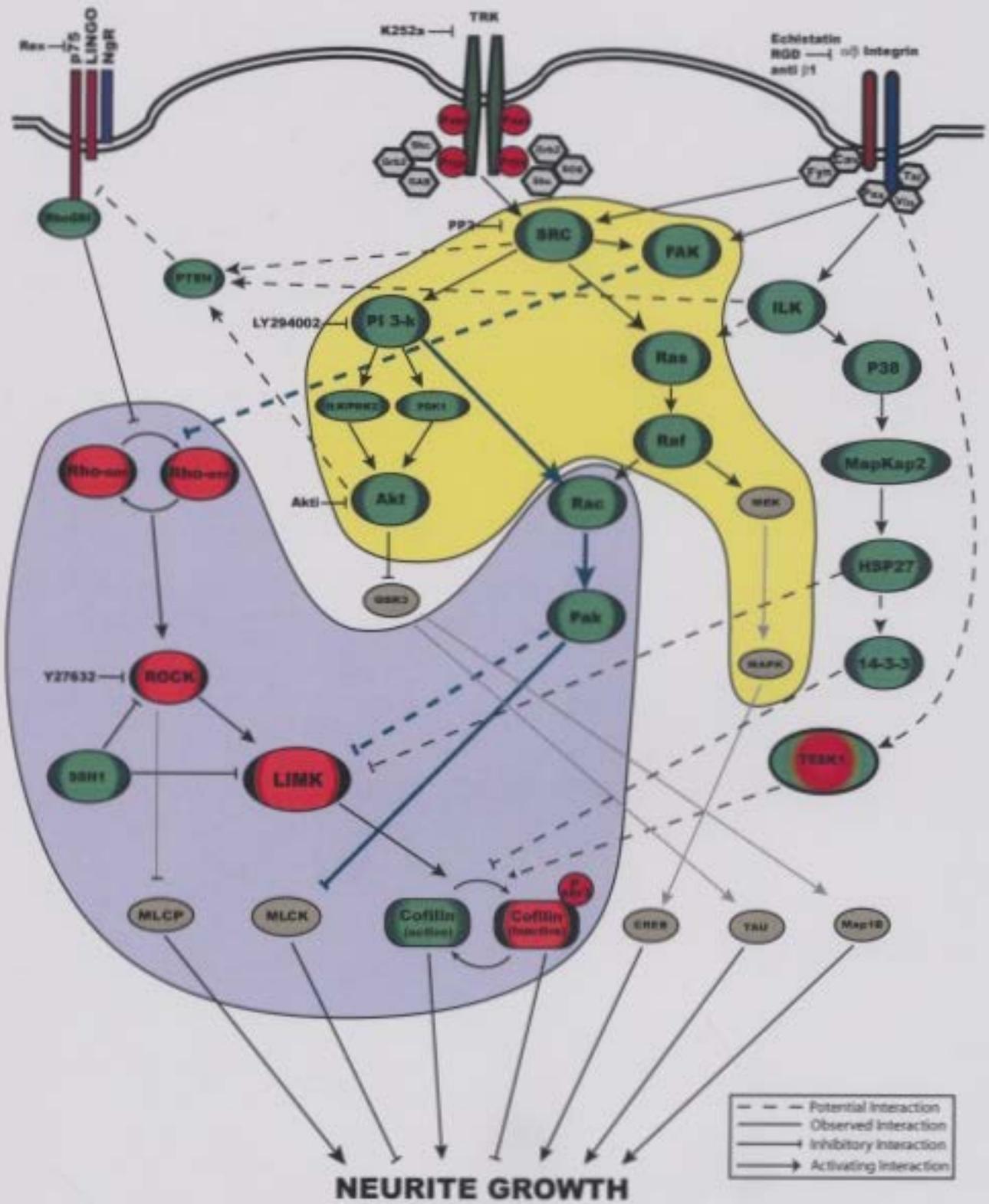


Fig 6.2

effectors that have been shown to be essential for growth cone remodelling and axonal extension. For example, it is possible that PI-3K may activate Rac, which could then activate Pak inhibiting LIMK and myosin light chain kinase (MLCK). Both of these intermediates have been shown to be important for retrograde flow of actin and growth cone retraction (Dent and Gertler, 2003; Huber et al., 2003). It has also been suggested that Fak, which we have shown to be activated by both NGF and LN, may inhibit activation of Rho and its downstream effector Rock, thereby stimulating growth cone extension and neurite growth (Dent and Gertler, 2003; Huber et al., 2003; Ren et al., 2000). Although these possibilities exist, further work is necessary to confirm these interactions.

2) As suggested in Section 6.2.3, our findings may be useful when attempting to overcome the inhibitory effects of components found in both myelin and the glial scar. Thus, it is important to determine how these inhibitory substrates signal to inhibit neurite growth and whether or not they can be influenced by the signalling events induced by laminin and NGF. It is well known that these inhibitory molecules exert their effects by signalling via RhoA and its downstream effector ROCK, however, the influence that activation of the Src/PI 3-K/Akt pathway may play in these signalling events are unknown. Similarly, it is also unclear how molecules of the glial scar actually activate these inhibitory signalling intermediates; for instance, the cell surface receptor for the chondroitin sulfate proteoglycans has not yet been identified (Schweigreiter *et al.*, 2004). The possibility exists that they exert their effects by interrupting cellular adhesion to positive extracellular matrix molecules such as laminin.

3) We have yet to identify the mechanisms that underlie the differential laminin-induced growth response between the IB4+ve and NGF-responsive populations of DRG neurons. Although I have studied the interaction between laminin and NGF in the NGF-responsive cell population only, the finding that the IB4+ve neurons do not respond to laminin in the absence of added GDNF is still of interest. Specifically, I would like to know if the observed difference between these cells is due to different integrin receptor expression. My initial immunocytochemistry experiments suggest that the integrin receptor subunits, $\alpha 1$ and $\beta 1$, are expressed by all populations of DRG neurons. However, the degree of receptor subunit expression across the different populations has not been identified. For example, it is possible that the IB4+ve cells, which don't respond to laminin in the absence of GDNF, may express fewer of these receptors. Similarly, as indicated in the introduction, laminin has the ability to bind to more than one integrin receptor, and thus it may be that there is a different pattern of integrin expression seen between the two cell populations. To study these possibilities, I have performed some preliminary work using oligo microarray analysis (oligo arrays used in these experiments are provided by SuperArray Bioscience Corporation, Frederick, MD, USA, and are called Oligo GEArrays). To carry out these types of experiments, total RNA from each population was used to synthesize biotin tagged cRNA. The tagged cRNA was bound to microarrays that have oligo sequences for the different integrin receptors attached to their surfaces. These arrays were visualized using xray film and analyzed using the provided software. Fig 6.3 is a representation of the different genes that have been observed on these arrays and what they actually look like when

Figure 6.3. ECM array gene expression and representative clustergram from NGF-responsive and IB4+ve cell populations. Representative microarray gene expression and analysis for NGF-responsive and IB4+ve adult DRG neurons before (T-0) and after being plated on LN coated dishes for 24 hrs. A, A2: Clustergram and microarray of NGF-responsive cells plated on LN for 24hrs. B, B2: Clustergram and microarray of IB4+ve cells plated on LN for 24hrs. C, C2: Clustergram and microarray of NGF-responsive cells before plating. D, D2: Clustergram and microarray of IB4+ve cells before plating. I: Map of gene probes and array location.

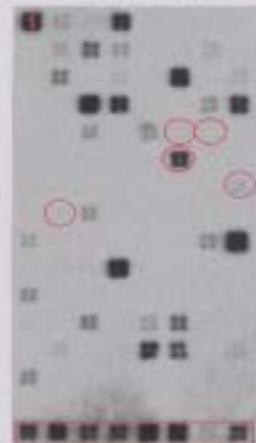
A B C D



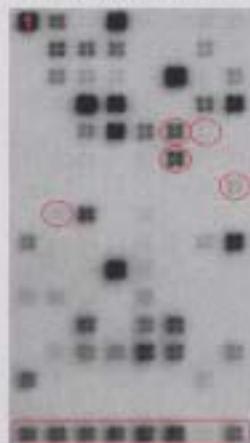
A2 NGF-Responsive +LN



B2 IB4+ve +LN



C2 NGF-Responsive T-0



D2 IB4+ve T-0



E

Ppia	Acta1	N/A	Rgs	Casp8	Casp9	Cxcl	Cdh3
1	2	3	4	5	6	7	8
Cav	Col4	Cdh1	Cdh2	Cdh3	Cdh4	Cdh5	Cxcl
9	10	11	12	13	14	15	16
N/A	Cxcl	Col1a1	Col1a2	Col1a3	Cxcl	CTNNA1	N/A
17	18	19	20	21	22	23	24
N/A	N/A	Cdh	Cdh	Cxcl	N/A	Cdh	Cdh
25	26	27	28	29	30	31	32
Dec	Ecm1	Fg	Fg	Ig	(Cdh)	(Rgs)	N/A
33	34	35	36	37	38	39	40
N/A	Ig	N/A	Ig	Ig	(Rgs)	(Rgs)	Ig
41	42	43	44	45	46	47	48
Ig	N/A	Ig	Ig	Ig	Ig	Ig	(Ig)
49	50	51	52	53	54	55	56
Cdh	Ig	Ig	Ig	Ig	Ig	N/A	PTT
57	58	59	60	61	62	63	64
Ldha	(Ldha)	Mmp	Mmp	Mmp	Mmp	Mmp	Mmp
65	66	67	68	69	70	71	72
LOC201348	Mmp	N/A	Mmp	Mmp	Mmp	MMP	Mmp
73	74	75	76	77	78	79	80
Mmp	Mmp	Mmp	Mmp	Mmp	Nrcam	Nrcam	Nrcam
81	82	83	84	85	86	87	88
Serpins2	Pocam	Plas	Plas	Plas	RT1-Aw2	Sell	Sell
89	90	91	92	93	94	95	96
Selp	Serpins5	Serpins1	Serpins2	Sparc	Spp1	N/A	Tfbs
97	98	99	100	101	102	103	104
Timp	Timp	Timp	Timp	Tac	Tfbs	Vcam1	Va
105	106	107	108	109	110	111	112
Blank	Blank	PUC18	Lac1	Lac2	ASTR2	ASTR1	AS1
113	114	115	116	117	118	119	120
Rpl32	Ldha	Alka	Alka	Gapd	Gapd	BAS2C	BAS2C
121	122	123	124	125	126	127	128

Fig 6.3

performing the analysis. Although these results are preliminary, differences in integrin expression have been identified; specifically, both $\alpha 1$ and $\beta 1$, subunits that we have identified by ICC, seem to be differentially expressed between the two cell populations (Fig 6.4). As noted, these results are preliminary and subsequent replication has not been performed, however this technique does look promising.

4) It has been suggested that the results obtained from flat 2D surfaces could be quite different from what would occur in a more physiologically relevant space. With this in mind, I have begun using synthetic 3D matrices to further study axon growth and behavior. Specifically, I began by plating neurons on 3D nanofibril cover slips that were either uncoated (NANs), poly-amine coated (SANs), or laminin coated, and cultures were stained and imaged using confocal microscopy to determine if the 3D structure alone aids in significant neurite outgrowth (The Ultra-Web Synthetic Nanofibrillar surfaces, used in these experiments were provided by Donaldson Company Inc., Minneapolis, MN, USA). Preliminary results suggest that adult DRG neurons still require laminin and/or NGF to elicit a growth response (Fig 6.5 A, C). However, laminin- and/or NGF-induced neurite growth seems to be enhanced by the 3D structure of these matrices (Fig 6.5 B, D, E, F). As these results are preliminary, neurite growth analysis needs to be performed to confirm these observations. However, if this is the case, it may provide us with more insight into the environmental conditions required for neurite growth *in vivo*. As these 3D structures provide a model that more closely resembles the *in vivo* environment, they will also be used in experiments in which we will try to overcome the previously mentioned inhibitory substrates. For instance, they can be coated with inhibitory myelin

Figure 6.4. Analysis of ECM arrays from NGF-responsive and IB4+ve cell populations. RNA from NGF-responsive and IB4+ve adult DRG neurons was collected either immediately after isolation (T-0) or after being plated on LN coated dishes for 24 hrs and analyzed via microarray analysis (the array location of each component for this experiment have been circled in Fig 6.3). $\alpha 1$ integrin (A) and ICAM (H) RNA expression was significantly higher in NGF-responsive cells than in IB4+ve cells after being plated on laminin for 24 hrs ($p < 0.001$). However, LN plating stimulated a significant increase in $\alpha 1$ integrin and ICAM expression in both cell groups ($p < 0.001$). $\beta 1$ (F) and αv integrin (E) RNA expression was significantly higher in NGF-responsive cells than in IB4+ve cells before LN plating ($p < 0.001$), however, no significant difference was seen after being plated on LN for 24 hrs ($p > 0.05$). Laminin (G) RNA expression was significantly higher in NGF-responsive cells than in IB4+ve cells both before and after laminin plating ($p < 0.001$). No significant difference was seen in $\alpha 3$ (B), $\alpha 5$ (C), or $\alpha 7$ (D) integrin RNA expression. To control for between experiment variation (exposure/CRNA loading, etc.) densitometric analysis for each probe was corrected against internal housekeeping genes located on each microarray. Significance was tested using one-way ANOVA with Tukey post-hoc testing. * $p < 0.05$, ** $p < 0.001$.

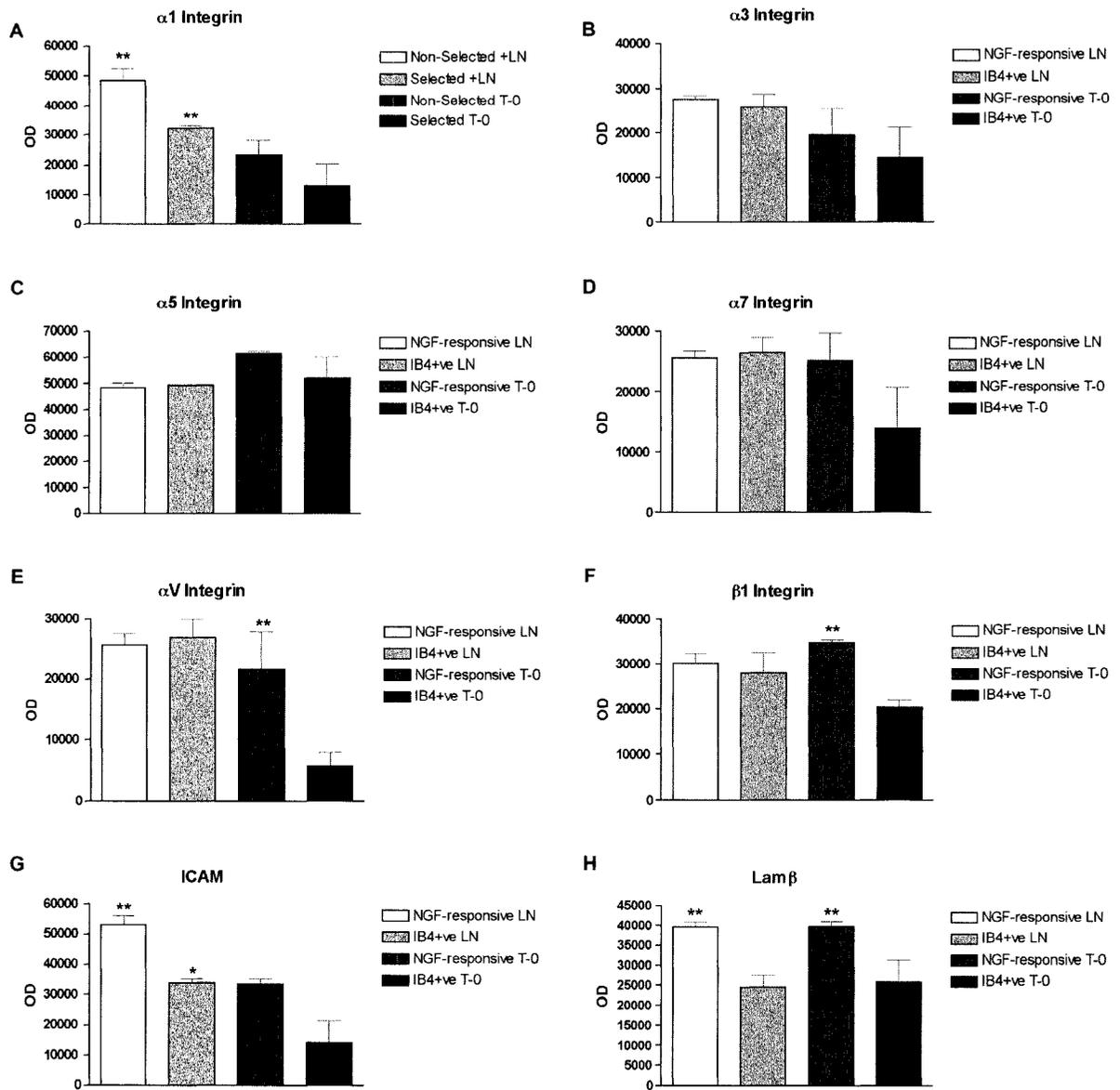


Fig 6.4

Figure 6.5. Adult DRG neurons plated on various Ultra-Web synthetic nanofibrillar cell growth surfaces and immunostained with β 1 integrin and total tubulin. Cells were plated on untreated (NANS), polyamine treated (SANS) or LN coated (SANS+LN) nanofibers plus or minus NGF for 24hrs prior to fixation and immunostained using antibodies against β 1 integrin (red) and total tubulin (green). Cells were imaged at 40x using laser scanning confocal microscopy. No significant amount of neurite growth was observed for cells plated on the NANS (A) or SANS (C) for 24 hrs minus NGF. However, significant neurite growth was observed when cells were plated on NANS (B) or SANS (C) plus NGF. A significant amount of neurite growth was observed for cells plated on LN coated SANS minus NGF (E), which was potentiated when plated plus NGF (F). Note the local accumulation of β 1 integrin in the growth cones of cells plated on both NANS plus NGF (B) and SANS plus NGF (D) (arrows), characteristic of cells plated on LN (E, F), but not normally seen on similar 2D surfaces. However, the 3D surface alone is not sufficient to stimulate neurite growth in the absence of added NGF. Scale bar – 50 μ m.

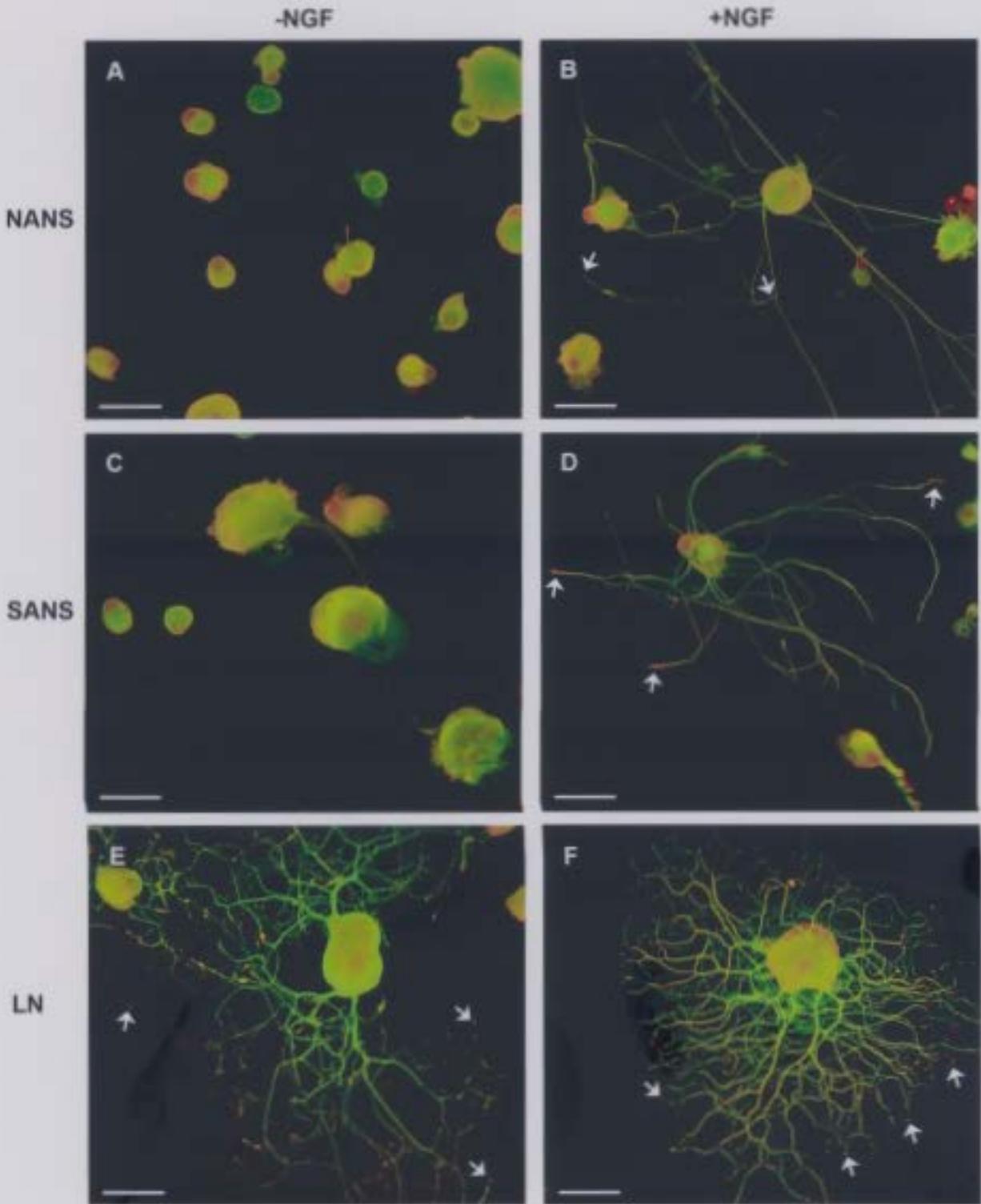


Fig 6.5

and glial scar proteins, and the effects of laminin and NGF can be assessed. We are also interested to see if the signalling pathways activated within neurons plated on these 3D structures are the same as those identified on 2D surfaces. To show the structure of these 3D matrices and how neurites grow when plated on them, I have included a composite confocal micrograph with accompanying light microscopy (Fig 6.6). As shown, neurons plated on these nanofibrillar surfaces extend neurites that climb over and penetrate into the 3D matrix.

Figure 6.6. 3mm Z-stack of adult DRG neurons plated on LN coated Ultra-Web synthetic nanofibrillar cell growth surfaces and immunostained with β 1 integrin and total tubulin. Cells were plated on LN coated (SANS+LN) nanofibers minus NGF for 24hrs prior to fixation and immunostaining using antibodies against β 1 integrin (red, C, F, I) and total tubulin (green, B, E, H). Sequential 1 μ m slices were imaged at 100x using laser scanning confocal microscopy, and transmitted light (to show nanofiber structure) and arranged from apical (A) to basal surfaces (G). The 3D nature of the nanofiber surface can be identified in panels A, D, and G as well as in subsequent enlargements J, K, and L (J, K, L are enlargements of the area of A, D, and G respectively as indicated by the red circle). The neurite indicated by the arrow is growing over a nanofiber however as the scans become more basal a gap in the neurite begins to appear indicating that the neurite has climbed over the fiber and fallen to continue on the other side. Neurite growth on this LN coated 3D surface is more extensive than that seen on similar 2D plating conditions. Cell body is indicated by an asterisk. Scale bar for – 50 μ m.

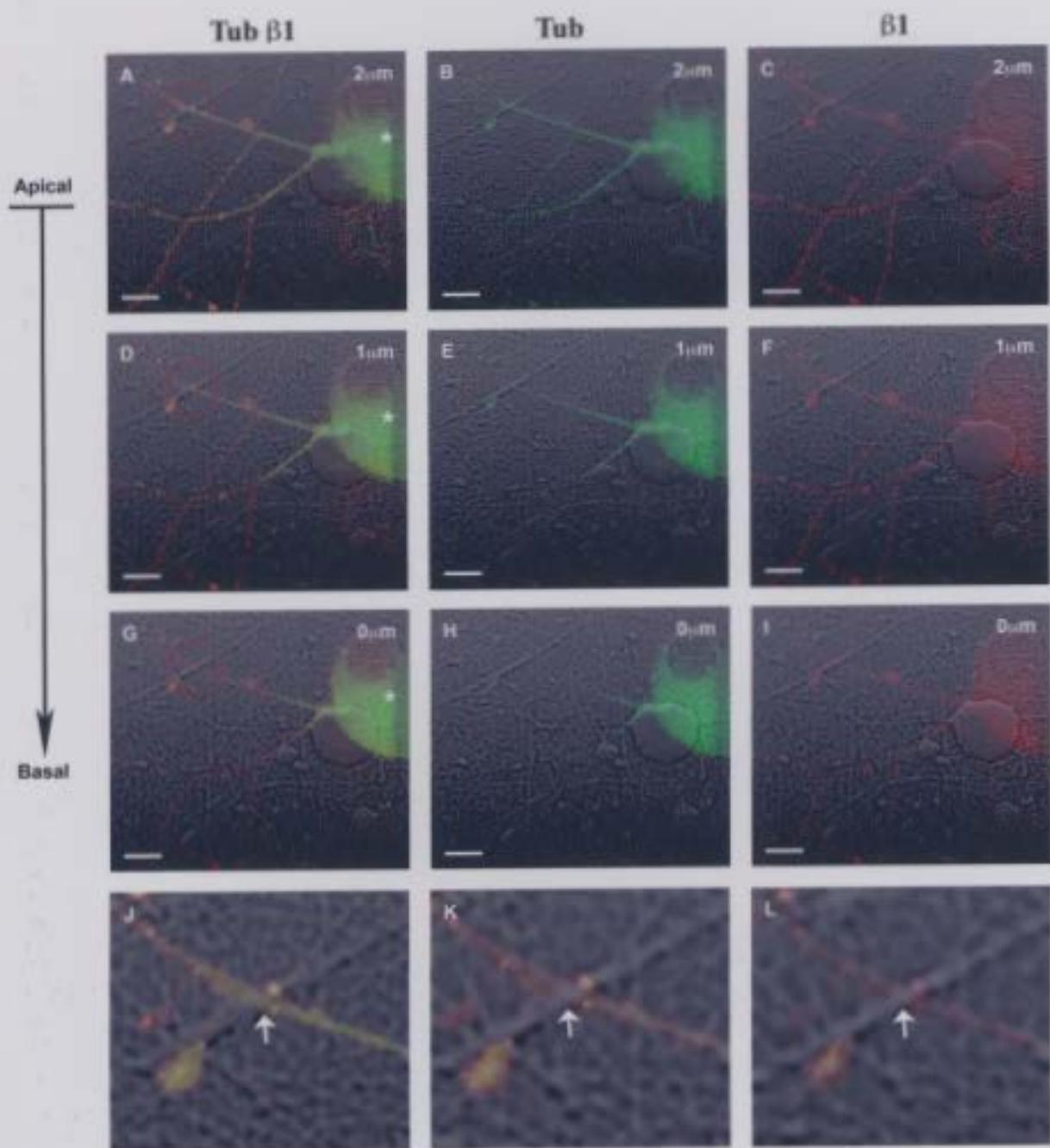


Fig 6.6

6.4 Conclusion

To highlight my results I will conclude by summarizing my major findings.

- 1) The extracellular matrix molecule laminin is sufficient to promote significant amounts of neurite growth from NGF-responsive adult DRG neurons in the absence of added growth factors.
- 2) Laminin induced neurite growth in the NGF-responsive populations of DRG neurons is potentiated by the addition of NGF and unaffected by the addition of GDNF.
- 3) The extracellular matrix molecule laminin is not sufficient to promote significant amounts of neurite growth from IB4+ve adult DRG neurons plated in the absence of added growth factors, although it appears to be required for GDNF-induced neurite growth.
- 4) Both NGF-responsive and IB4+ve cell populations are dependent on the PI 3-K/Akt signalling pathway for neurite growth, but only the IB4+ve cell population also requires the MEK/MAPK signalling pathway.
- 5) Enhanced neurite growth in the NGF-responsive cell population induced by laminin and NGF results from crosstalk between integrin and Trk activated signalling pathways.
- 6) Src acts as a key upstream point of collaboration between integrin and Trk induced activation of the PI 3-K/Akt signalling pathway in the NGF-responsive cell population.

- 7) Enhanced neurite growth stimulated by laminin and NGF in the NGF-responsive cell population is potentially due to a sustained activation of the downstream signalling intermediate Akt.

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