INTRACELLULAR SIGNALING UNDERLYING NEURITE GROWTH IN ADULT SENSORY NEURONS

DAVID M. JONES

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Intracellular Signaling Underlying Neurite Growth In Adult Sensory Neurons

By

David M. Jones

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Abstract

Regeneration in the peripheral nervous system (PNS) is relatively successful compared to that in the central nervous system. A major reason for this may be the permissive growth environment. In the PNS, a variety of factors contribute to promote neurite regeneration. These can be divided into two general categories i) trophic factors released by their targets, or supporting cells like Schwann cells and ii) components of the extracellular matrix (ECM). Generally, these factors can influence neurite growth by binding cell surface receptors and subsequent activation of intracellular signaling cascades.

I have employed a tissue culture system of adult rat dorsal root ganglion (DRG) neurons to study the contribution of trophic factors (specifically NGF and IGF-1) and the ECM protein, laminin, in promoting neurite growth. Using computer-assisted analysis of neurite growth and analysis of protein expression and post-translational modifications with Western blotting, I have attempted to correlate the biological response (eg. neurite growth) with the biochemical response (signaling intermediates and activation). Through the use of pharmacological intervention, I have further tried to characterize the requirement for specific signaling cascades in the growth response.

In Ch. III, further evaluation of this growth synergism with neuron tracing revealed that having both NGF and IGF (N+I) in the environment of regenerating neurites does not increase the number of neurons with neurites (neuritogenesis) but does enhance neuronal elongation and neurite branching above that seen with either growth factor alone. This synergistic effect correlates with the activation of the PI3-K>Akt>GSK-3 pathway more so than with the ras>Raf>MEK>ERK pathway.

Pharmacological inhibitors of PI3-K (LY294002), GSK (LiCl), and ERK activity (UO126) confirmed the importance of the PI3-K>Akt>GSK-3 pathway. These experiments suggested that NGF and IGF signaling converge on the PI3-K>Akt>GSK-3 pathway to exert their growth synergism. To complement these experiments, I examined the biochemistry of two possible downstream effector proteins of both these signaling pathways; the microtubule associated protein tau, and the transcription factor cyclic adenosine monophosphate response element binding protein (CREB). Phosphorylation of tau appears to correlate with growth.

In Ch. IV, preliminary data on the contribution that laminin has on NGF-dependent neurite growth was collected. The contribution of ECM components to neurite growth is well recognized. These experiments demonstrated that laminin can enhance NGF-dependent neuritogenesis. Like growth factors, the growth contribution of laminin is , at least, in part mediated by activating specific signal transduction cascades. The β1 integrin subunit, paxillin, and FAK were phosphorylated when grown on laminin as compared to a polylysine substrate. However these integrin signaling proteins were not further phosphorylated by NGF when cultures were grown on laminin. The enhanced NGF-dependent neuritogenesis by laminin is however associated with decreased and increased activation of GSK-3 and ERK, respectively. This correlation suggests a convergence of both integrin and NGF signaling on the PI3-K>Akt>GSK-3 pathway.

Taken together these results provide further evidence to the notion that many different factors are required for maximal neurite regeneration. In addition they suggest that signaling convergence upon the PI3-K>Akt>GSK-3 pathway and ras>Raf>MEK>ERK pathway underlies environmental factor growth synergism.

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List of Abbreviations

Ab, antibody
AC, adenylate cyclase
Akt, protein kinase B
BDNF, brain-derived neurotrophic factor
$[Ca^{+}]_i$, intracellular calcium
cAMP, cyclic adenosine monophosphate
CNS, central nervous system
CREB, cAMP response element binding protein
DRG, dorsal root ganglia
ECM, extracellular matrix
ERK, extracellular regulated kinase/mitogen activated protein kinase (MAPK)
FAK, focal adhesion kinase
FRS2, fibroblast growth factor receptor substrate 2
GAP-43, growth associated protein-43/neuromodulin
GF, growth factor
Grb2, growth factor related binding protein 2
GSK, glycogen synthase kinase
IGF, insulin-like growth factor
IGFR1, insulin-like growth factor receptor 1
ILK, integrin linked kinase
IRS, insulin receptor substrate
K252a, tyrosine kinase inhibitor
LiCl, lithium chloride
LIF, leukemia inhibitory factor
LY294002, PI3-K inhibitor
MAP, microtubule associated protein
NGF, nerve growth factor
NT-3, neurotrophin-3
$p75^{NTR}$, low affinity p75 neurotrophin receptor
PDK, 3-phosphoinositide-dependent kinase
PKA, protein kinase A
PKC, protein kinase C
PI3-K, phosphoinositide-3-kinase
P-L, polylysine
PLC, phospholipase C
PNS, peripheral nervous system
RSK, ribosomal S6 kinase
SH2, src homology 2
SOS, son of Sevenless
TrkA, high affinity NGF receptor
UO126, MEK inhibitor
VEGF, vascular endothelium growth factor

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Technically speaking both Masuma and Karen helped me immensely. Masuma was at my aid for all the detailed questions, while Karen was there to answer every other question and to lead me along the way. The great thing is that I gained two more friends along the way. Karen, thanks for giving me the opportunity to work with you and making it an enjoyable experience along the way. My lab mates of old (Arun, Michelle, Sarah, Melanie, Chuck) and new (Elaine, Budd) helped me through the minute to minute struggles of completing a MSc. They helped to make it fun.

Emotionally speaking my friends and family (Mom, Wayne, Ward) were always there for encouragement and to lend an ear during the troughs. This gratitude is especially extended to the person who was there day in and day out during the last two years of an extended writing period. She tipped the scales to balance me out, gave me a reason, for which one became enough. I appreciate and value everything all you have done Anna, especially one- being.

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CHAPTER I: INTRODUCTION

1.1. Neurite Growth Events in PNS regeneration

1.1.1. Adult PNS *In vivo* Regeneration

Once a peripheral nerve has been injured the individual axons go through fairly well defined phases as written, for example, in Sunderland- "The peripheral nerve". The microenvironment surrounding the injured PNS axon is conducive to regrowth, unlike that in the central nervous system (Schwab, 2002; Bray et al, 1991). Before the environmental factors that make for this permissive environment are identified, an overview of *in vivo* regeneration events will be presented to provide a background for what regeneration entails and what affects it.

Post Injury Survival

Transection of a peripheral nerve and the neurite it houses does not always lead to successful regeneration. In fact there are some estimates that up to 50% of the neurons will die following injury (Fu and Gordon, 1997). This is dependent on both the proximity of the lesion (with the more proximal the lesion the worse the outcome), and the degree of injury, where crush injuries result in more successful regrowth than partial or complete transection. Neurotrophins and other growth factors appear to be crucial for post injury survival and are supplied at the site of injury by target tissues, Schwann cells, fibroblasts, and macrophages (cf. Lundborg et al, 1994; Fu and Gordon, 1997, for review). Other factors including the components of the surrounding extracellular matrix and vascular support are likely to influence post injury survival as well. Obviously survival of the neuron of origin is key in order for regeneration to occur.

Post Injury Degeneration

After damage, the axons distal to the lesion undergo Wallerian degeneration (cf. Sanes and Jessel, 2000; Fu and Gordon, 1997, for review). During this process neuronal and myelin debris are phagocytosed by macrophages and Schwann cells. In the CNS, myelin inhibitory molecules such as myelin associated glycoprotein (MAG) and Nogo pose a significant barrier to regeneration (Caroni and Schwabb, 1988 a,b; Chen et al, 2000; Tang et al, 2001). It has been suggested that clearing the lesion site of myelin inhibitory molecules makes the local environment more permissive to growth (Fu and Gordon, 1997; Shen et al, 1998). The PNS has less myelin inhibitory proteins than in the CNS.

Post Injury Cellular Reaction

The cell body of surviving neurons has a characteristic response to injury called chromatolysis (for review see Reiner, 1995; Sanes and Jessell, 2000). The term is based on the histological description of a post injury neuron and refers to the dissolution of the Nissl substance (cf. Berdan et al, 1990, for review). This chromatolysis process could represent the initiation of regeneration activity in the neuron, particularly synthesis of important growth related proteins such as growth associated protein-43 (GAP-43), and cytoskeleton proteins like tubulin and actin (for review see Kreutzberg, 1996; Reiner, 1995). The neuron can then begin to extend its damaged proximal axon much like it does during development. In short, a growth cone is formed, it "detects" cues along a path, and cytoskeletal and membrane extension provide axonal growth. The regenerating axon detects many environmental factors that have both growth promoting and inhibitory actions. As well, the formation of a peripheral scar provides mostly a physical obstacle to

as compared to the physical and chemical inhibitory influence of the glial scar found in the CNS. (McKerracher, 2001).

Post Injury Trophic Support

In the distal stump at the site of Wallerian degeneration, Schwann cells proliferate, undergo phenotype modifications, and become motile, all in effort to make the regenerative environment conducive to neurite growth (Reiner, 1995; Fu and Gordon, 1997; Sanes and Jessell, 2000). Schwann cells represent an immense source of external support for regeneration. In addition to the secretion of both survival and growth promoting factors such as NGF, IGF-1, and GDNF, they also become motile and align in parallel arrays within which regenerating neurites will grow. These permissive tubes are known as the "bands of Bunger". In addition to being permissive, Schwann cells also secrete laminin, a major component of the ECM that is highly favorable for neurite growth (Chiu et al, 1991). Having both soluble and insoluble promoters of growth make the PNS regeneration more successful than that in the CNS, where barriers to successful regeneration have been shown to include myelin-associated inhibitory proteins (eg - MAG, Nogo) and potentially a lack of appropriate trophic factors (Tuszynski et al, 1994; Schwab and Bartholdi, 1996; Stichel and Muller, 1998; Fawcett and Asher, 1999).

1.1.2. The PNS is a Permissive Environment for Neurite Growth

As this overview suggests, and investigations are continually supporting, external factors are the major determinants of regeneration and multiple external factors are likely to be required for maximal regeneration.

The first hint of the control external factors have over regeneration came from the classic transplantation studies by Aguayo and colleagues, demonstrating that a PNS environment can support axonal regeneration in CNS neurons (David and Aguayo, 1981; Bray et al, 1987). These experiments suggested that the environment can override the regenerative predisposition of neurons. The two front running candidates believed to be responsible for influence of the environment are soluble growth factors and the insoluble ECM upon which neuronal processes grow (Nicholls et al, 2001). Logically then modifying the environment should be able to entice growth. Numerous experiments suggest that this is the case. One in particular has demonstrated that functional recovery at all levels including growth, synapse formation and neurotransmission can be encouraged by appropriate growth factor application soon after injury (Ramer et al, 2000). In these experiments, completely resected cervical roots were treated via intrathecal administration of GDNF, NGF, or NT-3. Specific subgroups of sensory neurons responsive to these growth factors demonstrated quite impressive growth. Not only was the pattern of reinnervation relatively accurate, but the regenerated afferents also had some functional value. Electrophysiological studies confirmed electrical connectivity, while behavioral testing established that noxious stimuli could elicit the appropriate reflex compared to sham-operated animals. This method of neurotrophin treatment is perhaps one of the most successful attempts at complete regeneration to date.

As for the ECM, its importance has long been known. Laminin is a major component of the PNS extracellular matrix associated with regeneration (Le Beau et al, 1995), and suspected to oppose the inhibitory influence of inhibitory factors such as MAG (Mckerracher et al, 1994; David et al, 1995). Many have suggested that the laminin

is a major contributor to the growth permissive properties of the PNS, and that the lower amounts seen in the CNS may contribute to poor regeneration attempts in the brain and spinal cord (Nicholls, 2001). Perhaps then manipulation of the environment may be able to override the intrinsic response of neurites to injury.

1.1.3. Adult *In vitro* Neurite Regeneration

The more intricate details of axonal regeneration are best studied *in vitro* as opposed to the *in vivo* observations at the peripheral nerve level as described above. It is often difficult in culture to determine which neuronal process is the axon and which the dendrite. This is particularly hard with sensory neurons because of their pseudounipolar morphology. For this reason the process is usually called a neurite and will be referred to as such from herein. With this reductionalist approach to studying regeneration, a closer observation of neurite morphological behavior and analysis of the signal transduction responsible for growth is possible.

Smith and Skene (1997) have divided the regenerative process of mature DRG neurons into two temporal phases: branching and elongation. Arborizing growth occurs within 24h post injury, is responsive to neurotrophins, and is characterized by a branchy neuritic pattern. The genes expressed in naïve (uninjured) neuons are sufficent for this growth, a mode of growth that may represent the changes in target field arborization aforementioned to be seen *in vivo*. In contrast, the elongation mode of growth is characterized by a lower frequency of branching and is dependent upon transcription. Interruption of trophic support is necessary to provide the transition to this more regenerative type of growth (Smith and Skene, 1997).

The experiments presented in my thesis use dissociated adult rat DRG neurons (see Fig. 2.6 for details). The unique advantage of these neurons is their lack of dependence upon any growth factors for survival (Lindsay, 1988; Kimpinski et al, 1997). This model allows the growth effects of growth factors to be confidently dissected from the known survival effects they display in neonatal DRG neurons. This is a very important distinction because it is sometimes difficult to dissect growth from survival effects in other culture systems. The use of mitotic inhibitors creates an enriched neuronal environment by eliminating non neuronal cells while sparing post mitotic neurons. Therefore the environment exposure can be more easily controlled than *in vivo*. Other *in vitro* models of neurite growth that have been used include PC12 cells, a pheochromocytoma cell line, SH-SY5Y cells, a neuroblastoma cell line, and embryonic or neonatal primary neurons. These models have their own advantages, but the cells are quite different from adult primary neurons. For the reasons outlined here I feel that the primary sensory neurons are suitable to investigate the signaling events underlying neurite growth.

1.2. Growth Factor Signaling and Neurite Growth

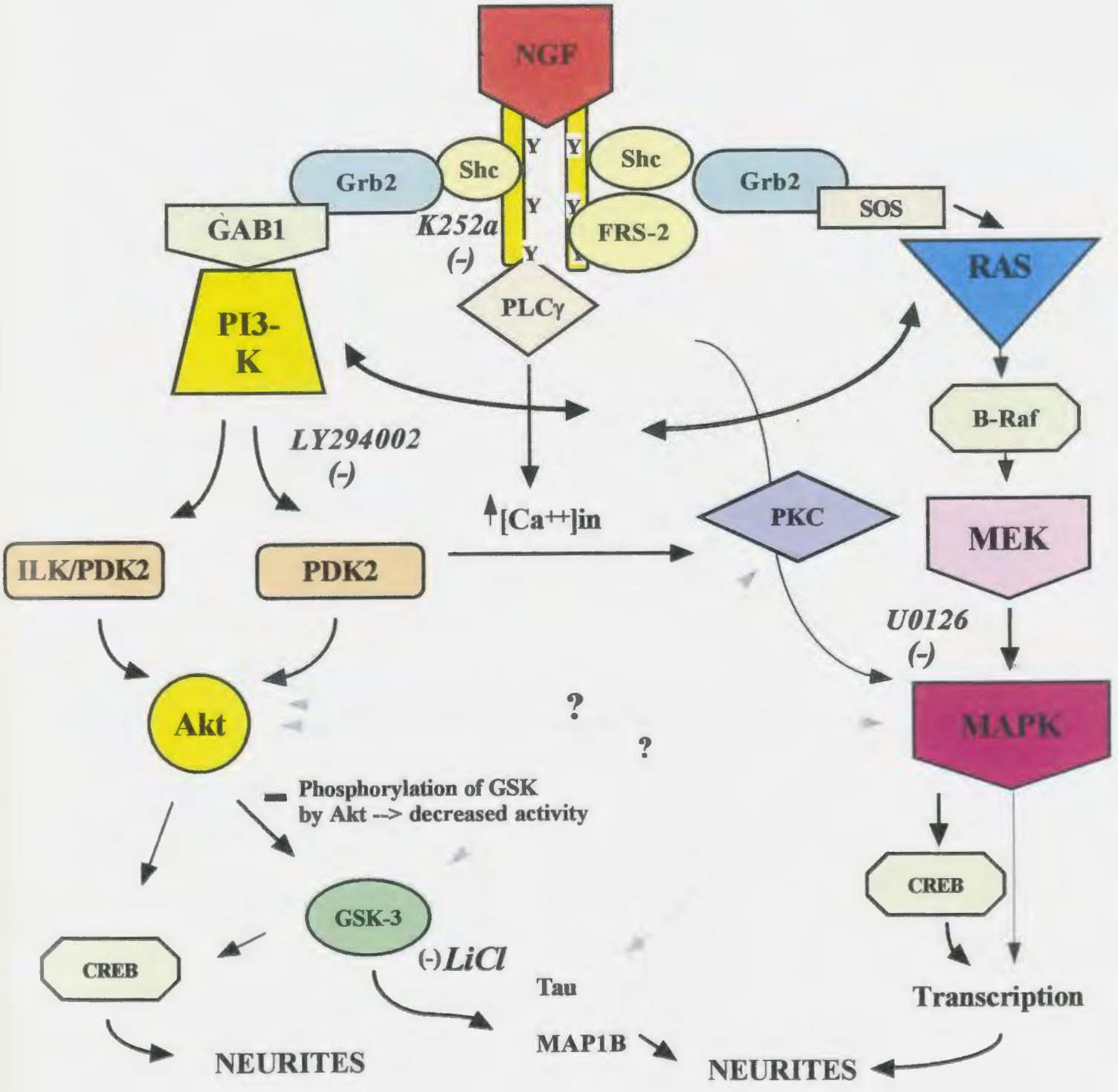
The last decade has seen an explosion in the understanding of signaling mechanisms, and growth factor signaling has been at the forefront. The technical advances in both genetic and biochemical analysis has helped this campaign tremendously. In the neurotrophin field, the signaling events underlying NGF growth effects in sensory neurons are perhaps one of the most extensively studied and relevant.

1.2.1. NGF Signaling

NGF signaling is initiated by its binding to its highly affinity receptor, TrkA (Kaplan et al, 1991, see figure 1.1). A NGF dimer binds a single TrkA receptor, initiating receptor dimerization and leading to the tyrosine phosphorylation of TrkA at three tyrosine (Y) sites in the autoregulatory domain (Y670, Y674, Y675) that promotes TrkA activity (reviewed in Segal and Greenberg, 1996; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Upon activation, the subsequent autophosphorylation of TrkA at other tyrosine residues creates docking sites for proteins such as the adapter protein Shc at Y490, FRS-2 (fibroblast growth factor receptor substrate) also at Y490, and phospholipaseC-γ1 (PLC-γ1) at Y785 (Kaplan and Miller, 1997).

The recruitment of Shc to TrkA results in association of growth factor related binding protein 2 (Grb2) to Shc. This complex can either bind the adapter protein, Gab-1 which is bound to and activates phosphatidylinositol-3-kinase (PI3-K), or the guanine nucleotide exchange factor (GEF), son of Sevenless (SOS) causing the transient activation the small G protein Ras. Activation of Ras can also lead to the stimulation of PI3-K activity. The recruitment of PLC-γ1 leads to its phosphorylation by TrkA and subsequent activation (Kaplan and Miller, 1997, Kaplan and Miller, 2000; Patapoutiano and Reichardt, 2001). Considering that both Shc and FRS-2 binds the TrkA receptor at the same site, an interesting molecular model of how NGF can lead to both differentiation and proliferation has been proposed (Meakin et al, 1999). Cell cycle arrest and neuronal differentiation is suggested to be mediated by FRS-2, while proliferation by Shc. Perhaps then FRS-2 could be involved in neurite growth as this process is central to neuronal differentiation.

Figure 1.1: NGF-TrkA Signaling Solid arrows or attachment indicates activation of the downstream substrate. Dashed lines indicated postulated interpathway interactions. The (-) symbol indicates inhibition of the adjacent signalling protein. Explanation of acronyms for all signaling proteins is provided in the list of abbreviations section.



Adapted from Kaplan and Miller, 2000

PI3-K and growth

Phosphorylation and release of membrane phophoinositides attract signaling proteins containing pleckstrin-homology domains. The most important of which are the 3-phosphoinositide-dependent kinases (PDKs), signaling proteins that are required for protein kinase B/Akt activation (for review see Burgering and Coffer, 1995; Coffer et al, 1998; Downward, 1998). There are a wide variety of Akt substrates, the majority of which have been demonstrated to be involved in various apoptosis/survival paradigms. Such effector pathway members include IκB, forkhead transcription factor, and Bcl-2/Bcl-x-associated death promoter (BAD) (Kumari et al, 2001; Datta et al, 1999). Another substrate of Akt is glycogen synthase kinase-3 α/β (GSK-3 α/β). Phosphorylation of GSK-3 α/β at serine 9 in the α isoform and 21 in the β isoform by Akt inactivates GSK-3, whereas constitutive phosphorylation at tyrosine 216 leads to maintained activation in most resting cells (Grimes and Jope, 2001a; Woodgett, 2001).

In addition to its role in promoting survival PI3-K signaling is thought to also play a role in regulating neurite growth. The majority of the cell lines, and primary neurons investigated display at least some PI3-K-sensitive neurite growth (Encinas et al, 1999; Korhonen et al 1999; Namikawa et al, 2000; Kaplan and Miller, 2000; Kimpinski and Mearow, 2001). Within these studies inhibition of certain members of the PI3-K signaling pathway inhibit growth, whereas activation supports growth. Although Akt (Namikawa et al, 2000) and GSK (Takahashi, 1999) are certainly involved in growth, it remains unclear exactly how these signaling proteins function in the growth process.

In the developing brain, GSK-3 expression is highest during neurite growth and concentrated in the distal region of the neurite (Takahashi, 2000). This region is

considered the most active area during neuritogenesis. Many of the studies implicating GSK-3 as a potential effector in growth came from experiments using lithium, a direct inhibitor of the kinase, to disrupt growth (Takahashi, 1999). Oddly enough, neurite retraction is critical to neuritogenesis, a process believed to rearrange the cytoskeleton to permit neurite growth (Sayas et al, 2002). Molecules that induce this process, such as lysophosphatidic acid (LPA), lead to tau phosphorylation through GSK-3 activation (Sayas et al, 1999). This suggests that the function of GSK-3 in neurite growth mediated by its influence over tau. More recently, an inactive (phosphorylated) form of GSK-3 has been demonstrated to be colocalized with F-actin and to mediate the neurite inhibition elicited by semaphorine 3A, an inhibitory guidance molecule (Eickholt et al, 2002). The issue is further complicated by the observation that factors, such as NGF, that phosphorylate GSK-3 inactivate the kinase yet support neurite growth. Before the function of GSK-3 in growth can be established the specifics on its activity must be further elucidated.

Effector proteins of GSK-3 α/β such as the microtubule associated proteins MAP1B (Lucas et al, 1998), and the transcription factor CREB (Bullock and Habener, 1998; Grimes and Jope, 2001b) have been implicated in the growth process. The phosphorylated form of MAP1B is found within the growth cone and is upregulated by NGF, thereby suggesting a possible role in neurite elongation (Gordon-Weeks, 1993). In addition, NGF leads to the activation of CREB leading to the expression of immediate early genes and inducing transactivation (Ginty et al, 1994; Finkbeiner et al, 1997). The influence that GSK-3 has on tau, MAP1B, and CREB suggests that GSK can be involved

in both cytoskeletal reorganization and protein synthesis, two crucial events in neuritogenesis.

Ras-ERK Pathway and Growth

The degree of involvement of the Ras-Raf-MEK-ERK pathway in neurite growth is somewhat controversial. The activity of Ras is necessary and sufficient for both the induction and elongation of neurite growth in PC12 cells (Burry, 2001), and necessary in conjunction with PI3-K signaling to provide local neurite growth in sympathetic neurons (Atwal et al, 2000). However in differentiated SH-SY5Y, a neuroblastoma cell line commonly used in neurite growth studies, ERK is not associated with neurite outgrowth (Olsson and Nanberg, 2001). As well in primary DRG sensory neurons the importance of ERK signaling in neurite growth is minimal compared to PI3-K signaling (Kimpinski and Mearow, 2001). The Ras-Raf-MEK-ERK pathway is involved in growth, but exactly how is yet to be clearly defined.

Like the PI3-K-Akt-GSK pathway, this pathway has been demonstrated to phosphorylate and thereby exert some control over two growth effector proteins, tau (Buée et al, 2000) and CREB (Xing et al, 1998). As mentioned above, the activation and function of tau in relation to phosphorylation is complex but well recognized as being important in neurite growth. Phosphorylation of tau by ERK could be how ERK activity is involved with neurite growth. Another result of ERK activation is the phosphorylation of CREB through Rsk2 activation at a site required for CREB transactivation (Xing et al, 1996).

While most of the signaling associated with NGF-dependent growth is likely mediated by the PI3-K-Akt-GSK signaling arm, activity of the ras-Raf-ERK signaling

arm may have some role in neurite growth of adult sensory neurons. This influence could be mediated by the control the ras-Raf-ERK pathway has over tau and CREB.

PLC-γ1 and growth

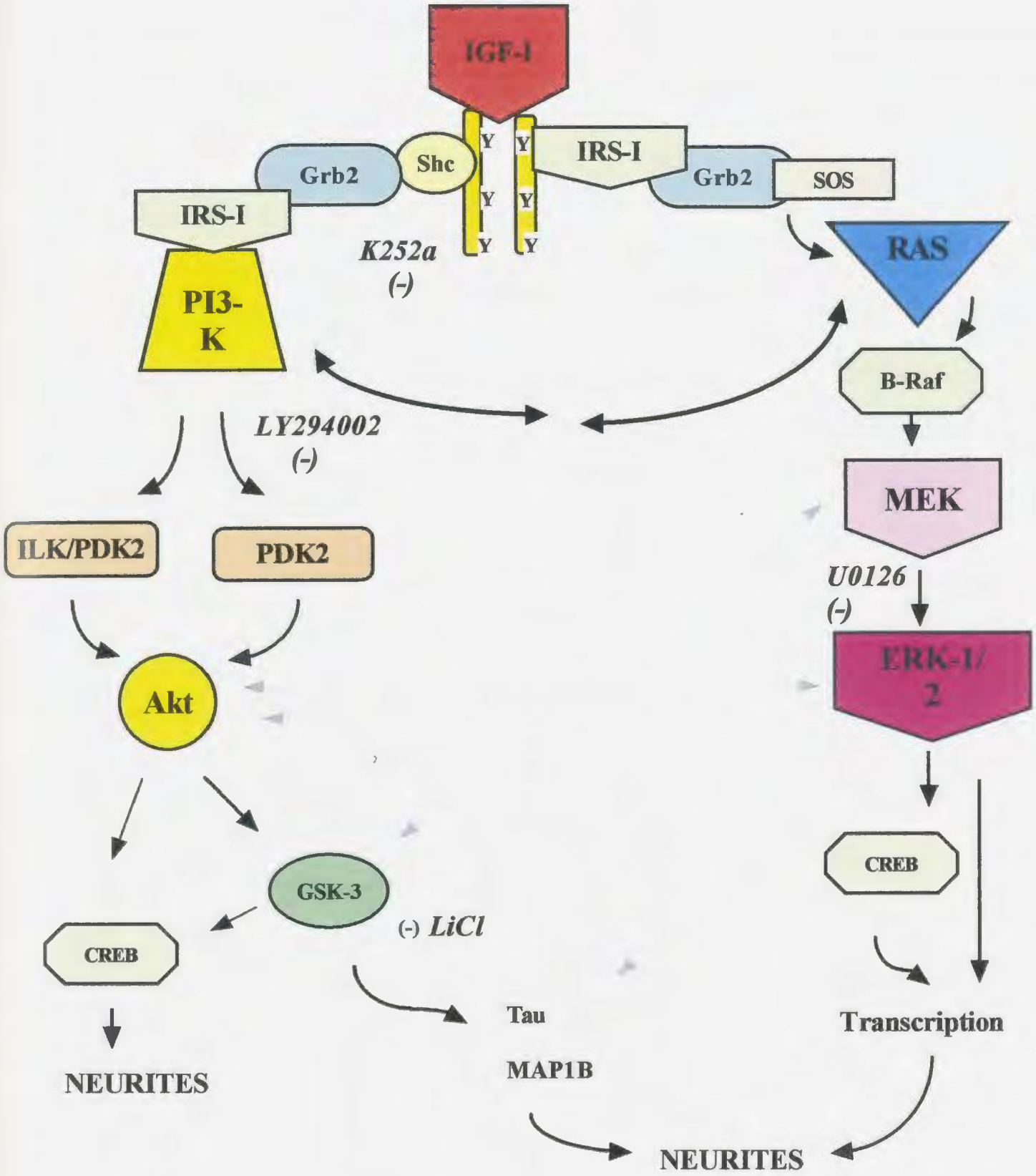
Activation of PLC-γ1 leads to the hydrolysis of phosphatidylinositides in the membrane causing the release of diacylglycerol (DAG) and inositol 1, 4, 5 triphosphate (IP_3). The former is required for the activation of protein kinase C-δ (PKC-δ) and other PKC family members, while the later increases the concentration of intracellular Ca^+ (Battiani, 2001). Both PKC-δ and $[Ca^+]_i$ contribute to neurite growth signaling in certain cells (Hundle et al, 1995; Burry, 1998; Doherty et al, 2000; Gomez and Spitzer, 2000).

This signaling system can be involved with neurite growth at many levels. Whether it is through its activation of PKC-δ or generation of an intracellular Ca^+ gradient, PLC-γ1 is needed for the molecular mechanisms required for chemoattraction of the growth cone towards NGF gradients during neurite pathfinding (Ming et al, 1999). The activity of PKC is regulated by many upstream proteins and PKC participates in the regulation of many substrates, including MEK and GAP-43 (Hundle et al, 1995; Rosner and Vacun, 1999). Both of these proteins can influence neurite growth, and represent another way that PLC-γ1 activity can contribute to neurite growth.

1.2.2. IGF signaling

The pleiotrophic actions IGF-1 begins with binding to its receptor IGF receptor –1 (IGFR-1) on the cell surface (see Fig. 1.2). Like TrkA, IGFR-1 is a tyrosine kinase that

Figure 1. 2: IGF-IGFR Signaling Solid arrows or attachment indicates activation of the downstream substrate. Dashed lines indicated postulated interpathway interactions. The (-) symbol indicates inhibition of the adjacent signalling protein. Explanation of acronyms for all signaling proteins is provided in the list of abbreviations section.



Adapted from Singleton and Feldman, 2001

becomes activated upon ligand binding, then autophosphorylates to create docking sites for signaling proteins (White and Kahn, 1994), the two major proteins being insulin receptor substrate (IRS) and Shc (reviewed in Feldman et al, 1997; Burtscher and Christofori, 1999). Recruitment of IRS differs from that of GAB in NGF-TrkA signaling as it is directly recruited to IGFR1. Once recruited, IRS is phosphorylated and becomes a docking protein for SH2 domain containing proteins like PI3-K and Grb2, leading to their activation. Upon binding to IGFR-1, Shc becomes phosphorylated and can then associate with Grb2, eventually leading to ERK activation (Baltensperger et al, 1993; Skolnik et al, 1993; Pronk et al, 1993). One major difference between NGF and IGF signaling is that PLC is not activated by IGF (Zapf-Colby, 1999; Kimpinski and Mearow, 2001).

IGF and Growth

The actions of IGF-1 are not classically thought of in the context of neurite growth, as much as they are in mediating the local hormonal influences of growth hormone. Despite this, the functional similarity of IGF-1 with insulin, and the finding that insulin can enhance neuritic growth (Snyder and Kim, 1980; Bothwell, 1982; Collins and Dawson, 1983) led to the discovery that IGF-1 can stimulate neurite growth in embryonic peripheral ganglion neurons (Recio-Pinto et al, 1986). Furthermore, reduced levels of IGF-1 in the diabetic state may contribute to diabetic neuropathy (for review see Ishii, 1995), a theory supported by the protective effect of IGF against neuropathy (Ishii and Lupien, 1995).

Research in the SH-SY5Y neuroblastoma cell line has pointed to the role of IGF signaling pathways in cell differentiation. In these cells IGF-1 stimulates both proliferation and differentiation. Neurite growth is an example of differentiation that is

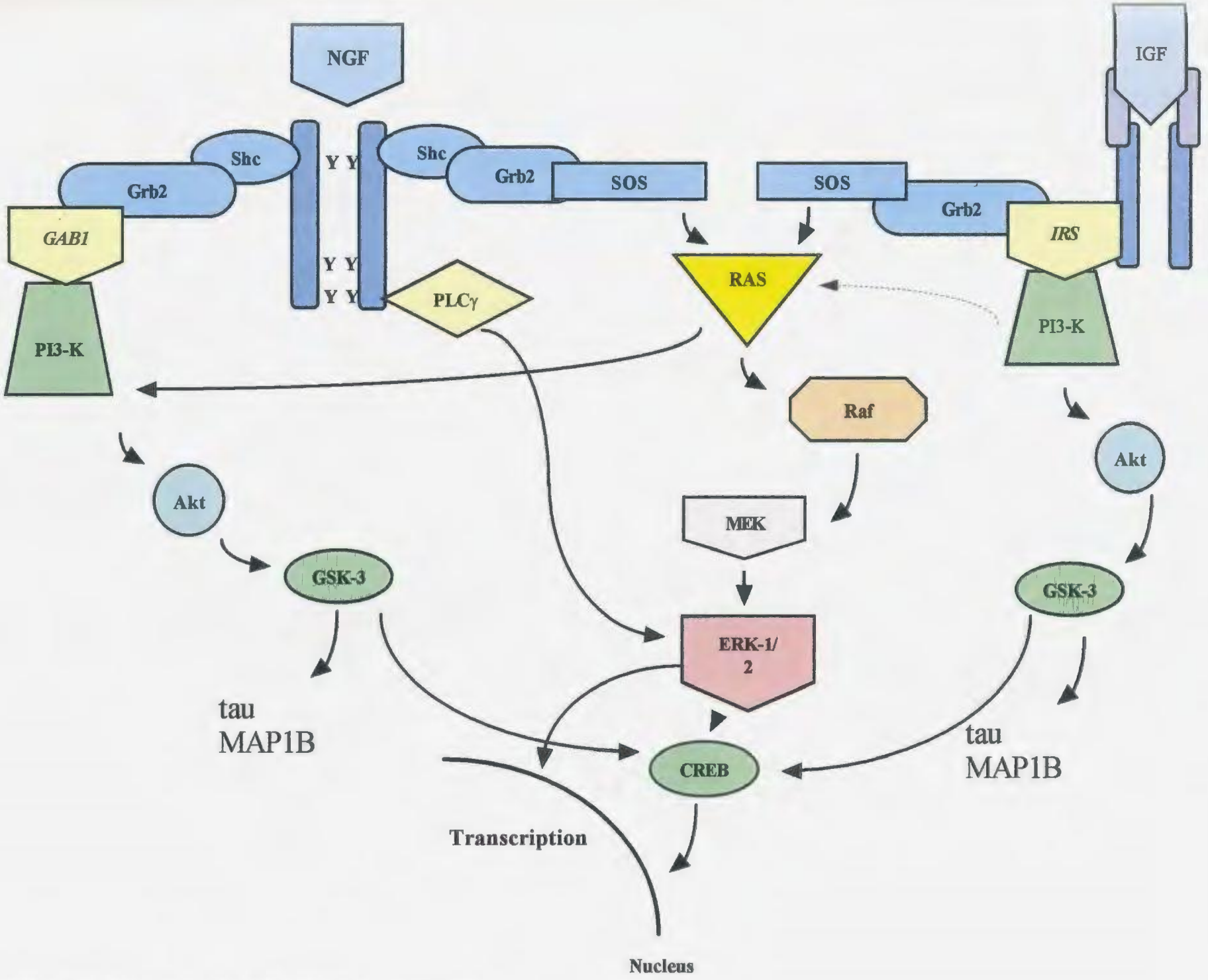
mediated by either the ERK or PI3-K pathway (Kurihara et al, 2000). Another group has proposed that ERK is required for IGF-1 stimulated neurite outgrowth in these cells, while PI3-K regulates growth cone extension and membrane ruffling (Kim et al, 1998).

Much like growth effects of NGF, both the ras-ERK and PI3-K signaling pathway contribute to the neurite growth effects of IGF-1. Considering this and other evidence presented in Ch. III, it is entirely possible that both NGF and IGF sigaling pathways may converge to influence growth (See Fig 1.3).

1.3. Extracellular Matrix Molecule (ECM) Signaling and Neurite Growth

The function of the adhesive properties of the ECM (substrate *in vitro*) in neurite growth is well established. Components of the ECM can direct, stimulate, and at times inhibit growth by providing adherence and via activation of specific signaling cascades. The importance of integrin signaling in neurite growth is highlighted by influence that transgenic integrin expression has on neurite growth (Werner et al, 2000; Condic, 2001) and the involvement of integrins in nervous system development. Transgenic deletion of the $\alpha 7\beta 1$ subunit in mice renders integrin signaling dysfunctional and results in incomplete PNS regeneration (Werner et al, 2000). More recently, the high degree of integrin control over neurite growth was demonstrated using gene transfer-mediated expression of α-integrin subunits (Condic, 2001). In these experiments, inducing the expression of certain α-integrin subunits dramatically improved the regenerative growth of adult DRG neurons.

Figure 1. 3: Possible NGF and IGF Signaling Interactions Solid arrows or attachment indicates activation of the downstream substrate. Dashed lines indicated postulated interpathway interactions. Explanation of acronyms for all signaling proteins is provided in the list of abbreviations section.



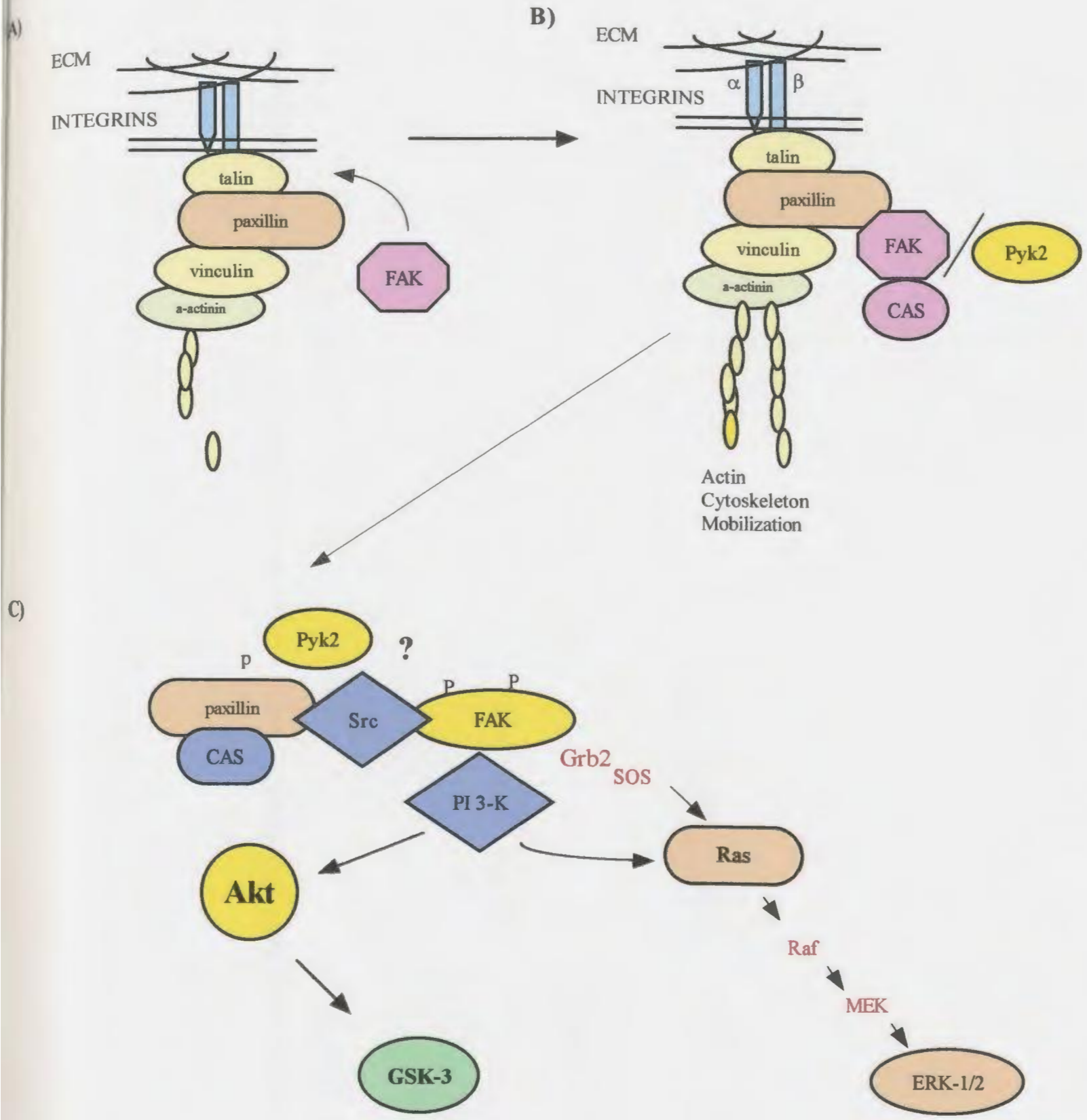
Adapted from Kaplan and Miller, 2000; Singleton and Feldman, 2001

Like the growth factors, components of the ECM convey much of their growth promotion ability through activation of signaling pathways. Many of the ECM components involved in growth bind to a ubiquitously expressed, transmembrane glycoprotein family called the integrins. Integrins are the principle receptors that mediate the influence of substrate on neurite growth (Jones, 1996; Condic, 2001). Briefly, binding of certain ECM components to integrins leads to the recruitment and subsequent activation of signaling proteins to the cytoplasmic tail of the integrin receptor complex (see Fig 1.4). The two major groups of proteins activated are signaling kinases (eg. focal adhesion kinase, FAK, and src) and cytoskeleton modulating proteins (eg. paxillin, and talin). Being major regulators of both these types of signaling proteins places integrins as key players in neurite growth. In addition, the ability of these integrin signaling to affect that of NGF raises the possibility of signaling convergence with integrins and NGF influence growth (Fig. 1.5). Refer to Chapter III Section 1 for a more complete description of integrin signaling events.

1.4. Objectives, Hypothesis, and Specific Aims:

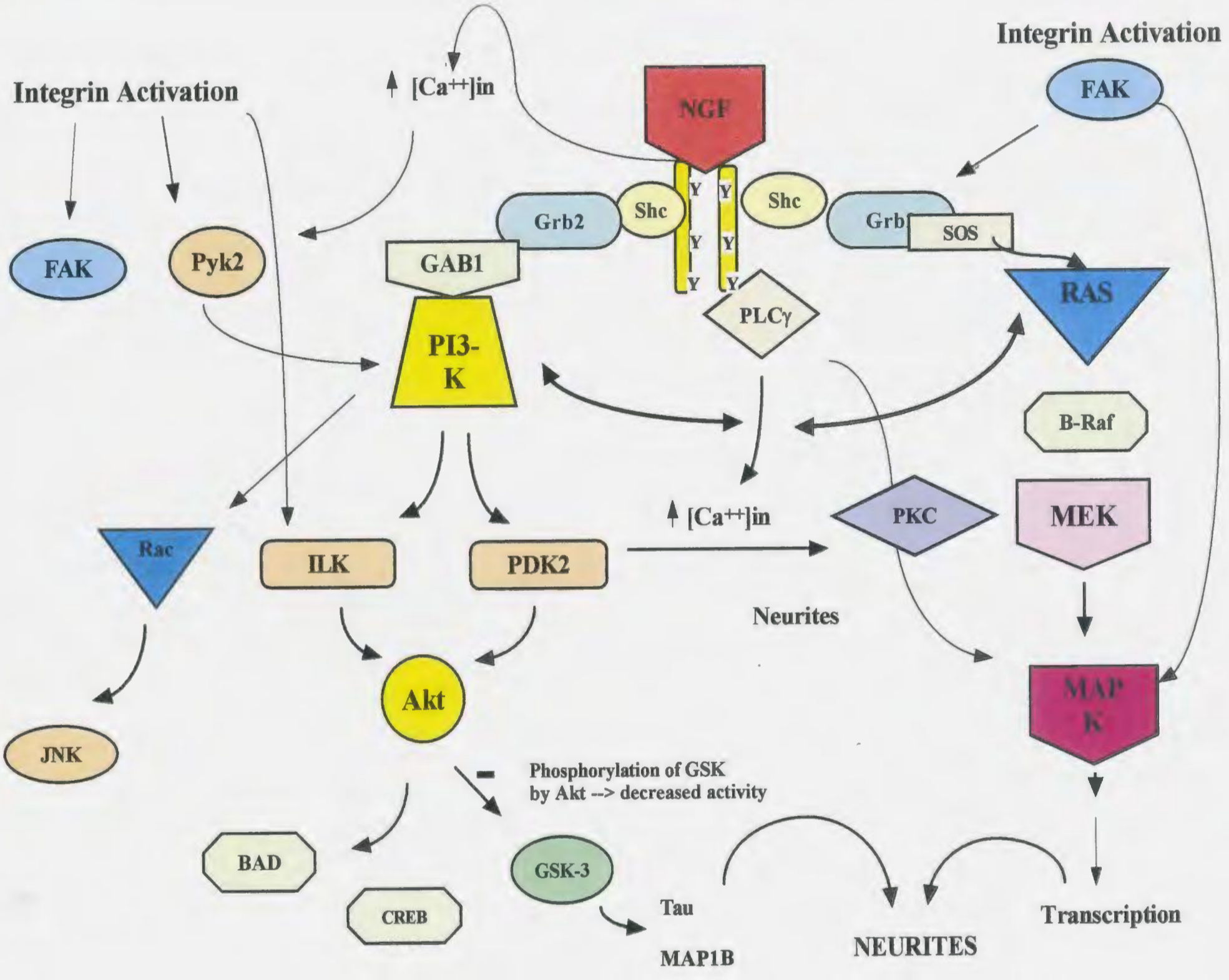
The goal of this thesis was to further investigate the trophic effects that environmental factors have on growth and the signaling underlying their actions. In Chapter III the synergistic effect that NGF and IGF-1 have on neurite growth in the adult rat dissociated DRG cultures was investigated. Our laboratory has previously shown that NGF, but not BDNF or NT-3, promoted distal neurite extension in the compartmented culture system and further demonstrated that IGF-1 can potentiate neurite growth into an

Figure 1. 4: Integrin Signaling The events underlying integrin action in neurons divided into steps. **(A)** Various integrin subtypes engage specific components of the ECM and thereby activate integrin receptors. **(B)** Upon activation cytoskeletal modulating proteins (talin, paxillin, vinculin, a-actin) are recruited and begin to moblize the actin cytoskeleton. FAK, or the neuronal homologue Pyk2, are attracted to paxillin and commence various integrin signaling cascades. **(C)** Though many of the details are currently under investigation, FAK/Pyk2 is speculated to activate growth factor signaling pathways such as the Ras-Raf-MEK-ERK and PI3-K-Akt-GSK pathways.



Adapted from Giancotti and Ruoslahti, 1999

Figure 1. 5: Possible NGF and Integrin Signaling Interactions Solid arrows or attachment indicates activation of the downstream substrate. Dashed lines indicated postulated interpathway interactions. The figure represents a synthesis of Fig 1.1 and 1.4 to show potential cross-activation and convergence of previously described signaling cascades. Explanation of acronyms for all signaling proteins is provided in the list of abbreviations section.


Integrin Activation
[Ca++]in
NGF
Integrin Activation
FAK
Grb2
Shc
Y
Y
Y
Y
Y
Y
Shc
Grb
SOS
FAK
Pyk2
GAB1
PLCγ
RAS
PI3-
K
B-Raf
[Ca++]in
PKC
MEK
Rac
ILK
PDK2
Neurites
MAP
K
JNK
Akt
Phosphorylation of GSK
by Akt --> decreased activity
BAD
GSK-3
CREB
Tau
MAP1B
NEURITES
Transcription

NGF environment (Kimpinski et al, 1997; Kimpinski and Mearow, 2000). My experiments were designed to first, investigate this growth potentiation, and then to elucidate the signaling events responsible for it. The contents of Ch III are the basis of a manuscript submitted to Experimental Neurology. In Chapter IV the potential role of the culture substrate in promoting adult neurite growth and underlying signaling events was investigated, although experiments here are of a more preliminary nature.

The hypotheses underlying my studies were as follows:

1.4.1. Hypothesis 1

The presence of both IGF and NGF in the environment will result in a synergistic promotion of neurite growth compared to either factor alone.

To test this, I assayed two sorts of growth parameters.

Specific Aim #1 was to examine the percentage of cells bearing neurites in control (no growth factor), NGF, IGF, NGF+IGF (N+I). This parameter gives a gross estimate of the percentage of plated responding to the culture conditions.

Specific Aim #2 was to analyze the neurite growth patterns in more detail within each of the culture conditions using computer-assisted tracing of individual neurons and their neurites. This parameter provides information about the length and branching/complexity of neurite growth.

1.4.2. Hypothesis 2

Interactions and/or convergence of NGF and IGF activated signaling pathways underlie the growth factor synergism on neurite growth.

Since NGF and IGF elicit growth, both stimulate similar signaling pathways, and both have receptors co-expressed in a substantial population of rodent DRGs (see Chapter III), it seemed logical to suspect that the mechanism of their growth synergism lies within an interaction between or convergence of their signaling pathways.

Specific Aim#1 was to correlate the activation of recognized growth factor signaling proteins with the observed growth effects. Using standard Western blotting with phospho-specific antibodies the relative activation of these proteins were examined. The signaling proteins include such intermediate proteins as Akt, GSK-3 and ERK, and the putative effector proteins they regulate such as tau, and CREB.

Specific Aim #2 was to assess the effect that pharmacological inhibition of these signaling proteins had on the percentage of neurite bearing neurons, neurite length and branching.

Specific Aim #3 was to assess the biochemical signaling in the presence of the inhibitors in an attempt to define the relative importance of the individual signaling in growth.

1.4.3. Hypothesis 3

Substrate-activated signaling, such as that initiated by laminin binding to integrins, can further contribute to growth factor signaling pathways and provide another convergence point to result in optimal growth.

Specific Aim #1 was to assess differences in growth when neurons were plated on poly-lysine compared to laminin, and then attempt to outline the signaling components activated in each of these conditions.

CHAPTER II – MATERIALS AND METHODS

2.1. Neuronal cultures

Dorsal root ganglia (DRG) from young adult (4-5wk) Sprague-Dawley rats (Memorial University of Newfoundland Vivarium) were dissected and dissociated essentially as described previously (Kimpinksi et al., 1997,1999; See Fig. 2.6). Briefly, ganglia from all spinal levels were removed and the roots trimmed. They were subsequently incubated in 0.25% collagenase (Invitrogen/ Gibco BRL, Burlington, Ont) for 45 min, followed by 0.25% trypsin for 20 min. Cells were then mechanically dissociated by trituration in 2 ml of modified NeuroBasal (NB, Invitrogen) medium, and pelleted at 1000 rpm for 5 min. The cell pellet was suspended in NB supplemented with 100 U penicillin/streptomycin, B27 medium supplement (Invitrogen), and 20uM cytosine arabinoside.

Neurons from one animal were plated in three Lab-Tek 16 well chamber slides (Nunc International, Naperville, NC) for neurite growth assessment, 12-well plates for Western blotting, or 96-well plates (3 animals/plate) for survival assays and incubated at 37°C, 95% O_2 and 5% CO_2. Slides and culture plates were coated with poly-lysine (1 μg/ml, BD Bioscience, Bedford, MA), 1:3 dilution of rat tail collagen, and laminin (5 μg/ml, Invitrogen). Neurons were cultured in modified NB alone or in NB supplemented with NGF (25 ng/ml, Cedarlane Labs, Hornby Ont) , IGF-1 (25 ng/ml, Sigma, St. Louis, MO) or both.

2.2. Experimental treatments

In growth factor experiments of Ch. III, two sorts of experiments were undertaken for analyses of effects on signal transduction pathways. First, stimulation experiments were performed as follows: neurons were plated for 24h in the absence of growth factors and then washed over 2-3 hrs with fresh NB and subsequently stimulated with 25ng/ml of the appropriate GF for 10min, 30 min, 1h, 6h or 24h. Alternatively, in chronic experiments the neurons were plated with 25 ng/ml of either NGF, IGF or both and sampled at 24h or 48h.

Chronic pharmacological inhibitor studies were identical to the chronic cultures except that 2-3h post plating appropriate concentrations of the selected inhibitors were added. This procedure gave the neurons time to recuperate from the dissociation protocol and attach to the culture substrate, two processes that the inhibitors could potentially disrupt. Inhibitors used were LY294002 to inhibit PI 3-K (Calbiochem, La Jolla, CA), U0126 to inhibit MEK (Calbiochem) and LiCl to inhibit GSK (Sigma). The inhibitors were used at the following concentrations, which we had empirically determined to be appropriate for inhibiting neurite growth without influencing survival: LY294002 – 10 μM; U0126 – 10 μM; LiCl- 3mM.

For the substrate experiments of Ch. IV, adult DRG dissociated cultures were plated on a substrate composed of either 1hr coat and dry of poly-lysine (1 ug/ml, BD Bioscience, Bedford, MA), the P-L condition, or 1hr coat and dry of P-L followed by two coats of 1:3 dilution of rat tail collagen, and laminin (5 ug/ml, Invitrogen). Growth factor treatments are identical to Chapter III.

2.3 Immunocytochemistry

Neurons were first washed with Tris-buffered saline (TBS) and then fixed in 4% paraformaldehyde (pH 7-7.4) in TBS for 20 minutes. Neurons were permeabilized with 0.1% Triton-X-100 (Sigma) and blocked with 10% normal goat serum. For neuronal and neuritic identification, antibodies directed against peripherin or PGP9.5 were employed (1:250, Chemicon, Temucula, CA). To distinguish between various neuronal populations we used the following antibodies or lectins: mTrks, IGF-R-α1 and -β1 (Santa Cruz Biotech, Santa Cruz, Ca) and isolectin IB4 (Sigma) at concentrations of 1:250-1000. Cells were incubated with the primary antibodies or IB4 at 4^{o}C for 16-20 hrs. For detection, two methods were employed. For analysis of populations and growth, Cy2 or Cy3- tagged secondary antibodies were employed (Jackson Immunoresearch Labs, West Grove, PA). In these experiments double staining was employed wherein cells were incubated with antibodies to both receptors, or a combination of the receptor antibody and biotinylated-IB4 lectin; the IB4 was visualized with FITC-conjugated avidin (Sigma). These slides were coverslipped with glycerol and imaged with confocal laser scanning microscopy or photographed with a SPOT RT digital camera.

For neuronal tracing analyses, HRP-tagged secondary antibodies at 1:50 were used followed by visualization with the ABC system (Vector Laboratories, Burlingame, CA) and diamino-benzidine (DAB) as the developing agent. For the identification of neuronal populations expressing the different growth factor receptors, a minimum of 2 fields (at low magnification) per experiment (n=3) were imaged and photographed for analyses. Growth factor receptor expression was calculated as the percentage of the total

number of neurons being positive or double positive. Digital images were collected and composite figures prepared using Adobe Photoshop (Adobe Corp).

For the substrate experiments, neurons were fixed and then permeabilized with 0.1% Triton-X-100 (Sigma) and blocked with 10% normal goat serum. For neuronal and neuritic identification, antibodies directed against peripherin or PGP9.5 were employed (1:250, Chemicon, Temucula, CA). Neurons were then incubated at 4°C overnight. The next day, neurons are washed 3 X 10 minutes with TBS-T (0.1% Tween). Biotinylated secondary antibody diluted 1:200-1:1000 was added for 1 hour and washed with TBS-T as before. To quench endogenous peroxidase 0.3% H_2O_2 in TBS was added for 20 minutes followed by a 15 minute wash in TBS-T. Visualization was performed using the ABC system (Vector Laboratories, Burlingame, CA) and diamino-benzidine (DAB) as the developing agent.

2.4. Measurements of Neurite Growth

Neurite initiation (neuritogenesis) was first quantified by determining the percentage of the total phase bright neurons with neurites twice the length of their cell bodies. The two observers were aware of the conditions because the results are clear and objective thereby obviating the need for double blind analysis. The data was normalized to percentage of the control (no growth factor) condition and plotted as the mean $\pm$ SEM. Preliminary experiments indicated there was little growth before 48h after plating, suggesting that 48h would be the optimal time point to obtain growth measurements.

Individual neuron tracings of DAB-stained neurons were carried out using the Neurolucida (MicroBrightField, VT) tracing program; data analyses were carried out

using the Neuroexplorer component of the program to attain Scholl analysis (intersection point and length) and total neurite length data. As I was interested in the effect the condition has on growth promotion, representative cells were those with the maximal growth promotion. Scholl 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. This analysis gives a measure of the extent of branching. 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.

2.5. Protein Analysis

For Western analyses, neurons plated in 12 well plates were stimulated or cultured with growth factors in the presence or absence of the inhibitors for varying periods of time, as described above. Neurons were subsequently processed according to our established procedures (Kimpinski and Mearow, 2001). In brief, medium was aspirated from the cells, the cells gently washed with ice –cold TBS containing 200 mM sodium vanadate and collected using a rubber policeman in TBS. Following pelleting of the cells at 3000 rpm for 5 min, the cell pellet was suspended in protein lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus protease inhibitors, 1 mM sodium vanadate, and sodium fluoride). After cell lysis and centrifugation of the lysate at 10,000 rpm for 5 min, the supernatants were used to determine protein concentrations using the BCA protein assay (Pierce Chemicals, Rockford, IL.). Equivalent amounts of protein (50 ug) were subjected to SDS-PAGE (10% or 7.5-15% gradient gels). Following transfer to

nitrocellulose, the blots were first stained with Ponceau Red to assess the equivalency of protein loading.

Following washing with TBS, the blots were subsequently probed with the following antibodies: phosphospecific Akt (Ser 473), phospho-p44/42 MAPK, phospho-GSK3β, Akt and GSK3 (NEB/Cell Signaling, Beverley, MA), phospho-Creb (Ser133), Creb and phosphotyrosin 4G10 (Upstate Biotech Incorp, Lake Placid, NY), ERK (Santa Cruz). Antibodies direct against the S199 and S396 sites of tau were obtained from BioSource International and total tau (tau 5 clone) from CalBiochem. Blots were cut and reprobed sequentially such that any given gel experiment could be probed with up to 15 different antibodies. Blots were visualized with ECL reagents (NEN, Boston, MA) and exposure to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC), and subsequently digitized and densitometrically analysed with a cyclone ChemiImager and composite figures prepared using Adobe Photoshop graphics software.

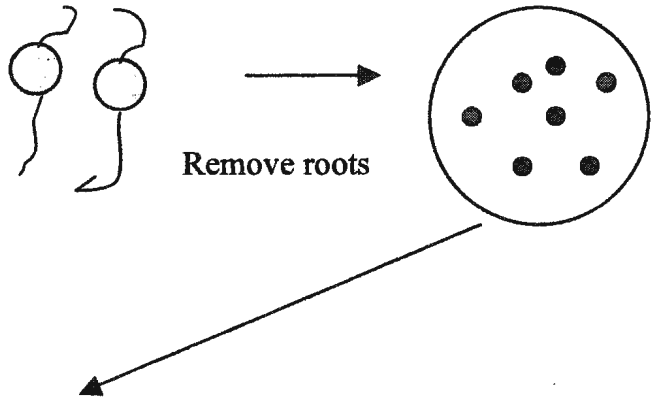
2.6. Statistical Analysis

All reported data quantification is based on at least 3 separate experiments. Data are plotted as mean $\pm$ SEM and significance is noted only if $p<0.05$ as determined by ANOVA using Tukey or Newman-Keuls testing for post hoc comparisons for multiple group analyses.

Figure 2.6: Preparation of DRG Cultures Graphical summary of the steps involved in the culturing of neurons used in all experiments. See text for further experimental details.

A. Dissection

DRGs are removed from young adult rats



B. Dissociation

Collagenase – 45 min at 37°C

Trypsin – 20 min at 37°C

Sequential mechanical trituration, followed by pelleting cells at 1000rpm for 5 min

C. Plating

Cells are resuspended in appropriate culture medium and plated

→ 12- well plates → biochemical analyses

→ Chamber slides → growth analyses

CHAPTER III: SIGNALING CONVERGENCE UNDERLIES GROWTH FACTOR SYNERGISM

3.1 Introduction

Factors contributing to the regeneration of peripheral nerves have been the subject of numerous investigations. In the PNS both motor and sensory axons undergo regeneration in most instances. However, full functional recovery rarely occurs and this could be due to both the long distances involved and factors primarily associated with the microenvironment of the peripheral nerves (Fu and Gordon, 1997; Zochodne and Cheng, 2000).

Adult sensory neurons can survive *in vitro* in the absence of added neurotrophins, though neurotrophins like NGF are required for both maintenance of phenotypic characteristics and for the promotion of neurite growth (Lindsay, 1988; Mulderry, 1994; Malgrange et al, 1994; Edstrom et al, 1996; Kimpinski et al, 1997; Gavazzi et al, 1999). In addition to the neurotrophins, other growth factors and cytokines are increasingly being shown to influence axonal growth from adult sensory neurons, either acting alone or modulating the responses of the neurons to trophins like NGF (eg., Ferneyhough et al, 1993; Gavazzi et al, 1999; Sondell et al, 1999; Cafferty et al, 2001; Kimpinski and Mearow, 2001). These include GDNF, IGF, VEGF, EGF, and LIF, all of which can be detected in peripheral nerves or the DRGs at some point after nerve injury, although their exact role(s) in the repair and regenerative processes are not well defined (Zigmond et al, 1996; Fu and Gordon, 1997; Terenghi, 1999; Zochodne, 2000). We have previously shown that IGF-1 can elicit extensive neurite growth from adult sensory neurons, and

furthermore, appears to potentiate NGF-induced neuritogenesis (Kimpinski and Mearow, 2001).

These growth factors signal through specific receptor tyrosine kinases, with the subsequent activation of common intracellular signaling intermediates including Ras, mitogen activated protein kinase (MAPK or ERK), phosphoinositide 3-kinase (PI 3-K), the serine-threonine kinase Akt (reviewed in Folli et al, 1996; Kaplan and Miller, 2000). The need or sufficiency of the various components for neuritogenesis has been studied most extensively in cell lines, although emerging studies in primary neurons indicate that the relative contributions of these major pathways differ with both cell type and developmental age. For example, both the PI3-kinase and Ras-MAPK pathways have been shown to be required for growth from sympathetic neurons (Atwal et al, 2000), while MAPK inhibition has little or no effect on neurite growth from sensory neurons (Klinz et al, 1996). In our previous study we provided evidence that the PI 3-K pathway plays an important role in adult sensory axonal growth (Kimpinski and Mearow, 2001).
In the current study we have investigated the potential mechanisms underlying the synergistic effects of NGF and IGF-1 on neurite growth from adult DRG neurons. We hypothesized that the increased growth observed in the presence of both growth factors was due to a convergence of the signaling pathways. Using both biological and biochemical assays, we show that the individual growth factors elicit similar patterns of growth, yet activate the signaling pathways in somewhat different ways. Furthermore, in the presence of both NGF and IGF-1 there is some convergence in terms of the signaling pathways, and our results further point to the importance of the PI 3-K→Akt→ GSK3β pathway in neurite growth.

3.2. Results

3.2.1. NGF and IGF act synergistically to promote neurite growth of adult DRG neurons above either growth factor alone.

To first evaluate the influence of NGF and IGF on adult DRG cultures we used immunostaining to detect peripherin, an intermediate filament protein. This aids visualization of neurons since peripherin is only expressed in neurons. Representative micrographs (Fig. 3.7) depict the different neurite growth patterns promoted by the various growth factor conditions used. Although neurites in the IGF-1 (B) and NGF (C) treated cultures display more branching than control, in the presence of both IGF-1 and NGF (N+I, D) there is a more extensive network of neurite growth than in either growth factor alone. In addition, the neurites in the N+I cultures appear not only to be longer, but also display a much more elaborate branching pattern than either in NGF or IGF alone.

The percentage of neurons extending neurites was next counted at 48h post-plating (Fig. 3.8A). This demonstrates the influence that NGF and IGF-1 and the combination of the two have on neurite sprouting. Only 17% of the neurons extended neurites when plated without any growth factor influence (control), while IGF (I) and NGF (N) promoted neurite growth from 31% and 40% of the neurons, respectively, a level significantly higher than control. In the presence of both N+I, the situation that promotes the more elaborate neuritic network (Fig 3.7), 45% of the neurons extended neurites. The combination of both growth factors has a significantly increased influence over control, but not over NGF or IGF alone. These numbers were surprising at first

because they did not seem to reflect the extent of the neurite growth observed in the N+I cultures.

3.2.2 Exposure to a NGF and IGF environment promotes more elaborate neurite branching than either growth factor alone

To further assess the influence that NGF and IGF-1 have on growth an analysis of the neurite growth pattern was undertaken. Neurite branching was quantified by tracing representative cells from the same cultures used to quantify neurite initiation followed by analysis of the tracing data. The two parameters measured were total neurite length (Fig. 3.8B) and a Scholl analysis, which calculates the number of intersection points (3.8C) and amount of neurite length (3.8D) within 20 μm radius circles placed concentrically over the cell soma. As is shown in Fig 3.8B, longer neurites extend from all growth factor treated cultures as compared to control (c=526 $\pm$ 97 μm). The total length of neurites growing in NGF alone or IGF alone did not differ significantly, reaching lengths of 1101 $\pm$ 57 and 1288 $\pm$ 354 μm, respectively. This is similar to data obtained in our previous compartmented culture studies where neurites growing into IGF-containing side compartments showed slightly longer extension than into NGF-containing side compartments (Kimpinski and Mearow, 2001). Neurites growing in the N+I environment exhibited the greatest total neurite length reaching lengths of 2492 $\pm$ 378 μm. Thus the dual growth factor environment supported significantly ($p \leq 0.05$) longer neurites than either of the growth factors alone.

The second parameter analyzed was the extent of branching of the neuritic growth. The number of intersection points (data from the Scholl analyses) was greater in

all growth factor groups than control (Fig 3.8C). Like the observations for neurite length, neither growth factor alone was a better promoter of neurite branching than the other. However, when exposed to N+I, neurites extend more densely than either growth factor alone as shown by the larger number of intersections with concentric circles radiating from the soma. This effect begins from the number of neurites extending from the soma, continues with increasing number of branches on the neurites until the termination of the neurites. Perhaps a better representation of the extent of branching, the total neurite length (Fig 3.8D) within contiguous circles reveals the area occupied by the neurite network. Thus, the closer analysis of the neurite growth patterns reveals that the dual growth factor environment significantly enhances the amount of neurite branching with N+I>NGF=IGF>Control.

So although the total number of neurons extending neurite was not significantly different in the N+I condition, the amount of growth from the individual neurons was significantly enhanced.

3.2.3 DRG neurons coexpress IGF-IR and TrkA

We then attempted to determine the potential mechanism(s) underlying the growth factor synergism. Considering that NGF and IGF have overlapping biological functions in neurons and elicit these responses through similar signaling pathways, it would seem reasonable that signaling convergence could be responsible for the enhanced neurite growth. This hypothesis implies that a substantial number of adult DRG neurons co-express TrkA and IGFR-1. We employed double labeling of the receptors (with immunofluorescence) to confirm this expectation. In 3 experiments with at least 100

neurons countedin each experiment (n=3), TrkA was found to be expressed by 65% ± 11 and IGFR-1 in 62% ± 10 of the plated neurons. More importantly, 41% ± 8 of the total population were observed to coexpress TrkA and IGFR-1, roughly the same percentage of neurons that extend neurites under the influence of N+I. Furthermore, we found that this receptor expression profile did not change in any of the different growth factor environments; that is, there was little effect of NGF or IGF-1 on the number of cells expressing these receptors over the course of our experiments (24-72 hrs).

3.2.4. Signaling pathways contributing to the growth response

Having established that neurons were responding to both NGF and IGF, we were interested in determining the potential biochemical mechanisms contributing to the observed biological response, and undertook a biochemical analysis of the signaling intermediates activated in each of the conditions described above, i.e., in the absence of added growth factors, plus NGF or IGF, and N+I. In these experiments then, we first plated neurons in serum-free defined medium in the absence of any growth factors for 24 hrs, and then stimulated the cultures with either the addition of NGF or IGF, or N+I for 30 min or 1 hr, and then collected the cell lysates, and carried out immunoblotting experiments. The rationale for this was to provide baseline data for the short term effects of the growth factors, and to be sure that we could detect any activation events that we were interested in, eg., phosphorylation of the receptors, and downstream intermediates like Akt and MAPK. In our previous work with adult DRG neurons, we found that we were able to detect such events by 30 min and that these were generally still detectable at 1hr (Kimpinski et al, 1999; Kimpinski and Mearow, 2001; also unpublished

observations). However, since we were interested in the longer term effects of growth factor exposure, which would more likely be influencing the neurite extension and branching patterns, we also carried out experiments where the neurons were plated directly into either the control condition or the appropriate growth factor condition and sampled 24 or 48 hrs later.

Representative blots of short term stimulation are shown in Fig 3.9. Firstly, we demonstrate that there is little difference in either TrkA or IGFR-1 tyrosine phosphorylation with the short term stimulation of either growth factor. We examined several downstream intermediates, all known to be phosphorylated in response to growth factor receptor activation. Increased phosphorylation of Akt is detectable in all growth factor conditions by 30 min, although in the presence of either NGF or IGF this diminishes slightly by 1 hr. In the N+I condition, the pAkt levels remain elevated at the 1 hr time point. The phosphorylation pattern of MAP kinase (also referred to as ERK) is somewhat different in that increased levels of phosphorylation are mainly observed in the NGF and the N+I conditions; the IGF condition does not differ significantly from the control. From other experiments where we examined 5, 10 and 15 min stimulations, we know that we have not missed a potentially earlier phosphorylation events. Both blots were sequentially probed with the non-activation state dependent antibodies to Akt and MAPK to control for protein loading.

We also investigated the activation of these intermediates in longer term exposure experiments as outlined above. Here we sampled cells 12, 24 and 48 hrs after plating in the presence of NGF, IGF or N+I compared to the control cultures where there was no growth factor added. Figure 3.10 presents representative blots from this series of

experiments. It is interesting to note that in the presence of IGF alone there is still little detectable pMAPK compared to the NGF or N+I conditions at any of the time points examined. This observation suggests that the ras-MAPK pathway is not a major pathway activated by IGF in these cells, despite the fact that there is quite substantial neurite growth, supporting our hypothesis that MAPK is not required for neurite growth in adult sensory neurons.

3.2.5. Specific contributions of individual signalling intermediates to neurite growth

There are conflicting reports concerning the signaling events underlying growth factor dependent growth and the importance of specific signaling proteins is yet to be fully understood. Furthermore many reports in the literature suggest that both the PI 3-K and Ras>MAPK pathways are equally associated with neurite growth. In an effort to define the responsible signaling proteins involved in neurite growth from adult DRG neurons, pharmacological inhibitor agents K252a, LY294002, and U0126 were used to inhibit TrkA, PI 3-K, and MAPK, respectively. The latter is designed to block both the activation and activity of MEK thereby disabling activation of MAPK. A downstream target of PI 3-K and Akt is GSK3β, whose role in neurite growth appears to be associated with its ability to phosphorylate tau and microtubule-associated protein 1B (Goold et al, 1999; Goold and Gordon-Weeks, 2001). LiCl has been used as a selective and competitive inhibitor of GSK3 activity (eg., Klein and Melton, 1996). The inhibitors were added to the neurons 2-3h post-plating to avoid any interference with attachment and administered at a concentration that we had empirically determined was sufficient to inhibit growth but not deleteriously influence survival.

We used immunostaining with peripherin in order to be able more easily visualize the effects of the inhibitors on neurite growth. Fig 3.11 presents representative micrographs of neurons grown in the N+I condition plus the various inhibitors; the effects were somewhat more pronounced in the single growth factor conditions. In the presence of K252a (Fig. 3.11A), neurite growth is almost totally attenuated. The processes of those neurons that do extend neurites are very short and do not range much beyond the soma. Inhibition of PI 3-K (Fig. 3.11B) or GSK3 (Fig. 3.11C) results in a similar effect. Neurite growth is inhibited with shorter neurites and considerably less branching. However inhibition of MAPK activation with U0126 does not inhibit growth to the same degree as that seen with inhibition of PI 3-K or GSK3 (Fig 3.11D).

We further quantitated these effects for cultures grown in NGF, IGF and N+I plus LY294002, U0126 and LiCl. We assayed the impact that the inhibitors had on growth factor-promoted neuritogenesis by assessing the percentage of neurite-bearing neurons in each of the growth conditions 48 hrs after plating. Fig 3.12 presents the summary data from these experiments, with summary data from the control, NGF, IGF and N+I conditions presented in panel A, and the effects of the various inhibitors shown in the remaining panels B-D. In Fig 3.12A, it is clear that all growth factor additions had a significant influence on promoting neurite initiation compared to the control ($p< 0.001$ for NGF and N+I,; $p< 0.05$ for IGF). The selective inhibition of tyrosine kinase activity with 200 nM K252a significantly blocks neurite growth in all growth factor conditions, with less than 5-7% of neurons exhibiting neurites (data not shown). Inhibition of the PI 3-K arm also provides a significant blockade of NGF–dependent neurite growth (Fig 3.12B, 10 μM LY294002 = 20% vs. 1mM LiCl =19%); blocking the actions of MAPK

was less effective (25 %) although it was still significantly different from NGF alone. IGF-dependent growth (Fig. 3.12C) was similarly attenuated by the inhibitors. In the dual N+I condition (Fig. 3.12D) inhibition of growth was also observed in the presence of all the inhibitors ($p \leq 0.05$).

Like the previous experiments, the neurite initiation quantification did not clearly represent the obvious visible growth effects. In an attempt to quantify the effect of the inhibitors on growth we again used tracings of individual neurons to analyse total neuritic length and number of intersections in each of the culture conditions and the summary data for these analyses are presented in Fig 3.13.

In the NGF-treated cultures (Fig 3.13A, left panel), the presence of both LY294002 and LiCl resulted in a significant inhibition of neurite growth (LY, $p < 0.001$; LiCl, $p < 0,05$); although U0126 did diminish growth, this effect was not statistically significant. The plot showing the intersection points (Fig 3.13A, right panel) provide an indication of the decrease in the amount of branching and extension that occurs in the presence of both LY294002 and LiCl.

In the IGF-treated cultures (Fig 13B, left panel), only the LY294002 significantly decreases the amount of neurite growth ($p < 0.001$), and it very clearly inhibits the branching pattern of growth as seen by the decrease in the number of intersection points (Fig 13B, right panel). The extent of growth and the branching patterns in IGF plus LiCl or U0126 were quite similar and did not differ significantly from the IGF only condition. The increase in intersection points observed at distances greater than 200 um out from the cell body is due primarily to several neurons (out of 20) whose growth patterns were quite extensive and elaborate compared to the others in the group analyzed.

In the N+I condition, all three inhibitors had a significant effect on neurite growth (Fig 13C, left panel, $p< 0.001$), although in terms of the branching patterns there were differences detectable between the conditions, with the number of intersection points being less in the LY294002 and LiCl conditions than in either the U0126 and the control N+I condition (Fig 3.13C, right panel)

Thus to summarize, inhibiting PI 3-K activity by LY294002 dramatically lowered both the total neurite length and the amount of branching elicited by each of the growth factor environments. Blocking GSK3 activity was also effective in reducing both neurite length and branching. In contrast, inhibition of MEK activity was much less effective in attenuating growth in any of the conditions.

3.2.6 Correlative Western blots confirm the involvement of suspected signaling proteins

Western blotting was used to assess the biochemical effects that the inhibitors had on the signaling intermediates, essentially to be sure that the concentrations used were effective in inhibiting their appropriate substrates. Representative blots are presented in Fig 3.14, with panel A showing the control cultures plus the inhibitors, B – NGF plus inhibitors, C - IGF plus inhibitors and D – N+I plus inhibitors.

In all conditions the blots show that LY294002 predictably blocks the kinase activity of PI 3-K resulting in less phosphorylation of Akt but only minimally lowering GSK-3 phosphorylation. The phosphorylation of GSK-3 was consistently elevated by LiCl, as seen in other studies (Chalecka-Franazek and Chuang, 1999). Furthermore, LiCl also upregulated the amount of total GSK. U0126, the MEK inhibitor, blocks both

constitutive and growth-factor activated phosphorylation of MAPK. Thus, the inhibitors do indeed block the activity of the expected signaling proteins thereby confirming their effectiveness in previous experiments.

However, we also observed several unexpected results. In all conditions, including the control condition, treatment with LiCl resulted in increased pMAPK, even greater than that due to the growth factor stimulation. The reason for this is not clear, although it may point to some sort of feedback regulation by inhibition of GSK or, alternatively, via the effects of lithium on other signaling intermediates (Davies et al, 2000). Furthermore, inhibition of MEK activity with U0126 lead to an elevation of the phosphorylation of Akt. We have also observed a similar effect in other experiments, where we have been investigating parameters that regulate neurotrophin-independent survival of these neurons. In those experiments where we have assayed cell survival, we consistently observed an increase in the amount of survival in the presence of either U0126 or PD98045 as well as an increase in the pAkt levels compared to controls. We have been unable to find any other examples of this sort of effect in the literature, although a recent publication suggests that it may have more to do with sustained activation of Akt, rather than a direct influence of MAPK on Akt (Shaw and Cohen, 1999).

3.2.7. Activity of growth related substrates of GF signaling intermediates correspond to neurite branching

The signaling proteins analysed thus far are intermediaries rather than the ultimate effectors of the growth factor actions. In an attempt to complete the growth associated

signaling pathway we probed for possible substrates of GSK and MAPK that could be involved in the neurite growth events. The microtubule-associated protein tau is one GSK-3 substrate known to stabilize microtubules (Reynolds et al, 2000). Two recognised sites of GSK-3-mediated tau phosphorylation are serines 199 and 396 (Billingsley and Kincaid, 199). Another well known effector of growth factor signaling is CREB. This transcription factor is a substrate of many signaling kinases, including MAPK via its activation of the pp90 ribosomal S6 kinase (RSK) family of Ser/Thr kinases and by GSK-3 by direct phosphorylation subsequent to priming phosphorylation by p90RSK (Xing et al, 1998). NGF and other growth factors can phosphorylate CREB at serine 133, a necessary primary activation site (Xing et al, 1998; reviewed in Shaywitz and Greenberg, 1999).

As shown in Fig 3.15, with chronic exposure (24h and 48h) to all growth factor treatments, phosphorylation of tau at ser199 is minimally altered. In contrast, phosphorylation at ser396 is elevated in all growth factor environments at 48h and especially in the presence of N+I. The phosphorylation of CREB on the other hand is greater in NGF and IGF at 24h, but barely dectectable in the N+I condition; phosphorylation in the N+I environment is induced at 48h as in the other growth factor conditions.

Both tau and CREB are substrates of GSK-3 and MAPK. After realising that the inhibitor treatments were uncovering interactions between pathways, we subsequently investigated the effect of the inhibitors on these downstream targets in the absence or presence of the growth factors (Fig 3.16). Lithium diminished the phosphorylation of tau at ser396 in control and all growth factor environments without any effect on the

phosphorylation at ser199. It also decreased the phosphorylation of CREB stimulated by NGF and IGF. Surprisingly, inhibiting PI 3-K activity lead to an enhanced phosphorylation of CREB above control and growth factor induced levels. Also surprising was the observation that lithium lowered both NGF- and IGF-induced CREB phosphorylation, yet U0126 did not affect CREB phosphorylation. These results were somewhat unexpected, given that MAPK (via p90RSK) phosphorylates CREB at ser133, and again point to more complex interactions between signaling pathways than any linear representation can depict. The site detected by our antibody is phosphorylated by MAPK (ser133), but not GSK3 (ser129) (Grimes and Jope, 2001b; Shaywitz and Greenberg, 1999).

3.3 Discussion

3.3.1 Signaling Convergence underlies the synergy of NGF and IGF on neurite growth

In this study we have investigated the influence of combinations of growth factor treatment on cultured adult DRG neurite growth and the underlying signaling activity. This model lends itself nicely to investigations of growth factor influence over neurite growth because of the NGF-independence for survival. I find that exposure to the dual growth factor environment of NGF and IGF (N+I) synergistically promotes growth. Observation of these N+I-treated cultures reveals a much more complex neurite growth pattern, highlighted by both longer neurites and greater arborization. Through tracing analyses of individual neurons, we were able to quantify this complexity and demonstrate that N+I treatment leads to a significantly greater total neurite length and more neurite

branching than either growth factor environment alone. Both these growth factors have been previously described to exert considerable influence over neurite growth in a variety of neural cell types (Feldman et al, 1997; Torres-Aleman, 1999; Patel et al, 2000; Kaplan and Miller, 2001). Considering the multiple environmental cues (substrate, growth factors, cytokines, etc.) that govern neurite growth and the coordinated activity required, it seems reasonable that just such a synergism between two of these factors (NGF and IGF), and possibly more, exists.

The mechanism underlying this growth factor synergism could be at many levels. For one, Schwann cell motility and function is activated by IGF and is also a likely component of the regenerative response (Cheng et al, 2000). However, in our cultures the percentage of Schwann cells did not appear to differ with any of the growth factor treatments. As well, one of IGF's diverse effects is to increase gene expression. IGF could then potentially elevate TrkA expression and thereby increase NGF-TrkA signaling, or act to increase the levels of intermediates acting on theTrk-signaling cascade. However we do not see any increase in the amount of receptor or receptor activation at any time point (data not shown for 24h and 48h). Considering that both NGF and IGF activate similar signaling pathways we hypothesized that some kind of signal strengthening may underlie the observed N+I growth synergism. Both NGF (Kaplan and Miller, 2001) and IGF (Feldman et al, 1997) activate PI3-K-Akt and ras-MAPK intermediate signaling pathways. For this signaling convergence to occur both growth factor receptors would have to be concurrently expressed. Fluorescence double labeling for TrkA and IGFR-1 revealed that a substantial portion of the cells in our cultures dually express these receptors thus making signaling convergence at least

plausible. As well the addition of IGF to NGF environment did not alter the percentage of neurons responding to NGF (see Fig 3.14A), suggesting that there is not a separate population of neurons responding to the presence of IGF; if there were two distinct populations, we would have expected to see an additive effect on the percentage of cells with neurites.

Our acute stimulation experiments confirmed this and revealed that NGF and N+I environments elicit similar activation of MAPK and Akt. When neurons are exposed to chronic growth factor environments (24h and 48h), paralleling our growth data, the activation of Akt and phosphorylation of one of its key downstream substrates, GSK-3, is further enhanced in the N+I environment more so than in the presence of NGF. This activation pattern begins to show at 24h and fully develops at 48h, paralleling the time frame of the N+I synergistic growth pattern. It is well established that both NGF and IGF phosphorylate GSK, and Akt seems to be the primary mechanism involved though many more exist (for review see Grimes and Jope, 2001a). The binding of NGF to TrKA and IGF to IGFR-1 recruits intracellular proteins creating a scaffold. Adapter proteins such as GAB bind to TrkA and IRS to IGFR-1 (Feldman et al, 1997; Kaplan and Miller, 2000). Since PI3-K binds both of these adapter proteins it seems reasonable that simply more Akt could be recruited and thereby activated when both of these receptor scaffolds are created. Such a situation would occur in the presence of N+I and could explain the heightened signaling through the PI3-K>Akt>GSK signaling arm. Phosphorylation of GSK at serine 9, the site detected in our study, actually leads to inactivation of GSK, and it has been speculated that many of the downstream substrates of GSK are constituitively inhibited by GSK and only released from this inhibition upon GSK phosphorylation.

The relative importance of the major growth factor pathways in adult sensory neurons is not clear (Atwal et al, 2000; Liu and Snider, 2001). However, we see a stronger association between Akt activity and subsequent GSK inactivation and adult sensory neuron branching than MAPK signaling. Our results could also be interpreted to point out that both pathways are needed for growth. Not only do others support the notion that both major signaling pathways are involved in growth but also that these pathways are active in condition(s) where most growth is occurring.

3.3.2. Confirmation of the inhibitor specificity reveals novel interactions between two major growth factor signaling pathways

We used pharmacological inhibitors to ablate the activity of key signaling proteins to further validate the importance of PI3-K signaling in adult sensory neurite branching. Since the inhibitors block the activity of signaling proteins also involved in adult neuronal survival, I used low concentrations of the inhibitors that have been reported not to affect survival (Dodge et al, 2002). K252a (200nm) selectively blocks TrkA activity and also almost completely blocks neurite growth even in the N+I condition. The influence of N+I on branching is similarly attenuated upon addition of both LY294002 and LiCl, but seems to be less drastically affected by U0126. Measuring the total neurite length of these cultures is supportive of this observation, showing that blocking either PI 3-K→Akt (with LY294002) or GSK (with LiCl) attenuates the N+I induced branching. MEK→ MAPK inhibition had little detectable effect on N+I branching. Although our inhibitor studies data are consistent with others (Liu and Snider, 2001) in that the PI3-K-Akt-GSK signaling is of prime importance in growth, it is in

contrast to others that have suggested the importance of MAPK in growth, and this may relate either to the cell type or the developmental age (Atwal et al, 2000; Kaplan and Miller, 2000).

The specificity of some of the inhibitors used in this study has been recently questioned (Davies et al, 2000). We show here that the inhibitor concentrations used blocked the phosphorylation of the appropriate kinases, and also suggests a novel link between the two major growth factor activated signaling pathways (PI3-K-Akt-GSK and ras-MAPK). As in other cellular contexts, LiCl does lead to the phosphorylation GSK (Stambolic et al, 1996). More specifically, our blots consistently reveal that blocking GSK activity leads to the phosphorylation of MAPK and that blocking MAPK leads to phosphorylation of Akt. Both observations could be explained by the non-specificity of inhibitors but this is not likely considering that the concentrations used have been shown to specifically block the intended kinases. Furthermore blocking MAPK has been shown to activate Akt in other culture systems (Shaw and Cohen, 1999), suggesting that inter-pathway signaling is plausible. These interactions could be due to either some activation or inhibition of upstream intermediates, or alternatively, rather than assuming that the increased phosphorylation is due to kinase activity, it is also quite possible that the effect is mediated by modulation of phosphatase activity. Signaling interaction has been speculated in most systems and thought to involve checks (inhibition and stimulation) that regulate the likely signaling balance underlying complex morphological events such as neurite growth. The functional significance of these previously unobserved links in the signaling network to underlying growth is not yet clear.

3.3.3. Identifying the phosphorylation state of potential down stream growth factor effector proteins

The signaling proteins described previously are essentially intermediates which connect growth factor receptor activation and subsequent signaling intermediate activation to downstream effector proteins that ultimately influence neurite morphology. Two well established effector proteins of growth factor responses are the transcription factor CREB (Shaywitz and Greenberg, 1999) and the microtubule associated protein tau (Billingsley and Kincaid, 1998; Mandelkow et al, 1995). Although the intracellular function of both these proteins are complex and not likely to be completely controlled by single site phosphorylation, we assessed their phosphorylation profiles at 24h and 48h in the various growth factor environments to gain an understanding of their possible activity during neurite growth. Phosphorylation of tau plays a key role in axonal growth and numerous kinases, including both GSK-3 and MAPK, can phosphosphorylate tau and thereby regulate its binding to microtubule (Billingsley and Kincaid, 1998; Mandelkow et al, 1995; Takahashi et al, 1999; Reynolds et al, 2000). In the presence of N+I, when neurite branching is maximal, phosphorylation of tau at ser396, but not ser199 is much higher than with the other growth factors alone. With many more neurites being laid down in the N+I environment perhaps this elevated tau phosphorylation is required to balance the ratio of stable:unstable microtubules in the extending neurites and permit further branching from the primary neurites, or to maintain the integrity of the neurites already present.

With respect to CREB phosphorylation, in other cellular systems NGF leads to the phosphorylation of CREB at ser133 that is required for the CREB-mediated

immediate early gene activation (Shaywitz and Greenberg, 1999). One route of phosphorylation at ser133 is by MAPK via ribosomal S6 kinases (Xing et al, 1998). However it appears that CREB is only fully active upon phosphorylation at ser129, accomplished processively by such kinases as GSK3, (Fiol et al, 1994). Although this phosphorylation hierarchy may exist in neurons, it was recently demonstrated that GSK inhibits CREB transactivation activity in neuroblastoma cells, and that inhibition of GSK with lithium or via Akt activation results in increased CREB activation (Grimes and Jope, 2001b). Therefore CREB is perhaps maximally active in the N+I environment where phosphorylation at ser133 by ERK is sustained and the inhibitory influence of GSK is minimized. This may be reflected in our Western blotting by less phosphorylated CREB due to consumption of the activated transcription factor or its binding to DNA both of which could lead to less pCREB detectable on our blots.

It remains difficult to place importance in neurite growth upon any one signaling pathway over another. It is speculated by many, and suggested in this study, that these pathways clearly do not exist in isolation. Furthermore, there are other growth factor-dependent and -independent pathways (intracellular Ca^{2+} levels, cAMP-PKA and PLC-γ-PKC) that have been shown to influence growth factor signaling. The data presented here points to the intriguing possibility that interpathway "crosstalk" does exist and may play an important role in the fine-tuning of cellular response to trophic factors and maintaining a delicate balance between events required for neurite growth.

3.4. Figures

Figure 3.7: The presence of both NGF and IGF-1 elicits a more elaborate neurite growth pattern than either growth factor alone. Representative micrographs of adult DRG neurons are shown; neurons were fixed with 4% formaldehyde and immunostained using an anti-peripherin antibody in conjunction with a Cy3-conjugated secondary antibody. **A**, control cultures not receiving any growth factors do not demonstrate much neurite growth; **B** & **C**, neurons maintained in 25 ng/ml of either NGF **(B)** or IGF-1 **(C)** extend more diffuse neurites. **D**, in the presence of both NGF and IGF-1, the neurites are longer and the network pattern is more elaborate. Scale bar, 50 um.

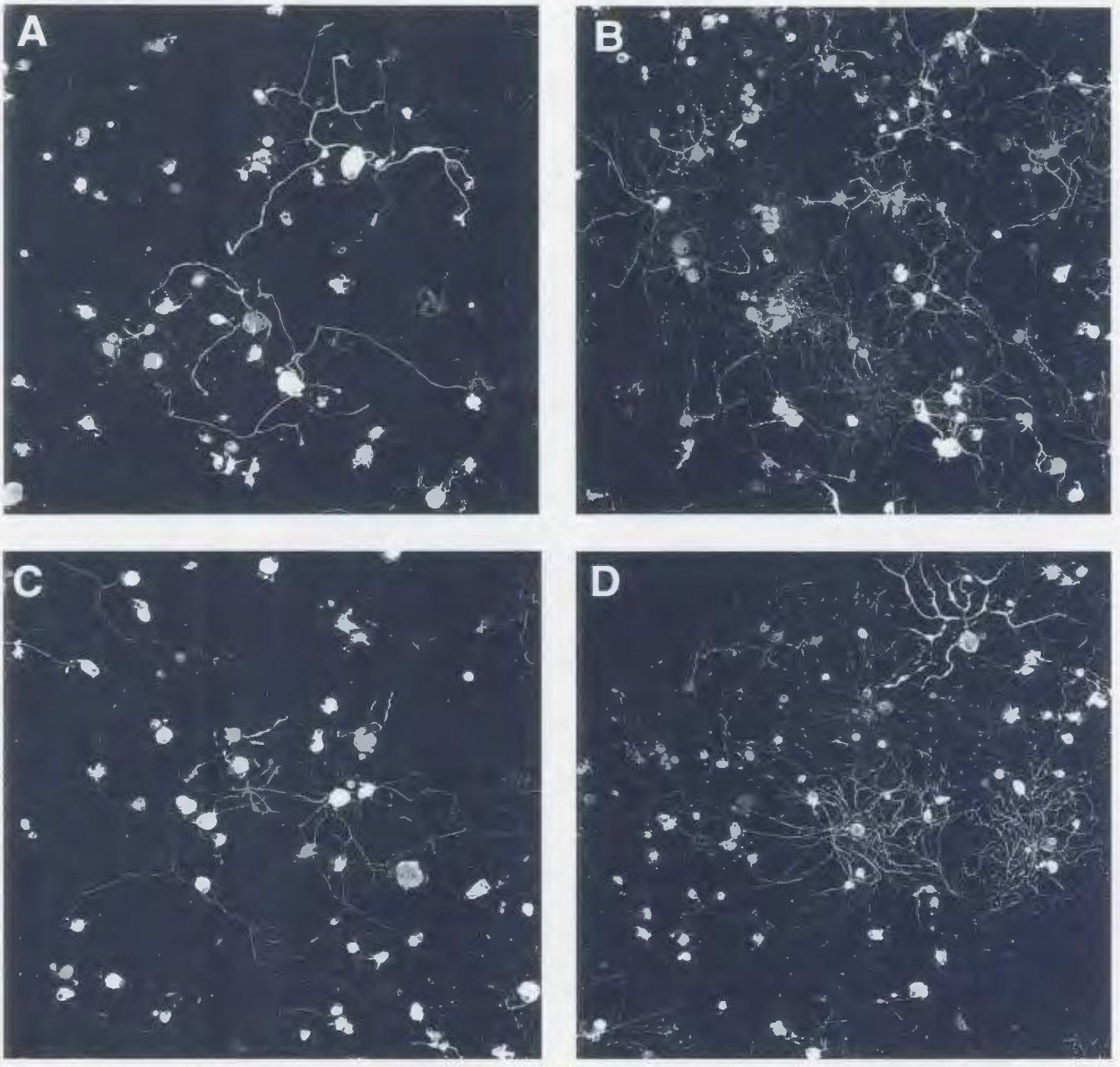

A
B
C
D

Figure 3.8: NGF and IGF-1 work synergistically to evoke maximal neurite growth. Dissociated mass adult DRG neuron cultures received either no growth factor (control), 25 ng/ml of NGF, 25 ng/ml of IGF-1, or 25 ng/ml of NGF and 25 ng/ml of IGF-1 upon plating. Cultures were maintained for 48hrs at which time various neurite growth parameters were measured. **A**, neurite initiation was assessed by counting the number of phase bright neurons that extend neurites twice the length of their cell body compared to the total number of neurons plated. Values represent 4 separate experiments with duplicates of at least 100 neurons. **B-D**, Sholl analysis data. Neurons were fixed and visualized with immunofluorescence or DAB detection of peripherin and subsequently traced using the Neurolucida software program. Data analyses of the tracings show the total neurite length and Sholl analysis. The total neurite length is plotted as the mean $\pm$ SEM in **(B)**, while the Sholl analysis data are separated into intersection points **(C)** and neurite length **(D)**. Significance noted (*) for $p < 0.05$, (**) for $p<0.001$.

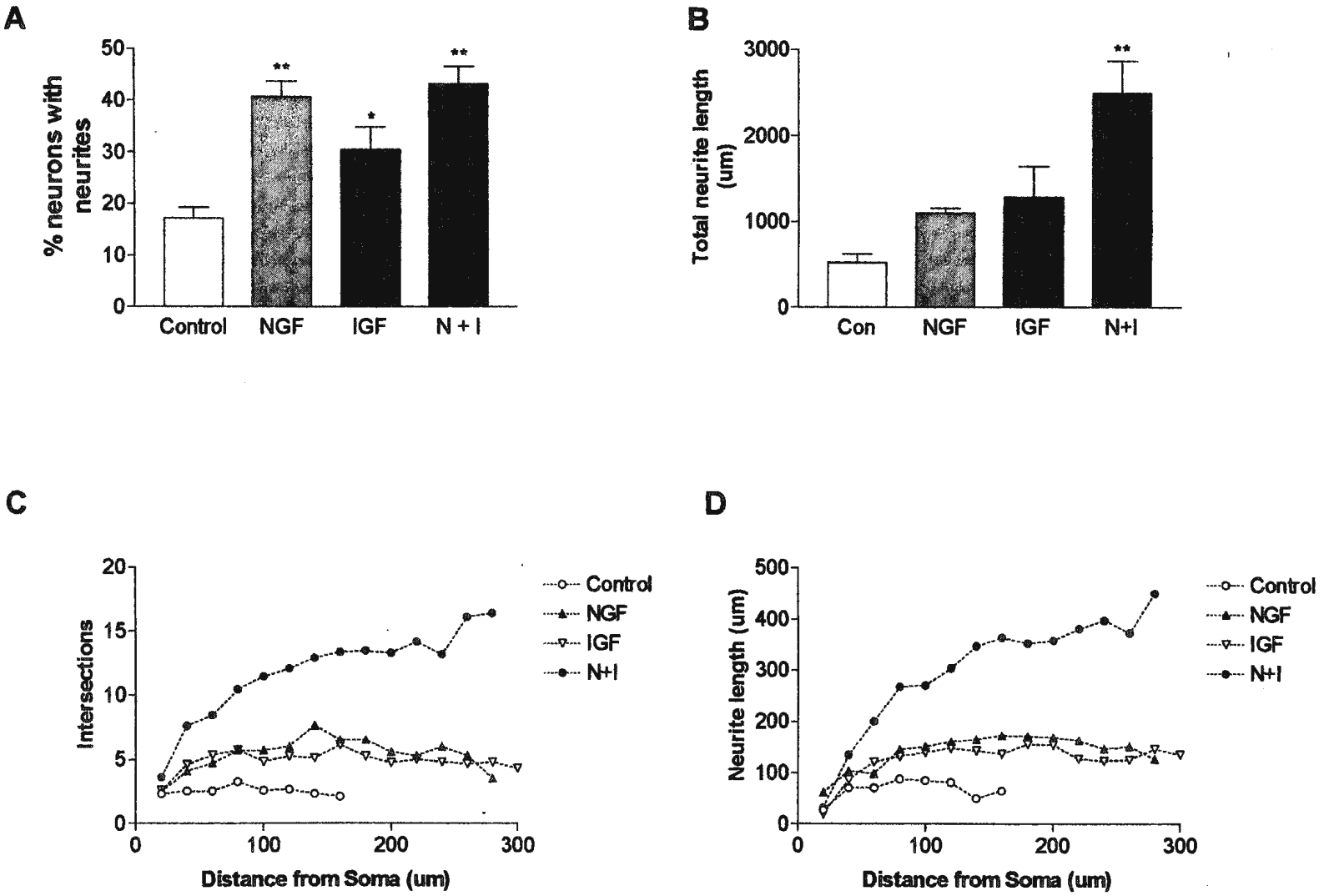

A
% neurons with neurites
50
40
30
20
10
0
**
*
**
Control
NGF
IGF
N + I
B
Total neurite length (um)
3000
2000
1000
0
**
Con
NGF
IGF
N+I
C
Intersections
20
15
10
5
0
0
100
200
300
Distance from Soma (um)
Control
NGF
IGF
N+I
D
Neurite length (um)
500
400
300
200
100
0
0
100
200
300
Distance from Soma (um)
Control
NGF
IGF
N+I

Figure 3.9: Signaling proteins activated by short-term stimulation with NGF and IGF-1. Lysates were prepared as described in Materials and Methods from cultures stimulated by various growth factor conditions for the indicated times (30', and 1h). Lysates were then subjected to standard Western blotting with phosphospecific antibodies, followed by activation state independent antibodies. **A & B**, blots from neurons stimulated for 30' **(A)** or 1h **(B)** were cut and initially incubated with an anti-phosphotyrosine antibody (4G10). The same blots were then sequentially stripped and probed for both TrkA and IGF-1R so that the phosphorylated forms could be compared to the total amount of protein. Alignment of the phosphotyrosine blot with the TrkA and IGF-1R blots confirmed that the top band (top arrow) is the phosphorylated form of IGF-1R, and the bottom band (bottom arrow) the phosphorylated form of TrkA. Akt and MAPK activation were assessed by comparing blots using activity dependent antibodies against Akt and MAPK with blots using antibodies directed against total Akt and MAPK protein. Blots shown are representative of several experiments (n=3-5).

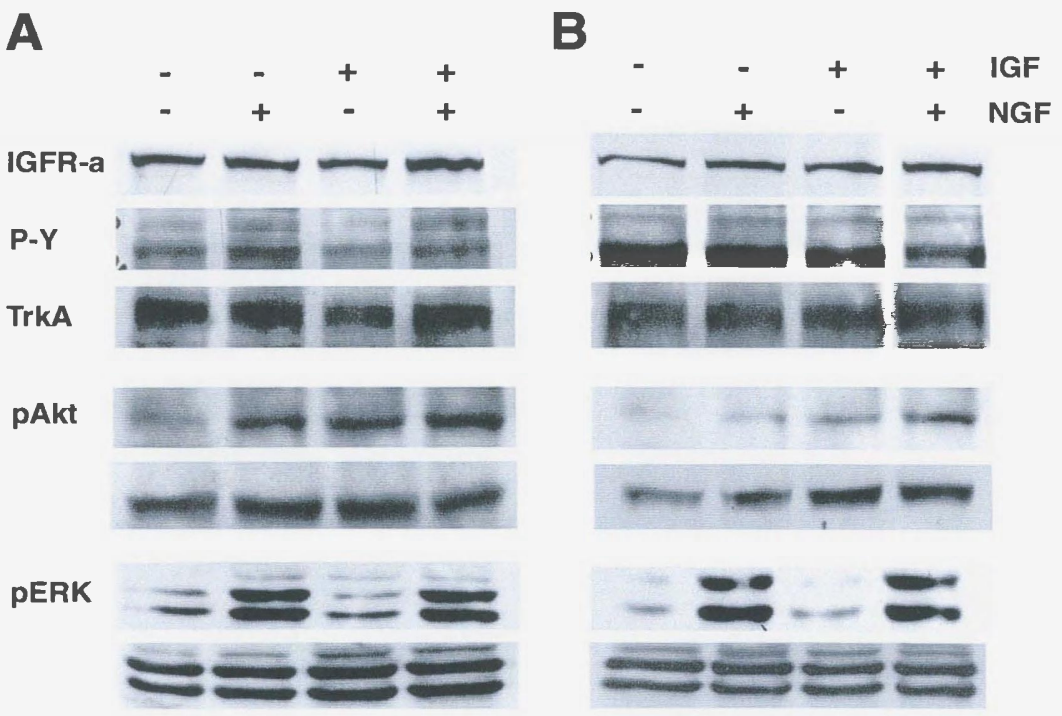

A
B
- - + +
- + - +
IGF
NGF
IGFR-a
P-Y
TrkA
pAkt
pERK

Figure 3.10: Profile of growth factor signaling intermediates following chronic exposure to the various growth factor environments. Neurons were exposed to the indicated growth factor environments upon plating and maintained for 6h **(A)**, 12h **(B)**, and 24h **(C)**. Lysates were prepared and subjected to standard Western blotting procedures as before. The activation states of Akt, GSK3α/β, and MAPK (ERK-1/2) were assessed by probing with activity dependent antibodies and comparing these levels to the amount of total signaling protein using non-activity dependent antibodies. The same blots were probed and sequentially stripped. Blots are representative of several experiments (n=3-5).

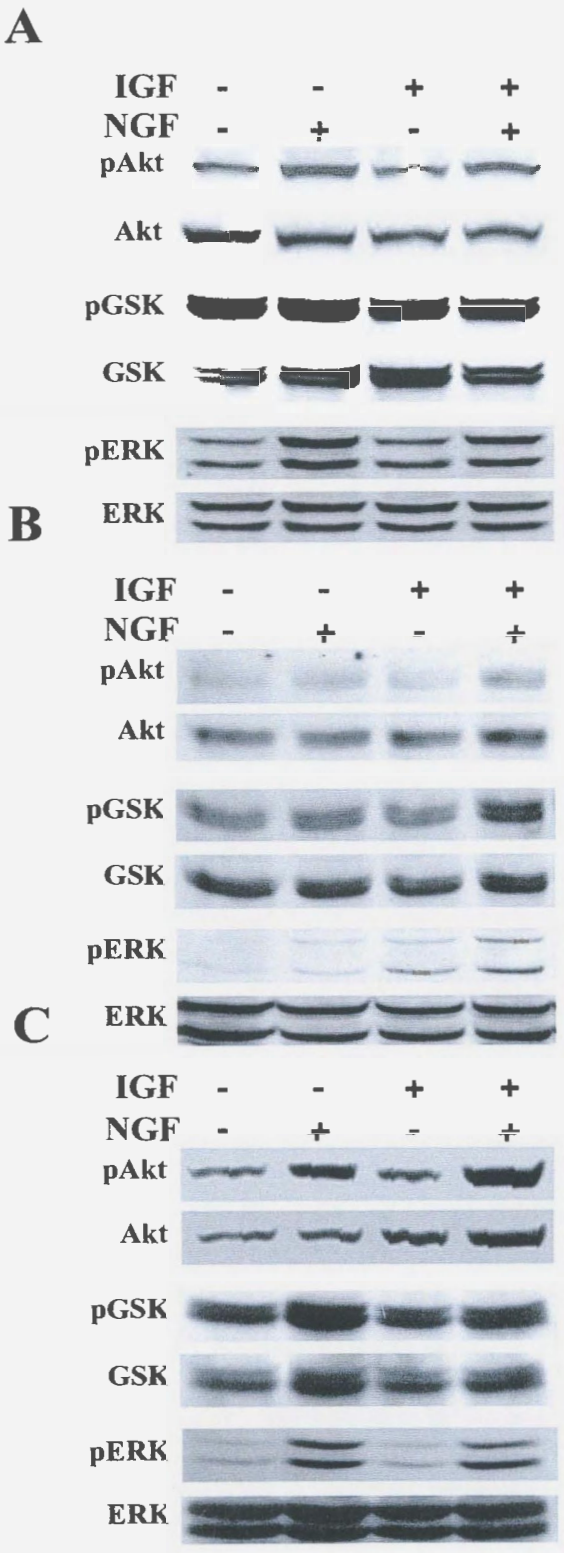

A
IGF - - + +
NGF - + - +
pAkt
Akt
pGSK
GSK
pERK
ERK
B
IGF - - + +
NGF - + - +
pAkt
Akt
pGSK
GSK
pERK
ERK
C
IGF - - + +
NGF - + - +
pAkt
Akt
pGSK
GSK
pERK
ERK

Figure 3.11: Inhibiting different signaling proteins leads to different neurite growth patterns. Adult DRG cultures were prepared as before and plated within N+I. Following 2-3h of incubation the indicated concentrations of pharmacological inhibitors were added to the cultures. At 24h post plating neurons were fixed and processed for immunofluorescent staining of peripherin as described previously. **A**, selectively blocking tyrosine kinase activity with 200nM K252a essentially blocks all extensive growth normally seen in N+I (see Fig. 1D). **B & C**, representative micrographs of N+I cultures grown with PI 3-K inhibition (10 uM LY294002) and GSK3α/β inhibition (1mM LiCl) show limited neurite length and branching. **D**, blocking MEK activity (10 uM UO126) minimally obstructs neurite length and branching. Scale bar, 50 um.

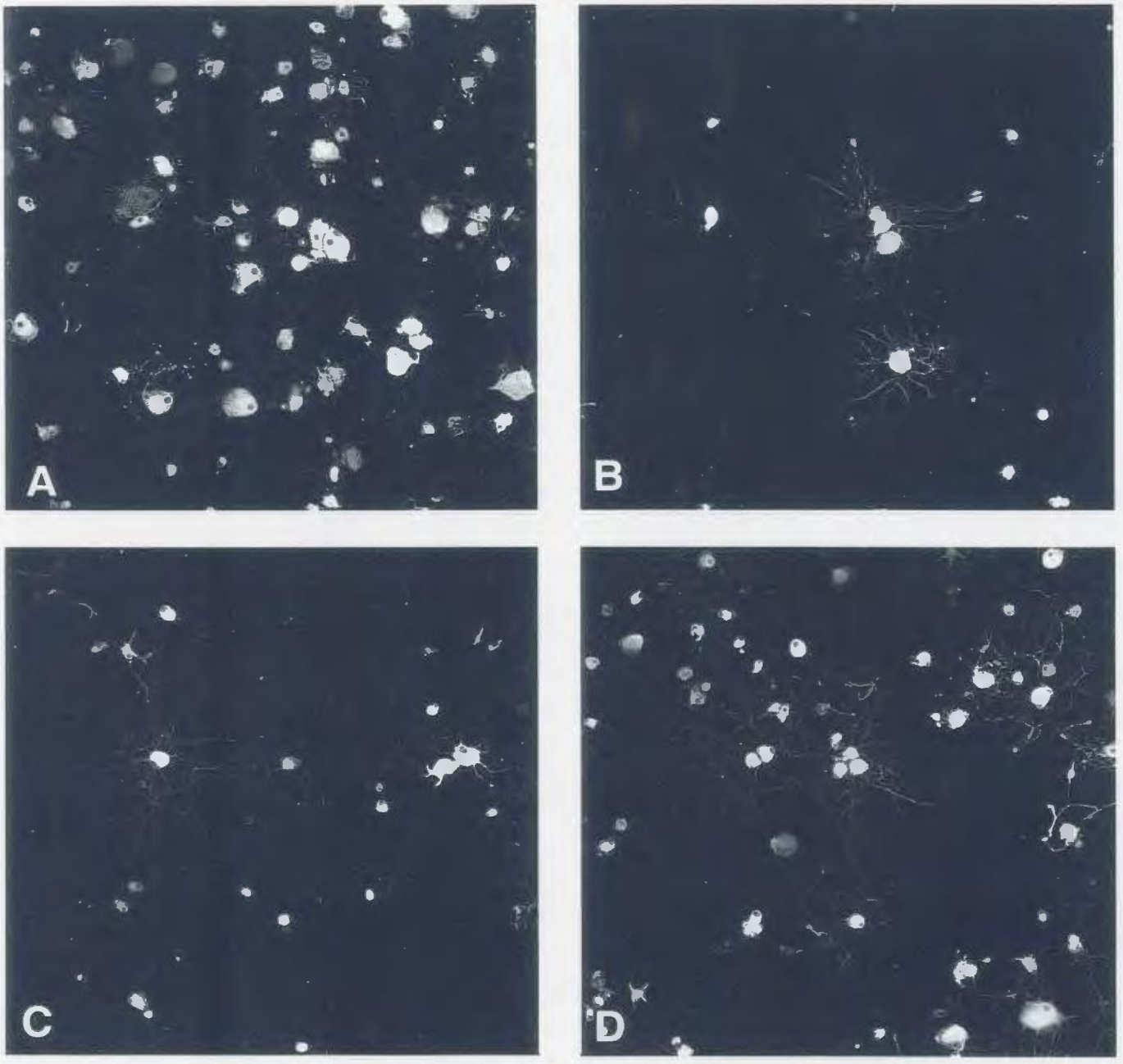

A
B
C
D

Figure 3.12: Blocking signaling intermediates leads to less growth factor-dependent neuritogenesis. Cultures were treated as described in Fig. 4 and the percentage of neurons with neurites twice the diameter of the cell body were counted at 48h post-plating. The effects of inhibitors on control **(A)** NGF **(B)**, IGF **(C)** and N+I **(D)** dependent growth was investigated. Inhibiting either PI3-K (10uM LY294002) or GSK3α/β (1mM LiCl) results in decreased neurite initiation and inhibiting MEK (10uM UO126) has less effect. Data represent the mean ± SEM for three separate experiments with duplicate cultures in each experiment and at least 100 cells counted per duplicate. Significance was tested using one-way ANOVA with Tukey post-hoc testing; *- $p<0.05$; **- $p<0.001$.

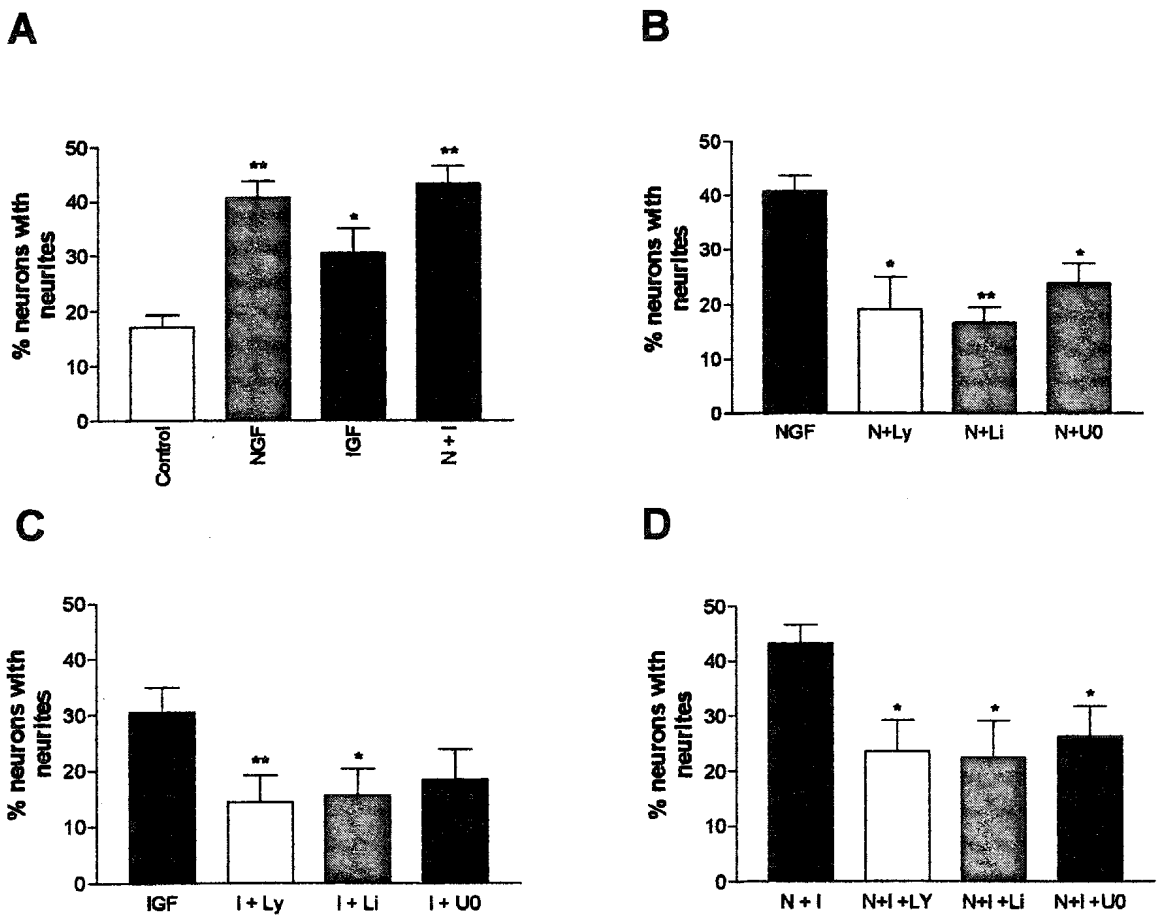

A
% neurons with neurites
50
40
30
20
10
0
**
*
**
Control
NGF
IGF
N + I
B
% neurons with neurites
50
40
30
20
10
0
*
**
*
NGF
N+Ly
N+Li
N+U0
C
% neurons with neurites
50
40
30
20
10
0
**
*
IGF
I + Ly
I + Li
I + U0
D
% neurons with neurites
50
40
30
20
10
0
*
*
*
N + I
N+I +LY
N+I +Li
N+I +U0

Figure 3.13: Disruption of PI 3-K or GSK3 activity provides the most effective inhibition of neurite length and branching. Cultures were exposed to LY294002, UO126, or LiCl as in Fig. 4, fixed and immunostained with anti-peripherin at 48h. Growth was assessed using the Neurolucida program neuron tracing program to examine the total neurite length (histogram) and Sholl analysis (line plot) from neurites promoted by NGF **(A)**, IGF **(B)** and N+I **(C)**. Blocking PI 3-K and GSK3α/β activity diminishes total neurite length and the number of intersection points in Sholl analysis more so than blocking the activity of MAPK. Data are from tracings of 15-20 cells from 3 experiments and are the length ± SEM or number of intersection points in 20 um radial increments from the soma. Significance was tested using one-way ANOVA with Tukey post-hoc testing; *- $p<0.05$; **- $p<0.001$.

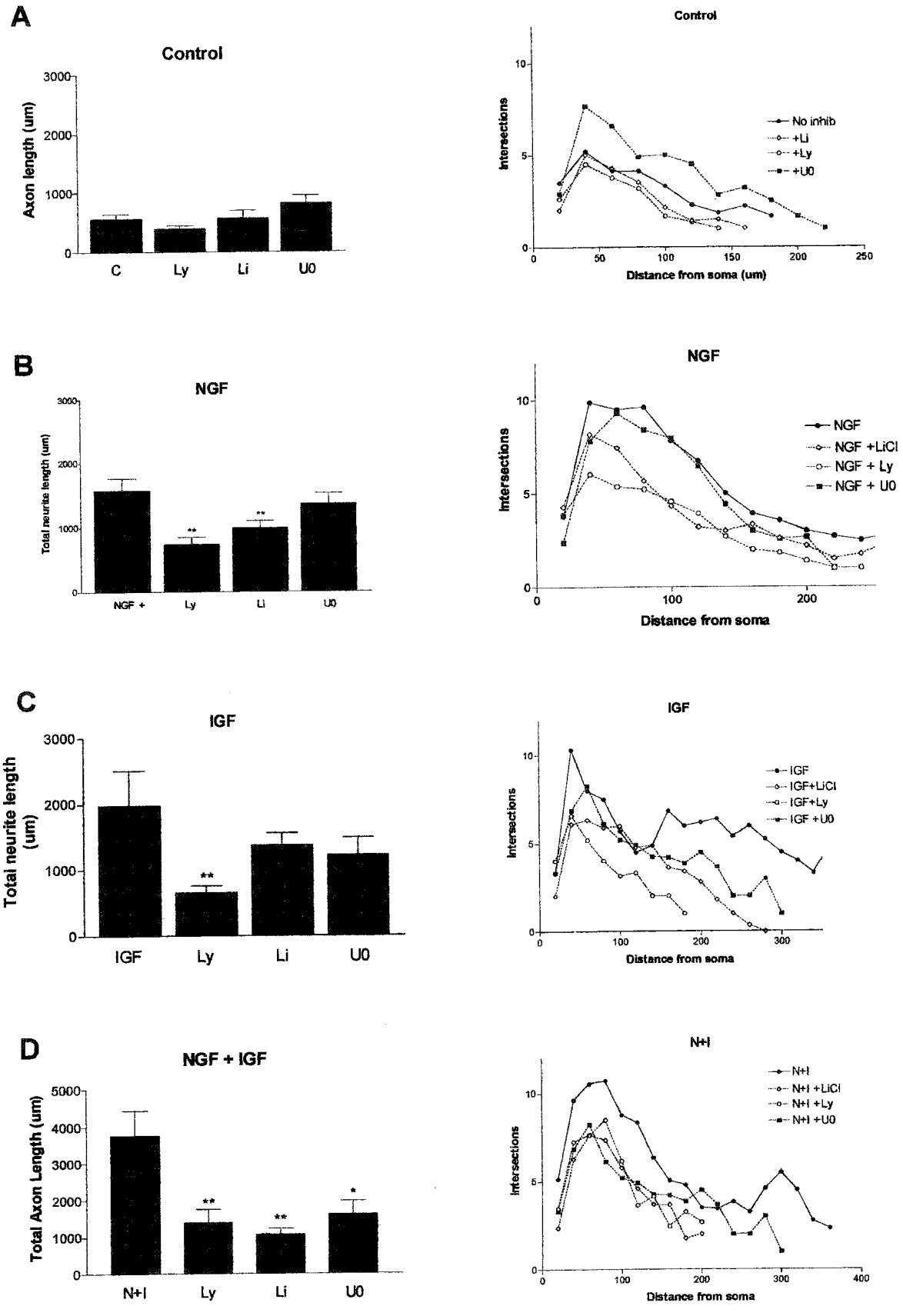

A
Control
Axon length (um)
3000
2000
1000
0
C
Ly
Li
U0
Control
Intersections
10
5
0
0
50
100
150
200
250
Distance from soma (um)
No inhib
+Li
+Ly
+U0
B
NGF
Total neurite length (um)
3000
2000
1000
0
**
**
NGF +
Ly
Li
U0
NGF
Intersections
10
5
0
0
100
200
Distance from soma
NGF
NGF +LiCl
NGF + Ly
NGF + U0
C
IGF
Total neurite length (um)
3000
2000
1000
0
**
IGF
Ly
Li
U0
IGF
Intersections
10
5
0
0
100
200
300
Distance from soma
IGF
IGF+LiCl
IGF+Ly
IGF +U0
D
NGF + IGF
Total Axon Length (um)
5000
4000
3000
2000
1000
0
**
**
*
N+I
Ly
Li
U0
N+I
Intersections
10
5
0
0
100
200
300
400
Distance from soma
N+I
N+I +LiCl
N+I +Ly
N+I +U0

Figure 3.14: Pharmacological inhibitors have the expected effects, but also reveal unexpected interpathway signaling. Immunoblotting was carried out on lysates prepared from cultures treated with the indicated inhibitors and growth factors. The effect of the inhibitors on the phosphorylation state of Akt, GSK3α/β, and MAPK (ERK) was assessed in control **(A)**, NGF **(B)**, IGF **(C)** and N+I **(D)** treated cultures sampled after 24 hrs of treatment. Note that inhibitors block the activity of the expected proteins: that is, LY294002 decreases Akt phosphorylation due to inhibition of PI 3-K, LiCl increases GSK3α/β phosphorylation, and UO126 blocks MAPK phosphorylation. In addition, LiCl results in increased phosphorylation of MAPK and UO126 results in an increased phosphorylation of Akt. These blots are representative of experiments repeated in triplicate.

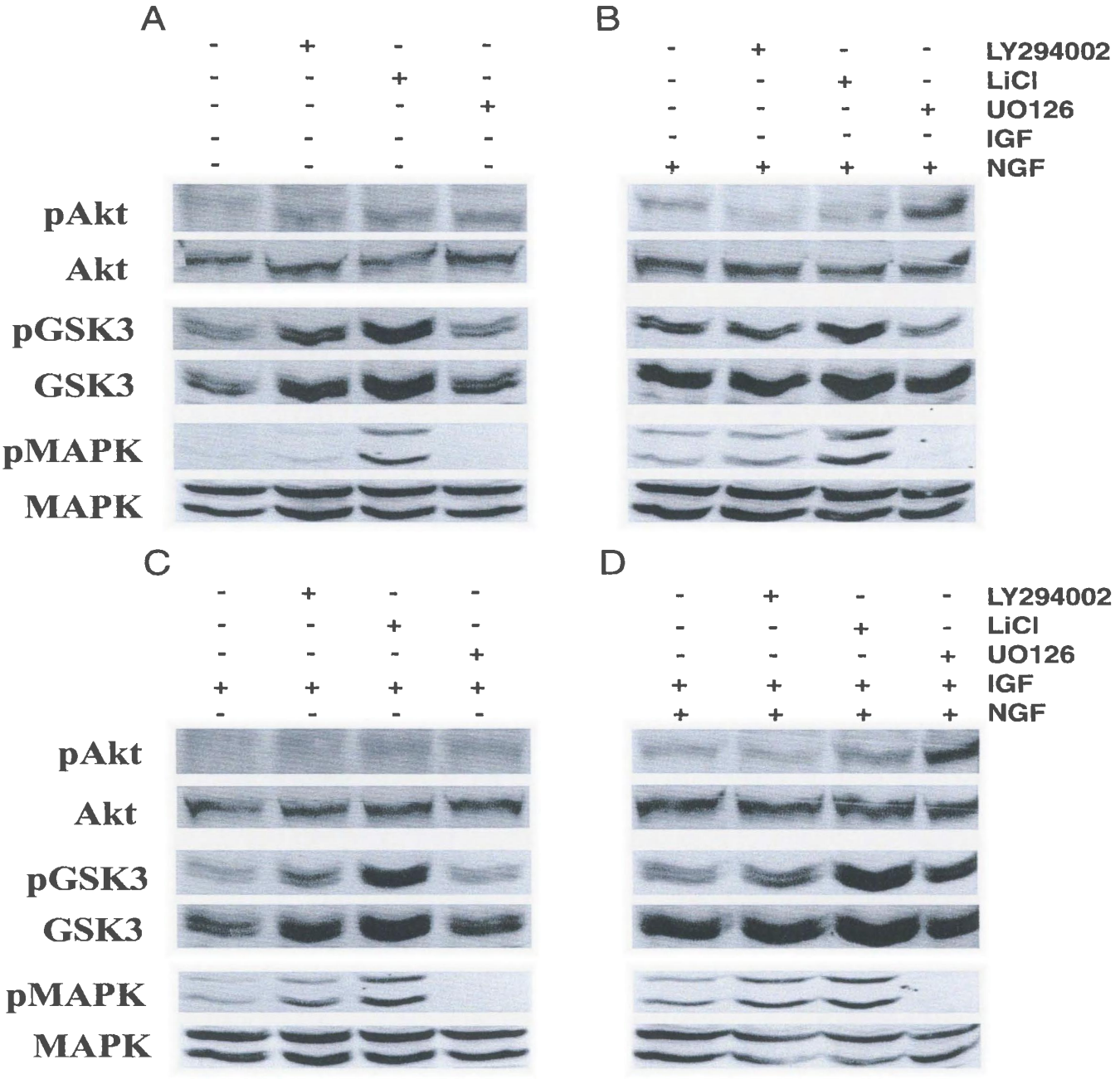

A
B
C
D
LY294002
LiCl
UO126
IGF
NGF
pAkt
Akt
pGSK3
GSK3
pMAPK
MAPK

Figure 3.15: Tau is phosphorylated in the presence of growth factors. Tau is a microtubule associated protein that has been shown to play a role in process outgrowth, and we have examined the phosphorylation of tau at 2 sites known to be targets of both GSK3 and MAPK. Immunoblotting with phospho-specific antibodies directed against serines 199 and 396 of tau was carried out. **A & B**, Adult DRG neurons were exposed to the indicated growth factor environments for either 24hr **(A)** and 48hr **(B)**; there is an increase in the tau phosphorylation in the presence of the growth factors compared to controls. **C-F**, Neurons were exposed to either no growth factor **(C)** or NGF **(D)**, IGF **(E)** or NGF+IGF **(F)** in the presence of the indicated inhibitors. Cultures were sampled at 24 hrs after plating. These blots are representative of experiments repeated in triplicate.

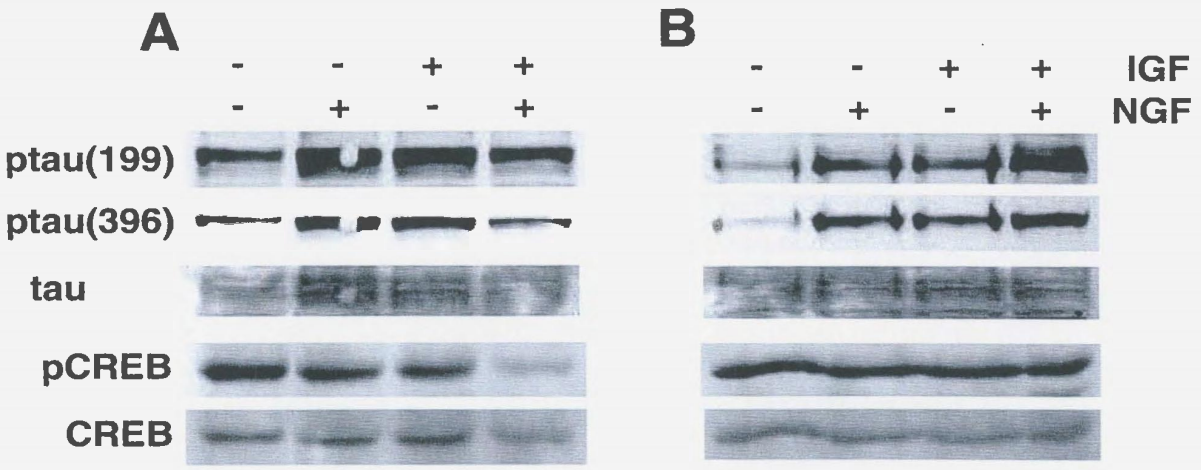
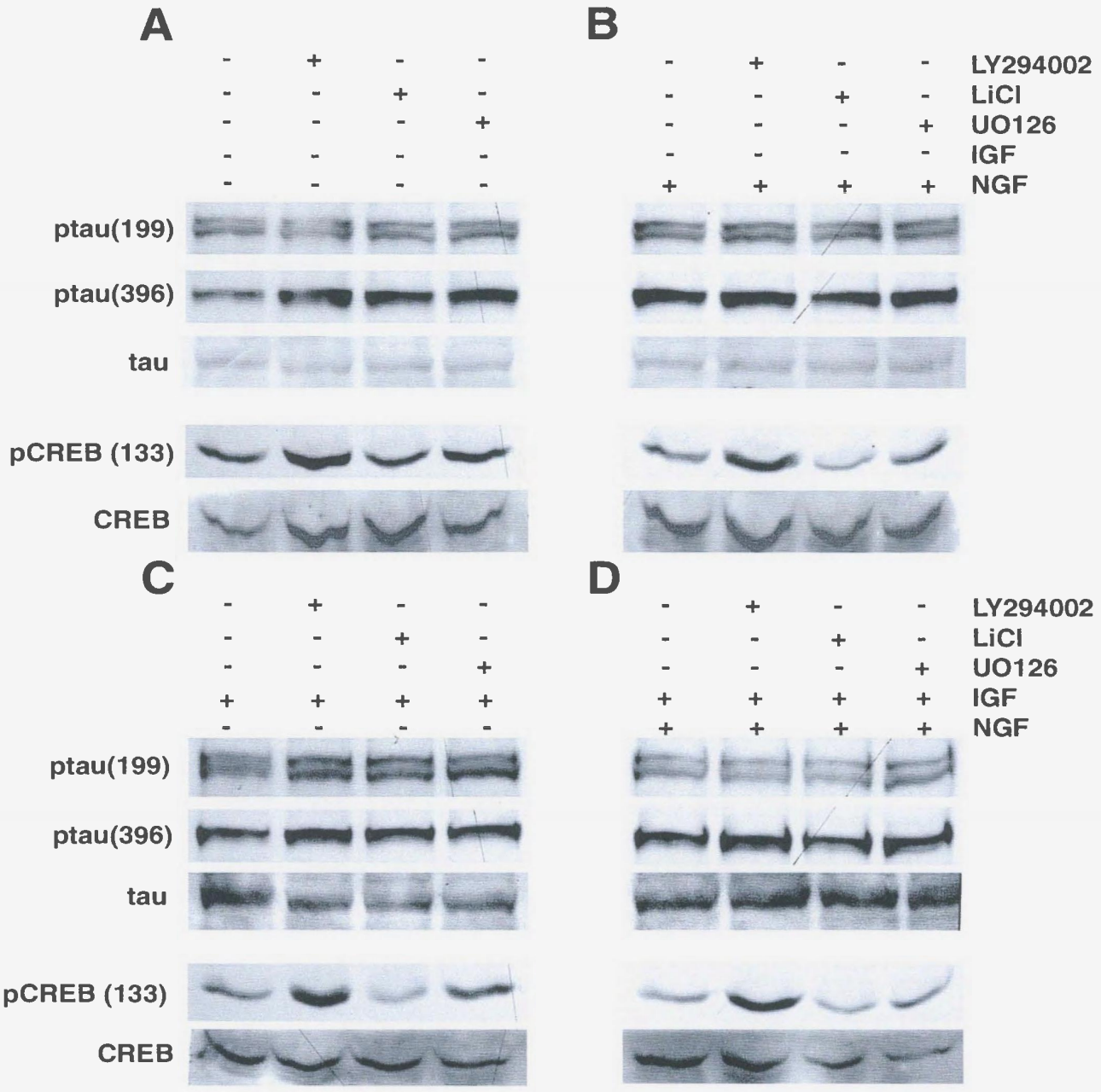

A
B
- - + +
- + - +
IGF
NGF
ptau(199)
ptau(396)
tau
pCREB
CREB

Figure 3.16: Effects of inhibiting upstream growth factor activated intermediates on the phosphorylation of the potential downstream effectors tau and CREB. Immunoblotting of the same samples were carried out as in Fig. 8. Blots represent the effect of inhibitors on the phosphorylation of tau at serine 199 and 396 as well as on serine 133 of CREB in cultures grown for 24h in control **(A)**, NGF **(B)**, IGF **(C)**, and N+I **(D)** cultures. Blocking the activity of GSK leads to less phosphorylation of tau at serine 396, but not serine 199. While blocking Akt activity enhances growth factor phosphorylation of CREB. This is a representative blot of at least 3 separate platings of the same experiment using the same samples as in Fig. 4 and 8.


A
B
C
D
LY294002
LiCl
UO126
IGF
NGF
ptau(199)
ptau(396)
tau
pCREB (133)
CREB

CHAPTER IV: INTEGRATION OF NGF AND INTEGRIN SIGNALING

4.1. Introduction

4.1.1. ECM and Growth

Like nearly every cell in the body, neurons are surrounded by a complex extracellular matrix (ECM) which they adhere to and are profoundly influenced by throughout their development and maturation. These crucial neuron-ECM interactions regulate processes such as neuronal migration during development in addition to survival, differentiation, and neurite outgrowth in adulthood. The major adhesive proteins found within the peripheral nervous system ECM are collagen, fibronectin, proteglycans, and laminin (for review see Previtali et al, 2001). Cell surface glycoprotein receptors called integrins mediate much of the neuron-ECM interaction, and the ECM constituent with the greatest influence in the peripheral nervous systems (PNS) seems to be laminin (McKerracher et al, 1996). The presence of laminin in the PNS and minimal amount in the CNS in the adult has been suggested to be partly responsible for more successful regeneration in the PNS (David et al, 1995; Reiner, 1995).

The ECM is intimately involved in a diverse array of neuronal processes throughout the life of a neuron. During the development of the PNS, the ECM-integrin interaction participates by regulating Schwann cells polarity and migration ultimately leading to mylein formation upon developing neurites (Previtali et al, 2001). This process is crucial in the establishment of peripheral nerves. Developing neurites are drawn along a specific pathway through the selective expression of laminin and subsequent adherence via surface integrins (Mckerecker et al, 1996; Previtali et al, 2001). In the adult PNS,

integrins not only influence but may actually determine neurite regeneration. It was recently demonstrated that transgenic expression of integrins in adult DRG to levels observed in growing embryonic DRG neurons yields not only longer neurites, but also more neurites per neuron (Condic, 2001). Similarly, integrin deficient mice failed to reinnervate the whiskerpad following facial nerve crush *in vivo* (Werner et al, 2000). Although laminin and integrins are clearly involved in peripheral nerve regeneration the exact mechanism remains unclear.

4.1.2. Integrin Activation and Effector Signaling

The neuronal response to ECM-integrin engagement is accomplished by 1) direct cellular physical adhesion to the ECM and 2) modulation of intracellular activities such as signalling pathways and cytoskeletal reorganization. This dual influence over intracellular activity translates into a profound effect on neuronal behaviour. There have been some 22 different integrins described, each a heterodimer comprised of various possible combinations of 17 α and 8 β subunits (Kumar, 1998). In the PNS, integrin receptors mainly consist of a $\beta1$ subunit paired with different α subunits. The major laminin receptors for sensory neurons are $\alpha1\beta1$ and $\alpha3\beta1$ (Tomaselli et al, 1993). However, integrin subunit expression is specific to the neuronal subtype and distinct functions have been ascribed to different subtypes. The $\beta1$ subunit has the additional complexity of having 4 different spliced variants (β_{1A}, β_{1B}, β_{1C}, β_{1D}) that differ at the cytoplasmic domain (Fornaro et al, 2000). This domain is responsible for much of the intracellular function of integrins and as such each of these spliced variants appear to have some unique functions.

Upon laminin-integrin binding, integrins aggregate, phosphorylate and attract specific proteins to eventually form a local signaling unit called a point contact (Kumar, 1998; Giancotti and Ruoslahti, 1999). Point contacts have been suggested to be analogous to focal contacts in other cells, areas are believed to represent the signaling foci whereby the ECM message is transmitted through integrins to affect the intracellular signaling (Renaudin et al, 1999). During growth cone progression, point contacts provide the link by which the ECM can influence neurite morphology by altering cytoskeletal dynamics (Renaudin et al, 1999). They may also act as focal adhesions formed during cell spreading in motile cells that provide physical stability necessary for migration (Van Veen and Van Pelt, 1994). In this way, the ECM can influence lamellipodia and filiopodia guidance and provide stability for elongating neurites previously laid down by strengthening the ECM-integrin bond. Members of the rho-family of small guanine nucleotide-binding proteins Cdc42, Rac and Rho are thought to be important in this process (Giancotti and Ruoslahti, 1999). Therefore, integrins likely provide physical and signaling support during neurite growth.

Much of integrin intracellular physiology first involves association with intracellular proteins followed by the activation of specific signaling pathways. Extracellular integrin occupancy has been called *In-trans* binding. This type of integrin binding activates two distinct signals 1) inside-out signals that modulate the strength of the ECM-integrin binding and 2) outside-in signals that activate intracellular signaling pathways responsible for many the processes regulated by integrin (Previtali et al, 2001).

Upon attachment to an extracellular substrate, integrins undergo many structural changes. These include phosphorylation, conformational change, and finally aggregation

of adjacent integrins. Though not yet clearly defined, all these changes affect the adherence ability of integrins (inside-out signals) and the subsequent activation of signaling proteins (outside-in signals). Upon activation, integrins provide membrane localized scaffolding for the recruitment and subsequent activation of specific integrin signalling proteins and activate the many different functioning proteins bound to integrins, for a cartoon version see Fig 1.4.

Two proteins activated upon integrin activation are focal adhesion kinase (FAK) and paxillin. Integrin physiology is controversial and differs depending on the cell type and context. A general agreement on integrin function in neurons has not yet been reached and many researchers attempt to draw parallels between cellular migration and neurite growth. Upon integrin binding, the c-terminal of β1 is phosphorylated and provides a binding site for FAK (Giancotti and Ruoslahti, 1999). This huge protein contains many domains that attract further proteins such as cytoskeletal proteins (talin, vinculin, paxillin), adapter proteins (Grb-SOS complex), and other signaling kinases (Src, PI3-K). Therefore, FAK represents an opportunity for integrin activity to alter many different neuronal functions. Many of substrates of FAK have been implicated in neurite growth, thus providing another means whereby integrin activation can modulate neurite growth. Another non-receptor tyrosine kinase related to FAK, Pyk2, also participates in integrin signalling (Ivankovic-Dikic et al, 2000). This kinase is structurally similar to FAK and is believed to share overlapping functions as well as having other unique signaling tasks. For example, Pyk2 can compensate for the absence of FAK to a degree, but in contrast to FAK it was found that Pyk2 is regulated by intracellular Ca^{+} ($[Ca^{+}]_i$) and activated by different stimuli (Schlaepfer et al, 1999)

Integrin stimulation also activates paxillin. This protein influences actin polymerization (Sattler et al., 2000; Kumar, 1998), a process necessary for neurite growth. Although controversial, the classic description of paxillin function includes attraction to point contacts via binding with FAK. Autophosphorylation of FAK at tyrosine 395 creates a Src homology 2 (SH2) domain thus permitting binding and activation of Src. Paxillin is a target of Src. However, others contend that integrin-linked kinase (ILK) provides the link between integrins and paxillin (Wu and Dedhar, 2001). However stimulated, once activated paxillin acts as an adapter protein to affect actin polymerization and regulate cytoskeletal organization during various cellular activities (Ivankovic-Dikic et al, 2000). This control over the cytoskeleton has made paxillin an attractive protein to study in integrin regulation of neurite growth. It should be apparent that integrin signaling can influence a variety of other signaling systems and potentially modulate many different cellular behaviors.

4.1.3. Physical Consequences of Integrin Activation in Neurite Growth

Neurotrophin function is similar to the activity of integrins discussed thus far; both direct growth cone guidance through its influence over the cytoskeleton and activate signaling proteins. Integrins exhibit fairly uniform expression within neuronal membranes but become more concentrated at the tips of growth cone filopodia upon exposure to NGF (Grabham and Goldberg, 1997). This observation lead to a model proposing that NGF and integrins work together to direct growth cone advancement (Grabham and Goldberg, 1997). In this model, integrins direct the pathfinding of neurite growth through their adhesion properties, essentially consisting of balancing of "pulling"

and "releasing" the growth cone. The integrins at the growing edge pull while those more proximal release. Inside-out signaling like participates in this process by modulating the local adhesive properties of integrins to strengthen the ECM connection at the growing tip. This represents yet another means by which integrins are involved in neurite growth.

4.1.4. Integrin Interaction with GF Physiology and Signaling

Integrins and growth factors also co-ordinately function through their respective signalling pathways. Not only are overlapping signalling pathways activated, but integrin signalling is suspected to intimately modulate growth factor signalling and fine tune the eventual message (Eliceiri, 2001; Kumar, 1998). This intimate relationship between integrin and growth factor signaling has been identified in many non-neuronal cells, and underlies such processes as cell migration and angiogenesis (Eliceiri, 2001).

Considering the role of growth factor signalling in neurite growth (see Ch. III), it is possible that integrins can also direct growth by influencing growth factor signalling. The two major growth factor pathways integrins are believed to govern are the PI3-K>Akt>GSK and the ras-Raf-ERK pathway. Although exactly how integrins lead to activation of these pathways is not fully understood, they are believed to be activated either directly or through one or all of the front running candidate mediators ILK (for review see Wu and Dedhar, 2001), FAK (Schlaepfer et al, 1999) or Pyk2 (Ivankovic-Dikic et al, 2000). Similar signalling intermediates are associated with integrin and growth factor-activated signalling proteins.

Though it is certain that integrins are involved in neurite growth, the exact mechanism is not known. It is of keen interest to further investigate this involvement to maximize the regeneration potential of neurites.

4.1.5. Hypothesis and Specific Aims

In these experiments I revisit the role of laminin in neurite growth and I hypothesize that laminin would provide a favorable substrate for neurite growth and that the growth effects of growth factors would be additive to that of laminin. In other words, I predict that there will be convergence on integrin activated with growth factor activated signalling proteins leading to increased neurite growth. One problem with most of the current data on the relationship between integrins and peripheral regeneration is that most experiments were performed in non-neuronal cells. All procedures and materials are described in Ch. II. In order to test this hypothesis, my objectives were to examine integrin signalling by measuring phosphorylation at key sites associated with activation of the β1 integrin subunit, FAK, and paxillin, using standard Western blotting and phosphospecific antibodies.

Like the integrin signaling proteins above, the activation of key GF signaling proteins involved in neurite growth such as Akt, GSK, and ERK was assessed using phosphospecific antibodies in standard Western blotting. I suspected that the presence of both laminin and NGF in the environment would stimulate greater phosphorylation of these proteins than either one alone.

In accordance with my hypothesis, my results show that optimal growth is achieved when both NGF and laminin is in the environment. Although laminin leads to

phosphorylation of β1, FAK, and paxillin, NGF does not lead to additional activation of integrin activation and signaling. However, the NGF and laminin environment does lead to additional activation of the both Akt and GSK-3 than with laminin alone.

Although only correlative, this data suggests that both NGF and laminin is required for optimal adult neurite regeneration and that this requirement lies within the integration of integrin signalling with growth factors. I suggest that this integration of signalling strengthens a major neurite growth signal (PI3-K>Akt>GSK), much like IGF integration with NGF signaling potentiates the growth effects of NGF.

4.2. Results

The results represent a summary of the preliminary data that was collected in a series of experiments. More notable differences include, the neurite initiation studies were performed on immunostained neurons and not live cultures. As well, the numbers are quite small. The neurite initiation data compiled from only two separate experiments (Fig. 4.18) and the Western blots (Figs. 4.19 & 4.20) from 1-2 experiments. Because of the small numbers statistical analysis could not be performed. However the trends described in this section are holding true in on going experiments being conducted by our laboratory.

Lysates were collected from cultures plated on P-L or laminin, in the presence or absence of NGF. In these experiments I only examined the influence of NGF, rather than the other growth factor combinations used in Ch. III. Western analysis of the blots was first done using an anti-phosphotyrosine antibody. The same blots were then reprobed with antibodies directed against the β1 subunit, FAK, and paxillin.

Sec 4.2.1. Neurite Growth Analysis

To identify the influence that substrate components have on neurite growth, cultures plated on poly-lysine compared to those grown on laminin. Representative micrographs of cultures grown on poly-lysine (Fig. 4.17A), poly-lysine in the presence of 50ng/ml NGF (Fig. 4.17B), laminin (Fig. 4.17C), and laminin + 50 ng/ml NGF (Fig. 4.17D) are presented.

The most obvious observation is the sparse density of neurons cultured on P-L, although the adherence appears to be increased in the presence of NGF. In the P-L condition, the neurite growth pattern is characterized by short neurites that do not branch frequently. The presence of NGF also enhances these growth characteristics but not as impressive as that seen in the laminin condition. In contrast, neurons plated on laminin are much more plentiful, and display much longer neurites that undergo extensive arborisation. NGF dramatically enhances the neurite growth supported by the laminin substrate (Fig 4.17D). Like the neurons plated on P-L, NGF + laminin stimulates longer neurites that extend greater branching patterns. Thus, the presence of both laminin and NGF in the growth environment provides the best environment to support neurite growth in these experiments.

To evaluate growth quantitatively the impact of substrate and NGF on percentage of neurons with neurites twice the cell body length was calculated (Fig. 4.18). When control (no growth factor) cultures are plated on a substrate composed solely of P-L only about 9% of the neurons extend neurites as compared to 33% of the neuronal population when grown on laminin. When exposed to NGF only 7% of neurons plated on P-L extend

neurites, while the addition of laminin to the substrate permits NGF to stimulate roughly 50% of the neurons to do so. Laminin also promotes the optimal trophic influence of a NGF and IGF-1 (N+I). Only 9% of the neurons grown on P-L in the presence of N+I sprout neurites, while almost 50% undergo sprouting when grown on Lam.

It should be noted that the data from these experiments were collected after immunostaining the cultures; this resulted in some loss of weakly adherent neurons, such as those in the P-L condition. The result is likely an underestimate of the percentage of neurite bearing neurons. In Ch. III, I used estimates of non-fixed cultures.

Sec 4.2.2. Integrin Signaling

In an attempt to discern differences in signaling in neurons plated on P-L vs laminin, I carried out biochemical studies of the expression and phosphorylation of particular intermediates know to be involved in integrin signaling. Western blot analysis was used to assess the degree of phosphorylation in the corresponding bands of the phosphotyrosine blot with the blot of the total β1 subunit, FAK, and paxillin protein levels.

A representative blot (Fig. 4.19) demonstrates that in neurons plated on laminin substrate there is increased phosphorylation of β1 subunit as compared to one composed solely of P-L. The addition of NGF to cells plated on poly-lysine results in the phosphorylation of β1 subunit, although no further phosphorylation occurs when cells plated on laminin are exposed to NGF.

A similar phosphorylation pattern occurs with FAK and paxillin. More specifically, adding either laminin to the substrate or NGF to the soluble environment

leads to more phosphorylation of FAK and paxillin than in the P-L substrate. However the addition of NGF to neurons cultured on laminin does not lead to any further phosphorylation than induced by laminin alone.

Sec 4.2.3. Integrin Signaling Converges Upon Growth Factor Signaling

Engaging integrin receptors stimulates the same intracellular pathways as growth factors. In fact, it has been suggested that the cellular response to integrins is through modulation of growth factor signalling (Kumar, 1998). As outlined in the introduction, the two major signalling pathways activated by both integrins and growth factors are the ras>Raf>MEK>MAPK and the PI3-K>Akt>GSK signalling systems. Using Western blotting analysis with phosphospecific antibodies the relative degree of phosphorylation of Akt, GSK, and ERK was assessed in the same lysates described above.

In Fig. 4.20, there is little difference between the phosphorylation of Akt when neurons are cultured on laminin as compared to P-L. However, NGF leads to greater Akt phosphorylation when neurons were plated upon the P-L substrate, and the greatest phosphorylation is seen in the laminin + NGF environment.

Fig. 4.20 also shows that the substrate composition appears to modulate GSK phosphorylation slightly different. In contrast to Akt, there is greater phosphorylation of GSK when laminin is the substarte as compared to P-L. However like Akt, when in the presence of NGF neurons supported on a P-L substrate phosphorylate GSK to a greater degree, and NGF similarly induces additional phosphorylation of GSK than neurons plated on laminin.

In Fig. 4.20, it is evident that phosphorylation of ERK has yet another pattern. The laminin substrate also leads to more phosphorylation of ERK than a P-L substrate. Interestingly, the addition of NGF to the media does not alter the ERK phosphorylation when neurons are grown on P-L, and would appear to be the case when neurons are grown on laminin, suggesting perhaps that a threshold of activation has been achieved.

4.3. Discussion

It should be noted that the data reported in this chapter are a summary of only 2 experiments. The reason for this shortcoming is primarily time constraints. The experiments I have reported on are currently being extended in the Mearow Laboratory (Tucker, Jones Rahimtula, Mearow, 2002 Neurosci Abs).

4.3.1. Results Summary and General Conclusions

Collectively, these experiments emphasize the requirement of a permissive substrate for neurite growth to be optimal. More specifically, in these experiments neurons are dependent on laminin as the major substrate component to reach full growth potential elicited by NGF. Ch. III demonstrates this as there is a substantial amount of growth observed in all growth factor conditions, which could be due to the presence of laminin in the substrate. The trophic benefit of NGF demonstrated in various studies and in Ch. III could not overcome the lack of laminin, as illustrated by the relative lack of neurite growth in neurons grown on poly-lysine.

4.3.2. NGF and Laminin Promotes Optimal Neurite Growth

The impact of an ECM containing laminin is quite apparent by simple observation or by quantifying neuritogenesis. Without laminin neither the control, NGF, or N+I environments are capable of promoting neurite growth to their potential. It would be helpful to analyze the effect that substrates have on the neurite growth pattern. As outlined in Ch. III, the neurite initiation measurements only provide data about how the population of neurons respond to a particular growth modulator. Judging from the representative micrographs, laminin does seem to induce longer neurite growth and perhaps an even more extensive branching pattern. Laminin would likely also be necessary for the neurite elongation and extensive branching stimulated by a NGF and IGF environment.

Indeed, in current experiments being conducted in our laboratory, it has been seen that laminin enhances both total neurite extension and branching compared to neurons plated on P-L. In addition, placing a β1 subunit-blocking antibody in the media blocks this enhanced growth. As expected, integrins appear to intimately involved in neurite growth.

Sec 4.3.3. Phosphorylation of β1, FAK, and Paxillin on Poly-lysine and Laminin

Integrin activity is associated with phosphorylation of the β1 subunit (Aplin et al, 1998; Giancotti and Ruoslahti, 1999). Recently, a genetic study revealed that β1 subunit and involved integrin signalling is required for proper myelination (Relvas et al, 2001). Most of the studies examining the role and activity of integrins have been performed on

very different populations of cells. Neuronal research in this area is lacking and given the unique nature of cells the data is likely to be equally as unique.

In my experiments NGF leads to the phosphorylation of the β1 subunit, FAK, and paxillin in neurons plated on P-L. However NGF does not seem to enhance the growth response in these neurons. In contrast, when neurons are plated on laminin the story is quite different. NGF does not cause any additional phosphorylation of either the β1 subunit, FAK, or paxillin, but NGF does lead to substantially more growth. This observation suggests that NGF can modulate the activity of the β1 subunit, FAK, and paxillin, and that the cross talk between integrin and growth factor signaling is bidirectional. Maybe growth factor phosphorylation of the β1 subunit represents inside out signalling and modulates the avidity that integrins has for the ECM (Giancotti and Ruoslahti, 1999).

My data suggests that further experiments need to be carried out to more clearly analyze the putative interaction between substrate-dependent and growth factor-dependent proteins and its importance in the neurite growth process. Signaling proteins that could mediate this interaction include Pyk2 and FAK (Ivankovic-Dikic, 2000).

Sec 4.3.4. Integrin and Growth Factor Signaling Convergence Maximizes Growth

Integrins and growth factors could synergistically participate in neurite growth in three ways: 1) NGF directing integrin aggregation at the growth cone, a physical interaction; 2) integrin associated kinases functioning independently of or interacting with growth factor signaling, or 3) by activation of growth factor signaling through growth factor receptor association with integrins (*in cis* binding). My experiments did not

address this possibility 1), but it is likely that this mechanism is at work (Grabham and Goldberg, 1997). Though it is possible that all these integrin functions are involved in neurite growth, a more likely explanation is that a coordinated balance of all functions, based on both timing and strength, are necessary for the optimal neurite growth observed in the laminin + NGF environment.

These data again reiterate the relative importance of the PI3-K>Akt>GSK-3 signalling system over the ras>Raf>MEK>MAPK in neurite growth. This speculation is based on the observation that in the environment displaying the greatest amount of growth (laminin + NGF) the greatest phosphorylation of both Akt and GSK-3 occurs. Using intervention studies with pharmacological inhibitors and/or transfections of implicated signalling proteins would strengthen this speculation. Expected results would see less disruption of growth with UO126, the MEK inhibitor, than with LY294002 and lithium, inhibitors of PI3-K and GSK-3, respectively.

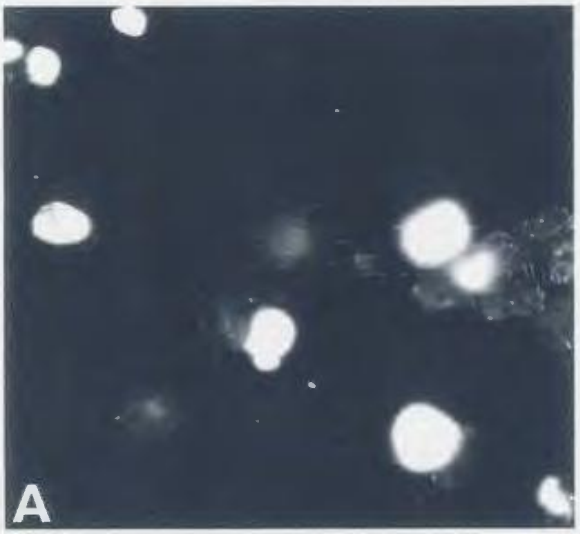
Phosphorylation of GSK-3 is actually more closely associated with the amount of growth rather than Akt. Like growth, GSK-3 is phosphorylated greater in the laminin vs P-L, and even more in the laminin+NGF environment vs laminin. It is strange that Akt is not phosphorylated by laminin even though GSK-3, one of its major substrate, is. This may suggest that other kinases that phoshorylate GSK-3 such as p70RSK, or protein kinase A, may mediate GSK-3 phosphorylation in this setting (Grimes and Jope, 2001a).

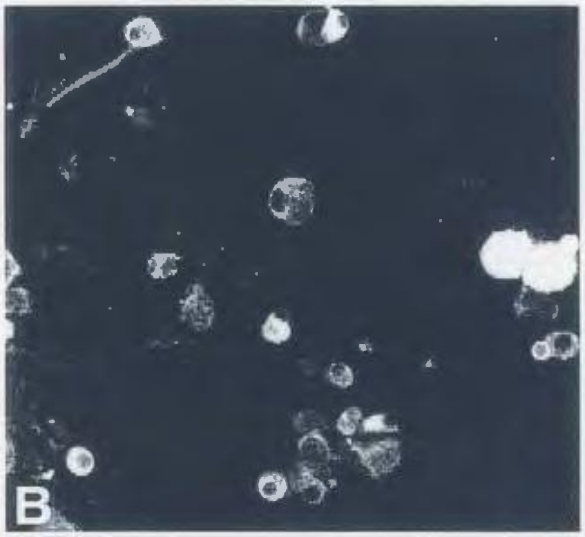
The actual mechanism may be that an environment with laminin and NGF does two things to lead to growth. One is the cycling of integrin receptors to the growth cone, which essentially extends the membrane and growth cone, and subsequently propelling the growth cone towards a laminin substrate (Grabham and Goldberg, 1997). The second

is that the integration with growth factor signaling provides the signaling needed to provide the contents of the extending neurite. The PI3-K>Akt>GSK signaling systems could regulate the cytoskeletal activity necessary to support growing neurites (Takahashi, 1999) while the ras>Raf>MEK>MAPK system may regulate the expression of gene products needed in the formation of new neurites such as membrane proteins, vessicles, etc (Perron and Bixby, 1999).

It is apparent from this study that integrins and growth factors do function co-ordinately to provide maximal environmental support of neurite growth in adult DRG neurons. Further work is required to fully understand potential mechanisms and interactions.

Figure 4.17: Laminin induces neurite growth and is required for NGF induced neurite growth. Representative micrographs of dissociated adult DRG cultures grown on poly-lysine **(A)**, laminin **(B)**, poly-lysine in 50 ng/ml NGF **(C)**, or laminin in 50 ng/ml NGF **(D)**. Neurons were cultured for 48h, fixed, and detected using an immunofluorescence probe against peripherin. Neurons were later visualized with a fluorescent microscope and converted to black and white digitally.


A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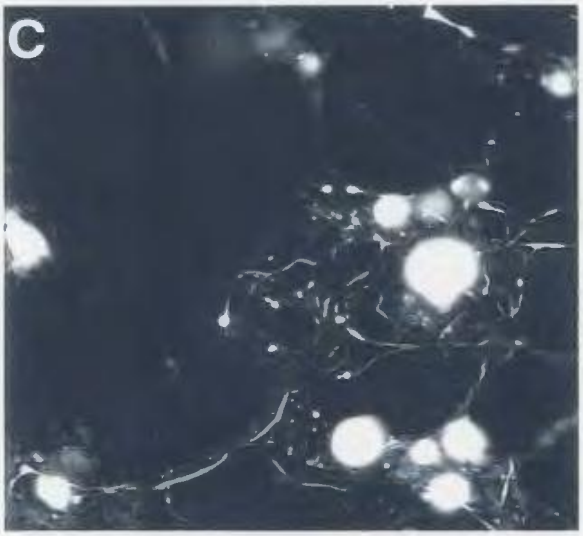

C

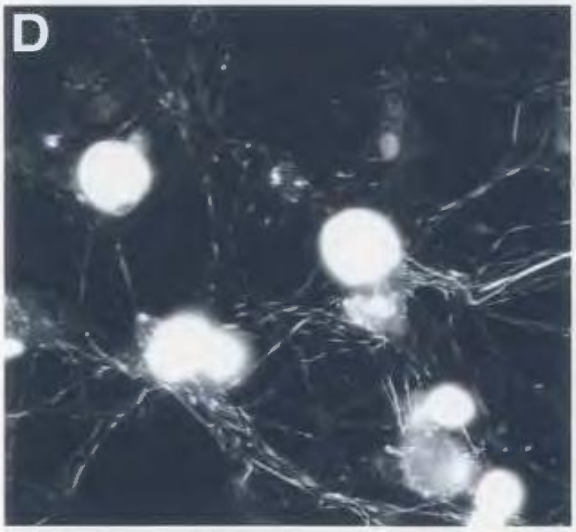

D

Figure 4.18: Laminin is the ultimate substrate and is necessary for NGF dependent neuritogenesis. Adult DRG cultures were treated as before and grown upon the same substrates and within either no growth factor (control), 50 ng/ml NGF, 50 ng/ml IGF, or 50 ng/ml NGF + IGF. Again, neurons were grown for 48h, fixed, and probed with an antibody probe against peripherin and detected with DAB. Neurons with neurites twice the cell body length were counted and scored as the percentage of neurons with neurites. Graph represents average values of two experiments.

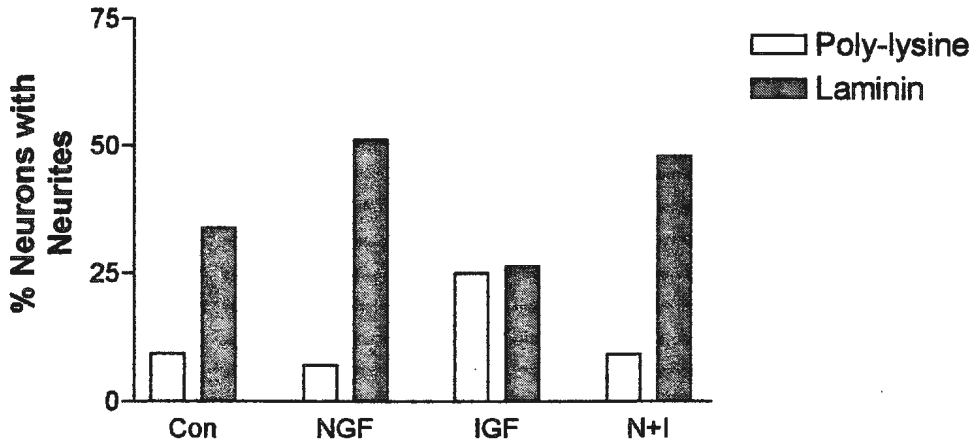

% Neurons with Neurites
75
50
25
0
Con
NGF
IGF
N+I
Poly-lysine
Laminin

Figure 4.19: Laminin but not NGF activates integrin signaling. Adult DRG neurons were cultured as before, but now in 12 well plates. At 24h post plating neurons were collected, lysed and the cytosol contents separated by centrifugation of the nuclear and membrane extract. Later the cytosol lysate was separated on 8-10% acrylamide SDS electrophoresis gels, transferred to a nitrocellulose membrane and probed with various integrin signaling proteins and detected with HRP based chemoluminesce as performed in standard Western blotting procedures. The entire blot was first probed with a phosphotyrosine primary, then the blot was stripped, cut, and probed with β1 integrin subunit, FAK, paxillin. The phosphorylation of the integrin signaling proteins are presented here above the non-phosphorylated forms .

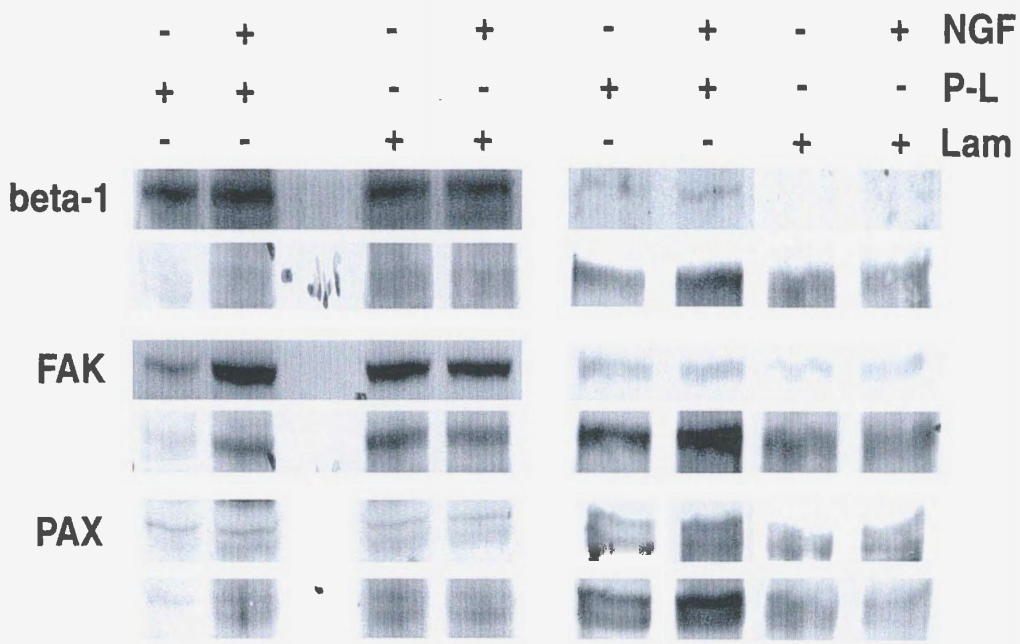
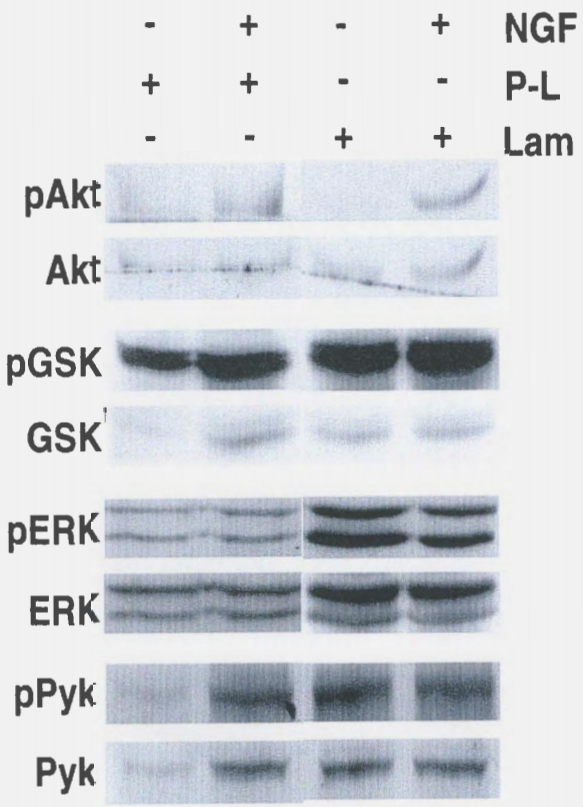

- + - + - + - + NGF
+ + - - + + - - P-L
- - + + - - + + Lam
beta-1
FAK
PAX

Figure 4.20: Laminin and NGF convergence upon the PI3-K-Akt-GSK pathway corresponds to neurite growth. Adult DRG neurons were cultured and harvested for standard Western blotting techniques as described in previous figure and in Material and Methods section. The blot was cut appropriately to minimize the number of strip needed. Phospho-specific antibodies for Akt, GSK-3 α/β, and ERK-1/2 were used as a measure of activation. The levels of the non phosphorylated forms are presented directly below.


- + - + NGF
+ + - - P-L
- - + + Lam
pAkt
Akt
pGSK
GSK
pERK
ERK
pPyk
Pyk

CHAPTER V –DISCUSSION

5.1. Hypothesis 1- Growth Factor Synergism

The potential for multiple factors to maximize axonal growth/regeneration has become obvious as an increasing number of trophic factors have been identified (Fu and Gordon, 1997; Zochodne, 2000). In certain situations within the nervous system it is not valuable to salvage as many connections as possible. Forming aberrant connections post-injury could lead to more dysfunction than not forming any connections at all. Having multiple requirements for growth would help to avoid this situation. The proposal of multiple growth influences is supported not only because it makes physiological sense, but also by the relatively limited success with attempts at optimizing regenerative growth (Tatagiba, 1997; Gimenez y Ribotta et al, 1998). To date the many attempts consist of using a single type of growth influence, most often one trophic factor, to stimulate regenerative growth. Most single modality treatments have failed, and considering that multiple factors influence growth and that multiple factors are physiologically sensible, it seems logical that only with multiple modality treatment will regenerative growth be maximized.

Despite this, relatively few investigations have explored the additive effects of growth influencers and none has proposed an explanation for any additive growth. Schwab and associates demonstrated that the combination of both an antibody that blocks recognized inhibitory proteins and NT-3 yields better cortiospinal tract long-distance regeneration than either alone (von Meyenburg, 1998), an effect attributed to NT-3 boosting sprouting from the lesion site as opposed to enhanced elongation. With this

clever strategy, investigators intervened on two levels of growth influences- they blocked inhibitory proteins and enhanced trophic support. Using these two distinct influences on growth resulted in additive effects on growth.

The various growth factors that influence growth are arguably the most influential environmental stimuli to promote growth. With this in mind, our laboratory has uncovered another additive treatment to promote growth- NGF and IGF-1 (Kimpinski and Mearow, 2001). In these experiments IGF-1 was shown to potentiate neurite growth into NGF containing side compartments of Campenot cultures beyond that achieved by NGF alone. This was specific to NGF and IGF-1 as attempts to combine the growth promoting abilities of other recognized growth factors failed (Kimpinski and Mearow, 2001).

The results of my experiments further support the interaction between these two factors, showing a synergistic effect that could result from convergence of the growth factor activated signaling pathways. Observations mentioned in concluding remarks of Chapter III suggest that whatever the mechanism, both somehow act directly on individual neurons and not through non-neuronal support. Interactive intracellular signaling or "cross talk", such as convergence, offers one opportunity for the actions of all influential environmental factors to culminate and ultimately determine neurite growth.

It seems then that it is essential to maximize the trophic support offered by soluble growth factors to elicit maximal neurite regeneration. There are other soluble environmental contributors to growth such as neuroactive cytokines (IL-6, LIF) that if added to the soluble environment may further enhance regeneration. It has become

evident that immune cells like the macrophages not only invade the area of injury during degeneration and phagacytose debris, they also release cytokines that can modulate growth (Zigmond et al, 1996; Fu and Gordon, 1997). Examples of these neuroactive cytokines include leukemia inhibitor factor (LIF), interleukin (IL)-6, and ciliary neurotrophic factor (CNTF). The timing and location of presentation of these soluble factors are likely to be important. Perhaps it would be beneficial to apply IGF to the ganglia and NGF at the local site of injury. Whatever the correct combination, it seems that multiple strategies will be required to maximize regeneration.

5.2. Hypothesis 2- Signaling Convergence Underlies Synergistic Growth

5.2.1. Convergence Upon PI3-K Pathway

In general, the data points towards the emergence of the pattern of sustained ERK and increasing GSK phosphorylation that correlates with an increasingly more neurite branching and elongation. The inhibitor data further supports the notion that the PI3-K-Akt-GSK pathway is most important in growth, but does not discount the involvement of the ras-Raf-ERK pathway. At this point it still remains difficult to attribute a certain degree of importance or specific growth event to either pathway. However, considering that blocking members of the PI3-K>Akt>GSK-3 pathway is more disruptive to regeneration than blocking ras-Raf-ERK pathway members and that the activation of signaling proteins in correlate better with growth, the importance of this pathways is assured.

One possible explanation of the molecular mechanism underlying NGF and IGF synergism are a strengthening of the signaling through PI3-K-Akt-GSK pathway. This is

especially possible since both NGF and IGF activate PI3-K leading to Akt activity and GSK deactivation, but take different route. In fact, the two growth factors activate PI3-K similarly, but appear to do so via at least two different adapter proteins binding to their respective receptors. The adapter protein GAB-1 associates with and thereby activates PI3-K upon TrkA activity (Kaplan and Miller, 2000), while IGFR-1 phosphorylation recruits IRS-1 to the receptor, which acts identical to GAB-1 to activate PI3-K (Butler et al, 1998). This could increase the activity of more Akt molecules leading to the phosphorylation of more GSK molecules. The inhibitor data suggests that these pathways do not signal linearly, but rather have an ongoing interaction between ERK and Akt and GSK and ERK. In this way the two pathways may only function maximally if activated simultaneously. Another possibility is that both pathways are needed to activate separate effector proteins. The activated form of ERK is present at 24h in all the growth factor conditions, but only remains high at 48h in the NGF and N+I condition. Active ERK may provide the protein synthesis needed for a continually growing neurite (Encinas et al, 1999; Adams et al, 2000). GSK phosphorylation remains elevated at 48h in the NGF and N+I conditions as well, but more so in the N+I. Maybe this extra deactivation of GSK provides that extra control over tau activity to permit extra growth in the N+I (Takahashi et al, 1999; Sang et al, 2001). Like the environmental factors that activate them, more than one signaling pathway contributes to neurite growth, although some may be more important than others.

5.2.2. The low affininty p75 neurotrophin receptor and growth

The signaling activity stimulated by NGF-TrkA does not occur in isolation when a neuron is subjected to NGF. Another receptor that must also be considered, is the low affinity p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor family of receptors that possesses a low avidity for all neurotrophins (Kaplan and Miller, 2001; Barrett, 2000). Although it lacks cytoplasmic kinase activity, through association with intracellular proteins, $p75^{NTR}$ has been shown to modulate trophic actions of neurotrophins through direct interaction with the TrkA receptor, modulation of TrkA ligand affinity and interaction with TrkA signaling (Davies, 2000). Additional intracellular pathways implicated in the action of $p75^{NTR}$ include activation of sphingomyelin hydrolysis (Dobrowsky et al, 1994) and NF-κB nuclear translocation (Kimpinski et al, 1999).

A perplexing finding in the investigation of $p75^{NTR}$ function was that its activation can lead to apoptosis depending on the developmental context and cell type (Kaplan and Miller, 2000). Many suspect that some sort of balance between the survival promotion via TrkA activation and the pro-apoptotic effect of p75 underlies the influence that NGF has on the fate of certain neurons and glial cells (Casaccia-Bonnefil et al, 1999; Majdan and Miller, 1999). However the role of p75 is not limited to its apopotic capabilities. A more direct role in neurite growth has been uncovered.

Neurotrophin binding to $p75^{NTR}$ has recently been identified to have a more direct role in neurite growth. Immunoprecipitation demonstrated that $p75^{NTR}$ binds to RhoA, an interaction that was shown to lead to the constitutive activation of this small GTPase (Yamashita et al, 1999). Although RhoA has a defined role in neurite growth, the

investigators confirmed that its activation by $p75^{NTR}$ is specifically involved using both a Rho-inactivating enzyme and overexpressing a constitutively active form of RhoA. It is also possible that the activation of ceramide by engagement of NGF with p75 may mediate the growth promoting abilities of NGF in hippocampal neurons (Brann et al, 1999). In cultures of adult DRG neurons, activation of the $p75^{NTR}$ or adding ceramide to the culture, resulted in decreased neurite growth (Kimpinski et al, 1999; McMarthy and Mearow, unpublished observations). These examples of a more direct role of $p75^{NTR}$ in neurite growth add yet another degree of complexity to the nature of the growth promoting ability of NGF.

The overlapping nature of survival and neurite growth is seen also in the overlapping nature of the intracellular signaling elicited by NGF. Where survival ends and neurite growth begins, along with the details of the cumulative NGF signal via both TrkA and p75, are essential questions that need to be answered in order to understand the trophic action that NGF has on neurite growth.

5.2.3. Other Growth Associated Signaling Pathways

Other intracellular signaling events are well known to be associated with growth. However the extracellular stimuli that activate them are either not well characterized or so numerous that it would be impossible to delegate a stimuli to a particular intracellular signal associated with growth. Three examples of these growth associated signaling signals are the adenylate cyclase-cyclic adenosine monophosphate-protein kinase A (AC-cAMP-PKA) signaling system, intracellular Ca^{+} ($[Ca^{+}]_i$) shifts, and phosphatases. Each of these signals have a plethora of activators and more importantly are able to modulate

and activate a variety of other signaling systems.

There is evidence to suggest that one of first well characterized and highly conserved signaling pathway, the adenylate cyclase>cAMP>protein kinase A cascade (PKA), is also activated by neurotrophin signaling (Yao et al, 1998; York et al, 1998). These studies suggest that PKA is activated by a Ras related small G protein Rap1, and that PKA is able to modulate NGF control over Erk signaling. The adapter protien Crk may associate with TrkA, recruit the C3G, a guanine exchange factor specific for Rap1 (Grewal et al, 1999). However these experiments were performed in PC12 cells and they demonstrated that PKA is not required for direct NGF induced neurite growth, but may be involved in some the events required for growth.

There is also evidence that cAMP levels may be important in determining the impact of guidance molecules on growth cone direction (Song et al, 1997). In addition to its role in guidance, the AD-cAMP-PKA system has also been implicated in hormonal stimulation of neurite growth in various CNS populations. For example, estrogen enhances neurite growth in midbrain dopaminergic cells (Beyer & Karolczak, 2000), and corticotrophin releasing factor acts similarly in an immortalized cell line resembling locus coeruleus (Cibelli et al, 2001). Considering that CREB, the token transcription factor activated by the AD-cAMP-PKA system is also involved in growth, it is likely that the AD-cAMP-PKA system may help to activate CREB as well.

Local, transient shifts in the concentration of intracellular Ca^+ ($[Ca^+]_i$) are involved in a host of cellular events, including those important during neurite regeneration (Doherty et al, 2000; Gomez and Spitzer, 2000). Recently, it was found that local elevations in $[Ca^+]_i$ induce growth cone formation through protease activation

(Spira et al, 2001). Considering the numerous possibiltity for $[Ca^{+}]_i$ to influence other signaling systems involved in growth, it is likely that $[Ca^{+}]_i$ could further modulate growth through signaling interactions (Song and Poo, 1999; Rizzuto, 2001).

Phosphorylation of signaling proteins is not a unidirectional means of regulating the activity of signaling proteins. Rather, a balance between the dephosphorylation of proteins by phosphatases and the converse by kinases is likely to deem the ultimate phosphoylation state and hence activity of a protein (Oliver and Shenolikar, 1998). Phosphatases are under equally stringent control in cells as kinases, and they likely have an equivalent impact on neuronal function (Arregui et al, 2000), including neurite formation (Stroker, 2001).

Thus, there are likely ongoing, overall signals necessary to ultimately alter the ability of a neuron to respond to changes in its environment. These signals result from contributions from all the factors that influence growth, and it is the balance of growth promoting and growth inhibiting factors that ultimately determines neurite advancement, maintenance, or even retraction.

5.3. Hypothesis 3- Physical environmental contributors

5.3.1. The Substrate

The profound influence of the surrounding substrate on neuronal behavior and the intimate link between integrins and growth factors meant that the requirement of laminin for maximal NGF action comes as no surprise. Although neurons were attached to poly-lysine, neurons were plated on this non-biological substrate failed to respond to NGF to the extent they did on laminin. A similar study to that of Ch. IV examined the survival

and growth effects of the cooperative actions of laminin and/or fibronectin with NGF in embryonic DRG neurons (Millaruelo et al, 1988). However, developing neurons are much different from post-injury adult neurons. The experiments presented in Ch. III revisit these findings in adult rodent DRG neurons and attempted to delineate the mechanism of this cooperative action.

The experiments were designed to examine the contribution that laminin had on growth, and to identify any additive growth to that seen with NGF. As summarized in Ch. IV, my data pointed to a need for a permissive substrate like laminin, for neurons to fully respond to the trophic influence of NGF.

Other physical environmental factors such as the cellular adhesion molecules found on the non-neuronal cells surrounding the lesion also impact growth (Kandal et al, 2000; Walsh and Doherty, 1996). For the most part neurites either grow on a substratum or on other cells. The membrane bound receptors in this form of intercellular connection are called cell adhesion molecules (CAMs). Perhaps the three most recognized CAMs are neural CAM (NCAM), N-cadherin, and L1 (Doherty et al, 2000). As the name suggests each of these molecules provide a link between the cells they are expressed in and neurons. Until recently the adhesion property was believe to convey most of their growth promoting potential (Long and Lemmon, 2000; Skaper et al, 2001). Much like the adhesive properties of ECM constituents. The demonstration that soluble versions of these CAMs can equally promote growth suggests that more than just the mechanical force generated by attachment to a stable CAM is at play (Doherty et al, 2000). Yet another environmental factor conveys at least a part of its message via intracellular signaling.

5.3.2. Activation of Integrin Signaling

In the preliminary data that comprise Ch. IV, the phosphorylation of β1, paxillin, and FAK all occur concurrently. The phosphorylation of these proteins on tyrosine (Y) residues corresponds to integrin activation and could be described as representing the activation of integrin signaling (for review see Panetti, 2002). In motile cells, the cycling between phosphorylated and dephosphorylated forms of the β subunits is thought to underlie the focal contact remodeling required for cell migration (Sakai et al, 1998). The cytoskeletal modulating protein, paxillin, requires tyrosine phosphorylation for localization to the focal contact, and is critical for the role paxillin has in cell migration and spreading (Schaller and Parsons, 1995). The signaling kinase FAK not only acts as a non-receptor tyrosine kinase and autophosphorylates at tyrosine resides, but it requires phosphorylation at Y397 to bind and thereby activate PI3-K (Eliceiri, 2001). Tyrosine phosphorylation of β1, paxillin, and FAK is a critical regulatory step necessary for the complete function of integrin signaling (Giancotti and Ruoslahti, 1999).

If the tyrosine phosphorylation I detected is indicative of the relative activity of the observed integrin proteins, then my results could be explained as follows. Integrin signaling is activated by NGF when neurons are grown on poly-lysine or by laminin alone. The addition of NGF to the media does not provide any additional integrin signaling activation over laminin. This could be interpreted to mean that NGF attempts to activate integrin signaling when neurons are upon a sub-optimal regeneration substrate such as poly-lysine. When neurons are on a substrate conducive to regeneration such as

laminin, then NGF itself does not activate integrin signaling over and above what would be activated by ECM engagement of integrins.

The activation of integrin signaling largely has two consequences: (1) modulation of the cytoskeleton (2) activaton of kinases that modulate other signaling proteins. It was outside the scope of this thesis to identify the effect that paxillin phosphorylation has on actin dynamics. However if it is speculated that FAK mediates the modulation of growth factor signaling pathways as is the case in other cells (Ivankovic-Dikic, 2000), then any activation of growth factor signaling proteins associated in the presence of integrin signaling could be attributed to FAK. Such is the case with the two members of the PI3-K pathway, Akt and GSK (Ch. IV, Fig. 4). Upon activation of FAK by laminin adhesion, FAK then phosphorylates at Y397, binds PI3-K and thereby leading to the activation of Akt and phosphorylation of GSK. Since NGF-TrkA binds PI3-K differently it can possibly activate a separate pool of the members of the PI3-K pathway, similar to how NGF and IGF synergism is suggested to work in Ch. III.

The effect that environmental stimuli have on cellular behavior has been hypothesized to be sorted out by the interactions between the signaling pathways they stimulate, a concept known as network or interactive signaling (Bhalla and Iyengar, 1999; Weng et al, 1999). Considering the innumerable possible signaling interactions and the different consequences they result in, the concept of network signaling could explain how many cellular behaviors are controlled by environmental stimuli, or at least, how neurite regeneration is dictated by the many environmental factors that influence it.

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