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The Role of Tyrosine Phosphorylation in Neurite Outgrowth

by

MATTHEW LAROUCHE

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ABSTRACT

In this thesis, I examined the role of tyrosine phosphorylation during axonal regeneration of adult neurons from the pond snail, *Lymnaea stagnalis*. The role of tyrosine phosphorylation in initiation and elongation, the first stages of the regenerative process, were investigated using tyrosine kinase (TK) inhibitors (lavendustin A and K252a), and a tyrosine phosphatase inhibitor (sodium orthovanadate). When TK inhibitors were present at the time of cell plating, neurite initiation and elongation were suppressed. Later application of TK inhibitors resulted in decreased inhibition of initiation, but no inhibition of elongation. Taken together, the data suggest that both initiation and elongation are activated by TK activity but subsequently these processes become independent of TK activity.

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LIST OF ABBREVIATIONS

ABS	Antibiotic Solution
ATP	Adenosine Triphosphate
ANOVA	Analysis Of Variance
BDNF	Brain Derived Neurotrophic Factor
Ca / CaMIIK	Calcium Calmodulin Kinase II
c.f.	Compare With
CM	Brain Conditioned Medium
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CRNF	Cysteine Rich Neurotrophic Factor
DM	Defined Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
e.g.	For Example
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
GDNF	Glial-Derived Neurotrophic Factor
h	Hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
i.e.	That Is
Ltrk	*Lymnaea* Trk-like Receptor
MLCK	Myosin Light Chain Kinase
µm	Micrometer
mt	Microtubule
NGF	Nerve Growth Factor
NT 3	Neurotrophin 3
NT 4/5	Neurotrophin 4/5
NT 6	Neurotrophin 6
NT 7	Neurotrophin 7
$P75^{ntr}$	Low-affinity Neurotrophin Receptor
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PNS	Peripheral Nervous System
RPA	Right Parietal A Neuron
S/T	Serine/Threonine
TK	Tyrosine Kinase
Trk	Neurotrophin Receptor Tyrosine Kinase

INTRODUCTION

Axonal Regeneration

When a neuron is axotomized, the cell will generally initiate attempts to recover from this injury. This process of axonal regeneration has been studied for over 100 years. In the case of mammals, it has been determined that the ability of a neuron to successfully recover from axotomy is generally limited to the peripheral nervous system (PNS). Many studies have concluded that there is a potential for axonal regeneration in the mammalian central nervous system (CNS), but this possibility appears to be severely limited (Schwab et al., 1993; Lu and Waite, 1999). Studies using PNS nerve grafts transplanted into the CNS show that neurons in this system are capable of axonal regeneration but factors inherent to the CNS seem to prevent this process from occurring (Benfey and Aguayo, 1982). For example, there are a variety of proteins, such as NI 35, associated with oligodendrocytes, that limit axonal regeneration in the mammalian CNS (Kapfhammer and Schwab, 1992).

Many animals however, including invertebrates and lower vertebrates such as amphibians, are able to restore functioning capacity to most parts of their nervous system, including their CNS (Bulloch and Jones, 1988; Bulloch and Ridgway, 1989; Farel and Meeker, 1993; Moffett, 1995). Since the mammalian CNS has a restricted capacity to regenerate, these differences in ability to repair damage are worth understanding. To uncover the basis of these differences, it is essential first to understand how the process of axonal regeneration is controlled in organisms that are capable of this process.

Regenerating neurons produce projections known as neurites and this process can be broken down into four stages:

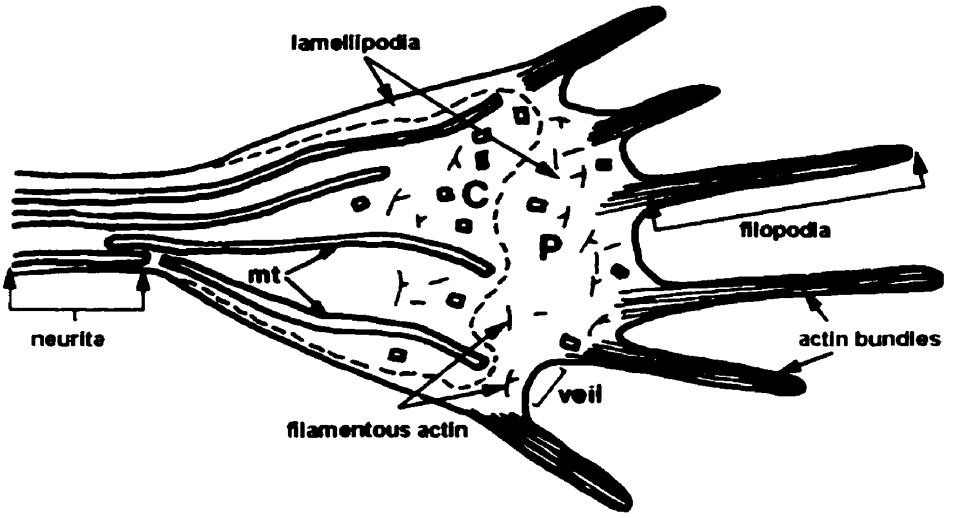
1. Initiation of neurite outgrowth.
2. Elongation of neurites.
3. Neurite turning or guidance.
4. Cessation of outgrowth, and formation of connections.

Axons usually regenerate by extending one or more neurites from the proximal axon stump. If the axon is completely removed by proximal axotomy, however, neurites regenerate from the cell body. This has been observed both *in vivo* and *in vitro* (Bulloch and Kater, 1982; Schacher and Proshansky, 1983). This regeneration of neurites from the cell body *in vitro* is the type of axonal regeneration that is studied in this thesis.

For the regenerating neuron to become functional again, it must extend and properly navigate towards the target before reestablishing a synapse with it. The growing neurite is led by a motile structure originally observed by Ramon y Cajal which he called the "cone of growth" (Ramon y Cajal, 1928). In modern times this structure is known as the growth cone.

Growth cones have a characteristic hand-shaped profile that consists of a central (C) region that is supported by both actin and tubulin, and a peripheral (P) region which is supported only by actin (Fig. 1). The P region is subdivided into two areas: the flat lamellipodia, in which a meshwork of actin supports the overlying cell membrane, and filopodia, the finger-like projections that have actin bundles as their core. Filopodia, compared to the rest of the cell, have a relatively small volume and seem capable of

Figure 1: Schematic drawing of a growth cone. The growth cone is composed of two regions, the central region (C) and the peripheral (P) region. The border between the two regions is demarcated by the dotted line. The C region contains microtubules (mt), actin, and organelles, while the P region is supported by an actin meshwork that also contains organelles, and is composed of two areas, the lamellipodia and the filopodia. Figure is adapted from Letourneau et al., (1991).


lamellipodia
C
P
filopodia
mt
neurite
actin bundles
veil
filamentous actin

sensing and responding to minute changes in the surrounding environment. They have therefore been termed the 'sensory organs' of the growth cones (Davenport et al., 1993).

Surface-bound receptors are at least partly responsible for cueing the cell on how to respond to signals in the environment. These receptors are generally specific for one factor, or a small family of factors that may be found surrounding the cell. Binding of these factors to their cognate receptors dictates to a cell when and where to produce outgrowth. These factors are classified into two functional categories. Trophic factors, or neurotrophic factors, provide survival and differentiation cues to both adult and developing neurons, while tropic factors give guidance cues to the growth cone.

Systemic knockouts of neurotrophic factors have shown that certain proteins are responsible for developmental aspects of whole groups of neurons. By genetically removing the ability of an animal to produce a certain factor, commonly known as a knockout approach, it has been shown that animals that do not produce a particular neurotrophin, may develop a nervous system missing key areas. For example, mice unable to make nerve growth factor (NGF) fail to form dorsal root ganglia and lose sympathetic ganglia by 10 days following birth, demonstrating that this protein is critical for the development and/or survival of both ganglia (Snider, 1994). NGF and its homologues are grouped into a family known as the neurotrophins. Other members of this family include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), NT 4/5 (Snider, 1994), as well as the recent additions NT-6 and 7 (Barde, 1994; Nilsson et al., 1998, Lai et al., 1998). These proteins are known to bind to a family of receptor tyrosine kinases known as Trks, as well as a low-affinity receptor known as p75 (Barbacid, 1994; Chao, 1994). Other examples of trophic factors include epidermal

growth factor (EGF), fibroblast growth factor (FGF), ciliary-derived neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF).

Trophic factors exist in all types of animals including vertebrates and invertebrates. EGF, for example, has been cloned from mammals such as mice as well as invertebrates such as the pond snail, *Lymnaea stagnalis* (Plata-Salamán, 1991; Bulloch et al., 1999). A novel neurotrophic factor was also recently cloned from *Lymnaea* and named cysteine-rich neurotrophic factor (CRNF). As of yet, this factor has no known homologues in any other animal (Fainzilber et., 1996). CRNF has been shown to induce outgrowth in a variety of cultured *Lymnaea* motoneurons in an otherwise serum-free medium (Fainzilber et al., 1996).

Traditionally a protein was considered to be either a growth factor (or mitogen) or a neurotrophic factor (i.e. one that supports growth and differentiation of postmitotic cells). In recent years this distinction has grown less clear. Proteins such as EGF or FGF, which are usually regarded as mitogens, have now been shown to confer survival and differentiation on neurons (Yamada et al., 1997). Factors like EGF, which are known to have effects on actively dividing cells as well as postmitotic cells, are now known as multi-potent growth factors.

A second group of factors are tropic molecules. These proteins and peptides are not mutually exclusive of the neurotrophic molecules, but are a continually expanding group of factors involved in influencing the direction of neuronal outgrowth. Examples of these proteins include, but are not limited to, NGF, netrin-1, semaphorins, ephrins, and may also include the neurotransmitter molecule acetylcholine (Mueller, 1999).

Extrinsic factors are available to the neuron in two basic forms, either bound to the substrate or in a soluble form in the surrounding environment. These factors may also be attractive and/or repulsive. For many neurons, the effect a factor has depends on the cell type and consequently on the receptors it expresses. In addition, a factor may be either attractive or repulsive to a single cell depending on the internal state of the second messengers of the cell (Mueller, 1999). Examples of soluble chemotropic molecules may also include the neurotrophins as well as some proteins which are predominantly known to be repulsive, such as some members of the semaphorin family of proteins (Van Vactor and Lorenz, 1999). Examples of bound factors include proteins found in the extracellular matrix (ECM), such as laminin and/or fibronectin, as well as certain semaphorins (Kapfhammer and Schwab, 1992).

Most soluble tropic and trophic factors signal through TK receptors. In contrast, substrate-bound factors may signal directly through TK receptors, as in the case of cell adhesion molecules, or indirectly through TKs, as in the case of the ECM-receptor family, the integrins. The integrins are thought to activate TKs receptors indirectly through clustering that occurs upon ligand binding (Guiancotti and Ruoslahti, 1999).

Cell Signaling

Trophic and tropic factors confer their message to the cell by attaching to a specific membrane-bound receptor (Schlessinger and Ullrich, 1992). The binding event causes a change in the conformation of the receptor resulting in its activation. All receptors of this type are kinases, which are enzymes capable of catalyzing the phosphorylation of a substrate (Schlessinger and Ullrich, 1992). The receptors themselves

are thought to be activated through autophosphorylation, and they in turn phosphorylate signaling molecules. This phosphorylation cascade is the mechanism that transfers the message from extracellular binding event to the appropriate intracellular target(s). The resulting action varies, but neurotrophic molecules act to influence the survival, growth direction or cell morphology of neurons.

In general, all kinases have three major functional regions: a substrate-binding region, an adenosine triphosphate (ATP)-binding region, and a transferase region that catalyzes the translocation of a phosphate group (PO_4^{2-}) from the bound ATP to the kinase substrate. Kinases can be divided up into two major groups, named after the amino acid residues that they phosphorylate: tyrosine kinases (TKs) and serine/threonine (S/T) kinases. In addition to being fundamental components of many receptors, tyrosine and S/T kinases are involved in signal transduction. Examples of S/T kinases involved in signal transduction include protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG).

Calcium and small GTPases are two examples of second messengers known to be linked with TKs. Intracellular free calcium is a tightly controlled cellular messenger that is involved in regulating many aspects of neurite outgrowth, including elongation and guidance (e.g. Gomez and Spitzer, 1999). Calcium release from intracellular stores may be caused by TK receptors such as Trks.

The small GTPases are a family of GTP binding proteins related to Rho, that are known to regulate the actin cytoskeleton (Luo et al., 1997). These proteins have been demonstrated to control neurite outgrowth characteristics such as filopodia and lamellipodia formation, and growth cone guidance (Luo et al., 1997). Small GTPases are

also known to be involved in transducing signals from both the FGF and GDNF TK receptors (Kuo et al., 1997; Hiwasa et al., 1997)

TK receptors are present in a wide range of organisms, and in many cases these receptors show extensive homology, suggesting conservation of function. For example, the insulin receptor in *Aplysia* is thought to mediate changes in potassium currents in neurons, a role that is akin to their mammalian receptor counterparts (Jonas et al., 1996; Wischmeyer et al., 1998). Such conservation of function may hold true for Trk receptors as well. A receptor TK (Ltrk) recently cloned from *Lymnaea* has striking overall homology, including conservation of many functional domains, to the mammalian Trks (van Kesteren et al., 1998). This receptor is expressed during development as well as after axotomy in the adult nervous system, suggesting a role in both of these processes (van Kesteren et al., 1998). Immunological experiments suggest the existence of further Trk homologues in *Lymnaea* (Melvin and Bulloch, 1999). A partial clone of a Trk-like receptor has also been obtained from the sea snail, *Aplysia californica* (Giustetto, 1999) and functional and immunological experiments have suggested the presence of a Trk-like receptor in squid (Moreno, et al., 1999).

As for receptor TKs, many cytoplasmic TKs are structurally conserved in both vertebrates and invertebrates. Homologues to cytoplasmic TK, such as MAPK, have been cloned in *Drosophila* and *Aplysia* (Perrimon, 1994; Ambron and Walters, 1996). Conservation of structure has also been shown for S/T kinases. Homologues to at least two isoforms of PKC have been cloned from neurons in *Aplysia* in recent years. These two isoforms share not only structural characteristics with their vertebrate homologues, but also have similar mechanisms of activation (Kruger, et al., 1991; Sossin et al., 1993).

The levels of both tyrosine and serine/threonine phosphorylation are subject to constant regulation. Tyrosine phosphorylation for example, is regulated on one side by both the receptor and cytosolic TKs that phosphorylate cellular proteins. On the other side of this balance are protein tyrosine phosphatases (PTP), which are enzymes that remove phosphate groups from tyrosine. Therefore PTPs are also involved in controlling the basal level of tyrosine phosphorylation in the cell. Changes in the level of cellular phosphorylation are brought about by the activation of either a kinase or a PTP.

The PTP family is composed of a number of subclasses of phosphatases (Jia, 1997). The low molecular weight subclass is comprised of proteins that have molecular weights of about 18 KDa. These proteins have little sequence homology with other members of the PTP family, apart from the catalytic site. Receptor-like and dual-specificity PTPs make up the other two subclasses of this family. Proteins in these two subclasses have molecular weights that range from 30-50 KDa (Jia, 1997). As their name indicates, receptor-like PTPs are membrane-associated proteins (Stoker and Dutta, 1998). Dual specificity PTPs are able to dephosphorylate phosphotyrosine residues, as well as serine/threonine residues (Jia, 1997). All PTPs have a conserved intracellular catalytic domain of approximately 240 residues, with the conserved motif C-X_5-R (C = cysteine, R = asparagine, x = any amino acid) (Jia, 1997). In this motif it is the cysteine residue that is critical for enzyme function (Stoker and Dutta, 1998).

The Function of Tyrosine Phosphorylation

One approach to examining how kinases and phosphatases regulate phosphorylation is to use inhibitory reagents. These reagents take advantage of structural

characteristics which are necessary for the function of the protein. Specificity to a certain protein or family of proteins is conferred by targeting critical regions of a protein such as a kinase that is common to the group of interest. In the case of kinases, one mechanism of inhibition is to prevent ATP from binding to the appropriate region on the kinase. Some targeting to a specific family of kinases is possible since the ATP binding region is thought to differ between the TK and S/T kinase families. K252a and lavendustin A are two examples of inhibitors that exert their actions by competing with ATP for binding to a TK (Table 1) (Kase et al., 1987; Onoda et al., 1989). However, the TK inhibitor K252a is also able to inhibit S/T kinases such as PKA and PKG. Since the specificity of these reagents is not absolute, it is often helpful to employ multiple reagents. I selected three inhibitors to study the role of TKs, K252a, lavendustin, and genistein (genistein data are not presented but are described in the Discussion). These inhibitors were chosen on the basis of previous literature which demonstrated their ability to inhibit TKs (Akiyama et al., 1987; Koizumi et al., 1988; Onoda et al., 1989)

PTPs may be manipulated in a similar fashion using inhibitors (Table 1). Sodium orthovanadate (Na_3VO_4) is capable of preventing the functioning of a wide spectrum of PTPs by acting as a phosphate analogue to these PTPs (Seargent et al., 1979). Vanadium (V) is found in a number of forms in nature. In biological systems, vanadium in the extracellular fluid is principally found in VO_4^{1-} form (vanadate), and is reduced to vanadyl form (VO^{2+}) intracellularly (Dafnis and Sabatini, 1994). It is likely that the vanadyl form of this compound inhibits PTPs (Morinville et al., 1998). It is unknown how vanadate enters cells but it is likely that it is through facilitated or active transport since this compound is charged in solution (Morinville, et al., 1998).

Table 1: Summary of inhibitory reagents used in this thesis. Abbreviations: PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; MLCK, myosin light chain kinase; Ca / CaMKII, calcium calmodulin kinase II; ATP, adenosine triphosphate; p'tase, phosphatase; and EC, concentration reported to inhibit a kinase type in the reference cited.

Reagent	Inhibits	**EC**	**Mechanism of Action**	**Nonspecific Effects**	**EC**
K252a	Tyrosine kinases	50nM[1] 200nM[2]	Competes with ATP for binding on kinase.[3]	PKA PKC PKG MLCK Ca / CaMKII	18nM[3] 25nM[3] 20nM[3] 20nM[4] 1.8nM[4]
Lavendustin A	Tyrosine kinases	44 nM[5] 0-100μM[6]	Competes with ATP for binding on kinase.[5] Noncompetitive inhibition with src.[5]	Low PKA and PKC effects	>100μM[5]
Orthovanadate	Tyrosine p'tases	20μM[7] 100μM[8]	Competes with phosphate group.[9]	Actin depolymerization Exchanger inhibition[10]	

[1] Wang, 1994
[2] Koizumi et al., 1991
[3] Kase et al., 1987
[4] Hashimoto et al., 1991
[5] Onoda et al., 1989
[6] Worley and Holt, 1996
[7] Wu and Bradshaw, 1992
[8] Mandell and Banker, 1998
[9] Seargeant and Stinson, 1979
[10] Dafnis and Sabatini, 1994

Finally, as with other proteins, some functions of kinases can be studied using immunochemical methods such as immunocytochemistry and Western blotting. Labeling activated proteins with antiphosphotyrosine antibodies can yield information about the phosphorylation state. Immunocytochemistry using a phosphotyrosine antibody is an effective method of determining when and where proteins are phosphorylated in the cell. Western blots using phosphotyrosine antibodies yield information concerning the size and phosphorylation state of proteins by separating proteins on the basis of mass.

A pharmacological approach was taken in this thesis to study how TKs, PTPs, and ultimately tyrosine phosphorylation are involved in neurite outgrowth. This method of analysis lacks some specificity, in that some of the inhibitors are capable of inhibiting other proteins aside from the desired ones (Nonspecific Effects, Table 1). However, it is the first of many steps, and does give some insight as to the role of these proteins in regulating the production of neurites. In the case of TKs, inhibitors with different nonspecific effects were used in an effort to implicate these kinases instead of S/T kinases.

Alternate approaches to pharmacological approaches include methods that alter protein expression or effectiveness, such as antisense RNA or dominant-negative. In an antisense approach, translation of a protein is reduced by introducing antisense mRNA. This method could be used to determine the specific role of proteins such as Ltrk, in the regulation of outgrowth. Dominant-negative approaches are usually performed with a protein that dimerizes, as in the case of the Trks (Kaplan, 1998). A plasmid coding for an ineffective protein (e.g. one that lacks a kinase domain) is introduced into cells. When

translation of the inserted protein occurs, the result is an inhibition of the wild type protein because the ineffective protein dimerizes with the wild type version.

Function of TKs in Neurite Outgrowth.

Evidence that points to the involvement of tyrosine phosphorylation in the regulation of neurite outgrowth is extensive yet sometimes contradictory. Trophic factors, such as BDNF and FGF, are known to signal through TK receptors, and cause neurite outgrowth (Bosco and Linden, 1999; McFarlane et al., 1996). *In vitro*, in postnatal rat hippocampal cells, tyrosine phosphorylation likely regulates outgrowth in axons and dendrites differently. Incubation with the PTP inhibitor orthovanadate resulted in a suppression of axonal initiation while the outgrowth of dendrites was unaffected (Mandell and Banker, 1998). If orthovanadate was added early enough, outgrowth could be prevented altogether. Orthovanadate also suppressed axonal elongation, suggesting that the process of axonogenesis and elongation in embryonic hippocampal neurons is dependent on a decrease in tyrosine phosphorylation (Mandell and Banker, 1998). Worley and Holt (1996) showed, *in vivo*, that herbimycin and lavendustin A prevented frog retinal ganglion axons from properly extending towards their midbrain target, the optic tectum. They concluded that tyrosine phosphorylation was responsible for regulating the neurite extension.

However there is also evidence that TKs negatively regulate neurite outgrowth. Embryonic chick forebrain neurons incubated with TK inhibitors (genistein or lavendustin A) *in vitro* potentiated neurite outgrowth, suggesting that tyrosine phosphorylation was playing an inhibitory role in neurite extension from these neurons

(Bixby and Jhabvala, 1992). These examples suggest that tyrosine phosphorylation likely regulates the initiation and elongation aspects of neurite outgrowth.

Studies of neurite outgrowth in cultured motoneurons neurons from the snail, *Helisoma trivolvis,* suggested that TK activity was required for both the initiation and elongation processes of neuronal regeneration *in vitro* (Wang, 1994). Wu and Goldberg (1993) have shown that TK activity is necessary for the formation of filopodia after axotomy of cultured neurons from *Aplysia.* This group went on to show, using immunocytochemistry, that the punctate phosphorylation seen in the filopodia of neurons from this snail was colocalized with β integrin. Furthermore, the appearance of integrins virtually never occurred without being first preceded by phosphotyrosine immunoreactivity. Finally, they showed that in cultured mouse neurons, tyrosine phosphorylation was also associated with proteins that immunochemically resemble members of the ezrin-radixin-moesin family of proteins. This group of proteins is involved in regulating actin dynamics in cells, which suggests that TKs are in a position to facilitate communication between the ECM and the neuronal cytoskeleton.

A number of recent reports have implicated TKs in the guidance of neurites both *in vivo* and *in vitro.* For example, when grasshopper embryos were incubated in TK inhibitors (herbimycin A or genistein) there was an increase in the number of neurons which crossed the midline of the CNS. This suggests that tyrosine phosphorylation is responsible for transducing an inhibitory signal that prevents some axons from crossing the midline (Mennon and Zinn, 1999). This conclusion is consistent with a growing body of evidence that molecules traditionally known only as trophic factors, are also able to act as tropic molecules to certain groups of neurons (e.g. NGF; Letourneau). Keeping in

mind that most known receptors of trophic factors are TKs, this suggests that TKs are involved in the guidance of neurites.

The gastropod molluscs, (i.e. slugs and snails) have contributed significantly to the understanding of synaptic plasticity and axonal regeneration of adult neurons. Molluscs of the subclass opisthobranchs, e.g., the marine snail *Aplysia,* have been particularly instrumental in understanding synaptic plasticity and learning. Molluscs of the pulmonate subclass (e.g. *Lymnaea* and *Helisoma*) have been the subject of many studies of axonal regeneration and synapse formation (Bulloch and Syed, 1992; Moffett, 1995).

This study of axonal regeneration uses neurons isolated from adult snails (*Lymnaea stagnalis*) to establish whether tyrosine phosphorylation regulates the first two stages of neurite outgrowth: initiation and elongation. Pharmacological inhibitors were employed to investigate the role of TKs and PTPs. This research indicates that TKs regulate both the initiation and elongation stages of axonal regeneration. Inhibitor experiments conducted at two time points, specific for both initiation or elongation, suggest that TK activity is only necessary to initiate these two processes and is not required for their maintenance.

MATERIALS AND METHODS

ANIMALS

A laboratory stock of the mollusc *Lymnaea stagnalis* was raised in aerated artificial pond water (0.25 g/L Instant Ocean, Aquarium Systems) in a 12 hour light/dark cycle at room temperature, and fed lettuce and Trout Chow (Purina). Snails that had shell lengths of 18-25 mm (3-4 months in age) were used for cell culture, whereas snails with shell lengths of 25-30 mm (4-5 months in age) were used for preparing brain-conditioned medium. This animal stock was originally derived from the Free University of Amsterdam, and has been inbred for at least 100 generations.

SOLUTIONS AND MEDIA

CELL CULTURE

Normal saline was made up in water from a Super Q filtration system equipped with deionisation and activated carbon filters (Millipore Inc) and contained the following (in mM): NaCl, 51.3, KCl, 1.7, $CaCl_2$, 4.1, $MgCl_2$, 1.5, N-3-hydroxyethlypiperazine-N'-2-ethanesulfonic acid (HEPES), 5.0, final pH 7.9. Antibiotic saline (ABS) was prepared by adding 150 μg/ml of gentamycin (Sigma G-3632) to autoclaved normal saline. Defined medium (DM) was 50% diluted, serum-free, Leibovitz L-15 (GIBCO) medium containing final concentrations of the following: (in mM) NaCl, 40.0, KCl, 1.7, $CaCl_2$, 4.1, $MgCl_2$, 1.5, HEPES, 5.0, glutamine, 1.0 and 20μg/ml gentamycin (final pH 7.9). The medium was specially purchased in a salt-free form (containing only vitamins and amino acids) so that salts could be added, to ensure that the medium was appropriate for

Lymnaea. The 50% dilution of L-15 media was required because this medium was originally designed for mammalian cell culture. This dilution factor has been shown to produce successful results in the single cell culture of *Lymnaea* and *Helisoma* neurons (Wong et al 1981, Ridgway et al., 1991; Syed et al., 1996).

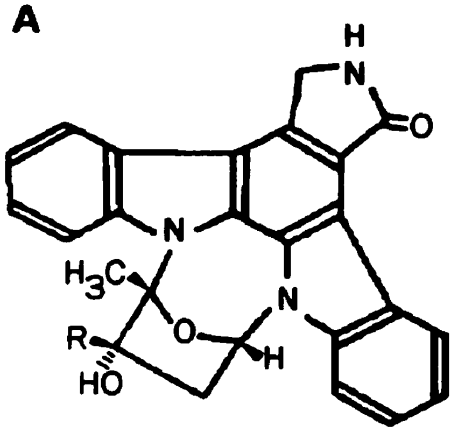
To prepare brain-conditioned medium (CM), *Lymnaea* CNS were isolated under sterile conditions, by deshelling and anesthetizing the animal in 25% (v/v) menthol-containing-Listerine (Warner-Lambert) in saline for 10 minutes. Snails were pinned, dorsal surface up, in a sterile dissecting dish containing ABS. The central ganglionic ring was removed through a midline incision and washed at least 5 times for 5 minutes each in ABS. The CNSs were then incubated in DM (2 CNSs/ml) in silicon-coated dishes (Sigmacote, Sigma) for 72 hours. The CNSs were subsequently removed, and deionized water was added to replace medium lost to evaporation. This mixture was then filtered through a 0.2 μm filter (Acrodisc), divided into aliquots, and frozen at –80 ^{0}C until needed. CM was thawed as required for experiments and diluted 1:1 with DM. This dilution has been empirically determined to be an optimal concentration to elicit neurite outgrowth from cultured *Lymnaea* neurons (Ridgway et al., 1991).

REAGENTS

Reagents were obtained from the following sources: K252a (Kamiya), K252b, (Kamiya), Lavendustin A (Sigma), and sodium orthovanadate (Aldrich; Fig. 2). K252a, K252b, and lavendustin A were each dissolved in dimethyl sulfoxide (DMSO), and divided into aliquots. These concentrated stock solutions were then stored at -20 ^{0}C until use.

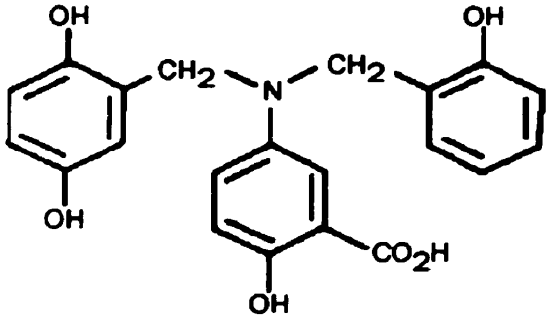
Figure 2: Molecular structure of molecules related to the current thesis. ***A-C.*** Structures of inhibitory reagents. ***A.*** The staurosporine alkaloid derivatives, K252a and K252b. K252a inhibits tyrosine kinases by competing with ATP (***D.***) for binding (Kase et al., 1987). K252b is an inactive analogue of K252a. ***B.*** Structure of Lavendustin A. This compound inhibits tyrosine kinases by competing with ATP for binding to the kinase (Onoda et al., 1989). ***C.*** Structure of sodium orthovanadate. This reagent is thought to inhibit a broad spectrum of tyrosine phosphatases by acting as a phosphate analogue (***E.***) (Seargeant and Stinson, 1979). ***D.*** Chemical structure of adenosine triphosphate (ATP). *A-C.* act by mimicking structural aspects of ATP that allows them to compete with ATP for binding to a tyrosine kinase. ***E.*** Chemical structure of a phosphotyrosine residue.

A

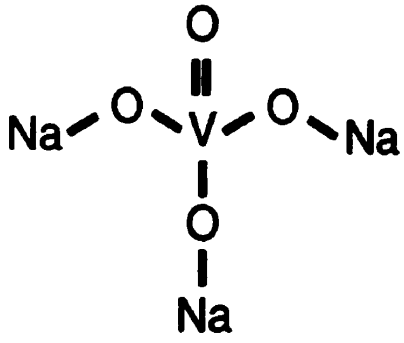


Compound	R
K252a	$COOCH_3$
K252b	COOH

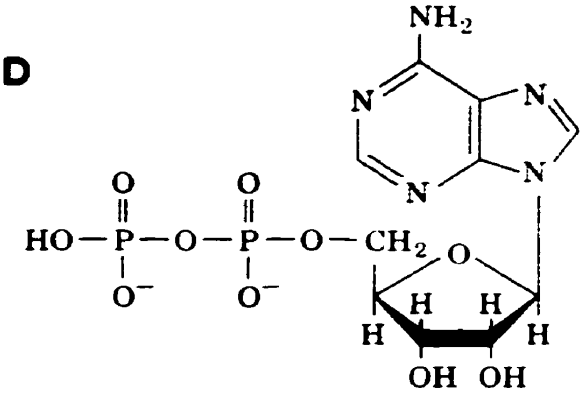
B



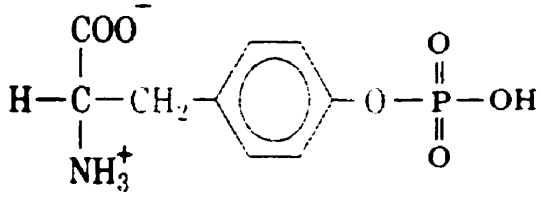
C



D



E



When required, a frozen aliquot was thawed, and DM was added to make a 10x stock solution. 200 µl of 10x concentrate was added to 1800 µl of culture medium. Control for these reagents was an equivalent concentration of DMSO (0.05-0.5 % final concentration), in culture medium.

K252a and lavendustin A were chosen for their ability to inhibit the function of tyrosine kinases by competing with ATP for binding to the kinase (Kase et al., 1987; Onoda et al., 1989). Both of these inhibitors have been shown to be effective in molluscan systems. Using Western blots, Wu and Goldberg (1992) demonstrated that lavendustin A was effective at inhibiting src, which is a cytoplasmic TK enriched in the growth cone of neurons from *Aplysia* and mammals. A thorough literature search indicates that lavendustin A has never been employed as anything but a TK inhibitor. Moreno et al (1999) demonstrated that K252a was effective at preventing NGF induced tyrosine phosphorylation in molluscan neurons, suggesting that this reagent is an effective inhibitor of TK activity in these cells.

Although K252a has been shown to inhibit S/T at concentrations in the range of those used in the current experiments. Lavendustin A has been shown to not inhibit S/T kinases at the concentrations used in these experiments (Table 1). Therefore, similar consequences resulting from incubation with either TK inhibitor, suggest that the effect is due to TK inhibition, and not the inhibition of an S/T kinase.

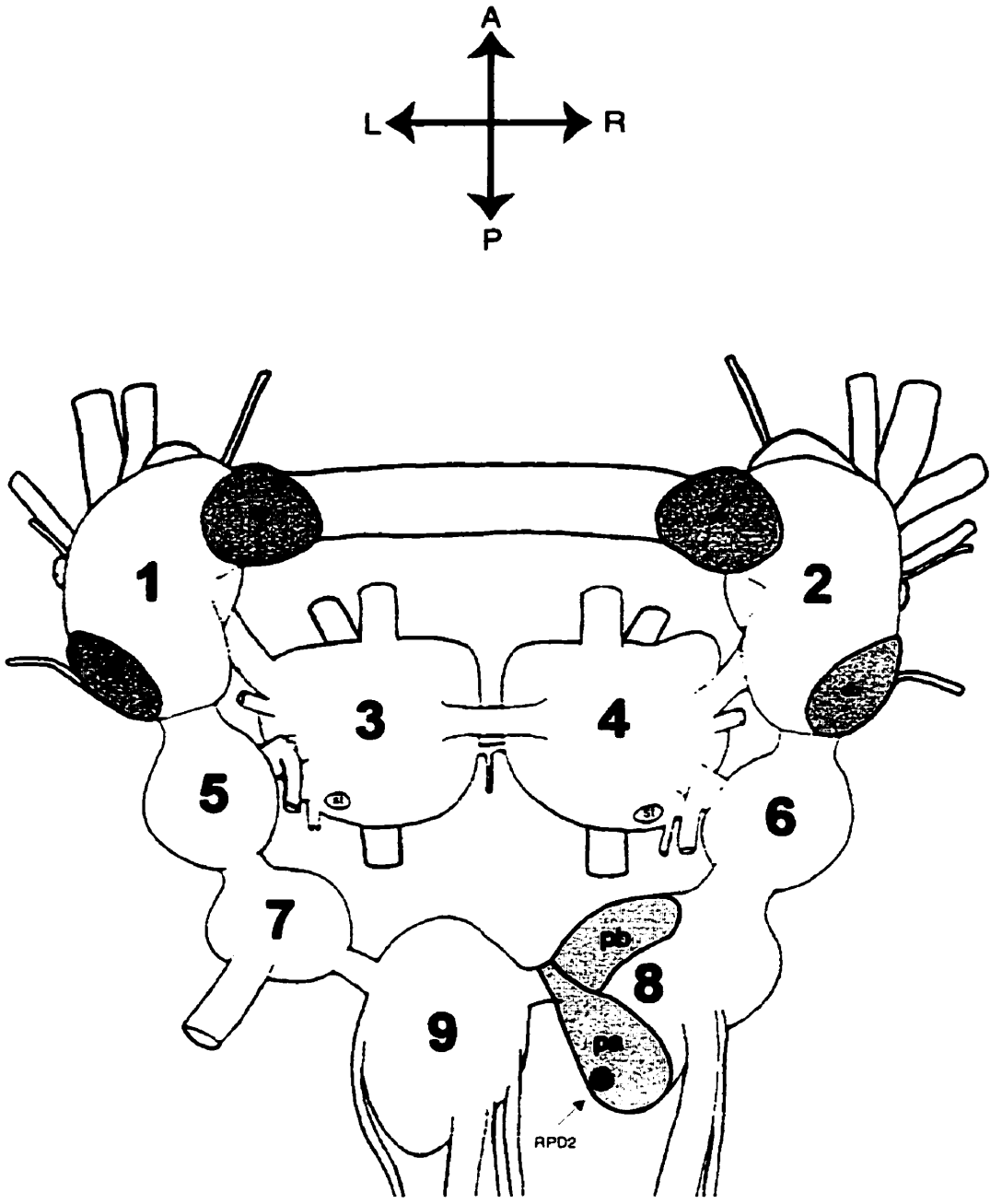
Sodium orthovanadate was chosen for its ability to inhibit tyrosine phosphatases (Kodota et al., 1987; Laniyonu et al., 1994; Mandell and Banker, 1998). This reagent was prepared fresh daily by dissolving it in DM to produce a 10x solution, and diluted 1:10

with culture medium when added to the culture dish. Controls for orthovanadate were prepared by adding 200 µl of DM to 1800 µl of culture medium.

CELL CULTURE

Cells of a specific group in the right parietal ganglion of the *Lymnaea* CNS were chosen as the focus of this study (Fig. 4). These neurons, known as the RPA cells, have been investigated in a number of cell adhesion, outgrowth, and physiological studies (Wildering et al., 1995; Syed et al., 1992). Although they do not express the recently cloned molluscan receptor tyrosine kinase, Ltrk, these cells do respond to the mammalian growth factors, NGF and CNTF, by producing outgrowth (Fainzilber et al., 1996; Syed et al., 1992: Syed et al., 1996). Considering that the majority of growth factors, both for vertebrates and invertebrates, signal through tyrosine kinase receptors, it is likely that sprouting in these and other *Lymnaea* neurons is regulated by tyrosine phosphorylation. These cells were chosen as the subject of this study partly because they have been well characterized (Wildering et al., 1998, 1995; Syed et al., 1992). Additionally, these cells are both plentiful and hardy, two factors that facilitate the removal of single cells. Wildering et al. (1998) detailed the methods used for culture of single *Lymnaea* neurons. Briefly, the CNSs were removed from snails as previously described above and incubated in DM containing 0.67-1.0 mg/ml collagenase/dispase (Boehringer Mannheim) and 0.67 mg/ml trypsin (Sigma, type III) for 15-18 minutes at room temperature (20-23°C), subsequently followed by incubation in 0.67 mg/ml soybean trypsin inhibitor (Sigma, type I-S) for 17 minutes. Enzyme incubation times were determined empirically on a day-to-day basis. The amount of time that a CNS was digested depended on how easily cells

Figure 3: Dorsal view of the CNS of the pond snail *Lymnaea stagnalis*. The CNS is composed of 9 ganglia joined together to form a ring-like structure. Numbered ganglia represent the following: 1-2, L and R cerebral ganglia, 3-4, L and R pedal ganglia, 5-6, L and R pleural ganglia, 7-8, L and R parietal ganglia, and 9, visceral ganglion. Abbreviations: R = right, L = left, db = dorsal body, st = statocyst, A = anterior, P = posterior, D = dorsal, V = ventral, pa = parietal A cells, pb = parietal B cells, RPD_2 = right parietal dorsal 2 (a large white cell which is usually a visible landmark in the parietal ganglia). RPA cells are a cluster of about 30 light-coloured cells found caudal to the RPB cells on the dorsal side of the CNS.


A
L
R
P
1
2
3
4
5
6
7
8
9
Pb
Pa
RPD2

were removed and/or on their condition upon removal. All experiments were done by digesting equal number of CNSs in two sequential digests. If removal of the neurons was difficult after the first digestion, the digestion times were adjusted to improve cell quality as needed in the second digestion. These nervous systems were placed in high-osmolarity DM (DM + 30 mM D-glucose; HO) for 30-40 minutes. Following this incubation the CNSs were pinned, dorsal surface up, to a sterile silicone-rubber-lined dissection dish also containing HO. The connective-tissue sheaths were removed from the parietal ganglion with fine forceps. Single cells were individually removed from the desheathed ganglia by applying light suction to the cell using a silicon-coated (Sigmacote), fire-polished pipette (40-70 μm diameter) attached to a volume micrometer syringe (Gilmont, GS 1100). Cells were then placed into 35mm culture dishes (Falcon 3001) previously coated with 0.1 % poly-L-lysine, as described below, containing either 2 ml DM or CM. Plated cells were incubated in a dark humidified chamber at room temperature (20-23 °C) for 24-72 hours.

CULTURE DISHES

Falcon 3001 dishes were incubated with a 1 mg / ml sterilized (0.2 μm filter) solution of poly-L-lysine (Sigma) in saline for 8-16 h. Following this incubation, the lysine solution was removed by aspiration, and dishes were washed 3 times using sterile, Super Q water and allowed to dry. Dishes were stored at 4 ^{0}C for up to three months until required for use.

MICROPHOTOGRAPHS

All bright-field photographs of cells were taken on a Zeiss inverted microscope, equipped with the following objectives (numerical apertures listed in brackets): 2.5 x (0.05), 10 x (0.25), 20 x (0.25) and 40 x (0.75). Film used was Kodak Techpan (50 ASA).

CELL VIABILITY

DYE EXCLUSION ASSAY

Cells were cultured as above and incubated for 72-96 h. Culture medium was gradually replaced, over the span of 5 minutes, with saline containing 0.4 % trypan blue (w/v). Cells were incubated in this solution for an additional 10 minutes, and the dye was subsequently washed out with normal saline.

Cells which were not attached to the substrate for the full length of the experiment were not scored. All cultured cells were assessed for viability by judging brightness when viewed under phase-contrast conditions on a Zeiss inverted microscope. Cell attachment to the substrate was assessed by lightly tapping on the body of the microscope.

OUTGROWTH ANALYSIS

PRIMARY NEURITE NUMBER AND RADIAL LENGTH

Neurite outgrowth was assayed using a modified version of a method originally developed by D.A. Sholl (1953). A photograph of a 1 mm micrometer was taken using the 2.5 and 10 x objectives on the same Zeiss inverted microscope used to photograph all cultured cells. These photographic negatives were used to calibrate a measuring graticule, which had ten equally spaced concentric rings, as well as measuring gradations up to the

equivalent of 15 additional rings. The radial distance between adjacent rings was determined to be equivalent to 120 and 30 μm for the 2.5 x and 10 x objectives, respectively. For analysis, the smallest ring (equal to about 30 μm in diameter, under the 10x objective) was centered on a microphotographic negative of each cell, and sprouting criteria, which will be described shortly, were determined for each neuron (Fig. 4).

Neurite Initiation Studies

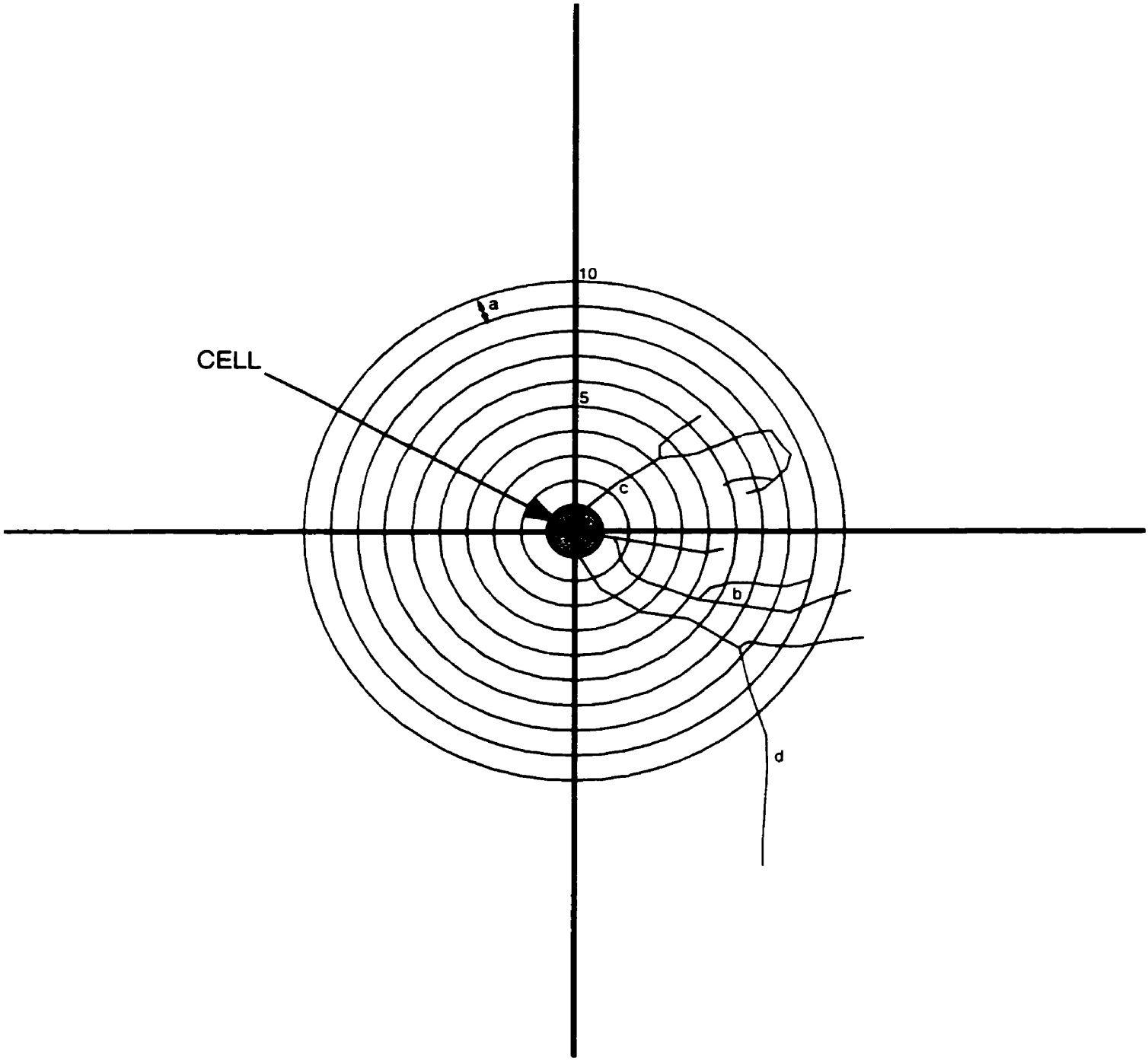
The purpose of these experiments was to determine whether treatment with a pharmacological inhibitor (either a tyrosine kinase inhibitor or a tyrosine phosphatase inhibitor) had an effect on the number of primary neurites that a cell could produce. For all experiments, a primary neurite was defined as a projection which originated directly from the cell body and extended for a distance of at least 30 μm

To study the effects of inhibitors on neurite initiation, neurons were cultured in CM containing a tyrosine kinase inhibitor (K252a or lavendustin A), tyrosine phosphatase inhibitor (sodium orthovanadate), or vehicle (control), for 24-72 h. Microphotographs of each cell were taken at specific times. The 35 mm microphotographic negatives of cells were analyzed as described above. The number of primary neurites produced by each cell was determined, and an average number of primary neurites per cell was calculated for all the cells in each condition.

Neurite Elongation Studies

Changes in neurite elongation were determined using photographs of neurons taken before and after a period of incubation with CM containing either an inhibitor or

Figure 4: Analysis of neurite outgrowth. A graticule with 10 equally spaced concentric rings was centered on the microphotographic negative of a cell. The radial distance between adjacent rings was equivalent to 30 μm ('a'), once magnification was taken into account. Primary neurite length was measured by determining the farthest ring from the cell body that was crossed by the neurite (radial length), e.g. 'b' = 10 (300 μm), 'c' = 8 (240 μm). The determination of radial length of a neurite such as 'd' was assisted by graduations on the crosshairs of the graticule (not shown).


10
a
CELL
5
c
b
d

dissolving vehicle (control). From these photographs the total radial extension for all primary neurites, at each time, was determined and an average calculated for all cells in a condition.

For studies concerning neurite elongation which occurred 0-24 h following cell plating ("early" elongation), inhibitors or solvent only (i.e. control) were added to the CM contained in a culture dish at the time of plating. Microphotographs were taken of cells in each condition at 24 h intervals following cell plating. The negatives of the microphotographs were used to determine the total radial primary neurite length for cells at each time. Cells were numbered so that analysis could be performed on the same cell over a period of time.

For studies of the neurite elongation that occurred 24-72 h following cell plating ("ongoing" elongation), cells were cultured in CM for 24 h, at which point microphotographs were taken. Immediately afterward, inhibitors or control reagent (DMSO) were added to the culture dishes in appropriate concentrations, and the cells were again incubated. After 48h and 72 h total incubation time, the neurons were photographed again. In both studies of elongation, cells which had not produced neurites by 24 h were not included in the statistical analysis.

STATISTICS

Significance in studies concerning both neurite initiation and neurite elongation experiments was tested using two way ANOVA. This method was chosen for statistical analysis since it compares trends in the data and not specific points, making it an appropriate test of significance for this type of data. All data from elongation experiments

were log-transformed to normalize the data. (Sokol and Rohlf, 1981). Significant differences in the number of cells sprouted in each condition (a binomial quantity) in the initiation experiments were tested using Fisher two by two contingency tables. Differences were considered significant if $p < 0.05$. Levels of significance were represented in the following manner: $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = ***. SEMs were used to represent data variance in graphs since this measure displays the variance of the mean instead of variance of the sample.

RESULTS

Neuronal regeneration involves a number of steps, each of which might be independently controlled by different signaling mechanisms. The signaling pathways that are actually involved in processes such as initiation and elongation are relatively unknown, but there is a great deal of evidence to suggest that tyrosine kinases play key roles as transducers of growth cues. For example, neurons from *Lymnaea stagnalis* sprout neurites when isolated and cultured in brain conditioned medium (CM). However, cells that are cultured in defined medium (DM), which contains no exogenous growth factors, produce no signs of organized outgrowth (e.g. Ridgway et al., 1991; Syed et al., 1992; Wildering et al., 1998). This is true of all types of neurons examined to date in *Lymnaea*, which include 6 groups of motoneurons, 3 classes of interneurons, and 8 groups of neurosecretory cells (Ridgway et al, 1991; Syed et al., 1996). In the present study, I examine the role of tyrosine kinases in both the initiation and elongation phases of neurite outgrowth. To this end, cultured neurons were exposed to inhibitors affecting the regulation of tyrosine phosphorylation.

Previous studies of neurite outgrowth involving both *Helisoma* and *Lymnaea* neurons, *in vitro,* used a nonquantitative scoring procedure which designated a cell as sprouted or not sprouted. The criterion for scoring a cell as sprouted was the presence of one of more neurites of at least one soma-diameter in length (e.g. Wong et al., 1981; Ridgway et al., 1991; Wildering et al., 1998). This method was inadequate for the present study, which required more and better quantitative measurements of, for example, neurite length.

For this reason I introduced a new method of analysis (see Material and Methods) that quantifies both the number and the radial length of each primary neurite.

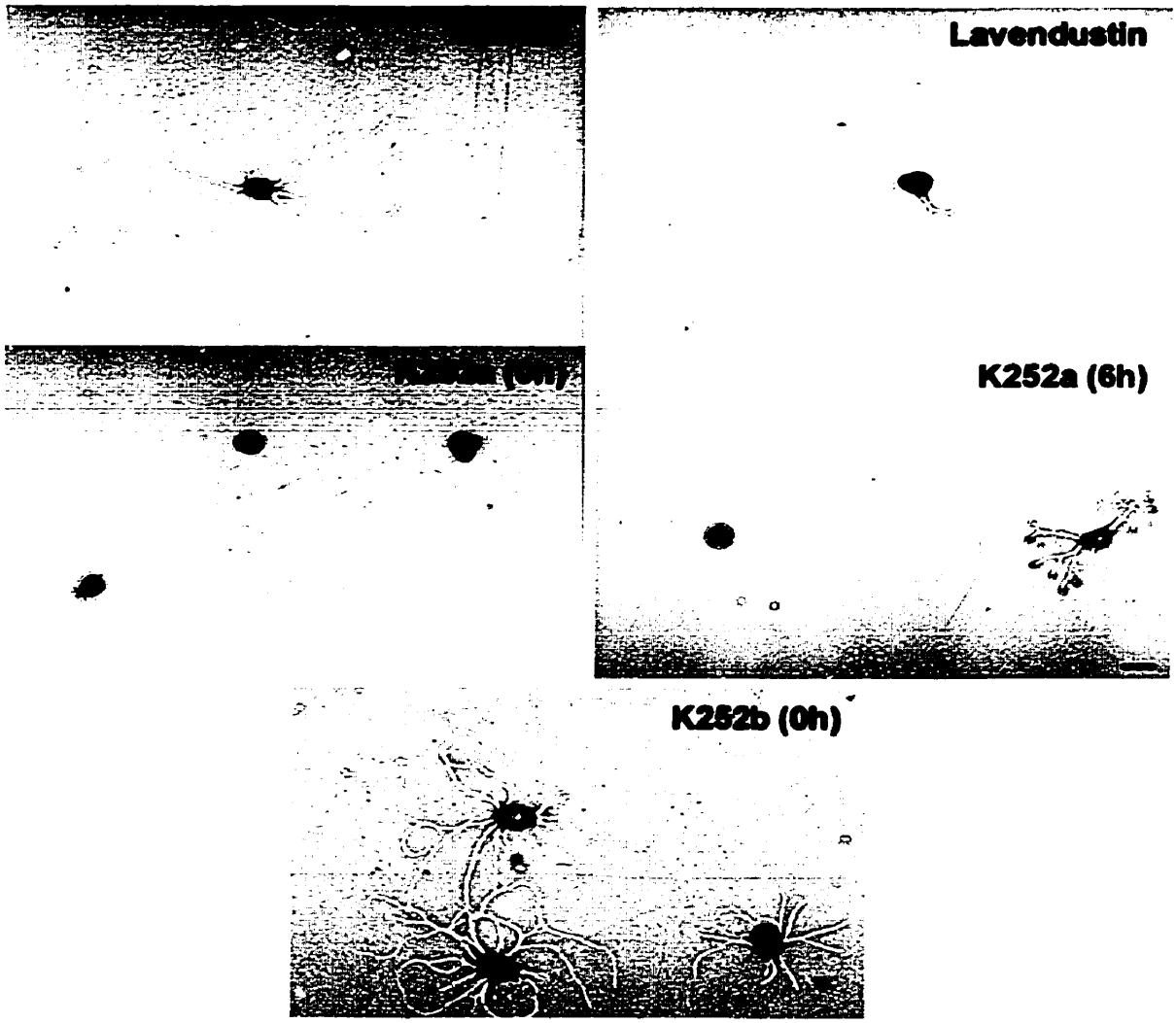
Neurite Outgrowth

To understand how changes in the levels of tyrosine phosphorylation affect outgrowth, it is necessary first to define the pattern of outgrowth exhibited by neurons in CM. Previous investigations have shown that when right parietal A (RPA) neurons are cultured in CM, 70-80 % will exhibit robust outgrowth after 48 h in culture (Ridgway et al., 1991; Wildering et al., 1998). This result was confirmed by the current study. Sprouting was usually initiated about 10-12 h following plating. Typically, sprouted neurons developed 1-12 primary neurites (average 2-4) that reached up to 600 μm in length, after 24 h in culture. By 72 h, neurite outgrowth approached a plateau of 4-5 neurites per cell that were up to 900 μm in length (control, Fig. 5 and 9). The extent of outgrowth varied between experiments; for this reason a control condition was always included in each experiment, and all the outgrowth was scored blindly.

The Effects of Tyrosine Kinase Inhibitors on Neurite Initiation

Cells were plated in CM with or without one of the following tyrosine kinase inhibitors: 100 nM K252a or 20 μM lavendustin A. These concentrations were based on previous studies of K252a with cultured neurons from the related snail *Helisoma* (Wang, 1994). Lavendustin A concentrations were based on values commonly used in previous studies with various cell types (e.g. Wu and Goldberg, 1993; *Aplysia*; Worley and Holt, 1996; *Xenopus*). Control neurons were cultured in CM containing an equivalent amount

Figure 5: The effects of 20 μM lavendustin A or 100 nM K252a on neurite initiation. Neurons were cultured in CM containing 0.02% DMSO (Control), 20 μM lavendustin A (Lavendustin), 100 nM K252a added before cell plating (K252a, 0 h), 100 nM K252a added 6h after cell plating (K252a, 6 h) or 100 nM K252 (K252b) added before plating. Photomicrographs were taken 48h after cell plating. Scale bar in bottom right photo = 50 μm. Contrast and brightness of the photos were altered to enhance print quality.

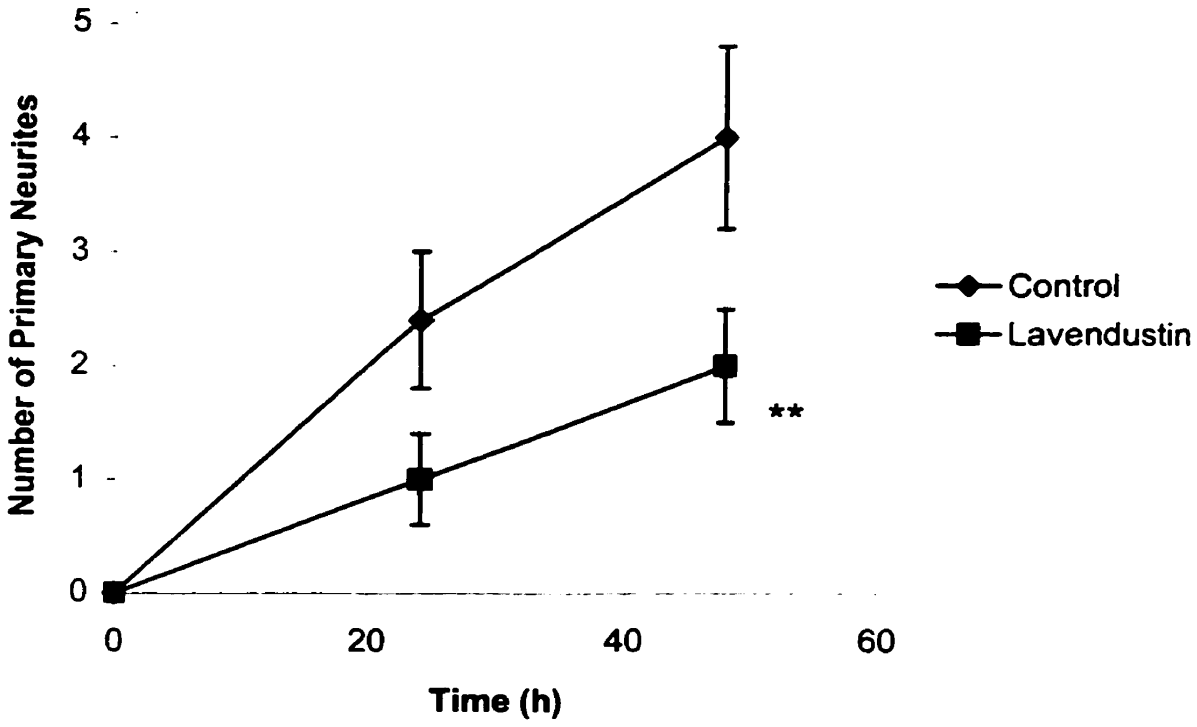

Lavendustin
K252a (6h)
K252b (0h)

of the vehicle (DMSO) used to dissolve the inhibitors. In this case the final concentration of DMSO in CM was 0.02% (v/v). Control cells incubated for 48 h in CM produced robust outgrowth similar to that described above (Fig. 5). Conversely, neurons incubated in either 20 μM lavendustin A or 100 nM K252a either did not respond to CM, or did so by producing short neurites (Fig. 5).

We next quantified the effects of lavendustin A on the initiation of neurite outgrowth as described in the Materials and Methods. Control neurons exhibited 2.4 ± 0.6 and 4.0 ± 0.6 primary neurites after 24 h and 48 h in culture, respectively. Numerically, the extent of this outgrowth was comparable in all control neurons throughout this study. In contrast, neurons incubated in 20 μM lavendustin A produced 0.9 ± 0.4 and 1.8 ± 0.6 primary neurites after 24 h and 48 h, respectively ($p < 0.01$, Fig. 6, Table 2). Thus, cells incubated for 48 h in lavendustin A, exhibited 50% fewer neurites than controls. While 56% and 76% of cells responded under control conditions after 24 and 48 h, respectively, only 20% and 50% of the lavendustin A-treated cells produced outgrowth after 24 h and 48 h (Table 2). Therefore, neurons cultured in lavendustin A were less likely to initiate neurites after 24 h incubation, and those that did sprout produced fewer processes than controls, after 24 and 48 h.

The next series of experiments involved the TK inhibitor K252a, a reagent that has often been employed to study the function of tyrosine kinases including some families of receptor tyrosine kinases (e.g. Trks: Koizumi et al., 1988; Levine, et al., 1995; de Bernardi, et al., 1996). I expanded the scope of this investigation in two respects: First,

Figure 6: The effects of 20 μM lavendustin A, added before cell plating, on the initiation of neurite outgrowth. Cells were cultured in CM plus one of the following (final concentrations), 0.02% DMSO (control) or DMSO + 20 μM lavendustin. Primary neurites for each cell were counted using the photographic negatives taken at 24 and 48 h. Each conditioned combined the results fromthree separate plating sessions. Cell numbers for each condition are as follows, Control, n = 25, and lavendustin = 20. Error bars represent SEM. ** = $p < 0.01$ vs control.


5
4
3
2
1
0
Number of Primary Neurites
0
20
40
60
Time (h)
**
Control
Lavendustin

24h Treatment With 20 μM Lavendustin A

A	Control	Lavendustin A	p vs. control
Primary neurites (PN) per cell	2.4 ± 0.6	0.9 ± 0.4	< 0.01
Cells With PN (%)	56	20	< 0.05
Cell Number	25	20	N/A

48h Treatment With 20 μM Lavendustin A

B	Control	Lavendustin A	p vs. control
Primary neurites (PN) per cell	4.0 ± 0.6	1.8 ± 0.6	< 0.01
Cells With Primary Neurites (%)	76	50	n.s.
Cell Number	25	20	N/A

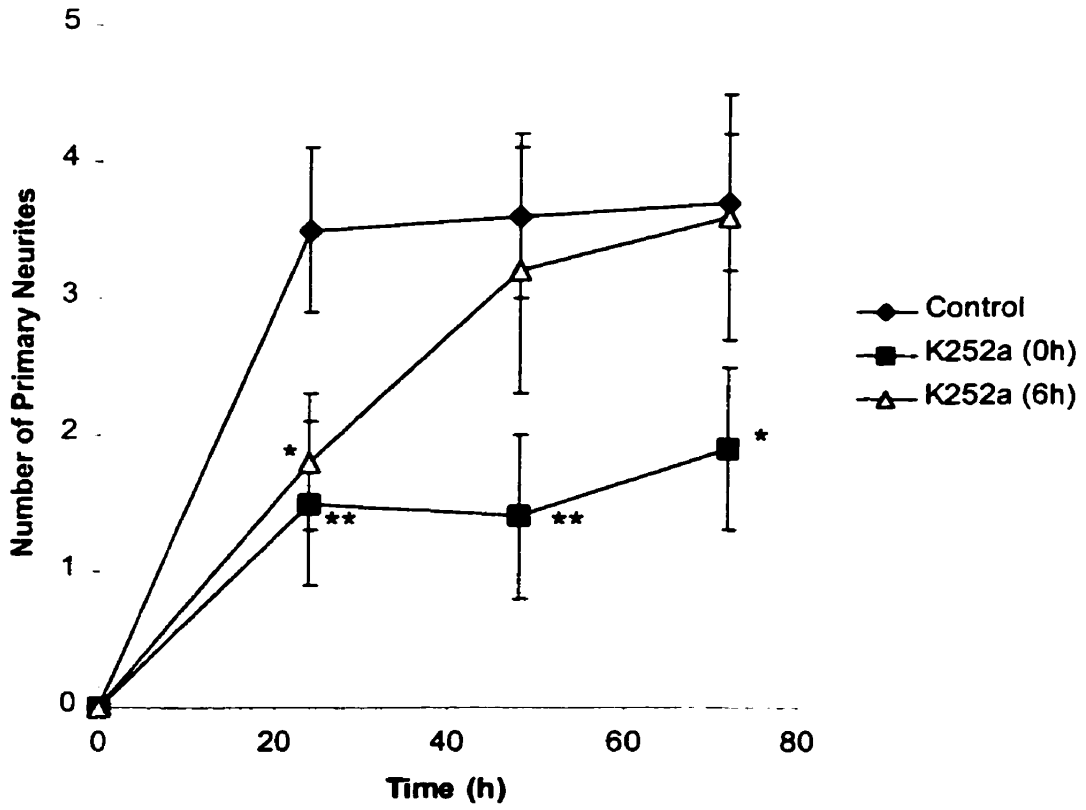
Table 2: Summary of the effects of lavendustin A on neurite initiation after 24 h (***A***) and 48 h (***B***). N / A = not applicable

the time was extended to 72 h; second, the reagent was applied either before ('0 h') or after completion of cell plating ('6 h').

In this series of experiments, the neurite outgrowth of cells under control conditions reached a plateau at 24 h, reinforcing the importance of matched controls (Fig. 7). The number of primary neurites developed by control cells was 3.5 ± 0.6, 3.6 ± 0.6, and 3.7 ± 0.5 after 24, 48, and 72 h respectively. Fewer primary neurites were produced when neurons were plated into medium containing 100 nM K252a (i.e. '0 h' data set). When exposed to 100 nM K252a after 0 h, 1.5 ± 0.6, 1.4 ± 0.6 and 1.9 ± 0.6 primary neurites were produced by neurons by 24, 48, and 72 h respectively (Table 3). Thus, by 72 h, neurons plated in medium containing K252a had produced 51% fewer neurites than controls. As in the case of lavendustin A, neurons plated in K252a exhibited a reduced overall level of responsivity, since only 46 % of experimental neurons sprouted neurites compared to 80 % of controls.

The results were strikingly different when 100 nM K252a was added to the CM after neurons were plated. Cells that were exposed to K252a 6 h after plating developed 1.8 ± 0.5, 3.2 ± 0.9 , and 3.6 ±0.9 primary neurites after 24, 48 and 72 h respectively (Fig. 7). It is important to note that neurons had not yet produced visible neurites by 6 h, when this reagent was added. By 24 h, neurons cultured in K252a had developed primary neurites, but significantly fewer than controls (3.5 ± 0.6, $p < 0.01$), and only half (48%) of these neurons had produced neurites, compared to 70 % of controls ($p < 0.05$, Table 3). However by 48 h, the number of primary neurites produced by cells cultured in K252a (6 h) reached the control level, and 56 % of experimental cells had initiated outgrowth

Figure 7: The effects of 100 nM K252a, added before or 6h after cell plating, on the initiation of neurite outgrowth. Neurons were plated in CM containing 0.02 % DMSO (Control), or DMSO + 100 nM K252a (K252a (0 h)) added before cell plating, or DMSO + 100 nM K252a (K252a (6 h)) added about 6h after beginning cell plating. Primary neurites for each cell were counted using the photographic negatives taken at 24, 48 and 72 h. Each conditioned combined the results fromtwo separate plating sessions. Cell numbers for each condition were as follows, Control n = 25, K252a before, n = 24, and K252a after n = 25. Error bars represent SEM. * = $p < 0.05$, ** = $p < 0.01$ vs control.


5
4
3
2
1
0
Number of Primary Neurites
0
20
40
60
80
Time (h)
Control
K252a (0h)
K252a (6h)
*
**
**
*

A	Control	K252a (0h)	p vs. control K252a (0h)	K252a (6h)	p vs. control K252a (6h)
Primary neurites (PN) per cell	3.5 ±0.6	1.5 ± 0.6	< 0.01	1.8 ± 0.5	< 0.05
Cells With PN (%)	70	33	< 0.01	48	< 0.05
Number of Cells	25	24	N/A	25	N/A

B	**Control**	K252a (0h)	p vs. control K252a (0h)	K252a (6h)	p vs. control K252a (6h)
Primary neurites (PN) per cell	3.6 ± 0.6	1.4 ± 0.6	< 0.01	3.2 ±0.9	n.s.
Cells With PN (%)	76	33	< 0.01	56	n.s.
Number of Cells	25	24	N/A	25	N/A

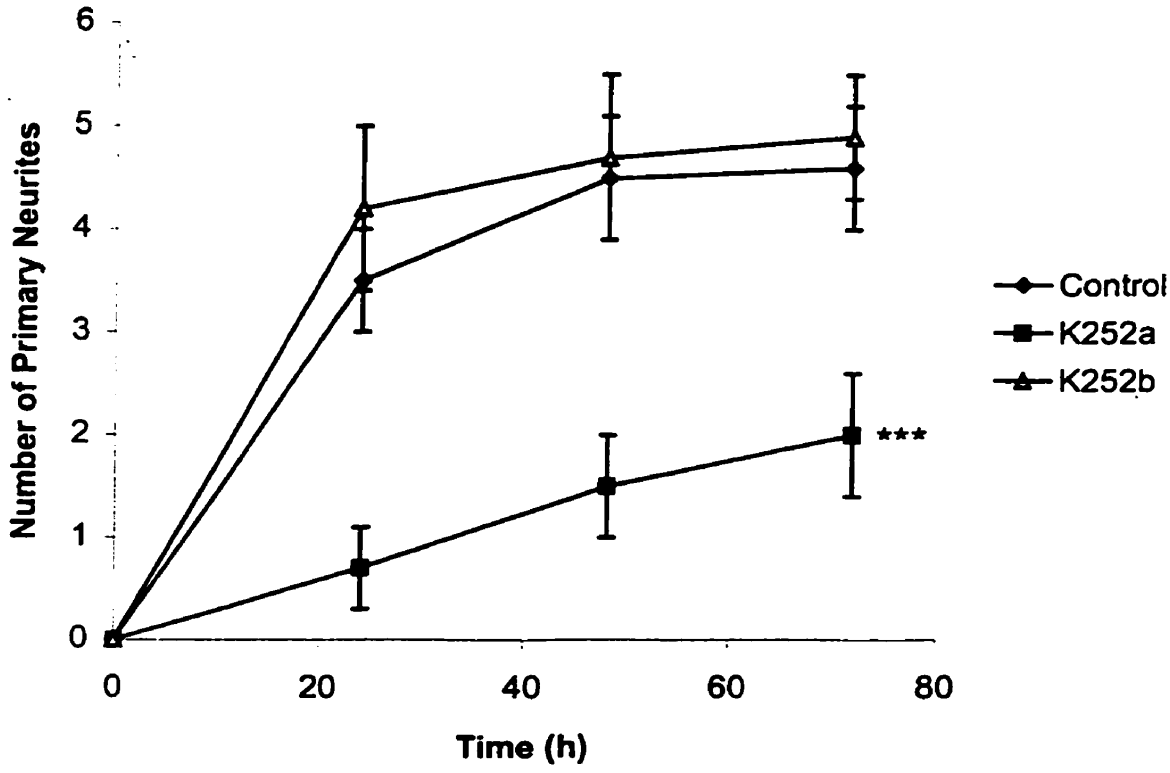
C	Control	K252a (0h)	p vs. control K252a (0h)	K252a (6h)	p vs. control K252a (6h)
Primary neurites (PN) per cell	3.7 ± 0.5	1.9 ± 0.6	< 0.05	3.6 ± 0.9	n.s.
Cells With PN (%)	80	46	n.s.	56	n.s.
Number of Cells	25	24	N/A	25	N/A

Table 3: Summary of the effects of K252a on neurite. RPA neurons were cultured in 100 nM K252a added to the CM before plating (K252a (0 h)) or following cell plating (K252a (6 h)). Control neurons were cultured in CM containing 0.02% DMSO (v/v). ***A.*** Outgrowth of cells after 24 h in culture. ***B.*** Outgrowth of cells after 48 h in culture. ***C.*** Outgrowth of cells after 72 h in culture. n.s. = not significant. N / A = not applicable.

(Table 5). This value was not significantly lower than that for control neurons, 76 % of which had produced neurites by this time ($p > 0.05$). These findings suggested that K252a is less effective at suppressing neurite initiation when added to culture medium 6 h after plating than when present at the moment of plating.

In a separate set of experiments, an inactive analogue of K252a, K252b (Fig. 4), was applied to cultured cells, to serve as an additional control (Fig. 5). In these experiments the reagents were added to CM before the neurons were added, and subsequently incubated. After 24, 48 and 72 h, control neurons exhibited 3.5 ± 0.5, 4.5 ± 0.6, and 4.6 ± 0.6 neurites per cell, respectively (Fig. 8). Similarly, neurons treated with 100 nM K252b had developed 4.2 ± 0.8, 4.7 ± 0.8, and 4.9 ± 0.6 neurites after 24, 48, and 72h, respectively (Fig. 8). These values were not significantly different from those for controls ($p>0.05$, ANOVA). In this series of experiments, neurons that were plated in CM containing 100 nM K252a exhibited a suppression of initiation that was similar to that shown by neurons cultured in the same conditions discussed above (compare Fig. 7 and 8). Neurons treated with K252a had developed 0.7 ± 0.4, 1.5 ± 0.5, and 2 ± 0.6 neurites after 24, 48, and 72h respectively. This was significantly different from the rate of initiation in neurons cultured in control conditions or treated with 100 nM K252b ($p < 0.001$, ANOVA). These experiments further support the notion that TKs are responsible for initiating neurite outgrowth. Incubation with the TK inhibitor K252a, again suppressed the initiation of neurites. Neurons incubated with K252b, a reagent similar to K252a, that is incapable of inhibiting TKs but still able to inhibit S/T kinases at the same concentrations as to K252a, exhibited the same number of primary neurites as controls.

Figure 8: The effects of 100 nM K252a or 100 nM K252b added to CM before cell plating on the initiation of neurite outgrowth. Neurons were plated in CM containing 0.02 % DMSO (Control), or DMSO + 100 nM K252a (K252a) added before cell plating or DMSO + 100 nM K252b (K252b) added before cell plating. Primary neurites for each cell were counted using the photographic negatives taken at 24, 48 and 72 h. Each condition combined the results from two separate plating sessions. Cell numbers for each condition is as follows, Control, n = 17, K252a, n = 21, and K252b, n = 17. Error bars represent SEM. * = $p < 0.05$, ** = $p < 0.01$ vs control.

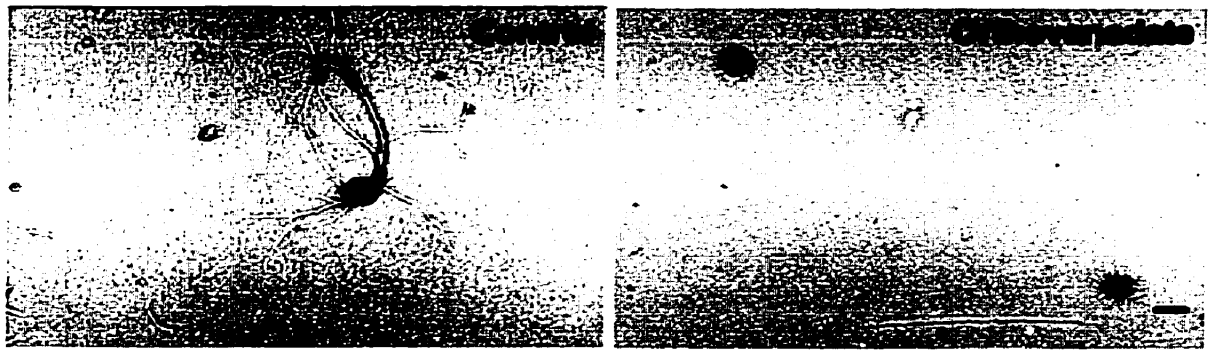

Number of Primary Neurites
6
5
4
3
2
1
0
0
20
40
60
80
Time (h)
***
Control
K252a
K252b

The Effects of Tyrosine Phosphatase Inhibition on Neurite Initiation.

To further explore the role of tyrosine phosphorylation in the initiation of neurite outgrowth, neurons were cultured in CM with orthovanadate, a tyrosine phosphatase inhibitor (Fig. 9). In this experimental series, control neurons had developed 2.9 ± 0.5, 3.8 ± 0.5, and 4.2 ± 0.5 primary neurites after 24, 48 and 72 h respectively (Fig. 10). Addition of 100 μM orthovanadate to CM, before cells were plated, resulted in a potent inhibition of neurite outgrowth. These neurons exhibited only 0.1 ± 0.01 primary neurites after 72 h, with only 8 % of the cells sprouting neurites compared to 86 % of controls (Table 4).

The dramatic effect of orthovanadate raised the question as to whether the effects seen above were a result of toxicity. A trypan bluedye-exclusion assay was performed on control and orthovanadate-treated cells cultured for 72 h. Absence of staining for all cells in either control or orthovanadate conditions demonstrated that the cell membranes were still intact (data not shown, 1 experiment, n = 15 for each condition). Also, the orthovanadate concentrations used in these experiments were similar to concentrations previously used on cultured cells, and shown to have no toxic effects (Mandell and Banker, 1998; Laniyonu et al., 1994). Finally, neurons cultured in orthovanadate remained phase-bright, spherical, and adherent to the culture dish for the duration of the experiment (> 72 h). All of these characteristics are indicators of healthy cells (Wildering et al., 1998).

Figure 9: The effect of 100 μM orthovanadate on the initiation of neurite outgrowth in RPA neurons. Cells were cultured in CM containing 10% DM (Control) or DM + 100 μM sodium orthovanadate (Orthovanadate). Microphotographs were taken after 48h in culture. Scale bar in the bottom right = 50 μm. The brightness and contrast of the photos were adjusted to enhance print quality.

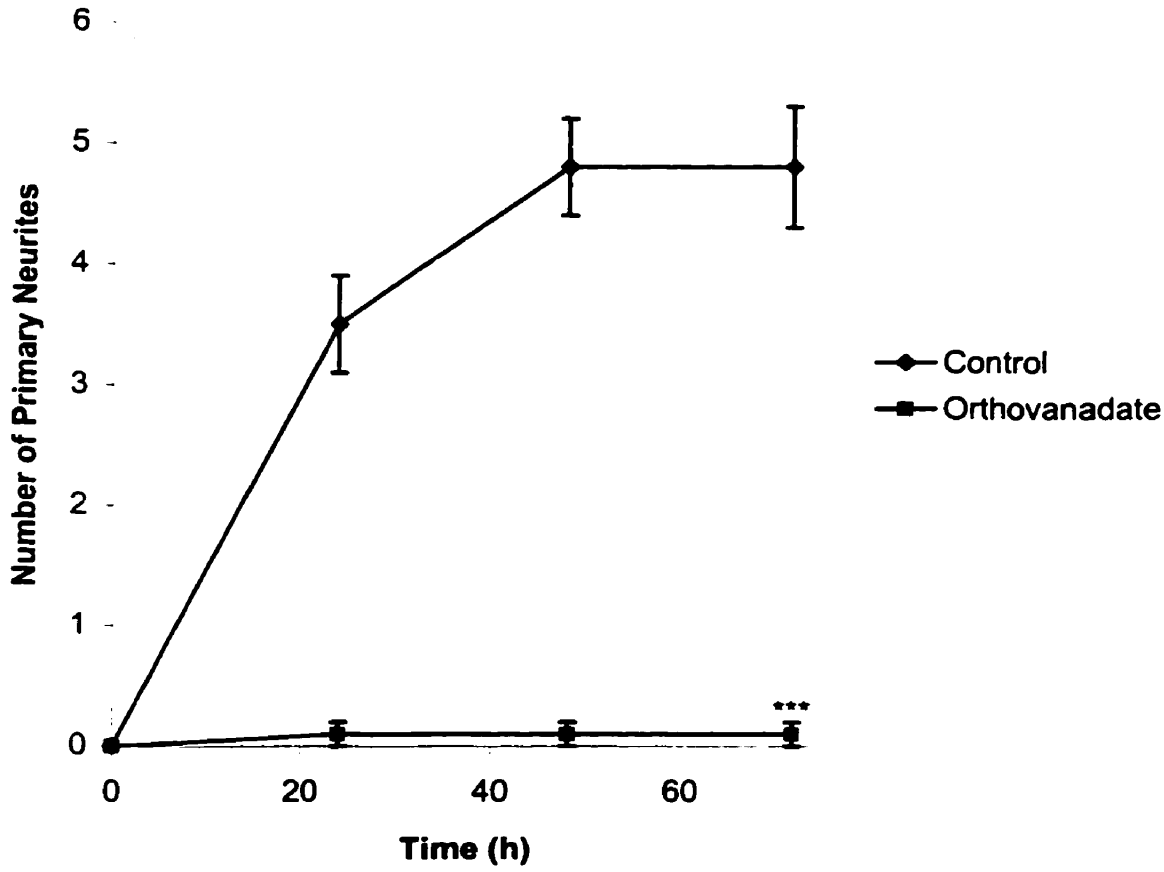


72h Treatment With 100 μM Orthovanadate

	Control	Orthovanadate	p vs. control
Primary neurites (PN) per cell	4.2 ± 0.5	0.1 ± 0.1	< 0.001
Cells With PN %)	86	8	< 0.001
Cell Number	28	36	N / A

Table 4: Summary of the effects of orthovanadate on neurite initiation. Cells were cultured in orthovanadate and control conditions for 72h. N / A = not applicable.

Figure 10: Effects of 100 μM sodium orthovanadate on the initiation of neurite outgrowth. Cells were plated in CM containing 10% DM (Control), or DM + 100 μM sodium orthovanadate (added before plating). Photomicrographs were taken 48h following cell plating. Each conditioned combined the results fromthree separate plating sessions. Cell numbers for each condition are as follows: control n = 28, orthovanadate n = 36. Error bars represent SEM. *** = $p < 0.001$ vs control.


6
5
4
3
2
1
0
Number of Primary Neurites
0
20
40
60
Time (h)
***
Control
Orthovanadate

The Role of Tyrosine Phosphorylation in Neurite Elongation

The next phase of neurite outgrowth examined was elongation. Elongation was examined after two different intervals: immediately after neurite initiation (early elongation), and 24 h after cell plating (ongoing elongation).

The Effects Of Inhibiting Tyrosine Kinases On Elongation

To examine the role of TKs in regulating the early elongation, I analyzed the same neurons for which data on neurite initiation were presented above. All elongation studies tracked changes in the total radial primary neurite lengths (see Materials and Methods). Any neurons that had not produced neurites by 24 h were not included in the analysis.

After 24 h incubation in lavendustin A, only 4 of 20 (20 %) neurons had sprouted, compared with 14 of 25 (56%) of controls (Figs. 5 and 11, Table 2). The low experimental number was problematic for realistic statistical analysis. Thus, although the lengths of the primary neurites of the experimental neurons were only 41% of that of controls after 48 h, this difference was not statistically significant.

Analysis of the radial length of primary neurites of neurons cultured in K252a, added either before or following cell plating, demonstrated that this inhibitor effectively suppressed early elongation irrespective of the time in culture before addition (Fig. 12). The total radial length of primary neurites of control neurons was 906 ± 101, 1243 ± 122, and 1238 ± 112 μm after 24, 48, and 72 h, respectively. When K252a was added to the CM before plating, the total radial primary neurite lengths were 296 ± 45, 326 ± 73,

and 349 ± 80 μm after 24, 48, and 72 h in culture, respectively. This inhibitor clearly suppressed the ability of the neurites to elongate.

A similar suppression of early elongation was seen in neurons plated in CM with K252a added 6 h after cell plating. Under this condition, primary neurite length was 420 ± 43, 553 ± 67, and 636 ± 69 after 24, 48, and 72 h, in culture, respectively (Fig. 12). Thus, after one day in culture, the total radial primary neurite length of K252a (0 h) experimental neurons was 67% less than controls. Later K252a addition (6 h) resulted in a 55% reduction of primary neurite length. This difference was still evident after 72 h incubation, when the neurites of K252a (0 h)-treated neurons were only 28% as long as in controls, while those of the K252a (6 h) group were 52% of control lengths. This demonstrated that regardless of the time of addition, K252a effectively inhibited early elongation, and that inhibition persisted for at least 72 h after cell plating.

A separate series of experiments were performed in which neurons were exposed to 100 nM concentration of K252a, its inactive analogue K252b (100nM), or 0.02% DMSO (control) in CM. Neurons incubated in either control conditions or 100 nM K252b exhibited total radial primary neurite lengths that were not significantly different up to 72 h following plating (ANOVA, Fig. 13). The total radial primary neurite lengths of neurons cultured in control conditions were 700 ± 102, 911 ± 121, and 949 ± 132 μm after 24, 48, and 72 h respectively, while those of neurons treated with 100 nM K252b were 640 ± 132, 670 ± 105, and 853 ± 126 μm, after 24, 48, and 72 h respectively.

Neurons treated with 100 nM K252a exhibited total radial neurite lengths of 360 ± 94, 270 ± 76, and 256 ± 81 μm after 24, 48, and 72 h respectively. Although the total radial primary neurite lengths of neurons treated with 100 nM K252a in this experiment were similar to the previous experiment (c.f. Fig. 12 and 13), the neurons in these experiments exhibited primary neurites that were significantly shorter than controls or K252b-treated neurons at 48 and 72 h. As in the case of the early elongation studies using 20 μM lavendustin A (Fig. 11), a sample size may have limited the ability of the statistical analysis to discern differences between control and experimental neurons at 24 h. Therefore after 24 h neurites of neurons treated with 100 nM K252a were 49 and 44 % shorter than those of control and K252b treated neurons respectively. This difference becomes more evident after 72 h in culture where K252a-treated neurons were 73 and 70 % shorter than control and K252b-treated neurons respectively.

Next, I examined whether tyrosine kinase signaling was involved in the ongoing elongation of neurites. Thus, TK inhibitors were added to culture dishes after 24h and incubated for an additional 24 h, as described in the Materials and Methods (Fig. 14).

The total radial primary neurite length for control neurons (0.02 % DMSO added at 24 h) was 977 ± 213 and 1257 ± 274 μm after 24 and 48 h, respectively (Fig. 15). Neurons that were cultured in CM for 24 h, and subsequently incubated with 20 μM lavendustin A for an additional 24 h, exhibited comparable primary neurite lengths of 608 ± 105, and 1054 ± 143 μm after 24 and 48 h respectively (not statistically different from those of controls, Fig. 15).

Figure 11: The effects of 20 μM lavendustin A, applied at plating, on neurite length. Neurons were plated and incubated in CM containing 20 μM lavendustin A. Photomicrographs of the cells were taken at 24 and 48 h, and radial neurite lengths for each primary neurite were measured as described in the Materials and Methods section. Data presented are the combined results of three separate plating sessions. Total cell number is as follows: Control, n = 14, Lavendustin A, n = 4. Error bars indicate SEMs.

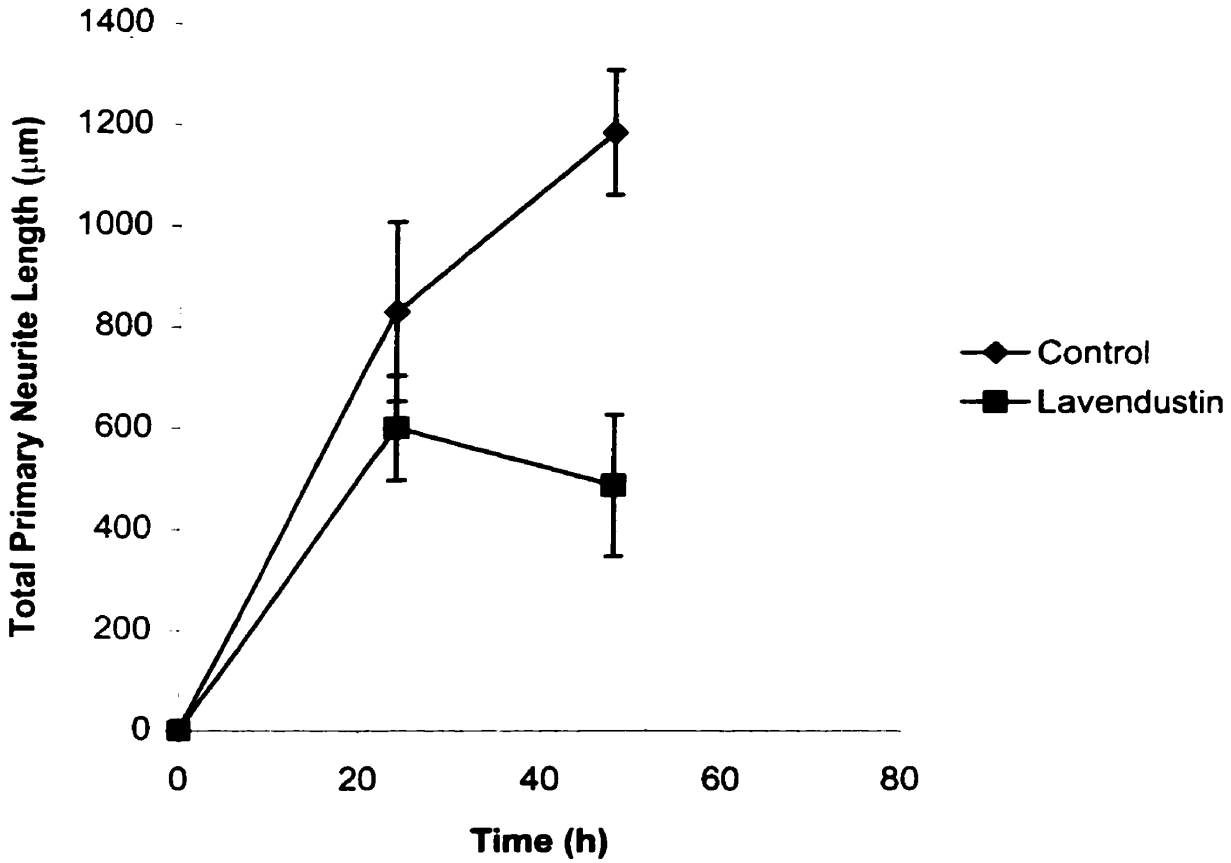

1400
1200
1000
800
600
400
200
0
Total Primary Neurite Length (µm)
0
20
40
60
80
Time (h)
Control
Lavendustin

Figure 12 : The effects, on neurite length, of 100 nM K252a, added to CM before (0 h) and after plating (6 h). Photomicrographs were taken at 24, 48, and 72 h after cell plating, and radial neurite lengths were measured for each cell as described in the Materials and Methods. Each point represents combined data from two separate plating sessions. Cell numbers are as follows: Control, n = 19, K252a (0 h), n = 8, and K252a (6 h), n = 12. Error bars indicate SEMs. *** = $p < 0.001$ vs control.

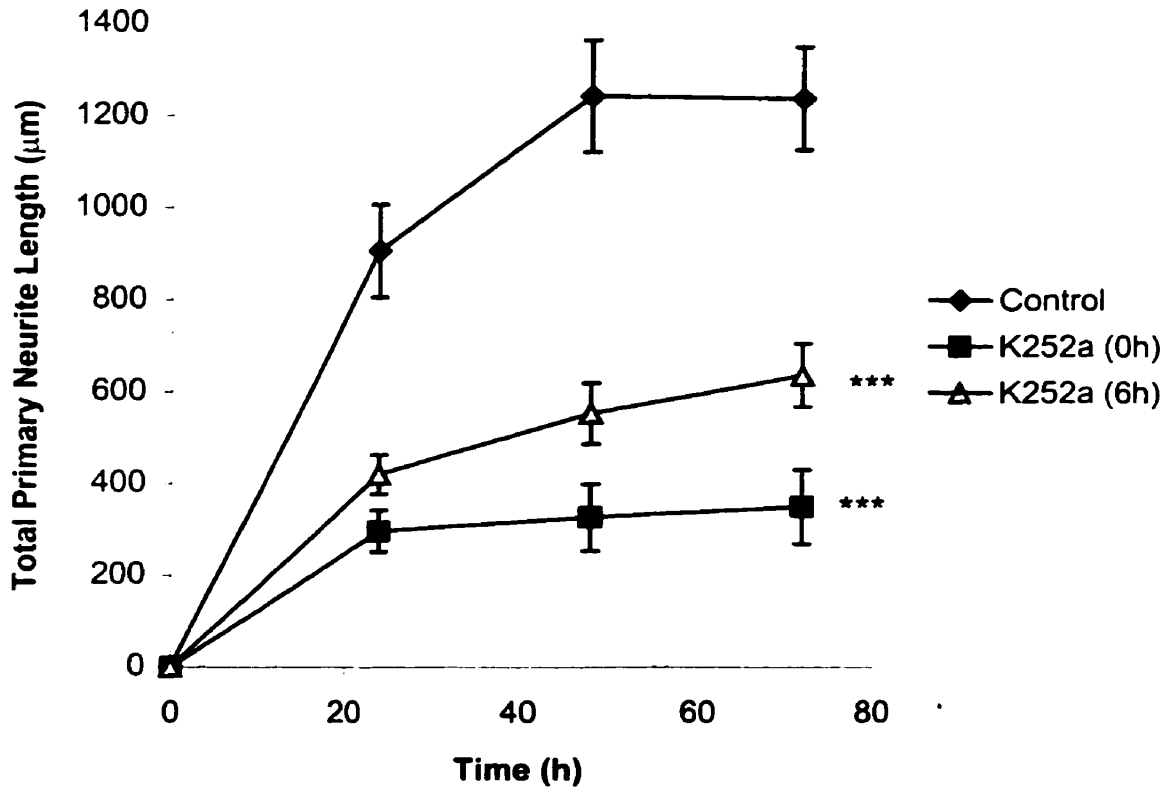

1400
1200
1000
800
600
400
200
0
Total Primary Neurite Length (μm)
0
20
40
60
80
Time (h)
Control
K252a (0h)
K252a (6h)
***
***

Figure 13: The effects of tyrosine kinase inhibition on neurite length. 100 nM K252a (K252a), 100 nM K252b (K252b), or 0.02% DMSO added to CM before plating. Photomicrographs were taken at 24, 48, and 72 h after cell plating, and radial neurite lengths were measured for each cell as described in the Materials and Methods. Each point represents combined data from two separate plating sessions. Cell numbers are as follows: Control, n = 15, K252a, n = 4, and K252b, n = 15. Error bars indicate SEMs. ** = $p < 0.01$, * = $p < 0.05$ vs control

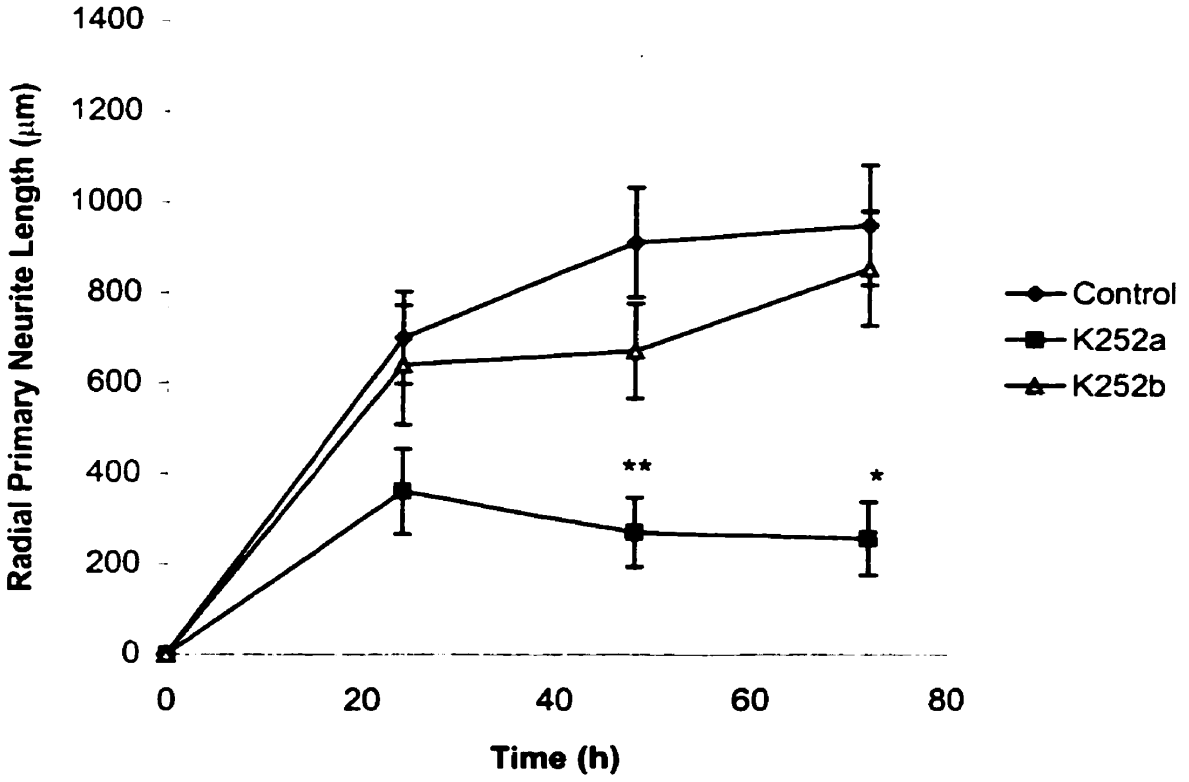

1400
1200
1000
800
600
400
200
0
Radial Primary Neurite Length (μm)
0
20
40
60
80
Time (h)
**
*
Control
K252a
K252b

Figure 14: The effects of tyrosine kinase inhibitors on neurite elongation. Cells were cultured in CM alone for 24h and microphotographs were taken (left panels). The right-hand column shows the same cells after an additional 24h in culture, in CM + 0.02% DMSO (Control), 100 nM K252a (K252a), or 20 μM lavendustin (lavendustin). Scale bar in bottom right = 50 μm. Contrast and brightness of the photos were adjusted to enhance print quality.

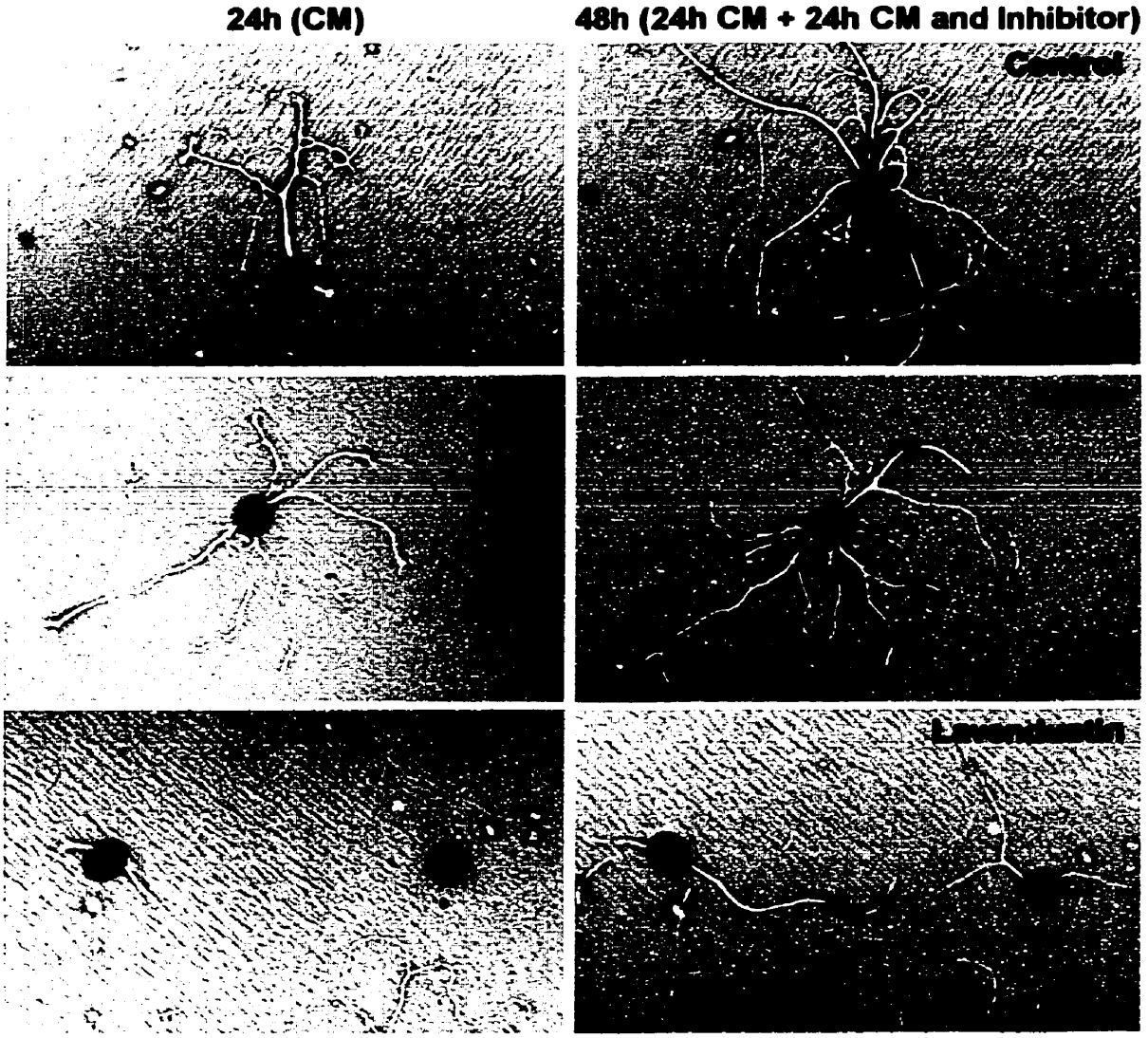
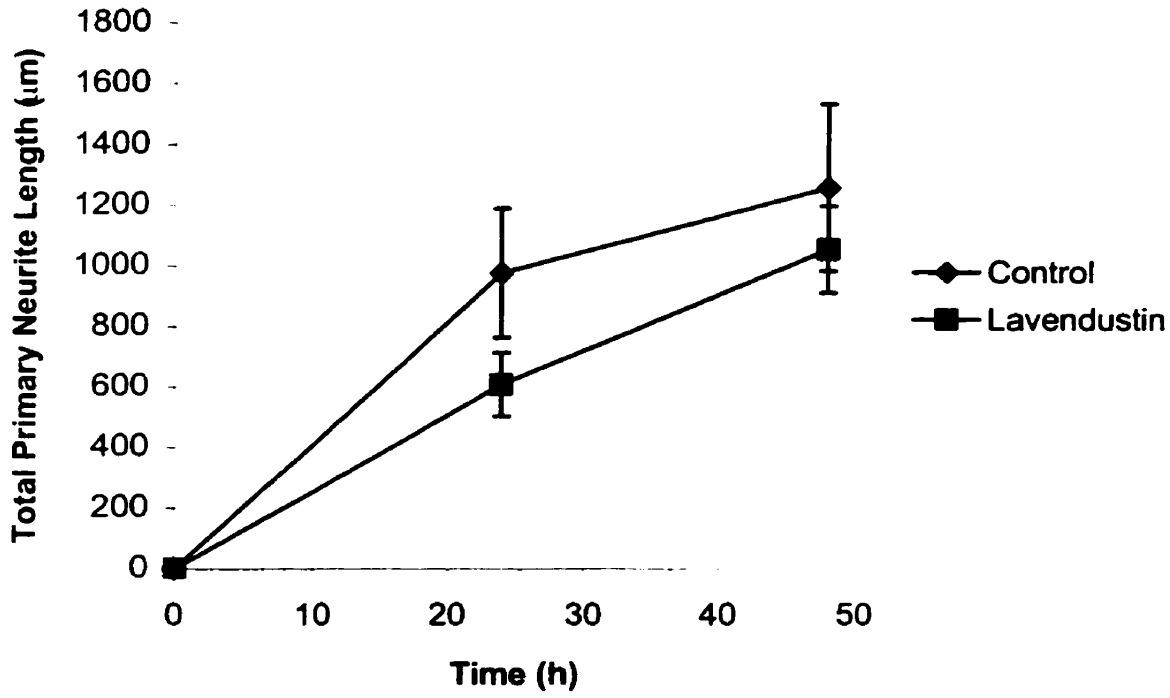

24h (CM)
48h (24h CM + 24h CM and Inhibitor)
Control

Figure 15: The effects of 20 μM lavendustin A on neurite length. Neurons were plated in cell culture dishes containing CM and incubated for 24 h. Cells were subsequently incubated with lavendustin for an additional 24 h, and photos were taken after 48h in culture. Radial length of each neurite was measured as described in the materials and methods. Cell number for each condition is as follows: Control, n = 21, and lavendustin, n = 16. Graph represents data collected from two plating sessions. Error bars indicate SEM. There was no significant difference between neurite lengths of lavendustin A-treated cells and control neurons.


Total Primary Neurite Length (µm)
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Time (h)
Control
Lavendustin

It is important to note that neither lavendustin A nor control-treated neurons experienced a significant increase in neurite length over the 24 h treatment period. However, since lavendustin A-treated neurons exhibited a doubling in neurite length, it suggested that this reagent was ineffective at inhibiting ongoing neurite elongation. The effects of K252a on neurite elongation at 24 h are unclear at this point. In this experiment, control neurons exhibited total radial primary neurite lengths of 742 ± 193 after 24, and 819 ± 207 μm after 48 h, while neurons incubated for 24h in 100 nM K252a added at 24 h produced total radial primary neurite lengths of 523 ± 101 after 24h, and 606 ± 105 μm 48 h (Fig. 16). Although apparently similar to the results of lavendustin A experiments (Fig. 15), this data set is difficult to interpret since the absolute amount of elongation after 24 h was minimal in both experimental and control conditions.

The Effects of Tyrosine Phosphatase Inhibition on Neurite Elongation

The effects of tyrosine phosphatase inhibition on neurite elongation were also explored. Again, two stages of this process were studied, early and ongoing. Orthovanadate proved to be a potent inhibitor of early elongation (Fig. 10). In these experiments, control neurons exhibited primary neurites that were 675 ± 84, 1024 ± 111, and 1084 ± 114 μm in total radial length after 24, 48, and 72 h, respectively (Fig. 17). In contrast, neurons incubated in 100 μM orthovanadate had produced primary neurites with total radial lengths of 90 ± 13, 30 ± 1, 30 ± 1 μm after 24, 48, and 72 h respectively. After only 24h in culture control neurons exhibited primary neurites that were 7 times longer than neurites produced by orthovanadate-treated neurons. Orthovanadate-treated

Figure 16: Effects of the tyrosine kinase inhibitor, K252a, on ongoing elongation of neurites. Neurons were incubated in CM for 24h, and then 100 nM K252a (final concentration) was added and cells were incubated for an additional 24h. The average total primary neurite length is shown for each condition. Numbers of cells for each condition were as follows: Control, n = 14, and K252a, n = 16. Graph represents data from one plating session. Error bars indicate SEM. There was no statistically significant difference between neurite lengths of K252a-treated and control neurons.

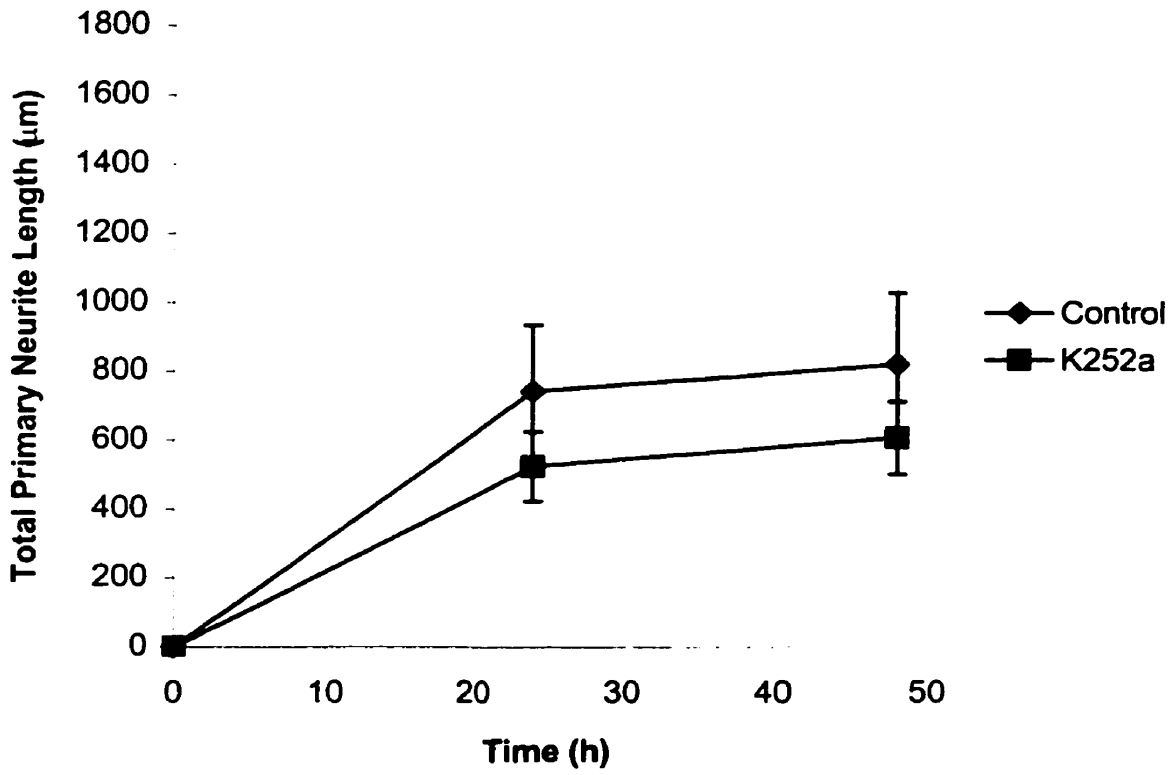
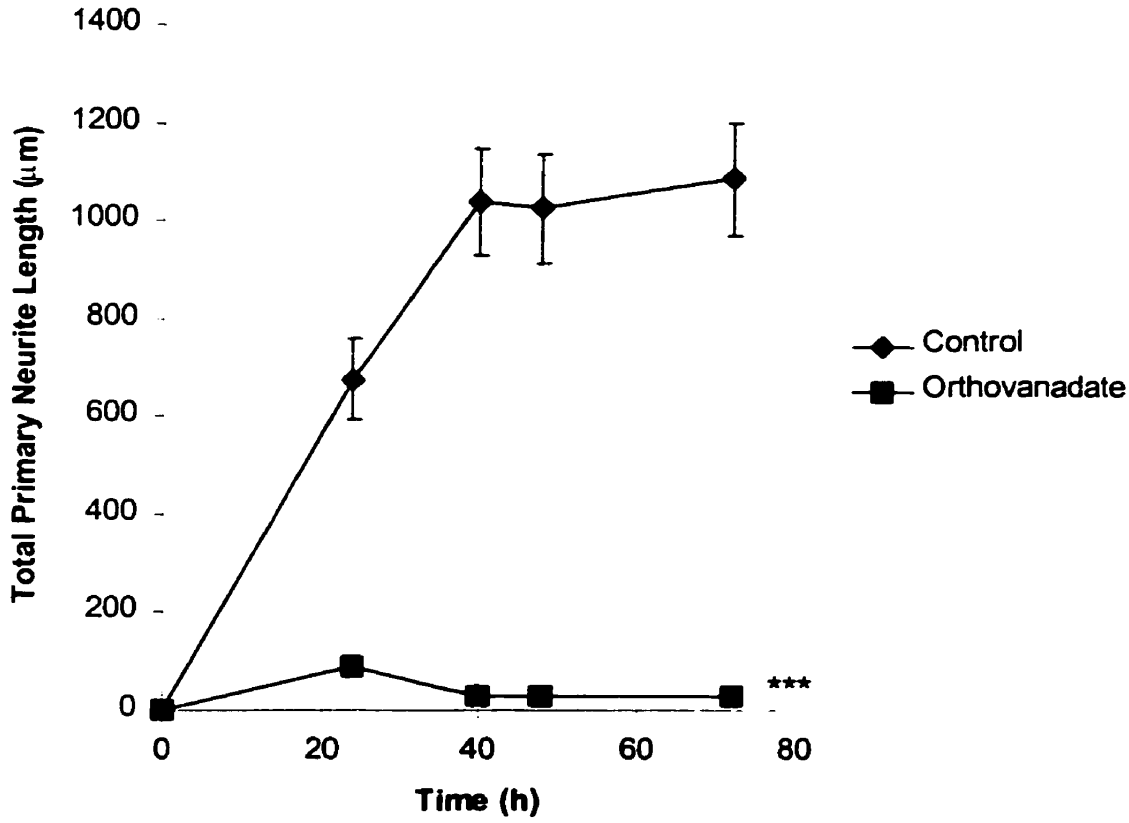

1800
1600
1400
1200
1000
800
600
400
200
0
Total Primary Neurite Length (µm)
0
10
20
30
40
50
Time (h)
Control
K252a

Figure 17: The effects of tyrosine phosphatase inhibition on neurite length. Neurons were plated in CM containing 100 μM orthovanadate and incubated. Photomicrographs of the cells were taken at 24, 40, 48 and 72 h, and radial neurite lengths for each primary neurite were measured as described in the Materials and Methods section. Data presented are the combined results of three separate plating sessions. Total cell number is as follows: Control, n = 30, Orthovanadate, n = 3. *** = $p < 0.001$ vs control. Error bars indicate SEMs.


1400
1200
1000
800
600
400
200
0
Total Primary Neurite Length (μm)
0
20
40
60
80
Time (h)
***
Control
Orthovanadate

neurons did not show any signs of elongation after the first 24 h, suggesting that inhibition of tyrosine phosphatases prevented early elongation from occurring in these neurons.

Cells that produced neurites after 24 h in CM and were then incubated in 100 μM orthovanadate for an additional 24 h exhibited a neurite retraction of about 50 % (Fig. 18). After 24 h in culture with CM, control neurons produced primary neurites with radial lengths totaling 770 ± 100 μm and after 48 h incubation these primary neurites had increased to 884 ± 103 μm in total radial length (Fig. 19). Neurons that were treated with 100 μM orthovanadate after 24 h in culture, exhibited primary neurites with a total radial length of 748 ± 137 μm at 24 h. After a subsequent 24 h incubation with 100 μM orthovanadate, total radial length of these primary neurites was reduced to 300 ± 64 μm. Since the effects of 100 μM orthovanadate on the initiation and elongation processes described were so marked, a lower concentration of orthovanadate (10 μM) was also tested. Similarly, neurons treated with 10 μM orthovanadate after 24h, exhibited primary neurites that were reduced from 1006 ± 235 μm in total radial length to 754 ± 135 μm (Fig. 19). Therefore treatment with 100 μM orthovanadate for 24h resulted in a 60% reduction in total neurite length, while treatment with 10 μM orthovanadate produced a 25% reduction in overall neurite length. Thus in both cases orthovanadate induced a significant reduction in total primary neurite length.

Figure 18: Effects of tyrosine phosphatase inhibition on neurite length. Neurons were cultured in CM for 24h (left column). The right column shows the same cells after an additional 24h culture following the addition of one of the following (in final concentrations) to the CM: Control; 10% DM, Orthovanadate 10 μM; 10 μM sodium orthovanadate, and Orthovanadate 100 μM; 100 μM sodium orthovanadate. Scale bar in bottom left hand corner = 50 μm. The contrast and brightness of the photos were adjusted to enhance print quality.

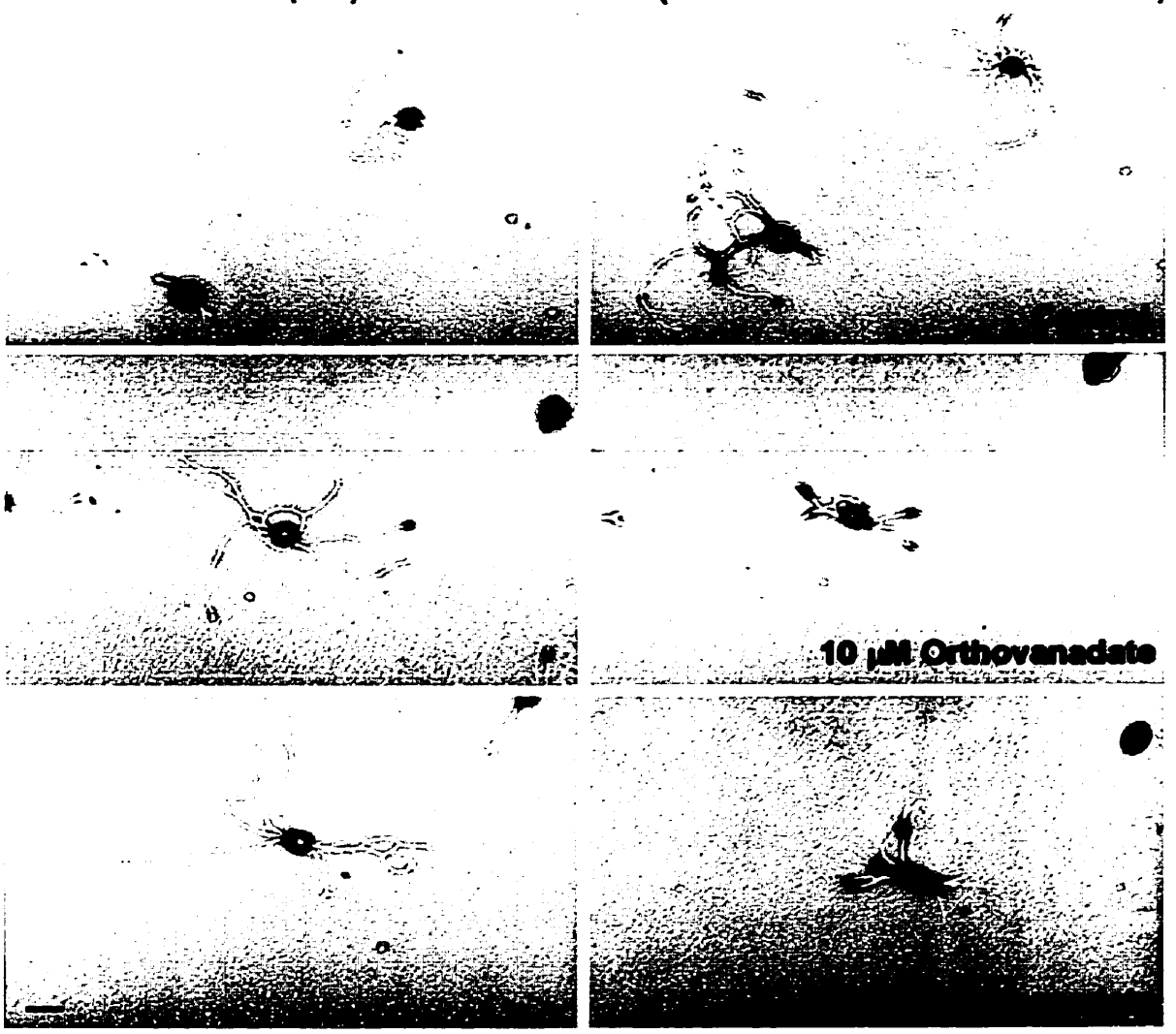
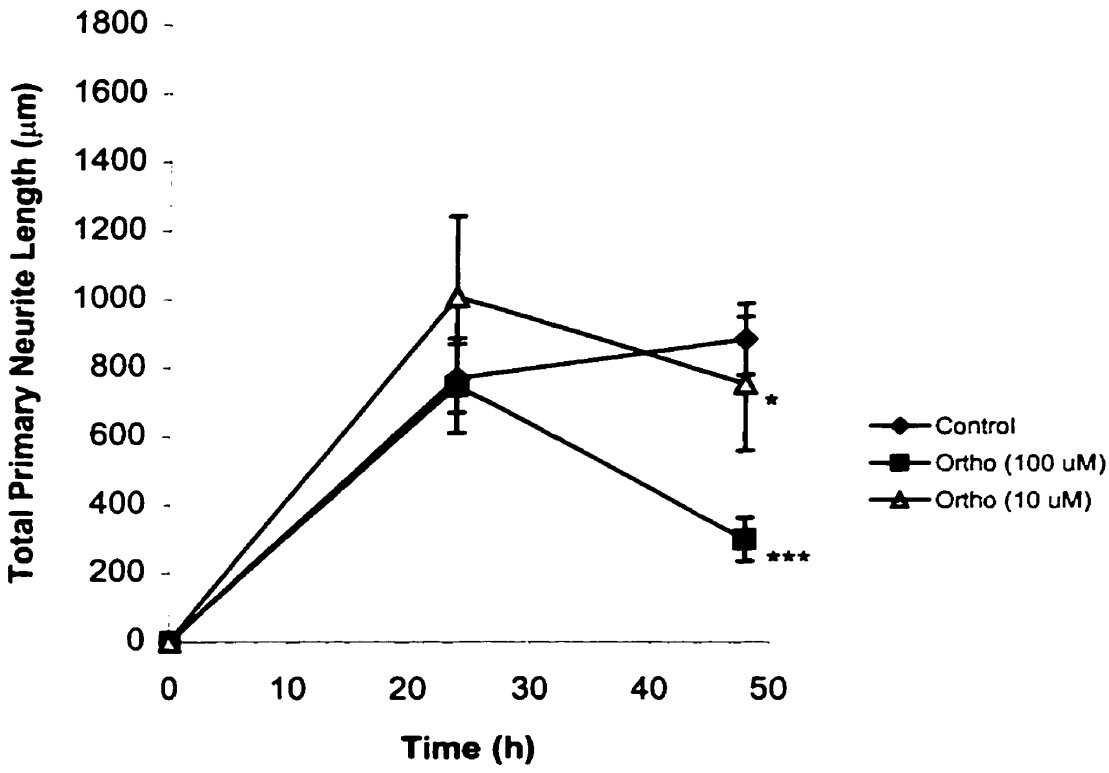

24h (CM)
48h (24h CM + 24h CM and Inhibitor)
10 μM Orthovanadate

Figure 19: The effects of tyrosine phosphatase inhibitor on neurite length. Neurons were plated in cell culture dishes containing CM and incubated for 24h. At this time, one of the following (in final concentrations) was added to the culture dish, and the cells were subsequently incubated for an additional 24h. 10% DM (Control) 10 μM orthovanadate (Ortho 10 uM), or 100 μM orthovanadate (Ortho 100 uM). Numbers of cells for each condition were as follows: Control, n = 35, and Ortho 10 μM, n = 23, Ortho 100 μM, n = 13. Graph represents data from two plating sessions. Error bars indicate SEM. Significance determined with two way ANOVA tested difference in the trends of inhibition. * = $p < 0.05$, and *** = $p < 0.001$ vs control.


1800
1600
1400
1200
1000
800
600
400
200
0
Total Primary Neurite Length (µm)
0
10
20
30
40
50
Time (h)
*
***
Control
Ortho (100 uM)
Ortho (10 uM)

DISCUSSION

This study examines the process of axonal regeneration using neurons from the pond snail *Lymnaea stagnalis*. Neuronal regeneration studies commonly utilize molluscan models since they offer unique advantages for *in vitro* manipulation. For example, *Lymnaea* neurons are relatively large, compared to those in many other animals, and therefore easily identified and isolated. These neurons, in common with neurons from invertebrates in general, have only axonal projections through which all cellular input and output is mediated (Bulloch and Jones, 1988; Bulloch and Ridgway, 1989). These neurons have no dendrites, instead collateral branches originating from the axon act as conductors for input from other cells and mediate output, along with the axon (Bullock and Horridge, 1965). The axonal regeneration exhibited by these neurons in culture closely resembles the regeneration exhibited by the same neurons *in vivo*. Regeneration usually occurs from the proximal axon stump when neurons are axotomized *in vivo*. When proximal axotomy occurs at the soma, neurites are produced directly from the cell body, and thus this study is an examination of the process of axonal regeneration *in vitro*. In the current study, I have used pharmacological inhibitors of tyrosine kinases (lavendustin A and K252a) and protein tyrosine phosphatases (PTPs; sodium orthovanadate) to investigate the role of tyrosine phosphorylation in neurite outgrowth.

Initiation

I found that when neurons were plated and incubated with TK inhibitors (either lavendustin A or K252a) for 48 h, fewer primary neurites were produced than in controls.

This suppression of neurite initiation suggested that the activation of tyrosine kinases was required for outgrowth to begin.

When the TK inhibitor K252a was added to the culture medium, neurons produced fewer neurites than matched controls. Subsequently, I tested the hypothesis that the amount of outgrowth exhibited by neurons cultured in this inhibitor depended on the timing of K252a addition. Interestingly, when K252a was added to the CM before plating, fewer neurites were produced than when cells were cultured in CM in which K252a was added 6 h after plating. Incubation with K252b, an analogue of K252a thought to be incapable of inhibiting TKs at the concentration used in these experiments (Knusel and Hefti, 1992), resulted in no inhibition of sprouting. This further suggests that the effects of K252a on neurite initiation are due to inhibition of TKs.

This suggested that some cells were able to initiate a growth program that became independent of TKs by this time, i.e. that inhibition of TKs was less effective in inhibiting neurite outgrowth once a growth program was initiated in these cells. This conclusion is supported by the fact that neurons treated with K252a after cell plating exhibit similar numbers of primary neurites as controls by 48 h (Fig. 7). The number of neurites produced by cells that were treated with K252a before plating were still significantly different compared to controls up to 72 h after plating, which was the longest interval tested.

The PTP inhibitor, sodium orthovanadate, was employed in an effort to support the conclusion that TKs and phosphotyrosine were responsible for initiating neurite outgrowth. I expected that incubation with a phosphatase inhibitor, a reagent that should increase cellular phosphotyrosine levels, would result in an increase in sprouting in these

neurons. Surprisingly, incubation with orthovanadate produced the greatest suppression of neurite initiation. These inhibitor-treated cells showed few signs of neuritic outgrowth, suggesting that PTP inhibition also suppressed it.

Alternatively, orthovanadate may affect initiation through inhibiting exchangers such as the sodium/potassium exchangers (Morinville et al., 1998). This effect may upset the ion homoeostatic balance enough to prevent activities such as neurite outgrowth.

Elongation

I next sought to determine the role of TKs in the neurite extension that occurs during 0-24 h following plating (early elongation), using the TK inhibitors, lavendustin A and K252a. For this study, changes in the length of primary neurites produced by cells was measured.

Primary neurites produced by cells treated with K252a were substantially shorter than primary neurites in controls, up to 72 h following plating, suggesting that TK activation was required for early elongation (Fig. 12). In contrast, the effect of 20 μM lavendustin A on early elongation was not conclusive. Although there appeared to be an inhibitory effect of the drug, the small sample size did not permit a conclusive statistical analysis (Fig. 11). In this case, less than 10% of experimental cells sprouted by 24 h leaving only 4 experimental cells to be tested, whereas 56% of control neurons (n = 14) sprouted by this time.

Orthovanadate was employed to help support conclusions that TKs, and ultimately tyrosine phosphorylation, were involved in regulating early elongation. By using this inhibitor to prevent the action of PTPs, and presumably increase cellular

phosphotyrosine levels, we expected to see the opposite effect of TK inhibition. This inhibitor proved to be effective at suppressing elongation, as well as initiation. In the neurons that did sprout, there was a marked inhibition in early elongation that lasted for at least 72 h following plating. This suggested that inhibiting PTPs, and thus increasing levels of phosphorylation, suppressed neurite elongation in addition to neurite initiation. Similar to initiation, the suppression of early elongation by orthovanadate may have been due to its ability to inhibit exchangers in these neurons.

In contrast to early elongation, it appears that ongoing elongation was independent of tyrosine kinase activity. Addition of 20 μM lavendustin A to neurons cultured in CM for 24 h had no apparent effect on neurite elongation: in fact, the neurites of these cells doubled in length over the next 24 h (Fig. 15). Although neither control nor lavendustin A-treated neurons exhibited a significant increase in neurite length during the period of treatment, this data suggests that the ability of neurites to elongate was not suppressed by lavendustin A.

In ongoing elongation experiments with K252a, neurite elongation appeared to cease in the neurons in both the control and experimental dishes prior to the application of the drug (Fig. 16). However, it may be argued that this reagent did not have an inhibitory effect on neurite length since total primary neurite length did not decrease during the period of treatment. Another approach would be to add inhibitors earlier, somewhere in the 12-24 h interval, since it takes about 12 h for neurite initiation to begin (unpublished observation).

In addition to effects on early outgrowth, PTPs also had an effect on ongoing elongation. In fact, treatment with either concentration of orthovanadate (10 or 100 μM)

caused a net retraction over 24 h. The ability of this the PTP inhibitor to affect neurite elongation suggested that PTPs were still involved in regulating neurite elongation at this stage. Again, an alternate explanation for these observations may be that since orthovanadate induced a reduction in neurite length, PTPs may play a role in regulating the stability of existing neurites.

In the TK-inhibitor experiments, neither lavendustin A nor K252a had an effect on ongoing elongation suggesting that tyrosine kinases are not involved in regulating this process. The results of the orthovanadate experiments appear to contradict this conclusion, since the application of this inhibitor caused a dramatic retraction of neurites. This observation may be explained a number of ways. Orthovanadate is known to affect the cytoskeletal dynamics, but this appears to be mediated through a tyrosine phosphatase-dependent mechanism (discussed below).

The ability of orthovanadate to inhibit initiation and elongation may be due to effects of orthovanadate on transporters in the cell unrelated to PTPs (Dafnis and Sabatini, 1994). Inhibiting transporters such as sodium/potassium ATPase may upset the ion balance, resulting in depolarization of the neuron.

As with all pharmacological reagents, there is always the question as to whether they are specifically affecting only the desired targets in the cell. It is the author's belief that the TK inhibitors are effective on TKs in *Lymnaea* neurons, and several lines of evidence support these conclusions. Regarding the issue of invertebrate neurons, lavendustin A has been shown to be effective at inhibiting tyrosine phosphorylation in lower vertebrates and invertebrates (e.g. Amphibians: Worley and Holt, 1996; Molluscs: Wu and Goldberg, 1995; Annelids; Catarsi et al., 1995). As for K252a, this reagent has

also proven to be effective at inhibiting tyrosine phosphorylation in molluscs (Moreno, 1999). The concentration of this inhibitor used in these experiments is similar to those used previously. Of course a definitive answer concerning the specificity of TKs inhibitors for specific proteins would be through quantitative phosphotyrosine Western blotting.

The problem of determining whether these inhibitors preferentially affect TKs over S/T kinases was approached in a number ways. First, two TK inhibitors, with differing side-effects (Table 1) were employed in this study. The similarity in results, suggests that effects observed were due to inhibition of TKs, the only kinase(s) that both of these inhibitors are known to affect. Secondly, a thorough literature search shows that lavendustin A has never been shown to inhibit anything but tyrosine kinases (e.g O' Dell, et al., 1991; Wu and Goldberg, 1995; Catarsi et al., 1995; Worley and Holt, 1996). Finally K252b, a reagent similar in structure to K252a (Fig. 2), was shown to have no effect on neurite initiation or early elongation. Since K252b is incapable of inhibiting TKs (Kase et al., 1987; Knusel and Hefti, 1992), yet effective at inhibiting S/T kinases at concentrations similar to those used for K252a it further implicates K252a in inhibiting TKs. Furthermore, this suggests that the kinases are involved in controlling neurite initiation and early elongation are TKs. In the case of orthovanadate, immunocytochemical experiments suggested that this reagent does indeed inhibit PTPs and increase the level of tyrosine phosphorylation in these cells (see below).

An additional TK inhibitor, genistein, was used in preliminary experiments for this thesis. Although genistein has been characterized as a general TK inhibitor (Akiyama et al., 1987) it has also been found to be capable of inhibiting protein synthesis (Hu et al.,

1993). However, it is interesting to note that cells cultured in this inhibitor did exhibit a suppression of neurite initiation. Although this inhibitor is known to be capable of preventing protein synthesis as well as TK activity, suppression of neurite initiation was consistent with the effects of K252a and lavendustin A, suggesting that these observations were due to inhibition of TK(s) and not protein synthesis inhibition.

Pharmacological experiments, where the TK inhibitors K252a and genistein were incubated with cultured motoneurons isolated from the snail *Helisoma trivolvis*, a close relative of *Lymnaea*, demonstrated that TK activation is required for both initiation and elongation of these neurons(Wang, 1994). Wang employed a method of analysis that only classified a cell as either sprouted or unsprouted. Specifically, the method of analysis for initiation was a nonquantitative method that determined whether a neuron sprouted by producing one or more neurites in response to CM in the absence or presence of a TK inhibitor. The method of outgrowth analysis used in the current thesis determined the number of primary neurites as well as the total radial primary neurite length, which allowed a more comprehensive analysis of changes in the quantity of outgrowth produced. In terms of initiation, Wang's results were similar to those of the present thesis, i.e. few neurons sprouted in the presence of TK inhibitors.

Comparison of the results of Wang's study with mine reveals an apparent contradiction. Specifically, Wang's results indicated that neurite elongation stalled in the presence of TK inhibitors. However, Wang's studies used a completely different approach. Whereas I applied TK inhibitors for long periods (24 - 48 h), Wang examined a 2 h window of application and showed that growth cones collapse within 60 minutes of inhibitor application. Growth cone translocation resumed within 30 minutes of removal

of the inhibitor. It is possible that these observations reflect a transient action of TK inhibitors, which would not be observable under the present study. Alternatively, a difference of species or neuron type might be responsible for these differences.

My results imply that the signal cascade for long-term elongation involved either S/T kinases and/or TKs that were insensitive to inhibition by either lavendustin A or K252a. This conclusion is based on the fact that neither lavendustin A nor K252a were able to inhibit ongoing elongation when either was added after cells were exposed to CM. If this were not the case, incubation with either K252a or lavendustin A at any time during culture, should have been effective at suppressing outgrowth.

Finally, the findings in the present study suggest that, in both initiation and early elongation, TKs and PTPs are necessary, while PTPs seem to also be required for ongoing elongation. Although these studies have not been able to implicate specific proteins in these outgrowth processes, it is the first of many steps towards doing this.

FUTURE EXPERIMENTS AND CONCLUSIONS

An obvious experiment, to test the hypothesis that brief exposure to CM is enough to start a program that results in neurite outgrowth, would be to remove CM from a culture dish about 6 h after plating cells. Although a pilot experiment of this design showed that RPA neurons indeed develop neurites after a brief exposure to CM, the results of these experiments were not conclusive. This is probably because some outgrowth-promoting factor(s) in CM may adhere to the poly-L-lysine substrate of the culture dish. Thus CM can be removed and replaced with DM, and these plates will still support outgrowth (Bulloch and Hauser, 1989)

Additional pharmacological experiments will be required to characterize the targets of lavendustin A and K252a in *Lymnaea* neurons. These two inhibitors are also known to be capable of inhibiting S/T kinases (see Table 1). It is possible that effects on neurite outgrowth in the presence of lavendustin A or K252a were due to inhibition of S/T kinases instead or as well as inhibiting TKs. To investigate this possibility I could use a general inhibitor of S/T kinases such as H7 (Kawamoto and Hidaka, 1984). If the effects on outgrowth were quantitatively similar to our results with TK inhibition, it would suggest that the suppression of neurite outgrowth by lavendustin A or K252a was due to inhibition of S/T kinases and/or TKs. Alternatively, a different conclusion from this observation would be that S/T kinases may regulate neurite outgrowth downstream of TKs. For example, the neurotrophin Trk receptors have been shown to mediate elongation and survival signals through pathways involving both S/T kinases and TKs (Kaplan, 1998). If the pathways are divergent, they would likely regulate different aspects of neurite outgrowth, so one would expect to the two treatments to differ in outgrowth characteristics.

It may be difficult to draw conclusions regarding the specificity of orthovanadate for PTPs from results of using compounds that mimic its effects on substrates such as the cytoskeleton (Table 1). Orthovanadate has been shown to cause actin depolymerization, (e.g. Chintala, et al., 1999) which could have contributed to the retraction of neurites observed in this study. However, the PTP inhibitor, phenylarsine oxide (PAO), also has been shown to depolymerize actin (Chintala, et al., 1999). PAO inhibits PTPs through covalent modification of PTPs, which is a different mechanism of inhibition than orthovanadate (Table 1), suggesting that the effects of PTP inhibitors on actin dynamics

are mediated through an indirect TK-dependent pathway. Indeed, a large body of evidence suggests that actin dynamics are tightly regulated by tyrosine phosphorylation (Tanaka and Sabry, 1995).

Distinguishing between specific effects of orthovanadate on PTPs vs. nonspecific effects on actin would be facilitated by the use of a phosphatase inhibitor that does not affect actin dynamics. There are a number of reasons to believe that orthovanadate does inhibit PTPs in this system. For example, immunocytochemistry experiments clearly demonstrate elevated phosphotyrosine levels (detected with an antiphosphotyrosine antibody) after a 24 h incubation with orthovanadate (unpublished observation). Secondly, the concentrations of this inhibitor used here were similar to those used in other experiments in which Western blotting showed that it inhibits PTPs specifically (Wu and Blackshaw, 1992; Laniyonu et al., 1994; Mandell and Banker, 1998).

Western blotting using phosphotyrosine and/or protein-specific antibodies would be a possible way to examine the specificity of the inhibitory reagents and their effects on phosphotyrosine in the cell. The resolving ability of an electrophoretic gel would allow the investigator to determine to what extent the inhibitors used affect the tyrosine phosphorylation of specific proteins, defined by their molecular masses. More specifically, Western blots and the appropriate protein-specific antibodies, may give more insight into the involvement of receptor and/or cytoplasmic TKs which are involved in initiation and elongation. For example, the involvement of the Trk receptor family in these outgrowth processes could be determined using Western blots with Trk-specific antibodies, as well as phosphotyrosine-specific antibodies. Based on the experiments completed in this thesis, a first possible step would be to determine whether the

pharmacological inhibitors used in this thesis affect the phosphorylation state of Trk-antigen-containing proteins, as determined using phosphotyrosine and Trk-specific antibodies. Since more than one protein may contain Trk antigens and undergo changes in phosphorylation, further characterization with a method such as protein sequencing would be required to further determine the identity of these proteins.

As discussed in the introduction, it is known that tyrosine kinase receptors do exist in *Lymnaea* (van Kesteren et al., 1998). Ltrk, a homologue of the mammalian Trk receptors, has been cloned from this snail, and immunocytochemical evidence suggests that other homologues exist (van Kesteren et al., 1998; Melvin and Bulloch, 1999). It is as yet unknown whether these receptors are directly involved in modulating neurite outgrowth, although all identified growth factors to date have been shown to signal through tyrosine kinase receptors (Schlessinger and Ulrich, 1992).

Additionally, an EGF homologue has also been recently purified in *Lymnaea*, and although the receptor for this peptide has not yet been cloned, pharmacological evidence suggests that it promotes outgrowth in neurons through a TK-dependent mechanism (Hermann et al, 1999).

In this thesis, I have presented data to suggest that adult *Lymnaea* RPA neurons require TK activity to start both the initiation and elongation programs that occur during axonal regeneration. Data collected at two separate time points, for both initiation and elongation, suggest that once TKs signal the start of the neurite outgrowth program, neurite outgrowth is no longer dependent on TKs for propagation of this process.

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