HEPOXILINS AND NEURONAL REPAIR: Effects on SCG Neurons After In Vitro Injury

by

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A thesis submitted in the conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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I. Abstract

HEPOXILINS AND NEURONAL REPAIR:
Effects on SCG Neurons After In Vitro Neurite Injury

Rebecca K. Amer
Master of Science, 2000
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The objectives of this in vitro study were to examine the effects of hepoxilin A₃ on a) neurite outgrowth post-injury, and b) intracellular calcium in superior cervical ganglion (SCG) neurons. Two to 6 hours following injury, the % increase in neurite outgrowth (over NGF-treated controls) was 19.4% and 31.3% in NGF-treated cultures treated with 2.8 μM HxA₃ (HDHX) and 0.28 μM HxA₃ (LDHX), respectively. Six to 13 hours post-injury, outgrowth was 14.1% and 29.4% higher than in controls, for HDHX and LDHX groups, respectively. A rapid increase in fluorescence in the somata of uninjured, fluo-3 loaded neurons was evoked by the addition of LDHX or HDHX. By 8 seconds post-treatment with HxA₃, fluorescence peaked and subsequently decreased after 1 minute to a new stable plateau significantly above rest. Our research has demonstrated for the first time the involvement of a specific lipoxygenase metabolite in the modulation of NGF-stimulated neurite outgrowth.
II. Acknowledgements

I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Cecil Pace-Asciak, for his guidance and support of this research. Without his patience, kindness, and understanding, I never could have completed this work. I would also like to thank my advisor, Dr. Linda Mills, whom I consider to be a co-supervisor rather than an advisor. Her energy, wisdom, sense of humor, and great advice shall always be remembered. Furthermore, I am indebted to Dr. Gordon Chu, whose help setting up this culture system was invaluable. I would also like to thank Dr. Charles Tator, for the generous use of his lab and equipment, and both Kam Kassiri and Denis Reynaud for their contributions and assistance throughout this work. Finally, I would like to thank my family and friends for their loyalty and support.
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AHP</td>
<td>afterhyperpolarization</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxy methyl ester</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>API</td>
<td>average pixel intensity</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytosine-D-arabinofuranoside</td>
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<tr>
<td>bDNF</td>
<td>brain derived nerve factor</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
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</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DDH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DMPH₄</td>
<td>6,7-dimethyl-5,6,7,8-tetrahydropterine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<td>Term</td>
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</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>$\Delta F/F_0$</td>
<td>fractional change in fluorescence</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>HDHX</td>
<td>1.0 µg/ml hepoxilin A₁</td>
</tr>
<tr>
<td>HEPES</td>
<td>$N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HETE</td>
<td>hydroxy-5,8,10,14-eicosatetraenoic acid</td>
</tr>
<tr>
<td>HPETE</td>
<td>hydroperoxy-5,8,10,14-eicosatetraenoic acid</td>
</tr>
<tr>
<td>HX</td>
<td>hepoxilin</td>
</tr>
<tr>
<td>IF</td>
<td>initial feed</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>K-S</td>
<td>Kolmogorov-Smirnov test</td>
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<td>Leibovitz-15-Air dissociation/dissection medium</td>
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<td>L-15-CO₂</td>
<td>Leibovitz-15-CO₂ plating/feeding medium</td>
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<td>La³⁺</td>
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<tr>
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</tr>
<tr>
<td>LOX</td>
<td>lipoyxygenase</td>
</tr>
<tr>
<td>MF</td>
<td>maintenance feed</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
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**xi**
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>sodium dihydrogen phosphate</td>
</tr>
<tr>
<td>NDGA</td>
<td>nordihydroguiaretic acid</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>pheochromocytoma cell line</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandins</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>SCG</td>
<td>superior cervical ganglia</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TCPO</td>
<td>trichloropropene oxide</td>
</tr>
<tr>
<td>TX</td>
<td>thromboxane</td>
</tr>
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INTRODUCTION

1. Overview: The Problem of Spinal Cord Injury

Research on the treatment of spinal cord injury (SCI) is in an exciting phase. Progressing in close parallel are several approaches that hold promise of providing more effective treatment of acute injuries and restoring function in patients with chronically-injured spinal cords. The major causes of SCI are sporting and recreational activities, motor vehicle accidents, and falls at home or at work (Tator and Edmonds, 1979). About half of SCI patients suffer complete loss of motor and sensory functions below the level of the lesion, with about two thirds of these patients suffering cervical level injuries (Tator and Fehlings, 1991). With improved resuscitative efforts aimed at preventing any further injury to the central nervous system, most patients with acute SCI arrive at the hospital in a potentially treatable state. Early pharmacological intervention, improved imaging measures, and sophisticated care in specialized treatment units are all critical measures for ensuring the best possible outcome for the patient. However, for patients with severe SCI, these supportive measures will only lead to a modest improvement in neurological function and recovery.

SCI research remains an area of intense investigation. Recently, researchers have proposed that traumatic brain injury to the brain or spinal cord, be it the gray or white matter, is an evolving phenomenon (Tator and Edmonds, 1979; Tator and Fehlings, 1991). Initially, patients suffer from a ‘primary’ impact injury or a shear type injury to the neurons and supporting structures of the brain and/or spinal cord. The majority of functional deficits, however, are thought to be the result of a ‘secondary’ biochemical injury initiated by the primary mechanical trauma (Collins, 1983; Hall et al. 1989, Sandler and Tator, 1976; Tator and Fehlings, 1991).
Numerous biochemical mechanisms contribute to the manifestation of secondary injury (Figure 1), however the exact nature and causal relationships of these interconnected mechanisms remain poorly understood.

Improved understanding of both primary and second injury phenomena has occurred because of careful in vivo and in vitro modeling of the injury process. Under controlled and predictable conditions, it has become possible to dissect these individual mechanisms, and to study their respective interplay. The understanding of the underlying basic pathophysiology then provides the foundation on which to base potential therapeutic intervention, which may comprise simple alteration in normal physiology or complex pharmacotherapeutic interventions. Advances in the understanding of the basic pathophysiological mechanisms at the cellular level have facilitated the development of drugs with specific therapeutic goals and potential. Furthermore, this understanding sheds light on the most elementary of neurobiological principles and processes, thus enhancing the basic fund of knowledge, with potential utility in related areas of basic science or clinical research.

2. Neuronal Injury and Regeneration

2.1 Neuronal injury

There are primary and secondary components to both non-traumatic and traumatic injury to the nervous system. In spinal cord injury (SCI), at the time of the original trauma, there are immediate effects on the neural tissue known as the primary injury, which in the most severe cases is usually irreversible (Tator, 1996). Primary injury leads to both direct and indirect cellular damage. Direct disruption of the cell membrane of the somata or axon causes a loss of cell integrity, with resultant cytoskeletal and organelle disruption. Indirect injury can be caused by
Figure -1. Mechanisms of secondary spinal cord injury

**BIOCHEMICAL CHANGES**
- neurotransmitter accumulation
- arachidonic acid release
- free-radical production
- eicosanoid production (prostaglandins)
- lipid peroxidation

**VASCULAR CHANGES**
- loss of vascular autoregulation
- hemorrhage
- loss of microcirculation
- reduction in blood flow

**MECHANISMS OF SECONDARY INJURY**

**LOSS OF ENERGY METABOLISM**
- ↓ in ATP production

**ELECTROLYTE CHANGES**
- ↑ in extracellular potassium
- ↑ in sodium permeability
a direct injury to blood vessels, with the ensuing hemorrhage or ischemia leading to a pathological process similar to stroke.

Some cells and tissue constituents in the area of injury endure effects of the initial trauma over time, which subsequently leads to a secondary injury. A combination of pathophysiological processes, including ischemia, changes in regional blood flow, fluxes in the levels of various cations (i.e. calcium and potassium), and an increase in the concentration of excitatory amino acids such as glutamate and aspartate, have all been shown to lead to secondary injury as a result of acute SCI in humans (Young and Koreh, 1986; Panter et al. 1990). In addition, many of these physiological processes have been found to either directly or indirectly raise the levels of calcium ions within the somata or axons of injured neurons (Tymianski and Tator, 1996). The initial awareness that calcium ions are key mediators of secondary cellular injury to neurons as well as to other cells was based both on early experimental observations and fundamental theoretical considerations. Many important cellular activities, from signal transduction, to activation of intracellular enzymes, to signaling at the DNA level, were all found to depend upon the controlled rise and fall in the levels of intracellular calcium (Clapham, 1995; Simpson et al. 1995). Therefore, given the important and heterogeneous roles of calcium in a variety of cellular functions, abnormalities in calcium homeostasis became implicated in mediating the secondary injury to cells following a primary ischemic or traumatic insult (Tymianski and Tator, 1996).

2.2 Neuronal regeneration

Neuronal regeneration after neurite injury requires both regrowth of transected neurites and reconnection with former targets. After injury in the PNS, the transected axon first forms multiple short sprouts and growth cones at the tips by several hours. The majority of the sprouts
are later cropped as the axon elongates and eventually, the axon reaches its target where it must subsequently form a new synapse (Bisby, 1995). In the CNS, spontaneous regeneration has not yet been achieved after neuronal transection. If presented with the optimal environment and stimulants, however, several studies have demonstrated that CNS neurons can both regenerate and make functional connections subsequent to injury (Aguayo et al. 1990; Schwab, 1990; Cheng et al. 1996). One reason for the lack of success in regenerating neurons in the CNS is an environment which inhibits regeneration and/or lacks the factors necessary for regeneration post-axonotomy (Caroni, 1997; Bahr and Bonhoeffer, 1994; Chen et al. 1995; Fawcett, 1992; Davies, 19941-2). After axonal transection in the CNS, astrocytes, oligodendrocytes, and myelin form a barrier to regeneration (Bahr and Bonhoeffer, 1994; Schwab and Bartholdi, 1996). Astrocytes lack cell-surface and adhesion molecules that are necessary for axonal growth; oligodendrocytes and myelin contain growth inhibitory molecules, the neutralization of which results in limited axonal regeneration. Furthermore, a lack of induction of neurotrophic growth factors, which play a significant role in the regeneration response, may also contribute to poor nerve regeneration observed in the CNS (Schwab and Bartholdi, 1996; Kobayashi et al. 1997). In contrast, neuronal regeneration in the PNS is far more successful because the environment is significantly less inhibitory. Axotomy in the PNS leads to an activation of Schwann cells, causing proliferation and re-expression of cell surface adhesion molecules and extracellular matrix components which are favorable for neuronal regeneration (Bahr and Bonhoeffer, 1994; Schwab and Bartholdi, 1996). In addition, Schwann cells in the PNS increase their production of neurotrophic factors such as nerve growth factor (NGF) and brain derived nerve factor (bDNF) after nerve injury which is also believed to enhance regeneration.
2.3 Calcium and neuronal regeneration

The biochemical basis of neuronal outgrowth and regeneration has been an area of intense investigation. The pioneering work on NGF led the way to uncovering a variety of environmental conditions and specific molecules that regulate the initiation of neuronal outgrowth (Levi-Montalcini and Angeletti, 1968; Schubert et al. 1978; Greene and Shooter, 1980; Gundersen and Barrett, 1980). Second messengers, including cAMP (Schubert et al. 1978, Nirenberg et al. 1984), inositol phospholipids (Ishii, 1978; Spinelli and Ishii, 1983; Hama et al. 1986), and calcium (Schubert et al. 1978; Connor, 1986; Cohan et al. 1986, 1987), have all been implicated in the cellular mechanisms of neuronal outgrowth. Calcium, in particular, performs a myriad of functions in the neuron from induction of gene expression to neurotransmitter release. Calcium is an important intracellular regulator of neurite elongation and motile growth cone structures (Mattson and Kater, 1987), and directs the assembly of both microtubules (Shliwa et al. 1981) and microfilaments (Adelstein and Eisenberg, 1980). Its specific role in signaling neuronal regrowth subsequent to nerve injury, however, has not yet been clearly defined.

Recent studies have shown that a localized elevation of intracellular calcium at the transected end of the molluscan Helisoma and Aplysia neurons is important for initiating regeneration of neurites through growth cone formation (Rehder et al. 1992; Ziv and Spira, 1997). In addition, some researchers have theorized that growth cone formation and maintenance requires an optimal level of intracellular calcium. For example, neuronal survival is poor at very low calcium levels, growth cone structure collapses at higher levels, and neuronal death occurs at extremely elevated levels (Kater and Mills, 1991). These studies have associated a calcium rise in neuronal fibers with growth cone formation and have implied that a localized calcium rise at the transected end of the neurite alone is sufficient for growth cone formation. However, several
other studies have shown that decreasing the rise of intracellular calcium at the cell body can inhibit fiber initiation in freshly plated rat DRG neurons possibly by inhibiting a calcium induced calcium release phenomenon. Since *in vitro* transection of neuronal fibers may increase the intracellular calcium level in both nerve fibers and in the somata (Strautman et al. 1990; Ziv and Spira, 1993; Ziv and Spira, 1995; Sattler et al., 1996), it is possible that intracellular calcium at the cell body may also be important for the initiation of neurite regeneration.

3. Nerve Growth Factor

3.1 Nerve growth factor

Neurotrophic factors are required for the development, survival, and maintenance of distinct populations of neurons. Among various growth factors which have been proposed to function as neurotrophic agents in the central nervous system is nerve growth factor (NGF) (Levi-Montalcini and Angeletti, 1968), a prototype member of the neurotrophin family of growth factors, which includes brain-derived nerve factor (bDNF), neurotrophin-3, and neurotrophin 4/5 (Chao, 1992). NGF is required for the differentiation and survival of sympathetic and some sensory neurons in the peripheral nervous system and provides trophic support for the cholinergic neurons of the basal forebrain (Lewin and Barde, 1996; Ebadi et al. 1997; Davies, 1994; Snider, 1994). NGF may also be involved in some neuroimmune interactions (Levi-Montalcini et al. 1996). NGF exerts its neurotrophic activity by binding to a receptor complex comprised of a low affinity component, p75 (Chao et al. 1986; Radeke et al. 1987), which has been postulated to interact with G-proteins (Feinstein and Larhammer, 1990; Knipper et al. 1993), and a high affinity component, trkA (Kaplan et al. 1991; Klein et al. 1991; Weskamp and Reichardt, 1991; Meakin et al. 1992), which contains a cytoplasmic domain with tyrosine kinase activity. TrkA undergoes
autophosphorylation upon NGF binding, and it has been shown to be essential for the biological activity of NGF (Loeb et al. 1991; Loeb and Greene, 1993).

A great deal of information is now available on the various biological responses elicited by NGF. The primary actions of NGF on susceptible neurons include a) neuronal survival and b) neurite outgrowth. NGF is necessary for the survival of developing and adult sympathetic neurons, and developing sensory neurons. With respect to neurite outgrowth, NGF has been shown to have an effect on collateral sprouting in sensory neurons in vivo (Diamond et al 1992\(^1\)-\(^2\)), percentage of process-bearing cells in sensory neurons in vitro (Lindsay, 1988), and arborization of sympathetic ganglion neurons both in vivo and in vitro (Snider, 1988; Campenot, 1992).

3.2 Nerve growth factor and neurite outgrowth

The role of NGF in neurite outgrowth has been explored in experiments which have demonstrated increased sprouting or arborization of sympathetic and DRG neurons with NGF in vivo (Diamond et al. 1992; Snider, 1988; Levi-Montalcini, 1968; Adams et al. 1997). The effect of NGF on regeneration in vivo after axonal transection, however, is not as clearly defined. While some studies have reported that there is no beneficial effect of NGF on neurite outgrowth (Derby et al. 1993; Oudega and Hagg, 1996; Grill et al. 1997), others have stated that NGF produces no effect or a negative effect on neurite outgrowth (Diamond et al. 1992; Gold, 1997). In primary cultures of neurons, NGF has been shown to increase neuritogenesis and arborization (Snider, 1988; Campenot, 1992; Yasuda et al. 1990; Hu-Tsai et al. 1994; Kimpinski et al. 1997; Belliveau et al. 1997). The majority of these in vitro studies, however, fail to distinguish between increased neurite outgrowth from the cell bodies (i.e. neuritogenesis) from outgrowth.
from pre-existing neurites which have been axotomized in vitro (i.e. branching or arborization). This distinction is important in the study of regeneration of axons following traumatic injury because in both spinal cord and peripheral nerve injury, the main objective is not to stimulate new axonal growth from the cell bodies but to encourage the regrowth of pre-existing axons which have been transected or damaged. Two studies utilizing the compartmented culture model developed by Campenot (Campenot, 1977) have attempted to define the role of NGF on neurite regrowth after in vitro axotomy. In one study, SCG neurons were grown in a petri dish which had been divided into 3 compartments by a teflon divider. The cell bodies were located in the center and their neurites extended outward into the two side (distal) compartments. Neurites were transected in the distal compartments and the media in those compartments were replaced with media containing varying levels of NGF. It was concluded from this study that higher NGF concentrations at the distal ends of neurites increase neurite arborization but not elongation (Campenot, 1992). A similar conclusion was reached with a second study using DRG neurons, but as in the previous study, the center compartment contained no NGF while the concentrations in the distal compartments varied from 0 to 100 ng/ml (Belliveau et al. 1997).

The role played by either receptor component in the NGF signal transduction mechanism(s) or the second messenger(s) leading to specific NGF responses are still under investigation. Some studies have reported that a variety of second messengers are affected by NGF, including cAMP (Knipper et al 1993; Nikodijevic et al 1975; Schubert and Whitlock, 1977), cGMP (Laasberg et al. 1988), phosphoinositides (Knipper et al. 1993; Contreras and Guroff, 1987), glycosylphosphatidylinositol metabolites (Chan et al. 1989), and arachidonic acid (De George et al. 1988, Fink and Guroff, 1990). Ca$^{2+}$ as a putative mediator of NGF responses has also been investigated. Some studies suggest that NGF increases Ca$^{2+}$ uptake, possibly through
a unique Ca\(^{2+}\) channel (Nikodijevic and Guroff, 1991; Nikodijevic and Guroff, 1992). Other studies have demonstrated, however, that intracellular Ca\(^{2+}\) is the main source for NGF-evoked cytosolic free Ca\(^{2+}\) concentration increase (De Bernardi et al. 1996; Pandiella-Alonso et al. 1986; Lazarovici et al. 1989). In a 1996 study by De Bernardi et al., NGF increased the cytosolic free Ca\(^{2+}\) concentration in C6-2B cells expressing trkA. In these cells, NGF (50 ng/ml) evoked a 3-fold increase in cytosolic free Ca\(^{2+}\) concentration. While cells were shown to be insensitive to chelation of extracellular Ca\(^{2+}\), the response was abolished following depletion of Ca\(^{2+}\) stores or blockade of intracellular Ca\(^{2+}\) release providing strong evidence that intracellular Ca\(^{2+}\) (released from the IP\(_3\)- and thapsigargin-sensitive calcium pools) is the primary origin for NGF-evoked cytosolic free Ca\(^{2+}\) concentration increase. Furthermore, there is evidence to suggest that this NGF-induced increase in cytosolic calcium plays a role in the induction of neurite outgrowth.

In a 1994 study by Tsukada et al., activation of phospholipases C and A\(_2\) by NGF-activated tyrosine kinase, caused both a release of calcium from intracellular stores and arachidonic acid from cellular phospholipids, leading to the induction of neurite outgrowth in PC12 cells. Pretreatment of cells with selective inhibitors of tyrosine kinase (ST638 and genistein) and phospholipases C (neomycin B) and A\(_2\) (p-Bromophenacyl bromide and indomethacin) depressed neurite outgrowth following 60 minutes treatment with NGF. In addition, TMB-8, an inhibitor of intracellular calcium mobilization, also significantly depressed NGF-induced neurite outgrowth, which supports earlier findings of an important relationship between NGF, calcium, AA mobilization and metabolism, and neurite outgrowth.
4. Arachidonic Acid and Neuromodulation

4.1 Overview of prostaglandin formation and metabolism

Prostaglandins are fatty acid derivatives into which two molecules of molecular oxygen have been incorporated through the enzyme cyclooxygenase. They are derived from the intermediate cyclic endoperoxides, PGG₂ and PGH₂. The ‘primary’ prostaglandins (PG) are PGE₂, PGD₂, and PGF₂α, prostacyclin (PGI₂) and thromboxane (TXA₂ and its stable product TxB₂). Other products are derived from the lipoxygenase family of enzymes which incorporate only one molecule of molecular oxygen into the fatty acid structures. Recent animal and human studies have described the use and alterations of the prostaglandins in relation to several clinical problem areas in the neurosciences. These include cerebrovascular circulation, cerebral ischemia, migraines, neurotransmission and nociception (Leslie and Watkins, 1985).

The biosynthetic pathways of prostaglandin formation are illustrated in Figure 2 using arachidonic acid (AA) as an example. Ingested dietary fatty acids (polyunsaturated) are bound to albumin and then incorporated into cell membranes and subcellular structures as components of phospholipids and cholesterol esters. In response to various stimuli, including mechanical, chemical, or humoral factors, phospholipase A₂ is activated. The phospholipase then cleaves AA from cellular phospholipids, which then undergoes a site-specific incorporation of molecular oxygen by either of two enzymes, cyclooxygenase (COX) or lipoxygenase (LOX). The initial products formed from the COX-catalyzed reaction of AA are the ‘cyclic endoperoxide intermediates,’ designated PGG₂ and PGH₂. COX exists as two distinct but similar isozymes, COX-1 and COX-2. The two isoforms of COX are two separate gene products. They are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Protective prostaglandins which preserve the integrity of the stomach
**Figure -2. Arachidonic acid metabolism**

Dietary Fatty Acids → Phospholipids → ARACHIDONIC ACID

- CYCLOOXYGENASE (COX) → PGG₂ → TxA₂, PGH₂, PGI₂
- LIPPOXYGENASE (LOX) → HPETE → HETE

- PROSTACYCLIN SYNTHASE → PGI₂
- THROMBOXANE SYNTHASE → TxA₂

Other LOX derivatives (i.e. LIPOXINS, HEPXILINS)
lining and maintain normal renal function in a compromised kidney, are synthesized by COX-1 (Vane J et al, 1998). COX-2 is inducible and is found in inflammation and cancer. The inducing stimuli include pro-inflammatory cytokines and growth factors, implying a role for COX-2 in both inflammation and control of cell growth. Although COX-2 is believed to be inducible, it is present constitutively in the brain and spinal cord, where it may be involved in nerve transmission and in fever (Vane J et al, 1998).

The site-directed LOX pathways yield positional homologs of hydroperoxyeicosatetraenoic (HPETE) acid which are further metabolized into the leukotriene family of compounds, lipoxins, or hepoxilins. The HPETEs are unstable biologically and are reduced enzymatically (via glutathione peroxidase) or nonenzymatically to the corresponding HETE (Figure - 2) (Leslie and Watkins, 1985). The family of LOXs involved in this branch of AA metabolism is divided into four subtypes: 5-, 8-, 12-, and 15-lipoxygenase. 5-LOX catalyzes the two-step conversion of AA to leukotriene A₄. The first step consists of the oxidation of AA to the unstable intermediate 5-HPETE, and the second step is the dehydration of 5-HPETE to form leukotriene A₄. These events are the first committed reactions leading to the synthesis of all leukotrienes and play a critical role in controlling leukotriene production (Silverman E and Drazen J, 1999). The 12-LOX isoform catalyzes the conversion of AA to 12-HPETE and linoleic acid to 13-hydroperoxyoctadecadienoic acid (13-HPODE). 12-LOX, but not 5- or 15-LOX, is specifically expressed in pancreatic cells and is involved in regulating glucose-stimulated insulin secretion (Bleich et al, 1998). Products of 12-LOX metabolism have also been linked with inflammatory pathways in endothelial cells, kidney mesangial cells, inflammatory bowel disease, and corneal epithelial cells (Bleich et al, 1998). The direct action of 12-LOX on complex lipids and cellular membranes also links them to the process of reticulocyte maturation, low-density
lipoprotein oxidation in atherosclerosis and pulmonary host defenses (Conrad D, 1999).

4.2 Neuromodulatory effects of arachidonic acid

The excitable membranes of the CNS are enriched in 20-carbon unsaturated fatty acids, particularly arachidonic acid (20:4; AA). It is well known that the amounts of free fatty acids in the brain increase during ischemia (Abe et al. 1987; Abe et al. 1989; Hsu et al. 1989) and in other pathological states (Bazan, 1989; Siesjo et al. 1989) possibly via activation of phospholipase A2 or phospholipase C (Irvine, 1982). The amount of free AA decreases faster than other free fatty acids during the post-ischemic period (Yoshida et al. 1986). Some biologically active substances are generated from AA by COX or LOX activity, which may be a factor in the development of post-ischemic brain edema (Chan et al. 1982; Dempsey et al. 1986). In contrast, AA and its metabolites have been implicated as intracellular or intercellular messengers for the action of a number of hormones and growth factors on neural as well as non-neural cells. In the brain, stimulation of several neurotransmitter receptors has been shown to increase the release of AA and its eicosanoid metabolites. N-Methyl-D-aspartate (NMDA) stimulates the release of AA in striatal cells (Dumuis et al. 1988), cerebellar granule cells (Lazarewicz et al. 1990) and hippocampal cells (Sanferiu et al. 1990). α-Adrenergic receptor stimulation causes AA release from spinal cord, hippocampal and cortical neurons, but not from glial cells in primary culture (Kanterman et al. 1990). Acetylcholine and serotonin also stimulate AA release from hippocampal neurons (Kanterman et al. 1990; Felder et al. 1990). AA released in such a manner has a wide variety of effects on ion channels in excitable tissues (Fraser et al. 1993; Keyser and Alger, 1990; Schweitzer et al. 1993). It has also been shown to modulate synaptic excitability (Fraser et al. 1993; Pellmar, 1991). In the hippocampus, there is convincing evidence for AA
acting as an intercellular messenger relaying information from postsynaptic to presynaptic neurons, which is required for a sustained increase in neurotransmitter release following the induction of long-term potentiation (Lynch and Boss, 1990; Lynch et al. 1989; Williams et al. 1989).

Several studies have also indicated an effect of AA on the survival and morphology of neuronal cells. The toxicity of AA has been implicated using neuron-like cell lines (Dehault et al. 1993; Murphy et al. 1989), and embryonic chick motor neurons (Dehault et al. 1993). The promoting effect of AA on neurite outgrowth has also been demonstrated on the pheochromocytoma cell line (PC12 cells) and chick motor neurons (Dehault et al. 1993). A 1994 study by Williams et al. demonstrated that AA and/or one of its metabolites is the second messenger that activates calcium channels in the cell adhesion molecule (CAM) pathway leading to axonal growth. Fibroblasts transfected with CAMs promote neurite outgrowth by activating a second messenger pathway that culminates in calcium influx into neurons through N- and L-type calcium channels. In this study, the same neurite outgrowth response was directly induced by AA (10μM); the response was subsequently inhibited by an inhibitor (RHC-80267) of diacylglycerol lipase (an enzyme which is known to generate AA in cells) acting at a site upstream from calcium channel activation (Williams et al. 1994¹). Moreover, during differentiation of PC12 cells to a neuronal phenotype and prior to the extension of neuronal fibers, there is a rapid and sustained increase in the capacity of the cells to synthesize and release eicosanoids. Drugs that inhibit arachidonic acid liberation from membrane phospholipids (i.e. mepacrine or 4-bromphenacyl bromide) block NGF-stimulated neurite outgrowth by PC12 cells and DRG neurons (DeGeorge et al. 1988). In addition, selective inhibitors of COX metabolism of arachidonic acid (i.e. indomethacin and aspirin) fail to block outgrowth. Inhibitors of LOX metabolism (baicalein,
BW755, and eicosatetraenoic acid) are potent blockers, however, suggesting that LOX metabolites may be important for NGF-stimulated fiber growth by PC12 cells and DRG neurons (DeGeorge et al. 1988).

AA and its metabolites also exert both trophic and toxic influences on hippocampal neurons in primary culture. In a 1994 study by Okuda et al., AA at a concentration of $10^{-5} \text{ M}$ showed a profound toxic effect on cultured hippocampal neurons. This toxicity was found to be related to AA metabolism by LOXs. Toxicity was markedly and significantly prevented by the LOX inhibitor nordihydroguaiaretic acid (NDGA) ($10^{-4} \text{ M}$). In addition, AA861 and baicalein (each at $10^{-6} \text{ M}$), selective inhibitors of 5- and 12-LOX respectively, also showed significant protective effect, whereas the COX inhibitor indomethacin ($10^{-5} \text{ M}$) had no effect. The presence of AA metabolism by the LOX pathway in neuronal cells has also been reported in a number of other studies, which have shown that the effects of AA on ion channels are exerted by its LOX metabolites, including the 12-LOX metabolite 12-HPETE (Buttner et al. 1989) and the 5-LOX metabolite leukotriene C$_4$ (Schweitzer et al. 1993). At lower concentrations, AA was also found to exert trophic effects on hippocampal neurons (Okuda et al. 1994). In addition to a survival-promoting effect, AA was also effective in promoting neurite elongation. At a concentration of $10^{-6} \text{ M}$, AA significantly potentiated neurite elongation in two-day old primary cultures. The presence of the COX inhibitor indomethacin was found to potentiate the survival-promoting effect of $10^{-6} \text{ M}$ AA, suggesting a shift of AA metabolism toward the LOX pathway. LOX metabolites themselves may therefore exert trophic effects when they are present at appropriate concentrations. To further advance this hypothesis, it will be necessary to identify the metabolite(s) in question and show that they alone, or in combination, stimulate nerve fiber growth.
5. Hepoxilins

5.1 Formation and metabolism

Hepoxilins are hydroxy epoxide metabolites of arachidonic acid (AA), formed through the rearrangement of 12S-hydroperoxy-5Z, 8Z,10E,14Z-eicosatetraenoic acid (12S-HPETE), the initial product resulting from the action of 12-lipoxygenase on AA (Pace-Asciak, 1984; Pace-Asciak et al. 1983). Two hepoxilins are formed as a result of this reaction: 8(S/R)-hydroxy-11S,12S-trans-epoxyeicosa-5Z,9E,14Z-trienoic acid (hepoxilin A₃, HxA₃) and 10(S/R)-hydroxy-11S, 12S-trans-epoxyeicosa-5Z,8Z,14Z-trienoic acid (hepoxilin B₃, HxB₃) (Figure - 3). Hepoxilins contain a trans-epoxide whose configuration is 11S, 12S. The ‘A’ and ‘B’ designation relates to the carbon position of the hydroxyl group; ‘A’ refers to a hydroxyl group at C8 and ‘B’ refers to one at C10 (Pace-Asciak, 1994). Hydrolysis of the epoxide generates the trihydroxy metabolites 8(S/R),11R,12S-trihydroxy-eicosa-5Z,9E,14Z-trienoic acid (trioxilin A₃, TrxA₃) and 10(S/R),11S,12R-trihydroxy-eicosa-5Z,8Z,14Z-trienoic acid (trioxilin B₃, TrXB₃). Formation of hepoxilins was first reported in human platelets and lung, liver, pancreas, brain, aorta and pineal gland of the rat (reviewed in Pace-Asciak, 1994; Pace-Asciak, 1993). Their synthesis has also been reported in tropical marine algae (Moghaddam et al. 1990) and Aplysia brain (Piomelli et al. 1989). Furthermore, formation of HxA₃ and its metabolism via the epoxide hydrolase pathway is also shown to occur in mammalian brain.

HxA₃ is unstable biologically and is metabolized into trioxilin A₃ through the reaction of an epoxide hydrolase. HxA₃ exhibits increased stability and potency in its methyl ester form. The activity of the free acid form of hepoxilin A₃ is dependent upon the type of vehicle used (i.e. HxA₃ is active in releasing calcium when used in an ethanol vehicle but not in DMSO), whereas the methyl ester is equally active in either vehicle (Reynaud et al. 1999). The trioxilin metabolite is
Figure -3. Arachidonic acid metabolism: Hepoxilin pathway

Arachidonic Acid

12-Lipoxygenase

(12S)-HPETE

Hepoxilin Synthase

Hepoxilin A₃

Epoxide Hydrolase

Hepoxilin B₃

Glutathione S-Transferase

Trioxilin A₃

Hepoxilin A₃-C
inactive. When the activity of hepoxilin epoxide hydrolase is inhibited by trichloropropene oxide (TCPO), HxA₃ is further metabolized through a glutathione transferase pathway to form a glutathione conjugate of hepoxilin termed HxA₃-C. This metabolite is produced via the addition of glutathione to the epoxide moiety to form the 11-glutathionyl-12-hydroxy metabolite (Pace-Asciak et al. 1989; Pace-Asciak et al. 1990; Pace-Asciak et al. 1990; Pace-Asciak et al. 1990; Pace-Asciak et al. 1990).

HxA₃ is also unstable chemically as it is rapidly hydrolyzed in acid media into trioxin A₃. In contrast, HxB₃ is resistant to both enzymatic and nonenzymatic hydrolysis (Pace-Asciak, 1994). Most of the biological activities are exerted by HxA₃, which therefore appears to be the most important bioactive hepoxilin (Pace-Asciak, 1993; Pace-Asciak, 1994; Pace-Asciak et al. 1998).

5.2 Biological actions of hepoxilins

Hepoxilins have been shown to exhibit a variety of biological actions, including the secretion of insulin from rat pancreatic islets (Pace-Asciak and Martin, 1984), regulation of cell volume through activation of K⁺ channels in intact human platelets (Margalit et al. 1993), potentiation of bradykinin-evoked vascular permeability in rat skin (Laneuville and Pace-Asciak, 1991), and potentiation of norepinephrine-induced vascular contraction on de-endothelialized rat aorta (Laneuville et al. 1992). In addition, HxA₃ formed through the hypotonic- or shear stress-induced activation of human platelets, inhibits the aggregation of normal human platelets (Margalit and Granot, 1994). Furthermore, HxA₃ stimulates the release of the second messengers AA and diacylglycerol from human neutrophils (Nigam et al. 1990), inhibits the NECA-induced stimulation of adenyl cyclase and subsequent formation of cAMP in the rat pineal gland (Reynaud et al. 1994). Most importantly, and central to this thesis, HxA₃ evokes a receptor-mediated release of calcium from intracellular stores in human neutrophils (Pace-Asciak, 1994).
Hepoxilins have actions on neuronal function. Endogeneously formed by the mammalian and *Aplysia* brain, HxA$_3$ is reported to simulate the actions of 12-HPETE, which causes presynaptic inhibition of evoked potential in neurons (Pace-Asciak, 1988; Piomelli et al. 1987). In electrophysiological studies using rat hippocampal CA1 neurons, transient exposure to HxA$_3$ results in hyperpolarization, increase in amplitude and duration of the post-spike train afterhyperpolarization (AHP), increase in amplitude and duration of the inhibitory postsynaptic potential (IPSP), and inhibition of the 4-aminopyridine-induced release of norepinephrine in hippocampal slices (Carlen et al. 1994; Pace-Asciak et al. 1990). These effects exhibit a sharp dose-response relationship, with minimal threshold or no effect occurring at 3 nM and maximal effects occurring at 10 nM. Carlen et al. suggested that these effects were achieved through increased K$^+$ conductance during hyperpolarization and increased GABA release and GABA binding during IPSPs. Taken together, these findings provide strong evidence that hepoxilins have significant neuromodulatory actions.

### 5.3 Calcium-mediated effects of hepoxilins

The primary biological action of hepoxilins appears to relate to their ability to release calcium from intracellular stores through a receptor-mediated mechanism. The receptor appears to be an intracellular hepoxilin-specific binding protein (Reynaud et al. 1996), and may be G-protein coupled, since its actions are inhibited by pertussis toxin (Dho et al. 1990). Studies using human neutrophils, pancreatic β-cells and vascular tissue show that HxA$_3$ causes a dose-dependent rise in intracellular calcium. Two components have been observed, an initial rapid phase of intracellular calcium rise, followed by a slow decline to plateau levels that remain above the original baseline calcium level. Studies have suggested that the initial rapid phase is caused
by a release of calcium from intracellular stores in the endoplasmic reticulum; the slower rate of decline (plateau phase) is thought to be caused by calcium influx as it is abolished in zero calcium medium (Pace-Asciak, 1994; Mills et al. 1997). Addition of lanthanum chloride (1uM), an inorganic calcium channel blocker, prior to or post-addition of hepoxilin, eliminates the second phase completely, with the calcium peak returning rapidly to normal baseline levels (Reyanud et al. 1999) as observed in extracellular calcium-free medium. These results further confirm the belief that the plateau phase is due to calcium influx.

Calcium dynamics in response to HxA, varies in different subcellular compartments within the cell. In a 1997 study by Mills et al., hepoxilin-evoked calcium sequestration was eliminated by prior exposure to CCCP, a mitochondrial uncoupler. CCCP also eliminated the plateau phase of the calcium response in cell suspension, suggesting that this phase was associated with mitochondrial function rather than calcium influx alone. To exclude the possibility that hepoxilin caused an accumulation of calcium in the nucleus, hepoxilin was added to cells previously labeled with the carbocyanine dye, DiI (a marker of both the plasma and nuclear membranes) and fluo-3. Addition of hepoxilin caused an increase in intracellular calcium, manifest as a marked increase in cytosolic fluorescence, but not in the clearly visible nuclear compartment. In summary, these results demonstrate that hepoxilins evoke the release of calcium from the endoplasmic reticulum, and this calcium is subsequently taken up by organelles and is tightly sequestered. Moreover, these findings offer an explanation for observations previously made with cell suspensions in which HxA was shown to inhibit the receptor-mediated release of calcium by several chemotactic agents, including tetrapeptide, fMLP, platelet-activating factor (PAF) and leukotriene B4 (Laneuville et al. 1993).
6. Rationale

Hepoxilins, 12-lipoxygenase metabolites of AA, have been isolated in both the rat and mammalian brain (reviewed in Pace-Asciak, 1994), and are known to elicit significant neuromodulatory effects on *Aplysia* and hippocampal CA1 neurons (Piomelli et al. 1987; Pace-Asciak et al. 1990; Carlen et al. 1994). The literature suggests that AA and its LOX metabolites also exhibit significant neuromodulatory actions, specifically in the area of neuronal outgrowth (DeGeorge et al. 1988; Okuda et al. 1994; Williams et al. 1994). In a 1998 study by DeGeorge et al., NGF-stimulated fiber growth in PC12 cells and DRG neurons was blocked by inhibitors of LOX metabolism (baicalein, BW755). Furthermore, the presence of the COX inhibitor indomethacin was found to potentiate the survival-promoting effect of AA (10^(-6) M) in two-day old primary cultures, suggesting a shift of AA metabolism toward the LOX pathway (Okuda et al., 1994). The isolation of specific LOX metabolite(s) involved, however, has yet to be reported. Are the hepoxilins the LOX metabolites mediating these effects (Figure-4)?

In addition to their neuromodulatory actions, hepoxilins also mediate calcium release from intracellular stores within the cell (reviewed in Pace-Asciak, 1994). Calcium is an important intracellular messenger in the neuron, and is known to play a role in the induction of gene expression and neurotransmitter release, direction of microtubule and microfilament assembly, neurite fiber elongation, growth cone formation, and initiation of neuronal regeneration post-injury (Rehder et al. 1992; Ziv and Spira, 1997). The effects of hepoxilins on neuronal outgrowth have not previously been studied. After careful review of the literature, therefore, the potential role of hepoxilins in the modulation of neuronal regeneration was investigated.
Figure -4. Schematic of the involvement of AA and its metabolites in neurite outgrowth

- NGF
  - PLC and PLA₂ activation
  - AA release from phospholipids
  - LOX metabolites
  - Ca²⁺ release from intracellular stores

↑ in [Ca²⁺]ᵢ

- Induction of neuronal gene expression
- Remodeling of cytoskeleton (i.e. microtubule and microfilament assembly)
- Growth Cone Formation

NEURITE OUTGROWTH/REGENERATION
7. **Hypothesis**

Hepoxilins modulate neurite outgrowth of regenerating rat Superior Cervical Ganglion (SCG) neurons after *in vitro* neurite transection.

8. **Specific Objectives**

a) To identify the effects of hepoxilin A₃ (HxA₃) on NGF-mediated outgrowth following acute neuritic transection of rat SCG neurons *in vitro*.

b) To determine the effects of hepoxilin A₃ (HxA₃) on intracellular calcium dynamics in rat SCG neurons.
METHODS

1. Animals

In accordance with the model system proposed by Campenot (Campenot, 1977) and the methodology of culturing neurons from the rat superior cervical ganglia (SCG) developed by other groups (Mains and Patterson, 1973; Hawrot and Patterson, 1979), we utilized newborn Sprague-Dawley rats, 24 to 48 hours post-natal, for the dissociated culture of sympathetic neurons (Figure-5).

2. Cell Culture Methods

2.1 Cell culture media

The cells were plated and maintained in Leibovitz-15 (L-15) medium. The media was prepared according to methods previously described (Mains and Patterson, 1973; Campenot, 1993). Powdered L-15 media containing L-glutamine but no bicarbonate was obtained from Gibco Laboratories, USA. A 14.4 g package was reconstituted in 1.25 liters DDH$_2$O with 1ml of stable vitamin mix. The latter contained 13 amino acids and vitamins, including: L-aspartic acid, L-glutamic acid, L-proline, L-cystine, p-aminobenzoic acid, B-alanine, vitamin B12, myo-inositol, choline chloride, fumaric acid, coenzyme A, d-biotin, DL-6,8-thiociic acid. The solution was subsequently divided into two separate solutions, L-15-Air and L-15-CO$_2$. Sodium bicarbonate was added to the media solutions at a final concentration of 4 mM in L-15-Air, and 20 mM in L-15-CO$_2$. After adjusting the pH to 7.35, four media constituents were added to both media solutions: D-glucose, L-glutamine, penicilln-streptomycin, and fresh vitamin mix. (All chemicals hereafter, unless otherwise specified, were obtained from Sigma Chemicals, Oakville,
Figure - 5. Schematic of SCG dissection and culture techniques

Isolate superior cervical ganglia from 24-48hr post-natal rat pups

Trypsinization of ganglia for 30 min

Neutralization with rat serum and L-15-Air media

Dissociation of ganglia via trituration

Plating of isolated SCG neurons on collagen-coated petri dishes

Incubation of neurons in Initial Feed at 37°C and 5% CO₂ for 10-14 days
Ontario.) Glucose was prepared as a 30% w/v stock solution in DDH₂O, and was added to the media at a 2% v/v final concentration. A 200 mM stock solution of glutamine was added to the media at a 1% v/v final concentration. Penicillin-Streptomycin in powdered form was reconstituted in 20 ml sterile DDH₂O, and added to the media at a 1% v/v final concentration, yielding a final penicillin concentration of 100 units/ml and a final streptomycin concentration of 0.10 mg/ml in the medium. The fresh vitamin mix consisted of 6,7-dimethyl-5,6,7,8-tetrahydropterine or DMPH₄ at 0.05% w/v and glutathione at 0.025% w/v, in DDH₂O. This solution was added to the media at a 1% v/v final concentration. Both solutions were subsequently sterilized using vacuum filtration, using a 0.22 μm porous cellulose acetate membrane (Corning, USA). Solution osmolality was always determined to be between 280-320 mOsm.

The L-15-Air medium was used during the early stages of SCG isolation, for it contained enough nutrients to support the sympathetic neurons during SCG dissection and trypsinization. The L-15-CO₂ medium, however, required other essential media additives to maintain the viability of cells and to support their growth during cell dissociation, plating, and maintenance feedings. Such additives included nerve growth factor (NGF), rat serum, vitamin-C, and cytosine arabinoside (ara-C).

Sympathetic neurons have very well defined requirements for growth in culture, and the presence of NGF in the medium is the most critical of these requirements (Mains and Patterson, 1973; Higgins et al. 1991). NGF (Cedarlane Laboratories, Canada) was obtained lyophilized as the 2.5S form and reconstituted in PBS. 1 mg of 2.5S NGF was dissolved in 50 ml PBS (20 μg/ml PBS) and was stored in 1 ml aliquots at -20°C. NGF was added to the L-15-CO₂ medium at a final concentration of 10 ng/ml, in accordance with the protocol from other laboratories (Mains and Patterson, 1973; Campenot et al. 1992). Another essential requirement for the
viability and growth of sympathetic neurons in culture is rat serum. Rat serum was prepared from blood drawn by cardiac puncture from male retired-breeder Sprague-Dawley rats as previously outlined (Higgins et al. 1991). The serum was added to the L-15-CO₂ media at 2.5% v/v final concentration (Campenot et al. 1991). Vitamin-C is a required co-factor for noradrenaline synthesis by sympathetic neurons in culture, and it was added to the medium at a final concentration of 1% v/v. Cytosine-D-arabinofuranoside (ara-C), an antimitotic agent, was added to the L-15-CO₂ medium at a final concentration of 1% v/v (or 5 μM). This compound was added to prevent the growth of non-neuronal cells, whose presence often influences the growth and development of neuronal cells in culture (i.e. secretion of growth factors other than NGF). By the fourth or fifth day, few to no non-neuronal cells remained in the cultures dishes.

2.2 *Isolation of superior cervical ganglia*

Each post-natal rat pup was decapitated using a pair of surgical scissors and the head was pinned onto a cork board for dissection. Using a dissecting microscope with a 16X objective lens, the two superior cervical ganglia, grey in color and egg-shaped in size, were easy to locate, each lying beneath the common carotid artery at its bifurcation. With the aid of forceps, the ganglia were gently removed and subsequently placed in L-15-Air dissection medium. A total of 15-20 neonatal rats were used for each dissection, therefore yielding 30-40 ganglia in total.

2.3 *Dissociation of sympathetic neurons*

Following their removal, the ganglia were dissociated enzymatically in a 10% v/v Trypsin (Sigma T-2904) / PBS solution at 37°C with periodic manual agitation. After 30 minutes, the effect of trypsin was neutralized with a 10% v/v rat serum / L-15-Air solution and ganglia were
subsequently suspended in Maintenance Feed (L-15-CO2, no ara-C) (MF) and triturated using a fire polished Pasteur pipette. Approximately 30 triturations were required to completely dissociate the neuronal cells from the ganglia.

2.4 Preparation of collagen matrix and plating of cells

The cells were plated and maintained on a collagen matrix. Type-1 collagen was extracted from rat tails using methods and techniques previously described (Bornstein, 1958). The tails were first sterilized in 70% ethanol and then rinsed in sterile double distilled water (DDH₂O). The tendons were teased away from the tails, and were subsequently extracted in a 0.1% v/v acetic acid / DDH₂O. The extraction of collagen was carried out with gentle agitation using a magnetic stirrer at 4°C for 48 hours. The mixture was centrifuged at 10,000 rpm for 30 minutes and then stored at 4°C for up to four months. Dishes were coated with collagen 24 hours prior to plating, and were left to dry at 37°C and 5% CO₂. Previous studies by the laboratory of Dr. Charles Tator demonstrated that for the purposes of coating tissue culture dishes, the extracted collagen solution was found to be more effective when diluted in DDH₂O to yield a 25-30% v/v collagen solution.

Dishes were rinsed with DDH₂O to remove traces of acetic acid prior to plating. The bottom of each culture dish was then scratched using a 21-pin rake (Tyler Research Instruments Co., Canada) which yielded 20 parallel tracks of 250 µm width. The cells were plated in the center of the dish which allowed the growing neurites to extend linearly along the tracks during incubation at 37°C and 5% CO₂ (Campenot et al. 1991). After 10-14 days, neurites measured up to 10 mm in length.
2.5 Maintenance of cultured neuronal cells

As previously mentioned, the cells were plated in the center of the dish in a drop of MF. After approximately 20 hours at 37°C and 5% CO₂, the dishes were fed with 2 ml of Initial Feed (IF). This feeding medium comprised L-15-CO₂, media additives and ara-C (5 μM). The presence of ara-C in the medium prevented the growth of any glial cells, and led to cultures almost solely composed of sympathetic principal neurons. Thereafter, cells were fed with IF every 3-5 days. The cultures were maintained at 37°C with 5% CO₂ and 95% air present in the incubator.

3. Confocal (laser scanning) Microscopy

3.1 Instrumentation - hardware and software

The confocal imaging system used during both calcium dynamics and regeneration experiments consisted of an MRC-600 confocal (laser scanning) microscope (Bio-Rad Microsciences LTD., UK). Phase-contrast microscopy was used to acquire non-confocal transmission images during injury-regeneration experiments. Both the 10X (Nikon Ph1 DL 10/0.25 160/-) and the 20X (Nikon Ph2 DL 20/0.4 160/1.2) phase objectives were used. The Fluor20 objective (Nikon 0.75 160/0.17) was used for fluorescence microscopy. Image acquisition and processing was performed with Bio-Rad’s proprietary software, either the DOS® based SOM, or the Windows® based CoMOS. The images acquired were either first stored on the network, or were directly stored onto a 3.5" magneto-optical disk, with a 230 Mb storage capacity (disk and disk-drive manufactured by Fujitsu Corporation, Japan). During the course of the calcium experiments utilizing fluo-3, a fluorescein isothiocyanate (FITC) filter block was used on the microscope and the laser excitation setting kept at 488-DF10, with a neutral density
of 1 or 2 filter in place. Emission was detected through a low-pass emission filter with cut-off at 515 nm. The confocal aperture was kept 2/3 open, and the black level and gain settings were kept constant for all experiments. All experiments were carried out at room temperature in viewing media (see below).

3.2 Drugs and solutions

For injury-regeneration experiments, Initial Feed supplied with NGF (10 ng/ml), was used as the control cell-bathing or external solution. For calcium-dynamics experiments, the external medium was a balanced salt solution, consisting of the following (in mM): 135.0 sodium chloride (NaCl), 5.0 potassium chloride (KCl), 5.0 glucose, 10.0 N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 4.0 sodium bicarbonate (NaHCO₃), 2.0 sodium dihydrogen phosphate (NaH₂PO₄), 1.8 calcium chloride (CaCl₂), and 1.0 magnesium chloride (MgCl₂); all chemicals were obtained from Sigma Chemical Company, USA. The solution was pH balanced to 7.35 (7.3-7.4) and osmotically balanced at 310-320 mOsm. Drugs added to the external solution in the course of both calcium and regeneration experiments included the following: anhydrous dimethyl sulfoxide (DMSO) (41648, Fluka Chemika, Switzerland) used at a final concentration of 1 µl/ml (0.1%); calcium ionophore A-23187 free acid (Molecular Probes Inc., USA) was made up as a stock solution in DMSO, and used at a final concentration of 1.65 µM; and 8(S)-HxA₃-methyl ester (C. Pace-Asciak and P. Demin, The Hospital for Sick Children, Toronto, ON) was made up as a stock solution in DMSO and was used at final concentrations of 0.1 µg/ml HxA₃ (LDHX) and 1.0 µg/ml HxA₃ (HDHX). Both A-23187 and 8(S)-HxA₃-methyl ester were stored as stock solutions at -20°C.
3.3 Cellular loading of calcium indicator dye fluo-3

The calcium indicator dye, fluo-3 AM, was obtained as an acetoxymethyl ester (AM) (F-1242, Molecular Probes Inc., USA). As the cell-permeant AM ester, fluo-3 AM is able to cross cellular membranes. Once inside the cell, fluo-3 AM is hydrolyzed by intracellular esterases, thereby releasing the Ca^{2+}-sensitive indicator, which is then capable of binding intracellular calcium. Upon its binding, fluo-3 reportedly undergoes a 100-fold increase in fluorescence; it is relatively non-fluorescent when not bound to calcium. Fluo-3 AM, as a 50 μg solute, was dissolved in 50 μl of anhydrous DMSO. Given the stoichiometry, 5 μl of this solution, dissolved in 1 ml of the control external solution, yielded a 5 μM concentration of fluo-3 AM in the solution. To enhance dye loading, Pluronic F-127 (P-1572, Molecular Probes Inc., USA) was used as a 20% w/v stock solution in anhydrous DMSO at a volume of 1 μl per ml control external solution. The solution was sonicated for 5 minutes prior to use, and this represented the loading medium. The cells to be loaded were first washed in the control external solution at 25°C for 5 minutes, and thereafter suspended in the same solution. To effectively remove the serum, the cells were superfused with the loading medium, and the culture dish was returned to the incubator at 37°C. After 45 minutes, loading medium was aspirated, the culture dish was superfused with the control external solution, and was kept at 25°C for 5 minutes. The cells were subsequently maintained in either normal (1.8 mM CaCl₂, 20 mM HEPES) or zero extracellular calcium (0 mM CaCl₂, 20 mM HEPES, 5 mM EGTA). Although calcium is required for proper cell attachment, the introduction of EGTA into the media did not appear to cause any disruptions (i.e. cause cells to lift off from the bottom of the dish).
4. Experimental Protocol

4.1 Injury-regeneration experiments

For these experiments, SCG cultures were used 10 to 14 days following initial plating, at which time neurites extended more than 10 mm from the cell bodies. Cell somata were confined to the center of the dish with neurites growing in a linear fashion along the tracks towards the periphery of the culture dish. The cell population was predominantly neuronal in origin, having been maintained in IF prior to the experimental manipulations. Prior to the experiment, the site of injury in each culture was chosen and marked with a pen on the bottom of the dish. The injury site was approximately 10-15 soma width (soma width was measured at 25-30 μm) from the most peripherally located cell body, from among the cluster of cell bodies situated centrally on each track. The neurites were injured with a motor driven rubber impactor which descended vertically downwards perpendicular to the surface of the culture dish. This device was designed and constructed by the Biomedical Engineering Department at the University of Toronto and consisted of an electrically driven vertical shaft to which was affixed a sharp edged rubber impactor (Figure-6). Both the starting height and the rate at which the impactor descended could be adjusted independently, but for these experiments were held constant. The rubber-impactor injury device produced neurite transection in approximately 75% of the available tracks, or 15 tracks per culture dish. This was a direct result of the length discrepancy between the impactor tip and the collective track width, the latter slightly exceeding the former. Tracks selected for long term viewing were proximate and exhibited complete neurite transection. A distance of at least 2 mm between the injury site and the cell body was selected because at this distance 1) the neurites would be more axonal like; 2) there is minimal cell death due to the transection; and 3) to further simulate traumatic axonal injuries in patients where the cell body is far from the injury.
Figure - 6  Injury device for injury-regeneration experiments

These photographs illustrate the injury device described in the METHODS section. The injury device for the injury-regeneration experiments is illustrated in (a), with progressive close-up views of the rubber-impactor as it descends upon the tracks, inflicting injury to the neurites (b) and c). The injury apparatus was designed to enable precise adjustments of the impactor’s starting height. The speed of descent of the impactor was controlled by the centrally mounted turn-knob, and was kept constant during the course of the injury-regeneration experiments. Note the width at the impactor’s tip in (c), which was fashioned to approximate the combined width of the 20 tracks.
Immediately following transection, cultures maintained in Initial Feed (with 1.8 mM CaCl₂) were exposed to one of the following treatments: 1) NGF (10 ng/ml) alone (Control); 2) 0.1% DMSO (1 μl/ml) + NGF; 3) 0.1 μg/ml or 0.28 μM HxA₃ (LDHX) in 1 μl DMSO + NGF; and 4) 1.0 μg/ml or 2.8 μM HxA₃ (HDHX) 1 μl DMSO + NGF. Each treatment group consisted of 3 sister cultures. DMSO, the vehicle for HxA₃ addition, was tested in all experimental paradigms and acted as an internal control for cultures treated with HxA₃. All compounds were mixed with a small volume of IF (=50 μl) and subsequently added to the dish of interest with a siliconized glass pipette. Phase images of the culture dishes were subsequently made at 2, 6, and 12 hours post-injury. The images always spanned the same region, in the vicinity of the transection injury, thus enabling sequential observations of specific changes in neurite behaviour, including dieback and neurite regeneration. During dish removal and image acquisition, the cultures were not exposed to atmospheric conditions for more than 3-4 minutes at a time. This prevented fluctuations in the culture medium temperature and/or the pH, both of which can adversely affect various aspects of cell morphology, physiology, and growth.

4.2 Calcium-dynamics experiments

Sister cultures were used for calcium-dynamics experiments. Cultures were maintained in Initial Feed (with 1.8 mM CaCl₂ and 10 ng/ml NGF). Fluo-3 loading was carried out as described above. Images were acquired at a resolution of 384 x 256 pixels at 8 bits/pixel, with 488nm excitation, 515nm emission, neutral density of 1, and constant black level and gain settings between experiments. Cultures were maintained in either normal or zero extracellular calcium, and were subsequently exposed to one of the following treatments: 1) 0.1% DMSO (i.e. 1 μl/ml
DMSO) (Control); 2) 1.65 μM calcium ionophore (A-23187); 3) 0.1 μg/ml HxA \(_3\) (LDHX) in 1 μl DMSO; and 4) 1.0 μg/ml HxA \(_4\) (HDHX) in 1 μl DMSO. All compounds were mixed with a small volume of media (=50 μl) and subsequently added to the dish of interest with a siliconized glass pipette. Each treatment group consisted of 3 sister cultures. With respect to the image acquisition program SOM, consistency was maintained in two important variables: the scan speed was kept at F2-Normal, corresponding to 1 second/frame, and 512 lines/frame; and the image size was kept at 4 horizontal boxes/screen. The four boxes generated during the course of image acquisition represented the following: first image, pre-treatment; second image, 4 seconds post-treatment; and the third and fourth images at 8 and 12 seconds post-treatment, respectively.

Changes in calcium in cell somata were monitored for up to 6-7 minutes using confocal microscopy. It should also be noted that images were acquired and displayed on the screen in the 'geog' pseudo-color mode, using the 'geog' look-up table. In this geographical color representation scheme, blue, green, and yellow depict lower pixel intensities, whereas red and white respectively, represent increasingly higher pixel intensities.

Under high-intensity illumination conditions, the irreversible destruction or 'photobleaching' of the excited fluorophore often becomes a factor which limits fluorescence detectability, thereby resulting in a decreased observed fluorescence. For this reason, therefore, photobleaching of excited fluo-3 was monitored in loaded neurons of untreated cultures at 5 minute intervals for up to 30 minutes. No significant change in fluorescence (i.e. average pixel intensity) was observed during the entire 30 minute period, from the time of cellular loading of fluo-3.

Fluorescence detection sensitivity may also be compromised by background signals, which may originate from endogenous sample constituents (referred to as autofluorescence) or from
unbound or nonspecifically bound probe (referred to as reagent background). Therefore, background fluorescence in fluo-3 loaded cultures was monitored over the duration of all experiments; no significant change in background fluorescence was ever observed.

5. Data Analysis and Statistics

5.1 Injury-regeneration experiments

The quantitative data obtained from the images of cultures taken at specified times post-injury include: 1) neurite length; 2) the rate of regeneration (μm/hr) from 2-6, and 6-12 hours after neurite transection; and 3) the % increase in rate of regeneration over controls (i.e. transected neurons treated with NGF alone). Image analysis software (DOS® based SOM) was utilized to make neurite length measurements, which were reported as mean length (in μm) ± standard error of the mean (sem). The rate of regeneration was calculated using the total distance regenerated divided by the time interval required to grow that distance. The total distance regenerated was determined by measuring the distance between the lead 3 neurites per track and the site of transection at 2, 6 and 12 hours post-injury. Although it was difficult to distinguish particular neurites, careful observations were made so as to follow the same lead neurites at each successive time point for rate of outgrowth measurements. Fiduciary markers along the tracks helped to keep track of the location of specific neurite tips or 'ends' of growing neurites at 2, 6, and 12 hours following transection. It was necessary to begin calculating the rate at 2 hours post-transection, because neurite dieback was evident after transection and initiation of regeneration was not consistent across treatment groups at earlier time points.
The percentage increase in rate of regeneration over controls was calculated using the following formula:

\[(R_t - R_c/R_c) \times 100\%\]

where \(R_t\) is the rate of regeneration of the treatment group of interest, and \(R_c\) is the rate of regeneration of the control group (i.e. transected neurons treated with NGF alone). Rates of regeneration were compared to one another using a one-way ANOVA. As this data did not meet assumptions for normality, the non-parametric Kruskal-Wallis one-way ANOVA on ranks had to be performed. In instances where comparisons had to be made to a single control group, after the data had been subjected to a Kruskal-Wallis one-way ANOVA on ranks, multiple comparisons versus the control group were carried out using Tukey’s Test. For either of the above tests used to analyze the injury-regeneration data, the level of significance, or the p-value, was set at 0.05. Derivation of the descriptive statistical parameters, execution of the various statistical tests, as well as plotting of the data, were all performed using SigmaPlot and SigmaStat statistical software packages (Jandel Corporation, USA).

5.2 Calcium-dynamics experiments

In fluo-3 loaded neurons, changes in intracellular fluorescence both pre-and post-treatment were measured using image analysis software (DOS® based SOM). Once an area or region of interest within the cell (excluding the nucleus) was selected for measurement, a rectangle was drawn around the area using the ‘Stats’ command, and the average pixel intensity (API) (a measure of the average fluorescence of individual pixels in a given region) was subsequently obtained. The program’s calculative output consisted of the API and the standard deviation between the individual pixel intensities. The fractional change in fluorescence (\(\Delta F/F_0\)),
an expression used to normalize the data, was defined as:

\[(\text{Post-Pre})\text{API}/(\text{Pre})\text{API}\]

The \(F_0\), \(F_1\), and \(\Delta F\) data presented as the API, as well as \(\Delta F/F_0\), were all reported as two significant figures after the decimal. The \(\Delta F/F_0\) data was averaged from all the individual experiments within a treatment group (n), and the data for each group expressed in the form of mean ± sem. The \(\Delta F/F_0\) data were compared to one another using a One Way ANOVA. As this data did not meet assumptions for normality, the non-parametric Kruskal-Wallis One Way ANOVA on ranks had to be performed. In instances where comparisons had to be made to a single control group, after the data had been subjected to a Kruskal-Wallis One Way ANOVA on ranks, multiple comparisons versus the control group were carried out using Tukey’s Test.

For either of the above tests used to analyze the calcium-dynamics data, the level of significance, or the p-value, was set at 0.05. Derivation of the descriptive statistical parameters, execution of the various statistical tests, as well as plotting of the data, were all performed using SigmaPlot and SigmaStat statistical software packages (Jandel Corporation, USA).
RESULTS

1. General Features of Sympathetic Neuron Cell Culture

Sympathetic neurons were plated in the center of the culture dishes that had been scratched with a pinrake to produce parallel tracks (Figure 7a-d). Immediately after plating, the neuronal bodies aggregated into groups, with each group comprising up to 10 somata. Neurites and growth cones emerged from the cell body clusters within 24 hours of cell plating, strongly attaching themselves to the collagen matrix. Almost all of the neurite outgrowth was directed in a linear manner along the tracks, and towards the periphery of the dishes, as shown in Figures 7c and 7d. Neurite morphology was assessed by phase-contrast microscopy in sympathetic principal neurons well established in culture for 10-14 days. Neurites terminated their growth upon reaching the end wall of the culture dish, and none were found to turn around and grow in the reverse direction.

2. Injury-Regeneration Experiments

Neurite outgrowth was examined in 10-14 day old SCG cultures at 2, 6, and 12 hours post-injury. An age of 10 to 14 days was chosen because the ability of these neurons to produce catecholamines increases dramatically in the second week of culture. At this point, neurons have matured and are more similar to the in vivo situation where growing neurites have reached their targets (Chun and Patterson, 1977). Cultures were exposed to Initial Feed containing NGF (10 ng/ml) 24 hours post-plating and during experiments. Earlier injury-regeneration studies conducted in our lab utilized SCG cultures maintained in NGF at final concentrations of 200 and 50 ng/ml, respectively (data not shown). Comparable rates of outgrowth post-injury, however, were observed in cultures maintained in NGF at a concentration of 10 ng/ml. All subsequent
**Figure - 7** Sympathetic principal neurons from the rat SCG growing on collagen

(a) Dissociated sympathetic principal neurons from the rat superior cervical ganglion (SCG) growing on a collagen-coated 35 mm plastic tissue culture dish. The cells were imaged at 10 days in culture through a 10X objective lens. The initial feeding medium contained the antimitotic agent ara-C, thus eliminating most of the non-neuronal cells, and yielding an almost purely neuronal cell culture.

(b) A 35 mm tissue culture dish with 20 parallel tracks in the center of the dish. Tracks were produced with a pinrake to ensure the linear outgrowth of extending neurites.

(c) A 10X phase-contrast image of a 10-day old culture. This image is taken over the center of the dish, showing aggregated cell somata and the bipolar outgrowth of neurites on the tracks.

(d) Same culture as in (c), but viewed with a 20X objective lens. This image was taken over the periphery of the dish, in the zone free of cell somata and containing only the neuritic processes. Note the variation in neurite size and the extensive pattern of branching. The neurites are seen to be strictly confined to the track, with no growth across the adjacent scratches into the adjoining tracks.
studies, therefore, were carried out using NGF at a concentration of 10 ng/ml. Immediately following transection, cultures maintained in Initial Feed (with 1.8 mM CaCl₂) were exposed to one of the following treatments: 1) NGF (10 ng/ml) alone (Control); 2) 0.1% DMSO (1 μl/ml) + NGF; 3) 0.1 μg/ml HxA₃ (LDHX) in 1 μl DMSO + NGF; and 4) 1.0 μg/ml HxA₃ (HDHX) 1 μl DMSO + NGF. Each treatment group consisted of 3 sister cultures. All compounds were mixed with a small volume of IF (≈50 μl) and subsequently added to the dish of interest with a siliconized glass pipette. Dieback began immediately following transection, and was complete by 1-1.5 hours post-injury. At two hours, distinct growth cones could be identified emerging from the proximal neuritic segments. Phase images of the culture dishes were subsequently made at 2, 6, and 12 hours post-injury. The images always spanned the same region, in the vicinity of the transection injury, thus enabling sequential observations of specific changes in neurite behaviour, including dieback and neurite regeneration. Fiduciary markers along the tracks helped to identify the location of initial transection injury, the degree of dieback distance, as well as the degree of outgrowth at different time points. Given that neuronal regeneration ensued only after the completion of dieback at 1-1.5 hours following transection, and that growth cones were evident at 2 hours post-injury, the onset of regeneration most likely occurred 1-2 hours after the neuritic transection injury. At 24 hours post-injury, neurites often grew into the distal cut end which made measuring neurite length extremely difficult. For these reasons, therefore, rates of regeneration were obtained from 2 to 6 and 6 to 12 hours post-injury.

2.1 Neurite regeneration 2-6 hours post-injury

Two to 6 hours post-injury, neurite outgrowth increased significantly in cultures treated with LDHX and HDHX compared to controls (NGF alone). Mean rates of outgrowth (± sem)
for controls and neurons treated 0.1% DMSO (1 μl/ml) were 39.69 ± 1.59 μm/hr (n=135 cells, Figure - 8) and 41.82 ± 1.53 μm/hr (n=132 cells, Figure-9), respectively. Mean rates of outgrowth for neurons treated with LDHX and HDHX were 52.13 ± 1.49 μm/hr (n=129 cells, Figure-10) and 47.40 ± 2.18 μm/hr (n=123 cells, Figure-11), respectively, corresponding to increases in outgrowth of 31.3% and 19.4% over controls (Figure-12b). When the mean rates of outgrowth were subjected to statistical comparison using the Kruskal-Wallis one way ANOVA on ranks, a significant difference between groups was revealed (p=0.001, Figure-12a). Multiple pairwise comparisons using Tukey’s Test were performed, and outgrowth rates for LDHX and HDHX treatment groups were found to differ significantly from both the control (NGF alone) and the DMSO groups (p<0.05). There was a significant difference between LDHX and HDHX groups (p<0.05), while the DMSO group was not found to differ significantly from the control group (p>0.05).

2.2 Neurite regeneration 6-12 hours post-injury

Six to 12 hours post-injury, neurite outgrowth increased significantly in cultures treated with LDHX compared to controls (NGF alone). The mean rate of outgrowth for neurons treated with NGF alone was 24.11 ± 1.74 μm/hr (n=135 cells, Figure-8). Mean rates of outgrowth for neurons treated with LDHX and HDHX were 31.20 ± 2.23 μm/hr (n=129 cells, Figure-10) and 27.52 ± 2.14 μm/hr (n=123 cells, Figure-11), respectively, corresponding to increases in outgrowth of 29.4% and 14.08% over controls (Figure-13b). When the mean rates of outgrowth were subjected to statistical comparison using the Kruskal-Wallis one way ANOVA on ranks, a significant difference between groups was revealed (p= 0.05, Figure-13a). Multiple pairwise comparisons using Tukey’s Test showed that the LDHX group differed significantly from both
Figure - 8

Phase-contrast images of regenerating neurites post-treatment with NGF alone. A representative control culture (NGF alone) from an injury-regeneration experiment, imaged using phase-contrast microscopy (20X objective). Only the proximal end of transected neurites are evident. The entire injury site, including distal end of transected neurites are not shown in the image sequences. The sequence of events are illustrated in each of the images, which are sequentially labeled from $t = 2$ to $12$ hours post-injury. The phase image at $t = 2$ hours clearly shows the location of neurite transection injury, carried out 10-15 soma widths to the left of the most peripherally located cell somata, from amongst the central cluster of cell somata. Neurite outgrowth is evident at $t = 6$ and $12$ hours post-injury, as evidenced by growth cone development and extending neurites. Red arrow indicates location of initial injury. Initial location of regenerating neurites after the completion of dieback at 2 hours post-injury is indicated by the yellow line. Green arrows indicate regenerating neurites. Black arrows demonstrate fiduciary markers along the tracks.
Figure - 9

Phase-contrast images of regenerating neurites post-treatment with DMSO alone. A representative neuronal culture treated with 0.1% DMSO from an injury-regeneration experiment, imaged using phase-contrast microscopy (20X objective). The sequence of events are illustrated in each of the images, which are sequentially labeled from $t = 2$ to 12 hours post-injury. Neurite outgrowth is evident at $t = 6$ and 12 hours post-injury, as evidenced by growth cone development and extending neurites. Red arrow indicates location of initial injury. Initial location of regenerating neurites after the completion of dieback at 2 hours post-injury is indicated by the yellow line. Green arrows indicate regenerating neurites. Black arrows demonstrate fiduciary markers along the tracks.
Figure - 10

Phase-contrast images of regenerating neurites post-treatment with LDHX + NGF. A representative neuronal culture treated with LDHX (0.1 \( \mu g/ml \) HxA) from an injury-regeneration experiment, imaged using phase-contrast microscopy (20X objective). The sequence of events are illustrated in each of the images, which are sequentially labeled from \( t = 2 \) to 12 hours post-injury. The phase image at \( t = 2 \) hours clearly shows the location of neurite transection injury, carried out 10-15 soma widths to the left of the most peripherally located cell somata, from amongst the central cluster of cell somata. Neurite outgrowth is evident at \( t = 6 \) and 12 hours post-injury, as evidenced by growth cone development and extending neurites. Red arrow indicates location of initial injury. Initial location of regenerating neurites after the completion of dieback at 2 hours post-injury is indicated by the yellow line. Green arrows indicate regenerating neurites. Black arrows demonstrate fiduciary markers along the tracks.
Figure - 10

The figure shows three images labeled T=2hr, T=6hr, and T=12hr.
**Figure - 11**

Phase-contrast images of regenerating neurites post-treatment with HDHX + NGF. A representative neuronal culture treated with HDHX (1.0 μg/ml HxA₃) from an injury-regeneration experiment, imaged using phase-contrast microscopy (20X objective). The sequence of events are illustrated in each of the images, which are sequentially labeled from t = 2 to 12 hours post-injury. The phase image at t = 2 hours clearly shows the location of neurite transection injury, carried out 10-15 soma widths to the left of the most peripherally located cell somata, from amongst the central cluster of cell somata. Neurite outgrowth is evident at t = 6 and 12 hours post-injury, as evidenced by growth cone development and extending neurites. Red arrow indicates location of initial injury. Yellow lines indicate initial location of regrowing neurites after the completion of dieback at 2 hours post-injury. Green arrows indicate regenerating neurites. Black arrows demonstrate fiducial markers along the tracks.
Figure - 12

(a) Rate of neurite outgrowth between 2hr and 6hr post-transection injury. The bar graph illustrated here displays the rate of neurite outgrowth, or regeneration, 2 to 6 hours following acute neuritic transection injury. The respective data for neurons exposed to the following treatments post-injury are shown: 1) NGF alone (Control); 2) 0.1% DMSO (1 μl/ml) + NGF; 3) 0.1 μg/ml HxA₃ (LDHX) + NGF; and 4) 1.0 μg/ml HxA₃ (HDHX) + NGF. The data is presented as the mean rate of outgrowth (μm/hr) with error bars indicating the standard error of the mean. Each treatment group consisted of 3 sister cultures; injury-regeneration experiments were repeated 3 times. The number of cells for each treatment group was therefore derived from cultures used in all three experiments (ie. in 3 experiments, 12 cultures per treatment group). Asterisks denote a statistically significant difference between the control and treatment groups. The respective p-values were derived using a Kruskal-Wallis One Way ANOVA on ranks, and multiple comparisons versus the control group performed using Tukey’s Test. The level of significance is 0.05. Data is summarized in Table-1.

(b) Increase in rate of neurite outgrowth over control (NGF alone) between 2hr and 6hr post- transection injury. The bar graph presented here displays the % increase in neurite outgrowth of DMSO, LDHX, and HDHX treatment groups, over control, 2 to 6 hours post-injury.
Figure - 12

(a) Bar graph showing the rate of outgrowth (μm/hr) for Control, DMSO, LDHX, and HDHX treatments post-injury. The bars indicate a significant increase in rate for LDHX and HDHX compared to Control.

(b) Bar graph showing the % increase in neurite outgrowth for DMSO, LDHX, and HDHX treatments post-injury. The bar for LDHX is significantly higher than that for DMSO and HDHX.
Figure - 13

(a) Rate of neurite outgrowth between 6hr and 12hr post-transection injury. The bar graph illustrated here displays the rate of neurite outgrowth, or regeneration, 6 to 12 hours following acute neuritic transection injury. The respective data for neurons exposed to the following treatments post-injury are shown: 1) NGF alone (Control); 2) 0.1% DMSO (1 μl/ml) + NGF; 3) 0.1 μg/ml HxA₃ (LDHX) + NGF; and 4) 1.0 μg/ml HxA₃ (HDHX) + NGF. The data is presented as the mean rate of outgrowth (μm/hr) with error bars indicating the standard error of the mean. Each treatment group consisted of 3 sister cultures; injury-regeneration experiments were repeated 3 times. The number of cells for each treatment group was therefore derived from cultures used in all three experiments (ie. in 3 experiments, 12 cultures per treatment group). Asterisks denote a statistically significant difference between the control and treatment groups. The respective p-values were derived using a Kruskal-Wallis One Way ANOVA on ranks, and multiple comparisons versus the control group performed using Tukey’s Test. The level of significance is 0.05. Data is summarized in Table-1.

(b) Increase in rate of neurite outgrowth over control (NGF alone) between 6hr and 12hr post-transection injury. The bar graph presented here displays the % increase in neurite outgrowth of DMSO, LDHX, and HDHX treatment groups, over control, 6 to 12 hours post-injury.
Figure 13

(a) Rate of Outgrowth (um/hr)

(b) % Increase in Neurite Outgrowth

Control  DMSO  LDHX  HDHX
Treatment Post-Injury

([Rate_{Tx}-Rate_{Control}]/Rate_{Control})*100]
## Table - 1

Rate of neurite outgrowth post-transection injury

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of neurons (n)</th>
<th>Rate of Outgrowth 2-6 hours Post-injury (µm/hr)</th>
<th>Rate of Outgrowth 6-12 hours Post-injury (µm/hr)</th>
<th>% in Outgrowth 2-6 hours Post-Injury</th>
<th>% in Outgrowth 6-12 hours Post-Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NGF alone)</td>
<td>135</td>
<td>39.69 ± 1.59</td>
<td>24.11 ± 1.74</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.1% DMSO (1 µl/ml)</td>
<td>132</td>
<td>41.82 ± 1.53 (p&gt;0.05)</td>
<td>24.76 ± 1.82 (p&gt;0.05)</td>
<td>5.34%</td>
<td>2.68%</td>
</tr>
<tr>
<td>LDHX (0.1 µg/ml)</td>
<td>129</td>
<td>52.13 ± 1.49 (p&lt;0.05)</td>
<td>31.20 ± 2.23 (p&lt;0.05)</td>
<td>31.30%</td>
<td>29.40%</td>
</tr>
<tr>
<td>HDHX (1.0 µg/ml)</td>
<td>123</td>
<td>47.40 ± 2.18 (p&lt;0.05)</td>
<td>27.52 ± 2.14 (p&gt;0.05)</td>
<td>19.40%</td>
<td>14.08%</td>
</tr>
</tbody>
</table>

- the respective p-values were derived using Kruskal-Wallis One Way ANOVA on Ranks; multiple comparisons versus the control group performed using Tukey’s Test. The level of significance is 0.05.
the control (NGF alone) and DMSO groups (p<0.05). The mean rate of outgrowth of the HDHX group, however, did not differ significantly from control or DMSO groups (p>0.05). LDHX-enhanced neurite outgrowth after 6 hours, was significantly less than that elicited during the first 6 hours post-injury (p<0.001). HxA₃ was not found to substitute for NGF-mediated outgrowth, for treatment of injured neurons with HxA₃ in the absence of NGF produced little to no regrowth (data not shown). The data from sections 2.1 and 2.2 is summarized in Table-1.

3. Calcium-Dynamics Experiments

3.1 Calcium-dynamics in uninjured SCG neurons (in normal extracellular calcium)

Following successful fluo-3 loading, experiments were undertaken to examine hepoxilin-evoked changes in intracellular calcium in the somata of uninjured neurons. Cultures were maintained in normal extracellular calcium (1.8 mM CaCl₂, 20 mM HEPES) and were subsequently exposed to one of the following treatments: 1) 0.1% DMSO (i.e. 1 μl/ml DMSO) (Control); 2) 1.65 μM calcium ionophore (A-23187)(positive control); 3) 0.1 μg/ml HxA³ (LDHX) in 1 μl DMSO; and 4) 1.0 μg/ml HxA³ (HDHX) in 1 μl DMSO. All compounds were mixed with a small volume of media (=50 μl) and subsequently added to the dish of interest with a siliconized glass pipette.

A) Effects of 0.1% DMSO vehicle (1 μl/ml)

Firstly, the response to an addition of DMSO, the vehicle for addition of HxA₃, was assessed to determine whether it affected the fluorescence intensity of fluo-3 within the somata. Addition of 0.1% DMSO did not lead to a significant increase in the neuronal calcium response, as noted by a lack of a qualitative change in the fluorescence intensity of fluo-3 (Figure-14).
Figure - 14

Confocally-derived images of intracellular fluorescence in uninjured neurons post-treatment with DMSO. Representative changes in the fluorescence signal intensity in uninjured SCG neurons at $t = 0\text{ sec}$, $8\text{ sec}$, $30\text{ sec}$, and $2\text{ min}$ post-treatment with $0.1\%$ DMSO ($1\, \mu l/ml$). Images were obtained using confocal (laser-scanning) microscopy in conjunction with the calcium-binding fluorophore fluo-3-AM. Experiments were carried out in normal extracellular calcium ($1.8\text{ mM CaCl}_2$). The fluorescence signal is presented in pseudo-color using a calcium lookup table (i.e. calcium.lut), as shown by the wedge on the upper-left image.
Calculation of the API were made using the pre- and 8 seconds post-treatment images. The ΔF/F₀ was calculated to be 0.04 ± 0.03 (n=40 cells) (Figures 18a and 19). This mean ΔF/F₀ served as the control group for subsequent comparisons with the other treatment groups.

B) Effects of calcium ionophore A-23187 (1.65 μM)

Calcium ionophore (A-23187) was introduced into the extracellular medium at a concentration of 1.65 μM, and within 8 seconds, a rapid increase in fluorescence was observed (Figure 15). Measurements were made using the pre- and 8 seconds post-treatment images. The ΔF/F₀ was calculated to be 1.54 ± 0.07 (n=20 cells) and was significantly different from the control group (p<0.05) (Figures 18b and 19). By 8 minutes post-treatment, fluorescence levels had decreased to a new stable baseline significantly above rest (ΔF/F₀ = 0.33 ± 0.12).

C) Effects of HxA₃: LDHX (0.1 μg/ml) and HDHX (1.0 μg/ml)

LDHX (0.1 μg/ml HxA₃) evoked a rapid increase in fluorescence signal intensity in the somata by 4 seconds. By 8 seconds post-addition, signal intensity peaked (Figure-16), with a mean ΔF/F₀ of 0.87 ±0.13 (n=82 cells) (Figures 18c and 19). Two minutes post-addition, fluorescence levels decreased to a new stable baseline significantly above rest (ΔF/F₀ = 0.43 ± 0.09). HDHX (1.0 μg/ml HxA₃) also evoked a rapid increase in intracellular fluorescence by 4 seconds. Fluorescence peaked at 8 seconds post-addition (Figure-17), with a mean ΔF/F₀ of 1.26 ± 0.10 (n=93 cells) (Figures 18d and 19); by 2 minutes post-addition, fluorescence levels decreased to a stable plateau significantly above rest or baseline calcium levels (ΔF/F₀ = 0.54 ± 0.07). Data for control (i.e. DMSO), A-23187, LDHX, and HDHX groups was subjected to the Kruskal-Wallis one way ANOVA on ranks, and there was a significant difference among groups
Figure - 15

Confocally-derived images of intracellular fluorescence in uninjured neurons post-treatment with calcium ionophore (A-23187). Representative changes in the fluorescence signal intensity in uninjured SCG neurons at $t = 0$ sec, 8 sec, 30 sec, and 2 min post-treatment with calcium ionophore A-23187 (1.65 μM). Images were obtained using confocal (laser-scanning) microscopy in conjunction with the calcium-binding fluorophore fluo-3-AM. Experiments were carried out in normal extracellular calcium (1.8 mM CaCl$_2$). The fluorescence signal is presented in pseudo-color using a calcium lookup table (i.e. calcium.lut), as shown by the wedge on the upper-left image.
Figure - 16

Confocally-derived images of intracellular fluorescence in uninjured neurons post-treatment with LDHX. Representative changes in the fluorescence signal intensity in uninjured SCG neurons at t = 0 sec, 8 sec, 30 sec, and 2 min post-treatment with LDHX (0.1 μg/ml HxA₃). Images were obtained using confocal (laser-scanning) microscopy in conjunction with the calcium-binding fluorophore fluo-3-AM. Experiments were carried out in normal extracellular calcium (1.8 mM CaCl₂). The fluorescence signal is presented in pseudo-color using a calcium lookup table (i.e. calcium.lut), as shown by the wedge on the upper-left image.
Figure - 17

Confocally-derived images of intracellular fluorescence in uninjured neurons post-treatment with HDHX. Representative changes in the fluorescence signal intensity in uninjured SCG neurons at t = 0 sec, 8 sec, 30 sec, and 2 min post-treatment with HDHX (1.0 µg/ml HxA₃). Images were obtained using confocal (laser-scanning) microscopy in conjunction with the calcium-binding fluorophore fluo-3-AM. Experiments were carried out in normal extracellular calcium (1.8 mM CaCl₂). The fluorescence signal is presented in pseudo-color using a calcium lookup table (i.e. calcium.lut), as shown by the wedge on the upper-left image.
Figure - 17
Figure - 18

Fractional change in fluorescence ($\Delta F/F_0$) in SCG neurons post-treatment with DMSO, calcium ionophore (A-23187), LDHX, or HDHX. The graphs a)-d) illustrate the fractional change in fluorescence in neurons exposed to the following treatments, respectively: a) 0.1% DMSO (i.e. 1 $\mu$l/ml DMSO) (Control); b) 1.65 $\mu$M calcium ionophore (A-23187); c) 0.1 $\mu$g/ml HxA$_3$ (LDHX); and d) 1.0 $\mu$g/ml HxA$_3$ (HDHX). Experiments were carried out in normal extracellular calcium (1.8 mM CaCl$_2$). The data is presented as the mean $\Delta F/F_0$ (as explained in the METHODS section), with error bars indicating the standard error of the mean, versus time in ‘seconds’ post-treatment. Data is summarized in Table-2.
Figure - 19

Peak fractional change in fluorescence ($\Delta F/F_0$) in neurons post-treatment with DMSO, calcium ionophore (A-23187), LDHX, or HDHX. This bar graph illustrates the peak $\Delta F/F_0$ values in uninjured neurons 8 seconds post-treatment by DMSO (control), LDHX, HDHX, or calcium ionophore A-23187. The data is presented as the mean $\Delta F/F_0$, with error bars indicating the standard error of the mean. Each treatment group consisted of 3 sister cultures; calcium dynamics experiments were repeated 3 times. The number of cells for each treatment group was therefore derived from cultures used in all three experiments. Asterisks denote a statistically significant difference between the control and treatment groups. The respective p-values were derived using a Kruskal-Wallis One Way ANOVA on ranks, and multiple comparisons versus the control group performed using Tukey’s Test. The level of significance is 0.05. Data is summarized in Table-2.
Figure - 19

The graph shows the effect of different treatments on a normalized parameter (F_1 - F_0)/F_0. The treatments are labeled as DMSO, LDHX, HDHX, and A-23187. The bars indicate the mean values with error bars depicting the standard deviation. The asterisks (*) denote statistically significant differences compared to the DMSO control.
Table - 2

Calcium-dynamics in the somata of uninjured neurons

<table>
<thead>
<tr>
<th>Nature of Treatment</th>
<th>Number of neurons (n)</th>
<th>Pre-Treatment (API)</th>
<th>Post-Treatment (API)</th>
<th>Fractional change (ΔF/F₀)</th>
<th>~One Way ANOVA on Ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (Control)</td>
<td>40</td>
<td>35.47 ± 2.73</td>
<td>37.10 ± 2.19</td>
<td>0.04 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>20</td>
<td>58.97 ± 2.18</td>
<td>149.96 ± 2.26</td>
<td>1.54 ± 0.07</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>LDHX (0.1 μg/ml)</td>
<td>82</td>
<td>50.34 ± 4.61</td>
<td>94.14 ± 9.70</td>
<td>0.87 ± 0.13</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>HDHX (1.0 μg/ml)</td>
<td>93</td>
<td>53.96 ± 5.19</td>
<td>121.81 ± 9.62</td>
<td>1.26 ± 0.10</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

- the respective p-values were derived using Kruskal-Wallis One Way ANOVA on Ranks; multiple comparisons versus the control group performed using Tukey's Test. The level of significance is 0.05.
Multiple pairwise comparisons using Tukey's Test revealed a significant difference between the control and both the LDHX and HDHX groups (p<0.05). There was also a significant difference between LDHX and HDHX groups (p<0.05). The calcium ionophore group differed significantly from the LDHX group (p<0.05), but not from the HDHX group (p>0.05). The data from sections A)-C) are summarized in Table-2.

**D) Comparison of the distribution of peak fluorescence post-treatment with LDHX or HDHX**

In addition to there being a significant difference in mean peak fluorescence between LDHX and HDHX groups, there also appears to be a significant difference in calcium responsiveness of individual neurons to differing concentrations of HxA₃. Fractional change (ΔF/Fₒ) values were obtained to determine the mean fluorescence response of neurons post-treatment with HxA₃. The distribution of peak fluorescence in neurons post-treatment with HxA₃ was also determined, to examine the response of individual neurons in a given population. Fluorescence values from experiments were placed into three separate groups: a) neurons at 'rest'; b) 'peak' fluorescence in neurons treated with LDHX; and c) 'peak' fluorescence in neurons treated with HDHX. Data from all three groups were plotted as % frequency of response versus raw fluorescence (API) (Figure-20). Using the Kolmogorov-Smirnov (K-S) test for normality, rest fluorescence data was found to exhibit a normal distribution (p=0.22), whereas peak fluorescence data from LDHX or HDHX groups were found to exhibit non-normal distributions. When the K-S test was used to compare peak fluorescence distributions for both treatment groups with rest fluorescence distribution, a significant difference in distribution was observed between: a) LDHX and HDHX groups (maximum difference between the distributions as defined by the K-S test, 'D', was 0.29, p=0.001), and b) LDHX treatment group and rest...
Figure 20

Distribution of peak raw fluorescence in SCG neurons at rest, and post-treatment with LDHX or HDHX. This graph illustrates peak calcium fluorescence in uninjured SCG neurons at rest (dotted line), 8 seconds post-treatment with LDHX (broken line), and 8 seconds post-treatment with HDHX (solid line). The x-axis represents raw fluorescence values (API), and the y-axis represents % frequency of response. Using the Kolmogorov-Smirnov (K-S) test for normality, rest fluorescence data was found to exhibit a normal distribution (p=0.22), whereas peak fluorescence data from LDHX or HDHX groups were found to exhibit non-normal distributions. When the K-S test was used to compare peak fluorescence distributions for both LDHX and HDHX groups with rest fluorescence distribution, a significant difference in distribution was observed between: a) LDHX and HDHX groups (D=0.29, p=0.001), and b) LDHX treatment groups and rest (D=0.71, p<0.001); and c) HDHX treatment group and rest (D=0.82, p<0.001).
Figure - 20

![Graph showing raw fluorescence (API) vs. percentage frequency of response.](image)
(D=0.71, p<0.001); and c) HDHX treatment group and rest (D=0.82, p<0.001). Fluorescence data was further analyzed to determine whether or not there was a difference in 'peak fractional change' (ΔF/F₀) between treatment groups. Peak ΔF/F₀ values from LDHX and HDHX groups were plotted as % frequency of response versus fractional change in fluorescence (Figure-21a). (The peak ΔF/F₀ value for DMSO was very small (i.e. 0.04 ± 0.03), and therefore was disregarded with respect to HxA-induced increase in fluorescence.) When the K-S test was used to compare the peak ΔF/F₀ distributions, a significant difference in distribution was observed between the two treatment groups (D=0.26, p=0.004). To further compare the difference in peak ΔF/F₀ distributions, the data for each of the two treatment groups was sub-divided into four groups: A) % frequency of neurons with no to small increase in fluorescence (ΔF/F₀=0-0.799); B) % frequency with a moderate increase in fluorescence (ΔF/F₀=0.8-1.599); C) % frequency with a large increase in fluorescence (ΔF/F₀=1.6-2.399); and D) % frequency with saturating levels of fluorescence (2.4-2.999) (Figure-21b). By grouping the data as such, we were able to examine the responsiveness of subpopulations of fluo-3 loaded neurons post-treatment with LDHX or HDHX. (Ranges for groups A-D were selected based on the heterogeneous fluorescence responses for both treatment groups observed in Figure - 21a.) The percentage of data (for LDHX and HDHX groups, respectively) in each of the four groups was: A) 24.39% vs. 5.38%; B) 59.76% vs. 67.74%; C) 13.41% vs. 23.66%; and D) 2.44% vs. 3.23%. These results show that 24% of the neuronal population treated with LDHX were group ‘A’ neurons; the percentage of neurons in group ‘A’ decreased to 5% upon treatment with HDHX. This may indicate a subpopulation of neurons which are refractory or non-responsive. 60% of the population treated with LDHX were group ‘B’ neurons; the percentage of neurons in group ‘B’ increased to 67% upon treatment with HDHX. A similar shift in fluorescence response was
Figure-21

(a) Distribution of peak fractional change in fluorescence (ΔF/ΔF₀) in SCG neurons post-treatment with LDHX or HDHX. This graph illustrates the distribution of peak fractional change in fluorescence in uninjured SCG neurons post-treatment with LDHX (dotted line) or HDHX (broken line). The x-axis represents fractional change in fluorescence (ΔF/ΔF₀), and the y-axis represents % frequency of response. When the K-S test was used to compare the peak ΔF/ΔF₀ distributions, a significant difference in distribution was observed between the two treatment groups (D=0.26, p=0.004).

(b) Distribution of peak fractional change in fluorescence (ΔF/ΔF₀) in groups A-D for SCG neurons post-treatment with LDHX or HDHX. This graph illustrates distribution of peak fractional change in fluorescence in groups A)-D) in SCG neurons post-treatment with LDHX (solid bar) or HDHX (hatched bar). Data for each of the two treatment groups was sub-divided into four groups: A) % frequency of neurons with no to small increase in fluorescence (ΔF/ΔF₀=0-0.799); B) % frequency with a moderate increase in fluorescence (ΔF/ΔF₀=0.8-1.599); C) % frequency with a large increase in fluorescence (ΔF/ΔF₀=1.6-2.399); and D) % frequency with saturating levels of fluorescence (2.4-2.999). The percentage of data (for LDHX and HDHX groups, respectively) in each of the four groups was: A) no to small increase (24.39% vs. 5.38%); B) moderate increase (59.76% vs. 67.74%); C) large increase (13.41% vs. 23.66%); and D) saturating levels of fluorescence (2.44% vs. 3.23%).
observed between LDHX and HDHX treatment groups in group ‘C’ neurons. 13% of the population treated with LDHX were group ‘C’ neurons; the percentage of neurons in group ‘C’ increased to 24% upon treatment with HDHX. There was no apparent shift in fluorescence between treatment groups in Group ‘D’ neurons, which suggests that this neuronal subpopulation is not dose-dependent. Together, these results demonstrate a dose-dependence fluorescence response exists in group A, B, and C neurons. The drop in fluorescence in group ‘A’ neurons and the corresponding increases in fluorescence in group ‘B’ and particularly group in ‘C’ neurons upon treatment with hepoxilin, suggest that there is significant heterogeneity in SCG neurons with respect to HxA3-induced increase in fluorescence.

3.2 Calcium-dynamics in uninjured SCG neurons (in zero extracellular calcium or using lanthanum chloride (La 3+))

Studies using human neutrophils, pancreatic β-cells and vascular tissue show that HxA3 causes a dose-dependent rise in intracellular calcium. Two components have been observed, an initial rapid phase of intracellular calcium rise, followed by a slow decline to plateau levels that remain significantly above baseline calcium level. Studies have suggested that the initial rapid phase is caused by a release of calcium from intracellular stores in the endoplasmic reticulum; the slower rate of decline (plateau phase) is thought to be caused by calcium influx as it is abolished in zero calcium medium (Pace-Asciak, 1994; Mills et al. 1997). Addition of lanthanum chloride (1μM), an inorganic calcium channel blocker, prior to or post-addition of hepoxilin, eliminates the second phase completely, with the calcium peak returning rapidly to normal baseline levels (Reyanud et al. 1999) as observed in extracellular calcium-free medium. These results further confirm the belief that the plateau phase is due to calcium influx.
To determine whether or not extracellular calcium played a role in the rise and subsequent decrease of intracellular calcium levels observed when HxA₃ was added to the somata of uninjured neurons, cultures were maintained in zero extracellular calcium (0 mM CaCl₂, 20 mM HEPES, 5 mM EGTA) and exposed to HxA₃ at a concentration of 1.0 µg/ml (HDHX). (It should be noted that zero calcium experiments were performed in conjunction with normal calcium experiments, to control for culture conditions, HxA₃ addition, etc.). Upon addition of HDHX, an increase in fluorescence was observed by 4 seconds, as seen under normal calcium conditions. Peak levels of fluorescence occurred 6-8 seconds post-treatment, producing a mean ΔF/F₀ of 0.93 ± 0.08 (n=20 cells) (Figure 22a). Approximately 1.5-2 minutes post-treatment, a new stable baseline of fluorescence was achieved (ΔF/F₀ = 0.20 ± 0.03) and was found to differ significantly from resting levels (p<0.05). Baseline fluorescence in zero calcium also differed significantly from baseline fluorescence in normal calcium (p<0.05), which suggests that hepoxilin-evoked calcium influx plays a role in the plateau phase.

The inorganic calcium channel blocker lanthanum chloride (La³⁺) was subsequently used to further investigate whether or not the plateau phase observed in uninjured neurons upon addition of HXA₃ was due to calcium influx. Under normal calcium conditions (1.8 mM CaCl₂), HDHX was added to cultures and a rapid rise in fluorescence was observed (ΔF/F₀ = 1.57 ± 0.27; n=10)(Figure 22b). After 5 minutes, a new stable plateau significantly above baseline calcium levels was achieved (ΔF/F₀ = 0.88 ± 0.20). La³⁺ was then added to the medium at a concentration of 100 µM. A small, but statistically insignificant decrease in fluorescence between 30 and 60 seconds post-addition of La³⁺ was observed (ΔF/F₀ = 0.70 ± 0.15), with a subsequent increase and stabilization of fluorescence 2 minutes post-addition of La³⁺ (ΔF/F₀ = 0.82 ± 0.20). Unlike the findings using zero extracellular calcium, these results suggest that calcium influx did not
play a role in sustaining the plateau level. However, unlike other experiments which were repeated 3 times with larger sample sizes, this experiment was conducted once at the end of our research study, using a small neuronal population (n=10 cells). This experiment should be repeated again, using a larger sample size and perhaps a larger concentration of La$^{3+}$. Larger concentrations of La$^{3+}$ (i.e. 500-1000 μM) have been used successfully in neurons used to block calcium channels (Mattson and Kater, 1987).
Figure - 22

(a) Fractional change in fluorescence (ΔF/F₀) in SCG neurons post-treatment with HDHX in zero extracellular calcium. This graph illustrates the fractional change in fluorescence in neurons treated with HDHX in: a) normal extracellular calcium medium (squares), and b) zero extracellular calcium (circles) (0mM CaCl₂, 20 mM HEPES, 5 mM EGTA). Peak levels of fluorescence in zero calcium medium occurred 6-8 seconds post-treatment, producing a mean ΔF/F₀ of 0.93 ± 0.08 (n=20 cells) (Figure 18a). Approximately 1.5-2 minutes post-treatment, a new stable baseline of fluorescence was achieved (ΔF/F₀ = 0.20 ± 0.03) which was found to differ significantly from resting levels (p<0.05). Baseline fluorescence in zero calcium also differed significantly from baseline fluorescence in normal extracellular calcium (p<0.05) (see Figure 16d).

(b) Fractional change in fluorescence (ΔF/F₀) in SCG neurons post-treatment with HDHX and lanthanum chloride (La³⁺). This graph illustrates the fractional change in fluorescence in neurons treated with HDHX in normal extracellular calcium. HDHX was added to cultures and a rapid rise in fluorescence was observed (ΔF/F₀ = 1.57 ± 0.27; n=10) (Figure 18b). After 5 minutes, a new stable plateau significantly above baseline calcium levels was achieved (ΔF/F₀ = 0.88 ± 0.20). La³⁺ was then added to the medium at a concentration of 100 μM. A small, but statistically insignificant decrease in fluorescence between 30 and 60 seconds post-addition of La³⁺ was observed (ΔF/F₀ = 0.70 ± 0.15), with a subsequent increase and stabilization of fluorescence 2 minutes post-addition of La³⁺ (ΔF/F₀ = 0.82 ± 0.20).
Figure - 22

(a) Graph showing data points and a line.

(b) Graph with data points and a label "La\(^{3+}\)".
DISCUSSION

1. Superior Cervical Ganglion (SCG) Cell Culture System

1.1 Selection of SCG in vitro cell culture system

In vivo models of SCI are comparable to the pathology of human SCI. These models have provided considerable information regarding tissue damage, changes in regional blood flow, fluctuations in glucose concentration, etc., all of which are known to neuronal and glial cells. In order to understand the specific nature of SCI, however, especially when testing new treatments and pharmacological agents prior to their implementation in human trials, it is also important to examine individual cells to understand the injury-repair process at the cellular level. In vitro systems offer the unique ability to precisely control the environmental conditions outside the cell. Correlation between cause and effect is much easier to establish in such systems, in the absence of the many confounding and overlying in vivo homeostatic influences, including blood flow and the presence of non-neuronal cells. In vitro studies are often conducted to aid in the interpretation of observations obtained in vivo, whereas at other times, they provide the very foundation upon which in vivo experiments are based (Tymianski et al. 1993).

In selecting an in vitro cell culture system for modeling injury-repair mechanisms in neural tissue, neurons from both the central and peripheral nervous systems were considered. Brain cortical neurons and mammalian spinal neurons have both been used in studies modeling hypoxic/ischemic injuries (Goldberg and Choi, 1993; Tymianski et al. 1993). These neurons are not only specific in their growth requirements, but often require a population of glial cells alongside the neurons for trophic support, therefore rendering the neuronal population 'heterogeneous' or 'mixed'. Peripheral neurons, either sensory or sympathetic, from both the
dorsal root and the superior cervical ganglia, have also been used extensively in several experimental paradigms. Under specified conditions, both culture systems yield a homogeneous population of neurons and are capable of being manipulated in culture for up to 4 weeks, producing an extensive arbor of neurites (Higgins et al. 1991).

Our decision to use sympathetic principal neurons was based primarily on the well-established cell culture techniques originally described and pioneered by Campenot (Campenot, 1977). One important feature of this model was the elimination of non-neuronal cells from cultures. Non-neuronal components, including glial cells, endothelial cells and fibroblasts, were effectively eliminated from the culture by the use of the antimitotic agent, ara-C (Campenot, 1993), creating an almost entirely homogeneous population of neuronal cells. Although it is becoming ever clearer that glial cells in the PNS (i.e. Schwann cells) are an important source of signals (i.e. release of growth factors) which aid in the regulation of neuronal survival and outgrowth (Jessen and Mirsky, 1999), the presence of such cells in culture could affect or modulate neurite regeneration in vitro, thereby interfering with experimental parameters, including rate of neurite outgrowth post-injury.

Campenot’s method of scratching the matrix prior to the plating of cells, to ensure the linear growth of neurites along the 15-20 tracks was an extremely appealing feature of this model. Moreover, the compartmentalization of cells in the center of the dish upon plating meant somata would be confined to the center, with only neurites occupying the periphery of the dish (Campenot, 1993). This arrangement is similar to neuronal organization in the spinal cord, whereby neuronal cell bodies comprising the gray matter are at a distance from the tracks in the white matter to which they give rise. It was an advantage to have this degree of organization in the culture system, with regeneration proceeding in a defined direction following injury. If the
somata had not been restricted to the center of the dish, the multi-directional growth of neurites would have made it difficult to identify regenerating neurites post-injury. The formation of somatal-neuritic synapses in a multi-directional fashion could have also affected or modulated neurite regeneration. Furthermore, using the Campenot approach, each of the 15-20 tracks contained more than 10 neurites, which allowed us to study several cells at any given time point, unlike other in vitro models of axonal injury, such as Lucas's laser dendrotomy model or Cohan's axotomy model, which are both limited to the study of one or two cells (neurites) at a time (Lucas et al. 1985; Cohan et al. 1983). Finally, the well established methods and techniques for the culture of sympathetic principal neurons were straightforward and consistently reproduced.

1.2 Rationale for selection of HxA, concentrations in regeneration and calcium dynamics assays

Unlike a variety of other potent endogenously synthesized mediator/modulator compounds, such as acetylcholine, norepinephrine, dopamine and others, eicosanoids are not stored pre-formed in cells. They are rather synthesized and released in response to various activating stimuli (Leslie and Watkins, 1985). The amount and type of eicosanoid synthesized and released by various cells depends on many factors, including: 1) the type and amount of available substrate; 2) the cell type in which specific enzyme systems are present; 3) the presence of enzymes to inactivate or convert a certain eicosanoid to other metabolites; and 4) the type of biosynthesis stimulus (trauma, ischemia, catecholamines, etc.) The release of AA and subsequent metabolism by the LOX pathway has been widely demonstrated in the mammalian central nervous system. The occurrence of the hepoxilin pathway in the CNS was first demonstrated in a 1988 study by Pace-Asciak. Incubation of homogenates of the rat cerebral cortex with exogenous AA
(10 \mu g) led to the stimulated appearance of HxA\textsubscript{3} from 5.0 \pm 0.2 ng/mg protein (basal level) to 12.9 \pm 1.5 ng/mg protein. HxA\textsubscript{3} was detected as its stable trihydroxy derivative, trioxilin A\textsubscript{3} (Pace-Asciak, 1988). The addition of exogenous AA (1 \mu M) and its subsequent metabolism into LOX products has also been shown to stimulate neurite outgrowth and inhibit neurite retraction in neural hybrid NG108-15 cells (Smallheiser et al. 1996). In a 1994 study by Okuda et al., AA was found to exert both toxic (10^{-3} M) and trophic (10^{-6} M) effects on hippocampal neurons. Moreover, in studies using rat hippocampal CA1 neurons, AA and its hepxiilin metabolites in both ‘\mu M’ and ‘nM’ concentrations were found to exhibit significant neuromodulatory effects (Carlen et al. 1989; Carlen et al. 1994). Furthermore, biochemical experiments using mass spectrometry detection techniques showed that HxA\textsubscript{3} is formed by intact hippocampal slices. HxA\textsubscript{3} formation was significantly enhanced by the addition of exogenous free AA to the bathing medium (Pace-Asciak et al. 1989\textsuperscript{2}). These experiments provide biochemical support that the previously demonstrated electrophysiological actions of AA (Carlen et al. 1989\textsuperscript{2}), which are identical to those of hepxiilins mentioned earlier, could be due to the transformation of AA into hepxiilins within the brain slice itself (Carlen et al. 1994).

For the most part, the biological effects of hepxiilins in previous studies have been elucidated at both \mu M and nM concentrations. HxA\textsubscript{3}, at concentrations ranging from 1-10 \mu M, has been shown to cause a rapid increase in cytosolic calcium by releasing it from intracellular stores (Dho et al. 1990; Pace-Asciak, 1994; Mills et al. 1997; Reynaud et al. 1999). In electrophysiological studies, hepxiilins have exhibited neuromodulatory effects on hippocampal neurons in the 3-10 nM range (Carlen et al. 1994). In our study, therefore, we selected concentrations of HxA\textsubscript{3} which were included in the concentration ranges for previously elicited neuromodulatory effects of both hepxiilins and AA. We demonstrated that HxA\textsubscript{3},
at concentrations of 0.28 μM (LDHX) and 2.8 μM (HDHX), both enhanced NGF-stimulated neurite regeneration after *in vitro* transection injury and increased intracellular calcium levels in fluo-3 loaded SCG neurons. Although effects of hepoxilins on neuronal regeneration have not been previously examined, the effects of HxA₃ (at the stated concentrations) on intracellular calcium dynamics in our study, parallel earlier findings in studies using human neutrophils, platelets, and vascular tissue which used similar concentrations of HxA₃.

### 2. Injury-Regeneration Experiments

Our injury-regeneration experiments demonstrated that neurite outgrowth increased significantly in cultures treated with LDHX and HDHX in the presence of NGF (10 ng/ml), when compared to controls (NGF alone) at 2 to 6 hours post-injury. Furthermore, neurite outgrowth increased significantly in cultures treated with LDHX when compared to controls at 6 to 12 hours post-injury. Neurons treated with LDHX exhibited greater increases in outgrowth at both time intervals post-injury than those neurons treated with HDHX, suggesting that a specific concentration of hepoxilin may required for the optimal enhancement of NGF-stimulated outgrowth. One important question which our research did not address was whether or not the stimulatory effects of HxA₃ on neurite outgrowth post-injury can be maintained upon re-addition of the compound at later time points. Future studies should address this question, by adding HxA₃ immediately post-injury, and again at both 6 and 12 hours, to determine whether or not the level of regeneration can be maintained.

HxA₃ was not found to substitute for NGF-mediated outgrowth, for treatment of injured neurons with HxA₃ in the absence of NGF produced little to no regrowth (data not shown). These findings are similar to those found in a study examining the biological effects of hepoxilin...
A3 on human neutrophils (Sutherland et al. 2000). The free acid of HxA3 caused strong chemotaxis of human neutrophils at concentrations as low as 30-40 nM. The dose dependence of HxA3 on chemotactic activity revealed a biphasic behavior in that it was attenuated at higher concentrations (i.e. 100-1000 nM). However, at these higher doses (1-10 μM), free HxA3 caused a rise in cytosolic Ca2+ by releasing it from intracellular stores and also significantly reduced the fMLP-induced liberation of AA, suggesting the modulation of certain cell signaling processes. HxA3-evoked chemotaxis was found to be independent of the cytosolic Ca2+ signal, since such a rise in intracellular Ca2+ was not observed at concentrations of HxA3 that exhibited chemotactic activity. A possible explanation for the bell-shaped dose-response curve for chemotactic activity was that the rise in cytosolic Ca2+ observed at higher concentrations of HxA3 counteracted the chemotactic activity of this eicosanoid (Sutherland et al. 2000). In order to determine whether or not there is a similar dose-response effect of hepoxilin on neurite outgrowth, additional concentrations of hepoxilin must be examined in this injury-regeneration model.

There is increasing evidence which implicates phospholipid metabolism in NGF-stimulated neuronal outgrowth (Hama et al., 1986; DeGeorge and Carbonetto, 1987; DeGeorge et al., 1988). In a 1988 study by DeGeorge et al., a direct correlation was made between nerve fiber growth in PC12 cells and DRG neurons, and arachidonate liberation from phospholipids and its subsequent metabolism by the LOX pathway. In PC12 cells and DRG neurons treated with NGF, inhibitors of both AA release (mepacrine or 4-bromophenacyl bromide) and AA metabolism by lipooxygenases (baicalin, BW755, nordihydroquaiaretic acid), were found to prevent the early morphological events associated with neurite outgrowth, suggesting a link between NGF-stimulated arachidonate liberation and metabolism and nerve fiber growth. Furthermore, a 1993 study by Carman-Krzan and Wise demonstrated that the phospholipase A2-LOX pathway is an
early mediator of the interleukin-1 beta (IL-1) signal transduction process leading to the stimulation of NGF secretion from cortical astrocytes. It is widely acknowledged that astrocytes are involved in the trophic support of neurons through the release of various growth factors such as NGF and glial neurotrophic factor (GDNF) without which neurons cannot survive (Lu et al., 1991; Rudge et al., 1994). Previous studies have demonstrated that IL-1 is a potent activator of NGF secretion from rat cortical astrocytes and hippocampal cells maintained in primary culture (Carman-Krzan et al. 1991; Friedman et al. 1990; Spranger et al. 1990; Vige et al. 1991). IL-1 treatment of astrocytes elevated NGF mRNA content followed in time by an increase in cellular and secreted amounts of NGF. Upon investigation of the intracellular second messenger mechanisms involved in IL-1 induced NGF secretion by treatment of astrocytes, it was concluded using a series of phospholipase inhibitors (i.e. 30 μM mepacrine, 10 μM NDGA), that activation of the LOX pathway is one possible mechanism by which IL-1 stimulates NGF secretion (Carman-Krzan and Wise, 1993). Our studies demonstrate that the 12-LOX metabolite HxA₃ enhances the NGF-induced outgrowth of injured SCG neurons, which parallels these other studies showing that LOX metabolites may be key mediators in NGF-stimulated neurite outgrowth (DeGeorge et al. 1988; Carman-Krzan and Wise, 1993).

The presence of AA metabolism by the LOX pathway in neuronal cells has also been reported in a number of other studies. These studies have shown that the effects of AA on ion channels are exerted by its LOX metabolites, including the 12-LOX metabolite 12-HPETE (Buttner et al. 1989) and the 5-LOX metabolite leukotriene C₄ (Schweitzer et al. 1993). At lower concentrations, AA was found to promote both survival and neurite elongation in hippocampal neurons (Okuda et al. 1994). At a concentration of 10⁻⁶ M, AA significantly enhanced neurite elongation in two-day old primary hippocampal cultures. The COX inhibitor indomethacin was
found to potentiate the trophic effects of $10^{-6}$M AA, suggesting a shift of AA metabolism toward the LOX pathway. The involvement of specific LOX metabolites in the enhancement of neuronal outgrowth, however, has not yet been determined. Could these be the hepxilins?

Considerable research has been conducted in the regulation of activity-dependent changes in synaptic efficacy by AA and/or its metabolites, as well as the possible involvement of such fatty acid derivatives in mediating some of the responses that follow insults to nervous tissue, including repair, neural plasticity and cell death (Attwell et al. 1993; Axelrod et al. 1988; Bramham et al. 1994; Goodman et al. 1994; Harish and Poo, 1992; Piomelli, 1993; Verity, 1993; Williams et al. 1994). Isolated neuronal growth cones are extremely rich in free AA, reflecting a high basal phospholipase A$_2$ activity (Negre-Aminou and Pfenninger, 1993). In both neuronal and non-neuronal cells, growth factors and neurite-promoting factors cause the rapid, transient activation of phospholipase A$_2$ and release of AA (Axelrod et al. 1988; Chun and Jacobson, 1992; Moolenaar, 1994; Peppelenbosch et al. 1993; Piomelli, 1993; Verity, 1993). Supporting the idea that this pathway might be involved in neurite outgrowth is the report that phospholipase A$_2$ inhibitors exert a biphasic effect on elongation over several days in DRG neurons, enhancing outgrowth at low concentrations and inhibiting outgrowth at higher concentrations (Suburo and Cei de Job, 1986). In addition, exogenous AA and other unsaturated fatty acids have neurite promoting activity over a day or more in a variety of neuronal cell types (Bertrand et al. 1993; Okuda et al. 1994; Williams et al. 1994; Williams et al. 1994). In a study by Smalheiser et al., phospholipase A$_2$ and lipoxygenase inhibitors delayed initial neurite extension on laminin, and decreased F-actin staining at cell margins. It has also been demonstrated that AA and its LOX metabolites regulate F-actin remodeling in other cell types, and that actin-based contractility is a requisite for both neurite extension (Letourneau, 1981; Letourneau and Shattuck, 1989; Miller
et al. 1992; O'Connor and Bentley, 1993; Sobue, 1993) and acute neurite retraction (Finnegan et al. 1992; Jalink et al. 1993; Jalink et al. 1994; Smalheiser and Ali, 1994). These assays demonstrate a significant role for phospholipase A2-mediated release of AA and its subsequent oxidative metabolism in modulating rapid changes in neurite behavior, possibly by regulating the ability of the cytoskeleton to remodel (Smalheiser et al. 1996). In a 1994 study by Williams et al., cell adhesion molecules (CAMs) were used as a culture substrate for cerebellar neurons. Transfected CAMs promote neurite outgrowth by activating a second messenger pathway that culminates in calcium influx through N- and L-type calcium channels. Neurite outgrowth was directly induced by AA (10 μM), and this response was inhibited by N- and L-type calcium channel antagonists. Furthermore, neurite outgrowth stimulated by CAMs was abolished by an inhibitor of diacylglycerol lipase acting at a site upstream from calcium channel activation. Together, these results suggest that arachidonic acid and/or one of its metabolites is the second messenger that activates calcium channels in the CAM signaling pathway leading to axonal growth, and this is supported by recent evidence that shows the same concentrations of AA can increase voltage-dependent calcium currents in cardiac myocytes (Huang et al. 1992). Taken together, these studies have demonstrated that AA and its LOX metabolites play a critical role in neuronal survival, synaptic efficacy, neurite morphology, and neurite outgrowth. To date, however, the role of specific LOX metabolites has not been identified in such neuromodulatory processes. Could hepxoxilins be such critical neuromodulatory substances?

Hepoxilins, 12-LOX metabolites of AA, have been isolated in both the rat and mammalian brains. They are known to exhibit significant neuromodulatory effects on Aplysia and hippocampal CA1 neurons (Piomelli et al. 1987; Pace-Asciak et al. 19901-3; Carlen et al. 1994). In electrophysiological studies using rat hippocampal CA1 neurons, transient exposure to HxA3,
results in hyperpolarization and an increase in amplitude and duration of the post-spike train afterhyperpolarization (AHP), increase in amplitude and duration of the inhibitory postsynaptic potential (IPSP), and inhibition of the 4-aminopyridine-induced release of norepinephrine in hippocampal slices (Carlen et al. 1994; Pace-Asciak et al. 1990). In our study, hepoxilin A3 increased the NGF-stimulated regrowth of transected SCG neurons between 2 and 12 hours post-injury. Increases in outgrowth over controls (NGF alone) for neurons treated with LDHX and HDHX were 31.3% and 19.4%, respectively, between 2 to 6 hours post-injury, and 29.4% and 14.08%, respectively, between 6 and 12 hours post-injury. These results expand on earlier findings, which have demonstrated the importance of AA and its LOX metabolites on neuronal modulation. The modulatory activity of a specific LOX metabolite on neurite outgrowth is a novel discovery, since the involvement of specific AA metabolites have not previously been elucidated (Okuda et al. 1994; Smalheiser et al. 1996). We now have a working injury-repair model which can be used to test the effects of a variety of prostaglandins and related eicosanoids on NGF-mediated outgrowth. In previous studies, pertussis toxin was found to suppress the activity of HxA3 on: 1) chemotaxis of human neutrophils, and 2) liberation of AA and diacylglycerol in human neutrophils, further confirming the belief that the putative hepoxilin receptor is G-protein coupled (Sutherland et al. 2000; Dho et al. 1990). Therefore in future studies, inhibition of the G-protein-coupled hepoxilin binding protein (by means of pertussis toxin) should be performed to block the effects of hepoxilin on neurite outgrowth post-injury, which would further confirm its modulatory effects on neurite outgrowth. In addition, the effects of AA on neurite outgrowth should be investigated, considering the substantial evidence which demonstrates the involvement of this parent eicosanoid in neuromodulation. Phospholipase A2 and diacylglycerol lipase inhibitors (i.e. mepacrine, 4-bromophenacyl bromide), which block the
release of AA, could be used in this model to confirm the potential role of AA in the modulation of neurite outgrowth. Specific LOX inhibitors (i.e. baicalein, BW755, eicosatetraynoic acid), which suppress the formation of lipoxygenase metabolites including hepoxilins, could then be used to isolate other specific metabolites involved in the modulation of neurite outgrowth. Furthermore, the effects of 12-HPETE, the precursor to hepoxilins as well as hepoxilin analogs (i.e. HxB3, HxA3-C, HxA3-D, trioilins) on neurite regrowth should also be studied, to evaluate the specificity of hepoxilins’ role in the modulation of neurite outgrowth post-injury.

3. Calcium-Dynamics Experiments

The results from our calcium dynamics experiments demonstrated that a rapid increase in fluorescence signal intensity in the somata of uninjured, fluo-3 loaded SCG neurons was evoked by the addition HxA3. By 8 seconds post-treatment, signal intensity peaked, with fluorescence levels decreasing to a new stable baseline significantly above rest after 1 minute. The mean fractional change ($\Delta F/F_0$) in fluorescence for LDHX and HDHX was $0.87 \pm 0.13$ and $1.26 \pm 0.10$, respectively. To determine whether or not extracellular calcium played a role in the rise and subsequent decrease of intracellular calcium levels observed when HxA3 was added to the somata of uninjured neurons, cultures were maintained in zero extracellular calcium (0 mM CaCl2, 20 mM HEPES, 5 mM EGTA) and exposed to HDHX. Upon addition of HxA3, an increase in fluorescence was observed by 4 seconds, as seen under normal calcium conditions. Peak levels of fluorescence occurred 6-8 seconds post-treatment, producing a mean $\Delta F/F_0$ of $0.93 \pm 0.08$. Approximately 1.5-2 minutes post-treatment, a new stable baseline of fluorescence was achieved ($\Delta F/F_0 = 0.20 \pm 0.03$) and was found to differ significantly from resting levels. Baseline fluorescence in zero calcium also differed significantly from baseline fluorescence in normal
calcium, suggesting that hepoxilin-evoked calcium influx contributes to the plateau phase. Previous studies using human neutrophils, pancreatic β-cells and vascular tissue have shown that HxA3 causes a dose-dependent rise in intracellular calcium as well. There is an initial rapid phase of intracellular calcium rise, followed by a slow decline to plateau levels that remain significantly above baseline calcium levels. Studies have suggested that the initial rapid phase is caused by a release of calcium from intracellular stores in the endoplasmic reticulum; the slower rate of decline (plateau phase) is thought to be caused by a redistribution of intracellular calcium among organelles and by calcium influx as it is eliminated in zero calcium medium (Pace-Asciak, 1994; Mills et al. 1997). HxA3-induced calcium increase in SCG neurons exhibit the same behavior, with HxA3 causing a rapid, dose-dependent rise in intracellular calcium, followed by a decline to plateau levels significantly above rest. Moreover, when maintained in zero extracellular calcium, the plateau phase in SCG neurons treated with HxA3 was reduced significantly, which parallels earlier findings from studies using human neutrophils maintained in zero calcium medium.

In addition to there being a significant difference in mean peak fluorescence between LDHx and HDHx treatment groups, the degree of responsiveness of individual neurons to HxA3 also appears to be dose-dependent. Our results show that approximately 75% of all neurons treated with LDHx exhibit a moderate-large increase in fluorescence, whereas 94% of all neurons exposed to HDHx exhibit the same moderate-large response (Figure 21). Furthermore, our results suggest that group A, B, and C neurons are greatly dose-dependent with respect to HxA3-induced increase in fluorescence. The drop in fluorescence in group ‘A’ neurons and the corresponding increases in fluorescence in group ‘B’ and particularly in group ‘C’ neurons upon treatment with HDHx, suggest that the SCG neuronal population is heterogeneous with respect to HxA3-stimulated increase in calcium. A possible explanation for this phenomenon could be that
a specific concentration of HxA$_3$ is required to elicit a significant increase in intracellular calcium in individual neurons. Also, cellular integrity and/or morphology could also be involved in the number of neurons responding to a particular treatment. In future studies, it will be necessary to determine if there is a dose-dependent effect of HxA$_3$-induced calcium increase on neuronal outgrowth and regeneration and how this phenomenon affects individual cells in the population. This could prove to be an important finding for future studies involving neuronal regeneration, for the level of regeneration may not only be dependent upon a particular cellular event or trigger (i.e. increase in intracellular calcium), but also on the number of cells in a population manifesting this phenomenon.

Addition of lanthanum chloride (1uM), prior to or post-addition of hepxitin, has been found to eliminate the plateau phase in human neutrophils completely, with the calcium peak returning rapidly to normal baseline levels. These results further confirm the notion that the plateau phase is caused by calcium influx (Reyanud et al. 1999). In our studies using neuronal tissue, the addition of La$^{3+}$ (100 $\mu$M) did not produce a significant decrease in fluorescence, which suggests that calcium influx was not a factor in sustaining the plateau level in our cells. As mentioned previously, however, this experiment was performed once and on a small neuronal population, unlike other calcium experiments which were repeated 3 times with larger numbers of cells. In order to confirm the possible contribution of calcium influx to the plateau phase, experiments should be repeated again, using a larger sample size and/or a larger La$^{3+}$ concentration or specific calcium channel blockers.

Several authors have suggested a role for Ca$^{2+}$ in the regulation of neurite outgrowth (Schubert et al. 1978; Kostenko et al. 1983; Kater and Mills, 1991; Rehder et al. 1992). Initial fura-2 studies on cultured mammalian CNS neurons gave the first substantiative data on
intracellular Ca\textsuperscript{2+} and outgrowth (Connor, 1986). Intracellular Ca\textsuperscript{2+} in neurons exposed to substances and conditions known to affect outgrowth (serotonin or induced action potentials) were subsequently measured and they demonstrated strong correlations between the level of the free cytoplasmic Ca\textsuperscript{2+} and the growth status of the neuron (Cohan et al. 1986; Cohan et al. 1987). In a 1997 study by Ziv and Spira, both axotomy and a transient elevation in intracellular calcium in cultured Aplysia neurons was followed by the extension of a new growth cone from a region along the axon which intracellular calcium had been elevated transiently to 300-500 \(\mu\)M. These findings strongly suggest that a transient elevation of intracellular calcium to several hundred micromolars may be sufficient to induce the dedifferentiation of an axonal segment into a growth cone, thereby triggering neuronal outgrowth. Furthermore, growth cone formation did not occur if the large elevations in intracellular calcium associated with axotomy were prevented, suggesting that elevations in intracellular calcium are both necessary and sufficient for the formation of a growth cone after mechanical injury (Ziv and Spira, 1997).

In general, neuronal regeneration after injury requires the following steps: (1) resealing of the membrane; (2) production of a new growth cone; (3) pathfinding of the regenerating process, and (4) reconnection with the appropriate target. Upon axotomy, the proximal stump which is connected to the soma, shows typical signs of degeneration, including the disappearance of microtubules and neurofilaments close to the site of transection. Degeneration is suggested to result from an influx of calcium through the cut end, since degeneration can also be elicited by application of the ionophore 4-bromo-A2\(_{187}\) and can be prevented by performing the transection in calcium-free medium (Schlaepfer, 1974; Yawo and Kuno, 1983; Yawo and Kuno, 1985). These phenomena are transient however, and are often followed by new outgrowth. For a new growth cone to form from the proximal stump of a transected neurite, the cytoskeleton
must be ordered in a characteristic way, with F-actin filaments predominantly in the lamellipodial veil and filopodia, and microtubules exiting from the neurite and radiating predominantly into the organelle-rich central domain (Bridgeman and Dailey, 1989; Forscher and Smith, 1988). Since the intracellular calcium concentration is strongly implicated in affecting the cytoskeleton (Bamburg and Bernstein, 1991; Cohan et al. 1987; Forscher, 1989; Lankford and Letourneau, 1989), it is believed that the proximal stump of a severed neuron is also influenced by potentially inhibiting levels of intracellular calcium. A 1992 study by Rehder et al. set out to test this hypothesis, by determining the level of intracellular calcium critical for the formation of new growth cones post-injury. A sensitive regeneration assay was employed, whereby *Helisoma* neurons were severed after systematically elevating their intracellular calcium to varying degrees, to determine whether this would influence the probability of formation of a functional neuronal growth cone. Findings of this study demonstrated a relationship between the rise and restoration of intracellular calcium levels and the ability to form a neuronal growth cone. Higher levels of intracellular calcium (i.e. 500-1500 nM) were found to be inhibitory to the formation of new growth cones. Such a rise in intracellular calcium arose due to the injury of a neuronal process and could be further enhanced by electrical activity evoked presynaptically. Growth cone formation occurred only after calcium levels had been restored to basal levels (post-calcium challenge) (Rehder et al. 1992). Actin filaments are also reported to destabilize in dorsal root ganglion growth cones after exposure to high intracellular calcium (Lankford and Letourneau, 1989; Lankford and Letourneau, 1991). Moreover, the motility of neuronal growth cones can be completely inhibited by high concentrations of intracellular calcium (Cohan et al. 1987). The neuronal cytoskeleton, therefore, must be an important target of intracellular calcium.

In this study, we demonstrated that HxA₃ caused a dose-dependent, rapid increase in
intracellular fluorescence in intact SCG neurons, in that a higher concentration of HxA\textsubscript{3} (1.0 μg/ml or 2.8 μM) caused a significantly higher increase in calcium than a lower concentration of HxA\textsubscript{3} (0.1 μg/ml or 0.28 μM). The opposite effect was observed in the injury-repair model, in which the lower concentration of HxA\textsubscript{3} enhanced NGF-induced neurite regrowth post-injury significantly more than the higher concentration of HxA\textsubscript{3}. Cytosolic calcium increase and neurite outgrowth appear to be inversely related in the SCG neuron, which is consistent with other studies examining the relationship between calcium and its effects on cellular actions. Sutherland et al. demonstrated that a large rise in cytosolic calcium in human neutrophils, which was observed at higher concentrations of HxA\textsubscript{3} (1-10 μM), counteracted the chemotactic activity of this eicosanoid observed at lower HxA\textsubscript{3} concentrations (10 -100 nM) (Sutherland et al. 2000).

Initial studies by Lankford and Letourneau investigated the effects of changes in intracellular calcium levels on growth cone behaviour and ultrastructure of chick DRG neurons. Increases in calcium levels (addition of A-23187) or decrease in calcium levels (removal of extracellular calcium) result in cessation of neurite outgrowth. These results, which they attribute to the action of calcium on the neuronal cytoskeleton, further suggest that there is a permissive range of intracellular calcium for growth cone motility and neurite elongation (Lankford and Letourneau, 1987). Furthermore, a working model developed by Kater and Mills states that depending upon resting calcium levels, a given stimulus could have opposite effects. For example, within a neuron that is growing slowly as a result of permissive but suboptimal levels of intracellular calcium, a given stimulus could raise intracellular calcium closer to the optimal level and, accordingly, stimulate neurite outgrowth. Conversely, within a neuron that is already growing at a maximal rate (due to an optimal calcium level), the same stimulus could raise the calcium level above the permissive range and inhibit growth cone motility and neurite elongation.
(Kater and Mills, 1991). This model may explain why in the SCG model, the lower concentration of HxA₃ enhanced NGF-stimulated outgrowth to a greater extent than the higher concentration of HxA₃. Could this suggest that the HxA₃-induced (LDHX) increase in cytosolic calcium is near the optimal levels? It should be noted here, however, that calcium experiments in this study were conducted on intact, fluo-3 loaded neurons, not on injured neurons. Therefore, in order to better establish a relationship between intracellular calcium and neurite outgrowth in this in vitro model, it is necessary to conduct experiments examining the effects of varying concentrations of HxA₃ on transected, fluo-3 loaded neurons.

4. Conclusions

It must be emphasized that this in vitro model of neurite transection may differ greatly from in vivo axonal injury, where neurons may be damaged but not necessarily transected. Axonal injury in vivo may be associated with excitotoxicity, ischemia, and deprivation of trophic factors, none of which are present in this model. However, this study indicates that: (1) NGF-stimulated regrowth of transected SCG neurons is enhanced to different degrees in the presence of different concentrations of HxA₃; (2) a dose-dependent, rapid, increase in HxA₃-stimulated cytosolic calcium takes place with a subsequent decline to a new plateau level significantly above rest in intact SCG neurons (3) a dose-dependent effect of HxA₃ is observed on the percentage of neurons with a significant increase in fluorescence post-treatment; and (4) zero extracellular calcium medium abolishes the plateau phase induced by HxA₃ (1.0 μg/ml or 2.8 μM). Our research has identified the involvement of a specific LOX metabolite in the regulation of NGF-stimulated neurite outgrowth. Furthermore, we have demonstrated that calcium may play a role in HxA₃-enhanced regeneration, and that an optimal level of intracellular calcium
produced by low doses of HxA₃ may be necessary for maximal outgrowth to occur (Figure-23). The effects of HxA₃ may be even more dramatic if neurites are only damaged but not completely transected.

5. Future Directions and Possible Implications

There are several critical experiments which are necessary to expand on the knowledge acquired from this study. HxA₃-enhanced neurite regrowth in this transection model was found to be the greatest in the first 2 to 6 hours post-treatment. The addition of HxA₃ may cause a rapid disorganization of the neuronal cytoskeleton in the first few hours post-treatment, leading to the stimulation of neurite outgrowth. Future studies should address this question, by adding HxA₃ immediately post-transection, and again at both 6 and 12 hours, to determine whether or not HxA₃-enhanced neurite outgrowth can be sustained. Moreover, experiments should be conducted on mildly injured as well as transected fluo-3 loaded neurons, to establish whether or not a true causal relationship exists between intracellular calcium and neurite outgrowth. Calcium experiments using intact neurons was a good first step, however the use of injured neurons is critical in identifying a possible mechanism by which HxA₃ induces outgrowth. Other metabolites of AA should be assayed with respect to their ability to modulate neurite outgrowth to determine if the results reported here are specific to HxA₃ and not other metabolites. Using this in vitro injury-repair model, a variety of compounds could be assayed, which would clearly establish the specificity of certain eicosanoids involved in neurite outgrowth. Furthermore, experiments should be performed to examine the calcium response to HxA₃ in SCG neurons at the subcellular level. Previous work using neutrophils suggests that HxA₃ causes the rapid release of calcium from endoplasmic reticulum, manifesting in a rapid increase in intracellular calcium, with subsequent
Figure - 23. Potential mechanism for hepxilins’ modulation of neurite outgrowth.
sequestration in the mitochondria (Mills et al. 1997). Other studies have suggested that LOX metabolites involved in the modulation of NGF-mediated neurite outgrowth act at a site upstream from calcium channel activation, suggesting the possible involvement of calcium influx in neuronal regeneration (Williams et al. 1994). Future studies may include the use of both organelle-specific inhibitors such as the microsomal Ca$^{2+}$-ATPase inhibitor thapsigargin, which acts to deplete internal calcium stores, or dantrolene, a muscle relaxant used as an inhibitor of intracellular mobilization and calcium channel blockers (i.e. La$^{3+}$, Cd$^{2+}$, Co$^{2+}$), to identify the particular mechanisms by which HxA$_3$ (and possibly other AA metabolites) modulate neurite outgrowth. Our study is the initiation of a comprehensive set of experiments detecting mechanisms involved in neuronal repair.

By establishing a role of a specific LOX metabolite in neuronal regeneration using the SCG injury-repair model, we have expanded on previous work which has strongly implicated AA and its metabolites in a variety of neuronal activities, including neural plasticity, neuronal survival, and neurite outgrowth and repair. The implications of our research on neuromodulatory processes in vivo is also considerable. AA and its metabolites may prove to be important modulators of nerve injury and repair processes, in addition to other neurological disorders. Future studies, therefore, should focus on the isolation of specific eicosanoids and their metabolites involved in the modulation of neurite outgrowth, and the specific mechanisms by which their neuromodulatory actions are mediated.
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