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SYNERGISTIC EFFECT OF RETINOIC ACID, NERVE GROWTH FACTOR AND LAMININ FOR ENHANCED NEURITE EXTENSION

A Thesis Presented for

the Master of Science

Degree

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I would like to thank everyone who was helped me make this thesis a success. I would first like to express my gratitude to my advisor Dr. Wei He, for her valuable insight and guidance throughout the course of this work. I admire her vast knowledge, methodical and diligent approach to research. I am really proud to have worked under her and submit this thesis as a token of my appreciation.

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Peripheral Nerve Injury is one of the most common and serious traumatic injuries. About 300,000 cases of peripheral nerve injury are reported annually in Europe and another 50,000 procedures performed annually in the United States. Peripheral nerve injury can lead to lifelong disability and also death in severe cases. Repair techniques remain a major challenge because of lack of efficient regeneration. Though techniques like autografts are highly efficient, they face a lot of drawbacks. Alternate methods focus on the use of growth supporting and enhancement components in nerve guidance channels. Current research necessitates the use of a combination of compounds for enhanced nerve growth. In this thesis we study the combined effect of Nerve Growth Factor (NGF), laminin and Retinoic acid (RA) on nerve growth. This study is aimed at attaining higher efficiency in nerve growth by using a combination of compounds not tested together before. We expect to see an enhanced outgrowth, supposedly caused by the synergistic effect of these components, which will be greater than the outgrowth caused when these compounds are used individually or in combination with another compound. Chick dorsal root ganglion cells were cultured for 36 hrs in the presence of 50ng/ml NGF, 50µg/ml laminin and 1µM RA, either used individually, or in combination with another compound, or synergistically. The efficiency of each condition was determined based on neurite extension and area of outgrowth. The triple component system produced significantly greater neurite extension and outgrowth compared to all when the compounds are used individually or in combination with one another. The neurite length in the triple component system was 5 fold that of control whereas the area of outgrowth was as high as 60 times that of control. This enhanced neurite growth achieved will pave way for efficient nerve guidance channels development.
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CHAPTER 1: INTRODUCTION

Statement of the Problem:

Peripheral nerve injury is one of the most serious clinical issues, accounting to about 2.8% of all traumatic injuries, out of which 65% is found to occur in the upper extremity and that too involving radial nerves. Commonly seen in males in the age group of 18-25, most injuries are caused by motor vehicle and industrial accidents. Data from the National Centre for health statistics show that there were more than 50,000 peripheral nerve repair procedure performed in the United States in the year 1995 [1]. In Europe there are well over 300,000 cases of peripheral nerve injury reported annually [2].

After injury, the axons spontaneously regenerate over relatively shorter gaps (< 5mm). Occurrence of regeneration does not by itself mean functional recovery and this may primarily be due to the axons regenerating toward non targets. When the gap is greater than 1cm, there is a lack of guidance cues for the generating axons and they end up growing backward into the proximal nerve stump, or enter a non target endonerium or end up forming neuromas [3, 4]. Failure by the injured axons to regenerate and its degeneration over time may eventually lead to atrophy of the target [5]. These are the two primary concerns regarding peripheral nerve regeneration. Any tissue engineering technique employed to aid nerve regeneration must have a directional cue so as to guide the sprouting axons towards the target site, and at the same time must be time efficient so as to prevent loss of function or atrophy of target tissue.
It is important that any tissue engineering approach aimed at resolving peripheral nerve regeneration should be able to match the gold standards set by autografts for smaller injuries. Any alternate approach should not only be able to match the performance of the autografts but should also be able to resolve issues arising with autografts. Though autografts are considered as the gold standard of nerve regeneration, tissue engineering provides ample opportunity to reach higher standards and match intact nerves.

Hypothesis

We propose that by using a combination of Nerve Growth Factor (NGF), laminin and retinoic acid (RA) we would be able to attain maximum neurite outgrowth for a given time period. We expect an enhanced outgrowth, supposedly caused by the synergistic effect of these components, which will be greater than the outgrowth caused when these compounds are used individually or in combination with any one another compound. We also propose to study the effect of a delayed release model and its effect on nerve outgrowth, from which we plan to acquire information about the effect of time dependent release on the nerve outgrowth.

Objective

The objective of this study is not to find a final solution for peripheral nerve injury, but rather an improvisation towards the goal of successful nerve regeneration. Through this study, we aim to attain faster nerve growth within a given period of time by using a mixture of compounds that can act synergistically to enhance growth. Through the delayed release study, we would be able to set a framework for future work on time dependent release for even more synergistically effective growth.
2.1. Peripheral Nerve Injury

2.1.1. An introduction to Peripheral Nerve Injury

Injury to the peripheral nervous system (PNS) is one of the most common and serious clinical conditions leading to the loss of motor function or sensory function or both. Based on his study of 5777 trauma patients, Noble et al [6] outlines that peripheral nerve injury accounts for almost 2.8% of traumatic injuries, mostly caused due to motor injuries and in many cases leading to lifelong disabilities. Other common types of injuries leading to peripheral nerve damage are penetrating injury, crash, traction, ischemia, laceration by sharp objects [7, 8].

Though a complete understanding of the anatomy of the nerve is not essential for the present study, a basic understanding would help gain an insight of the pathophysiology of peripheral nerves. This is required for better understanding of nerve injuries and to make a knowledgeable approach towards nerve injury repair. Figure 1 illustrates the anatomy of a peripheral nerve.

A nerve basically consists of individual myelinated axons and groups of unmyelinated axons surrounded by an endonerium. The perinerium surrounds the fascicles which is a collection of axons. The fascicles combine to form the nerve trunk. The external epinerium covers the nerve trunk. The perineurium and epineurium are circumferential oriented whereas the endoneurium is longitudinally oriented [9].
Figure 1: Anatomy of a nerve. Adapted from [9, 10]
**Table 1** Classification of nerve injury. Adapted from [10]

<table>
<thead>
<tr>
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<tr>
<td>Neurapraxia</td>
<td>First Degree</td>
<td>Occurrence of segmental demyelination</td>
</tr>
<tr>
<td>Axonotmesis</td>
<td>Second Degree</td>
<td>The axons are severed, but the endoneurium remains intact, and there are optimal chances for regeneration under this condition</td>
</tr>
<tr>
<td></td>
<td>Third Degree</td>
<td>Discontinuity of axons and the endoneurial tube, but the arrangement of the perinerium and fascicular arrangements are preserved</td>
</tr>
<tr>
<td></td>
<td>Fourth Degree</td>
<td>Neuroma occurs. There is a loss of continuity of the axons, perinerium, fascicle and the endoneurial tube. The epinerium is still intact</td>
</tr>
<tr>
<td>Neurotmesis</td>
<td>Fifth Degree</td>
<td>Continuity of the entire nerve trunk is lost</td>
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</table>
The two major classifications of peripheral nerve injuries have been given by Seddon [11] and Sunderland [9]. The table above is a comparison between the two classifications.

Most of these patients acquire lifelong disability due to nerve injury [6]. Injuries to peripheral nerves may also occur due to trauma or surgical procedures, which can result in the loss of muscle function, impaired sensation, and/or painful neuropathies [12, 13]. Peripheral nerve injuries (PNIs) lead to reduced muscular recruitment and sensation. It also disrupts coordination among organs through changes in both the PNS and the CNS.

2.1.2. Current Strategies to Treat Peripheral Nerve Injury

In cases where the gap caused due to injury is small, coaptation by suturing the ends of the severed nerves is followed. For gaps larger than 5mm, coaptation cannot be implemented and autograft is used. Autograft is the process in which an uninjured healthy nerve is taken from another part of the body, usually called the donor site and then used in the injured site. Autografts are considered the ‘gold standard’ for nerve repair, but pose a few disadvantages like limited availability, loss of function in the donor site, size mismatch, additional surgery time, potential neuroma formation and also frequent unsatisfactory results [12, 14]. Allografts and Xenografts have also been used but with less success. These techniques often have to address the issues of immuno-suppression and are almost always affected by graft rejection [5]. Such limitations and disadvantages have lead to the search for alternate methodologies such as artificial nerve grafts, tissues engineered nerve conduits, etc.

Earlier attempts in using these artificial grafts to aid nerve regeneration were basically studies that involved empty or silane-filled silicone tubes. Although these synthetic grafts had a few advantages compared to natural grafts, their use was restricted in many ways. Most of them
could be effectively used only up to a distance of up to 1cm [15]. They were often non biodegradable and were considered foreign and thus enveloping scar tissue [16, 17]. These disadvantages led to the development of bioresorbable grafts. These grafts were not only biodegradable, but were also effective in encapsulating and providing guidance channels to the growing axons [18]. To further improve the effect of these guidance channels, the tubes are filled with extracellular matrix components like laminin [19], trophic factors like NGF and other support cell types like Schwann cells. Various levels of success have been attained in this field, but these grafts are still trailing the “gold standard” autografts.

2.2. Nerve Growth Factor and Nerve Cell Regeneration

2.2.1 Nerve Growth Factor for Neuronal Growth, Survival and Maintenance.

The presence and role of NGF as a growth promoting agent came into light in 1948 when Bueker [20] observed innervations of fragments of mouse sarcoma 180 when they were placed in the body of chick embryos. Basically designed to study the effect elicited by target tissues on neurons, the study revealed one of the most important factors of nerve growth. Experimental results showed that the neurons emerging from the embryos bearing the sarcoma were bigger in size. This was not restricted to neurons close to the sarcoma but was also observed in distant neurites. It was evident from the results that there was a growth promoting factor diffusing from the sarcoma tissue into the embryo’s circulation and was causing excessive growth and long nerve fibers. In an attempt to replicate these result in vitro [21], Levi – Montalcini and Meyer cultured sympathetic and sensory ganglia dissected from chick embryo in a medium containing fragment of the sarcoma 180 or 37. Observation after hours of incubation revealed dense, fibrous, halo of nerve fibers extending out of the ganglia adjacent to the sarcoma fragments. This
kind of growth was absent in any of the control cultures which were not supplemented with the sarcoma fragments. These results provided testimony to the release of growth promoting factors in vitro by the neoplastic tissues. This fibrillar halo kind of nerve growth from the explanted ganglia became the most important factors for identifying the presence of the growth promoting factor. Similar results were not obtained with other tumor tissues, ruling out a generalization of growth enhancement by tumor cells.

Since it was already established that NGF is present in the target tissues and body fluids [22], an attempt to study the effect of blocking NGF and observing its effect on neuronal cells was carried out by Levi-Montalcini [23]. They injected newborn mice with an antiserum for NGF. Controls were injected with normal serum. It was seen that by injecting a daily dose of antiserum for 8 days, almost 99% of the sympathetic nerve cells were irreversibly destroyed. A selective and permanent destruction of more than 90% of sympathetic neurons were observed in new born mice and other new born mammals. The antiserum seemed to have specifically affected organs and tissues related to these sympathetic neurons as all other tissues and organs were similar in functionality as the control. Only adrenergic sympathetic cells which innervate the male and female sex organs seemed to be resistant to the antiserum. But it should also be noted that these neurons did not show any marked effect with the addition of NGF. Levi-Montalcini and Angeletti [24] attempted to grow sensory and sympathetic neurons in medium with and without NGF supplemented medium. The experimental results showed that just Eagles medium and Eagles medium supplemented with 10% horse serum were inadequate for the survival and maintenance of neurons. Addition of NGF to the above mentioned medium not only resulted in neuronal survival but also showed enhanced neurite outgrowth when compared to the controls.
2.2.2 Importance of NGF in the Local Environment

While attempting to find out the importance of NGF for the survival and growth of these nerve cells, Robert B Campenot [25] found that it is the presence of NGF in the local growth environment, that is of primary importance. In his experiment, he had designed a three-chamber system well separated from each other to prevent passage of contents. It was seen that the neurites were able to cross the barrier into chambers containing a higher concentration of NGF. On removal of NGF from the chambers in which the cells were grown, the neurites stopped growing and at times seemed to degenerate. It was seen the growth had stopped and degenerated even though there was NGF present in chambers where the proximal part of the neurites were present. When NGF was removed from the chambers where the proximal part was found but there was still NGF contained in the chambers where the distal part was found, the cells still continued to extend and survive. This shows that the survival and growth of the extending neurite depends on the local NGF concentration, regardless of the concentration in other areas. This also reaffirms earlier finding that neurites seem to grow in directions where target tissues releasing NGF are placed, rather than in the opposite direction. As the author points out, this highlights the body’s mechanism to direct growing neurites towards target tissues. When neurites extend in direction other than towards the target tissues, they enter a region where there is no or very little NGF. The extending neurites stop extending and begin to degenerate even if there is enough NGF in the proximal end of the neurite or where its somas are present.

2.2.3 Nerve Growth Factor for Enhancement of Neurite Extension

Ronald M Lindsay [26] established through a series of experiments that though NGF enhances the regeneration and growth in sensory neurons, they are not required for the survival
of adult sensory neurons. He cultured adult rat dorsal root ganglion sensory neurons in the presence and absence of NGF and other neurotrophic factors. The reaction of these cells to the presence and absence of other non neuronal cells was also observed in this study. This was to rule out any factors secreted from the non neuronal cells from promoting neuronal cell growth. It was observed that even in the absence of NGF and other neurotrophic factors, about 70-80% of the cells survived. Though NGF was not seen to be necessary for the survival of the adult sensory neurons, they seem to promote the regeneration of axons from the adult DRG neurons.

In a similar experiment, Tanaka et al [27] tested the effectiveness of NGF on 10, 72- and 114-week old mice dorsal root ganglion. From the results it was seen that though NGF accelerated the growth of neurites in these cells, they were not necessary for the survival of the adult DRG neurons. It was seen that the NGF was able to significantly improve neurite growth and extension with even the 114-week old adult dorsal root ganglion proving that the growth promoting effect of NGF does not cease with aging, but it is probably just the dependence on NGF for survival that disappears with age.

The difference in the effect elicited by NGF in the early stages of embryonic stages was also being studied by a few researchers. In his study of the quantitative effect of NGF on chick dorsal root ganglion, Blood [28] used DRGs extracted from chick embryo at different embryonic development stages ranging from E8-E14. It was seen that there was an increase in the density of neurons in NGF treated cultures when compared to the control cultures and this effect was more pronounced in the earlier embryonic stages compared to the later embryonic stages. It was also seen that the number of neurons per unit area were significantly greater in the case of NGF treated samples in the earlier embryonic stages but the difference between the treated and control samples decreased with advancing embryonic levels. The length of the neurites extending out of
the neurons in samples treated with NGF was significantly greater compared to the controls in the earlier stages of development. A 2.05 fold significant increase has been reported for cells cultured from E10 embryonic cells. The most significant difference in the neurite length between NGF treated and control samples was observed between E9-E11 after which the difference started to become marginal compared to these stages.

2.2.4 Nerve Growth Factor as a Chemotactic Agent

Gundersan and Barret in their work [29] highlighted the role of NGF as a chemotactic agent. In their experimental work, they showed that the growing axons tend to move towards a source of higher NGF concentration. They showed that this change in direction of growth can be brought about by a concentration gradient of even as low as 1 biological unit. They also showed that the rate of growth of the axons was significantly greater when exposed to a higher concentration of NGF. It was demonstrated in their experiment, that the axons growing in a medium containing 1 BU of NGF had a growth of about 10μm in about 2 hours, whereas when a high NGF concentration (50 BU) was placed near the growing axons, the displacement was about 20μm within 30 minutes.

2.3 Role of Laminin in Neurite Extension

2.3.1 An Introduction to Laminin.

Laminin is a glycoprotein commonly found in the meshwork of basement membranes like the epithelial lining surrounding the nerves and blood vessels and also underlying pial sheaths of the brain. Apart from the basement membranes, it also occurs in the extracellular matrix at early stages of development. It is seen to have been localized along with specific types
of neurons of the central nervous system (CNS) and embryonic as well as adult stages. It is also expressed in the peripheral nervous system at various developmental stage [30]. This glycoprotein is a large multi-domained and cross shaped molecule synthesized and secreted by cells into their ECM, where it usually interacts with receptors found on the cell surface leading to morphological and migration variations with the cells.

2.3.2 Laminin for Enhanced Neurite Extension

The finding that laminin is localized in the basement membrane of the neuromuscular membrane [31] and around Schwann cells in the peripheral nerve lead to the investigation of the possible role of laminin in nerve growth. Baron-Van Evercooren et al investigated the effect of growing nerve cells on laminin coated surfaces and compared it to the growth of neurites on other surfaces like fibronectin and collagen [32]. Human fetal sensory ganglia cultures were grown on three different substrates namely laminin, fibronectin and collagen in a medium containing NGF. It was seen that of all three substrates, laminin produced the most extensive neurite outgrowth. Neurites extending out of the ganglia on a collagen surface were radially oriented and tended to form bundles. The Schwann cells were seen flattened under the neurites and were later seen migrating along the neurites. Fibronectin produced more growth on the neurites when compared to collagen and were seen spreading out radially on a heavy layer of fibronectin. Neurites growing on fibronectin showed more branching when compared to those grown on a collagen surface, and the Schwann cells had also migrated further. Laminin produced more extensive growth and branching when compared to the other two substrates. The branching was more extensive in all directions, Schwann cells were seen migrating along the neurites and the movement of the fibroblast seemed restricted. There were no major bundles formed on laminin like those on collagen. A study on the synergistic effect between laminin and NGF
revealed how these two worked together. Antibodies to NGF reduced growth to a great extent, though this growth was greater than that on a collagen surface. Thus NGF was essential for improvised neurite growth. Antibodies to laminin completely prevented growth on the surface in a serum free medium, though such drastic effects were not observed in a medium containing serum. This study showed how laminin was important for enhanced neurite extension and how NGF and laminin acted together to have a synergistic effect on neurite extension. The growth rate on laminin was 2.1 times that of control cultures.

2.3.3 Mechanism of Action of Laminin in Promoting Neurite Extension

To understand how laminin promotes the growth of neurites, Rivas et al [33] studied the effect of laminin on the growth cone of the growing neurites, as the growth cone is the specialized end of the growing neurite and thus must be a very important site of any growth promoting compound. Earlier it was believed that glycoproteins like laminin and fibronectin promoted neurite growth by promoting adhesion of the axons to the surface [34]. It was seen that there was an increased surface adhesion when these glycoproteins were used, and since there was also an increase in the neurite length, it was assumed that there was a connection between these two factors. Further work on this showed that surface adhesion does not necessarily have to be the factor promoting neurite extension as there was no way to prove how the adhesion helps the neurite to extend. Later it was shown that laminin promoted the neurite extension and enhanced adhesion to the surface, but these two effects were independent of each other and did not influence each other [35-37]. Thus Rodolfo et al attempted to study the effect of Laminin on the growth cone of a growing neurite. They first cultured a single rat sympathetic neuronal cell on a polylysine coated surface and supplemented it with serum free medium. A single thick neurite ending in a growth cone with a large lamellipodia was seen extending out of the cell. The rate of
extension was about 3 ± 1 µm/hr. It was seen that when laminin was added to the culture, it led to the formation of a multiple thin outgrowths with excessive branching at a rate of about 22±2µm/hr. Though there was a difference in growth rate, the morphology of the new neurites was similar in both cases. Since it was evident that laminin enhanced neurite extension, it was then decided to focus on the action of laminin on the growth cone. When the growth cone was observed on a polylysine surface, it was seen that the growth cone had a large lamellipodia and numerous filopodia which were moving constantly. This movement consisted of extension and retraction, especially the dorsal filopodia moving constantly towards the lamellipodia. The net movement was very small because the number of filopodia that were extending was only slightly greater than those retracting or staying still. Thus the overall forward movement was as slow as 4µm/hr on an average, although the extensions and retraction were themselves at about 2µm/min. This shows that there was no significant difference in the extensions when compared to the retractions. The advancing movement of the organelles into the lamellipodium was almost as slow as the extension of the filopodia. Addition of laminin to the culture produced a significant decrease in the number of filopodia and veils that were retracting. The rate at which the filopodia was extending out almost doubled, though this occurred gradually compared to the suddenly decrease in the number of retracting cells. The most important and dramatic change that laminin brought about to the growth cone was that the movement of the membranous organelles from the central region into the lamellipodium. The speed at which this movement happened was 6 times faster than laminin. This resulted in the complete filling of the original lamellipodium with membranous organelles within 20 minutes. Other notable differences in the growth cone 20 minutes before and 20 minutes after the addition of laminin are, the growth cone had advanced further after the addition on laminin compared to prior to and several of the
filopodia became larger after the addition of laminin. Though in some cases the original neurite proceed to grow as a single process, in most cases in resulted in the formation of multiple neurites from the peripheral end of the lamellipodium. The moving organelles filled the newly formed filopodia or the lamellipodia and these consolidated into neurites leading to the formation of growth cones at the tips. This forward movement of the membranous organelles causes forward growth of the neurites and it is a continuous process. The velocity of the filling of the lamellipodium and hence the neurite outgrowth is faster compared to the rate of retraction and thus there is an enhanced growth with laminin when compared to polylysine. Another finding that substantiates the usefulness of laminin is the observation that the extended neurites are retained longer on laminin than on other glycoproteins and growth promoting substrates [36]. It is believed that the action of laminin is mediated by integrin which acts as cell surface receptor and is essential for laminin to enhance the rate of neurite extension. The essential role of integrin was established when a polyclonal antibody for the $\beta_1$ subunit of integrin prevented the effects elicited by laminin on the morphology of the growing axons [38, 39]. The action of laminin is also selective, in the sense that it does not influence outgrowth by acting on the overall neurite, but rather acts on the growing tip of the growth cone.

2.3.4. Laminin for Directional Orientation

Kuhn et al. [40] devised a simple experiment to understand how laminin direct neuronal growth cones. DRG neurons were cultured on either laminin or fibronectin substrates and then were confronted with ‘guideposts’, which were polystyrene beads coated with the alternative substrate. That is when laminin is the substrate for culture, then fibronectin is the substrate for ‘guideposts’ and vice versa. It was seen that, if the cells are plated on a fibronectin substrate and confronted with a laminin-coated bead guidepost, a sustained acceleration in growth following
filopodial adhesion, dilation of growth cone, and translocation toward the laminin-coated bead could be observed. This acceleration in growth rate is sustained even beyond the guidepost. On the other hand, when cells are plated on a laminin substrate and filopodia contacted fibronectin-coated beads, then the cells experience a sustained deceleration in growth to that of fibronectin basal level. It was also seen that when the carboxyl terminal region of laminin is exposed on the guidepost bead, acceleration in growth cone navigation occurs on fibronectin substrate. An antibody pretreatment of laminin-coated guideposts nearly abolishes this acceleration thus pointing out the significance on the carboxyl terminal in directing the accelerated growth. The interactions between growing tips of neurites and laminin substrates or other ECM molecules are stereotypic and initiated by individual filopodia which thus detect a substrate on a guidepost and direct behavior even before growth cone behavior changes. The nature of the coating on guideposts is irrelevant for filopodial sampling, but a receptor–ligand interaction is required for long-term adhesion to the guidepost. Intracellular signaling is required for transduction of guidepost cues, since low concentrations of an inhibitor of protein kinases (protein serine/threonine kinase A and G, and calcium-dependent protein kinase C) reduce the number of growth cones responding even though the filopodial sampling and contacts appear normal. This result suggests that laminin model guidepost cues depend on protein kinase-dependent pathways for transduction of the laminin signal to the cytoskeletal apparatus.

2.4. Retinoic Acid and Nerve Cell Regeneration

2.4.1 Retinoic Acid for Enhanced Neurite Extension

Neil Sidell, Quinn, De Boni and Haskell were amongst the earliest people to study the effect of retinoic acid (RA) on nerve cell regeneration. Sidell was apparently the first person to
report the neurite outgrowth enhancement capability of RA in his study on the effect of RA on the growth and differentiation of human neuroblastoma cells in vitro [41]. He reported a dose dependent response in both the growth inhibition and morphological differentiation in the cells. From his results it was seen that RA showed increasing growth inhibition with increasing concentration. RA prevented cells from growing colonies on the agar surface on which they were seeded. Though there was inhibition of growth of the cells, there was no decrease in the viable cell count when compared to the control culture to which no RA was added. This suggested that RA inhibited any cell proliferation, but at the same time maintained the viability of the cells. An interesting outcome of this experiment was the morphological differentiation that the cells underwent upon treatment with RA. A large number of cells produced long neurites, maximum growth being observed in the concentration between $10^{-5}$M and $10^{-6}$M.

Quinn et al [42] attempted to study the effect that RA had on a culture containing different types of neuronal cells from neonate murine DRGs and fetal spinal cord and also from fetal human spinal cord explants. They also compared the results of using various concentration of RA ranging between 100µM to 1nM to find out the most optimal concentration of RA that produces maximum possible enhancement. From the results they were able to establish that RA primarily acted by increasing the mean neurite length and not the number of neurites. The results showed that RA produced a significant increase in neurite outgrowth length when compared to the controls. All the concentration studies produced an enhanced effect when compared to the controls which did not have RA in their growth medium. With respect to dorsal root ganglion neurons, it was found out that the effect of RA was more predominant at 10µM than the higher or lower concentrations. The neurite outgrowth enhancement at this concentration ranged between 18.1% to about 47.5% with a mean of about 30.7% over a period of 11 days through
which the cultures were observed. Though the enhancement noticed at other higher and lower concentrations tested was not as optimal as with 10µM, they were significantly greater than the control cultures. Enhancements of over 133% with respect to controls were also observed in a few cultures at this concentration. With the help of the representative length distribution histograms, they were able to conclude that RA acted primarily by increasing the length of most of the neurites in the population rather than acting on subset cells. In the case of fetal mouse spinal cord neurons, the optimal concentration of 10µM for DRGs produced an inhibitory effect in the fetal neurons. Inhibition of up to 12% was observed at 10µM for fetal mouse spinal cord neurons. At lower concentrations of 1µM and 0.01µM, RA was able to produce maximum outgrowth with fetal mouse spinal cord neurons. In the case of human fetal spinal cord neurons, optimal RA concentration for growth enhancement was 10µM and 0.1µM. From the data they observed that there was a dose dependent shift in mean length for the entire population and not a specific sub population and this helped them conclude that RA primarily acted by increasing mean neurite length rather than by increasing neurite sprouting. Though Haskell et al [43] observed optimal growth at 1 to 0.1µM with chick DRG, the growth was insulin dependent. But irrespective of the concentrations used, RA was found to produce enhanced neurite growth.

2.4.2. Retinoic Acid elevates NGF Receptor Concentration

Haskell et al [43] attempted to examine the effects of RA on cell surface receptors for NGF, since RA is known to have the capacity to modify glycoprotein synthesis. RA produced extensive extension in neurite length at the end of 4 days with 10^{-5}M with LA-N-1 human neuroblastoma cells. The morphology of the cells treated with RA was not very different from the morphology of cells treated with NGF. RA did not produce any morphological changes to the cells. They reported that RA produced a decrease in viable cells at the end of the 4 day treatment.
But the major aim of this study was to see if RA causes any significant increase in the number of receptors for NGF. An immune assay performed for NGF revealed intense fluorescence with RA treatment. There was rarely any fluorescence observed without RA. Since the immunofluorescence assay had the disadvantage that there could be non-specific binding of NGF, Haskell et al used soluble LA-N-1 cells with $^{125}$I-NGF to validate that RA increased the number of receptors of NGF. Experimental results showed that retinoic acid produced a 7 to 11 fold increase in the number of high affinity receptors for NGF and a 3 – 8 fold increase in the number of low affinity receptors. These results clearly showed that RA induced an increase in the number of NGF receptors apart from producing enhanced neurite extension.

In a similar kind of study, Scheibe et al [44] attempted to study the effect of RA on clonal PC12 cells derived from rat pheochromocytoma. The main aim of the study was to see the effect of RA on NGF receptors on the cell surface, and this was done by tracking p75$^{NGFR}$ which is a NGF receptor (NGFR). It was seen from the results that within 3 hr of addition of 1µM of RA, the expression of p75$^{NGFR}$ mRNA doubled and by the end of 12 hrs it was more than 3 times its initial value. It was seen that this level of elevated receptor concentration was maintained for about three days. In an attempt to determine if high level of p75$^{NGFR}$ mRNA was caused by modulations to the stability of the mRNA or by increased transcription, the half-life of the mRNA from control and cells treated with RA was compared after 8 hours. It was seen that the half-life of the control and the treated group was not significantly different, pointing out that the RA acted more through transcription rather than by just merely causing mRNA stability. To further determine if RA increased the binding of NGF to the cells, a Scatchard plot analysis was performed using $^{125}$I-NGF. It was seen that pretreatment with RA increased the number of low affinity binding sites by up to 3 folds, but no significant increase in the levels of high affinity
binding sites were noted. It was seen that there was no significant increase in this level after 24 hours. It was concluded that in the case of PC 12 cell line, RA increased the number of low affinity receptors.

2.4.3 Retinoic Acid for Nerve Cell Survival

Apart from enhancing the extension of neurites, it was also found out that RA is essential for the survival of neurons at early embryonic stages. This was established by Rodriguez al [45] when they cultured sympathetic neurons with NGF and RA. It was seen that the neurons from E7 (Embryonic development day 7) were not able to survive in a medium supplemented with just NGF and not any RA. Neurons from E7 embryos were able to survive when supplemented with both NGF and RA. Cells from later stages (E8 onwards) were able to survive in the presence of just NGF. Since it was already established that RA increases the number of receptors for NGF, they attempted to see if RA was essential for neuronal cell survival at earlier embryonic development stages. It is known that there are two kinds of receptors for NGF, a low affinity (K_d 2X10^{-9} M) receptor and a high affinity receptor (K_d 2X10^{-11} M) [46]. A steady state binding study carried out with E7 sympathetic neurons showed the presence of low affinity receptors but no detectable amounts of high affinity receptors. Since it is a prerequisite for NGF receptors to be presenters for the cell to be NGF responsive and because the cells had survived in the presence of NGF and RA, experiments were carried out to see if RA induced any NGF receptors. Experimental results showed that only the cells cultured in the presence of RA showed detectable amounts of the high affinity receptors and these cells were also able to survive. Since the low affinity receptors are fast dissociating, they were not able to elicit any response to NGF. The induction of high affinity receptors by RA made the cells NGF responsive and this helped them survive in the culture. The neuronal cells attain high affinity receptors on their own with
embryonic development. It was seen that cells from later stages of development exhibited detectable amount of the high affinity receptors, and these cells were able to survive in the presence of NGF alone. It should be noted that earlier works had reported survival of cells in culture mediums containing just NGF [24]. It should be noted in this particular reference that the cells did not survive when they were grown in eagles medium supplemented with horse serum, but survived when NGF was added to it. It is to be noted that there is a substantial amount of all-trans RA present in serum. The exact amount of RA present in horse serum is currently unknown, but there is a substantial amount of RA present in the serum of animals, about 0.01623 µM in humans and about 0.0067µM in rat serum [42]. This RA concentration in plasma falls within the range of RA concentration that can induce cell survival and growth.

The key role of RA for neurite survival was further established by Plum et al [47]. In his study he not only explains the importance of the presence of RA during earlier embryonic stages, he also affirms the necessity for continued presence of RA. They cultured DRG from chick explants from different embryonic stages with and without all-trans retinoic acid in a culture already supplemented with NGF. They found that, apart from NGF, all-trans RA was also essential for the survival of cells explanted from embryos at early development stages between 6.5-7 days. Their data showed that unless both NGF and all-trans RA were present, most of the cells were dead, showing that both NGF and RA was essential for long term survival of cells at this embryonic stages. At later level of embryonic development (7.5 – 11 days) RA was not essential for the survival of cells, though the presence of RA enhance the neurite outgrowth from the cells. Thus they were able to show that RA can produce enhanced neurite extension at all ages. Even at the later stages of embryonic development, NGF was essential for the survival of the cells, the absence of which caused the cells to perish. This reinstated the finding by others
about the requirement of NGF for neuronal cell survival. Plum et al also recorded another interesting change to the culture when RA was removed from the culture, upon which the neurites started to decrease in length and disintegrate, leaving behind tracks through which they had previously extended into. They were thus able to conclude that RA was necessary for the maintenance of the extended neurites to survive.

2.4.4. Mechanism of Action of Retinoic Acid

RA is known to act both in an autocrine and paracrine manner. RA can be enzymatically metabolized from retinol in target cells or it can directly enter the cell from the plasma. In blood it is usually transported as retinol bound to a retinol binding protein (RBP). This RBP retinol complex is taken up by the cells and converted to RA. RA then attaches to the Retinoic Acid Receptor (RAR) / Retinoid X Receptor (RXR) complex. It then attaches to the Retinoic Acid Response Element (RARE) found in the promoter region of the target gene. The mechanism has been schematically represented in Figure 2.
Figure 2 Mechanism of action of retinoic acid. Adapted from [48]
CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents

NGF stock solution was prepared by dissolving 10µg of NGF in 100 ml of sterile PBS. 50µl of this solution was dissolved in 950µl of sterile PBS, and the resulting solution was split into aliquots of 0.5ml and stored at -80°C. It should be noted that all pipette tips and eppendorf tubes used were pre-coated with bovine serum album (BSA) to minimize NGF adsorption onto the surfaces. NGF from these aliquots was used in the final reaction mixture. These aliquots were also coated with BSA for the above mentioned purpose.

RA stock solution was prepared by dissolving 2.3mg of RA in 700ml of absolute ethanol. This was divided into 5 tubes. 10µL of this stock solution was dissolved in 990µl of NEUROBASAL™ Medium (1X) (Invitrogen) to give a solution of 10^{-4}M concentration. This was used as the stock solution. The eppendorf tubes containing the stock solution were covered with aluminum foil because RA is light sensitive. The stock and aliquots containing the RA solution were stored at -80°C.

Uridine stock solution was prepared by diluting 0.01875 g of Uridine in 10.7 ml of PBS. This was then split into 10 aliquots of 1 ml each with a concentration of 1.75mg/ml. FDU (5-fluro-2-deoxyuridine) stock solution was prepared by dissolving 0.00809g of FDU powder in 10.8 mL of PBS. This gives a stock solution of 0.075mg/ml. This was divided into 10 vials of 1ml each. Both FDU and Uridine were covered with aluminum foil because they are slightly
light sensitive. Uridine and FDU were added to prevent the proliferation of non-neuronal cells (e.g. Schwann cells).

3.1.2. Substrate Preparation

A PLL stock solution of 0.1mg/ml was prepared by diluting 25mg of Poly-L-Lysine hydrobromide in 250 ml of sterile Phosphate Buffer solution and stored at -20°C.

Laminin stock solution was prepared by diluting 1mg of laminin (BD Biosciences; cat. no. 354232) in PBS to attain a final concentration of 1mg/ml. This was split into smaller aliquots of 0.5ml eppendorf tubes and stored at -80°C. The aliquot tubes were first pre-coated with BSA solution which was prepared by dissolving 50µg of BSA (Sigma Aldrich) in Distilled water. The aliquots are coated with BSA so as to prevent the laminin from attaching to the surface of the eppendorf tubes, which will lead to a reduction in the concentration of laminin in the solution, which would decrease the protein concentration in the final culture. The concentration of laminin that was used for substrate coating is 50µg/ml and this was prepared by diluting 100µl of laminin stock solution in 1900 ml of sterile PBS.

3.1.3. Culture Medium

A 50mL stock solution of the Neurobasal Culture medium was prepared by mixing 48.375mL of NEUROBASAL™ Medium (1X) (Invitrogen; Gibco 21103-049), 1mL of B-27 Supplement (Gemini Bioproducts, Calabasos, CA, USA; cat. no. 400-160), 500µL of Penicillin Streptomycin (Invitrogen Corp./Gibco, cat. no. 15140-122) and 125µL of L-Glutamine. This Neurobasal culture medium was stored at 4°C.
3.1.4. Eggs

Fertilized chicken eggs were obtained from The University of Tennessee Agricultural Experimental Station. The eggs were stored at 12°C and were then incubated at 100°F until they reached the desired embryonic stages. The eggs were placed on an automatic tilter to ensure proper shaking and the incubator was also supplemented with air and water to maintain appropriate humidity inside the incubator.

3.2. Experimental Protocols

3.2.1. Substrate Coating

3.2.1.1. Poly-L-Lysine Coating

The custom prepared well inserts were soaked in 70% ethanol for 30 minutes and then washed twice with fresh 70% ethanol and then allowed to air dry. The air dried wells were washed in sterile deionized water and allowed to dry for a few minutes. The wells were then loaded with 100µl of 0.1mg/ml PLL and left undisturbed overnight for approximately 12 hours. Afterwards, the PLL from the wells was removed, and the wells were washed thrice with sterile water. This is performed to remove any excess PLL because extra PLL may be inhibitory to cell survival. After washing with sterile distilled water, the wells were air dried for a few minutes.

3.2.1.2. Laminin Coating

To ensure uniform laminin coating requires the wells to be pre coated with PLL. Before applying the laminin coating, the PLL-coated wells were washed and air dried as described above. 100µl of 50µg/ml laminin was then added to the wells and allowed to stand for about 1hr.
These wells can either be left in an incubation chamber maintained at 37°C, or left over in the laminar hood. After 1 hr of incubation, the laminin solution from the wells was removed. After the laminin was completely removed, the wells were washed with sterile water to remove any excess laminin, as excess laminin can promote biased directional growth towards it, which may skew the data.

3.2.2. Embryo Dissection

Eggs between the embryonic developments stages 9 – 11 were used for this purpose. DRGs were explanted from chicken embryo between the embryonic stages E9-E11. A way of checking if there is an embryo in the incubated egg is by holding it against the flash light in a dark room. If a thin vasculature is to be spotted on the wall of the shell, it is an indication that the egg might have an embryo. Another possible indication of the presence of an embryo is the appearance of two dark spots when viewed with the flash light. These two spots are actually the eyes of the embryo.

Though there might be a possible indication for the presence of embryo within an egg, the possibility that the embryo is not formed or is deformed exists. The embryo can be removed from the egg by carefully cracking open the broader side of the eggs as illustrated in Figure 3. The arrow head shows the zone where the egg can be typically cracked open. We crack the broader size of the embryo because the air gap is greater at this side and this makes it easier to crack the egg without damaging the embryo. All tweezers and tongs used in the dissection process are sterilized using 70% ethanol before the dissection process. The tweezers are used to break the membranes and reach for the embryo. Once the embryo is removed from the eggs, it is placed in a petridish plate containing HBSS (Hanks balanced salt solution). This plate is placed
Figure 3 Sketch of a typical E10 egg (Adapted from science-art.com)
on ice so as to maintain a low temperature. This helps maintain the viability of the cells until it has been explanted and transferred to the culture plates. The whole dissection process is always carried out while the embryo is immersed in HBSS. The embryo is cut open from the top side and the organs are carefully removed. The DRGs can be seen on either side of the spine. They are carefully explanted and placed onto a petridish plate containing HBSS. The explanted DRGs are split into half so as to remove the surrounding membrane which inhibits sprouting of the neurites. These split DRGs are used in the cell culture.

3.2.3. Nerve Growth Factor Concentration Determination

The explanted DRGs were cultured on 2 sets of PLL coated wells and two sets of PLL and laminin coated wells. Each well was supplemented with the culture medium, 17.5μg/ml Uridine and 7.5μg/ml FDU. 1ng/ml of NGF was added to one set of PLL coated wells and 50ng/ml of NGF was added to the other set of wells. Similarly 1ng/ml of NGF was added to one set of wells coated with both PLL and laminin and 50ng/ml was added to the other set. These cells were incubated at 37°C for 36 hrs.

3.2.4. Cell Culture Conditions.

3.2.4.1. Control Groups

The basic control group consists of DRG cultured with the culture medium, 17.5μg/ml Uridine and 7.5μg/ml FDU on PLL coated wells with no additional growth enhancement molecules. A second control group has DRGs cultured under similar experimental conditions but on wells with an additional laminin coating.
3.2.4.2. Nerve Growth Factor as a Single Agent

DRGs were cultured in the culture medium supplemented with 50ng/ml NGF, 17.5µg/ml Uridine and 7.5µg/ml FDU, on PLL coated custom prepared wells. These were incubated at 37°C for 36 hours.

3.2.4.3. Nerve Growth Factor with Laminin

DRGs were cultured in the culture medium supplemented with 50ng/ml NGF, 17.5µg/ml Uridine and 7.5µg/ml FDU, on wells which have an additional laminin coating. These were incubated at 37°C for 36 hours.

3.2.4.4. Retinoic Acid as a Single Agent.

DRGs were cultured in the culture medium supplemented with 1µM RA, 17.5µg/ml Uridine and 7.5µg/ml FDU on PLL coated wells. The petridish plated containing the cultures were covered with aluminum foil to prevent degradation of RA. These were incubated at 37°C for 36 hours.

3.2.4.5. Retinoic Acid with Laminin

The DRG cells were cultured in the culture medium supplemented with 1µM RA, 17.5µg/ml Uridine and 0.75µg/ml FDU on custom prepared well plates which have an additional laminin coating apart from the PLL coating. The petridish plated containing the cultures were covered with aluminum foil to prevent degradation of RA. These were incubated at 37°C for 36 hours.
3.2.4.6. Nerve Growth Factor along with Retinoic Acid.

DRGs are cultured in the culture medium supplemented with 50ng/ml NGF, 1µM RA, 17.5µg/ml Uridine and 7.5µg/ml FDU on PLL coated wells. The petridish plated containing the cultures were covered with aluminum foil to prevent degradation of RA. These were incubated at 37⁰C for 36 hours.

3.2.4.7. Nerve Growth Factor, Retinoic Acid and Laminin

The DRG cells were cultured in the culture medium supplemented with 50ng/ml NGF, 1µM RA, 17.5µg/ml Uridine and 0.75µg/ml FDU on custom prepared well plates which have an additional laminin coating apart from the PLL coating. The petridish plated containing the cultures were covered with aluminum foil to prevent degradation of RA. These were incubated at 37⁰C for 36 hours.

The reaction mixtures were designed in such a way that it would be possible to test the individual effect of a single component on the growth of neurites, as well as the synergistic effect of any two compounds and that of all the compounds together. This would help us to compare and evaluate if any difference exists between the reaction conditions and if so the significance in the difference and the possible advantages. All the experimental condition can be summarized as a tabular column as follows.

3.2.5. Delayed Release Study

To test the significance of the time of addition of NGF to the culture, NGF was added at different time points and the neurite growth was examined for all the reaction conditions. The DRG cells were cultured in the culture medium supplemented with 1µM RA, 17.5µg/ml Uridine
and 0.75µg/ml FDU on custom prepared well plates which have an additional laminin coating apart from the PLL coating. The petridish plates containing the cultures were covered with aluminum foil to prevent degradation of RA. NGF was added at 0 hours, 12 hours and 24 hours after the initial addition of culture medium. These were incubated at 37°C for 36 hours.
<table>
<thead>
<tr>
<th>CONDITION</th>
<th>SUBSTRATE</th>
<th>URIDINE</th>
<th>FDU</th>
<th>NGF</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PLL</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>PLL</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>50ng/ml</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>PLL</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>--</td>
<td>1µM</td>
</tr>
<tr>
<td>4</td>
<td>PLL</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>50ng/ml</td>
<td>1µM</td>
</tr>
<tr>
<td>5</td>
<td>PLL+LN</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>PLL+LN</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>50ng/ml</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>PLL+LN</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>--</td>
<td>1µM</td>
</tr>
<tr>
<td>8</td>
<td>PLL+LN</td>
<td>17.5µg/ml</td>
<td>7.5µg/ml</td>
<td>50ng/ml</td>
<td>1µM</td>
</tr>
</tbody>
</table>
3.3. Image Acquisition and Data Processing

3.3.1 Image Acquisition.

All the wells were imaged using an Olympus inverted phase contrast microscopy at 4X magnification. The images were acquired using a Nikon camera attached to the microscope and were processed using Wasabi. All images were obtained as 8-bit images of 1000 X 1000 pixels.

3.3.2. Image Processing and Data Collection

The images were processed using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, MD) software and MATLAB 7.0 (Mathworks, Cambridge MA). For analysis of the length, 10 of the longer neurites were chosen at random. This was done manually by a blindfold mechanism, where a volunteer not associated with the project were asked to pick 10 neurites which they thought were the longest. They were not given any reaction condition details or any information about the study. The segmented line selection of ImageJ was used for measuring the length of the neurite outgrowth. This allows tracing the distance between various points. The outgrowth of the neurite was traced from the cell tip till its distal end (Figure 4). All values are obtained in pixels and are then converted into micrometers. The normalized area was calculated by measuring the overall area of coverage and subtracting the area occupied by the cell body from that value (Figure 5). Freehand selection of ImageJ was used for measuring the area occupied by the neurites and by the cell. The values were obtained in pixels and then converted to micrometers. The enhancement of image quality of quantification was carried out using various features in ImageJ and MATLAB.
Figure 4 Length measurement using ImageJ
Figure 5 Normalized area measurement using ImageJ
3.4. Statistical Analysis

All statistical analysis was conducted in JMP (JMP 6.0 SAS Institute, Cary, NC). Seventy longest neurites from each condition were considered for data analysis. A univariate analysis was performed to test for significance at $p \leq 0.05$. A post-hoc Tukey’s test was performed for conditions where significant difference was noted.
CHAPTER 4: RESULTS

4.1. Nerve Growth Factor Concentration Study

A study between the effect of two different NGF concentrations of 1ng/ml and 50ng/ml revealed that 50ng/ml produced more clearly defined neurite outgrowth when compared to 1ng/ml. Though a lot of cellular material was seen spreading around the cells treated with 1ng/ml NGF, the actual length of the neurite was significantly less ($p \leq 0.05$) than the neurite extension that was achieved with 50ng/ml (Table 3). The growth with 50ng/ml was more than twice of what was achieved with 1ng/ml (Figure 6).

Table 3 Neurite extension at different NGF concentrations

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF 1ng/ml</td>
<td>289.564 ± 22.2169</td>
</tr>
<tr>
<td>NGF 50ng/ml</td>
<td>594.495 ± 45.343</td>
</tr>
</tbody>
</table>

Figure 7 depicts the effect of 1ng/ml and Figure 8 represents the growth achieved at 50ng/ml. It can be seen that at 1ng/ml the spreading of cellular material is more profound whereas formation of defined neurites is minimal. Comparatively with 50ng/ml clearly defined neurites can be seen arising from the cell body. It can also be seen that with 50ng/ml the growth of the neurite is very clear, defined and directional.
Figure 6 Nerve growth factor concentration determination study
Figure 7 Nerve growth at 1ng/ml
Figure 8  Nerve growth at 50ng/ml
4.2. Study of Neurite Extension

Varying levels of extension were observed when Dorsal Root Ganglion (DRG) cells were cultured for 36 hours using all the reaction conditions discussed earlier. The growth obtained using NGF, RA and laminin (triple component system) together was significantly greater ($p \leq 0.05$) than the extension observed in cultures using any other reaction condition. Figure 11 compares the mean neurite length of the triple component system with all the other reaction conditions. Figure 12 illustrates the percentage difference in growth between the triple component system and all the other reaction conditions. The growth obtained with the triple component system was $1047.79\pm112.11\mu$m and was almost 5.4 times of Control 1 (PLL alone) and around 2.5 times of that obtained with Control 2 (PLL+LN). Table 4 lists the mean neurite length in µm achieved with each reaction condition. The triple component system also produced significantly greater neurite extension ($p \leq 0.05$) when compared to the best growth achieved by a double component system (laminin and RA). A significant difference in growth ($p \leq 0.05$) was observed between most of the reaction conditions. Extension of neurites on PLL substrate was the least. When NGF was present in the culture the extension was significantly greater than that observed with just PLL. The extension with NGF alone was also significantly greater than the extension observed with laminin alone. RA produced a greater level of extension when compared to either NGF or laminin. Absence of significant difference was only noted in the following two cases. The extension obtained using NGF and RA on a PLL substrate was not significantly different from that observed when NGF was used with a laminin substrate. Likewise there was no significant difference in the extension produced when RA was used on a PLL substrate and when NGF was used on a laminin substrate. Figures 9 and 10 illustrate the mean neurite length for each reaction condition. Figures 13-20 represent the growth achieved for each condition.
**Table 4** Neurite extension for various reaction conditions

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>LENGTH (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>194.6 ± 23.94</td>
</tr>
<tr>
<td>PLL+NGF</td>
<td>528.23 ± 51.68</td>
</tr>
<tr>
<td>PLL+RA</td>
<td>738.74 ± 185.82</td>
</tr>
<tr>
<td>PLL+NGF+RA</td>
<td>793.3 ± 110.57</td>
</tr>
<tr>
<td>PLL+LN</td>
<td>416.15 ± 106.44</td>
</tr>
<tr>
<td>PLL+LN+NGF</td>
<td>768.24 ± 93.18</td>
</tr>
<tr>
<td>PLL+LN+RA</td>
<td>927.5 ± 83.55</td>
</tr>
<tr>
<td>PLL+LN+NGF+RA</td>
<td>1047.79 ± 112.11</td>
</tr>
</tbody>
</table>
Figure 9: Average maximum neurite extension on PLL substrate.
Figure 10  Average maximum neurite extension on laminin substrate
**Figure 11** Comparison of neurite extension with triple component system
Figure 12: Percentage lower in growth compared to the triple component system
Figure 13 Nerve growth on treatment with PLL
Figure 14 Nerve growth on treatment with NGF
Figure 15 Nerve growth on treatment with RA
Figure 16 Nerve growth on treatment with both NGF and RA
Figure 17 Nerve growth on treatment with laminin
Figure 18 Nerve growth on treatment with NGF and laminin
Figure 19 Nerve growth on treatment with laminin and RA
Figure 20 Nerve growth on treatment with NGF, laminin and RA
4.3. Area of Outgrowth

A significantly greater area of outgrowth was achieved by using the triple component system in comparison with all other reaction conditions. Table 5 lists the area of outgrowth achieved with six reaction conditions. The cultures containing only RA were not taken into consideration for this study as RA in the absence of NGF only produced a small number of neurites but these were substantially long. Since the actual area of coverage would be significantly different from the area of outgrowth, these two conditions were omitted to avoid biased data. Least area of coverage was obtained with PLL alone, which is the control. Comparing to the control condition, presence of NGF increased the area of coverage by approximately 22 folds, whereas coating with a laminin substrate increased the area of outgrowth by approximately 11 folds (Table 5). The triple component system increased the area of outgrowth by 60 folds. Figure 21 illustrates the significant difference in normalized area of outgrowth between the different reaction conditions being studied (lack of significance between conditions is denoted by a symbol ‘a’ in the graph). Figure 22 illustrates the area of outgrowth of various reaction conditions with respect to the triple component system.
### Table 5  Area of neurite outgrowth

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>NORMALIZED AREA (µm²)</th>
<th>MEAN ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>17673 ± 6316</td>
<td></td>
</tr>
<tr>
<td>PLL+NGF</td>
<td>390152 ± 18725.6</td>
<td></td>
</tr>
<tr>
<td>PLL+NGF+RA</td>
<td>546888 ± 52779.8</td>
<td></td>
</tr>
<tr>
<td>PLL+LN</td>
<td>195168 ± 30066.7</td>
<td></td>
</tr>
<tr>
<td>PLL+LN+NGF</td>
<td>583820 ± 77365.3</td>
<td></td>
</tr>
<tr>
<td>PLL+LN+NGF+RA</td>
<td>1060919 ± 81365.3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 21 Normalized area of neurite outgrowth
Figure 22 Comparison of neurite outgrowth with triple component system
4.4. Time Delayed Release Study

The neurite growth observed with cultures where there was a delayed dose of NGF was significantly smaller when compared to the culture where NGF was added initially. There was no significant difference in growth when comparing the cultures where NGF was added after 12 hours and 24 hours after initial treatment with RA. In Figure 23, ‘a’ indicates absence of significant difference. It is seen that the length observed with a delayed addition of NGF is similar to the PLL+LN+RA in the main study (Figure 10).
<table>
<thead>
<tr>
<th>CONDITION</th>
<th>LENGTH (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN+RA+NGF(t24h)</td>
<td>938.43 ± 29.0166</td>
</tr>
<tr>
<td>LN+RA+NGF(t12h)</td>
<td>923.55 ± 49.1367</td>
</tr>
<tr>
<td>LN+RA+NGF(t0)</td>
<td>1067.65 ± 24.5531</td>
</tr>
</tbody>
</table>
Figure 23 Time delayed release study
CHAPTER 5: DISCUSSION

The addition of NGF to control culture significantly improved both the area of outgrowth and neurite length. Similar results have been observed with previous experiments. This difference in outgrowth and neurite extension can be attributed to the growth promoting activity of nerve growth factor. It was also observed that the number of cells surviving in the presence of NGF were greater compared to conditions where NGF was absent. This illustrates the importance of the presence of NGF in the culture not only for the enhanced growth of the cells but also for the survival of the cells. Laminin was able to produce neurite outgrowth in the absence of NGF. This substantiates the fact that laminin can support cell growth on its own. The exact mechanism by which laminin is able to help the cells survive is not well documented. Though the cells survive on a laminin substrate even in the absence of NGF, the growth achieved in the presence of NGF with laminin substrate is significantly greater compared to controls without NGF. Laminin produced greater outgrowth compared to PLL because of the fact that laminin is a better substrate for the neurites to adhere and it also promotes rapid forward movement in the growth cone.

RA produces long neurites on both PLL and laminin substrate, but the number of neurites is much smaller. The addition of NGF to a culture containing RA increases the number of neurites growing out of a cell. This may be due to the fact that retinoic acid increases the number of cell surface receptors for NGF. This means that more neurites extend out of the cells thereby increasing the area of coverage and the area of outgrowth. RA also increases the growth achieved with NGF. This is probably mainly an intracellular mechanism, where receptors for RA trigger a transcriptional process for enhancing neurite growth achieved by NGF.
A few synergistic steps could have been involved in achieving the enhanced neurite extension that was observed with the triple component system. Laminin as a substrate would have initially aided in proper adhesion of the cell. The presence of NGF helped in the survival of the cells and also initiated neurite extension. Laminin aided the neurite extension by rapidly increasing the forward movement of intracellular material in the growth cone. RA acted on the transcription machinery, increasing the number of receptors for NGF and also on the extension on the neurite. All the components acted synergistically with one another to produce the enhanced neurite extension.

The time delayed release study was based on the fact RA increases the number of receptors for NGF and these receptors would be active for 30 hours [44]. The study was designed to observe if addition of NGF to a solution containing pre activated NGF receptors would help achieve better outgrowth. The results showed that the extension achieved with the delayed addition of NGF was similar to the one containing just RA. There are a few possible reasons for why the delayed addition of NGF did not produce significant outgrowth. One of the most basic reasons that cannot be sidelined would be experimental errors. There are chances that the addition of NGF at a later stage was not properly devised and failed in the proper mixing of NGF to the reaction mixture. Though the experiment was carried out carefully to ensure proper mixing, chances of errors still exist. Another possible reason could be the amount of time given to the cultures with delayed addition of NGF. In the condition where NGF was added after 12 hours and 24 hours, they were incubated with NGF for only 24 hours and 12 hours respectively. This could also mean that enough time was not given for NGF to elicit its effect. This is based on a pilot study carried out by us to determine the best optimal imaging time. We observed a significant difference in the neurite growth between 24 hours and 48 hours. NGF had at times
shown minimal growth at 24 hours, but even after 48 hours the growth was significant. Thus there is a possibility that enough time was not given to the delayed addition cultures. Another possible reason could have been the fact that the receptors for NGF could have been present but might not necessarily be active. Previous works had only shown that the immune labeled receptors could have been observed for 30 hours, but this does not necessarily mean that these receptors might be active. There exists a possibility that these receptors were inactivated but continued to persist in the medium. Since no literature has substantiated this, future experiments have to be designed to confirm this.
CHAPTER 6: SUMMARY AND FUTURE WORK

The main purpose of the thesis was to verify a multi-component system could synergistically work in order to enhance the neurite extension and neurite outgrowth. A three component reaction mixture was designed and experiments were performed to verify that the effect elicited by these three components together is significantly greater compared to that elicited by the components individually or in combination with just another component. Experimental results showed that the triple component system produced significantly greater neurite extension and outgrowth compared to all other reaction conditions.

This thesis reveals a new dimension for enhanced neurite extension by the use of a combination of compounds. This could mean that any growth enhancement compound could be potentially used in combination with essential compounds to achieve enhanced neurite growth. This gives ample scope for researchers to test alternate compounds and their effect on nerve growth. The greater outcome of the whole thesis would be the implementation of the reaction model into a nerve guidance conduit. The experiment clearly identifies and associates each compound with a specific function relative to a guidance channel. This means that future research can look at alternate sources of functionally related compounds and test their significance on nerve growth. This will lead to the design of an efficient nerve guidance conduit.

The outcome of this thesis opens up a large number of areas of experimental scope for the future. The concentrations of the components used here were based on earlier works. But these compounds were used individually in earlier works. This means that there is a scope for a more optimal concentration when these compounds are used synergistically. One such study could try
to optimize the concentration of all the components for better enhancement of nerve extension. Studies can also concentrate on the time delayed release study. Future experiments can be carried out at various time points instead of 12 hours and 24 hours. There is also scope for adding RA at a later stage instead of NGF.

Future experiments could also concentrate on adding an alternative growth enhancing component instead of the components used here or try the effect of a fourth component on this system and see if the growth can be further enhanced.
REFERENCE

1. National Centre for Health statistics based on Classification of Diseases, t.r., Clinical modifications for the following categories: ICD-9 CM Code: 04.3, 04.5, 04.6, 04.7.


VITA

Sharath Ashwin was born on August 6th 1985 in Chennai, India. He obtained his Bachelors in Technology in Industrial Biotechnology from Anna University, India in 2006. He came to the University of Tennessee, Knoxville in Fall 2006 to pursue his Masters in Biomedical Engineering. After graduating from the Biomedical Engineering program, he is currently pursuing his Masters in Business Administration in the University of Tennessee, Knoxville.