The Effect of Laminin Concentration and Sildenafil Citrate on the Outgrowth and Elongation of Neurites and Effective Formation of Synaptic Structures Among Sympathetic Neurons

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Introduction

The human body is a beautifully complex system. Scientists have striven through thick and thin to understand its makeup, mechanisms, and how it has successfully managed to maintain balance through its various biological networks. The human body has a remarkable ability to recover, adapt with diseases and infections, refuse bones, mend wounds, and conform to various types of environments - yet, the one task our body cannot endeavor is the recovery from nerve damage. For decades scientists have researched methods of recovering nerve functionality - but have failed to do so. The implausibility concerning nerve recovery in our central nervous system rooted questions regarding the cellular and molecular mechanisms involved in the lack of recovery of physical nerve injuries and in neuropathic disabilities.

A recent 2018 census estimated that the annual incidence of spinal cord injury (SCI) is roughly 54 cases per one million people in the United States, or approximately 17,700 people who experience some form of SCI every year (National, 2018). According to the World Health Organization, individuals with a spinal cord injury are five times more likely to die prematurely than people without spinal cord injuries due to neurological and physical deficits caused by these SCIs (WHO, 2013). Accordingly, neuropathic/neurodegenerative diseases -- diseases that degenerate neurons structure and function in the brain -- such as Alzheimer disease, Parkinson's disease, and Huntington's disease account for more than 6.4 million cases in the United States (Harvard, 2018). Although attempts have been made to uncover the causes of these diseases and address the effects of SCIs - such as surgery to treat nerve roots and allow them to heal - they have merely been effective.

Here neurons are discussed and their corresponding structures, why neurons fail to regenerate and exhibit axon and neurite outgrowth after SCI and/or through neuropathic disabilities, the research previously conducted corresponding to neuron growth and outgrowth, and ultimately the role laminin and Sildenafil citrate plays in terms of neuronal growth, neurite outgrowth, and potential synapse formation.

Neuromorphology

In order to effectively understand the functionality behind the nerve cell, it is essential to visualize and comprehend the corresponding morphology and anatomy of a neuron. Dendrites and axons are known as neurites - or any projection from the body of a neuron. These neurites function as extensions to the soma, or body, of the cell as seen in *figure 1*. The soma attaches to the dendrites, which prompt information to the neuron, while the axon prompts information to other neurons. It has been recognized

that the nerve cell is a polarized cell that consists of axons, extensions, and classical neuronal output systems which, along with dendrites, function to transmit information through synapses (a joint between neurites of two or more neurons) and gap junctions. These neurites "communicate" with other neurons with recognition of synapses as structures specialized for one-way transmission (Guillery, 2005).

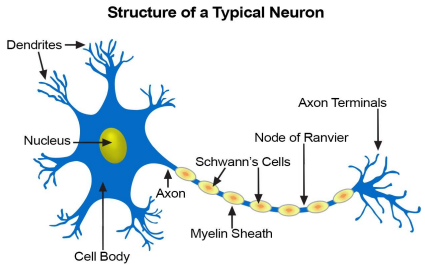

Figure 1. Structure of a Typical Neuron. The typical neuron encompasses a cell body (soma) with extensions known as neurites. These neurites can be both dendrites and axons which both function as neural communicators of neurons. These neurites form synapses (connections) with the neurites of other axons to allow electrical and chemical pathways. The myelin sheath (made up of Schwann cells) support and promote the transmission of these pathways in the neurite.
Image Source: website of the National Cancer Institute (https://www.cancer.gov)

Signal Pathways and Communications Between Neurons

However, it is now known that mammalian neurites have a rich repertoire of electrical and chemical dynamics to the point where Guillery's (2005) conclusion that chemical signals travel in only direct, one-way, linear transmission can be disputed due to neurons' sophisticated information processing capabilities (Mohan et al., 2015)**.** Key evidence has shown that the computational properties of neurons involve nonlinear synaptic outputs as well which means that electrical and chemical communication between neurons can occur back and forth between synapses.

A major factor in the integration of these properties is computed by dendritic spikes (Górski et al., 2018). Neurons generate a spike that develops within the cell - if a high enough electrical impulse is generated - and travels outward in order to communicate with other neurons. This is known as an action potential. With dendritic spikes, the dendrites generate enough electrical impulses to prime the neuron to fire. Ultimately, the dendritic spike allows for a less proximal stimulus and integration to reach an action

potential. Action potentials are regenerative electrical events, usually mediated by voltage-activated sodium ion channels (Na^+). This threshold is achieved after summations of synaptic inputs are made largely on to, and travel among, a neuron's dendritic tree (Stuart, Spruaton, Sakmann, & Häusser, 1997). The importance of axons, dendrites - or neurites for that matter - in communication has been investigated for centuries and their vital role in signal transactions has been inevitably affirmed. Dendrites are constantly conducting these signals from postsynaptic terminals to the body of the cell. While alternatively, axons conduct signals from the cell body to presynaptic terminals. According to Ramon y Cajal's (1899) study and Chklovskii's (2004) review, were it not for these long and branching processes - which allow the corresponding action potentials, dendritic spikes, and most importantly, the synapses found between these long and branching processes - neurons would not sustain systematic interconnectivity to support communication.

Neurite Regeneration and Growth and its Corresponding Inhibitors

Neurite (axonal) Regeneration and Growth and its Corresponding Inhibitors in the Peripheral Nervous System

So what occurs when these neuron extensions (neurites) are breached during some form of physical damage or weakened by neuropathic disability? The mature mammalian nervous system encompasses two primary systems: the peripheral nervous system (PNS) and the central nervous system (CNS). Although both involve a dependent circuit of nerves, their corresponding effect of nerve trauma varies greatly. For instance, after axotomy, the severing of an axon, in the peripheral nervous system, axons can readily regenerate and re-establish synaptic formations. This is due in part to the PNS's accessibility of Schwann cells and also its ability to upregulate numerous regeneration-associated genes (RAGs). Many of these regeneration-associated genes, along with the production of myelin by these Schwann cells, have shown to be essential to neurite formation and outgrowth (Huebner & Strittmatter, 2009). These responses - these alterations in the neurons' physiology allows the cells' function to deviate from cell-cell communication and signaling to that of reconstructing a new neurite, or axon (Blesch et al., 2012; Doron-Mandel, Fainzilber, & Terenzio, 2015) with the ultimate goal of re-establishing and supporting a synapse (connection) with other neurons. Jessen and Mirsky (2016) supported the idea that when a peripheral nerve is damaged or completely cut, it triggers and activates changes in the differentiation of damaged neurons - thus the changes in the expression of hundreds of genes - but also in the Schwann cells found near the injury. Not only do the neurons upregulate RAGs -- the Schwann cells found near the injury also shift their physiological function of ensheathing (cushioning) and myelinating the axon to that of

supporting regeneration as well (Nagarajan et al., 2002; Bosse et al., 2006; Barrette et al., 2010; Arthur-Farraj et al., 2012; reviewed in Jessen & Mirsky, 2016).

Neurite (axonal) Regeneration and Growth and its Corresponding Inhibitors in the Central Nervous System

Contrary to the PNS, the regulation and increase of RAGs appear to be relatively lower in the CNS while conversely upregulating *inhibitors* of axonal growth after physical damage to nerves instead (Marklund et al., 2006). Bomze et al. (2001) demonstrated that increasing RAG expression in the CNS could upregulate neurite regeneration and formation. Albeit, Huebner & Strittmatter (2009) acknowledge that the largest inhibitors of Bomze et al.'s (2001) proposal to upregulate neurite regeneration and outgrowth and ultimately re-establish synaptic communication through RAGs are myelin-associated inhibitors (MAI): specific proteins generated by oligodendrocytes - cells that cushion and insulate neurites in the central nervous. Oligodendrocytes function similarly to Schwann cells in the peripheral nervous system and are both derived from myelin, as seen in *figure 1*. However, oligodendrocytes are *not* expressed in PNS neurons. Specific MAGs such as myelin-associated glycoprotein (MAG), among others, have shown to directly impair neurite growth in vitro and potentially limit axon growth and synaptogenesis (formation of synapses between neurons) in-vivo after damage to the CNS (Mukhopadhyay et al., 1994; McKerracher et al., 2016; Domeniconi et al., 2002; Huebner & Strittmatter, 2009).

Laminin as a Dominant Promoter and Stimulator of Neurite Outgrowth

Laminin Overrides MAG Inhibitory Effects and Promotes Neurite Outgrowth while Releasing Nitric Oxide

Albeit, it has been tested and determined that laminin, a basement membrane protein that governs critical cellular processes including growth, survival, migration, protein synthesis, development, and differentiation (DiGiacomo & Meruelo, 2015), can actually *override* the inhibitory effects of myelin-associated inhibitors (MAI), such as myelin-associated glycoprotein, and stimulate and promote neurite outgrowth (David et al., 1995); when neurons in-vitro are coated with laminin these neurons exhibit neurite growth that begins rapidly and progresses extensively (Baron-Van et al., 1985) while also releasing Nitric Oxide, meaning that MAI expression must somehow decrease.

Laminin's role as a promoter of Neurite Outgrowth in Pheochromocytoma Cells

Many researchers have attempted to tackle the issue of limited regeneration and recovery of damaged neurons in the central nervous system in the past. For one, Pheochromocytoma (PC12) cells have been used to model and parallel neurons in the sympathetic nervous system - which is part of the central nervous system - when induced with nerve growth factor (NGF), a neuropeptide that neurons use for growth. PC12 cells are an adrenal tumor; the adrenal gland is established by neuronal derivatives (thus, the adrenal gland is part of the neuroendocrine system). PC12 cells are dedifferentiated adrenal tumor cells, which puts them back to their pre-neuron days. However, it is now possible to use NGF to restem them (differentiate them) into neurons and use them as a model system for sympathetic (CNS) neurons. The PC12 cell is the best classical neuronal cell model system due to its ability to acquire the CNS neuron features and characteristics when dealt with nerve growth factor (Benjamin S. Weeks). These cells have been widely used because of their ability to produce typical maker proteins for axon and dendrites and form synapse-like structures that parallel CNS neurons once differentiated (Jeon et al., 2010). In Tashiro et al.'s (1989) study, they concluded that when exposed to laminin, the CNS cells expressed neurite outgrowth at 2 hours and reached maximum neurite length at 5 hours.

Sildenafil Citrate as an Inhibitor of Phosphodiesterase Type 5

Although Laminin is specifically used in neuronal cells for its neurite outgrowth stimulation (Rialas et al., 2000), the use of Sildenafil Citrate (SC) in treating pulmonary hypertension and erectile dysfunction could potentially imply implementing SC as a treatment in neurodegenerative diseases (Vallejo et al., 2016). SC is primarily responsible for the degradation of cyclic guanosine monophosphate (cGMP) through the inhibition of phosphodiesterase type 5 (PDE5). cGMP is a critical messenger that regulates fundamental physiological processes such as chronic gene expression, cell growth, and apoptosis (Kukreja et al., 2012). Therefore, combining laminin and SC, which both prompt the release of nitric oxide, could imply cell outgrowth with increased angiogenic properties.

It has been previously demonstrated that laminin can overcome the inhibitory effects of myelin-associated inhibitors such as myelin-associated glycoprotein and simultaneously promote neurite outgrowth in CNS neurons in-vitro. The extent to which this basement protein can effectively elongate and extend neurites to the point of establishing synapse formations in sympathetic neurons (CNS neurons) is unknown. This study will, therefore, be unique by specifically measuring synapse count in sympathetic

neurons with different concentrations of laminin and also considering the addition of sildenafil citrate on neutrino growth

Materials and Methods

Medium Preparation and Cell Culture

The Pheochromocytoma (PC12) cells were kept in a growth medium, Dulbecco's Modification of Eagle's Medium (DMEM), in order to allow the survival and growth of the cells. To the DMEM (1L), 75 mL of horse serum (horse blood), 75 mL of fetal bovine serum (bovine fetus blood), and 10 mL of the antibiotic Penicillin-Streptomycin were added (all obtained from ThermoFisher Scientific). Horse serum, fetal bovine serum, and the antibiotic Penicillin-Streptomycin were used because they support in-vitro antibody response in the cell culture against any bacterium or foreign substances (Levy, 1980). The final medium was sterilized with a 250 mL Nalgene™ Rapid-Flow™ Sterile Disposable Filter Unit (Fisher Scientific), in order to filter and remove any bacterium or unwanted microorganisms from the medium - with the ultimate goal of preventing contamination in the final cell culture. The PC12 cells had been previously stored at -80° C in 0.1% Dimethyl Sulfoxide (DMSO). DMSO is used to increase the viscosity of cell membranes, which allows the accommodation of ice crystals to form without damaging the cells' membrane when the cells are to be stored at cold temperatures. This ultimately allow the cells to revitalize once thawed out to room temperature.

The PC12 cells were left out to be thawed and were then centrifuged at approximately 1000 revolutions per minute (RPM) for 10 minutes in order to separate the DMSO from the cells themselves. Following, once all the DMSO was removed and the cells were isolated, they were placed along with 20 mL of the aforementioned Dulbecco's Modification of Eagle's Medium (DMEM) solution in addition to 100 ng/mL of nerve growth factor - in order to differentiate the PC12 cells into neurons - in a 175 mL tissue culture flask. The cells in medium were then incubated for approximately 24 hours at 37°C in a ~5% CO_2 incubator in order to allow the cells to grow. In order to maintain research accuracy the cells needed to be confirmed for confluence. In consideration of the studies effect in cell replication at a fast rate, the standard confluence was maintained between 20-80%. At 80% or higher, the study would experience misleading data given the likelihood of cell degeneration. After 24 hours the PC12 cells were split with 5 mL of trypsin that was let simmer for 10 minutes. The original tissue culture flask was

divided evenly (~10 ml of cells + solution in two new flasks) to maintain cell confluence. These two new flasks were then incubated at 37°C in a ~5% CO_2 incubator for approximately three days.

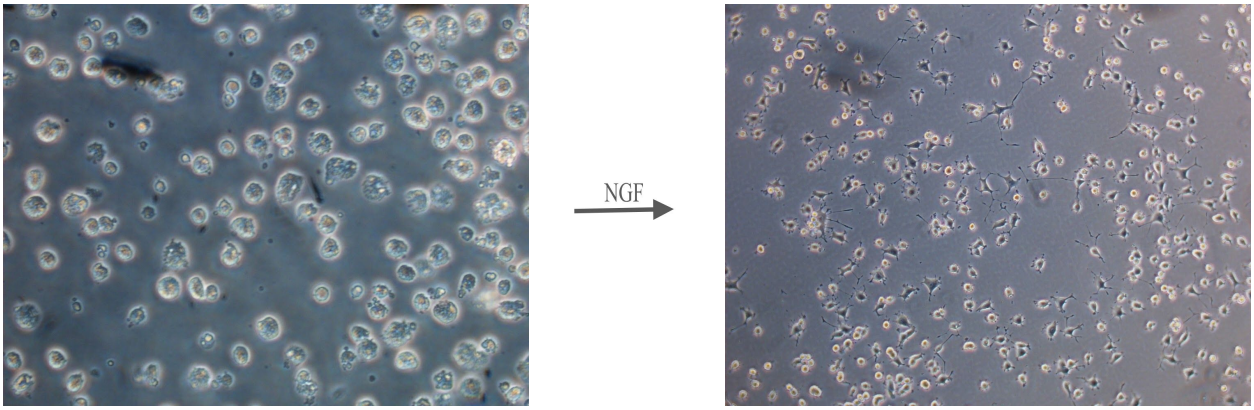

Figure 2a (left) and 2b (right). Before and After NGF. Images of the PC12 cells in the tissue culture flask before the addition of nerve growth factor (NGF) and after the addition of NGF which functions to differentiate the cells into sympathetic neurons. Figure 2a shows the cells with no neurite formations while figure 2b shows the cells with neurite formations demonstrating that the NGF had effectively differentiated the cells into neurons.

Treating the Pheochromocytoma (PC12) Cells with Laminin

In order to release and suspend the cells from the bottom of the cell culture flask and prepare the cells to be coated in laminin, the PC12 cells were released from the cell culture flask by agitation, run through a low speed centrifugation in order to discard the old modified medium, and resuspended in a medium consisting *only* of DMEM. This medium of cells was then seeded in 16-millimeter diameter wells that holds approximately 1.0 x 10^4 cells per 500 µl DMEM solution in each well, this becomes significant once the number of synapse-like formations in each well was statistically measured and analyzed - the number of cells in each well was expected to remain relatively constant in order to compare them to one another. Each of the wells were then coated with 0 ng/mL (control), 0.1 ng/mL, 1 ng/mL and 10 ng/mL of laminin respectively (obtained from Fisher Scientific), in addition to .5 mL of water, as seen in *figure 3* below, with three consecutive trials. The water acted as an activator of the laminin; since laminin is very fatty and concentrated, the water denatures the laminin compounds and opens up its biological domains --

easily allowing it to bind to the neurons' membranes.

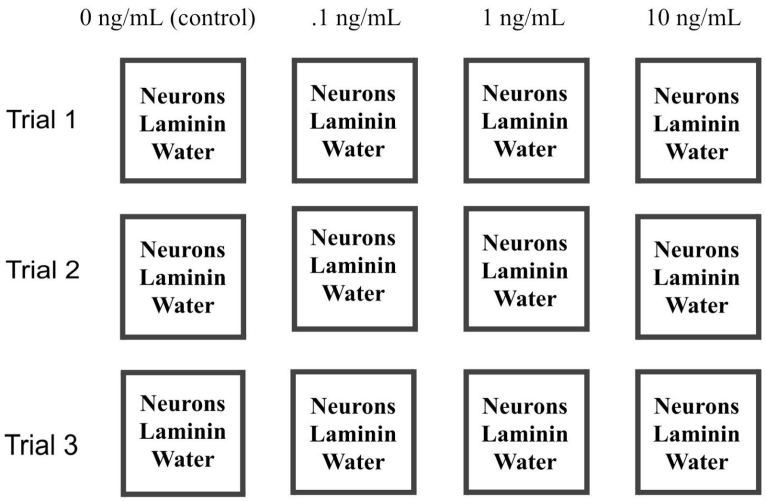

Figure 3. Well Plates of Laminin. Representation of the well plates. The neurons (PC12) were placed in the 16 mm wells in addition to 0, .1, 1, 10 ng/mL of laminin with .5 mL of water separately. This was run for three consecutive trials.

Following, after the wells were coated with laminin, the cells were then incubated at 4°C in a ~5% CO_2 incubator for 24 hours in order to allow the laminin molecules to bind to the cellular membranes of the neurons.

Adding Primed Cells to SC Quantities

cells were seeded into a different set of a 24-well tissue cluster plate containing 16-mm diameter well that had been either coated with 10 ng of laminin or left untreated using blank medium or medium containing 100 ng/ml of NGF. The PC12 cells were then treated with 50 uM Sildenafil Citrate (SC) and observed for neurite outgrowth.

Photomicrography and Neurite Outgrowth Assays

After both plates were coated with laminin and/or treated with SC and left to incubate for 24 hours, the .5 mL of water in each well was removed. The cells in each well were then observed under an Axiovert S100 Inverted Binocular Microscope (Obtained from SpectraServices) at a 320X magnification in order to capture and measure the lengths of the neurites extending from the PC12 cells and ultimately determine if the laminin and SC coating in the trials was effective in elongating and extending the neurites in the PC12 cells that were coated. A neurite outgrowth assessment was used in order to validify that the laminin used was functioning as it should. Each well was photographed under a total magnification of 320X and analyzed using Nikon's Universal Software Platform (NIS-Elements). For the neurite outgrowth assessment, the length of the neurites extending from the cell had to be *twice as much*, or more, the length of the diameter of the body of the neuron in order to constitute it as positive of neurite outgrowth.

Synaptic Formation Count

Morphometric Analysis

In order to quantify the effect of the different concentrations of laminin on the effective establishment of synapse formation among the neurons, a morphometric analysis of the PC12 cells coated only with laminin was conducted. Morphometric analysis, or morphometry, refers to the quantitative analysis of physical forms and changes. The focus was on the number of synapse formations in each of the trials solely coated with different concentrations of laminin. All of the wells were observed and photographed under a constant field of view and a constant total magnification of 320X with the Axiovert S100 Inverted Binocular Microscope and the Nikon's Universal Software Platform (NIS-Elements). Following, the total number of synapse-like formations in each view were recorded. Synapses were considered as any connection or attachment of neurites extending from any two or more neurons.

One-way Analysis of Variance (ANOVA) Test

After counting the number of synaptic structures in each of the images of each of the wells (which were kept at a constant magnification and field of view) a one-way analysis of variance of the number of synapse formations was conducted (using Excel), while assuming that the number of synapses among

these neurons were all independent, equally distributed, and normal. For normality, the mean values had to be approximately equal to the median values and their skewness had to be between -0.5 and 0.5. An analysis of variance (ANOVA) test is a statistical tool used to determine whether there is any significant quantitative variation between the means (average) of three or more independent groups. Since the goal was to compare the number of synapse formations based on varied laminin concentrations, in this experiment the ANOVA tested if there was any significant difference in the mean number of synapse formations among the neurons solely coated with the different concentrations of laminin.

A significance level of $\alpha = 0.05$ for the ANOVA test was used with the null hypothesis, H_O, being that there was no significant difference in the number of synapse formations among the neurons coated with the different concentrations of laminin, and the alternate hypothesis, H_A, being that there was a significant difference in the number of synapse formations among the neurons coated with the different concentrations of laminin. If the p-value generated from the one-way ANOVA was calculated to be above the $\alpha = 0.05$ level then H_A would be rejected in favor of H_O. However, if the p-value generated was calculated to be below the $\alpha = 0.05$ level then H_O would be rejected in favor of H_A deeming that there was a significant difference in the number of synapse formations in the neuron wells.

Tukey's Honest Significant (HSD) Test

Albeit, although the ANOVA test would determine whether there was - or was not - a significant difference in the average number of synapse formations of CNS sympathetic neurons coated with different concentrations of laminin, the statistical test would only determine if the results were significant overall - the ANOVA test would not determine where exactly the significant difference in synapse formations lies within the 0 ng/mL (control), .1 ng/mL, 1 ng/mL and 10 ng/mL of laminin used. For that reason, a post-hoc Tukey's Honest Significant (HSD) test was conducted (using Vassarstats) in addition to the ANOVA test. The HSD test compared all independent groups (the different concentrations of laminin) to one another; using an $\alpha = 0.05$ level, the HSD test ultimately determined where the statistical significance lies when all possible combinations of groups are compared. By using this test, comparing the number of synapse formations in the neurons coated with the lower concentrations to their constituent higher concentration and determine if there was any significant increase in the number of synapses as the concentration of laminin was progressively increased as well.

With this methodology, the hypothesis that increased concentrations of laminin not only promote and increase the elongation of neurites but also effectively promote increased numbers of synapse formations

among CNS sympathetic neurons to ultimately re-establish the electrical and chemical communications between these neurons could ultimately be accepted or rejected.

The methods used to answer my research question, that being a morphometric analysis along with an analysis of variance and post-hoc HSD test, align with my project goal because it directly compared the number of synapse formations within different concentrations of laminin. The experiment not only provides an overall conclusive determination of whether or not higher concentrations of laminin promote synapse formations, but also effectively demonstrates if progressively increasing the concentration of laminin can promote the formation of these neuronal connections significantly more.

Measuring Neurite Outgrowth Based on Sildenafil Citrate Addition

The PC12 cells were exposed to various conditions - they either lacked the addition of NGF, included NGF, or included NGF and laminin. Under these conditions Sildenafil Citrate was added and the neurite outgrowth was assessed based on the cellular morphologies.

Results

Synapse Formation Analysis

After counting the number of synapse formations in each well coated with the 0 ng/mL (control), 0.1 ng/mL, 1 ng/mL and 10 ng/mL concentrations of laminin for each trial, as seen in *figure 4*, and tested for normality as seen in *table 1*, the 0 ng/mL (control) concentration of laminin had synapse formations with a mean of 0.7, a median of 1, and skewness of -1.0003. The 0.1 ng/mL concentration of laminin had synapse formations with a mean of 10.3, a median of 10, and skewness of 0.4855. The 1 ng/mL concentration of laminin had synapse formations with a mean of 22, a median of 22.6, and skewness of 0.5365. Lastly, the 10 ng/mL had a mean of 42.3, a median of 42, and skewness of 0.4232.

0 ng/mL (control) **0.1 ng/mL** **1 ng/mL** **10 ng/mL**

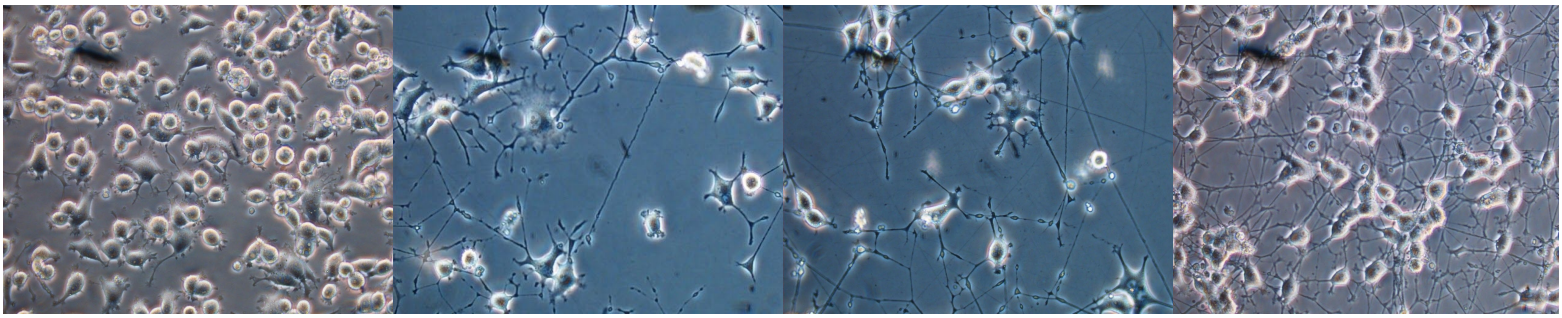

Figure 4. Single Trial of Laminin Coating. The CNS sympathetic neurons (PC12 cells as a model system) coated with no laminin (control), 0.1 ng/mL, 1 ng/mL and 10 ng/mL of laminin respectively demonstrating the outgrowth of neurites and formation of synapse-like structures.

	0 ng/mL (control)	**0.1 ng/mL**	**1 ng/mL**	**10 ng/mL**
Mean	0.7	10.3	22.6	41
Median	1	10	22	42
Skewness	-1.0003	0.4855	0.5365	0.4232
Normality	Yes	Yes	Yes	Yes

Table 1. Statistical Analysis of Synapse Formation. The table depicts the number of synapse-like formations counted in each well along with statistical analyses, including the mean values, median values, the skewness of the values, and whether they were considered normally distributed.

Based on the mean, median, and skewness presented in *table 1* above of the number of synapses counted of the neurons coated with different concentrations of laminin, it was determined whether or not the data collected was approximately normally distributed. Although the skewness of the synapse count in the neurons not coated with laminin is moderate, since the mean was relatively equal to the median it is reasonable to say that the distribution is approximately symmetric and normal. For the synapse count of the neurons coated with the 0.1 ng/mL, 1 ng/mL, and 10 ng/mL concentrations of laminin since all of their means values were ≈ to their corresponding median values, and their skewness were all between -0.5 and 0.5 it is reasonable to conclude that the synapse counts for these concentrations were also approximately normally distributed.

Since the data collected were all considered normally distributed, the analysis of variance (ANOVA) test could be conducted.

H_O (null hypothesis): $\mu_1 = \mu_2 = \mu_3 = \mu_4$

H_A (alternate hypothesis): *The mean number of synapses are not all equal.*

ANOVA	**Column 1**	**Column 2**	**Column 3**	**Column 4**	**Column 5**	**Column 6**
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2835	3	945	124.6153846	4.70E-07	4.066180551
Within Groups	60.66666667	8	7.583333333			
Total	2895.666667	11				

Table 2. Anova Analysis for Synapse Formations. The ANOVA test conducted for the number of synapse formations based on different concentrations of laminin demonstrating that the p-value generated ($4.70 \times 10^{-.07}$) was less than the $\alpha = .05$ level.

Therefore, since the P-value of the ANOVA test was calculated to be $4.70 \times 10^{-.07}$, this demonstrates to provide enough statistical evidence to reject H_O at the $\alpha = .05$ level. Thus H_O was rejected in favor of H_A at the $\alpha = .05$ level, which demonstrates that there is significant variability to the number of synapse formations among the neurons coated with different concentrations of laminin.

However, to determine where exactly the variability lies based on the different concentrations of laminin, the post-hoc Tukey's Honest Significant (HSD) test was conducted. Through this analysis, the control was compared to the .1 ng/mL, the .1 ng/mL concentration to that of 1 ng/mL, and ultimately the 1 ng/mL to the 10 ng/mL concentration, as seen in *table 3*. With the HSD test conducted based on the ANOVA results it shows that all the sample comparisons had all generated a significant p-value less than .05.

Tukey HSD Test	
HSD [.05] = 7.22, HSD[.01]=9.87	**P - Value**
0 ng/mL (control) VS. .1 ng/mL	P < .05
.1 ng/mL VS. 1 ng/mL	P < .01
1 ng/mL VS. 10 ng/mL	P < .01

Table 3. Tukey's HSD Test for Synapse Count. The post-hoc Tukey's HSD test demonstrating that the neurons coated with higher concentrations of laminin had significantly more synapse formations than their counterparts since all comparisons produced p-values < .05.

Neurite Outgrowth Analysis Based on Sildenafil Citrate Addition

Results of the addition of Sildenafil Citrate on the cells lacking NGF, including NGF, or Including both NGF and laminin demonstrated neurite outgrowth in the 24 hours after treatment with significant difference in the length of the neurites of those treated with SC and those not.

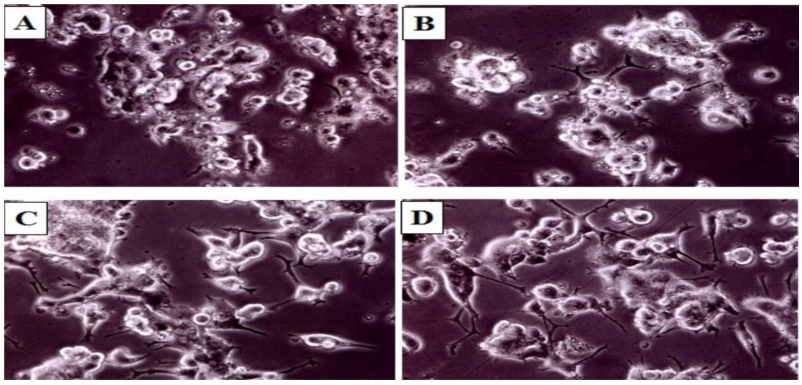

Figure 5. Sildenafil Citrate on PC12 cells with varying treatments. PC12 cells were untreated (A) treated with only SC (B), SC and NGF (C) SC, NGF and LN (laminin) (D).

Discussion

When treating the neurons with different concentrations of laminin after 24 hours, the results demonstrated positive results in terms of both neurite outgrowth and synaptic formation for all quantities. However, it was primarily the 1 ng/mL and 10 ng/mL quantities that expressed significantly more synaptic formations among the sympathetic neurons. As seen in table 2, an analysis of the ANOVA test conducted demonstrated that since the p-value generated was less than .05 ($p = 4.70E-07$) there is a significant variability to the number of synapse formations with the neurons coated with different concentrations of laminin. Therefore the null hypothesis, being that the number of synapse formations in neurons remained the same despite being coated with different concentrations of laminin, was rejected and the alternate hypothesis, deeming that there is a difference in the number of these synapses in the neurons, was accepted.

Further analysis with the Tukey's HSD test demonstrated that when the samples were compared with their constituent higher concentrations of laminin (i.e the 0.1 ng/mL VS. 1 ng/mL and then 1 ng/mL VS. 10 ng/mL) they all produced p-values of less than .05. Based on *table 3*, the .1 ng/mL concentration of laminin had a significantly higher count in synapse formations than that of the control, the 1 ng/mL

concentration of laminin had a significantly higher count in synapse formations than the .1 ng/mL concentration and the 10 ng/mL concentration of laminin had a significantly higher count in synapse formations than the 1 ng/mL concentration of laminin.

The addition of Sildenafil Citrate on the PC12 cells with varying conditions further demonstrated that it promoted neurite outgrowth in the cells even further with the addition of both NGF and Laminin to the cells.

Having understood the results of both the ANOVA test and the post hoc HSD test for the synapse formations and the extension of neurite due to the addition of SC, we can now discuss the implications of these findings. To begin, looking back at Baron-Van et al.'s (1985) study where they demonstrated that laminin prompts neurite outgrowth extensively in neurons, since the ANOVA test and the post-hoc Tukey's HSD test conducted both supported that there is, in fact, a significantly larger number of synaptic formations with neurons exposed to higher concentrations of laminin - this further demonstrates that progressively increasing the concentration of laminin thereby not only promotes neurite outgrowth, but must also be effectively overcoming the myelin-associated inhibitors - such as myelin-associated glycoprotein, that David et al. had previously mentioned - to the point where it is *also* allowing the increased establishment of synaptic connections between the neurites of neighboring neurons. Since the SC added to the cells demonstrated NO (neurite outgrowth), it is reasonable to conclude that Nitric Oxide activation in neuronal cells is sufficient for neurite formation. However, when added to cells already coated with laminin and NGF the SC stimulated NO even further. This implies that the SC compounds must be binding to different receptor molecules than those of laminin in order to stimulate the NO.

Furthermore, since the neurons used were sympathetic neurons, which are constituents of the central nervous system, these findings could further imply that if it were possible to upregulate the laminin protein in weakened and damaged neurons found in our bodies, it could potentially re-establish the chemical and electrical interconnectivity that Ramon y Cajal (1899) had originally deemed vital for neuronal communication between these neurons. These findings could also imply that the use of SC may potentially act as a valuable drug for use in repairing damaged nerves. By doing so, it could be possible to alleviate the effects of nerve trauma to the central nervous system and that of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, and Huntington's disease.

Limitations

There are, however, certain limitations to the procedure and experiment that need to be taken into account. For one, although the Pheochromocytoma (PC12) cells, which were extracted from the adrenal medulla gland of rats, were induced with nerve growth factor (NGF) to allow the cells to express neuronal cell characteristics such as neurites, the NGF only allowed a phenotypic (observable characteristic) change in the cells and not a genotypic one (relating to the genetic makeup of the cells). Thus there could be some confounding effect in terms of the number of synapses formed among these neurons when coated with laminin - with the question arising whether the effects were a result of neuronal characteristics or tumor genotypic expression. Moreover, since PC12 cells are extracted from the Adrenal medulla - which is established by neuronal derivatives -- it still the best neuronal model system to be used because the phenotypic characteristics expressed with these cells when induced with NGF appear to parallel true neuronal characteristics very closely.

Secondly, a limitation that arose as of the procedural portion of the experiment is that of the cell count. A hemocytometer along with the use of the 16-millimeter diameter wells were used in order to establish a constant cell density in each well with the goal of keeping the number of neurons in each well the same. However the number of cells in each field of view were not always equal and could have had some slight impact on the ANOVA + Tukey's HSD test since a lower, or higher number of neurons in each well could result in a lower/higher count of synapse formations. Albeit, in spite of such limitation, the number of cells in each field of view remained relatively constant with only slight variation in the cell count with the use of the hemocytometer and 16-mL diameter wells.

Future Research

Future research that should be pursued could address the genetic component of laminin and SC's role in allowing the extension of these neurite and ultimately allowing the re-establishment of synaptic structures among these neurons. When these neurons are coated with laminin, it has been demonstrated that two specific mitochondrial genes are expressed as a result: Chargerin II and Cytochrome B. (Weeks et al., 1996). Since neuronal mitochondria - organelles which function to provide energy for the cell - are known to concentrate and localize at synapses, there could potentially be some relation to the expression

of these two proteins and the ability of laminin to prompt synapse formations in these neurites. It is worth investigating what exact activation of genes in relation to the expression of these two proteins are prompting neurite elongation and synaptic formations. It is also worth investigating the exact signaling role that Nitric Oxide plays in neurite outgrowth and whether other Nitric Oxide stimulating compounds - among Sildenafil Citrate and laminin - could assist in nerve repair.

In continuation of further investigating how laminin prompts neurite outgrowth and synapse formation, it is planned to use a Reverse Transcription Polymerase Chain Reaction (RT-PCR). Through this reaction, it is possible to use DNA of the neurons coated with laminin to measure gene expression and isolate the specific gene(s) being upregulated in relation to the expression of the Chargerin II and Cytochrome B. proteins. The goal of such future research would then be to determine whether it would be possible to activate these specific genes in an attempt to allow neurite outgrowth and synapse formation in damaged neurons and ultimately alleviate the effects of spinal cord injuries and treat neurodegenerative diseases.

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