5-2021

The Role of Spider Silk in Peripheral Nerve Regeneration

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A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Arts / Science in Biology from
William & Mary

by

Langston Forbes-Jackson

Accepted for ____Honors_____
(Honors, High Honors, Highest Honors)

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May 10, 2021

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Abstract

Spider silk neural guidance channels (NGCs) are highly important innovations in the field of regenerative medicine. This paper will discuss the evidence in the literature that supports their function in regenerative medicine and provide a template for future experiments in the field. While many studies within the past 15 years have demonstrated the validity of spider silk as a scaffold for peripheral nerve regeneration, the molecular mechanics that facilitate regeneration are poorly understood. An emphasis on using silk from orb weaving spiders in particular may have caused researchers to overlook other spiders whose silk could prove to have vastly different results when used in an NGC. Techniques to directly visualize the actions of nerve adhesion molecules are scarce, although there is some innovation in this field that may prove promising. There is also the issue of human trials, as these devices have not yet been proven effective in humans despite successful trials in rodents and sheep. Ultimately, despite its proven usefulness as a scaffold for nerve regeneration, more research is needed to determine what makes spider silk NGCs effective in this capacity.

Introduction

The peripheral nervous system (PNS) performs two important functions: to carry signals from the brain to the rest of the body and to report sensory inputs to the central nervous system (CNS) (Hubener & Strittmatter, 2009). Damage to this system can leave people with long term disabilities that impair their ability to walk, maneuver, and generally interact with the world physically. It is estimated that PNS injuries, particularly in the upper extremities, disproportionately affect people doing manual labor, and can lead to extensive economic costs to those who are affected by them. A study of German workers with upper extremity PNS

injuries reported a calculated cost of an average sick leave of 147 days to their injuries, resulting in socioeconomic costs of 197€/day ($237.15/day) due to their injuries (Bergmeister et.al, 2020). Worldwide, the incidence rate of PNS injuries is estimated to be 1 out of 1000 individuals per year (Haidar et.al, 2020). Because of the severity of PNS injuries, procedures that enhance nerve regeneration are highly important in medicine, and advancements are continuously being pursued to this day (Carvalho et al, 2019). The human body does possess repair mechanisms in order to repair nerve damage, however, under certain circumstances these mechanisms can fail. In situations where a nerve has been severed such that there is a substantial gap between the two nerve ends, the body has great difficulty successfully reuniting both ends, which impairs communication between the brain and the affected organ/organs (Radtke et.al 2011, Radtke 2016). Thus, surgical methods are required in order to ensure successful healing of long distance nerve defects.

Nerve guidance channels (NGCs) represent one such technology that can help with this healing process. NGCs are surgically implanted channels that provide a guiding structure for regenerating neurons, literally bridging the gap between the two severed nerve ends (Carvalho et al, 2019). Many different materials have been utilized to support and enhance nerve regrowth inside the conduits; however, spider silk holds a special place in the literature due to its remarkable natural properties. This thesis will provide evidence that spider silk can be used to repair nerve damage, by reviewing the properties that make it successful in NGCs and reviewing previous studies that brought forth these insights. Important methods necessary to do this research will also be reviewed and further areas of research will be outlined. An experiment planned for this year will also be discussed, which aimed to test whether silk from *Loxosceles laeta* spiders could serve as an effective regeneration conduit.

Basic Properties of Nerve Regeneration

The following is an explanation of basic nerve anatomy and terminology for peripheral nerve regeneration. A neuron is composed of several parts: the axon, dendrites, and the soma. The axon is typically ensheathed by Schwann cells, which provide a protective myelin coating for the cell. Dendrites form branching structures from the main cell body, the soma, carrying signals to neighboring cells via electrical impulses. Gaps between the myelin coating are known as Nodes of Ranvier (Carvalho et.al, 2019). Each nerve in the body is a channel for thousands upon thousands of neurons and supporting structures, Schwann cells, growth factors, and other biological components necessary to facilitate signal transfer between the Central Nervous System and all innervated systems in the body (Lentz & Erulkar, 2020; Ray & Mackinnon,2010; Rotshenker, 2011).

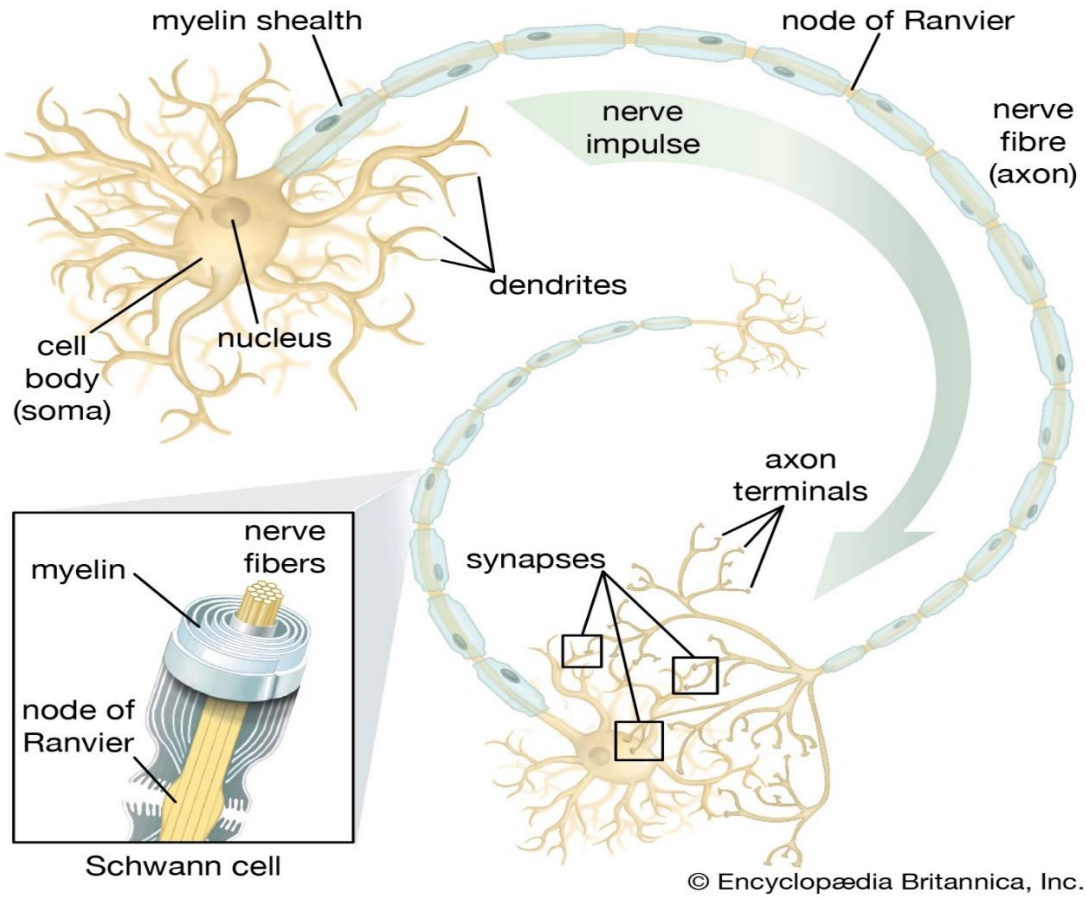

Figure 1: Model of a standard neuron. Schwann cells coated in myelin sheath. Adapted from Lentz & Erulkar (2020).

Nerves in the PNS have an intricate system of regeneration that researchers have worked to facilitate. When damage is incurred, a process called Wallerian Degeneration immediately begins (Huebner, 2009; Rotshenker, 2011). This process causes axons near the damage site to collapse, Schwann cells to discard their myelin sheaths, and recruits macrophages to degrade the released cells. While seemingly counterintuitive to the purpose of regeneration, this process is necessary because cell variation tends to occur near the site of injury, which can cause regenerative complications if not corrected (Carvalho et al, 2019). Once degeneration has taken place, the removed Schwann cells then de-differentiate and begin rapidly proliferating, resulting in the formation of highly aligned fibrous structures known as bands of Bunger, which serve as a guiderail for regenerating axons. Schwann cells have also been shown to secrete growth factors (GF) that enhance axonal regrowth and allow for complete regeneration of a damaged nerve (Carvalho et al, 2019). Once these structures are in place, axons should be able to proliferate along the bands of Bunger and successfully reattach the separate ends of the nerve strand.

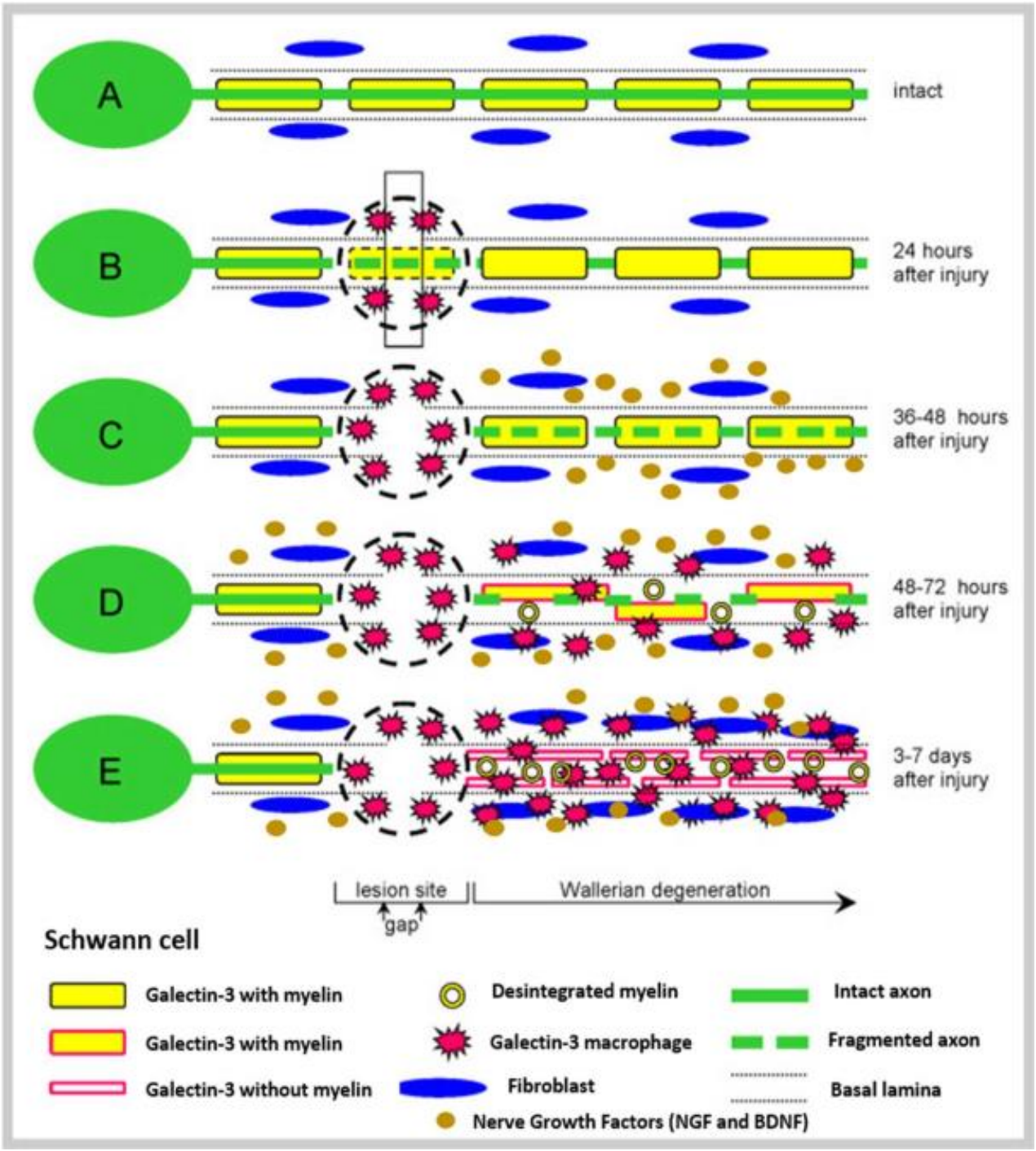

Figure 2: Process of Wallerian Degeneration. A-E shows the process of degeneration at specific time points. *Adapted from Rotshenker (2011).*

Unfortunately, this process does not always end in success. When the distance between nerve stumps is too long, approximately 4 cm or more, the process often fails (Radtke, 2016). At this point, surgical intervention is necessary. If the nerve ends are sufficiently close together,

they can be reattached via a technique known as end-to-end nerve suturing (Tateshita, 2018). When this is not possible, however, the current “gold standard” for nerve repair surgeries is autologous nerve repair (Radtke, 2016). This technique involves taking nerves from a separate source in the body, one with less functional significance, and utilizing them to form a graft to bridge the gap between damaged nerves (Meena et.al, 2021). While this technique has seen a great deal of success, it still has significant drawbacks. Donor site morbidity is a significant limiting factor, as well as a lack of available donors for this technique (Radtke, 2016). Because of this, better methods are being sought to facilitate PNS regeneration.

Basics of NGCs

Nerve guidance conduits (NGCs) represent a promising alternative to autologous nerve repair. Analogous terms in the literature include nerve guidance channels, nerve conduits, or nerve guidance conduits; however, for clarity’s sake the term NGC will be used for this paper. There are several material properties NGCs must possess to be effective. They must serve as a physical barrier to the environment while acting as a guide to direct regenerating neurons (Carvalho et al, 2019). They also need to be sufficiently durable in order to withstand the daily stresses that come with being on an extremity of the body, given that most PNS injuries tend to be located in such areas, such as the arms and legs. They should also promote a minimal immune response in order to prevent rejection from the body, and ideally be bioabsorbable, allowing them to be readily reabsorbed by the body once full regeneration of the nerve has taken place. Spider silk is important because it has been shown to meet all of these criteria successfully.

The process of creating NGCs to stimulate neuron growth began in 1881 with experiments on hollow bone conduits in dogs (Carvalho et al, 2019). More sophisticated techniques have followed since then, some following the same hollow tube model while others utilize techniques such as hydrogel fillings, micro/nano filaments, microchannels, freeze dried

fillings, and many others. Each of these approaches has been met with varying results. Hydrogels, gels made from specific proteins, peptides, extracellular matrix, or polysaccharides, have produced contradictory results. Some studies propose massive successes, while others fail to produce long term regeneration and even hinder axonal outgrowth (Carvalho et.al, 2018a; Wu et.al, 2017). Conduits using micro/nano filaments, on the other hand, have generally performed well with variance being dependent on the specific filament used (Carvalho et al, 2019). Most of the studies reviewed in this paper will be micro filament NGCs utilizing spider silk as the filament; however, recent studies have found success with other synthetic materials, with two notable examples being carbon nanofiber composites and silica aerogels (Farzamfar, 2019; Lynch et al 2018).

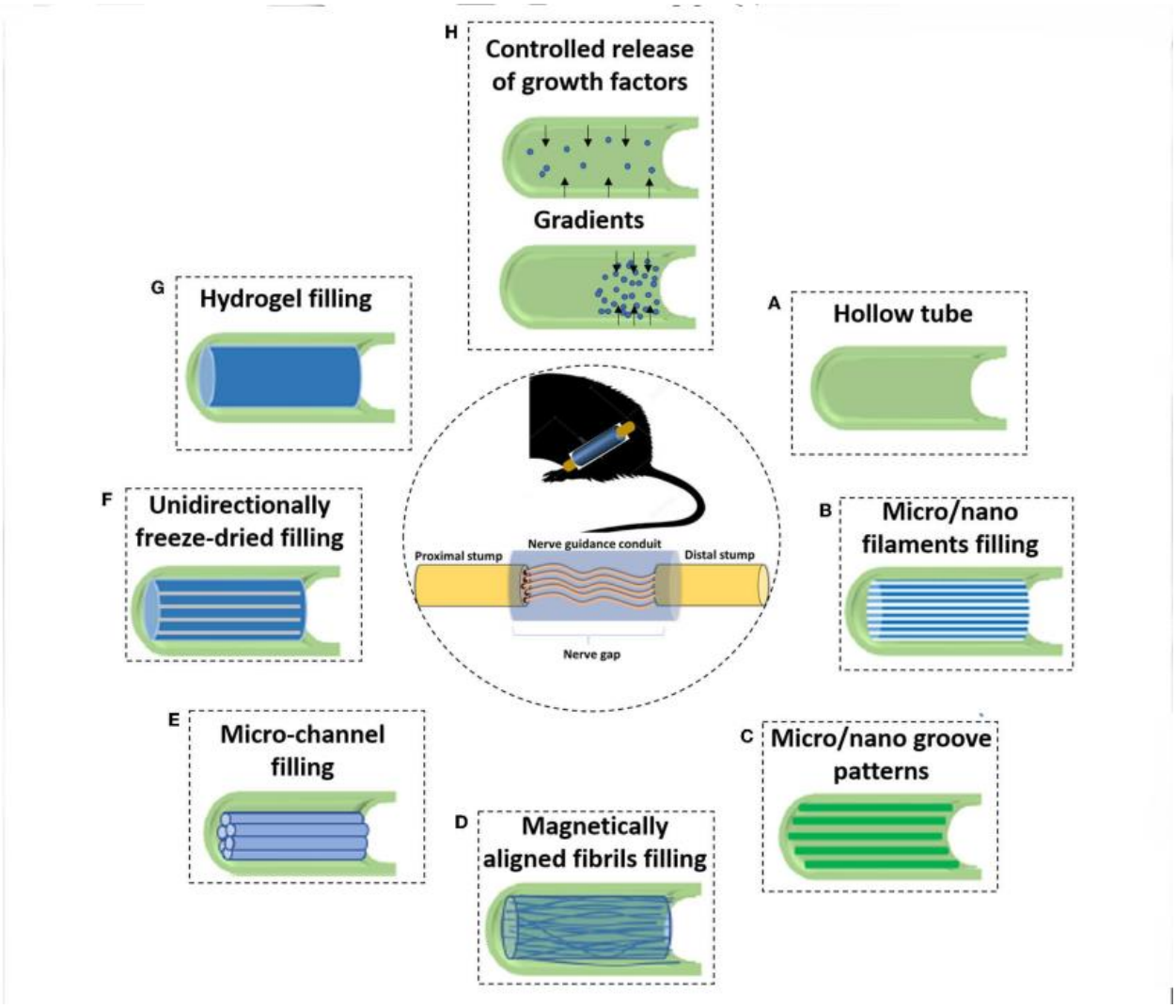

Figure 3: General mechanism of nerve guidance channels (NGCs) shown in center. NGCs bridge the gap between the proximal and distal nerve stumps, allowing for complete regeneration to occur. There are many different configurations NGCs can take that have been used in the literature: A) Hollow tube configuration: the initial strategy for NGC formation, B) Micro/nano tubes, C) Micro/nano groove-patterns, D) Magnetically aligned fibrils or cells, E) Micro-channel filling, F) Unidirectionally freeze dried filling, G) permissive hydrogel fillings, and H) controlled release of growth factors. Spider silk NGCs covered in this paper fall under category B. Adapted from Carvalho et al (2019).

Spider Silk Nerve Guidance Channels

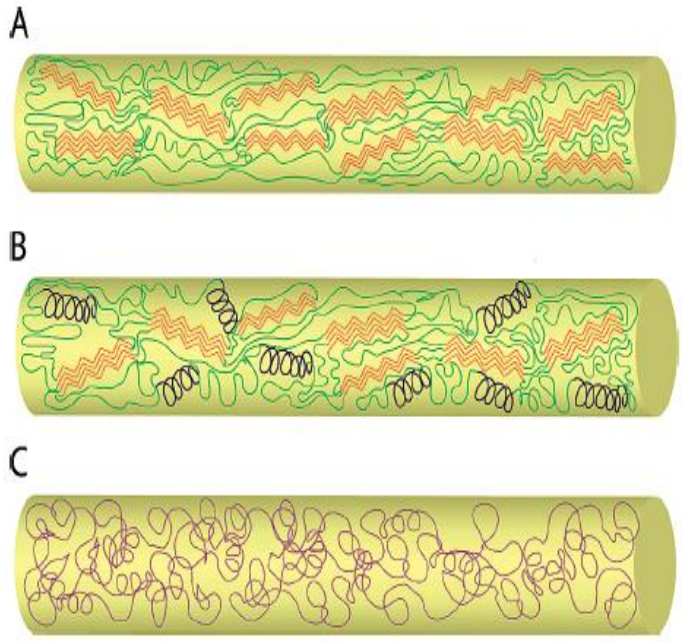

Figure 4: Protein Secondary structure of three classes of spider silk, as discussed by Rousseau et al. (2009). Model A corresponds silk originating from Major ampullate (MA), Minor ampullate (MI), and cylindriform silk glands. Red structures refer to Beta-sheets while green refers to the amorphous phase. Figure 2 part B corresponds to silk from the aciniform gland. Dark blue helices refer to alpha helices. Model C refers to flagelliform silk. The violet lines represent unordered regions of the silk. Adapted from Rosseau et al (2009).

Much of the current literature surrounding spider silk focuses on its incredible mechanical properties. Its durability allows it to outperform its weight equivalent in steel in strength tests and has proven tougher than Kevlar, while being significantly lighter and more flexible (Kiseleva, 2020). However, it is simply a complex of proteins at the end of the day, making these properties even more fascinating. It is important to note that there is not one, single, homogenous type of spider silk shared across all spider species. Individual spiders can use their silk glands to make different types of silk suited to their purposes in nature (Daves, 2013; Rousseau et.al, 2009). Dragline silk from the *Trichonephila* genus (formerly *Nephilia* genus, reclassified by Kuntner et al (2019)), particularly the Golden Orb Weaver, *Trichonephila clavipes*, is prevalent in the literature because it possesses particularly outstanding physical properties (Radtke, 2016). Methods to easily harvest the silk have also been developed. This is important to note because dragline silk has also become a very popular material of study for nerve regeneration; however, it is not known whether it possesses traits that enable it to be more effective at guiding regenerating nerve conduits as other materials (Naghilou, 2020).

Study	Materials used in conjunction with silk	Species of Spider used	Experimental Approach	Main findings
Allmeling et al 2006	Acellularized vein graft spider silk, Schwann cells, and Matrigel.	*T. clavipes*	Examined Schwann cell seeding capability of silk fibers in culture compared to polydioxanone monofilaments (PDS). Followed up with examination of seeding within acellularized nerve graft.	Complete Schwann cell seeding after 48 hours in culture. Full Schwann cell seeding after 7 days in acellularized graft.
Allmeling et al 2008	Multiple combinations of vein isografts, Schwann cells, spider silk, Matrigel,	*Trichonephilia genus**	Tested 5 different NGC compositions on rats to measure regenerative capability of each.	Most successful NGCs were group 4 (silk fibers, Matrigel, and Schwann cells) and group 2 (spider silk only).
Hafner et al, 2017	Dental pulps stem cells (DSPCs), spider silk.	*T. clavipes*	Examined whether exposure to *T. clavipes* dragline silk induced neural lineage differentiation of DSPCs/	DSPCs seeded on silk fibers successfully differentiated into neural lineages.
Resch et al, 2018	Adipose derived stem cells (ADSCs), spider silk.	*T. edulis*	Examined behavior of ADSCs in co-culture with Schwann cells using *T. edulis* spider silk as a guidance structure for both cell type.	Spider silk successfully guided and supported ADSC and Schwann cells proliferation. Equal distribution of both cells was found.
Zhang et al (2015)	Lysine-doped polypyrrole (PPy), regenerated spider silk, poly(L-lactic) acid (PLLA), nerve growth factor (NGF)	*A. ventricosus*	Tested whether a hybrid NGC combining PPy and spider silk could provide successful nerve regeneration in rats.	Successful exon regeneration and Schwann cell seeding found among treated rats.
Radtke et al, 2011	Acellularized nerve graft, spider silk	*T. clavipes*	Comparing success of silk NGC to autologous nerve repair in sheep with induced long distance nerve defect.	Both methods produced identical results, proving silk NGCs viability compared to a “gold standard” nerve repair technique.
Millesi et al, 2020	Rat Schwann cells, dorsal root ganglion neurons (rDRGs), nerve associated fibroblasts (rFBs), spider silk.	*T. clavipes*	Defining the behavior response of each cell group to spider silk fibers, compared to laminin coated plates.	Spider silk supported proliferation of each neural cell type without additional treatment. Cells exhibited in vivo morphology traits while in contact with silk fibers. rSCs formed bands of Bungner formed along silk fibers.
Kornfeld et al, 2021	Acellularized nerve graft (black headed mutton sheep). Spider silk.	*T. edulis*	Measured the speed of nerve regeneration for silk NGCs and compared to regeneration speed for autologous nerve repair.	Near identical regeneration rates were calculated for both methods.

Table 1: Summary of studies discussed in the literature review. Important materials that were used or investigated in the study and the species of spider used are listed as well. * This study did not explicitly state the species of spider used in it, only that it was of the *Nephila* genus. (Allmeling et.al, 2008).

The first known experiment using spider silk as a conduit for nerve cell proliferation was conducted by Allmeling et al (2006). This study tested the ability of *T. clavipes* silk to support Schwann cell growth when compared to polydioxanone monofilaments (PDS). To do this, they harvested dragline silk by pulling fibers directly from the spider abdomen, stimulating the major ampullate duct to produce dragline silk. Silk fibers were then arranged in a culture dish of human Schwann cells harvested from myocutaneous free flaps, tissue disconnected from its original blood supply, from human volunteers. After 48 hours, researchers found complete multilayer coverage of silk fibers with Schwann cells, and only partial coverage in PDS. This prompted the team to test the spider silk strands on an NGC fabricated from acellularized nerve cell veins, spider silk, and Schwann cells mixed with Matrigel. This experiment produced promising results, with the graft being completely seeded after 7 days in culture. These findings opened the door to further studies verifying and finding increased usages for spider silk as an NGC component.

Dr. Vogt's group followed up on this study by testing the ability of a spider silk NGC to repair a 20 mm sciatic nerve defect in rats (Allmeling et al, 2008). This experiment conducted tests on several different types of nerve isograft composed of different combinations of materials. The full list is a s follows: group 1, empty isografts (control); group 2, isograft vein with spider silk; group 3, vein, spider silk, and Schwann cells; group 4, veins, spider silk, Schwann cells, and Matrigel; and group 5, veins and Matrigel only. Each group was tested for defect closing on individual sets of 5 rats, with final measurements taking place after 6 months. Gastrocnemius degeneration was distinctly present in group 5, while groups 1, 2 and 4 showed significantly better ipsilateral and contralateral muscle ratios compared with group 5. Group 3 did not vary significantly from group 5 in this test. Groups 1-4 showed strong regeneration of sciatic nerve tissue, while sections of group 5 nerve constructs had extensive "void areas" throughout, where no tissue was present at all. Perhaps the most interesting finding from this

study was that the conduits with spider silk fibers and Schwann cells alone, group 3, resulted in inconsistent regeneration. Moreover, they did not find support for a previous finding by Donizelli et al (2006) that vein and Matrigel alone could promote nerve regeneration. These findings ultimately suggest that successful nerve regeneration can be achieved with a combination of silk fibers, Matrigel, and Schwann cells (group 4) or only spider silk (group 2). While it makes sense logically that group 4 would be successful, why group 2 also produced viable results is not well understood. More testing may be needed to determine the reasons for this interaction.

Further studies by other labs have confirmed the viability of spider silk as an NGC, supporting Schwann cells and stem cell lines useful for nerve regeneration. One such study tested *T. clavipes* silk's ability to support proliferation of dental pulp stem cells (DPSCs), a stem cell line that can be differentiated into neural lineages (Hafner et al, 2017). Micropatterning of surfaces onto which the cells are plated, in addition to other physical and biological cues, is known to affect the phenotype of the differentiated cells (Alberti et al, 2008; Irawan, 2013; Hafner et al, 2017; Pittenger et al, 1999). This experiment demonstrated that *T. clavipes* silk allows for DPSC differentiation into neural lineages, implying that the silk possesses the required micropattern for neural lineage differentiation. This was determined by comparing the spider silk to a synthetic silk produced using recombinant silk proteins. No proliferation was detected on the synthetic silk, leading to the conclusion that natural silk micropatterning is the key for differentiation. While the exact structural pattern needed for differentiation into the neural lineage is not known, the recombinant silk fibers showed much more varied morphologies and diameters than natural silk fibers. This may be a potential explanation for recombinant silk's ineffectiveness in supporting DPSC proliferation, however, a full understanding of the physical features needed drive DPSC differentiation into neural lineages is still necessary.

In addition to DPSCs, there have also been successful tests on adipose-derived human stem cells (AHSC) (Resch et.al, 2018). AHSCs can differentiate into neuronal cell lines, which

have been successfully utilized in regenerative nerve conduits (Dai, 2013). While the primary aim of the paper was to examine the behavior of AHSC co-cultures with Schwann cells, it does provide further evidence for spider silk's regenerative capabilities. Spider silk from this study was harvested from *Trichonephila edulis* and wrapped around a steel frame, then placed in a 6-well plate. The AHSCs and Schwann cells were mixed into a drop and gently placed on the well. Immunofluorescence was then used to identify both cell lines, using Schwann cell marker S-100 and ADSC marker CD 90, after 13 days in culture. Equal concentrations of Schwann cells and Adipose-derived stem cells were found.

Zhang et.al (2015) also demonstrated that experimental approaches utilizing hybrid constructs combining silk and other biomaterials can also be effective. They generated a combined lysine-doped polypyrrole (PPy), regenerated spider silk, poly(L-lactic) acid (PLLA), and nerve growth factor composite scaffold to compare to spider silk alone in an experiment repairing a 2 cm sciatic nerve defect in rats. PPy is an extensively studied material that has been shown to assist in nerve regeneration via electrical stimulation. The goal of this experiment was to combine the regenerative properties of PPy and spider silk using a composite produced via co-axial electrospraying. This is a technique utilizing electrohydrodynamic processes that form liquid droplets into microfibers, which have been utilized to generate a number of non-silk NGCs in the literature (Xue et.al 2019; Carvalho et al, 2019; Farzamfar et.al 2019). The ultimate result was a functional composite that successfully repaired a 2 cm sciatic nerve defect in rats within 10 months of implantation, with excellent Schwann cell seeding and axon regeneration.

A major success in this field comes from a study in which spider silk NGCs were compared to autologous nerve grafts (Radtke et al, 2011). In this study, a 6 cm tibial nerve defect was repaired in sheep using a spider silk based NGC vs. an autologous nerve graft. Spider silk from *T. clavipes* was used to fill a quarter of an acellularized nerve graft, which was surgically inserted to bridge the gap between severed nerve ends. Recovery of sheep receiving

the graft was compared to sheep receiving similar treatment but using autologous nerve graft to repair the defect. After 4 months, researchers saw that sheep were able to stand upright with no clear differences between groups with silk NGCs and autologous nerve grafts. No significant differences were found between the weight of the affected muscle between groups, and electrophysical data showed similar performance for both groups. Neurofilament staining indicated that axons fully repopulated the grafts without issues, and Schwann cell seeding was found to be successful as well. These findings indicate that spider silk based NGCs match the performance of the current “gold standard” in long distance nerve repair without the same drawbacks (Radtke, 2011).

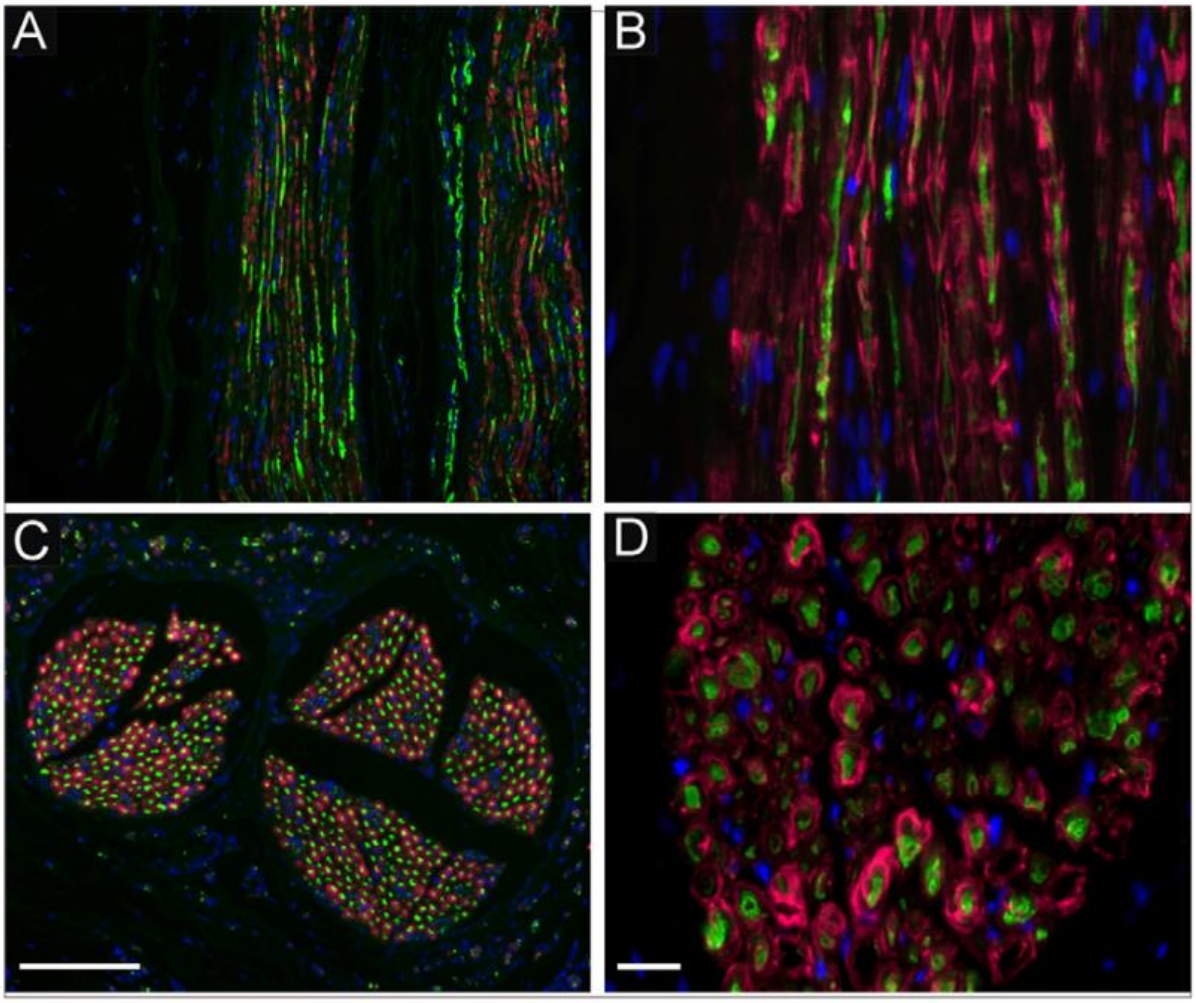

Figure 5: Histological analysis of regenerated fibers following silk NGC implantation. Immunostaining of regenerated nerve segments. Neurofilament in green, Schwann cells stained red, identified by S100, and cell nuclei stained blue with DAPI. Schwann cells are shown here to have successfully migrated through the conduit (A and B). Cross sectioning shows immunopositively staining for S100 and ensheathment of regenerated axons by endogenous Schwann cells shown though co-localization of neurofilament. Adapted from Radtke et.al (2011).

More knowledge is needed in order to determine why exactly spider silk performs well as an NGC. In pursuit of this, a recent study sought to define the behavioral response of primary rat Schwann cells (rSCs), dorsal root ganglion neurons (rDRGs), and nerve associated fibroblasts (rFBs) to native *Trichonephila* spider silk, as well as to narrow in on the regenerative effects of native spider silk (Millesi et.al, 2020). They did this by comparing the growth of these cells on *T. edulis* dragline silk fibers to laminin coated culture dishes. What they found confirmed a previous finding from Allmeling et al (2008) demonstrating that spider silk alone could support

attachment of Schwann cells, DRG neurons, and fibroblasts without any additional treatment. All of the cells additionally possessed their characteristic morphological features: rSCs were aligned in a bipolar fashion, rFBs spread across silk fibers, and rDRGs formed long axonal configurations, as they would naturally *in vivo*. Confocal image analysis also demonstrated that rSCs formed bands of Bungner along silk fibers, which may explain why spider silk performs so well as a regenerative conduit. As previously noted, bands of Bungner provide guidance structures for regenerating neurons, therefore, it stands to reason that a substance that induces their formation should create an effective medium for nerve regeneration (Carvallho, 2019; Hubener, 2009). Elongation and spreading of nerve associated rFBs was also noted, which could be potentially problematic as excessive FB spreading within NGCs may form a physical barrier to regenerating nerves. This was reflected in the group's findings as they found rFB presence significantly decreased the velocity of rSCs on silk fibers. This finding, in conjunction with the lack of migration directionality found on native silk, supports the view that recombinant silk fibers, that maintain the regenerative capabilities of natural silk but prevent intense fibroblast spread, is the next step in this field of study.

One final study, the most recent found as of time of writing, examined the time necessary for complete axonal regrowth (Kornfeld et.al, 2021). This study compared the speed of axonal regeneration of *T. edulis* silk NGCs to that of autologous nerve grafts in sheep. Electrophysiology recordings and immunochemistry was performed at group specific endpoints at 20, 30, 40, 50, 90, 120, 150, and 180 days after implantation surgery. Regeneration speed was calculated by estimating the average velocity of developing axons spreading through the graft, with 1.309 mm/day found for spider silk NGCs, and 1.57 mm/day found in autologous nerve grafts. There was a lag time of 10 days between experimental group end points, which may have caused the velocity calculations to be lower than the real values (autologous nerve transplant t=40 days, silk NGC t=50 days). The researchers discuss using Tinel sign evaluation

and radioactive axonal labeling as alternative methods for calculating regeneration velocity. The rate of silk degradation in the conduits was also quantified. Cross sections of the silk NGC were stained with Masson-Goldner-Trichrome to specifically visualize scar formation and connective tissue and compared with Toluidine blue stained semithin sections of Epon embedded nerve stumps. Toluidine Blue was used to specifically visualize semithin section contents, including cell nuclei. Degradation become fully apparent after 90 days in the Masson-Goldner-Trichrome stained segments, with complete degradation after three months. In Epon embedded segments, silk was not detectable after 50 days. Analysis of electrophysical results and axon regeneration at each time point demonstrated similar regenerative capabilities for both silk NGCs and autologous nerve grafts, confirming findings that both are near equally effective guides for nerve repair (Kornfeld, 2021; Radtke, 2011).

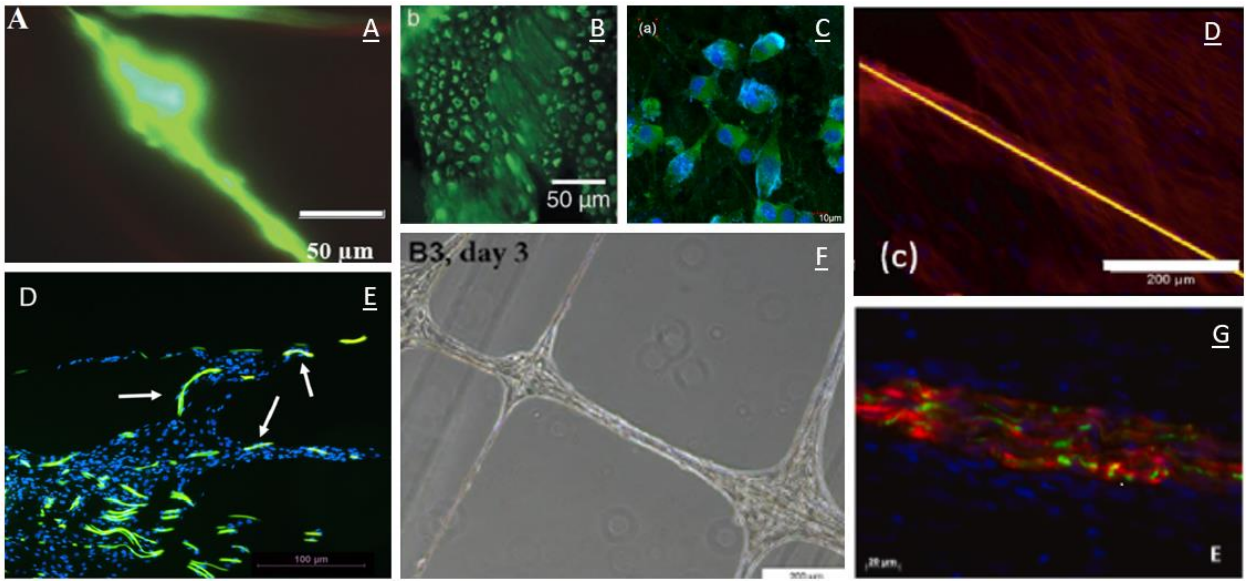

Figure 6: Examples of successful regenerative interactions between nerve cells and spider silk. A) Live/dead assay of Schwann cells on *T. clavipes* silk (Allmeling et al, 2006). Schwann cells are stained green; demonstrate multilayer seeding around fibers. B) Cross section of regeneration conduit containing acellularized nerve veins, spider silk, Schwann cells, and Matrigel. Immunostaining using anti-neurofilament antibodies showed regenerated axons staining positive for neurofilament, indicative of successful Schwann cell regeneration. C) PC12 cell adhesion to L-PRPN composite scaffold (Zhang et.al, 2015). D) Dental pulp stem cell (DPSC) adhesion to spider silk after sterilization with UV light for 30 minutes. Cells adhere well to the silk fibers. E) Schwann cells and adipose derived stem cell co-culture with spider silk (Resch et al, 2018). Immunostaining with autofluorescence of the silk (green) and cell nuclei stained with DAPI (blue). Cells adhered to silk marked by white arrows. G) Regeneration of spider silk nerve graft following 5 months of regeneration in sheep. Schwann cells stained with anti-S100 (red), nerve fibers with anti-neurofilament (green), and cell nuclei with DAPI (blue).

Commonly Used and Important Methods

Spider Silk Harvest: Spider silk harvest is typically done by affixing a spider in place and coaxing the silk out by mechanically puling the fiber from the silk gland (Allmeling, 2006; Naghilou, 2020; Radtke, 2011; Resch, 2019; Vehoff, 2007). The method of storing the silk varies depending on the experimental design. Some studies used the silk immediately after extraction, while others opted to store it for later use. Radtke et.al (2011) states that it should be kept at room temperature in closed containers, secluded from sunlight. Silk has been sterilized via UV light and autoclaving; however, not all studies made note of exactly how their silk was sterilized, so apparently a standard method for sterilization has not been established (Hafner, 2017; Resch, 2018). Allmeling et al, 2008 determined that sterilization was not necessary, due

to the silk being harvested in a sterile environment; however, Hafner et.al, 2017 tested UV sterilized silk strands against non-sterilized strands and found better proliferation on the sterilized group. Given this, it seems reasonable to sterilize silk before use, particularly before placing it in cell culture or NGC implantation.

Acellularized Nerve Ventricles: Standard procedure for preparing acellularized nerve ventricles is to extract nerve ventricles from pig lower extremities (Allmeling et al, 2006; Radtke et al, (2011). They are excised and freed from adhered fat, after which there are washed in PBS (phosphate-buffered saline) and incubated in trypsin/EDTA for 24 hours. Radtke et al (2011) repeated this procedure after washing with PBS again for two weeks. After washing, venules were histologically controlled by hematoxylin-eosin and trichome staining and frozen at -80 degrees Celsius until usage (Radtke et al, 2011). Veins were then filled with the appropriate NGC material, varying with experimental procedure.

Schwann cell harvest and cultivation: While the exact materials used in each study vary, the general principle for Schwann call harvest and cultivation is to isolate them directly from nerves belonging to the organism being studied and grow them in culture (Allmeling et al, 2006; Allmeling et al, 2008; Resch et al, 2018; Naghilou et al, 2020). Nerve samples from humans were obtained from surgery on free flaps, pieces of tissue disconnected from the body's blood supply (Allmeling et al, 2006; Resch et al, 2018). Allmeling et al (2006; 2008) cultured harvested cells using Dulbecco's modified Eagle's medium (DMEM/F12, PAA) supplemented with 20% FCS and 100 µg/mL penicillin and streptomycin (PAA) at 37 degrees Celsius and 5% CO_2 for three weeks in both studies. For the 2006 study the cells were then resuspended and cultured in melanocyte growth medium (M2, Promocell, Heidelberg, Germany) on poly-L-lysine (Biochrom) coated dishes, while the 2008 study purified cells on a glass surface for two hours following plating. Contaminating fibroblasts settled on the glass while Schwann cells remained in supernatant. These Schwann cells were then washed in PBS and used for experimentation

(Allmeling et al, 2008). Resch et al (2018) first washed the extracted nerve samples in PBS 1% antibiotic—antimycotic and transferred them into αMEM + (αMEM + 2.5% HEPES, 1% Pen/Strep + 10% FCS + 1% NaPyruvate) for fascicular dissection. αMEM is MEM (Minimum Essential Medium) without phenol red pH indicator color, used for live cell imaging. The fascicles were then transferred to 6-well plates with 6–10 cm fascicle tissue in each well, followed by overnight incubation in αMEM+ supplemented with 0.125% Collagenase Type IV, 1.25 U/ml Dispase II and 3 mM $Ca2Cl_2$.

Future Studies

While spider silk has been proven useful as a conduit for nerve regeneration, there are still a multitude of unsolved questions regarding the molecular mechanics that cause this interaction to occur. Neural cell adhesion molecule (NCAM) is a potential mechanism for binding that should be thoroughly investigated. NCAM is a member of the immunoglobulin (Ig) superfamily that is involved in cell-cell binding via homophilic interactions (Kasper, 2000). Other cell adhesion molecules (CAMs) of interest are L1 protein, N-cadherin, and integrin (Kiryushko et al, 2004; Liu et al, 2020). These molecules would be ideal targets for future study, to determine if they interact with spider silk proteins and the nature of those interactions.

Methods for measuring the interactions between CAMs and their substrates have been scarce. Imaging techniques to directly visualize these structures have yet to be discovered; however, there is a recently discovered method that may prove useful for visualizing NCAM interactions with spider silk. This method peels neurites from a substrate using an atomic force microscopy tipless cantilever, while monitoring for the role of NCAM using a laser total internal reflection fluorescence microscope (TIRFM) (Liu et al, 2020). Atomic force microscopy works by moving a tipped cantilever over a surface and measuring its deflection using a laser deflection system, wherein a laser is deflected off the cantilever surface and onto a position detection sensor (Dufrêne et.al, 2017). The behavior of the cantilever and the properties of the probe tip,

which is typically very sharp, can be modified to investigate a wide variety of physical properties (Dufrêne et.al, 2017, Schniepp et.al 2013). By using a tipless cantilever, this technique avoids disrupting the neurites, which allows them to re-adhere to the substrate. This repeated peeling process allowed for researchers to quantify the adhesion between NCAM molecules and the substrate by calculating the adhesion energy generated from the peeling process, as well as enabling them to determine that a 2-5 minute period is needed for adhesion to begin anew. TIRFM employs an evanescent wave to excite fluorophores rather than directly illuminating the surface, which allows for imaging of molecules close to the glass/system interface (Ockenga, 2012). This mechanism presents a potential method to observe neural adhesion to substrates and may be applied to spider silk substrates in the future. The researchers also suggest that this technique can be adapted to other adhesion molecules, such as L1 and integrin, that may have a significant impact on cell-substrate adhesion (Kiryushko et al, 2004; Liu et al, 2020).

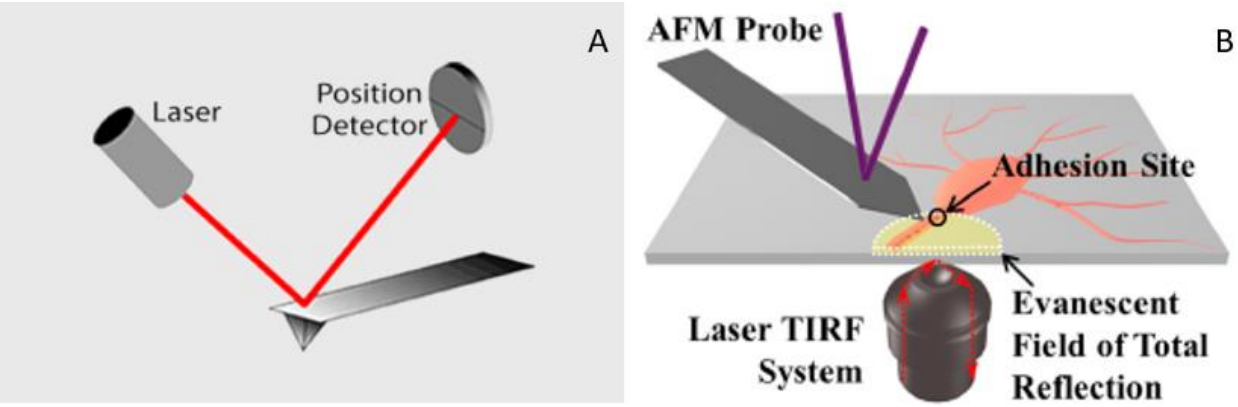

Figure 7: A) Basic diagram of Atomic Force Microscopy. Changes in the deflection of the tipped cantilever will be picked up by the sensor as the position of the light changes. Adapted from Atomic Force Microscopy (2019). B) AFM, Laser total internal reflection fluorescence microscopy setup used by Liu et al (2020). A tipless cantilever is utilized to peel neurites from the substrate without disrupting them, while that laser TIRF system visualizes the peeling and re-adhesion process. The purple bar above the AFM probe represents the laser beam used to measure cantilever position. Adapted from Liu et al (2020).

The exclusive focus on *Trichonephila* dragline silk may have caused the research community to overlook silk from other species that may have properties more conducive for nerve regeneration. While it has certainly proven to possess remarkable strength and toughness, there is currently no evidence that it outperforms other types of silk from different

species as an NGC. A recent study compared the effectiveness of 4 types of silk, *T. clavipes* dragline and cocoon silk and *A. avicularia* connecting and attaching silk, as scaffolds for Schwann cell growth (Naghilou, 2020). All four strands demonstrated similar ability to support Schwann cells. Despite their differences in morphology, Raman spectroscopy revealed similar protein secondary structures for each silk, which could be an explanation for their similar properties. The only major difference between strands was that *A. avicularia* attaching silk began to adhere to itself in culture, possibly due to its low beta-sheet count, which researchers believe imbued it with less rigidity than the other strands in the test. This experiment is the first step towards narrowing in on spider silk's neuroregenerative properties, and more should follow.

Loxaceles laeta silk may be a good candidate for future investigation due to its unusual morphology. It is very small, 40-80 nm thick and 6-9 μm wide, and ribbon-like, composed of many different nano-fibers linked together in a manner mimicking an electric cable, highly unusual compared to many other spiders (Schniepp, 2013; Wang & Schniepp, 2018). For comparison, *T. clavipes* dragline silk is approximately 3.2 μm in diameter; less wide but much thicker than *L. laeta* silk overall (Naghilou et.al, 2020). It also possesses structures dubbed "nano-papillae", point-like structures populating the silk's ribbon-structure, which may facilitate strong adhesive interactions with other surfaces (Schniepp, 2013). Because it is so unusual, and so little is known about how spider silks interact with nerve cells, learning how *L. laeta* silk interacts with the neural environment could be very useful in furthering our knowledge of the subject. An experiment that was planned but unable to be completed this semester due to the constraints of the pandemic would investigate whether *L. laeta* silk possesses the ability to function as an NGC by testing its ability to support PC12 cell proliferation and differentiation. PC12 cells were selected because they were readily available and have been used in prior experiments on silk NGCs due to their ability to differentiate into neural lineages (Zhang et.al, 2015). The essential plan for the procedure is as follows: silk fibers would be extracted from *L.*

laeta spiders as in previous experiments (Schniepp et al, 2013; Wang & Schniepp, 2018), silk would be placed in culture, seeded with PC12 cells, and left to incubate until the cells proliferate to a satisfactory confluency. At the end of the allotted growth period, the samples would be analyzed for PC12 proliferation and neural lineage differentiation using immunostaining to clearly identify PC12 cells in relation to the spider silk. We would look for clear evidence of cell proliferation around and along the fibers, and for differentiation into neural lineages. Other experiments could test Schwann cell adhesion and utilize it in NGCs using model organisms such as rats or sheep. One potential problem is that these silks tend to bend and wrinkle easily, which could lead to aggregations forming in cell cultures as with *A. avicularia* attaching silk strands in Naghilou et al's test (2020). Some might argue that this should disqualify *L. laeta* silk from being studied; however, given its unique properties and how little we know about how silk interacts with nerve cells, I still maintain that a study on this type of silk would be worthwhile in order to test our hypothesis and see what more can be learned about spider silk. Silk strands could be wrapped around a scaffold structure, perhaps mimicking the steel support frame used in Resch et al (2018), to hold them in place before being put in culture in order to counteract this property.

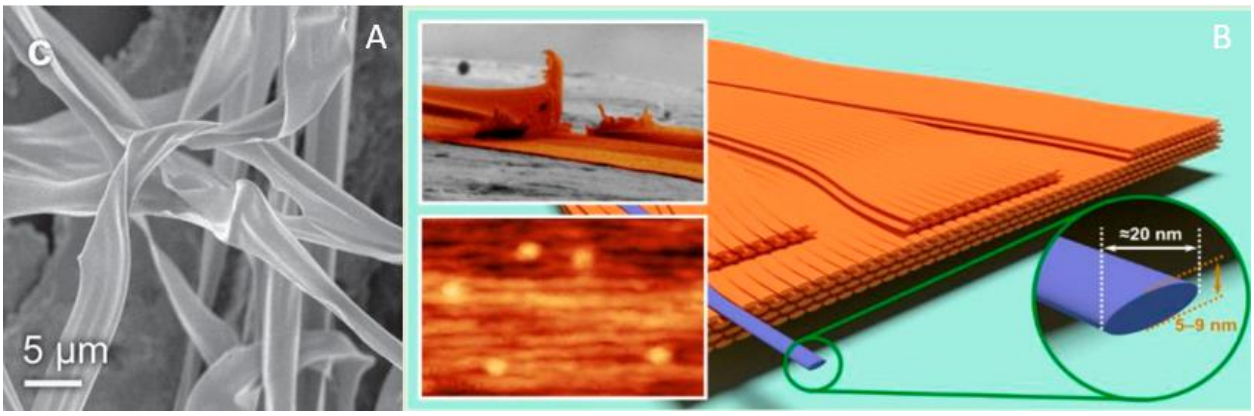

Figure 8: *Loxaceles laeta* silk. Designations for this paper are in the top right corner. A) Scanning electron microscopy of *L. laeta* silk. B) computer model of silk structure. Silk strands are composed of many linked nanofibrils forming cable-like structures. Image on the bottom left shows surface topography with nano-papillae visible as bright orange protrusions on the surface. Adapted from Schniepp et al, 2013 and Wang & Schniepp, 2018.

Additionally, new methods to create artificial nerve venules will help avoid having to rely on excised vein grafts from organisms such as pigs to create nerve grafts (Allmeling et al*,* 2006; Radtke et al, 2011). This material would need to possess the same properties as naturally generated nerve veins while being efficient to create. This may prove difficult to produce; however, there are advancements in the field of 3D bioprinting that are showing promising results. Ning et al (2018) utilized the technology to produce structures that can induce Schwann cell alignment and proliferation using hydrogels (Ning, 2018). While interesting in its own right, it would be interesting to see the results of a study that used spider silk or a printed substance mimicking its properties in place of the hydrogel. Such a study will provide further data on the effectiveness of silk NGCs vs other regenerative materials, while also further testing the viability of 3D printed nerve constructs.

Another useful study may be to compare the in vivo performance of spider silk NGCs to other successful nerve conduits, such as the electrospun carbon nanofiber conduit analyzed by Farzamfar et.al. (2019) or even against the lysine doped PPy spider silk composite formulated by Zhang et.al (2015). A complete comparative study will allow for a comparison between different NGCs under the same experimental conditions, allowing for a valid comparison between different materials. On a related note, while many studies use rat models to test new methods treating peripheral nerve injuries, the size of the organism and distinct differences in its regenerative biology pathways may make it an inefficient model for peripheral nerve regeneration in humans (Kaplan, 2015; Kornfeld, 2021). Sheep have been shown to be good models for peripheral nerve surgery experiments due to their nerve's being histomorphologically similar to human nerves, their size translating to human subjects more effectively, and the low cost associated with maintaining them (Forden et al, 2011; Ozturk et al, 2014). Despite this, there is still a need to define a model organism for this line of work, especially for a study comparing the effectiveness of multiple NGCs.

Finally, the next step in this line of research is to test silk NGCs on human patients. The aforementioned discrepancy in rat model results to humans highlights the need for in vivo testing in humans. As of writing there have been no trials for silk-based NGCs in humans. While spider silk has proven harmless for mammals in the tests outlined thus far, testing to ensure the same effect holds true for humans is necessary to confirm our biological response to the material. Non-human primates could potentially serve as a stepping-stone towards human trials; however, in addition to the controversial nature of such experiments, there is evidence that primate trials do not translate well to human experiments, which further complicates the issue (Carvalho et.al, 2018b). In addition, several studies have performed immunofluorescence and other imaging techniques to measure regeneration by excising the nerve from the host organism, sometimes killing it beforehand (Farzamfar, 2019; Kornfeld et al, 2021; Millesi et.al, 2019; Radke et al, 2011). This cannot be done in humans; however, analysis of functional recovery in human patients would likely be sufficient.

Conclusion

The PNS contains a highly sophisticated yet fallible response to nerve damage. Various repair methods have been tested, with autologous nerve repair being the “gold standard” for long distance nerve repair (Carvalho et al, 2019; Radtke, 2016). Spider silk has proven useful for this purpose due to its natural bio absorbability, physical ability to withstand daily wear and tear, and the lack of an immune response generated to its presence in biological systems. Multiple studies have confirmed its capability to support peripheral nerve regeneration, suggesting that the silk alone possesses the properties necessary to drive and/or facilitate the regeneration process, with multiple results demonstrating successful adhesion of axons, Schwann cells, and other nerve cells to spider silk strands (Allmeling et al, 2006; Allmeling et al 2008; Hafner et.al, 2017; Kornfeld et.al, 2021; Millesi et.al, 2020; Radtke et al, 2011; Resch et.al, 2018; Zhang, 2015). Ultimately, while this work is very promising, there are still questions

that need to be answered regarding how nerve cells adhere to silk. The molecular mechanics that underly this interaction are still not well understood, despite strong efforts to move in that direction (Kornfeld et.al, 2021; Millesi et.al, 2020). It stands to reason that cell adhesion molecules/proteins on nerve cells interact favorably with specific regions of silk secondary structure that, however, there is no data currently available to show if this is the case or what molecules/proteins are actually involved. Hopefully, in the near future we will be able to generate conduits that can be successfully used to treat patients with significant PNS injuries and enable them to live normally with their full range of ability.

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