UNIVERSITY OF CALIFORNIA SAN DIEGO

3D-Printed Multichannel Scaffold with Brain Derived Neurotrophic Factor Drug Delivery as a Functional Therapy to Peripheral Nerve Injuries

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Emily Karapetian

Committee in charge:

Professor Yacov Koffler, Chair
Professor Stacey Glasgow, Co-Chair
Professor Cory Root

2024

The Thesis of Emily Karapetian is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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by

Emily Karapetian

Master of Science in Biology

University of California San Diego, 2024

Professor Yacov Koffler, Chair
Professor Stacey Glasgow, Co-Chair

Peripheral nerve injuries (PNIs) are injuries to the peripheral nervous system, typically caused by a traumatic event, and are very common in a combat and trauma setting. The nerve autograft has been regarded as the "golden standard" for PNIs, where a piece of nerve tissue, sourced from an autogenous sensory nerve, is transplanted to the site of injury. However, the

nerve autograft has many shortcomings, such as pain and loss of function at the donor nerve site, neuroma formation, and risk of infection following surgery. Our lab developed a novel multichannel scaffold approach (MCS), which uses linear microstructures to restrict axonal regeneration to one direction. The MCS approach was then further strengthened by the addition of brain-derived neurotrophic factor (BDNF), which have been shown to help increase the rate of axon regeneration, and thus potentially eliminate the need for a donor nerve. In this study, the MCS approach was tested *in vivo* by injuring the sciatic nerve of rats and then implanting the following therapies: open tube, nerve autograft, multichannel scaffold, and the multichannel scaffold with the addition of BDNF. 12 weeks post implant we found that rats implanted with MCS, MCS/BDNF, or nerve autograft had similar number of regenerating axons. This was significantly higher than the number of regenerating axons observed in open tube control subjects (no channels). Our findings also showed that the MCS approach, both with and without BDNF, was successful in orienting regenerating axons into the proper pathway from proximal to distal aspect of the injury compared to nerve graft and open tube implants where axons were seen deviating from this path. Our experiment was able to demonstrate that the MCS with and without BDNF therapy performs significantly better in terms of axonal regeneration and axon alignment than the open tube therapy in the sciatic nerve rat model. Thus, a multichannel scaffold can be a replacement for the standard of care, which is the nerve autograft, preventing the serious side effects of this procedure and providing an off-the-shelf alternative. The development of the MCS approach allows researchers to apply similar approaches to peripheral nerve injuries throughout the body, such as cranial nerve injuries where donor grafts are needed.

INTRODUCTION

1.1 Peripheral Nerve Structure

The peripheral nervous system consists of all the nerves and ganglia outside the brain and the spinal cord, which make up the central nervous system. The peripheral nervous system is then further divided into the somatic nervous system and the autonomic nervous system. The somatic nervous system regulates voluntary movement through the skeletal muscles, while the autonomic nervous system regulates involuntary body responses, such as heart rate and digestion (Stewart, 2003).

The smallest subunit of a peripheral nerve begins with an individual nerve fiber. The nerve fiber can be just an individual axon, or it can be myelinated by the myelin sheath. The endoneurium is a thin layer of delicate connective tissue that covers the axons. Each fascicle is enveloped by the perineurium, which serves as a protective barrier for the nerve fibers. The epineurium, the outermost layer of dense connective tissue, embeds the fascicles. The number and size of fascicles in a nerve vary from one nerve to the next, but also throughout the nerve as well (Stewart, 2003).

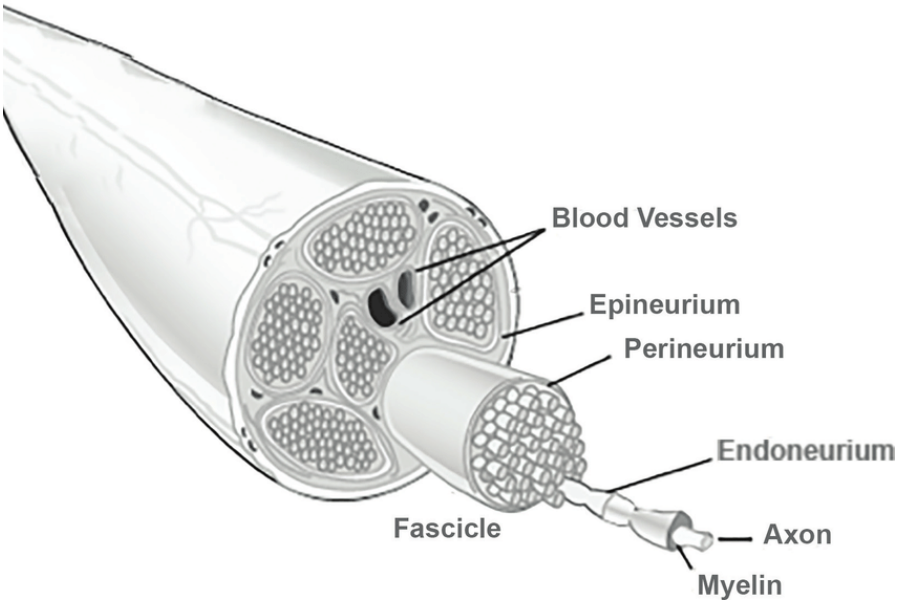

Figure 1: Diagram of peripheral nerve

1.2 Peripheral Nerve Injuries

Peripheral nerve injuries (PNI) are injuries that occur to the peripheral nervous system and are commonly caused by penetrative injuries, crushes, tractions, or ischemia (Toth, McNeil and Feasby, 2005). It is estimated that around 600,000 surgeries are performed annually in order to repair PNIs in various patients, making up about 3% of trauma cases and around 30% of all combat related injuries (Pawelec et al., 2018). The outcome of a PNI is determined by the level of cellular damage that is done at the site of lesion, in addition to the level of disruption to the surrounding connective tissue of the nerve, and personal factors, such as age and general health of the patient (Hall, 2005). Although there have been a few breakthroughs in therapies for PNIs throughout the past few decades, full functional recovery still remains slim, usually yielding unsatisfactory results (Zhang et al, 2022).

1.3 Wallerian Degeneration

Wallerian degeneration is the active process of anterograde degeneration of the distal end of an axon, that is the result of a nerve lesion or crush. In cases where there is a nerve transection or crush at the site of injury, two stumps are formed, the proximal stump, which is the stump that is closest to the neuron cell body, and the distal stump, which is the stump that is farther away from the neuron cell body. This series of changes is similar to the response seen at injuries occurring at various parts of the body, but there is a unique cascade of cellular responses at the extreme distal tip of the proximal stump and throughout the entire distal stump that has been coined "Wallerian Degeneration" (Hall, 1997). The uniqueness of the cascade stems from the fact that it reflects unique problems that arise with the repair of neurons. Wallerian degeneration occurs between 7 to 21 days following the injury and results in fragmentation and disintegration of the distal portion of the injured axon (Huebner and Strittmatter, 2009).

The first stage occurs soon after the nerve is injured, where the proximal and distal ends of the nerve are sealed until degeneration begins. Schwann cells toward the distal stump begin to dedifferentiate and assist in degradation of myelin debris. The second stage occurs when Schwann cells recruit macrophages, which clean up the axonal and myelin debris on the distal end, as well as promote growth factor production (Gaudet et al., 2011). The last stage, regeneration, occurs with regeneration of proximal axons and ends with reinnervation into their target, allowing for a functional recovery (Huebner and Strittmatter, 2009).

It was seen that, when no gap is present within the injury site, injured axons were readily able to regenerate across the lesion site, but once a gap was present, the regeneration of the axons was slowed down and the probability of regenerating axons successfully diminished. Various studies have been performed to understand the spatiotemporal sequence of cellular processes that

have to occur across the gaped injury site. It was found that when the proximal and distal stumps were inserted into opposite ends of a cylindrical or Y-shaped tube, deemed a “regeneration chamber”, neurotrophic molecules and matrix precursors filled the lesion site, which helped aid in the axon regeneration. When the size of the gap of the injury site increased, the chamber filled with less and less of these molecules and regenerating axons were not able to bridge the gap (Hall, 1997).

1.4 Misdirection of Regenerating Axons

One of the main reasons preventing patients from making a full functional recovery following a PNI, has to do with misdirection of regenerated axons (Sulaiman, 2013). In order for axons to be functional, they must reinnervate their original, correct target. During regeneration, axons elongate through the nerve, but they are not able to distinguish the correct fascicle that will lead them to their correct native target in order to make a fully functional axonal connection (Sabatier et al, 2011). Due to this phenomenon, muscles that are downstream of the peripheral nerve injury are commonly reinnervated by neurons that are not native and had previously innervated another muscle. This also leads to some targets having a loss of innervation, or denervation, which results in muscle inactivity and general morphological and physiological deterioration of those affected fibers (Borisov et al. 2001).

Injured peripheral nerves trigger central sensitization, which is hyperexcitable changes in the central nervous system (CNS). Central sensitization is one of the leading causes in the development of neuropathic pain (Leibovich et al., 2020).

1.5 Current Therapies for PNIs

Currently, the main therapy to treat PNIs is the nerve autograft, where a piece of nerve tissue, sourced from an autogenous sensory nerve, is transplanted to the site of injury. In order

for the surgery to be successful, the proximal and distal stumps must be completely free of tension when the nerve is grafted, and the sensory and motor components of the individual fascicles must also be matched as accurately as possible (Matsuyama, 2000). Because of these requirements, nerve autograft surgery can take well over several hours and requires a surgeon with sophisticated microsurgery skills. With a lengthy surgery, comes the increased possibility of infection and need for general anesthesia. Though nerve autografts can result in a functional recovery, other unwanted issues such as sensibility decrease, paresthesia, and neuropathic pain are common challenges patients face following surgery (Chou et al, 2022).

As nerve autografts require an autologous donor nerve, harvesting the donor nerve creates a new injury site, creating more opportunity for new challenges patients can face (Safa and Buncke, 2016). The sural nerve, a sensory nerve located in the lower leg, is one of the most frequently transplanted nerves for peripheral nerve injuries (Ducic, Yoon and Buncke, 2020). It was found that, of 214 patients surveyed following a sural nerve graft, 93% of patients reported sensory deficits at the intervention site, and complete sensory recovery was rare (Ducic, Yoon and Buncke, 2020). 23% of patients also experienced chronic pain, mainly in the form of neuroma formation at the site of harvest, which is a disorganized growth of nerve cells that can lead to chronic pain and decreased quality of life for patients.

Another common therapy to PNIs, is the use of an open hollow tube, which aims to help isolate redeveloping axons and protect the regenerating nerve from outside forces. The open hollow tube therapy allows for the regenerating axons to grow in multiple directions since it doesn't offer any spatial or physical guidance cues, limiting their use to injury sites with gaps < 1 cm. This limitation on gap size severely restricts the use of an open hollow tube as a functional therapy for PNIs. The direction the newly generated axons grow is important because if they do

not grow in the proper direction, then new axons will not be able to form a connection with the appropriate target such as denervated muscles.

1.6 Drug Delivery Systems

Drug delivery systems (DDS) are various systems that allow for therapeutic substances to be administered into the body in a safe and efficient way, through controlling the release rate and the time and initial place of drug release. DDS vary in how the therapeutic drug is administered (systemically or locally) and how its active ingredients are released and transported through the body. (Jain, 2020). The rate and overall amount at which the therapeutic is released is crucial, as administering too much can be wasteful, unnecessary, and could have toxic or aversive side effects (Paolino et al, 2006).

1.7 Nanoparticles

Nanoparticles and other various nanoscale drug delivery vehicles have gained popularity in the therapeutic industry because of their potential to increase the bioavailability and stability of protein therapeutics. Of those various nanoparticles, porous silicon nanoparticles (pSiNPs) stand out because of their ability to accommodate a vast array of therapeutics and their tunable pore size, allowing them to transport a variety of molecule sizes (Waggoner et al, 2023).

1.8 Neurotrophic Factors

Neurotrophic factors are a family of small proteins that have been found to be modulators of development and function for neurons in the peripheral and central nervous system. It has been observed that, following a nerve autograft coupled with an injection of a neurotrophic factor in the sciatic nerve of a rat model, BDNF (brain-derived neurotrophic factors), GDNF (glial cell line-derived neurotrophic factors), and NGF (nerve growth factor) enhance axonal regeneration in the graft, and grafts treated with BDNF and GDNF even had two to four fold

more motor axons than the controls. However, it was also seen that when BDNF, GDNF and NGF were overexpressed in these grafts persistently, it led to excessive axonal growth, with the axons not growing beyond the graft, and staying where the neurotrophic factors were located (Hoyng, Stefan A et al, 2014). Because of this phenomenon, the therapeutic quantity, 50 ng/mL, is commonly used in BDNF studies (Yuan et al, 2017).

1.9 3D-Printed Guided Scaffolds

Tissue engineering strategies that combine biomaterials and living cells have gained popularity in recent years in the field of regenerative medicine. One of these strategies is 3D-printed scaffolds, with or without the addition of living cells to aid in tissue regeneration. 3D-P scaffolds allows for the scaffold to mimic the 3D structure of the native target tissue. Cell delivery can be achieved with one of the two following ways in 3D-printed scaffolds: seeding cells after the printing process or embedding the cells within the bioink prior to printing the scaffold (Xue, Jianmin et al, 2023).

1.10 Multichannel Scaffold Approach

Due to the restrictions and shortcomings of the current therapies on the market, the Koffler lab designed a 3D-printed multichannel nerve guidance scaffold containing brain derived neurotrophic growth factors encapsulated in pSiNPs as a therapeutic alternative that resolves the issue of axons growing in multiple directions after repair and difficulty to collect donor nerve (Koffler et. Al, Nat. Med. 2019). The multichannel scaffold approach (MCS) offers large and open linear channels that extend over the entire lesion site in a honeycomb-like structure. Previous studies have shown that the MCS approach was not only successful in guiding axons, but also aided in guiding other important parts of the tissue, such as vascular and glial cells (Pawelec et al., 2018).

In addition to the linearly restrictive scaffold model to limit axon growth in one direction, we also loaded brain derived neurotrophic factor encapsulated in pSiNPs into the bioink to be 3D-printed into the scaffold walls and enhance axonal regeneration. It has been shown that neurotrophic factors, especially brain derived neurotrophic factor (BDNF) aid in regulation, signaling, and play an important role in normal development (McGregor and English, 2019). In peripheral nerves, BDNF is created by a subset of peripheral dorsal root ganglia neurons and Schwann cells, and when there is complete transection of a nerve or nerve crush, BDNF mRNA increases amongst these cells. This phenomenon can also be seen in the sciatic nerve, following injury. During the 3D-printing process of the multichannel scaffold, the bioink that is used to print the material was mixed with pSiNPs that encapsulated BDNF, so that silicon nanoparticles can effectively deliver the therapeutic over a sustained period of time (Kim et al, 2016). In our study, we tested the multichannel scaffold, multichannel scaffold releasing BDNF, nerve autograft and open tube therapy by transecting the sciatic nerve in a rat model and implanting the respective therapies into 9 rats per group. The animals survived 12 weeks before sacrifice. We hypothesize that the multichannel approach we have designed will be able to eliminate the possibility of axons growing in the wrong direction, as well as increase the rate of axonal regeneration with the multichannel releasing BDNF therapy.

CHAPTER 1 METHODS AND MATERIALS

3D printed scaffolds preparation: Scaffolds with linear microchannels were 3D-printed as reported previosuly[1]. Briefly, we use a bioink composed of 25% (w/v) Polyethylene Glycol Di Acrylate (Sigma-Alderich), 7% (w/v) Gelatin Methacrylate (Advanced Biomatrix), and 0.225% (w/v) LAP in Dulbecco's phosphate-buffered saline solution. We load the prepared design (a microchannel scaffold or open tube without channels (which is the control)) into the bioprinter interface and a continuous printing process is initiated. Following the printing process, the printed scaffold is removed from the printer and rinsed three times with sterile Dulbecco's phosphate-buffered saline and antibiotics (1% Pen Strep).

In-Vivo Studies

Adult female F344 Fischer rats were used in this study. All animal studies were carried out according to NIH guidelines for laboratory animal care and safety, adhering to protocols approved by the Institutional Animal Care and Use Committee of the Veterans Administration Health System, San Diego. Rats were allowed unrestricted movement after recovery and were housed with access to food and water ad libitum in VMU rooms maintained at constant temperature (19 °C- 22 °C) and humidity (40%-50%) on a 12:12h light/dark cycle.

A 20 mm long skin incision was made on the left lateral thigh and the left sciatic nerve trunk was exposed via a lateral gluteal muscle dissection. Epineural connective tissue surrounding the nerve trunk was separated with micro scissors and the nerve trunk was exposed from the hip to the sciatic trifurcation. A total of 15mm long nerve was exposed. The sciatic nerve and using micro scissors removed 10-mm section from the center region of the sciatic nerve trunk. The following groups were implanted: 1. Nerve autograft (n = 9). 2. Multichannel scaffold (n = 9). 3.

Multichannel scaffold releasing BDNF (n = 9). 4. Open tube (n = 9). The muscle was sutured with 9-0 polyglactin (Vicryl Ethicon Suture), and the skin was stapled.

Following surgery, rats received banamine (1 mg/kg) and ampicillin (0.2 mg/kg) in Ringer's lactate for three days. After recovery, the animals survived 12 weeks.

Tissue Processing

After 12 weeks, animals were perfused using transcardial perfusion. The sciatic nerve on the implantation side was harvested and placed into 30% sucrose for 48 hours. The tissues were then processed for histology. Implant, distal sciatic nerve, and gastrocnemius muscle sections were incubated overnight at 4ºC with the primary antibody mouse monoclonal NF200, (1:500, Millipore) to label axons, followed by incubation with Alexa 488 conjugated donkey secondary antibodies (1:250, Invitrogen) overnight at 4ºC, and then cover slipped. 2 sections per animal were quantified. Quantification was done using ImageJ.

Statistics

Multiple group comparisons were tested by one-tail ANOVA (JMP software) at a designated significance-level of $P < 0.05$, followed by post hoc analysis using Tukey's test.

CHAPTER 2 RESULTS AND DISCUSSION

Results

BDNF release

We 3D-printed nanoparticles into the scaffold, then performed drug delivery studies (Figure 2). In a 20-day release study, we found that most of the BDNF was released after 5 days, with a plateau around 9 days. However, over 14 days, we still have therapeutic quantities being released, over 50 ng/mL (Yuan, 2017).

In order to test the efficiency/effect of 3D printed silicon nanoparticles encapsulating BDNF, we performed a study *in vivo* in the sciatic nerve of a rat model. We performed an implant with the following experimental design: nerve autograft group, open tube group, multichannel group, and multichannel releasing BDNF group. There were 9 animals per each group. The experiment ran for 12 weeks.

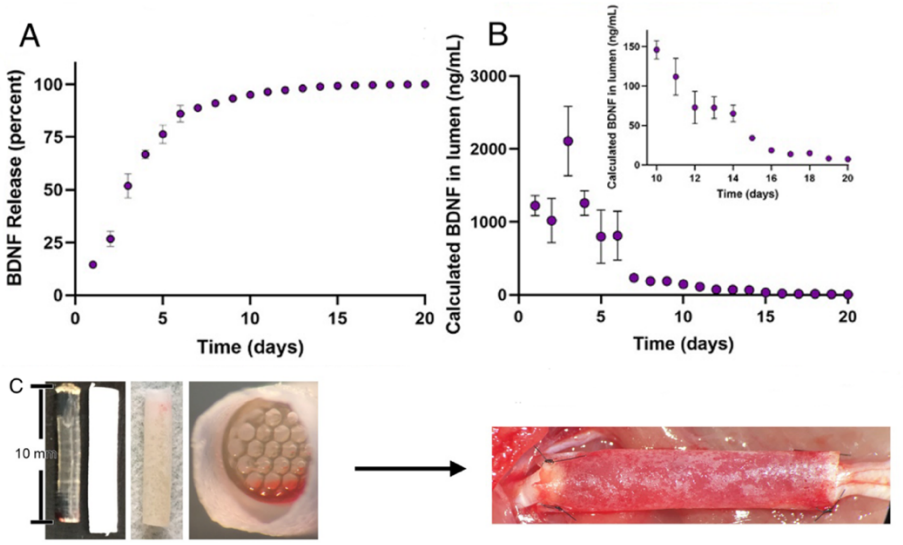

Figure 2: 20-day BDNF drug release study

A. Cumulative release profile of BDNF from the scaffold, presented as a percentage of total BDNF released over a period of 20 days. Data points represent mean values with error bars indicating the standard deviation. **B**. Calculated concentration of BDNF in the lumen of the scaffold over time, measured in ng/mL. The inset shows a magnified view of BDNF concentration changes between days 10 and 20. Error bars represent standard deviation. **C.** Images of the BDNF-releasing scaffold implant. The left image shows the scaffold's physical structure (scale bar: 10 mm), the middle image provides a closer view of the scaffold's inner architecture, and the right image depicts the scaffold implanted in a tissue environment, demonstrating its application *in vivo*.

Axon Counts (per location from proximal to distal)

Scaffold

After 12 weeks, we found that all groups had axons in the scaffold and distal aspect of the implant. Figure 3 quantification shows that there were significantly more axons in the autograft, multichannel and multichannel releasing BDNF scaffolds compared to the open tube group. We found that the autograft group had 3703 ± 186.3 (mean ± SEM) axons, the open tube group had 2211 ± 294.2 axons, the multichannel group had 3603 ± 305.1 axons, and the multichannel releasing BDNF group had 4252 ± 396.7 axons (Table 1). We found significantly more axons in the autograft group versus the open tube group (One-Way ANOVA, $p = 0.0080$), significantly more axons in the multichannel group versus the open tube group (One-Way ANOVA, $p = 0.0145$) and significantly more axons in the multichannel releasing BDNF group versus the open tube group (One-Way ANOVA, $p = 0.0002$).

Distal Nerve

Looking at the distal nerve, we found that the autograft group had 4492 ± 787.5 (mean ± SEM) axons, the open tube group had 2297 ± 419.9 axons, the multichannel group had 2499 ± 350.9 axons, and the multichannel releasing BDNF group had 4214 ± 303.3 axons (Table 1). We found significantly more axons in the autograft group versus the open tube group (One-Way ANOVA, $p = 0.0206$) and significantly more axons in the autograft group versus the multichannel group (One-Way ANOVA, $p = 0.0404$) (Figure 4). We also found significantly more axons in the multichannel releasing BDNF group versus the open tube group (One-Way ANOVA, $p = 0.0517$). There was not a significant difference between the multichannel group versus the multichannel releasing BDNF; however, it was trending that there were more axons in the multichannel releasing BDNF group (One-Way ANOVA, $p = 0.0953$). In addition,

multichannel releasing BDNF group axon counts were like autograft axon counts and there is no statistically significant difference between them (One-Way ANOVA, $p = 0.9794$).

Table 1: Average axon count following 12 weeks of the respective therapies in the Scaffold and Distal Nerve

	Scaffold		***Distal Nerve***	
	Mean	*SEM*	*Mean*	*SEM*
Autograft	3,703	186.3	4,492	787.5
Open Tube	2,211	294.2	2,297	419.9
Multichannel	3,603	305.1	2,499	350.9
BDNF	4,252	396.7	4,214	303.3

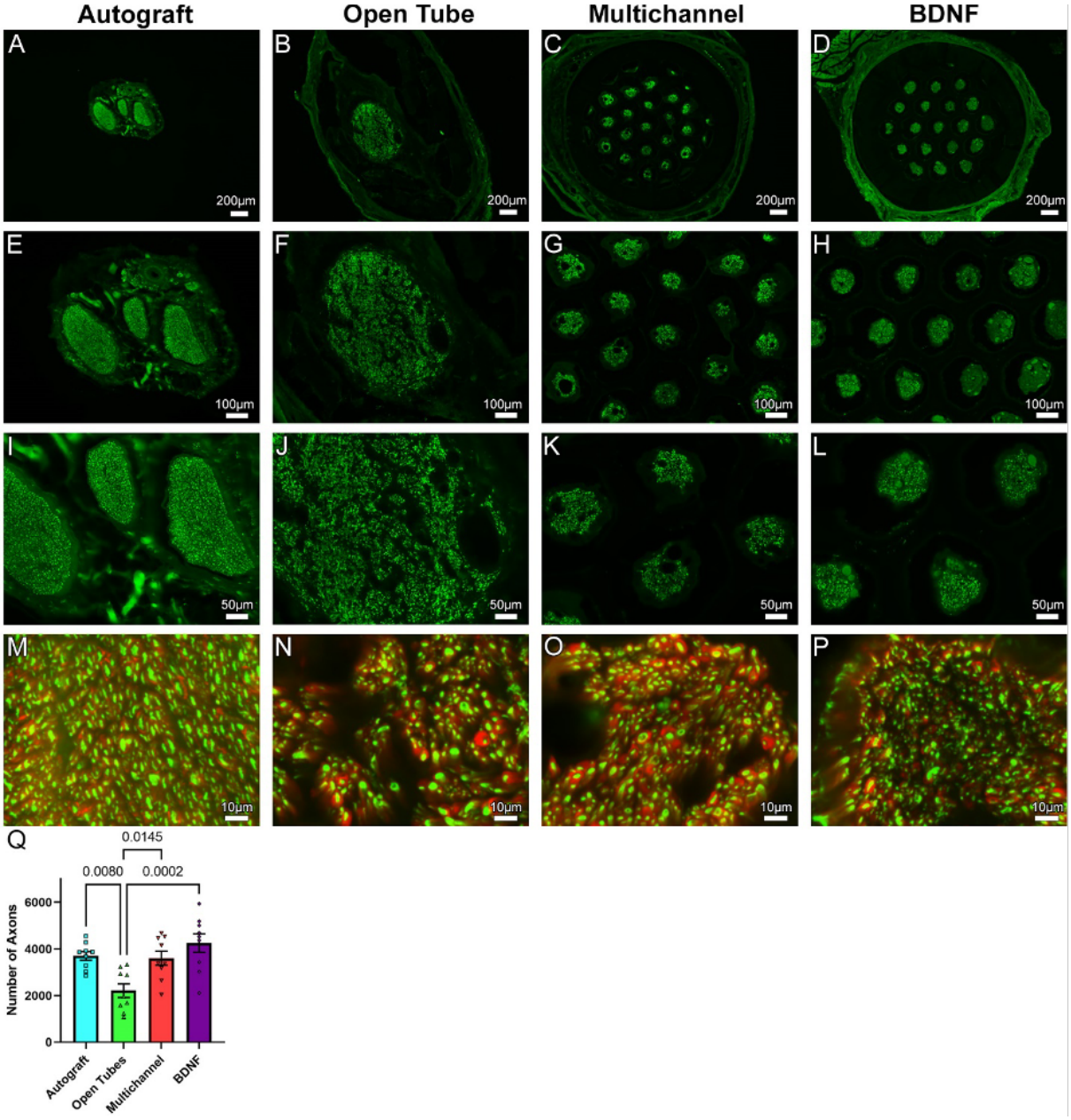

Figure 3: Axon Regeneration into scaffolds at 12 weeks
A,E,I,M – Autograft, B,F,J,N – Open Tube, C,G,K,O – Multichannel, D,H,L,P – BDNF. Immunolabeling with NF200 (green) to visualize axons and S100 (red) to visualize Schwann cells and then observed axons were counted and recorded. Q. Average axon counts observed following 12 weeks of Autograft (n = 9), Open Tube (n = 9), Multichannel (n = 9), Multichannel releasing BDNF therapy in the rat model (n = 9) into the scaffold.

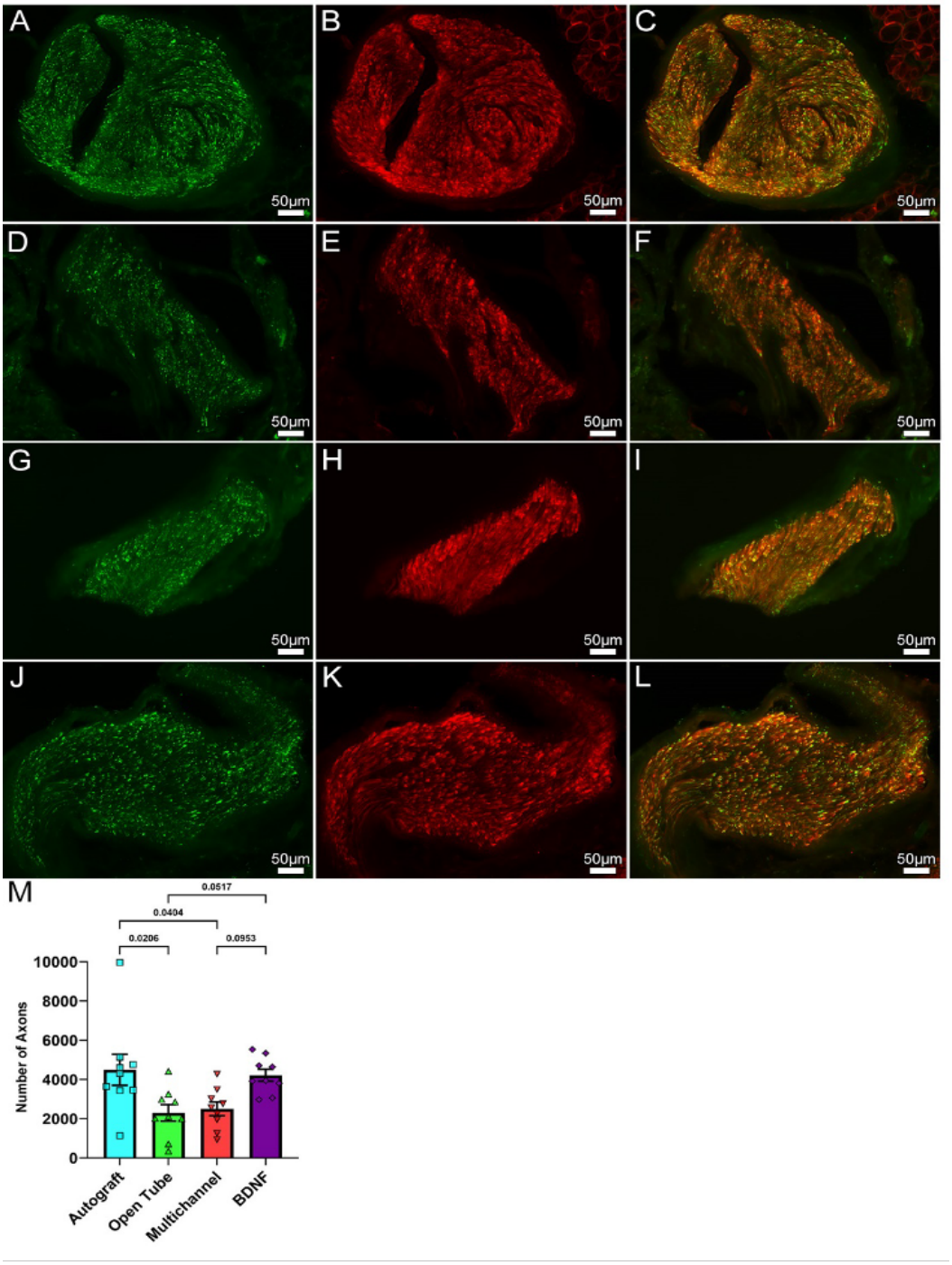

Figure 4: Axon Regeneration into distal nerve at 12 weeks
A-C – Autograft, D-F – Open Tube, G-I – Multichannel, J-L – BDNF. Immunolabeling with NF200 (green) to visualize axons and S100 (red) to visualize Schwann cells and then observed axons were counted and recorded. M. Average axon counts observed following 12 weeks of Autograft (n = 9), Open Tube (n = 9), Multichannel (n = 9), Multichannel releasing BDNF therapy (n = 9) in the rat model at the distal nerve.

Axon Alignment

Next, we characterized axon alignment with the proximal-to-distal regeneration axis (Figure 4) within the implant and found that the angle of deviation from the median for the autograft group was 6.985 ± 1.067 (mean ± SEM), the multichannel group was 5.878 ± 0.592, the multichannel releasing BDNF group was 6.477 ± 0.605, and the open tube group was 16.130 ± 1.196 (Table 2). Axons in the autograft, multichannel and multichannel + BDNF implants had a significantly lower angle of deviation from the median in comparison to the open tube group (One-Way ANOVA, $p < 0.0001$).

Table 2: Average angle of deviation from median of axons into the Distal Nerve

	Mean	*SEM*
Autograft	6.985	1.067
Open Tube	16.13	1.196
Multichannel	5.878	0.5918
BDNF	6.477	0.6054

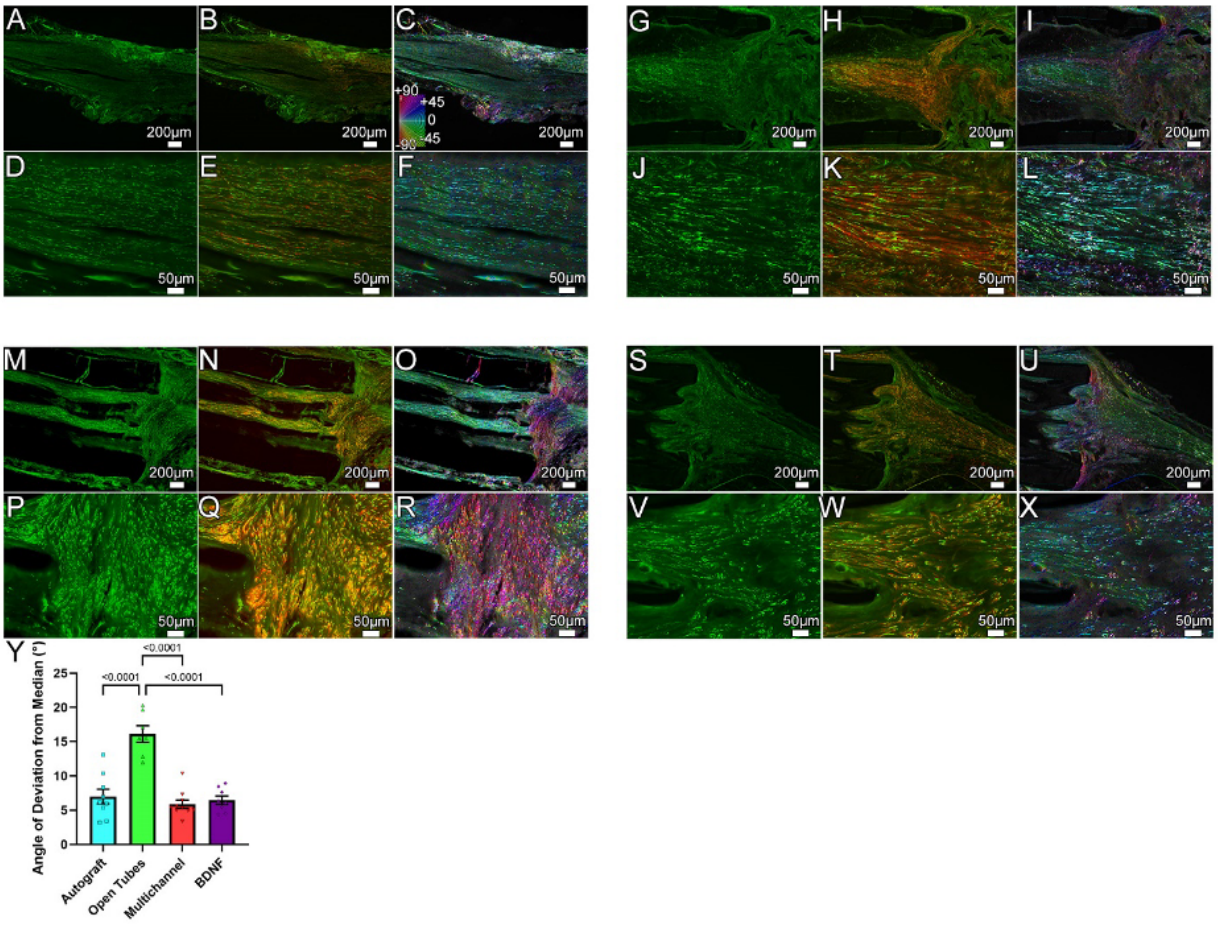

Figure 5: Axon alignment in the channels and interface with distal nerve
A-F – Autograft, G-L – Open Tube, M-R – Multichannel, S-X – BDNF. In each set, the left and middle images display immunolabeling with NF200 (green) to visualize axons and S100 (red) to visualize Schwann cells. The right image in each set shows the angle of deviation from the median of the axons. Y. Quantification of the average angle of deviation from median observed and recorded following 12 weeks of Autograft (n = 9), Open Tube (n = 9), Multichannel (n = 9), Multichannel releasing BDNF therapy (n = 9) in the rat model, at the distal nerve.

Discussion

Nerve guidance scaffolds for peripheral nerve injuries (PNIs) have been studied for many years as a replacement/alternative to the current "gold standard", the nerve autograft. Autografts require an autogenous donor nerve to be removed from a less needed site and transplanted to the site of injury. Problems arise because of the removal of the donor nerve, such as loss of function, chronic pain, as well as requiring a lengthy and intensive surgery. Recently, the use of multichannel guidance scaffolds has been introduced and researched for PNIs because of their

ability to remove the need for a donor nerve and allows for alterations, like the addition of neurotrophic factors and other things, to help aid in axonal regeneration.

In this study, we were interested in the addition of brain-derived neurotrophic factors (BDNF) to increase axonal regeneration at the injury site, resulting in a therapy better than the autograft. Our findings suggest that, after 12 weeks of therapy implantation, the axon regeneration into the scaffold is similar amongst the autograft, multichannel and multichannel releasing BDNF therapies. The average axon counts in the sampled areas were similar amongst all three of the therapies, suggesting that the multichannel and multichannel releasing BDNF approach performs at the same level as that of the "golden standard". However, the results of this study show that the addition of BDNF release into the scaffold did not support more axons compared to a multichannel scaffold without BDNF release in the scaffold. This was also seen in the distal nerve, but it was trending that the multichannel with BDNF release had more axons into the distal nerve. Though there was not a significant difference between the multichannel and multichannel with BDNF release, both therapies performed significantly better than the open tube therapy and comparable to the autograft.

Ensuring that the axons have proper alignment and are reaching their appropriate target is vital in returning full functionality. As axons regenerate at the proximal stump, it is very important that they stay on the correct pathway within the same fascicle as they were previously, to make sure they reinnervate the same fascicle at the distal stump. Axons may be able to reach the distal stump, but if the target nerve is not the appropriate one, it may result in reduced or even eliminated function at the injury site (Ducic, Yoon and Buncke, 2020). We found that axons in the open tube had a significantly higher angle of deviation from the median, than the autograft, multichannel and multichannel releasing BDNF scaffolds. The spatial organization that is

provided by the channels in the scaffold significantly reduced the deviation of regenerating axons from their appropriate pathway.

We couldn't find an observed effect for BDNF addition. Our hypotheses as to why there was no observed effect are: **A.** Not enough BDNF was loaded. **B.** Not enough particles were loaded. **C.** Particle distribution may have been skewed. Since we are loading nanoparticles containing BDNF into the scaffold, there are two areas where proper BDNF concentration may not have been reached: when loading BDNF into the nanoparticles and when loading nanoparticles into the scaffold. We have data that shows that with our nanoparticles, there is BDNF being consistently released from the nanoparticles at a concentration of 50 ng/mL after 14 days. Though 50 ng/mL of BDNF is known as the therapeutic concentration, it may not be a high enough concentration to enhance axonal regeneration (Lackington et al, 2017). The proper amount of nanoparticles containing BDNF may not have been loaded onto the scaffold during the 3D-printing process. Without enough nanoparticles loaded onto the scaffold, an effective amount of BDNF may not be reached after implantation. Particle distribution throughout the scaffold may have also been affected during the 3D printing process. During the printing process, the particles may have not been evenly distributed within the scaffold or may have sunk, possibly leading to one side, such as the proximal side, of the scaffold to have a high concentration of particles while the distal side lacked particles. This could lead to axons staying in the area with the higher BDNF concentration and not being pulled to the distal side to their axonal target (Hoyng, Stefan A et al, 2014). Particle distribution after printing was not observed, so that is something to keep in mind in future experiments.

Though there wasn't enhanced axonal regeneration observed with our multichannel releasing BDNF therapy, both the multichannel groups performed significantly better than the

open tube in axonal regeneration and alignment, as well as performed at similar levels to the autograft in axonal regeneration and alignment. The added advantage of 3D-printing eliminates the main drawbacks that are seen with the autograft therapy. These drawbacks, such as donor site loss of function and chronic pain, are completely removed in the multichannel approach, and don't require donor nerves (Pawelec et al, 2018). Although BDNF and silica-based nanoparticles were used for the drug delivery system, potentially looking into other nanoparticles and neurotrophic factors could lead to an increase in the rate of axon regeneration (Kim et al, 2016). The addition of periodic electrical stimulation can potentially help increase the rate of axonal regeneration when used with a nerve guidance conduit approach, such as the multichannel scaffold (Anderson et al, 2015). Future research could focus on loading higher concentrations of neurotrophic factors, as well as using a different nanoparticle for drug delivery.

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