INCORPORATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND PLEIOTROPHIN INTO A MULTILUMINAL BIOSYNTHETIC NERVEIMPLANT FOR THE REPAIR OF PERIPHERAL NERVE LONG GAP DEFECTS

by

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ABSTRACT

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To repair a fully transected nerve without putting undue tension on the nerve stumps, and too circumvent many of the obvious problems of using harvested nerve grafts synthetic nerve guides have been used in the clinical setting. Thus far this strategy has met with minimal success for the repair of short defects (i.e. 1 – 2.5 cm) while any defect larger than 3 cm have failed. We hypothesize that neurotorphic support, early vascularization, and contact guidance are needed to successfully regenerate a nerve across a long-gap defect. To this end we proposed a biosynthetic nerve implant (BNI) with a multiluminal design that could be incorporated with different neurotrophic factors. Pleiotrophin (PTN) and Vascular Endothelial Growth Factor (VEGF) have been demonstrated to exhibit growth promoting effects on neurons in the central nervous and peripheral nervous systems as well as other cell types within the nerve bundle, e.g. Schwann cells, fibroblasts, and endothelial cells.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

LIST OF TABLES

Table Page

CHAPTER 1

INTRODUCTION

1.1 Peripheral nerve injuries

Approximately 2.8 % of all trauma patients suffers from an injury to their peripheral nervous system (PNS) (Noble, Munro et al. 1998). The PNS are nerves that is utilized by the central nervous system (i.e. the brain and spinal cord) to communicate and control the peripheral organs (Deumens, Bozkurt et al. 2010). Injuries resulting in neurapraxia and axonotmesis are generally the most easy to repair (Sunderland 1951) injuries that results in neurotmesis (i.e. complete nerve transaction) are more challenging (Deumens, Bozkurt et al. 2010). Neurapraxia is a temporary loss of axonal conduction but not axonal continuity. Axonotmesis occurs when there is a loss of axonal continuity whiles the surrounding connective tissues, endoneurium, perineurium, and epineurium remains intact. Neurotmesis occurs when the entire nerve fiber is severed. Neurotmesis then be separated into three levels of severity depending on the integrity of the different layers of connective tissues; with the most sever being the complete severance of the axon, endoneurium, perineurium, and epineurium. Refer to Figure 1 for a schematic summary of the different classification of nerve damage.

The peripheral nerve can be injured in primarily two ways, inherited or acquired. Acquired peripheral nerve injuries can be the results of forces outside the body such as physical injury to the nerve or from forces inside the body such as tumors, autoimmune responses or some form of vascular or metabolic disorders. Inherited peripheral nerve injuries include any genetic conditions that are passed down from a previous generation such as tomoculus or neurofibrosacroma.

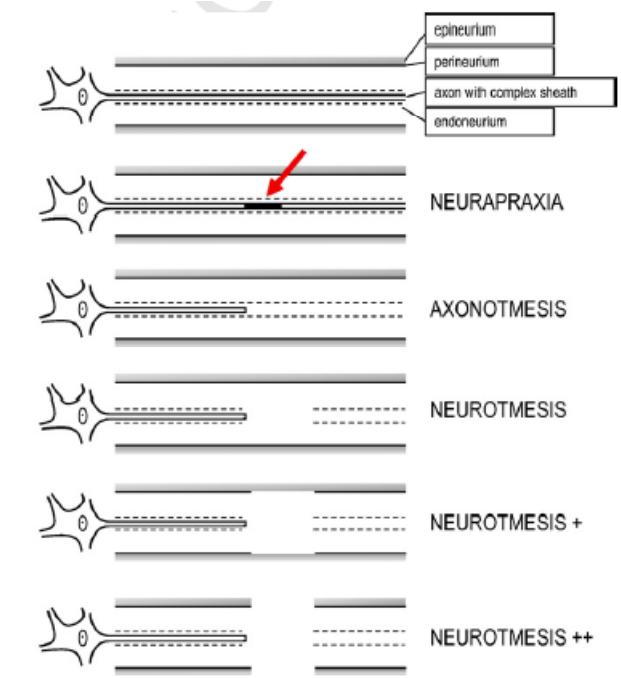

Figure 1. Seddon's classification of nerve damage (Jiang, Lim et al. 2010).

1.2 Clinical alternatives for nerve repair

Primary tensionless end-to-end attachment of a severed peripheral nerve resulting from neurotmesis is the preferred method of nerve repair as it is optimal for nerve regeneration and functional recovery, Figure 2 (Lee and Wolfe 2000). However, if the gap is too long for a tensionless reattachment the 'gold standard' would be to use an autologous nerve graft to bridge this defect (Pabari, Yang et al. 2010). Sensory nerves: such as the sural nerves (the most typically used), the superficial and deep peroneal nerves, posterior and lateral cutaneous nerves of the thigh, intercostals nerves, medial and lateral cutaneous nerves of the forearm, and dorsal cutaneous branch of the ulnar nerve (Pabari, Yang et al. 2010). This bridging method provides the regenerating axons with the required support to improve nerve regeneration.

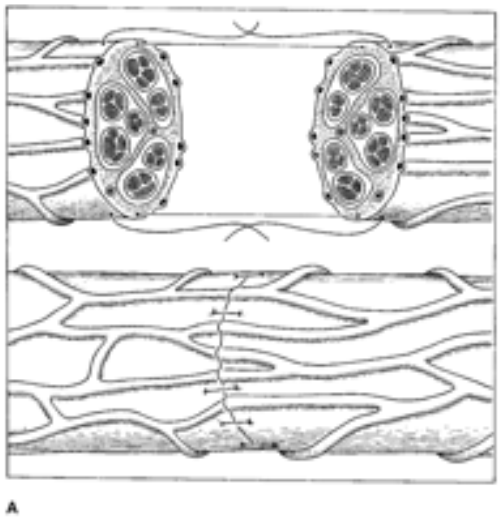

Figure 2. 'Gold standard' for the repair of a severed nerve. This could be two ends of a nerve being reconnected or a graft taken from a separate site being grafted in (Lee and Wolfe 2000).

While autologous nerve grafting provides an excellent biological bridge of mediating nerve regeneration, this method has some critical limitations. Donor site morbidity, the limited number of usable donor nerves, (Deumens, Bozkurt et al. 2010) and only 40 – 50 % of autograft patients shows functional recovery (Lee and Wolfe 2000). Furthermore, it has been reported that have there is a greater potential for motor nerve grafts to regenerate motor neurons than sensory nerve grafts (Brenner, Hess et al. 2006).

To alleviate the limitation of donor site morbidity and limited number of usable donor nerves, the use of allogenic (i.e same species) tissues (Pabari, Yang et al. 2010) or xenogenic (i.e. different species) tissues (Deumens, Bozkurt et al. 2010) have been investigated. However, the nature of these tissue requires an immunosuppresion regiment that while prevent graft rejection, impair nerve regeneration and functional recovery (Mackinnon, Doolabh et al. 2001). The possible transmission of disease is another concern (Deumens, Bozkurt et al. 2010). Recently, decellularized nerve allo/xeno-grafts have also been investigated, with good success (Deumens, Bozkurt et al. 2010; Pabari, Yang et al. 2010). However, when used to repairs long gaps these acelluar nerve grafts produced recovery that was inferior to an autograft (Ide, Tohyama et al. 1998). It have been reported that the poor outcome was due to the fact

that regenerating axons through longer defects requires signaling cues to direct and promote their growth and survival (Deumens, Bozkurt et al. 2010).

1.3 Synthetic nerve implants

An alternative to the use of harvested nerves is to use synthetic, manufactured conduits to guide and protect the regenerating nerve. Synthetic materials offer particular advantages in alleviating the need for secondary operation, donor site morbidity, and loss of sensation from the harvested site (Staniforth and Fisher 1978; Rappaport, Valente et al. 1993; Lundborg 2000). Silicone, an early synthetic material, was one of the first used and proved to be successful in the repair of short gaps in humans (Silva et al.), but was later abandoned because of its resistances to degradation and expansion (Pabari, Yang et al. 2010). Which caused long-term complications such as nerve compression or fibrosis (Pabari, Yang et al. 2010). Today, the US Food and Drug Administration (FDA) and the European Union Conformité Européenne (CE) have several approved conduits (SaluBridge, Neurotube, NeuroMatrix or NeuroFlex, Neurolac, NeuraGen) that are simple hollow tubes made up of non-functionalized polymer components (Meek and Coert 2008; Deumens, Bozkurt et al. 2010). Figure 3 are photographs of NeuraGen, Neurolac, and Neurotube, 3 of the 6 FDA approved nerve guides. The main limitation to the use of synthetic nerve conduits as nerve grafts is that the conduits are simple empty tubes. They do not present a normal nerve bundle and therefore does not mimic the microenvironment needed for the regeneration of a severed axon. In order to improve the efficacy of synthetic nerve conduits and enhance nerve regeneration several groups have proposed the use of luminal fillers.

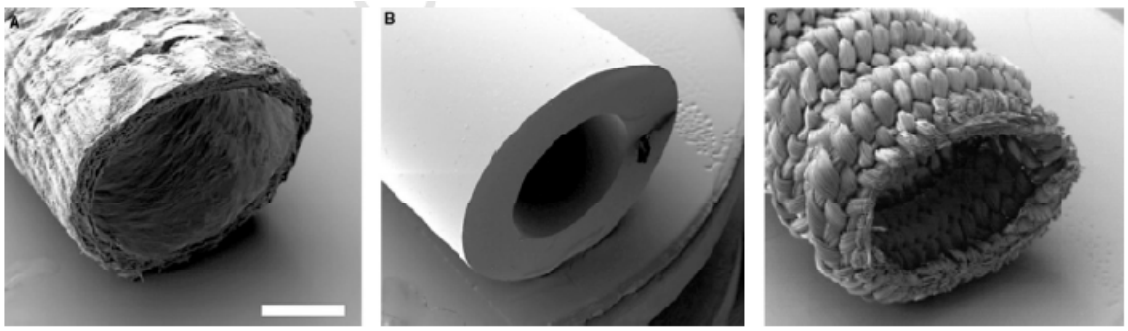

Figure 3. Scanning electron microscope photographs of three types of FDA approved nerve guides. A. NeuraGen from Integra Neuroscience (collagen), B. Neurolac from Polyganics BV (polylactide/caprolactone, C. Neurotube from Synovis Micro Companies Alliance (poly

1.4 Luminal fillers

All nerve fibers have different layers of connective tissue that protects and provide a framework, these are known as the epineurium, perineurium, and endoneurium Figure 4 (Pabari, Yang et al. 2010). Blood is supplied to the nerves from segmental extrinsic and longitudinal intrinsic blood vessels, but primarily from the intrinsic vessels and excessive tension can significantly compromise the flow of blood to the nerve (Bora, Richardson et al. 1980). For large gaps in a transected nerve a bridge that can mimic the different layers of the nerve is needed to connect the two sections.

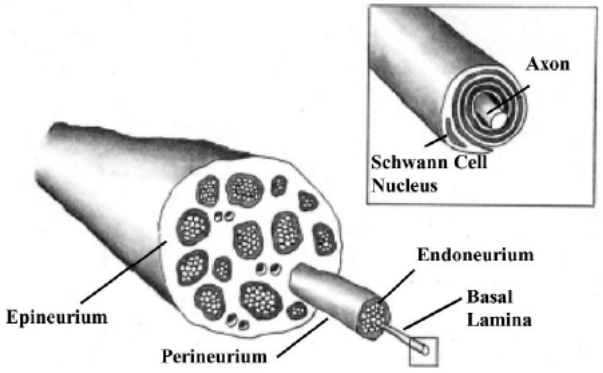

Figure 4. Schematic overview of the anatomy of a peripheral nerve(Schmidt and Leach 2003).

An improvement on nerve conduits was the inclusion of contact guidance, the main goal was to enhance Schwann cell alignment and encourage the formation of the Bands of Büngner following nerve injury (Jiang, Lim et al. 2010). Different fillers such as polyamide filaments, collagen filaments, polyester filaments, Matrigel supplemented with PLA wet-spun fibers, PGA filaments and gelatin fibers have all been investigated (Lundborg, Dahlin et al. 1997; Itoh, Takakuda et al. 2001; Gamez, Goto et al. 2004; Cai, Peng et al. 2005; Hu, Gu et al. 2008). This approach have shown promising results, however there were still several factors that is still under investigation: 1) there appears to be an optimal packing density of filaments within a lumen, 2) the size of each filaments is a factor, 3) it is possible that the only advantage to having fillers is that there is an increase in surface area for cell adhesion and growth and that contact guidance does not play role in nerve regeneration (Jiang, Lim et al. 2010).

A second approach was to include cellular components or transplanted cells tailored to secrete neurotrophic factors to support and guide nerve regeneration (Tohill and Terenghi 2004). Schwann cells (Cheng and Chen 2002; Mosahebi, Wiberg et al. 2003; Hsu, Chang et al. 2004; Rutkowski, Miller et al. 2004; Udina, Rodriguez et al. 2004; Phillips, Bunting et al. 2005), bone marrow stromal cell (Cuevas, Carceller et al. 2002; Cuevas, Carceller et al. 2004; Mimura, Dezawa et al. 2004; Zhang, He et al. 2005; Keilhoff, Goihl et al. 2006; Keilhoff, Stang et al. 2006; Chen, Ou et al. 2007; Hu, Zhu et al. 2007), olfactory ensheathing cells (Verdu, Navarro et al. 1999), neural stem cells (Heine, Conant et al. 2004), fibroblasts (Phillips, Bunting et al. 2005), skin-derived stem cells (Marchesi, Pluderi et al. 2007), hair follicle stem cells (Amoh, Li et al. 2005), ectomesechymal stem cells (Nie, Zhang et al. 2007) have all been studied at varying degrees and resulted in varying success (Jiang, Lim et al. 2010). Despite the promising results the need to find an autologous source, cost, risk of contamination, and the large time lag between injury and repair are some major obstacles that must first be overcome before these methods can become practical (Murakami, Fujimoto et al. 2003; Jiang, Lim et al. 2010).

Similar to the contact guidance theory a third approach was to use extracellular matrix molecules to mimic the post-injury microenvironment of a nerve inside the conduit (Jiang, Lim et al. 2010). Collagen

(Chen, Hsieh et al. 2000; Phillips, Bunting et al. 2005), laminin (Matsumoto, Ohnishi et al. 2000; Yu, Morgan et al. 2009), and fibronectin (Whitworth, Brown et al. 1995; Ahmed, Brown et al. 1999; Mosahebi, Wiberg et al. 2003) have all been investigated with varying success. The effects of ECM molecules are still inconclusive, controlled amounts of fibrous structure seem to have a positive effect but when introduce in bulk or hydrogel form the opposite effect is observe (Jiang, Lim et al. 2010).

The last and most common approach was the use of neurotrophic factors, because of their roles in controlling the survival, migration, proliferation, and differentiation of different neural cell types (Jiang, Lim et al. 2010). The most commonly studied factors are nerve growth factor (NGF) (He and Chen 1992; Lee, Yu et al. 2003; Xu, Yee et al. 2003), neurotrophin-3 (NT-3) (Sterne, Brown et al. 1997; Midha, Munro et al. 2003), Glial cell-derived neurotrophic factor (GDNF) (Barras, Pasche et al. 2002; Fine, Decosterd et al. 2002; Boyd and Gordon 2003; Boyd and Gordon 2003; Wood, Moore et al. 2009), and fibroblast growth factors (FGF) (Cordeiro, Seckel et al. 1989; Wang, Cai et al. 2003). Other not so common but still promising growth and neurotrophic factors that have been studied are ciliary neurotrophic factor (CNTF) (Zhang, Lineaweaver et al. 2004), glial growth factor (GGF) ((Mohanna, Terenghi et al. 2005), vascular endothelial growth factor (VEGF) (Hobson 2002), brain-derived neurotrophic factor (BDNF) (Terris, Toft et al. 2001), leukemia inhibitory factor (LIF) (McKay Hart, Wiberg et al. 2003), insulin-like growth factor 1 (IGF-1) (Fansa, Schneider et al. 2002), and platelet-derived growth factor (PDGF) (Wells, Kraus et al. 1997). These and other studies have shown that the inclusion of factors within a nerve conduit is effective, however, a more detailed understanding of factor dose response and their combinations on nerve regeneration is warranted in order for an optimal design of nerve repair conduits (Jiang, Lim et al. 2010).

Despite the advancements of nerve conduits and the use of nerve grafts clinicians and researchers are often limited to the treatment of nerve defects that are shorter than 5 cm in humans and 1.5 cm in rats; this is the critical defect size of a nerve gap (Jiang, Lim et al. 2010). However, this is not the case for injuries that results in a relatively short defect, typically less than 5 mm, in which axons of the

peripheral nerve will spontaneously regenerate without much help (Jiang, Lim et al. 2010). Any attempt to regenerate a nerve across a longer length has met with limited success, especially when the length is closer to the 5 cm defect size, illustrated by Table 1 by the fact that most research has been focused on shorter defect lengths.

Table 1. Successful nerve repair strategies. Strategies are sorted by defect lengths.

Defect Length	Tube material	Luminal filler	Clinical / Animal	Author
0.8 cm	PAN/PVC copolymer	Schwann cells	Rat	(Guenard, Kleitman et al. 1992)
1.0 cm	Polysulfone	Laminin conjugated to agarose gel	Rat	(Yu and Bellamkonda 2003)
1.0 cm	Polyurethane	Multiluminal collagen	Rat	Tansey et al., submitted
1.0 cm	PLGA	None	Rat	(Bryan, Holway et al. 2000)
1.0 cm	PVDF-TFE	None	Rat	(Fine, Valentini et al. 1991)
1.0 cm	PHEMA-MMA	PCL coils	Rat	(Katayama, Montenegro et al. 2006)
1.0 cm	PHB	Schwann cells	Rat	(Mosahebi, Simon et al. 2001)
1.0 cm	Silicone	Schwann cells	Rat	(Nilsson, Dahlin et al. 2005)
1.0 cm	Chitosan coated PDMS	NSCs	Rat	(Cui, Jiang et al. 2008)
1.2 cm	PLLA	None	Rat	(Evans, Brandt et al. 1999)
1.5 cm	Silicone	Fibroblast-like MSCs	Rat	(Chen, Ou et al. 2007)
1 - 1.8 cm	Silicone	PLLA filaments	Rat	(Ngo, Waggoner et al. 2003)
2.0 cm	Autologous muscle conduits	EMSCs	Rat	(Keilhoff, Stang et al. 2006)
2.0 cm	Collagen	Schwann cells	Rat	(Stang, Fansa et al. 2005)
2.0 cm	PLGA	Schwann cells	Rat	(Cheng and Chen 2002)
≤ 2.0 cm	Collagen	None	Human	(Ashley, Weatherly et al. 2006)
1.0 - 3.0 cm	PGA	None	Human	(Navissano, Malan et al. 2005)
2.0 - 4.0 cm	PGA	None	Human	(Rosson, Williams et al. 2009)
2.0 - 5.0 cm	Silicone	None	Human	(Braga-Silva 1999)
2.0 & 5.0 cm	PGA	None	Primate	(Mackinnon and Dellon 1990)
1.5 & 5.0 cm	Collagen	None	Primate	(Archibald, Shefner et al. 1995)
1.5 - 6.0 cm	ePTFE	None	Human	(Stanec and Stanec 1998)
8.0 cm	PGA	Laminin-soaked collagen fibers	Canine	(Matsumoto, Ohnishi et al. 2000)

The main reasons, observed by the author of this paper, are that a nerve conduit for a long nerve gap, 3 cm or more, does not have adequate surface area, for the adhesion and growth of satellite cells required to support the regenerating axons, and lacks signaling cues from neurotrophic growth factors.

We hypothesized that a synthetic nerve conduit incorporated with a multi-luminal design infused with the pleiotrophic factor Pleiotrophin (PTN) will be effective at axon repair across a large nerve defect, and that an combination of VEGF and PTN will be even more effective in axonal regeneration across a large gap. This hypothesis is based on the observation that by incorporating multiple channels inside the nerve conduit the surface area of said conduit is increased and will in turn be able to support the adhesion and migration of satellite cells required by the axons such as Schwann cells and fibroblasts. Secondly, by using pleiotrophic factors such as PTN and VEGF it would be possible to not only affect neuronal cell types but endothelial cells, for angiogenesis, as well.

1.5 Multiluminal nerve conduits

Dr. Mario Romero's lab and others have proposed tabularization techniques that mimic the multifascicular nerve anatomy (Tansey et al., submitted) as well as increase the surface area of a nerve conduit. The need for a nerve conduits that can mimic the microenvironment of a natural nerve bundle such as multiple channels, incorporation of growth factors, and control permeability to name a few are essential in regeneration (Pabari, Yang et al. 2010). If this bridge is synthetic, as it will be in this study, it must be able to support axons and neural cells, protect said cells from the environment but be patent enough to allow for nutrient and waste exchange. The biosynthetic nerve implant (BNI) proposed by this study is made up of a micro-renathane tubing filled agarose with collagen channels embedded throughout. The micro-renathane tubing mimics the epineurium of the nerve fiber, the agarose around the collagen will provide a substrate for the perineurium to grow on, and the collagen inside the channel will provide a substrate for the endoneurium, axons, and neural satellite cells. The tubing will have 500 µm holes placed throughout the tube allowing for nutrient/waste exchange. The pore size of the agarose

hydrogel was ~100 nm, determined by its weight/volume percent (1.5 wt%) (Narayanan, Xiong et al. 2006). The collagen channels will help regenerating axons form nerve cables that resemble the multifascicular anatomy of the normal nerve by way of a permissive contact guidance and favorable biological substrate. The overall design is schematically and photographically represented in Figure 5.

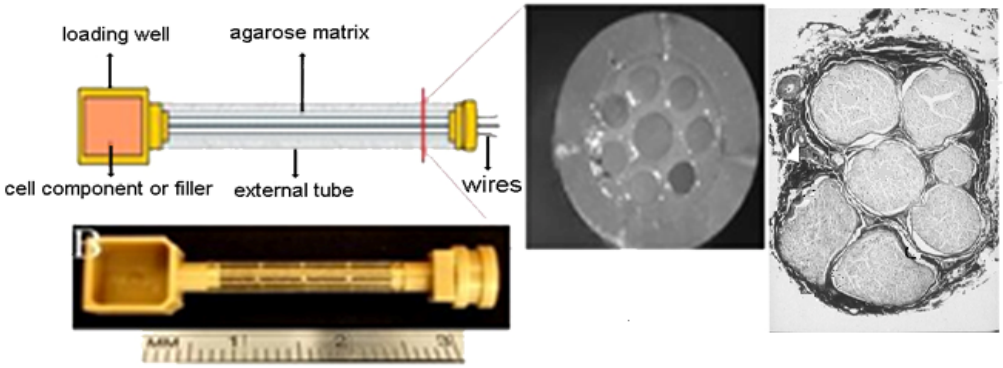

Figure 5. Side-by-side comparison of proposed design and a normal peripheral nerve. Image is that of a schematic representation and real life photograph of the casting device. To the right is the cross-section of the nerve conduit filled with agarose and collagen channels next to a cross-section of a normal peripheral nerve, sural nerve. Image of nerve was obtained from Imaging of Soft Tissue Tumors 2nd Edition by Dr Kransdor and Dr. Murphey, (Kransdorf and Murphey 2006).

A previous study performed by our lab also indicated that the multiluminal conduit is more effective than a simple tube filled with collagen. The study was performed by implanting 10 mm long nerve conduits, multiluminal, collagen filled or autograft, into the between the nerve stump of a transected sciatic nerve of a rat. The results showed that the multiluminal conduit performed better than the collagen-filled tube and was just as effective as the autograph. The side view of the nerve in each conduit after 10 weeks of repair can be seen in Figure 6. The conduits and BNIs used did not incorporate any neurotrophic or pleiotrophic support therefore it is concluded that the main reason for the enhanced axonal regrowth is due to the increased surface area provided by the multiluminal design of the BNI. Furthermore, the multiple channels helped the regenerating axons to form nerve fascicles that are very much similar to that of a normal nerve. The encouraging result of this previous study prompted the use of this design for the repair of a longer, 3 cm, nerve defect that is modeled in this present study.

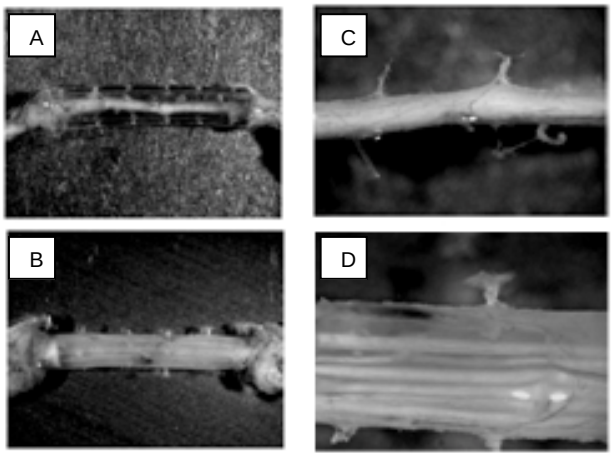

Figure 6. Collagen-filled conduit vs. multiluminal design. After 10 weeks post implantation a, and b is the nerve inside a 10 mm collagen filled conduit and c and d Is the nerve found inside the conduit made with a multiluminal design.

1.6 Pleiotrophins

With the exception of VEGF most growth factors investigated, summarized by Table 2, are mostly neurotrophic factors that only targets one single cell types. The use of neurotrophic support is the second element of the hypothesis proposed by this paper. However, it must be pointed out that neurotrophic support alone is not the most efficient. Aside from the regenerating axon a regenerating nerve fiber also contains many different cell types essential for a healthy nerve to function. Schwann cells, responsible for the mylenation and protection of axons, and endothelial cells, responsible for forming blood vessels, are two more important cell types that are present in a nerve fiber. Therefore, it would be beneficial to look at a different class of growth factors to provide neurotrophic support to the regenerating nerve, i.e. pleiotrophins.

Table 2. Neurotrophic supportive strategies visited by different researchers. Strategies are sorted by defect lengths

Defect Length	Tube material	Luminal filler	Growth factors	Clinical / Animal	Author
1.0 cm	Silicone	Matrigel	VEGF	Rat	(Hobson, Green et al. 2000)
1.0 cm	PHEMA-MMA	Collagen	NT-3, BDNF, FGF-1	Rat	(Midha, Munro et al. 2003)
1.0 cm	Chitosan	Laminin-1	GDNF	Rat	(Patel, Mao et al. 2007)
1.0 cm	Silicone	None	FGF-2	Rat	(Haastert, Ying et al. 2008)
1.0 cm	Silicone	None	FGF-2	Rat	(Haastert, Ying et al. 2008)
1.0 cm	PPE	None	NGF	Rat	(Xu, Yee et al. 2003)
1.0 cm	PHEMA-MMA	Collagen type I gel	NT-3, aFGF	Rat	(Midha, Munro et al. 2003)
1.0 cm	Fibronectin	None	NT-3	Rat	(Sterne, Brown et al. 1997)
1.0 cm	Heparin/alginate hydrogel	Heparin/alginate hydrogel	bFGF	Rat	(Ohta, Suzuki et al. 2004)
1.5 cm	PCLEEP	PCLEEP fibers	GDNF	Rat	(Chew, Mi et al. 2007)
1.5 cm	PDLLA	PDLLA polymer solution	bFGF	Rat	(Wang, Cai et al. 2003)
1.5 cm	PEVA	None	NGF, GDNF	Rat	(Fine, Decosterd et al. 2002)
2.0 - 4.0 cm	PHB	calcium alginate hydrogel	GGF	Rabbit	(Mohanna, Terenghi et al. 2005)

Pleiotrophins in particular are of interest because of their ability to affect many cell types at once. Unlike neurotrophins, whose effect is targeted to only one type of neurons, one pleiotrophins can and does affect multiple cell types (e.g. endothelial cells, fibroblasts, and Schwann cells). The pleiotrophins investigated by different groups for nerve regeneration includes: IGF-1, FGF1, FGF2, VEGF, and PTN (Cordeiro, Seckel et al. 1989; Fansa, Schneider et al. 2002; Hobson 2002; Wang, Cai et al. 2003; Blondet, Carpentier et al. 2005; Blondet, Carpentier et al. 2006). The two pleiotrophins of interest for this study includes Vascular Endothelial Growth Factor (VEGF) and Pleiotrophin (PTN). Both these pleiotrophic factors have been found to affect peripheral nerve axons as well as other cell types, mentioned above (Van Den Bosch, Storkebaum et al. 2004; Blondet, Carpentier et al. 2005).

1.6.1 Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a known angiogenic compound and has been extensively written about (Carmeliet 2003; Ferrara 2004; Kerbel 2008). Figure 7 is a “top-down” view of

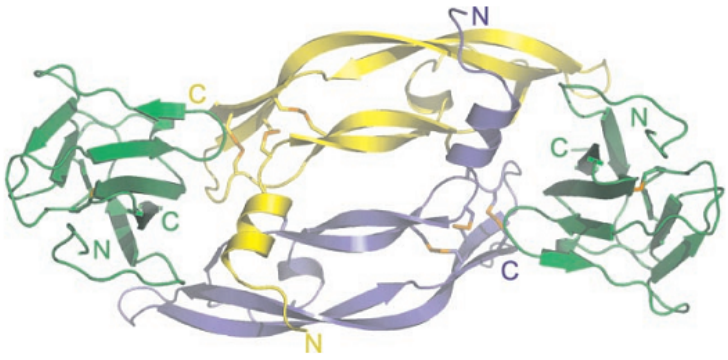

Figure 7. Ribbon depiction of VEGF-VEGFR1 complex. VEGF monomers are represented in yellow and blue, the two receptor molecules are green. This is a "top-down" view (Ferrara 2004).

two VEGF monomers interacting with two VEGFR1’s receptors. Known receptors include 3 VEGF receptors: VEGFR-1, VEGFR-2, VEGFR-3, and one coreceptor, believed to enhances VEGF signaling to the receptors, neurophilin-1 (NRP1) and neurophilin-2 (NRP2) (Carmeliet 2003; Ferrara 2004; Geretti, Shimizu et al. 2008). VEGFR-1 is thought to be a “decoy” receptor that traps excess VEGF in order to prevent over-activation of VEGFR-2 (Olsson, Dimberg et al. 2006). Recent studies, however, have shown that VEGFR-1 is able to exert neuroprotective effects during pathological conditions and stimulates postnatal angiogenesis (Li, Zhang et al. 2008; Ruiz de Almodovar, Lambrechts et al. 2009). VEGFR-2 is a signaling receptor responsible for angiogenesis by stimulating endothelial cells (EC) (Olsson, Dimberg et al. 2006). This receptor also stimulates neural cell types to migrate, proliferate and survive in the nervous system (Sondell, Lundborg et al. 1999; Jin, Mao et al. 2000; Sondell, Sundler et al. 2000; Jin, Zhu et al. 2002; Ogunshola, Antic et al. 2002; Wick, Wick et al. 2002). VEGFR-3 is responsible for inducing (lymph)angiogenesis, modulates angiogenesis by transmitting sprouting signals in endothelial tip cells, and induces proliferation of oligodendrocyte precursors and other neural progenitors (Jussila and

Alitalo 2002; Le Bras, Barallobre et al. 2006; Tammela, Zarkada et al. 2008). NRP1 and NRP2 can be found during the developmental phases, NRP1 is restricted to arteries and NRP2 can be found in venous and lymphatic vessels (Eichmann, Le Noble et al. 2005). Figure 8 summarized the interactions between VEGF and its corresponding receptors.

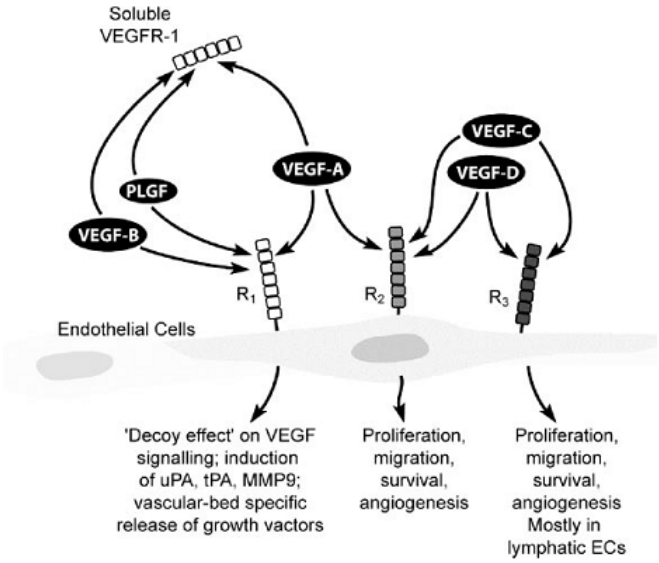

Figure 8. Interactions between VEGF and its receptor. GFR-2 and VEGFR-3 is responsible for proliferation, migration, survival, and angiogenesis in vascular EC and lymphatic EC, respectively (Ferrara 2004).

It have been VEGF increases angiogenesis to a transected nerve but also enhances Schwann cell proliferation and migration (Ruiz de Almodovar, Lambrechts et al. 2009). Acellular peripheral nerve isografts treated with VEGF had a significant increase in total number of axons and neural tissue when compared to ones without (Rovak, Mungara et al. 2004). Silicone chamber with Matrigel full of VEGF had 78% enhancement of myelinated axon and motor performance was significantly improved (Hobson, Green et al. 2000).

1.6.2 Pleiotrophin (PTN)

Before it was given the name Pleiotrophin (PTN), the 17-KD multifunctional growth factor was known by many different names: heparin-binding growth factor-8, heparin-binding growth-associated molecule, heparin-binding neurotrophic factor, heparin-affinity regulated peptide, osteoblast-specific protein-1, and p18 (Kovesdi, Fairhurst et al. 1990). Developing embryos have been found to express PTN in the peripheral nervous system (PNS) (Mitsiadis, Salmivirta et al. 1995). Since then it have been discovered that the protein's DNA sequence is 100 kb long, located on chromosome 7 band q33, contains five coding exons and one noncoding intron (Li, Hoffman et al. 1992). The protein contains two beta-sheet domains that are homologous with the thrombospodin type 1 repeat (TSR-1 repeat) (Kilpelainen, Kaksonen et al. 2000). Figure 9 is an illustration of the secondary structure of the PTN molecule with the two TSR-1 repeats, linker, and tails labeled.

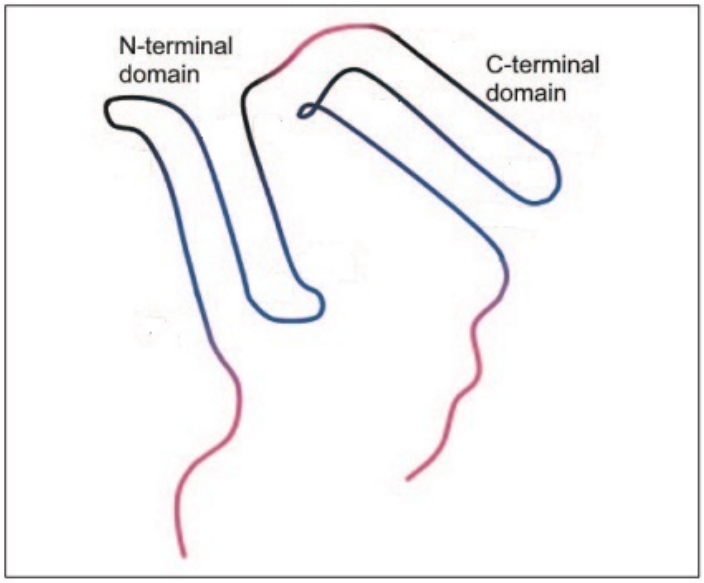

Figure 9. Secondary structure of PTN. Two β-sheet domains are labeled in blue, the random coils flexible linker and tails are labeled in red (Raulo, Tumova et al. 2005).

These repeats have been found to be associated with the binding of PTN to heparin and to exhibit its mitogenic, angiogenic, and neurotrophic activities (Raulo, Tumova et al. 2005; Hamma-Kourbali, Bernard-

Pierrot et al. 2008). PTN is a mitogen to endothelial cells, epithelial cells, fibroblasts, and others. It can also promote angiogenesis in both normal cells and tumor cells, PTN have been found to stimulate bovine capillary endothelial cells in culture to form stable tube-like structures. Most importantly, PTN have been found to promote outgrowth in neurons, glial progenitor cells and oligodendrocyte progenitors (Jin, Jianghai et al. 2009).

There are four transmembrane proteins that have been identified as PTN receptors: receptor protein tyrosine phosphatase (RPTP) β/ζ, anaplastic lymphoma kinase (ALK), N-syndecan, and low-density lipoprotein receptor-related protein-5 (Jin, Jianghai et al. 2009). Recently, Mi et al found that PTN can significantly enhance regeneration of myelinated axons across a nerve graft in an adult rat and have confirmed that ALK was expressed in the spinal cord motor neuron soma and neurite in vitro. Because the neurotrophic activity of PTN can be blocked by ALK antibody it was suggested that ALK is the receptor responsible for PTN's neurotrophic activity (Mi, Chen et al. 2007). ALK is a tyrosine kinase receptor that binds PTN with a dissociation constant of 32 ± 9 pM. PTN cause the receptor to self phosphorylate and other downstream effector molecules, such as: IRS-1, Shc, phospholipase C-γ, phosphatidylinositol 3-kinase resulting in the growth stimulatory effect of PTN (Stoica, Kuo et al. 2001; Mourali, Benard et al. 2006).

There are many findings that support the idea that PTN is important in the axonal regeneration process. Blondet et al found that in a crush sciatic nerve of a rat there were high levels of PTN in the nerve during the first week after injury; PTN was also found in the surrounding satellite cells, Schwann cells, macrophages, and endothelial cells in the distal portion of the nerve after injury. The cells mentioned above also exhibited immunoreactivity to PTN at varying times after injury (Blondet, Carpentier et al. 2006). Adding to the findings, the expression of PTN mRNA are much higher in motor nerve than in sensory nerves. Furthermore, when compared to other neurotrophic factors: nerve growth factor (NGF), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor, hepatocyte growth factor, fibroblast growth factor 2, GDNF, Ciliary neurotorphic factor, and IGF2

PTN is found to be more highly expressed in motor nerves than sensory nerve, in denervated muscle than denervated skin, and more so after an injured nerve (Hoke, Redett et al. 2006). This makes PTN a good candidate for study in axonal regeneration.

A study performed in our lab previous to this investigation demonstrated that PTN, when compared to BSA is able to produce larger and longer axons from disassociated motor neurons. PTN is relatively new and have not been extensively studied, Figure 10.

Referring to Table 1 above, VEGF, a growth factor with know pleiotrophic activities; have been investigated and showed good results. VEGF is an angiogenesis growth factor and has a secondary role of axonal regeneration (Rovak, Mungara et al. 2004; Ruiz de Almodovar, Lambrechts et al. 2009). So it stands to reason that a neurotrophic factor, such as PTN, that can also encourage angiogenesis and Schwann cell migration would be just as or more effective in guiding axons across a nerve conduit.

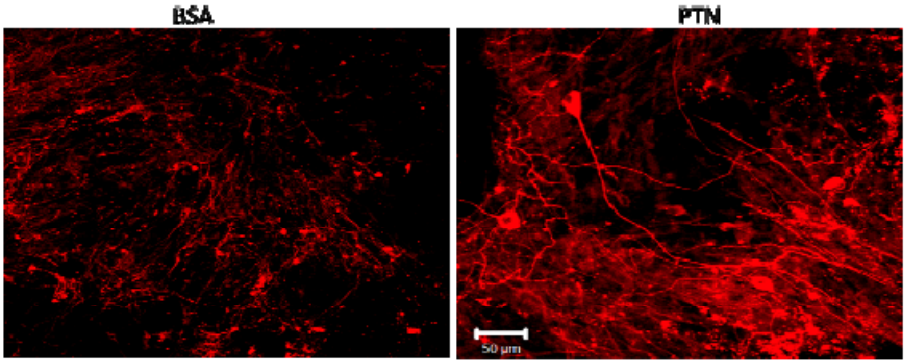

Figure 10. Confocal microscopy images of motor neurons treated with BSA and PTN. Axons were stained with α-β tubulin primary antibody and labeled with cy-3 conjugated secondary antibody.

1.7 Scope of this study

In order confirm the hypothesis of this study we have proposed two specific aims: 1) the advantage of a multi-luminal nerve conduit when compared to that of a simple nerve graft will be investigated. 2) To examine the effectiveness of PTN at encouraging axonal regeneration through a

synthetic nerve guide, and explore the synergistic effect resulting from the combining of two pleiotrophins, PTN and VEGF.

To this end 28 female New Zealand rabbits were divided into 4 groups: group 1 included 7 BNIs loaded with VEGF particles, group 2 included 7 BNIs loaded with PTN particles, group 3 included 5 BNIs loaded with VEGF and PTN particles, group 4 included 7 BNIs with just no particles, the pre-injured and un-operated on side animals were used as the positive control, and the negative control was of 3 animal implanted an empty tube loaded with collagen. Each member of the groups had their left peroneal nerve transected and grafted into their perspective nerve conduits. To quantify their recovery the subjects were tested for their ability to spread their hind toes, ability to feel an irritant on the dorsal part of their foot, and the number of axons in the distal part of the nerve was quantified.

This study is a continuation of a preliminary investigation in the effectiveness of the BNI and PTN at regenerating a severed nerve across a large gap headed by Abdul Dawood. The above mentioned study compared empty BNIs, BNIs filled with PTN, BNIs filled with VEGF, and empty tube conduits with an n of 2 each. The findings were that the BNIs filled with PTN were significantly more effective at axonal regeneration than the other test groups. This study seeks to verify this finding with a larger number of subjects in each group. The amount of growth factor used and the method of delivery, PLGA microparticles, were kept the same in both studies. Based on data not yet published my predecessor chose to use PLGA particles to encapsulate the growth factors. It was determined that a controlled release of growth factor over time was needed for the regeneration of axons across a large defect and that it would be more effective than simply placing growth factors directly in the BNI lumen. The concentration of particles was chosen to be 2.5 mg/ml because it was found that if the concentration was any higher the particles would block regeneration.

CHAPTER 2

METHODS

2.1 Growth factor particles

2.1.1 Fabrication

Particles loaded with VEGF and PTN were fabricated using the double emulsion method. Briefly, 0.2 g of polyvinyl alcohol (PVA) was dissolved into 20 ml of deionized (DI) water at 50^{o}C. At the same time 0.1 g of poly (lactic-co-glycolic acid) (PLGA) was dissolved in 2 ml of dichloromethane. Once the two solutions finished dissolving 10 µg of VEGF was dissolved into 0.2 ml of DI water and then added to the PLGA solution. The PLGA/VEGF solution was then sonicated at 15 W for 15 sec. This sonicated solution was then added to the PVA solution and sonicated at 30 W for 45 seconds. The final solution was left overnight with gentle stirring. The next day the solution was centrifuged at 3,000 rpm for 15 mins. The supernatant was collected and frozen for later analysis. The particles were washed three times with DI water and one time with phosphate buffered saline (PBS) and then resuspended in PBS and freeze dried. Washing involved vortexing the particles in a liquid, centrifuging the resulted solution at 3,000 rpm for 15 mins, and then discarding the dirty liquid. The final product was stored at -20^{o}C. These steps were repeated, and VEGF was replaced by 20 µg of PTN to fabricate PTN particles.

2.1.2 Characterization

The overall shapes of the particles were confirmed visually using the scanning electron microscope (SEM). A small drop of resuspended particles, before it was freeze dried, was placed on a glass cover slip and allowed to air dry. The dried particles were coated with silver using a sputter coating

machine. After which the coated sample was placed in the SEM and images were taken at different magnification.

A release profile of the VEGF particles was obtained using an ELISA kit obtained from Invitrogen. Four samples of particles, 1 mg each, were suspended in 1 ml of 1 % bovine serum albumin (BSA) in 1X PBS. The suspension was placed in a shaking incubator set at 37^{o}C and 60 rpm. At predetermined time points the supernatants were collected and the particles were resuspended in fresh BSA/PBS solution. The time points were 0 hrs, 1 hr, 6 hrs, 14 hrs, 24 hrs, 2 d, 4 d, 7 d, 10 d, 2 w, and 3 w after the initial suspension. Because there were no commercially available ELISA kits for PTN only the release profile of VEGF was acquired. The amount of PTN released from the particles was estimated by multiplying the results of the VEGF study by a factor of 4. This number was obtained based on the fact that twice as much PTN was use to make the particle and that each PTN molecules (18 kDa) are half the size of each VEGF molecules (45 kDa).

2.2 Biosynthetic nerve implant

2.2.1 Fabrication

The BNIs were fabricated using Micro-Renathane tubes purchased from Braintree Scientific, Inc. The tubes have an outer diameter of 0.24 cm and an inner diameter of 0.17 cm. A rotary tool was used to drill 500 µm holes down the length of the tubing on three of its sides. The holes were kept approximately 0.40 cm apart from each other and were evenly spaced around the tube. The tubes were then sectioned into 3 cm sections, washed with detergents, rinsed with DI water, soaked in 70 % ethanol and left inside a sterile laminar flow hood under UV light overnight.

The next day each tube was fitted into a casting device which included a well and an end plate on each end of the tube and metal dowel rods with a diameter of 500 µm were inserted through the length of the tube, guided by premade holes inside the well and end plate. Dowel rods, wells, and end plates were sterilized prior to this step. A 30 G needle was then used to inject 1.5 wt% agarose to the tube making

sure to fill the entire tube without bubbles. The tubes were left to cool and collagen, purchased from Millipore (atelomeric chicken collagen, 85 % type I, 15 % type II), was prepared following the manufacturer's instruction. When the agarose have sufficiently cooled and polymerized particles were added to the collagen, a small amount of the mixture was placed inside the well of the casting device and the rods were steadily withdrawn from the casting device. When the rods were all removed the end plate and well was removed and the BNI was placed in an incubator with a small amount of PBS to await implantation. The time between completion of BNI and implantation inside the animal was kept under one hour. Four groups of BNIs were made: pure collagen, 2.5 mg/ml VEGF, 2.5 mg/ml PTN, and 1.25 mg/ml VEGF and 1.25 mg/ml PTN combination. This was all done under sterile conditions.

2.3 Rabbit model of common peroneal nerve gap injury

2.3.1 Implantation

Prior to the procedure animals were preanesthetized with a mixture of 35 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. Once the animal goes down the left thigh was shaved and cleaned with 70 % ethanol and then betadine. The animal was then placed under anesthesia with the use of 3 % isoflurane inhalation. The left peroneal nerve was exposed by spreading the abductor cruris cranialis muscle and the biceps femoris muscle of the left thigh. The peroneal nerve was cleared from the underlying muscle and tibial nerve, fat and fibroblasts were cleared from it. The nerve was then bisected and the BNI was placed in between the two ends. Using 3-0 chromic gut sutures the BNI was tied to the underlying muscle and the nerve ends were placed inside the tube's openings and sutured into place using 9-0 monofilament sutures. In order to accommodate the BNI small sections of varying sizes were cut from the nerve. The muscles were then closed over the nerve and sutured together with 3-0 chromic gut sutures, the skin was stapled together and triple antibiotic ointment was applied to the wound. Gauze and adhesive bandages was wrapped around the wound. The animal was taken of anesthesia and allowed to wake before it was returned to its cage.

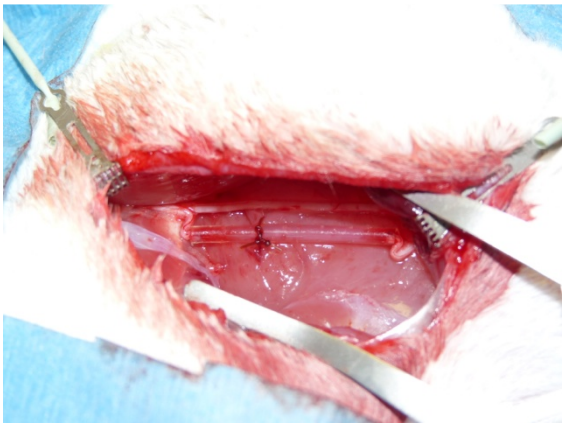

Figure 11. Implantation of BNI. A 3 cm implant placed in the between the nerve stumps of the transected left peroneal nerve.

2.3.2 Recovery

The animals were given four weeks to recover, the first two weeks the animals were fed apples and carrots as well as food pellets. Each animal was given 40 mg of sulfamethoxazole and 8 mg of trimethoprim orally and 1 mg of buprenorphine subcutaneously once a day. Extra pain medication was given if the animal exhibit additional pain symptoms such as refusal to eat or vocalization. At the end of the first week bandages were changed and the wounds were inspected for signs of infection, none were observed. At the end of the second week bandages and staples were removed. Starting from this time point each animal was given approximately 1 hour of exercise outside its cage once every three days. Exercise consisted of the animal being placed outside the cage and allowed to move around the room.

2.3.3 Behavioral testing: toe spread

At the end of the recovery period each animal was tested for functional recovery by the effectiveness of its toe spread once a week. This test was done by measuring the distance between the tip of the animal's outer most claws on the right and left foot. The figures were normalized by dividing the injured value with the uninjured value. To entice the animals to spread their toes the animal was held by

the scruff of its neck with one hand and support its rump with the other hand holding the animal's feet in the air. A puff of air was delivered to the animal's face and the resulting reaction was measured.

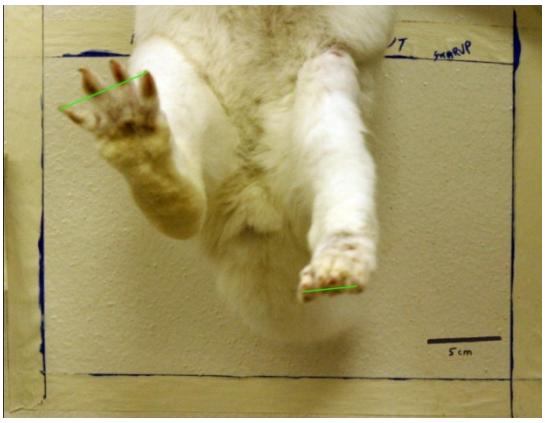

Figure 12. Weekly motor test, toe-spread. The distance between the first and last toe of each side of the animal is measured, green lines. To normalize the injured side was divided by the non-injured side.

2.3.4 Behavioral testing: formalin

After the recovery period each animal was tested for functional recovery of its sensory pathway once every two weeks with the help of the formalin test. The formalin test consisted of a 0.1 ml subcutaneous injection of 2 wt% of paraformaldehyde in PBS on the dorsal part of the subject's injured foot between the two inner most toes, approximately 1 cm up the foot. The total amount of time the animal licked its left foot in a span of 10 mins was recorded.

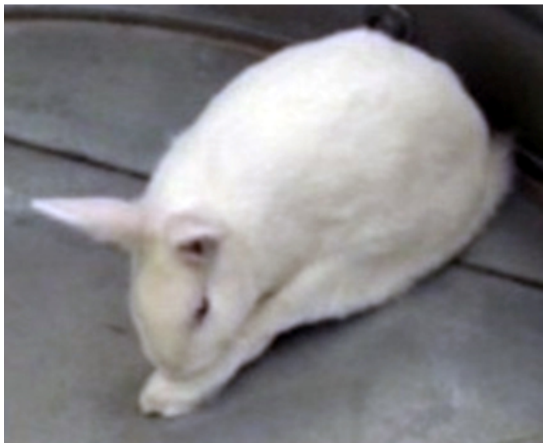

Figure 13. Biweekly sensory test, toe-licking. Each time the animal licked its foot and then pause or puts the foot down counts as 1.

2.3.5 BNI collection

At the end of the observation phase, 9 weeks, the animals were overdosed with pentobarbital, 120 mg/kg, intravenously. Once it was observed that the animal exhibits no pain response it was prepared for intracardial perfusion. Briefly, the animal's heart was exposed. Using a pump approximately 500 ml of 0.9 % sodium chloride was pumped into the body through the left ventricle and out the right atrium of the heart at 25 rpm. The sodium chloride was then replaced by 500 ml of 4 % paraformaldehyde. After all the paraformaldehyde was pumped through the animal the BNI was carefully harvested and placed in 4 % paraformaldehyde overnight and was replaced with PBS and kept in 4^{o} C for storage.

2.3.6 Immunohistochemistry

The BNIs were carefully cleaned and the outer micro-renathane tubing was cut away from tissue. The tissues were cut into sections and placed inside embedding cassettes. The sections were separated into groups: proximal end inside BNI, middle inside BNI, distal end inside BNI, and distal end outside BNI. The tissues were the dehydrated by serial submersion in 70 %, than 95 %, and finally 100 % ethanol for 1

hour each. At the end of the 3 hours the sections were submerged in molten paraffin, kept at 65^{o} C for 1 hour. At the end of the procedure each tissue section was removed, oriented in a mold and embedded in paraffin. Once the paraffin blocks cooled the sections were sliced into 10 µm slices using a microtome and fixed on glass slides and labeled. The slides were then allowed to air dried and then baked in an oven at 37^{o} C for 10 mins to fix tissues to the slide. The slices were then submerged serially in xylene, 100 % ethanol, 95 % ethanol, 70 % ethanol and then allowed to air dry.

The dried tissues were stained with primary antibodies mouse anti-neurofilament protein (NFP). The secondary antibodies used were goat anti-mouse Cy2. The staining process was as followed: the tissues were washed 3 times with washing solution for 10 mins in between. Then the tissues were submerged in a blocking solution for 1 hr. After this time the blocking solution was replaced by the primary antibody and kept at 4^{o} C overnight. The next morning the primary antibody was removed and the stained tissues were washed 3 times with washing solution for 10 mins in between. After the third washing the tissues were submerged in a solution containing the secondary antibody for 1 hr. After the hour the tissues were washed 3 more time with washing solution and a 4th time with PBS each at 10 in between. The final step was to fix a cover slip over the tissue. In between each washing the slides were placed on a shaker with a slow setting. Washing solution was 0.5 % triton in PBS; blocking solution was 4 % normal goat serum in washing solution. All steps after the second antibody was introduced were protected from light. Samples were stored at 4^{o} C.

2.4 Statistical analysis

Statistical analyses were performed with GraphPad Prism (Version 5, GraphPad Software, Inc.). All results are represented as the average ± standard deviation unless otherwise stated. Statistical differences were identified using a one-way ANOVA test. Differences were considered significant when $p < 0.05$.

CHAPTER 3

RESULTS

3.1 Particles characterization

Scanning electron microscope images of PLGA particles shows a mostly spherical morphology, Figure 14.

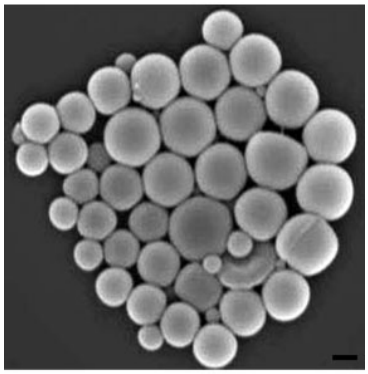

Figure 14. SEM image of PLGA particles. Scale bar = 5 µm.

There was no way to visually validate the presence of growth factors encapsulated within particles. In order to confirm growth factor encapsulation a drug release study was performed. Using the growth factor and concentration provided by the manufacturer of the ELISA kit a calibration curve was produced. The equation correlating the absorbance to the concentration was found to be: Concentration = (Absorbance – 0.062)/0.003, the R^2 value of the equation to the curve was 0.999. Using the curve and the data obtained from the release study it was found that there is an initial burst release of growth factors in the first 24 hours, up to 50 % is released. After this the profile shows a much slower release curve, approximately 5 % during the time frame between 24 hr to 500 hr after implantation. Using the supernatant, saved during fabrication of the particles, it was calculated that in each mg of particles there

is 184,422.3 pg VEGF. Each BNI contained approximately 200 µl of collagen loaded with VEGF particles at a concentration of 2.5 mg/ml, it can be estimated that approximately 0.5 mg of particles or 92,211.15 pg of VEGF is present in each BNI. According to the release study, Figure 15, it can be approximated that roughly 46,105.6 pg of VEGF is released during the first 24 hr and the rest is released during the 3 months time span.

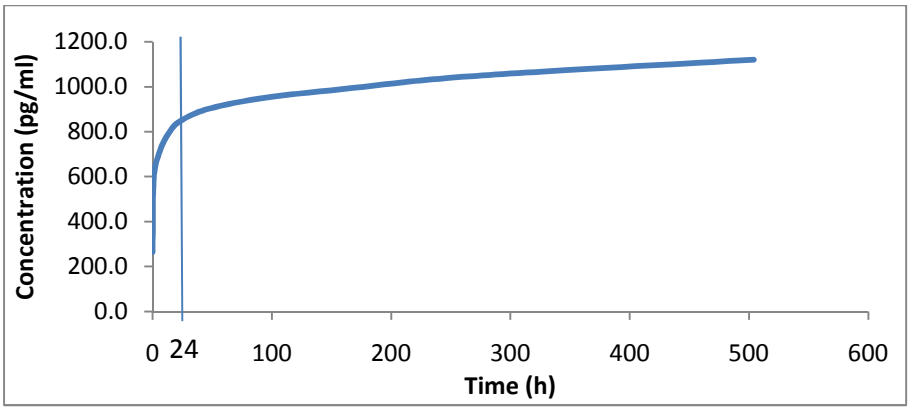

Figure 15. Release profile of VEGF particles.

Since PTN was not studied because of the lack of commercially available kits it was estimated that the number of PTN in each BNI was four times as much as the BNI with VEGF, or a total release of 184,422.4 pg during the first 24 hr with an additional 184,422.2 pg over the 3 months allotted for the study.

3.2 Rabbit model of common peroneal nerve gap injury

The ability of the animal to spread their toe was a way to assess the animal's recovery. Prior to the procedure the animals' ability to spread their toes was measured and normalized as described above. The average toe spread index (TSI) value for an uninjured animal is 0.99 ± 0.01 units. After the recovery period, at 4 weeks post implantation, TSI for all the groups dropped to approximately 0.64 ± 0.03 units. As the testing period progress the animal implanted with the simple tubing slightly decreased ending at

0.62 ± 0.061 units at the end of the 9th week. All the animal groups implanted with the BNIs increased over time. At the 9 week expiration date the empty BNI group had a TSI value of 0.72 ± 0.13 units, the VEGF loaded BNI group had a value of 0.77 ± 0.05 units, the PTN loaded BNI group had a value of 0.85 ± 0.10 units and the VEGF/PTN combination group had a value of 0.81 ± 0.0281 units, summarized in Figure 16.

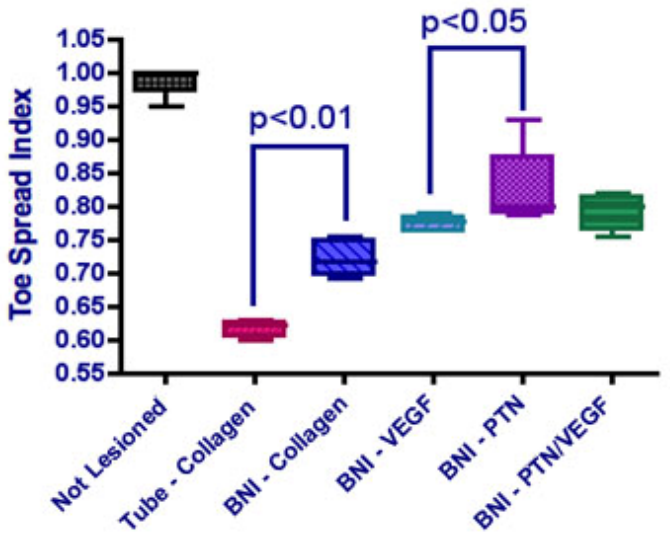

Figure 16. Comparison of the Toe-spreading Index of the different animal groups at week 9. The Toe-spreading Index is obtained by dividing the uninjured value by the injured value. There is a large difference between the collagen filled tube conduits compared to the BNIs. Between the BNIs the one with PTN performed significantly better than the one with VEGF.

By comparing the progress of the animals' ability to regain motor function over time it is possible to emphasize the efficacy of PTN at encouraging axonal regeneration. Figure 17 compares the progress of the animal group implanted with BNI filled with PTN vs. the animal group implanted with BNI filled with collagen alone vs. the animal group implanted with the simple tube filled with collagen. Out of the three groups the group implanted with the simple tube performed the worst, after injury the group's TSI value dropped to 0.64 ± 0.03 units and remained at this level throughout the entire test period. The group that

was implanted with an empty BNI filled with collagen performed slightly better, after the initial drop in TSI value the animals improved slightly, to a TSI value of 0.75 units, at week 5. However, as the time continues the animals' progress regressed slightly and their TSI value dropped to 0.7 units and remains so for the rest of the test period. For the first 7 weeks the group implanted with BNIs filled with PTN performed similar to that of the group implanted with the empty BNIs. However, starting at week 7 the PTN group's TSI value began to show an upward trend that ended at 0.8 units at the end of the 9 week test period.

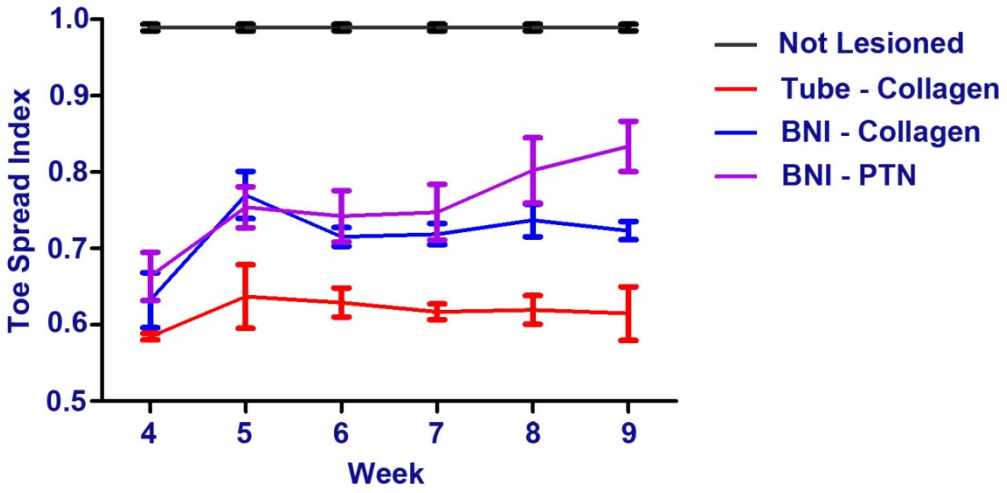

Figure 17. Toe-spread Index of animal groups over time.

The second behavioral test was to assess the animal's ability to sense and irritant on the dorsal part of its foot. Because of the caustic nature of test this test was performed once every two weeks instead of weekly. This test was not performed a third time at the 9 week time point because some of the animal developed sores at the injected site. It was found that uninjured animals would lick the injected site at an average of 17.33 ± 4.04 times in a ten minute time span. After the implantation of the nerve conduit the animals licked their foot only 1 or 2 times during the allotted time. At the end of the 7th week

the animal implanted with the empty tube filled with collagen did not improve, their lick response remained at 1 or 2 times during the allotted 10 mins time period. The group of animals implanted with BNIs performed significantly better in that they licked their foot at an average of 6.61 ± 2.05 times at the 7 week time point. However, there was no statistical difference between the different versions of BNIs; empty BNIs, BNIs filled with PTN, VEGF, or the combination of PTN and VEGF all performed similarly.

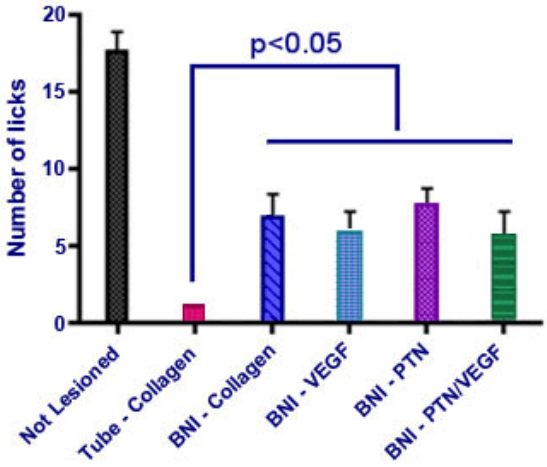

Figure 18. Comparison between animal's Toe-lick response within the different BNI groups and collagen filled tubes. The animals response to the stimulus was not significantly different between the different BNI groups.

The behavioral findings were later confirmed with the removal of the implants after the 9 week time span. The collagen filled tubes were found to have no regeneration. There was tissue growth into the conduit from both sides but after approximately 5 mm in the tissues abruptly stopped. All the other conduits were found to have tissue throughout the conduit. The differences were the amount of blood vessels and axon bundles in the different group. The VEGF containing BNIs had more noticeable blood vessels compared to the PTN containing BNI but vice versa with axon bundles. The BNIs incorporated with PTN and VEGF had about the same amount of axons and blood vessel as the BNI with PTN alone.

Figure 19 showed the photographs taken of the nerve conduits after they have been removed and fixed overnight in PFA.

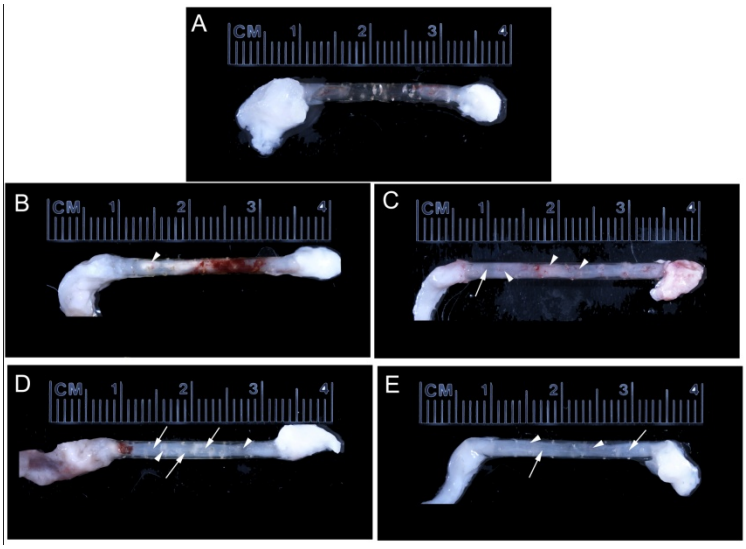

Figure 19. Gross anatomy of regenerated nerves. Nerves harvested from the 5 different groups: empty collagen tube (A), empty collagen filled BNI (B), VEGF filled BNI (C), PTN filled BNI (D), and PTN/VEGF filled BNI.

It was found that all the simple tube conduits failed to support axon regeneration. This was confirmed visually by the fact that the middle of the conduits was empty and did not contain any nerve tissue. These findings were later confirmed with immunohistochemistry staining of the distal end of the nerve outside the conduit. The staining showed that there were no axons present in the distal stump of the nerve. As Table 3 indicates the BNI group all performed better than the simple tube conduit did. Nearly half the empty BNIs had tissue all the way through its length. As expected the BNIs with neurotrophic support all had tissues throughout its length. Futhermore, there were visible blood vessels and axon fascicles inside the channels of the BNI. Nerve conduits that were counted as having axons in the distal end of the nerve had at least one noticeable fluorescent axon after immunohistochemistry staining. The few BNIs with trophic support that did not have complete tissue coverage had noticeable

bubbles in the agarose. These bubbles are the result of bad fabrication techniques and were removed from the group.

Table 3. Performance of all nerve conduits. Conduits with visible bubbles, resulting from bad fabrication techniques, were not counted. Blood vessels were recorded if clearly visible, However, unlike axonal density,

	Treatment					
	Not Lesioned	**Tube - Collagen**	**BNI - Collagen**	**BNI - VEGF**	**BNI - PTN**	**BNI - PTN/VEGF**
Tissue throughout conduit	N/A	0/3	4/6	5/6	5/5	4/5
Axons present at distal end	N/A	0/3	2/6	4/6	5/5	4/5
Blood vessel present in conduit	N/A	0/3	3/6	6/6	4/5	4/5

Immunohistochemistry was performed on paraffin sections taken from the distal section of the harvested nerve, outside the BNI. Because of Wallarian degeneration one can be fairly confident that any axon stained by the α-neurofilament protein antibody had regrew across the conduit. Figure 21 shows fluorescent images taken from the stained slides and the axon density found within each section.

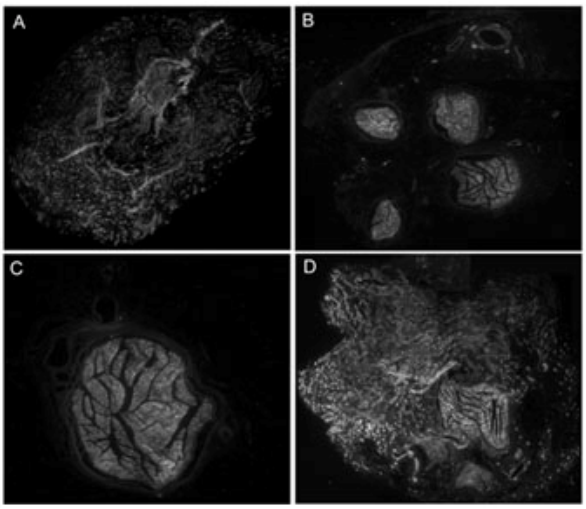

Figure 20. Immunohistochemistry staining of distal part of harvested nerves. Nerves were stained with mouse α-NFP primary antibodies and goat α-mouse Cy2 secondary antibodies. Images were taken with a 10x objective. A) BNI – Collagen B) BNI – VEGF C) BNI – PTN D) BNI - PTN/VEGF. Image of Tube – Collagen was not included because there was no fluorescence.

The density of the axons correlated well with the animal's motor behavior test. The fluorescent photograph of the distal stump connected to the simple tube was not included because there were not any axon present to be stained. The BNI filled with PTN performed the best, resulting in a nerve with the most amounts of axons growing through it. The BNI with the VEGF/PTN combination also had a significantly denser axon count when compared to the BNI filled only with collagen but it did not outperform the BNI containing PTN. Quantification results confirmed the absence of axons in the distal stump of the nerve repaired by the simple tube nerve conduit. The BNIs that contained PTN and the combination of PTN and VEGF, 40 ± 5 axons/1000 μm^2 and 38 ± 2 axons/1000 μm^2 respectively, performed significantly better that the BNI that contained collagen alone, 10 ± 1 axons/1000 μm^2.

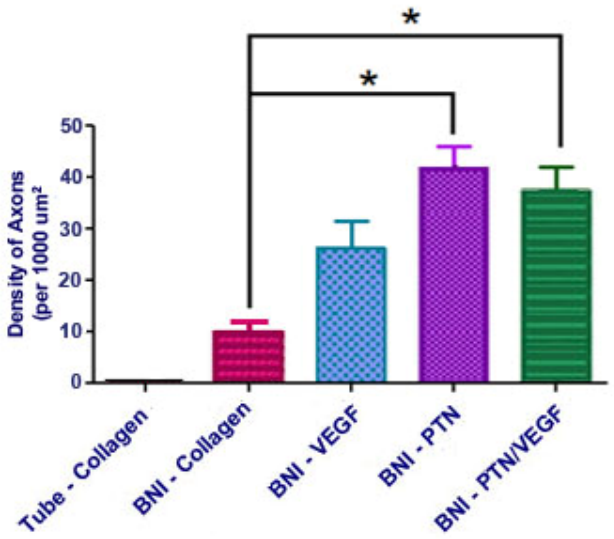

Figure 21. Quantification of axon density found in distal section of nerve. Five random 100 µm x 100 µm squares were chosen, number of axons inside each square was counted and then averaged with like groups.

CHAPTER 4

DISCUSSION

Currently the repair of a long nerve defect, 1.5 – 5.0 cm, in the clinical setting has seen little success. This is because all current FDA approved nerve conduits are simple hollow tubes (Meek and Coert 2008; Deumens, Bozkurt et al. 2010). These conduits cannot offer the physical and neurotrophic support required by the regenerating nerve. There is a need for an easily fabricated nerve conduit that mimics the natural anatomy of a nerve. The BNI presented in this work can be fabricated very easily and its design is similar to that of a normal nerve bundle. Furthermore, the incorporation of collagen channels within the agarose effectively increased the surface area of the conduits. A previous study demonstrated that BNIs can reproducibly achieve large fascicles of regenerating axons across a 10 mm gap in a rat model. When compared to a simple tube the BNI performed remarkably better, this was then confirmed by the current study. Functionally the BNI performed significantly better than the simple tube nerve conduit. There was a large statistical difference between the TSI and lick response when comparing the simple tube conduit group with the empty BNI group alone. Furthermore, when the nerve conduits were harvested all simple tubes were found to have failed completely while only half of the empty BNIs have failed. The success of the BNI can be partially attributed to its increased surface area created by the incorporation of channels through its lumen. The multiluminal design provided satellite cells and axons more surfaces to attach and migrate into the interior of the conduit. This design demonstrated that without making many changes it is possible to increase the effectiveness of a nerve conduit many fold when comparing to the current nerve conduit of today. While this seem to be the case base on our behavioral data, the number of animals with clear axonal regeneration in the tissue did not math the number of those showing fuctional recovery. Behaviorally all 7 animals improved over time and

performed significantly better than the negative control group. Immunohistochemistry, however, showed that only 2 of the 7 animals in the BNI-collagen group had axons present in the distal stump of the severed nerve. This inconsistency can perhaps be expaied by the limted sensitivity of the histological assay, In order to test this possibility, that a more sensitive visualization technique such as Electron microscopy can be implemented for the visualization of nerve axons.

A second advantage of the multiluminal design provides researchers with a way to uniformly distribute growth factors throughout the conduit to provide neurotrophic support to the regenerating axons. This is especially important in a long gap, the conduit itself is acelluar and will need to be infiltrated by all the different type of cells present in a normal nerve bundle. This was verified to be true when the BNIs were incorporated with the pleiotrophic compound PTN. The best performing group was from the animals that received BNIs filled with PTN loaded particles. The only test that did not showed a positive effect when PTN was added was the sensory test. While the BNI design did performed better than the empty tube design there was no statistical difference between the different BNI groups, PTN, VEGF or the combination of PTN and VEGF. This could be explained by the assumption that PTN and VEGF both only affect motor neurons and therefore did not have any neurotrophic affect on the sensory neurons of the peroneal nerve.

From the behavioral and immunohistochemistry data it is safe to conclude that a synthetic nerve conduit incorporated with a multi-luminal design infused with the pleiotrophic factor PTN was effective in axonal regeneration across a large nerve gap. However, the combination of VEGF and PTN did not seem to have a significant impact on axonal regeneration. Since PTN is able to stimulate axon regeneration and angiogenesis by stimulating endothelial cell. It is possible that PTN is masking the presence of VEGF, since VEGF's main role was to encourage angiogenesis.

In this study, we have shown that a conduit incorporated with a multiluminal design is effective in axon regeneration across a long gap. Furthermore, we have shown that by adding pleiotrophic factors, such as PTN, the effectiveness of the nerve conduit can be increased many fold. However, the addition

of multiple pleiotrophic factors that overlap in effects did not have a synergistic effect on the regeneration of axons across a large nerve defect.

At the moment the amount of PTN incorporated into a channel is limited by the size of the particle used to carry the factors. It might be beneficial if a way to increase the loading efficiency of PTN into PLGA particles and/or decreasing the size of the particle could be found. A second direction in the research could be to find a combination of growth factors that could encourage both motor, PTN, and sensory neurons, perhaps nerve growth factor. Combining VEGF and PTN was a novel idea but it was demonstrated that PTN did not need the help of VEGF to encourage axon and blood vessel growth, PTN was able to do this by on its own. However, neither PTN nor VEGF proved effective at repairing the subject's ability to feel. Incorporating a growth factor that is known to target sensory neurons might be beneficial. There are still many unknown variables in the use of PTN incorporated BNIs to repair a nerve with a large defect.Ideally it would be preferable for a BNI with no biologically active materials added to be used in the repair of a severed nerve. However, our data shows that an empty BNI, although better than an empty tube nerve conduit, cannot reliably support axon regeneration across a long nerve defect. The need for neurotrophic support, in our case PTN, is evident in the data reported above. A main limitation for the use of growth factors is the need forFDA approval In the case PTN, this may be complicate by some reports indicatedthe potential carcinogenic effect of PTN (Jin et al. 2009). Our data indicates that PTN did not cause tumor formation in the transected peripheral nerve, and thus this may not be a concern for long-gap repairs. In addition the neurotrophic factors inside the BNI are at at a very low concentration and restricted to the nerve, so that a general negative effect is unlikely. The restrictive volume of the individual channels where the factors are localized can effectively concentrate the neurotrophic factors to that of physiological levels while at the same time keeping the needed level of neurotrophic factors low enough that it will not greatly affect the bodye.

In summary, this work shows that a multiluminal design of the biosynthetic nerve implant does indeed provide the required support needed by a severed nerve to regenerate. Furthermore, we have

shown that neurotrophic support, such as Pleiotrophin, is essential for the regeneration of axons over a large, > 3 cm, gap. Success for the repair of a long gap nerve defect is therefore dependent on the inclusion of both components.

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BIOGRAPHICAL INFORMATION

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