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Effects of Electrical Stimulation and Testosterone in Translational Models of Peripheral Nerve Injury

Gina Monaco

Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE IN
TRANSLATIONAL MODELS OF PERIPHERAL NERVE INJURY

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY
GINA NICOLE MONACO

CHICAGO, ILLINOIS
MAY 2013
Dedicated to my parents,

Ruth and Antonio,

for their constant support.
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<tr>
<td>A</td>
<td>Ampere</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AR</td>
<td>Androgen Receptor</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
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<tr>
<td>BOS</td>
<td>Base of Support</td>
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<tr>
<td>cAMP</td>
<td>3’-5’-cyclic adenosine monophosphate</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>DC</td>
<td>Direct Current</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>DPO</td>
<td>Days Post-Operative</td>
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<tr>
<td>ES</td>
<td>Electrical Stimulation</td>
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<tr>
<td>EXP(B)</td>
<td>eB (odds ratio coefficient)</td>
</tr>
<tr>
<td>FMN</td>
<td>Facial Motor Nucleus</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth Cone-Associated Protein-43 kilodaltons</td>
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<tr>
<td>GDNF</td>
<td>Glial Derived Neurotrophic Factor</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary-Acidic Protein</td>
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<tr>
<td>GRBAS</td>
<td>Grade of Hoarseness, Roughness, Breathiness, Asthenia, and Strain</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IAC</td>
<td>Internal Auditory Canal</td>
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<tr>
<td>ID/OD</td>
<td>Inner Diameter/Outer Diameter</td>
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<tr>
<td>K/X</td>
<td>Ketamine/Xylazine</td>
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<td>L</td>
<td>Lumbar</td>
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<tr>
<td>NT-4/5</td>
<td>Neurotrophin-4/5</td>
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<tr>
<td>PACAP</td>
<td>Pituitary Adenylate Cyclase-Activating Peptide</td>
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<tr>
<td>Par-3</td>
<td>Protease activated receptor-3</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<tr>
<td>RLN</td>
<td>Recurrent Laryngeal Nerve</td>
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<tr>
<td>R2</td>
<td>Regression Coefficient</td>
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<tr>
<td>S</td>
<td>Sacral</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulins</td>
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<tr>
<td>SMF</td>
<td>Stylomastoid Foramen</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TENS</td>
<td>Transcutaneous Electrical Nerve Stimulation</td>
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<tr>
<td>TP</td>
<td>Testosterone Propionate</td>
</tr>
<tr>
<td>trkB</td>
<td>Tyrosine Kinase B</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>WPO</td>
<td>Weeks Post-Operative</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>VFM</td>
<td>Vocal Fold Mobility</td>
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CHAPTER I

INTRODUCTION

Peripheral nerve injuries constitute a large portion of neurotrauma seen in clinical settings and are associated with limb damage, skeletal fractures, and surgical interventions made to correct other medical problems. Though peripheral motoneurons are capable of regeneration following axonal injury, suboptimal functional outcomes usually follow from such damage in clinical situations. These suboptimal outcomes negatively impact patients’ quality of life, examples of which include the impairment of emotional expression after facial nerve paralysis, the difficulties in properly intoning speech following recurrent laryngeal nerve paralysis, and the altered stance and gait patients experience following paralysis of nerves innervating the leg. Animal and in vitro models that mimic the types of peripheral nerve injuries seen clinically need to be developed so that therapies, those in use as well as potential ones, can be evaluated side-by-side. Our laboratory has developed both extratemporal and intratemporal facial nerve crush models and has extensively explored the effects of treatments including the gonadal steroid testosterone and electrical stimulation, as well as their combination. Emerging from these studies is the idea that there are distinct post-injury stages of cellular repair that occur, and these stages can be augmented by a combinatorial treatment strategy to promote repair and increase functional recovery. The combinatorial therapy of
testosterone and electrical stimulation has been shown to be beneficial in two models of facial nerve injury; however, it is unknown whether this benefit applies in other peripheral nerve injury situations. This dissertation intends to develop distinct clinically relevant peripheral nerve injury models in order to investigate the generality of the effects of a combinatorial treatment consisting of testosterone and electrical stimulation on functional recovery from peripheral nerve injury.

**Aim 1: Determine the effects of the combination of brief ES and TP on functional recovery following intracranial facial nerve crush in rats.** *The working hypothesis is that administration of the combination treatment consisting of TP and brief ES soon after injury shortens recovery time after an intracranial facial nerve crush.* Immediately following an intracranial crush, gonadectomized adult male rats will receive implants of empty or TP-filled capsules and/or a one-time ES treatment 30min in duration or sham stimulation. Daily facial functional recovery and weekly motor nerve conduction will be assessed up to 12 weeks post-operative and will be compared between the different treatment groups (No TP/No ES and TP+ES).

**Aim 2: Determine the effects of brief ES and/or TP on functional recovery following recurrent laryngeal nerve crush axotomy in rats.** *The working hypothesis is that the administration of TP and brief ES soon after injury shortens recovery time after recurrent laryngeal nerve crush.* Gonadectomized adult male rats will undergo left recurrent laryngeal nerve crush axotomies, will receive empty or TP-filled capsule implants, and will receive a one-time ES treatment for 30min or sham-stimulation. The
rats will be divided into 4 treatment groups: 1) No TP/No ES, 2) TP-only, 3) ES-only, and 4) TP+ES. For each group, direct laryngoscopy will be used to compare vocal fold mobility between the different treatment groups at 1, 2, 3, 4, 5, and 6 weeks post-operative.

**Aim 3: Compare the effects of the combination treatment of brief ES and TP on functional recovery following both a sciatic nerve crush as compared to a sciatic nerve transection-surgical repair (autograft) injury in rats.** The working hypothesis is that the combination treatment of brief ES and TP will shorten recovery time after a crush injury to the sciatic nerve in a similar manner to that of an autograft injury of the sciatic nerve. Gonadectomized adult male rats trained to run on the CatWalk apparatus (Noldus) will undergo either a right sciatic crush or a right sciatic nerve transection with autograft repair at the level of the mid-thigh. Immediately afterward, rats will receive implants of TP-filled or empty capsules and/or a one-time ES treatment of 30min, and will be divided into the same 2 treatment groups as in aim 1. The CatWalk apparatus will be used weekly to compare gait changes between the different treatment groups, and assessments will be done at 2, 4, 8, and 16 weeks post-operative.
CHAPTER II
LITERATURE REVIEW

A. INTRODUCTION

The nervous system is divided into two parts; the central nervous system (CNS) consists of the brain and the spinal cord and the peripheral nervous system (PNS) consists of all of the remaining nervous tissue throughout the body. The functional unit of the PNS, the peripheral nerve, is commonly damaged during bodily trauma and can be damaged during surgery; often the final result is substandard recovery from the nerve injury. Though neurons in the PNS are capable of regenerating their injured axons, recovery is dependent on the amount of neuronal cell loss, regeneration rate, and successful target reinnervation. Currently no treatment regimen exists that reliably promotes regeneration following peripheral nerve trauma.

Three peripheral nerves are frequently injured in humans and can thus be used to design and test treatment strategies that enhance regeneration. These nerves are the facial nerve, the recurrent laryngeal nerve, and the sciatic nerve. The facial nerve has a complex anatomical course from the brainstem to the muscles it innervates. Recovery from facial nerve paralysis is dependent on the site of injury and so development of different animal models representing these different injury sites will be beneficial to both determine the degree of regeneration and to examine the effects of potential treatments. The recurrent laryngeal nerve, a branch of the Vagus nerve, also maintains a complicated course as its
axons journey from the brainstem through the neck and thorax and wind up back in the neck to innervate muscles in the larynx. This complicated course makes the nerve vulnerable to injury during neck surgeries and presents an ideal opportunity to examine the effects of potential treatments. The sciatic nerve is the largest nerve in the body and the consequences of its injury are debilitating, making it a prime target to test the recovery potential of new therapies.

Gonadal steroids and electrical stimulation (ES) are two interventions that have the potential to be translated to the clinic. Previous work indicates that their combination enhances axonal regeneration and improves functional recovery; however, since this has only been investigated in facial nerve injury paradigms it still remains to be seen if this combination therapy is widely applicable to peripheral nerve injury in general.

B. PERIPHERAL NERVOUS SYSTEM INJURY

Whereas the mature central nervous system (CNS) usually does not regenerate after injury, the peripheral nervous system (PNS) has the advantage of a permissive environment in concert with the activation of its intrinsic growth capacity which aids regeneration (Vargas and Barres, 2007). Despite this regenerative potential, functional recovery following peripheral nerve injury is often suboptimal. Recovery outcome is variable and depends on a multitude of factors: the proximity of lesion to the cell body, regeneration distance to target, and age of patient (Fu and Gordon, 1995a; Fu and Gordon, 1995b; Birch and Raji, 1991; White and Vaughan, 1991; Vaughan, 1992). Peripheral nerves are surrounded with fatty cushioning at locations that are vulnerable to mechanical perturbations, but they are still frequently damaged by physical insults. Such
insults include disruption of the myelin sheath causing conduction blocks or severe forces that crush, stretch, or sever the nerve.

1. ANATOMY OF A PERIPHERAL NERVE

   The epineurium is the outer sheath of an entire peripheral nerve; it consists of condensed layers of connective tissue made of collagens (Sunderland, 1991; Lee and Wolfe, 2000). Each peripheral nerve trunk is composed of multiple bundles or fascicles of nerve fibers. As the epineurium encircles and runs between fascicles, its main function is to sustain and protect them. Each nerve fascicle is surrounded by a well-defined sheath known as the perineurium; it contributes to nerve tensile strength. Within the fascicles, each individual axon and its Schwann cells are surrounded by the endoneurium and endoneurial fluid, a low-protein fluid that is the correlate of cerebrospinal fluid to the peripheral nervous system (Sunderland, 1991). The endoneurium functions to cushion and nourish the individual axons.

   A network of blood vessels supplies blood to a peripheral nerve. Of the two major arterial systems, one lies superficially on the nerve and the other lies within the interfascicular epineurium; the two are connected via anastomoses to a minor longitudinal system (Sunderland, 1991). The endoneurial capillaries act as an extension of the blood-brain barrier; the tight junctions between the endothelial cells create a system impermeable to a wide range of constituents and serve to separate the endoneurial fluid from the blood. Injury, ischemia, or toxins can disrupt this barrier.
2. INJURY CLASSIFICATIONS

In 1943, Seddon grouped nerve injuries into three major classifications in increasing severity: neurapraxia, axonotmesis, and neurotmesis. Neurapraxia is defined by local myelin damage which usually results from nerve compression. Axonotmesis is a loss of continuity of axons, with variable damage of the endoneurial and perineurial sheaths; however, the epineurium is intact. Finally, neurotmesis is characterized by a disruption of the epineurial sheath and the complete physiologic perturbation of the entire nerve trunk, such as in a complete transection injury (Seddon, 1943). After injury, functional losses occur in the following sequence: motor, proprioception, touch, temperature, pain, and sympathetic (Lee and Wolfe, 2000). Recovery usually occurs in the reverse order and incomplete injuries are more frequent than complete separation. Furthermore, mixed nerve injuries, in which all fibers are affected but to varying degrees, are fairly common.

3. CLINICAL PICTURE: COMMON PERIPHERAL NERVE INJURIES

The facial, recurrent laryngeal, and sciatic nerves are three commonly injured nerves which often lead to unsatisfactory or delayed recovery and thus serve as targets for new therapeutic interventions. Though they are disparate and in completely separate parts of the body, a common thread that binds them is that injury to each yields noticeable functional deficits.
a. Facial Nerve Injury

As the most commonly injured cranial nerve, lesions of the facial nerve occur at an incidence of ~30 cases per 100,000 (Gilden, 2004). Not only do such lesions result in noticeable distortion of the face, but other complications can occur. The eyelid droop characteristic of facial nerve palsy is due to the paralysis of the temporalis muscle; the temporalis muscle is then unable to raise the eyebrow and subsequently pushes the eyelid tissue down over the eye. Though the eyelid itself can open because cranial nerve III controls the levator palpebrae superioris muscle, it has difficulty closing because the orbicularis oculi is responsible for closing the eyelid and is innervated by the facial nerve. This increases exposure of the eye and can result in dry eye, infections, and in severe cases, corneal ulcers and loss of the eye from perforation (Kerrebijn and Freeman, 1998). Other complications can include drooling, speech difficulties, and inability to retain airway patency (Rosson and Redett, 2008). Another common complication in patients with facial nerve injury is synkinesis, or the involuntary movement of part of the face during the voluntary movement of another part of the face. (Kiese-Himmel et al., 1993; Mavrikakis, 2008).

i. Anatomy & Etiology

The facial nerve is particularly susceptible to injury because of its elaborate anatomical course from the brainstem to the muscles it innervates. The three main segments of the facial nerve are intracranial, intratemporal, and extratemporal. The intracranial segment of the nerve arises near the pons, travels within the cerebellopontine angle and enters the temporal bone through the internal auditory meatus (Figure 1). After
Figure 1: Course of the Facial Nerve. Upon exit from the facial nucleus, the facial nerve runs intracranially, then enters the internal auditory meatus (IAM), turns in the facial canal, and exits the mastoid bone at the stylomastoid foramen (SMF). Different types of injuries can occur at different segments of the nerve: 1) extratemporal 2) intratemporal and 3) intracranial. CPA (cerebellopontine angle).
entering the temporal bone, it is confined within a prolonged canal, which is often not much greater in diameter than the nerve itself (intratemporal segment). The nerve exits the skull at the stylomastoid foramen (SMF) and from thenceforth is termed extratemporal; it then travels through the parotid gland in its course towards the facial muscles. After branching into five main divisions and many smaller divisions, it exits the parotid gland to finally reach its targets. The course of the facial nerve through the posterior fossa, temporal bone, and parotid gland renders it vulnerable to many neoplastic, traumatic, and infectious events.

Lesions of the facial nerve within the cerebellopontine angle and the internal auditory meatus are usually due to compression by acoustic neuromas, meningiomas, or tumors of the jugular vein (Mavrikakis, 2008; Rosson and Redett, 2008). Surgical removal of these neoplasms can pose additional damage to the nerve. Lesions of the facial nerve within the temporal bone are due to Bell’s palsy, skull fractures, spread of inner ear infections, and petrous-temporal cancer. Bell’s palsy is the most common sudden cause of facial nerve paralysis and is believed to result from inflammation of the facial nerve during its course in the temporal bone due to reactivation of herpes simplex virus-1, leading to compression and possibly ischemia and demyelination (Tiemstra and Khatkhate, 2007). Extratemporal injuries of the facial nerve may result from traumatic injuries (lacerations and gunshot wounds) and complications arising from the removal of parotid gland tumors.
ii. Evaluation & Management

The House-Brackmann grading scale is the gold standard for grading facial nerve function (House and Brackmann, 1985). This system relies on evaluation of symmetry of the face during rest, degree of voluntary movement on the affected side, and degree of synkinesis, with scores assigned from 1-6 in increasing severity of dysfunction. The degree of nerve damage is often also assessed by nerve conduction studies. A reduction in the compound muscle action potential suggests axonal degeneration has occurred, and an increase in latency indicates the nerve has suffered demyelination (Kimura, 2006).

Several medical and surgical interventions are currently used for facial nerve injuries. Incomplete paralysis has an excellent prognosis and is managed purely by observation (Danner, 2008). Following complete facial nerve paralysis, the use of corticosteroids is suggested to minimize nerve edema and degradation in nerve function. Whether corticosteroids should be administered, however, is still controversial, as their efficacy has not been clearly demonstrated (Turk-Boru et al., 2005). The administration of corticosteroids to rats with experimental intratemporal facial nerve injury does not provide added benefit to other treatments studied (Sharma et al., 2010a); hence, the side effects of exogenous corticosteroids may outweigh the benefits. Current surgical interventions include decompression, anastomoses, and nerve grafting. Decompression involves removing bone to relieve pressure on the nerve from its surroundings. When the facial nerve is transected or sufficiently injured, requiring the injured portion to be removed, an end-to-end anastomosis (repair with suture or glue) is performed (Danner, 2008). When additional nerve length is needed, a cable nerve graft is used. Even though these surgeries re-establish connection between the ends of the facial nerve, regeneration
of axons across the reapposition site may be hindered. Therefore, the need to develop better treatments prevails as suboptimal functional recovery is often seen.

b. Recurrent Laryngeal Nerve (RLN) Injury

Recurrent laryngeal nerve lesions result in significant patient morbidity, causing vocal fold paralysis and incomplete glottic closure which can lead to changes in vocal pitch or a lack of voice, dysphonia, dysphagia, and in some cases aspiration (Araki et al., 2005; Tessema et al., 2009). The effects of these symptoms on patient quality of life can be devastating and recovery from this type of injury is variable as well as dependent on the degree of the initial damage; some patients recovery spontaneously within weeks while others never do (Myssiorek, 2004; Rosenthal et al., 2007). Changes in vocal pitch and quality, when accompanied with increased vocal effort, are particularly difficult to deal with for patients because the voice, like one’s face, is a distinguishing characteristic separate to each individual. A patient with a radial nerve injury experiences loss of function of a part of her body of which she possesses, whereas a patient with RLN paralysis often registers a more significant loss of self which can be considerably more distressing. Thus as with facial nerve paralysis, RLN paralysis can result in emotional disturbances subsequent to the change in one of the sufferer’s defining features.

i. Anatomy & Etiology

The RLN follows a long and indirect course from where it branches off the vagus nerve to its laryngeal entry (Figure 2). Its passage through the thorax and course through the thyroid gland make it particularly vulnerable to damage during surgery involving the
Figure 2: Course of Recurrent Laryngeal Nerves and Surrounding Structures. Both left and right RLNs pierce the thyroid gland, making them susceptible to damage during thyroidectomies. Figure adapted from www.ghorayeb.com
neck or thorax. The RLN is one of the longest branches of the cranial nerves, branching from the descending Vagus nerve at the level of the mediastinum, looping around the aortic arch on the left and the subclavian artery on the right, then traveling superiorly along the tracheo-esophageal groove. It enters the larynx on both sides behind the articulation of the inferior cornu of the thyroid cartilage and the cricoids, where it supplies sensory fibers to the internal surface of the larynx inferior to the vocal folds and innervates all of the intrinsic muscles of the larynx, except the cricothyroid muscle. The motor fibers stimulate abduction and adduction of the vocal cords for phonation, breathing, and protection of the lungs during swallowing.

RLN injury is a known complication of surgery in the neck and thorax. It has been reported that iatrogenic surgical injury to the RLN accounts for 46.3% of cases of unilateral vocal fold paralysis; the majority of these procedures are thyroidectomies (Rosenthal et al., 2007). Case series suggest that vocal fold palsy occurs in 1.8 – 4.7% of thyroid surgeries (Erbil et al., 2007; Chan et al., 2006). Other procedures during which the RLN is more prone to injury include anterior approaches to the cervical spine, open heart surgery, carotid endarterectomy, and other surgeries to the neck and thoracic regions. The nerve can also be compressed by tumor growth in the compact neck region: thyroid, laryngeal and esophageal carcinomas; paragangliomas, and lung cancer are some of the more common culprits (Myssiorek, 2004).

ii. Evaluation & Management

A variety of measures are used to assess vocal fold function, which includes voice production, vocal fold mobility, and glottic closure. Voice quality is assessed with the
vocal handicap index (Jacobson et al, 1997) and the grade of hoarseness, roughness, breathiness, asthenia, and strain (GRBAS) scale (Hirano 1981). The vocal handicap index is a questionnaire consisting of 3 subscales measuring functional, physical, and emotional handicaps in verbal communication, with scores ranging from 0-120 increasing in severity of dysfunction. The GRBAS scale assesses each of the five aforementioned vocal dysfunctions on a scale of 0-3 (normal to severe). In addition to these subjective measures, the vocal folds can be examined by laryngoscopy and videostroboscopy. Visualization of the larynx can be done with a flexible or rigid endoscope in order to observe the movement of the vocal folds. Videostroboscopy involves using a strobe light to illuminate the vocal folds and parse out individual vibration cycles. Stiffness, irregularity of movement, and glottic closure can all be visually observed.

Intraoperative monitoring of nerve conductivity is standard procedure in operating rooms during surgeries that put the RLN at risk; however, this has only been used as a pre-emptive measure. Current surgical interventions focus on bringing the injured vocal fold as medially as possible to improve contact with the other fold or directing another intact but less important nerve to the laryngeal muscles. The medialization techniques include injection laryngoplasty, which involves injecting a substance (collagen, tiny beads) into the fold to increase bulk, and medialization laryngoplasty, which consists of suturing the arytenoid cartilage in a medial position to the thyroid cartilage. These approaches are clever but do not promote recovery of the injured RLN or movement of the injured vocal fold. The anastomotic techniques all require the injury of a separate intact nerve in order to reconnect it to either the injured RLN or the muscles of the larynx. Unfortunately these methods require the sacrifice of a healthy, though
admittedly less vital, nerve and they yield variable success rates (Roh et al., 2009; Aynehchi et al., 2010; Hartl et al., 2005; El-kashlan et al., 2001).

c. Sciatic Nerve Injury

Sciatica is a general term describing pain and/or numbness of the hip and lower extremity due to pathologies affecting the sciatic nerve in its intraspinal or extraspinal course. Up to 40% of adults experience sciatica at some point throughout their lives; its most frequent cause is of intraspinal origin—a herniating lumbar disc pressing on the neural roots (Ergun and Lakadamyali, 2010). Patients who undergo sciatic injury lose motor function of the posterior compartment of the thigh as well as the entire leg in addition to suffering from pain and numbness. The classic motor symptom usually first reported in injuries involving the sciatic nerve is the foot drop, which is an inability to dorsiflex the foot resultant from an impaired anterior tibialis muscle, as the motor axons originating from the L4 and L5 nerve roots are the longest and the most susceptible to injury. Patients with this symptom compensate by exaggerating the lifting of the thigh during walking, producing a steppage gait which can produce balance defects. Impaired locomotion, pain, and impaired balance leads to great distress for patients.

i. Anatomy & Etiology

The sciatic nerve is the largest and longest peripheral nerve in the human (and rodent) body. Consisting of the axons from L4-S3, it measures approximately 2 cm in diameter at the sciatic notch. It curves slightly to begin its descent into the thigh and passes under the piriformis, where it can be trapped and compressed. From there, it
descends within the sciatic sheath, passes deep to the biceps femoris, after which it bifurcates at the apex of the popliteal fossa into the tibial and common peroneal nerves to innervate both the posterior and anterior muscles of the leg and foot, respectively (Figure 3). Though it appears to be one nerve that splits in two, both the tibial and common peroneal nerves have their own investing sheaths and axons from the two nerves do not co-mingle.

Injury to the sciatic nerve can arise from a wide variety of causes including traumatic injuries to the lower limb, neoplasms in the pelvis, thigh, and leg, lumbar instability, spondylolisthesis, slipped discs, vertebral facet hypertrophy, piriformis syndrome, herpes zoster, neurodegenerative diseases, and iatrogenic insults including hip joint surgery and intramuscular injections, particularly those to the gluteus maximus. Even a retroverted uterus can restrict and compress the nerve, causing damage (Ergun and Lakadamyali, 2010; Kulcu and Naderi, 2008).

ii. Evaluation & Management

Because the joints of the lower limb have such wide ranges of motion, the neuromuscular exam alone allows the clinician to localize nerve injury in the sciatic distribution with a good degree of accuracy. Subsequent to examination of the reflexes, strength, and range of motion, provocative tests have been devised to examine the sciatic nerve. Nerve conduction studies and electromyograms are typically performed to quantify nerve damage. Compound muscle action potentials and needle electromyograms are gathered from distal-most muscles first such as the extensor digitorum brevis and the abductor hallucis (Derr et al., 2009; Masakado et al., 2008). As
Figure 3: Course of the Right Sciatic Nerve. Arising from the lumbar plexus, the axons from L4-S3 travel inferiorly in the posterior compartments of the thigh and leg to innervate many target muscles. As it exits the pelvis, the sciatic nerve emerges from a small space between the piriformis and the obturator internus and can be trapped here. It is particularly vulnerable to injury as it enters the popliteal fossa; while it is traversing this fossa in its most superficial location along the lower limb, it is also dividing into the common peroneal and tibial nerves to supply the muscles of the leg. From Gray’s Anatomy.
with the facial nerve, a reduction in the compound muscle action potential suggests the occurrence of axonal degeneration while an increase in latency indicates demyelination (Kimura, 2006).

The sciatic and facial nerves share some medical and surgical interventions, namely the administration of corticosteroids, decompression, anastomoses, and nerve grafting. However, neurolysis, the removal of the outer layer of nerve sheath, has been used in cases of iatrogenic injury to the sciatic nerve (Senes et al., 2009) as well as electrical stimulation of the nerve to relieve pain. This stimulation is done with needle electrodes; they can be placed percutaneously with or without ultrasound guidance or into the area through an open surgical procedure (Chan et al., 2010). A portion of cases of weakness are due to chronic, repeated injury such as by crossing the legs one over another; these have good prognosis for spontaneous resolution. Injuries that require surgical reanastomosis or nerve grafting typically have different management strategies depending on the type of injury. In clean lacerating injuries in which the nerve ends are visible and or obvious deficits result, immediate end-to-end repair may be warranted. However, blunt transections due to lacerations tend to have better functional outcomes if surgery is delayed, even up to a year depending on the severity of the injury (Kim and Kline, 1996).
4. ANIMAL MODELS

a. Facial Nerve Injury

Models of experimental facial nerve injury in animals described in the literature include extratemporal transection and crush, intratemporal crush, and intracranial transection of the facial nerve (Mattsson et al., 1999; Jones et al., 2001; Sharma et al., 2009a). Thus far, an animal model of intracranial facial nerve crush injury has not been well characterized even though a majority of intracranial facial nerve injuries result from a stretching or compression of the nerve rather than a transection. Effects from extratemporal injuries were described by Nissl in 1892; since then, crush and transection injuries have been studied extensively in rodents with great success in elucidating molecular mechanisms underlying the injury response and subsequent repair process that can occur in peripheral motoneurons. Unlike humans, animals tend to recover functionality rather quickly from crush (axonotmesis) injuries (Hetzler et al., 2008) but complete functional recovery from transection (neurotmesis) injuries does not occur. Since most of the cases of facial palsy are due to Bell’s palsy plus intracranial tumors and complications regarding their removal, more clinically relevant models were needed. Sharma and colleagues described a crush injury to the intratemporal portion of the nerve while other groups developed ischemic models of facial nerve injury to model Bell’s palsy (Sharma et al., 2009a; Takeda et al., 2008). Yet another group developed an intracranial facial nerve transection model in rats; however, this model is not useful for studying the effects of therapy on functional recovery as it results in neurotmesis and the ends are left separated (Mattsson et al., 1999; Mattsson et al., 2006). One study involving
an intracranial crush was published by this group but they only observed their animals for three weeks after injury (Mattsson et al., 2001). To date, no reliable injury model has been demonstrated for the purpose of studying functional recovery after intracranial facial nerve injury.

b. Recurrent Laryngeal Nerve Injury

Animal models of experimental RLN injury include crush, transection, and transection with end-to-end repair. The phenomenon of synkinesis, due to aberrantly regrowing axons reinnervating the wrong muscles, has been documented in a transection-repair model (Flint et al., 1991). However, until recently, most of the literature in this model has focused on the transection or transection-repair models since rodents display spontaneous behavioral recovery following both types of injuries (Bamba et al., 2000; Nakagawa et al., 2004). The timeline of behavioral recovery in rats that have undergone a RLN crush injury has been established, but it is a relatively new model system as compared to the facial nerve axotomy (Tessema et al., 2009; Shiotani et al., 2007).

c. Sciatic Nerve

Experimental models of sciatic nerve injury in animals are varied, ranging from clip-compression to transection and repair to chronic denervation. Because of its large size as well as its ease of dissection and manipulation, it is ideal to work with in rodent models of nerve injury. Additionally, since a well-functioning sciatic nerve is vital to appropriate gait in rodents, injuries can be easily studied at both macro and micro levels and related to human function. Gait analysis post-injury has advanced from dipping hind
paws in paint in order to record the footprints to using video capture of rodents traversing illuminated walkways and dynamic analysis using treadmill walking (DeMedinacelli et al., 1982; Deumens et al., 2007; Beare et al., 2009). The sciatic nerve has also been used to examine the soma response to axonal disconnection as well as the molecular regenerative response post-injury, as there are some differences between spinal and cranial motoneurons (Powell et al., 1979; Schlaepfer and Hasler, 1979). Nerve grafting techniques have been practiced on rodent sciatic nerves, as well as promising graft substances been tested in rodents models of sciatic injury (Richardson et al., 1982; Waitayawinya et al., 2007). The sciatic nerve crush axotomy model has been well studied in rodents and the timeline for functional recovery is documented (Forman and Berenberg, 1978; Alberghina et al., 1983; Walker et al., 1994; Brown et al., 1999; Ferri and Bisby, 1999; Oliveira et al., 2001; Lago and Navarro, 2006). Sciatic transection, repair, and grafting techniques have been similarly studied; however, due the lack of a patent epineurium in these models, the functional recovery timeline has significantly greater variability (Hare et al., 1992; Ijkema-Paassen et al., 2002; Hamilton et al., 2011).

C. NEUROTHERAPEUTIC ROLE OF GONADAL STEROIDS

A variety of hormones, including gonadal steroids, glucocorticoids, and thyroid hormones, have been examined as neurotherapeutic agents for the injured or diseased nervous system. The many studies on gonadal steroids have distinguished them as potential agents for therapy. Estrogen affects not only learning, memory, behavior and cognition, but has also been shown to have neuroprotective and neuroregenerative effects (Green et al., 1997; McEwen and Alves, 1999; Matsumoto and Arai, 1981). Progesterone
has been shown to rescue motoneurons from degeneration and promote recovery from spinal cord injury (Thomas et al., 1999; Gonzalez et al., 2002). Androgens have been demonstrated to have a neurotherapeutic impact on various disease conditions and to enhance peripheral nerve regeneration, as described below.

1. ANDROGENS

Androgens have a wide variety of functions due to their ability to target numerous body tissues, including reproduction, development of primary and secondary sexual characteristics, increasing muscle mass, and affecting neuronal functioning. During development of the nervous system, the presence or absence of androgens determines gender-specific morphological and behavioral patterns of the adult (Kaiser and Morley, 1994; Beyer and Hutchison, 1997). Within the brain, androgens not only exert long-term organizational (permanent) effects during the developmental period, but they also have transient activation (reversible) effects during regenerative events (Arnold and Gorski, 1984).

Gonadal steroids are synthesized from cholesterol primarily in the adrenal cortex and the gonads (Champe and Harvey, 1994). The majority of testosterone production takes place in the Leydig cells of the testes. After it is secreted, testosterone is delivered via protein-bound transport to target organs. Approximately 60-70% of testosterone is tightly bound to sex hormone binding globulins (SHBG), 30-40% is bound loosely to albumins, and 0.5-2% is free (Iqbal et al., 1983). In addition to differences in production levels between the sexes, gender differences in SHBG levels influence the circulating levels of testosterone in males and females (Bialek et al., 2004). Also, the increase in
SHBG levels over time reduces the levels of free testosterone (Leifke et al., 2000). Free testosterone can cross the blood brain barrier and have effects on the CNS and the PNS (Champe and Harvey, 1994). Its lipid solubility allows the hormone to easily cross the cell membrane and bind to its intracellular receptor, the androgen receptor (AR). Testosterone can act either directly through an androgen pathway or indirectly through its aromatization to estrogen.

2. MECHANISM OF ACTION

Steroid hormones act on the nervous system using two different mechanisms, genomic and non-genomic. The genomic mechanism is slow-acting and involves binding of the hormone to its nuclear receptor. Subsequent binding of the hormone-receptor complex to DNA sites called hormone response elements acts to alter gene expression (Beato, 1989). The non-genomic mechanism is fast-driven and involves interaction of the hormone with membrane or neurotransmitter receptors (McEwen, 1991; Falkenstein et al., 2000). Steroid utilization of one or both of these mechanisms leads to changes in cells of the nervous system and effects on neurotropism and regeneration.

The presence of nuclear ARs in various regions of the brain and spinal cord allows testosterone to exert genomic effects. The distribution of ARs has been identified autoradiographically, biochemically and with in situ hybridization and immunocytochemical methods (Morrell and Pfaf, 1978; McEwen et al., 1982; Simerly et al., 1990). ARs are predominantly found in the medial hypothalamic area, ventromedial and dorsomedial nucleus, preoptic area, arcuate nucleus, amygdala, regions of the hippocampus, and lower motoneurons of the brainstem and spinal cord (Ozawa, 2005;
There are sex-related differences in AR distribution, with males having higher concentration of the AR protein than females (Lu et al., 1998). Exposure to gonadal steroids induces rapid ultrastructural changes, including alterations in the nucleolus and rough endoplasmic reticulum, increase in cell size, and increase in nuclear size and change in nuclear shape from normal ellipsoid to spherical (Cohen and Pfaff, 1981; Jones et al., 1990). The interval between rRNA transcription and processing is also shortened in the presence of gonadal steroids (Kinderman and Jones, 1993).

In the mouse facial motor nucleus (FMN), AR mRNA is localized to the cytoplasm but becomes concentrated to the nucleus upon treatment with testosterone (Tetzlaff et al., 2007a). Therefore, androgens may have genomic as well as non-genomic effects within the FMN. Unbound steroid receptors are known to complex with heat shock proteins (hsp; Pratt and Toft, 1997). AR has been shown to associate with hsp70 in prostatic cell lines (Veldscholte et al., 1992). Thus, the administration of androgens may mediate the cell response following neuronal injury by binding to ARs and releasing hsp70. Previous studies from our laboratory have shown that hsp70 mRNA levels increase by 364% following facial nerve transection in hamsters (Jones et al., 2000). The administration of testosterone, however, prevents this increase, suggesting that it substitutes the need to synthesize hsp70 by making available pre-existing hsp70. This mechanism would allow injured neurons to mount a regenerative response more immediately, without the need to mount a stress response first. Additional studies from our laboratory have demonstrated that application of testosterone increases the rate of axonal regeneration, but does not decrease the time to neuronal sprout formation,
suggesting that genomic mechanism predominates during the injury response (Sharma et al., 2009b). Furthermore, testosterone administration induces significant upregulation in the regeneration-associated genes βII-tubulin, brain-derived neurotrophic factor (BDNF), and neuritin and does so in a delayed fashion, between 2 and 7 days post-axotomy (Sharma et al., 2010b).

3. ROLE IN PERIPHERAL NERVE REGENERATION

Androgens’ therapeutic effects following peripheral nerve injury have been examined in several model systems. Our lab has investigated the role of gonadal steroids in the neuronal reparative process using a hamster facial nerve axotomy model, in which the facial nerve is crushed or transected at its exit from the stylomastoid foramen (SMF). Initial studies were aimed at determining the effects of a testosterone ester, testosterone propionate (TP), on functional recovery following a facial nerve crush in adult male Syrian hamsters (Kujawa et al., 1989). Varying doses and administration methods of TP were tested: 1) subcutaneous injection of TP in sesame oil vehicle every other day, 2) subcutaneous injection of TP in sesame oil vehicle every day, and 3) subcutaneous Silastic implants containing TP. Results demonstrated accelerated functional recovery in all groups treated with TP, along with a dose-response relationship. Higher and more frequent doses of TP had better effects on functional recovery, with the continuous dose achieved by Silastic implants having the most enhanced effects. Additionally, gonadectomized male hamsters did not display different recovery from gonadally intact animals, indicating that normal endogenous levels of testosterone were not effective.
Next, the effects of TP on rate of facial nerve regeneration following a facial nerve crush at the nerve’s exit from SMF were examined; it was found that testosterone administration increased the regeneration rate by 26-30% (Kujawa et al., 1991). Additionally, our lab has found that treatment with testosterone metabolites, dihydrotestosterone (DHT) and estrogen, increase regeneration rates as well (Tanzer and Jones, 1997). The effects of testosterone on molecular events have also been investigated in the facial nerve axotomy model. Results demonstrate that TP selectively upregulates regeneration-associated genes such as βII-tubulin, growth cone-associated protein-43 kilodaltons (GAP-43), BDNF, and neuritin and decreases expression of glial fibrillary-acidic protein (GFAP) in the FMN (Jones and Oblinger, 1994; Jones et al., 1997a; Jones et al., 1997c; Sharma et al., 2010b). TP increases the rate of axonal regeneration, but does not decrease the time to neuronal sprout formation (Kujawa et al., 1991; Sharma et al., 2009b). TP administration also has a pronounced effect on synaptic stripping following facial nerve transection in hamsters (Jones et al., 1997b). TP preserves approximately half of the axosomatic synapses that are otherwise removed in untreated animals, implying that TP treatment may reduce the initial shock to injured neurons.

Other peripheral nerve injury models also reinforce the neurotherapeutic role of gonadal steroids in nerve regeneration. Yu and Srinivasan (1981) have shown that TP administration enhances hypoglossal nerve regeneration in rats. TP also increases regeneration in rat sciatic motoneurons and accelerates functional recovery following hind limb paralysis (Kujawa et al., 1993; Brown et al., 1999). Vita et al. (1983) have also reported the accelerative effects of androgens on sciatic nerve regeneration in male rabbits. Apart from enhancing axonal regeneration, administration of DHT and estrogen
reduces loss of facial motoneurons following facial nerve transection in neonatal hamsters (Huppenbauer et al., 2005). Perez and Kelley (1996) also found that androgen treatment enhanced laryngeal motoneuron survival in *Xenopus laevis*. Therefore, treatment with androgens is a viable therapeutic strategy for peripheral nerve injuries.

D. NEUROTHERAPEUTIC ROLE OF ELECTRICAL SIMULATION

Electrical activity occurs naturally within the nervous system and plays an important role from development to adulthood. Application of electrical fields affects morphological and functional properties of neurons such as nerve branching, rate and orientation of neurite growth, rapid sprouting, and guidance during axon regeneration (McCaig, 1990; Borgens et al., 1981; Patel and Poo, 1982; Manivannan and Terakawa, 1994; Borgens, 1999; Wan et al., 2010). Electrical stimulation (ES) has been explored as a potential treatment for a variety of neurobiological diseases, including movement disorders, seizures, psychiatric disorders, chronic pain syndromes, and peripheral nerve and spinal cord injuries (Wan and Lin, 2009).

1. HISTORY OF ELECTRICAL SIMULATION

Early in vitro experiments investigated the effects of ES on morphological properties of neurons. Jaffe and Poo (1979) demonstrated that neurites react rapidly to an applied direct current (DC) field by orienting themselves parallel to the gradient. McCaig (1990) found that applying electric fields to *Xenopus laevis* further affected neurite morphology by increasing the number of filopodia at the growth cone and the number of cytoplasmic spines along a neurite shaft and by enhancing nerve branching. Application
of steady electric gradients across injured spinal cords of guinea pigs has been found to promote both anatomical and functional recovery (Borgens et al., 1987).

ES also improves survival of neuronal populations following axotomy in the CNS. ES of the transected optic nerve for 2 h promoted survival of retinal ganglion cells following transection of the optic nerve in rats (Morimoto et al., 2002). Okazaki et al., (2008) have further examined the different ES parameters of optic nerve stimulation. While 10 min of ES was found to be ineffective, 30 min of ES was as effective as 1 h and 2 h of ES in enhancing neuronal survival. Frequency of ES also affected neuroprotection, as a frequency of 20 Hz was found to be optimal, with lower (10 Hz) and higher (50 Hz) frequencies having no beneficial effect. Others have also confirmed the success of the 20Hz stimulation frequency (Ahlborn et al., 2007; Wan et al., 2010; Alrashdan et al., 2010). Our laboratory has found that a one-time, 30 min application of 20 Hz ES promotes recovery from both extratemporal and intratemporal facial nerve crush (Hetzler et al., 2008). In short, a variety of experiments over the past three decades have established that ES provides directional cues, influences orientation and regeneration in vitro and in vivo, promotes survival of CNS neurons, and accelerates functional recovery following spinal cord injury.

2. EMERGING ROLE IN PERIPHERAL NERVE REGENERATION

Though ES has been shown to affect neuronal properties and neuronal survival and regeneration post-injury positively, administration techniques vary widely ranging from daily, prolonged individual periods, to one-time brief stimulation (Nix and Hopf, 1983; Pockett and Gavin, 1985). Although such studies were essential in establishing
that ES accelerated functional recovery following peripheral nerve injury, they did not determine the anatomical reasons underlying the results. To achieve this, Al-Majed et al. (2000a) used the rat femoral nerve model of transection and surgical repair to examine the ability of ES to accelerate axonal regeneration and promote reinnervation specificity. The 20 Hz frequency was used because it is similar to the normal slow firing patterns of motoneurons. They found that 1 h to 2 weeks of electrical stimulation increased the number of regenerated axons farther from the site of injury as well as those that went into the appropriate branch. Other experiments using proximal labeling and histological techniques revealed that 1 h ES of the femoral and sciatic nerves increased the number of axons that regenerated across the suture gaps (Brushart et al., 2002; Vivo et al., 2008). ES has also been found to improve both motor and sensory recovery from sciatic crush injury (Alrashdan et al., 2010).

The above rat studies have been translated to humans in whom the median nerve had been damaged from carpal tunnel syndrome (Gordon et al., 2007). The median nerve was stimulated for 1 h during carpal tunnel release surgery in a randomized clinical trial, increasing the motor unit number estimates and accelerating functional recovery in patients. Given these findings, ES can be used as a strategy to improve the outcome of peripheral nerve injury.

3. MECHANISM OF ACTION

The mechanism of action of ES is not completely understood. An experiment using the rat femoral nerve model of transection and surgical repair determined that the regeneration-associated effects of ES were mediated by the cell body (Al-Majed et al.,
2000a). Transected and repaired femoral nerves in rats were stimulated with or without the application of tetrodotoxin (TTX; blocker of voltage-gated sodium channels) at a dose that completely blocks electrical transmission toward the proximal cell body. TTX completely abolished the previously seen effects of ES, suggesting that it acts to enhance the growth program initiated by the cell body after injury.

Subsequent studies demonstrated that ES first enhanced the upregulation of BDNF and its trkB receptor in axotomized rat femoral motoneurons (Al-Majed et al., 2000b) and then upregulated GAP-43 and cytoskeletal proteins (actin and α₁-tubulin) and downregulated neurofilaments (Al-Majed et al., 2004). Since neurofilaments interfere with axonal transport of actin and tubulin, this decrease in neurofilament to tubulin ratio was also associated with enhanced regeneration (Bisby and Tetzlaff, 1992). English et al. (2007) have further demonstrated that the lack of neurotrophin-4/5 (NT-4/5) in transgenic mice abolishes the enhanced regeneration induced by ES. Therefore, an increase in neurotrophic factor production appears to be an essential downstream effect of ES. Our laboratory has confirmed that ES treatment results in upregulated BDNF as well as other the regeneration-associated genes α₁-tubulin, GAP-43, neuritin, and pituitary adenylate cyclase-activating peptide (PACAP) after facial nerve crush (Sharma et al. 2010b). These genes have increased expression in the first few days after injury, which correlates well with the finding that the delay to sprout formation is decreased after application of ES (Sharma et al., 2009b).

ES is thought to mediate changes in the expression of regeneration-associated genes by increasing intracellular levels of cyclic adenosine monophosphate (cAMP), an intracellular second messenger (Gordon, 2009). This early increase in cAMP associated
with ES is most likely preceded by increased calcium entry into the cell body, the importance of which was suggested when Kerns and colleagues found that the application of verapamil, a calcium-channel blocker, reduced the regenerative effects of ES (Kerns et al., 1991). However, administration of a specific phosphoesterase IV inhibitor of the neuronal enzyme that hydrolyses cAMP, rolipram, mimics the enhanced regenerative effects of ES (Gordon, 2009). ES may work through additional mechanisms other than stimulating neural cells to synthesize regeneration-associated gene products. Wan and Lin (2009) recently postulated that ES of injured nerves may accelerate the transport of mRNAs to axons, which would likely improve axonal elongation; subsequently Wan and colleagues (2010) found that ES affects Schwann cell polarization by upregulating expression of P0 and Par-3, two genes involved in determining cell polarity, along with BDNF.

E. COMBINATORIAL TREATMENT STRATEGY

Based on the numerous studies reporting that gonadal steroids and ES positively affect nerve regeneration individually and the studies from our laboratory demonstrating that the two therapies approach different aspects of the regenerative process, it would be logical to examine the effects of combining both treatments. Sharma and colleagues found that the combination of TP and ES accelerated functional recovery in the extratemporal and intratemporal facial nerve injury models more than either alone. The effects of the two treatments were additive in the extratemporal injury, but were even more striking in the intratemporal injury: untreated animals only partially recovered within the time frame of the study, whereas 100% of the combinatorially treated animals...
completely recovered by the end of 8 weeks (Hetzler et al., 2008; Sharma et al., 2010a). The models of facial nerve injury currently in use do not simulate the most proximal injuries well, so there is a need to develop an animal intracranial model of facial nerve injury. It also remains to be seen whether this combinatorial treatment can improve other injured peripheral nerves other than the facial, such as the recurrent laryngeal and sciatic nerves. Therefore, the overall goal of this dissertation is to investigate the therapeutic effects of ES and testosterone in combination, in a variety of translational peripheral nerve injury models, the proposed intracranial facial nerve crush, the recurrent laryngeal nerve crush, the sciatic nerve crush, and the sciatic nerve transection-repair.
CHAPTER III
MATERIALS AND METHODS

A. ANIMALS

All animals used in the present study were adult, male Sprague-Dawley rats (2 months old or ~250 g), purchased from Harlan (Indianapolis, IN). Animals were allowed to acclimate to their environment for at least 3 days upon arrival, prior to any manipulation. Rats were housed under a 12 h light/dark cycle in microisolator cages and received a standard rodent diet and water ad libitum except during CatWalk apparatus behavioral training. All surgical procedures were completed in accordance with the National Institutes of Health guidelines on the care and use of laboratory animals for research purposes and approved by the institutional animal care and use committee.

Sterile, aseptic techniques were used throughout the study. Any animals displaying ≥15% weight loss were removed from the experiment. Wound clips and sutures were removed 10-14 days post-operative (dpo). Ketamine/xylazine overdose and intracardiac perfusion with room temperature saline followed by ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) was used to sacrifice all animals at the appropriate post-operative survival time.
B. SURGICAL PROCEDURES

1. GONADECTOMY

Gonadectomies were performed 3-5 days prior to facial, recurrent laryngeal, or sciatic nerve injury. Animals were anesthetized with 3.5% isoflurane and the surgical area was prepped with 70% ethanol and povidone-iodine. A 5 mm incision between the penile and the anal openings was made and both testicles were pulled through one at a time. The testicular and epididymal arteries were ligated bilaterally with 4-0 silk suture, and testicles were severed distal to the sutures. The wound site was closed with 9 mm wound clips and animals were allowed to recover under observation.

2. INTRACRANIAL FACIAL NERVE CRUSH AXOTOMY

Animals were anesthetized by intraperitoneal injections of Ketamine (100 mg/ml; 0.1 ml/100g body weight) and Xylazine (20 mg/ml; 0.025 ml/100g body weight). All crush-axotomies were performed on the right facial nerve, with the left facial nerve remaining intact and serving as an internal control in each animal. The right post-auricular area was shaved and prepped with 70% ethanol and providone-iodine solution and a curvilinear incision was made ~5 mm behind the ear with a #15 scalpel blade. The skin was elevated in the subdermal plane towards the ear canal. The extratemporal portion of the facial nerve was identified as it exited the stylomastoid foramen. The nuchal crest was identified between the superficial temporal muscle anteriorly and the splenius capitis muscle posteriorly. These two muscles were then incised and dissected off the skull. The splenius capitis muscle was resected back to the skull base and a small
portion of the temporals muscle was excised to provide improved exposure to the occipital squama and the mastoid process of the petrous temporal bone (Figure 4). The resected muscle was stored in room temperature 0.9% saline during the rest of the surgery to be used as a muscle plug at closing to prevent cerebrospinal fluid leak from the craniotomy defect.

Using a high-speed drill with a 2-mm diamond burr under microscopic visualization, the nuchal crest was thinned in a saucerized manner to reveal the transverse sinus. A #3 French suction-irrigator was used to bathe the operative field in room temperature 0.9% saline to prevent thermal injury to the temporal bone and the facial nerve from the drilling procedure. Next a craniotomy was made into the occipital squama until dura was seen, with the transverse sinus as the anterior limit (Figure 5). Using both epinephrine-soaked pellets and a small retractor, the parafloccular lobe of the cerebellum was retracted superiomedially. The drill was used to widen the craniotomy superiorly until the inferior cerebellar vein and mastoid cavity was identified. Posteriorly the craniotomy was widened to the end of the occipital squama bone. This created approximately a 3 x 3-mm craniotomy through which the petrous portion of the temporal bone was visualized (Figure 6).

With a 1-mm diamond burr, deeper dissection was performed to identify the petrous portion of the temporal bone. Staying posteriorly, the vestibulocochlear nerve was identified (Figure 7) and sacrificed. The vestibulocochlear canal was followed laterally until the cochlea was identified and entered. This area was widened anteriorly until the posterior wall of the facial nerve was skeletonized. A thin portion of bone was left over the facial nerve to protect it until final unroofing. Drilling was then performed
anterior to the facial nerve (Figure 8) to allow room for forceps. This allowed for a circumferential view of the labyrinthine segment of the facial nerve.

Once the facial nerve was sufficiently skeletonized, a Rosen needle was used to remove the remaining bone from the surface of the nerve (Figure 9). Because the nerve is anchored at the first genu, it is extremely susceptible to damage from microscopic movements during the crush injury. To solve this dilemma we ensured that the nerve was mobile within the canal so that microscopic movements could be absorbed by movement of the brainstem. Curved jewler’s forceps (#5 x 45) were then inserted into the craniotomy site and the intracranial portion of the facial nerve between the brainstem and meatal foramen was crushed firmly once for one minute. Due to limited access to the nerve, the nerve could only be crushed in one orientation (from the top). Care was taken to avoid suction or manipulation of the nerve immediately after the crush to avoid transection. A 3x3 mm small muscle plug was inserted into the craniotomy site to prevent cerebrospinal fluid leak. A 10x15 mm large muscle plug was placed over the craniotomy site to serve as a second layer of closure. The muscle and skin layers were closed separately.

Successful crush was verified by complete loss of the eyeblink reflex and loss of vibrissae orientation and movement in animals upon recovery from anesthesia. The injury paradigm severed the axons but left the neural sheath intact to provide a route for regenerating axons. The wound site was sutured with 4-0 prolene and coated with triple antibiotic ointment (Henry Schein).
Figure 4. Superficial Dissection of Muscle and Bone for the Intracranial Facial Nerve Crush – Layer 1. The temporalis and splenius muscles have been removed to expose the following structures: FN-ET: Facial Nerve extratemporal, NC: Nuchal Crest, STM: Superficial Temporal Muscle (Resected), SCM: splenius capitis muscle (Resected), OC: Occipital Squama, MP: Mastoid Process of the petrous temporal bone, Dotted line: Approximate area of initial craniotomy. L, lateral; M, medial; A, anterior and toward the snout; P, posterior.
Figure 5. Initial Craniotomy for Intracranial Facial Nerve Crush – Layer 2. The bone has been sufficiently thinned to expose brain tissue, yet the surrounding structures are intact: FN-ET: Facial Nerve extratemporal, STMr: Superficial Temporal Muscle (Resected), SCMr: splenius capitis muscle (Resected), MP: Mastoid Process of the petrous temporal bone, TS: transverse sinus, Dotted line: Approximate boundary between bone and brain during initial craniotomy. L, lateral; M, medial; A, anterior and toward the snout; P, posterior toward the tail.
Figure 7. Magnified View of Widened Craniotomy for Intracranial Facial Nerve Crush – Layer 4. Note animal rotated 90 degrees. The craniotomy measures 3 mm at its widest. FN-ET: Facial Nerve extra-temporal, TS: Transverse Sinus, STMr: Superficial Temporal Muscle (Resected), SCMr: splenius capitis muscle (Resected), 8th Nerve: Cochlear Nerve prior to sacrifice. L, lateral; M, medial; A, anterior and toward the snout; P, posterior toward the tail.
Figure 8. Craniotomy for Intracranial Facial Nerve Crush – Layer 5. Note animal rotated 90 degrees.
Drilling both anteriorly and posteriorly to the facial nerve is necessary for a complete crush. FN-ET: Facial Nerve Extratemporal, FN-IC: Facial Nerve Intracranial, TS: Transverse Sinus, STMr: Superficial Temporal Muscle (Resected), SCMr: splenius capitis muscle (Resected), 8th Nerve-S: Cochlear Nerve Sacrificed. L, lateral; M, medial; A, anterior and toward the snout; P, posterior toward the tail.
3. RECURRENT LARYNGEAL NERVE CRUSH AXOTOMY

Animals were anesthetized with isoflurane as described above and breathing rate was monitored throughout the surgery. The ventral neck area was shaved between the mandible and clavicle and then the animal was secured supine in a stereotaxic skull frame in order to stabilize the head and expose the neck. Direct laryngoscopy (described later in section 3-E-3) was performed before nerve exposure to document normal vocal fold motion prior to nerve injury. The neck area was prepped and a 2 cm midline incision was made in the skin between the hyoid and clavicle. Under a dissecting microscope, the midline strap muscles (sternothyroid and sternohyoid) were separated and retracted to expose the trachea and both left and right RLNs were visualized along the sides of the trachea. The left RLN was gently freed from the pretracheal fascia, its connective tissue attachment to the trachea, by #5 dumont forceps between the levels of the 5th – 9th tracheal rings. The nerve was crushed for 30 sec, twice with the same forceps used to dissect out the nerve at the 7th tracheal ring. After verification and documentation of successful crush with direct laryngoscopy, muscle layers were reapproximated and the overlying skin closed with wound clips as well as coated with triple antibiotic ointment.

4. SCIATIC NERVE CRUSH AXOTOMY

Animals were anesthetized with isoflurane and monitored as described above. After the right thigh was shaved and prepped, the right hind limb was secured to the operating surface with surgical tape to prevent movement. A 2.5 cm incision was made on the postero-lateral surface of the thigh along the grain of the biceps femoris beginning
5 mm distal to the greater trochanter of the femur. The cranial and caudal heads of the biceps femoris (corresponding to the short and long heads in humans) were separated and retracted to reveal the femur and the sciatic nerve. The sciatic nerve was then freed from the fascia securing it to the femur and adductor muscles and subsequently crushed twice for 30 sec each with jeweler’s forceps at the midpoint of the femur. Muscle layers were reapproximated and the overlying skin closed with wound clips.

5. SCIATIC NERVE AUTOGRAPH AXOTOMY

The sciatic nerve autograft surgery was conducted in the same manner as the sciatic crush injury up to the crush. However, instead of performing a crush injury, the nerve was transected through its circumference (including through the epineurium) at two places 7 mm apart and the epineurium reattached with sutures. In order to do this, before the transections took place, 2-3 knot preparations were placed around the circumference of the nerve so that immediately after the nerve was cut the nerve could be reapproximated. Retraction of the biceps femoris muscle used to expose the sciatic nerve also stretches it, so the nerve will shorten after it is cut. In order to minimize this shortening and thus the subsequent increase in tension needed to reapproximate the cut ends, the knot preparations were placed before each cut. A knot preparation consisted of a 10-0 nylon suture threaded lengthwise through the nerve twice (see Figure 10) with a slack loop of suture in between stitches, providing a base for the knot. These threads were then pulled taught together after the cut, held together with hemostatic clips, and tied individually. After both cuts were made and repaired with suture, the overlying muscle layers were reapproximated and the overlying skin closed with wound clips.
Figure 10. Knot Preparation. In order to facilitate suturing cut ends of the sciatic nerve back together for the autograft procedure, suture was threaded through the epineurium before the nerve was transected. 1 shows the needle piercing the epineurial layer. 2 shows the thread pulled through the path left by the need. 3 shows the full loop in place. 4 is after the transection occurs; the two nerve sections are still connected by the thread. The ends can be pulled taught in order to re-secure the nerve sections to one another.
C. ELECTRICAL STIMULATION

1. DESIGN OF ELECTRODE APPARATUS

A custom electrode apparatus was constructed in our laboratory for use in rats. Two Teflon-coated wires (Cooner Wire, Chatsworth, CA) were bared of insulation for 2-3 mm and soldered to two “male” connector pins (Wire Pro) in a connector strip (Allied Electronics). The connector pins soldered to the wires were covered with insulation tubing (Allied Electronics) and epoxy and allowed to dry overnight. One of the wires and its respective connector pin was marked with red insulation tape to designate it as the positive end of the electrode while the other wire and its connector pin were left unmarked to designate that as the negative end. All electrode apparatuses were tested with a voltmeter to ensure that they were functional prior to use in animals.

2. ELECTRICAL STIMULATION IN ANIMALS

In all nerve injury paradigms, electrode leads were sutured in place at the time of axotomy and removed immediately after the singular stimulation session was complete. Stimulation was done while the incision for the axotomy injury was open to simulate a human operating room situation. For the intracranial facial nerve crush axotomy, the positive wire was sutured proximal to the crush site adjacent to the facial nerve, while the negative wire was sutured ~3-5 mm away onto surrounding connective tissue. For the RLN crush axotomy, the positive wire was bent around the left RLN after it was dissected away from the larynx proximal to the crush site at or below the level of the 9th
tracheal ring and did not need suture to be held in place; the negative wire was sutured to the retracted strap muscles. For the sciatic nerve crush and resection-repair injuries, as with the RLN stimulation, the positive wire was bent around the sciatic nerve proximal to the crush injury or the more proximal resection-repair site and the negative wire sutured to the retracted biceps femoris. The connector pins of the electrode were connected to the leads of an isolated pulse stimulator (W-P Instruments, Inc.), and current was directed from the negative to the positive lead. Voltage was increased slowly and set at a threshold at which animals displayed a right ear flutter, a left vocal fold flutter, or a right hind limb twitch depending on the model. This voltage was measured to be between 200 and 300 mV for all animals and injury models. The supramaximal, monophasic pulses were delivered at a frequency of 20 Hz after the injury. Unstimulated animals were connected to the stimulator, but the voltage was kept at 0 V.

D. STEROID ADMINISTRATION

1. TESTOSTERONE PROPIONATE

TP was administered using subcutaneous capsule implants. Capsules were made of Silastic tubing (0.062 in. ID x 0.095 in. OD) and were 18 mm in total length. Of this length, 10 mm contained 100% crystalline TP (Sigma) while 4 mm wooden plugs sealed both ends of the capsule. Prior to use in animals, capsules were equilibrated overnight in physiological saline. In each animal receiving TP treatment, 2 capsules were implanted at the time of axotomy. A subcutaneous pocket for the capsules was created by making an incision on the mid-dorsal surface of animals or using the same incision site used for
electrode implantation. Capsules were left in animals until the time of euthanasia. This dose of TP has previously been shown to establish supraphysiological levels of systemic testosterone (Hetzler et al., 2008; Tanzer and Jones, 2004; Kujawa et al., 1989).

E. BEHAVIORAL TESTING

1. FACIAL NERVE FUNCTIONAL RECOVERY TESTING

In rodents, a complete crush or transection of the facial nerve results in ipsilateral loss of the eyeblink reflex, backwards vibrissae orientation, loss of vibrissae movement, and drooping of the mouth, and return of facial nerve function can be easily observed. Animals were allowed to roam freely in an empty cage, placed on a black background, to observe return of vibrissae orientation and movement. To elicit the eyeblink reflex, a puff of air was blown into the animal’s eye and the extent of eyelid closure was observed. Since symmetry of the mouth in rats was not obvious from observation alone and could not be easily scored, improvement in mouth droop was not used as an analysis parameter for facial nerve function.

For the facial functional recovery study described herein, function on the crushed, right side was always compared to the intact function on the left side using a 3-point recovery scale. A score of ‘1’ was used to indicate complete lack of function, ‘2’ to indicate the onset of any return in function, and ‘3’ to indicate complete, symmetrical function as compared to the left side. Recovery time for each functional parameter (eyeblink reflex, vibrissae orientation, and vibrissae movement) was defined as the number of dpo until the animals received a score of ‘3’. For eyeblink function, the
number of dpo until the onset of the reflex (score of ‘2’) was also analyzed. Behavioral observations were conducted daily in all animals until a score of ‘3’ was attained for all functional parameters. Complete functional recovery was defined as the number of dpo when all of the parameters had returned to normal.

2. FACIAL NERVE CONDUCTION TESTING

Motor nerve conduction studies were performed weekly, beginning on the day of axotomy and continuing until the day of sacrifice. Animals were anesthetized with intraperitoneal injections of Ketamine-Xylazine, as described in the previous section. A total of 5 electrodes (Grass Technologies, Astro-Med, Inc.) were used for testing. Two stimulating electrodes were placed below the infraorbital ridge to stimulate the facial nerve, while two recording electrodes were placed on the vibrissae pad to record the response. As the vibrissae pad in rats contains organized rows of indentations, the placement of electrodes could be easily replicated for each recording. A ground electrode was placed inferior to the external ear. Electrodes were connected to an electromyographic machine (Advanced Medical) that stimulated and displayed the response. The size of the response (amplitude) in mV and the time in ms (latency) for the electrical impulse to travel from the stimulation to the recording site were the two parameters used to analyze facial nerve conduction.

The optimal parameters used for stimulation and response recording of the facial nerve were determined previously as described by Sharma et al. to be 15 mA of stimulating current given at a frequency of 0.5 Hz; an average of 25 responses was recorded for each trial (2010b). Trials were repeated at least once to verify consistency.
of the recorded response. Recordings were conducted both on the right and the left sides, and percent change in amplitude and latency for the right facial nerve, relative to the left, was analyzed.

3. VOCAL FOLD MOBILITY TESTING

The technique of direct laryngoscopy was used to gather vocal fold mobility (VFM) measurements. After an animal was placed supine in a stereotactic operating system and the skull secured, a silk suture was used to retract the tongue while a 0° Pediatric ENDOGO® (Medtronic) operating endoscope with an epiglottis elevator was inserted into the larynx to observe and record vocal fold movement during respiration. The nose cone used to deliver isoflurane and oxygen remained over the nose during this procedure to maintain an appropriate plane of anesthesia. Videos were recorded with the ENDOGO® at the following points during surgery: pre-crush or pre-electrode placement (to confirm normal movement before crush), during determination of threshold ES, immediately post-crush (to confirm lack of mobility), and the day of euthanasia to assess recovery. Because of the tongue suture required for video capture of VFM, weekly measurements were not performed on the same set of animals; instead animals were assigned to a time-point and VFM measurements were only performed on the day of the crush and the day of euthanasia. This increased the number of groups and subsequently the number of animals required for the study.
The behavioral training regimen for the CatWalk XT consisted of food-depriving the animals for 12 hours a day, then placing them at one end of the walkway and a yogurt reward (Stonyfield Organic Strawberry Children’s Yogurt) at the other to encourage the animal to run across the walkway. The reward was removed as the animal approached the other end of the walkway and was given to the animal after 3 successful crossings of the walkway had been achieved. Training occurred once a day for the 14 days immediately prior to sciatic nerve injury.

Weekly testing of animals was done using the CatWalk XT apparatus. As during training, animals were food deprived for 12 hours the day of testing and tested during their active portion of the light cycle. The same yogurt reward was given to the animal after a minimum of 3 usable runs were achieved. A usable run had the following characteristics: at least 3 step cycles were recorded; the speed variability was low (less than 40%); and, there were no pauses as the animal crossed the walkway. All usable runs gathered for a particular animal on a specific day were analyzed and run statistics combined to create trial statistics; trial statistics were analyzed and compared across groups at various time points.

The following trial statistics have been shown to change following sciatic nerve injury and were thus chosen for analysis: base of support, print length, print width, print area, step pattern preference, and regularity index (Deumens et al 2007). Please see Figure 11 for visual representations of the parameter definitions. Base-of-support (BOS) is defined as the distance between paws attached to the same girdle as measured perpendicular to the walking direction; only the BOS for hind paws is affected by sciatic
Figure 11. **Parameters used to evaluate sciatic function.** (A) shows a bottom-up view of the outline of a rat resting on all 4 paws. The order of paw placement, stride length, print length, print width, print area, and base-of-support are all measurements that can be gathered from this view of the animal. The base of support can be determined for both the shoulder and pelvic girdles and is defined as the distance between paws attached to the same girdle as measured perpendicular to the walking direction. (B) shows a single rat paw that has been enlarged.
Figure 12. Normal Step Sequences. The six step patterns shown above can be grouped into three pairs: cruciate (an entire girdle is placed on the surface before the other one), alternate (paws from alternating girdles are placed), and rotary (circular pattern of paw placement).
nerve injury. Print length is defined as the length of the box which is artificially placed around the paw prints by the CatWalk XT software program. Print width is defined similarly to print length with the substitution of the width of the box for the length. Print area is the total surface area of the glass floor contacted by the hind paw during the complete stance duration. Changes in step pattern preference can be determined by comparing the predominantly used patterns among different animal groups. There are 6 normal step patterns defined for rodents and these can be grouped into the following 3 pairs as shown in Figure 12: cruciate (both paws of the shoulder girdle are placed before both paws of the pelvic girdle are placed), alternate (one paw from the shoulder girdle is placed first followed by a paw from the pelvic girdle; this is repeated with the remaining paws), and rotate (rotary paw placement). Animals that have received a sciatic nerve transection have been found to change their preferences in step patterns to those that have the affected hindpaw being preceded by the most distant paw; this keeps the center of gravity as far away as possible from the affected paw (Deumens et al., 2007). The regularity index expresses the number of normal step sequence patterns relative to the total number of paw placements.

H. STATISTICAL ANALYSES

Statistical analyses were performed using the SigmaPlot, Systat, and SPSS software packages. To determine significant differences between two groups, a two-tailed Student’s \( t \)-test with unequal variances and \( p < .05 \) was performed. To determine significant differences between three or more groups, a one-way analysis of variance
(ANOVA), with a Holm-Sidak multiple comparison post-hoc test at $p < .05$, was conducted. A two-way ANOVA was done when two independent variables, such as time and treatment, were present. This statistical test was again followed by a Holm-Sidak multiple comparisons post-hoc test to determine specific differences among groups.

In experiments where data values in a group did not fall along a normal distribution, the following non-parametric tests were used: for comparisons between two groups, the Mann-Whitney U test and for comparisons between 3 or more groups, the Kruskal-Wallis test with Mann-Whitney U post-hoc tests.

For the RLN experiment in particular, a logistic regression analysis of the percentage of animals achieving complete recovery was carried out to predict recovery status using time and treatment as predictors. This analysis was used because the outcome, being completely recovered or not, is a binary rather than continuous function. The resulting odds-ratios determined describe the odds of being completely recovered with one treatment as compared to the odds of being completely recovered given no treatment. Chi-squared analyses were subsequently used at individual time points to examine the predictive value of a treatment based on recovery status and to determine if there were significant differences between treatments in the likelihood of achieving complete recovery.
CHAPTER IV
EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE IN COMBINATION ON FUNCTIONAL RECOVERY FOLLOWING INTRACRANIAL FACIAL NERVE CRUSH INJURY

A. ABSTRACT

As functional recovery following peripheral nerve injury is dependent upon successful regeneration and target reconnection, combinatorial treatments that enhance different regeneration events may be required for recovery from severe injuries. The neurotherapeutic effects of nerve electrical stimulation and gonadal steroids have been demonstrated independently and in combination in extratemporal and intratemporal facial nerve injuries. The purpose of the first aim of this dissertation was two-fold: 1) to develop a reliable intracranial facial nerve crush model and 2) to investigate the therapeutic potential of combining ES with gonadal steroids in this most proximal model. Adult male rats were divided into three experimental groups: intracranial sham-operated, intracranial crush, and intracranial crush plus ES+TP combination therapy. Animals were observed daily for return of facial nerve function, including the eyeblink reflex, vibrissae orientation, and movement. Motor nerve conduction studies were done weekly to quantify the changes in peak amplitude and latency of evoked response. Rats treated with the combination therapy recovered partial facial function before untreated rats,
suggesting that the combination works in tandem to boost regenerative properties.

B. INTRODUCTION

Peripheral nerve lesions are common types of nervous system injuries that often lead to substandard functional recovery. Though the peripheral nervous system motoneurons have a robust ability to regenerate, recovery from nerve injury is dependent upon cell survival, repair, and mounting of a successful regenerative response. Despite the availability of surgical repair, complete functional recovery is often hindered by slow rates of regeneration over long distances and/or inadequate target reconnection (Valero-Cabre et al., 2004). Strategies to increase the rate of axonal regeneration and recovery are thus valuable therapeutic approaches.

The facial nerve in particular has been the subject of much investigation as it is a commonly injured nerve in humans (Gilden, 2004) and over the years various animal models have been developed to study the injury response in peripheral motoneurons and functional recovery. These animal models, which involve crush injuries either distal to the nerve’s exit from the stylomastoid foramen or throughout its course within the temporal bone, do not adequately mimic all conditions under which the facial nerve is subject to injury. The facial nerve is often compressed by the growth of acoustic neuromas and other tumors of the cerebellopontine angle or injured when such tumors are removed from the tiny and hard-to-access area (Mavrikakis, 2008). No animal model exists to recapitulate such injuries, which is unfortunate as the incidence of such tumors is increasing in the human population (Edwards et al., 2006).
It is well known that the more proximal to the cell body that a motoneuron is injured, the worse the functional outcome and the longer the timeframe for recovery. Our laboratory has demonstrated this directly by comparing recovery times between animals receiving extratemporal and intratemporal facial nerve crush injuries; it takes more than twice as long to begin to recover from the more proximal injury (Sharma et al., 2009a). Thus, it is important to have models of injuries proximal to the cell body in order to study the effects of potential therapies on severe injuries. Sharma et al. also found that the achievement of complete functional recovery from intratemporal facial nerve crush injury required a combinatorial treatment strategy (2010a) whereas individual treatments yield significant improvements in recovery from the more distal extratemporal facial nerve crush.

The combinatorial treatment strategy our laboratory has employed consists of the systemic administration of the gonadal steroid testosterone propionate and brief electrical stimulation (ES) of the crushed nerve. Throughout the study of the nervous system, gonadal steroids have been shown to exert significant trophic effects on the nervous system, including changes in cell survival, neurotransmitter metabolism, and neuronal regeneration (Schumacher et al., 1996; De Nicola, 1993; Jones, 1994; Jones, 1993a; Tetzlaff et al., 2006; Bialek, et al., 2004). Our lab has demonstrated that exogenously applied testosterone accelerates functional recovery and regeneration rates in hamsters subjected to extratemporal facial nerve crush as well as in rats subjected to sciatic nerve crush (Kujawa et al., 1989; Kujawa et al., 1991; Jones, 1993b; Tanzer and Jones, 1997; Brown et al., 1999). Since gonadal steroids primarily exert their effects on target tissue using a receptor-mediated mechanism and receptor-mediated mechanisms require
multiple steps for signal transduction, the therapeutic effects of gonadal steroids may be delayed in comparison to therapies that can exert more immediate effects.

In contrast, ES has been shown recently to have more immediate effects, as compared to gonadal steroids (Sharma et al., 2009b; Sharma et al., 2010b). Application of electrical fields has long been known to affect morphological and functional properties of neurons, such as nerve branching, rate and orientation of neurite growth, rapid sprouting, and guidance during axon regeneration (Borgens et al., 1981; Patel and Poo, 1982; Manivannan and Terakawa, 1994; Borgens, 1999). More recently, brief ES of the rat femoral nerve proximal to the injury site was shown to enhance axonal regeneration and specificity of motor re-innervation pathways (Al-Majed et al., 2000). ES also improves reinnervation and modulates spinal plastic changes following sciatic nerve injury in rats (Vivo et al., 2008). ES appears to exert these effects by influencing the neuronal soma response, in part by increasing expression of neurotrophic factors and their receptors and elevating levels of intracellular messengers such as cAMP soon after injury (Al-Majed et al., 2000; Al-Majed et al., 2004; Udina et al., 2008).

In the first aim of this dissertation, the effects of a combinatorial treatment strategy, administering both gonadal steroids and ES, on functional recovery were investigated in the newly developed intracranial facial nerve axotomy model. The rationale for this strategy was that, since the two treatments work through different mechanisms as well as during different phases of regeneration, then using them in combination may be required to achieve recovery in an injury model in which spontaneous recovery is limited. Our previous studies have demonstrated the positive effects of ES and gonadal steroids, alone and in combination, on functional recovery
following both extratemporal and intratemporal facial nerve crush injury in rats. Results from the more distal crush injury show that while either treatment alone accelerates recovery by ~8%, the combination treatment of electrical stimulation plus TP shortens recovery time by ~22% compared to untreated animals (Lal et al., 2008; Hetzler et al., 2008). Results from the intratemporal facial nerve crush injury established that both treatments are required to achieve complete recovery (Sharma et al., 2010a). In the present study, to further assess the therapeutic potential of the ES and TP combination, we used behavioral tests and motor nerve conduction studies following an intracranial facial nerve crush injury in gonadectomized adult male rats.

C. MATERIALS AND METHODS

1. ANIMALS & NERVE INJURY PARADIGM

Adult male Sprague-Dawley rats, weighing 250g, were purchased from Harlan (Indianapolis, IN) and used for all experiments, as described in Chapter III. Three to five days prior to nerve injury, rats were anesthetized with isofluorane and castrated. For facial nerve axotomy, rats were anesthetized by intra-peritoneal injections of Ketamine (100mg/ml; 0.1ml/100g body weight) and Xylazine (20mg/ml; 0.025ml/100g body weight). The right facial nerve was crushed at its exit from the pons as it entered the internal auditory canal (refer to Chapter III for details). One 60-second crush, was done with fine jeweler’s forceps to ensure a full crush.

The experimental design for this study is presented in Figure 13. Animals were divided into 3 experimental groups: [1] sham-operated, animals receiving no axotomy
Gonadectomy

3–5 d

Intracranial Facial Nerve Exposure

Sham

Crush +
No Treatment

Crush +
ES +TP

Daily Functional Recovery Assessment

Weekly Motor Nerve Conduction Assessment

12 wk

Figure 13. Experimental Design for Aim 1. This timeline will be followed to examine the effects of the combination treatment on recovery from intracranial facial nerve crush.
and no treatment, \( n = 5 \) [2] no treatment, animals receiving axotomy but no treatment, 
\( n = 5 \) [3] ES + TP, animals receiving axotomy and the combination of ES and TP treatments \( n = 4 \).

2. ELECTRICAL STIMULATION

A custom electrode apparatus constructed in our laboratory was used in all rats (refer to Chapter III). Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two “male” connector pins in a connector strip. At the time of axotomy, wires were run through subcutaneously and sutured ~2 mm proximal to the injury site (cathode) and ~3-5mm away from it (anode). The connector pins of rats were attached to leads of an isolated pulse stimulator, and rats were stimulated at a voltage at which they displayed a right ear flutter. Immediately post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20 Hz or sham stimulated for 30 min. Electrode wires were then removed and the animal’s wounds were closed.

3. HORMONE ADMINISTRATION

Immediately after injury, rats receiving testosterone treatment were subcutaneously implanted with two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (refer to Chapter III for details). The dosage given has previously been shown to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Tanzer and Jones, 2004; Hetzler et al., 2008).
4. FUNCTIONAL RECOVERY ASSESSMENT

Recovery of facial function on the right side was compared daily to the intact function on the left side, as described previously in Chapter III. Animals were observed for the return of eyeblink reflex and vibrissae orientation and vibrissa movement. For each of these functional parameters, the recovery time to reach function symmetric to that on the uninjured side was noted for analysis. For the eyeblink reflex, in addition to its complete recovery, the time till onset of the blink was also analyzed. Each group contained an $n$ of 4-6.

5. ELECTROMYOGRAPHIC RECORDINGS

Motor nerve conduction tests were done as described previously in Chapter III. Animals were anesthetized with intra-peritoneal injections of Ketamine-Xylazine, and recordings were taken pre- and post-operatively on the day of axotomy and weekly thereafter. Two stimulating electrodes were placed below the infraorbital ridge, and two recording electrodes were placed on the vibrissae pad to record the evoked muscle activity distal to stimulation. A reference electrode was placed inferior to the external ear. The facial nerve was stimulated at a frequency of 0.5 Hz and a 15 mA current, and an average of 25 responses was recorded. Percent change in peak amplitude and latency, relative to the response on the unoperated side, was calculated and plotted as a function of weeks post-axotomy. An $n$ of 4-6/time point was used for each of the 3 experimental groups.
6. STATISTICAL ANALYSIS

Significant differences in functional recovery parameters between treated and untreated groups were assessed using the non-parametric Mann-Whitney U test with \( p<0.05 \). To determine statistical significance among groups for percent change in amplitude and latency a two-way ANOVA (factors = dpo and treatment) was performed. Furthermore, it was decided \textit{a priori} to compare the untreated and treated animals at the endpoint of the study, 12 wpo.

D. RESULTS

1. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING TREATMENT WITH ES+TP COMBINATORIAL THERAPY

Figure 14 demonstrates the effects of the combinatorial therapy of ES+TP on the timing of the onset and full recovery of the facial functional parameters that returned throughout the time course of the study. Administration of the combination therapy significantly accelerated the onset of the eyeblink reflex as compared to no treatment (16.5 ± 0.3 dpo and 19.2 ± 1.0 dpo, respectively; \( p < .05 \)). Recovery time of the full eyeblink reflex was dramatically shortened in the group that received treatment. None of the untreated animals recovered the full reflex by 84 dpo, whereas all of the animals that had received the combination treatment recovered within the time frame of the study, 12 weeks. An alternative way to analyze these data, though highly conservative, would be to label all of the animals that exhibited an incomplete eyeblink reflex at the end of the study as being recovered at 84 dpo. Even this conservative approximation of recovery
Figure 14. Effects of brief ES and TP on onset and complete return of facial functional parameters following an intracranial facial nerve crush injury. Shown are recovery times in individual animals for the onset of the eyeblink reflex (Semi-Eyeblink), return of the complete eyeblink reflex, the onset of vibrissae movement, and return of the complete vibrissae orientation following administration of the combinatorial treatment of ES+TP or no treatment. As animals were followed for a maximum of 84 dpo, the asterisk over the complete eyeblink parameter indicates that all of the untreated animals experienced incomplete return of the eyeblink reflex. † = p < .05 comparing treated and untreated animals. n = 4-5/group.
time yields statistical significance when comparing the untreated and treated animals (treated animal recovery time of 76 ± 1.2 dpo), highlighting the powerful effects of ES and TP in combination.

Vibrissae orientation returned in untreated animals at 58.6 ± 1.7 dpo. It returned at 50.2 ± 1.2 dpo in treated animals indicating there was a trend toward decreasing recovery times with the addition of the combination treatment. Though this decrease did not achieve statistical significance, it came close with $p = 0.065$ and was probably due to the larger variability among untreated animals; it is also important to note that all of animals in the combinatorial treatment group recovered vibrissae orientation before the earliest animal in the untreated group, indicating this parameter is being positively affected by treatment.

Since complete vibrissae movement is the last facial functional parameter to return, the recovery time for vibrissae movement also determines the time for complete recovery. Unfortunately none of the animals in the study recovered completely within 84 days; however, there were significant differences between treated and untreated animals in the onset of the recovery of this parameter. In untreated animals, the vibrissae began to move at 46.6 ± 4.2 dpo. Administration of ES plus TP significantly reduced the time to begin movement to 23.5 ± 0.4 dpo.
2. EFFECTS OF TREATMENT WITH ES AND TP ON MOTOR NERVE CONDUCTION

Motor nerve conduction studies were performed weekly following axotomy and treatment with the combination therapy of ES and TP. Figure 15 demonstrates the percent change in peak amplitude of the evoked response for the crushed facial nerve in animals receiving no treatment and the combination of ES plus TP. Following axotomy at 1 wpo, peak amplitude dropped to ~80-90% in both groups that were injured. The amplitude was slightly decreased from baseline in sham animals, but this was not significant. By 12 wpo, peak amplitude in untreated animals remained significantly below normal. In comparison, in animals receiving the combined treatment of ES plus TP, the peak amplitude levels returned to near normal baseline values (~6.8 ± 28.5%) at 12 wpo (p < .05).

Figure 16 demonstrates the percent change in latency of the evoked response for the crushed facial nerve in animals receiving no treatment or the combination of ES plus TP. Following axotomy, at 1 wpo, latency of response increased by ~35-45% in all animals. Over the 12 weeks of the study, the latency gradually decreased and reached baseline/sham levels, ~5-10%, by 12 wpo. There was no difference in latency between the treatment groups; both remained elevated for the first 7 weeks post-injury and began to decline toward baseline starting around 8 wpo.
Figure 15. Effects of brief ES and TP on peak amplitude of evoked response following an intracranial facial nerve crush injury. Motor nerve conduction testing was done to record peak amplitude of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in peak amplitude on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. * = p < .05, comparing untreated and treated animals at a particular time point. Dotted red line at y-intercept represents baseline. n = 4-5/group.
Figure 16. Effects of brief ES and TP on latency of evoked response following an intracranial facial nerve crush injury. Motor nerve conduction testing was done to record latency of the evoked response in the vibrissal pad upon facial nerve stimulation. Shown are percent changes in latency on the injured side, relative to the unoperated side, over a time course. Vertical lines represent standard error of the mean. \( n = 4-5/\text{group}. \)
E. DISCUSSION

The present study found that the administration of a combination of ES and testosterone exerted positive effects on functional recovery from an intracranial facial nerve crush. Though the acceleration of complete recovery was not observed in any of the animals by the end of the 12 week time frame, it is possible that had the study been extended further complete recovery could have been achieved by the animals. The possibility also exists that complete recovery from this type of injury can never be realized. In that light, the benefits seen in the parameters indicative of partial functional recovery demonstrate the benefit of this combinatorial treatment. Since recovery from this proximal injury is protracted just as in human recovery from facial nerve palsy, any return of function brought about by this combination therapy – however little – is important as it gives hope to patients who have severe peripheral nerve injuries.

Investigators have described the uneven process of axonal regeneration across a transection and surgical repair site to be patchy as the axons cross the injury site at different rates and branch repeatedly before finding their way (Brushart et al., 2002; Al-Majed et al., 2000a). Since it has been shown that ES, when administered alone, does not promote the return of the normal evoked facial nerve response following intratemporal injury and does not yield complete recovery (Sharma et al., 2010a), ES is likely effective in amplifying the initial regeneration response but cannot sustain it. Testosterone, on the other hand, can accelerate the rate of regeneration as well as prevent atrophy of target muscles; its actions complement the actions of ES by sustaining the upregulated regeneration response through continued upregulation of genes associated with
regeneration (Sharma et al., 2009b; Sharma et al, 2010b). The effectiveness of the combinatorial treatment of ES and testosterone in accelerating functional recovery and the return of more normal motor nerve conduction may be explained by faster regrowth across the crush site, mediated by ES, in addition to a sustained enhanced regenerative response, mediated by TP. These findings have direct clinical relevance for cases of proximal intracranial facial nerve injury in which functional recovery is significantly prolonged.
CHAPTER V

EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE ON FUNCTIONAL RECOVERY FOLLOWING RECURRENT LARYNGEAL NERVE INJURY

A. ABSTRACT

Because suboptimal functional recovery often occurs following peripheral nerve, it may be valuable to employ therapies that enhance the intrinsic ability to neurons to regenerate. Using the injury paradigm of intracranial facial nerve crush, our previous study investigated the effects of a combinatorial treatment strategy, consisting of ES of the proximal nerve stump and systemic TP administration in Aim 1. Results indicated that the combination improves functional recovery. To begin to determine the generalizability of this treatment strategy, the second aim of this dissertation investigated the effects of ES and TP on recovery from a recurrent laryngeal nerve (RLN) crush. Following a left RLN crush at the level of the 7th tracheal ring, gonadectomized adult male rats were administered only ES, only TP, a combination of both, or left untreated and vocal fold mobility was assessed weekly. The two treatments were found to accelerate recovery individually as well as in combination.
B. INTRODUCTION

The RLN follows a long and indirect course from where it branches off the Vagus nerve to its laryngeal entry. Its passage through the thorax and course through the thyroid gland make it particularly vulnerable to damage during surgery involving the neck or thorax. RLN lesions result in significant patient morbidity, causing vocal fold paralysis and incomplete glottic closure which can lead to changes in vocal pitch or a lack of voice, dysphonia, dysphagia, and in some cases aspiration (Araki et al., 2005; Tessema et al., 2009). The effects of these symptoms on patient quality of life can be devastating and recovery from this type of injury is variable and dependent on the degree of the initial damage; some patients recovery spontaneously within weeks while others never do (Myssiorek, 2004; Rosenthal et al., 2007). Current treatments focus on medialization of the injured vocal fold through injection of collagen but do not address the lack of mobility (Rubin et al., 2003). Intraoperative monitoring of nerve conductivity is standard procedure in operating rooms during surgeries that put the RLN at risk; however, this has only been used as a pre-emptive measure. Previous work has demonstrated that a combination treatment consisting of brief electrical stimulation (ES) and supraphysiologic levels of testosterone, a gonadal steroid, improves functional recovery from both extratemporal and intratemporal facial nerve injury in rats (Hetzler et al., 2008; Sharma et al., 2010b) and from intracranial injury as shown in Aim 1. If this combination treatment proves to be effective in a second nerve injury model, it would provide more evidence for its potential use over a spectrum of human peripheral nerve injuries.
Electrical stimulation (ES) of injured nerves is associated with enhanced regeneration and thus it would be valuable to explore the translational potential of ES to RLN injury (Gordon et al., 2008). A summary of ES-mediated neuronal effects includes neuronal excitation, upregulation of regeneration-associated gene expression, promoting neurite growth after injury, improving selective targeting of regenerating axons, providing alignment and polarity instructions to myelinating Schwann cells, and enhancing the functional recovery of target organs (Gordon et al., 2008; Wan et al., 2010). Gonadal steroid hormones have also been explored as neurotherapeutic agents and have been shown to play trophic and protective role in conditions of injury or disease (Fargo et al., 2008). As gonadal steroids and ES may target regeneration events through different mechanisms or during different phases, combining both of these treatment strategies may be advantageous.

ES can be applied to a variety of places along the path of conduction. Target muscles innervated by the injured nerve can be stimulated or the neuron itself can be stimulated either distal or proximal to the injury site. End-organ stimulation has been clinically useful because it is a substitute for the usual neuronal input and thus keeps the denervated muscle alive while the injured axons are regenerating; however, it does not stimulate axonal regrowth itself (Williams, 1996). Stimulation to the injured nerve itself sends impulses in the forward direction along the myelin sheath as well as backwards toward the cell body to augment the internal distress signals carried by reverse axonal transport that increase expression of regeneration-associated genes such as growth-associated protein-43 (GAP-43; present at the tips of growth cones), α1-tubulin (a
structural protein within axons), brain-derived neurotrophic factor (BDNF), neuritin (enhances neurite extension in vitro), and pituitary adenylate cyclase-activating peptide (PACAP) (Sharma et al. 2010b).

In addition to effecting change when applied at different points along the neuronal conduction path, ES can be applied either once or multiple times within a wide range of safe stimulation parameters. In terms of clinical relevance, patients are much more likely to be compliant with a one-time application versus having to come back to clinic for multiple follow-up visits and both animal as well as human data indicate that one-time stimulation is at least as effective, if not more so, than frequent stimulations (Al-Majed et al., 2000; Gordon et al., 2009; Sharma et al., 2010a). ES treatment affects “early” events during regenerations: it reduces the initial delay before sprout formation begins, fails to accelerate the overall regeneration rate, and leads to rapid but short-term upregulation of regeneration-associated genes (Sharma et al., 2009b; Sharma et al., 2010b). Thus, the positive effects of ES on recovery have often been observed soon after treatment.

Testosterone, on the other hand, has been shown to accelerate the axonal regeneration rate through upregulation of genes involved in producing the structural building blocks of axons (including the tubulins) and leads to delayed but long-term upregulation of other regeneration-associated genes (Sharma et al., 2009b; Sharma et al., 2010b; Jones and Oblinger, 1994). We have previously combined this delayed effect of testosterone, using testosterone propionate (TP) which is a long-lasting form, with the more-rapidly acting ES to yield positive recovery results in facial nerve injury models (Hetzler et al., 2008; Sharma et al., 2010a). It is additionally important to note that the
RLN is similar to the facial nerve in that both cranial nerve nuclei from which the two nerves arise are abundant in androgen receptors (the nucleus ambiguus and the facial motor nucleus, respectively) (Perez and Kelley, 1996; Yu and McGinnis, 2001) and thus may be similarly sensitive to androgen treatment. However, cancers of the neck and throat are typically androgen-receptor negative (Voelter et al., 2008; Hagedorn and Nerlich, 2002) – an important factor to consider when it is often the removal of these cancers during which the RLN is injured.

It has been previously demonstrated that the natural timecourse for recovery from an RLN crush injury is up to 6 weeks (Tessema et al., 2009). The aim of the present study was two-fold: 1) to develop a model for therapeutic application of ES to an injured RLN; 2) to compare the effects of individual ES and testosterone treatments and the combination on functional recovery thereby adding generality to the efficacy of the combination treatment. Brief ES and TP were administered either alone or in combination and vocal fold mobility was assessed at different time-points in different groups of rats between 1 and 4 weeks post-operative (wpo).

C. MATERIALS AND METHODS

1. ANIMALS & NERVE INJURY PARADIGM

Adult male Sprague-Dawley rats (~2 months old) were purchased from Harlan (Indianapolis, IN) and used for all experiments, as described in Chapter III. Three to five days prior to nerve injury, rats were anesthetized with isofluorane and castrated. For
RLN crush axotomies as well as vocal fold mobility assessments, rats were anesthetized with isofluorane and breathing rate was monitored continuously during procedures. The left RLN was crushed at the level of the 7th tracheal ring, as described previously in Chapter III. Two successive 30-second crushes, on alternating sides, were done with fine jeweler’s forceps to ensure a full crush.

The experimental design for this study is illustrated in Figure 17. Animals were divided into 4 experimental groups: [1] no treatment, animals receiving axotomy but no treatment, [2] ES only, animals receiving axotomy and ES treatment, [3] TP only, animals receiving axotomy and TP treatment, and [4] ES + TP, animals receiving axotomy and the combination of ES and TP treatments.

2. ELECTRICAL STIMULATION

A custom electrode apparatus constructed in our laboratory was used in all rats (refer to Chapter III). Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two “male” connector pins in a connector strip. At the time of axotomy, wires were bent around the freed left RLN in place between the 5th and 9th tracheal rings (cathode) and sutured ~3-5mm away from the injury site (anode). The connector pins of rats were attached to leads of an isolated pulse stimulator, and rats were stimulated at a voltage at which they displayed flutter of the left vocal fold. Immediately post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20 Hz or sham stimulated for 30 min.
Figure 17. Experimental Design for Aim 2. This timeline will be followed to examine the effects of ES and TP on recovery from recurrent laryngeal nerve (RLN) crush. Untreated animals will be observed at 5 and 6 weeks in addition to the other time points.
3. HORMONE ADMINISTRATION

Immediately following injury, two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (Sigma), were subcutaneously implanted in rats receiving hormone treatment. The dosage given has previously been shown to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Hetzler et al., 2008; Tanzer and Jones, 2004).

4. VOCAL FOLD MOBILITY ASSESSMENTS

Direct laryngoscopy was achieved with a pediatric ENDOGO® and a user-modified sheath as described in Chapter III. Because of the tongue suture required for video capture of vocal fold mobility (VFM), weekly measurements were not performed on the same animals; instead animals were assigned to a time-point and VFM measurements were only performed on the day of the crush and the day of euthanasia. This increased the number of groups and subsequently the number of animals required for the study.

Recovery of vocal fold mobility on the left side was compared to the intact function on the right side. Paralysis was defined as the inability of the laryngeal muscle to abduct the affected side of the vocal fold. Videos were block-randomized, blinded, and scored by trained observers. An integral scoring scale of 0-4 was used.
5. STATISTICAL ANALYSIS

Significant differences in vocal fold motion among the various treatment groups were assessed using a two-way ANOVA (treatment x time) followed by the Holm-Sidak post-hoc test at p < .05 to examine the relationship between treatment and time on VFM scores.

A logarithmic regression analysis of the percentage of animals achieving complete recovery was carried out to predict recovery status using time and treatment as predictors (SPSS). Chi-squared analyses were subsequently used at individual time points to examine the predictive value of a treatment based on recovery status and to determine if there were significant differences between treatments in the likelihood of achieving complete recovery.

D. RESULTS

1. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING TREATMENT WITH ES, AND/OR TP

Figure 18 demonstrates the effects of ES alone on the timing of the recovery of vocal fold motion. Administration of brief ES significantly accelerated VFM recovery as compared to no treatment at one and two weeks post-crush (Average VFM = 0.5 and Average VFM = 2 at 1 wpo, respectively; p<0.01; average VFM = 2.25 and Average VFM = 3.67 at 2 wpo, respectively; p<0.05; Figure 18). However, as all animals were reaching the ceiling of full recovery by 3 and 4 weeks, this difference was no longer
Figure 18. Effects of brief electrical stimulation (ES) on vocal fold mobility (VFM) following a recurrent laryngeal nerve (RLN) crush injury. Shown are the average VFM scores at 1, 2, 3, and 4 weeks after RLN crush injury (WPO) comparing Untreated (CTL) and ES-treated animals. Each bar represents a separate cohort of animals. Vertical lines represent standard error of the mean. (** = p < .01, relative to no treatment; * = p < .05, relative to no treatment). n = 4-8/group.
Figure 19. Effects of brief electrical stimulation (ES), and/or supraphysiological testosterone propionate (TP) on vocal fold mobility (VFM) following a recurrent laryngeal nerve (RLN) crush injury. Shown are average VFM scores for 1, 2, 3, 4, 5, and 6 weeks after RLN crush injury (WPO) comparing all treatments. Vertical lines represent standard error of the mean. (** = p < .01, each treatment compared to no treatment; * = p < .01, ES and ES+TP treatments compared to no treatment). n = 4-9/group. Each time point represents a separate cohort of animals.
significant at these time points. Figure 19 demonstrates the effects of ES and/or TP on the timing of the recovery of vocal fold motion. TP administration by itself also produced increased VFM scores at 1 and 2 weeks post-crush as compared to controls, but there were no statistical differences between the ES-treated and TP-treated animals. (Average VFM for TP treatment = 1.9 and 3.4 at 1 and 2 wpo, respectively). Adding ES and TP produced the largest significant increase in average VFM at the early time-point of 1 week post-operative as compared to untreated animals (Average VFM = 0.5 and Average VFM = 2.4 at 1 wpo, respectively; p<0.05; Figure 19).

Vocal fold mobility scores were significantly increased in all treatment groups relative to the untreated group at 1 and 2 wpo. These differences became non-significant by week 3 as the untreated animals’ VFM scores began to move closer toward the upper limit of complete recovery. Within the untreated group, completely recovered animals began to be seen only at 3 wpo, whereas in the treatment groups fully recovered animals were seen as early as 1 wpo. The Two-way ANOVA with Holm-Sidak post-hoc tests revealed a statistically significant effect of treatment $[F(3, 80) = 6.57, p < .001]$ and a statistically significant effect of time $[F(3, 80) = 35.37, p < .001]$. No statistically significant interaction was observed between time and treatment $[F(9, 80) = .90, \text{n.s.}]$.

Table 1 breaks down the percentage of animals within each treatment group that did or did not attain complete RLN functional recovery at each time point. It required 6 weeks for all animals in the untreated group to recover which is consistent with previously published literature (Tessema et al 2007). The application of all treatments (TP, ES, or ES+TP) significantly decreases the time to recovery of vocal fold mobility
Table 1. Effects of brief electrical stimulation (ES), and/or supraphysiological testosterone propionate (TP) treatment on time required to complete functional recovery after recurrent laryngeal nerve (RLN) crush injury. Shown are the percentages of animals in each group that achieved a vocal fold mobility score of 4 (defined as complete functional recovery) at 1, 2, 3, 4, 5, and 6 weeks after RLN crush injury comparing Untreated, TP-treated, ES-treated, and ES+TP-treated animals. Listed below each percentage in parentheses is the number of recovered animals out of the entire set at each particular time point (n = 4-9/group). Each time point represents a separate cohort of animals.
from a RLN crush by two weeks which equates to a 33% reduction in overall recovery time. Unlike the treated animals, none of the untreated animals achieved complete recovery within the first two wpo. The logarithmic regression analysis was conducted to predict recovery status using time and treatments as predictors. A test of the full model against a constant-only model was statistically significant, indicating that receiving a treatment increased the odds of attaining full recovery ($Chi\ square = 53.358, p < .0005$ with $df = 2$). This corroborates the previously described result that VFM scores were higher among the treated animals at the earlier time points. Nagelkerke’s $R^2$ of .571 indicated a moderately strong relationship between prediction and grouping. Prediction success overall was 79.2% (76.7% for incomplete recovery and 81.1% for complete recovery) and improved from an initial 55.2%. The Wald criterion demonstrated that time, ES-treatment, TP-treatment, and ES+TP-treatment each made a significant contribution to prediction ($p < .0005$, $p = .001$, $p = .004$, and $p = .003$, respectively). EXP(B) values (odds ratios) for these parameters were 5.3 (wpo), 28.8 (ES-treatment), 15.6 (TP-treatment), and 18.7 (ES+TP-treatment), respectively. For example, this implies that the odds of an ES-treated animal being completely recovered at a particular time point are 28.8 times higher than if the animal is untreated. The Chi-squared analyses of cross-tabulations were performed to compare the treatments to each other and were not significant, suggesting that all treatments yielded similar results.
E. DISCUSSION

The present study found that administration of monotherapy of ES and the combination of ES and TP both had similar effects on functional recovery. ES alone accelerated the VFM functional recovery parameter and was sufficient to improve complete functional recovery as compared to untreated animals. In contrast to previous studies in the facial nerve, the combination of ES and testosterone in the RLN crush injury model, however, did not significantly shorten the time until complete functional recovery more than individual treatment. Though the benefit of a combinatorial treatment approach was not demonstrated in this model, this could be due to a few factors. One difference which could account for this is the particular nerve injured. Though both the facial nerve and recurrent laryngeal nerve derive from cranial motoneurons, the RLN is a branch of the vagus nerve – a nerve with numerous functions – whereas the facial nerve is completely motor after it exits the facial canal. The polyvagal theory has suggested that the myelinated and unmyelinated portions of the nerve serve different functions for neurogenic control of the heart (Porges, 2009). Since the heart is extremely sensitive to electrical activity, the vagal neurons and fibers that eventually compose the RLN may be more responsive to electrical stimulation as a therapy. This could account for the seeming lack of improvement with the addition of TP to the treatment regimen that is necessary in studies of regeneration of the facial nerve (Sharma et al., 2010a). Additionally, the distance to regenerate in the RLN model is almost twice as short as that required in an extratemporal facial nerve crush (the most distal crush our laboratory has examined). If the required regeneration distance is short,
to a certain degree maximal acceleration of regeneration may be achieved by one therapy alone and thus no extra benefit is seen by combining two effective treatments.

Electrical stimulation of many varieties has been demonstrated to accelerate behavioral recovery from facial and hindlimb paralysis after injury to the facial, femoral, and sciatic nerves (Al-Majed et al., 2000; Hetzler et al., 2008; Gordon et al., 2009); and, direct stimulation of the injured nerve stump has been shown to decrease the delay in axonal sprouting post-injury as well as upregulate the regeneration-associated genes GAP-43, α1-tubulin, BDNF, PACAP, and neuritin to enhance regeneration (Sharma et al., 2009b; Sharma et al., 2010b). Furthermore, this treatment would be easily applied in a surgery setting as a stimulator could be easily connected to the intraoperative monitors used to detect nerve damage during neck and thoracic operations. If nerve damage is noted, the stimulation could be applied while the patient is still under anesthesia. The downsides to a subsequent increase in operative time would be more than compensated for by the potential for improved speech, swallowing, and breathing outcomes with the use of ES therapy. Additionally, though the 30-min duration of stimulation has been shown to be effective in this study using the RLN and in others using the facial nerve, shorter periods have not be examined and it is entirely possible that less time may be required to see positive results in humans.

Gradual recovery in VFM after unilateral RLN crush injury was observed over a period of 6 weeks in untreated animals and 4 weeks in treated animals. The untreated animals recovered along a similar time course to that observed in other studies using a unilateral crush injury (Araki et al., 2005; Tessema et al., 2008; Tessema et al., 2009).
Tessema et al noted moderate recovery by 3 wpo (2008) with full recovery by 6 weeks post-operative. Our observation that none of the animals in the 2 wpo time point had recovered and only half of the animals had completely recovered at the 4 wpo time point confirms others reports (Araki et al., 2005; Shiotani et al., 2007).

It is also important to be aware of the relative differences in the distances that RLN motoneurons must regenerate their axons in humans versus rats when interpreting the results of the acceleration of behavioral recovery (both initiation and complete). Both human and rat nerve regeneration rates are in the same order of magnitude, approximately 1.7 mm/day in humans (Sunderland, 1947) and 4 mm/day in rats (Kujawa et al., 1993; Wang et al., 1997); however, the distance that a RLN motoneuron in a rat must regenerate its axon is on the order of millimeters whereas the same injury in a human will require the RLN motoneuron to regenerate centimeters of axon. Given this data, it is possible that humans will respond differently and will require the combination treatment to achieve more complete recovery because their RLN injuries are comparatively more severe than those delivered to the rats in this pre-clinical study.
CHAPTER VI

EFFECTS OF ELECTRICAL STIMULATION AND TESTOSTERONE IN COMBINATION ON FUNCTIONAL RECOVERY FOLLOWING SCIATIC NERVE CRUSH AS COMPARED TO SCIATIC NERVE TRANSECTION-SURGICAL REPAIR (AUTOGRAFT) INJURY

A. ABSTRACT

Injuries to the sciatic nerve can be caused by a vast array of etiologies; however, no matter the manner of injury, if it is severe enough the functional outcome will likely be poor. Differing types of injury have disparate associated management strategies based on the whether or not the injury is lacerating, clean, acute, or chronic. The purpose of the third aim of this dissertation was to further assess the effects of the combination therapy by comparing functional recovery in two types of injury to the sciatic nerve with or without ES plus TP. The sciatic crush injury was compared to a surgery mimicking a nerve graft procedure of the sciatic nerve (an autograft: a double transection-repair). Adult male rats were divided into four experimental groups: sciatic crush, sciatic crush with ES+TP treatment, sciatic transection-repair (autograft), and autograft with ES+TP. Animals’ gaits were observed with the CatWalk system at 2, 4, 8, and 16 weeks post-operative (wpo). Rats treated with the combination therapy recovered partial function earlier than untreated rats in both injury paradigms.
B. INTRODUCTION

Due to its extreme length, the sciatic nerve is subject to many insults. Sciatic nerve lesions are common clinical conditions that result in significant distortion of patients' gait, stance, and balance. Although function may return completely in some patients, others may demonstrate delayed recovery or residual deficits. Unsatisfactory recovery may be due to significant neuronal loss, inadequate reinnervation, misdirection of regenerating axons, or denervation-induced target atrophy.

As the nerve is formed within the lumbar plexus, it may be injured through compression by the overlying piriformis; this is known as piriformis syndrome. The sciatic nerve runs close to the femur as it extends down the thigh, so it can be severed or crushed when the femur is broken. When the sciatic nerve enters the popliteal fossa, its common peroneal and tibial branches separate and occupy relatively superficial locations in the knee region which render it vulnerable to mechanical insults including battlefield and gunshot injuries. Though the neuromuscular physical exam performed by physicians and nerve conduction studies can localize the majority of injuries to the sciatic nerve in humans and document functional recovery, complicated tools for gait analysis must be used in addition to study recovery from injury in animals (Derr et al., 2009; Masakado et al., 2008).

Current treatments for sciatic nerve paralysis following transection injuries include direct anastomosis or cable grafting (Kim et al., 2004). Additionally, ES and TP have been shown to have positive effects individually on both the experimental sciatic crush and the more clinically relevant transection-repair injury that closely mimics the insertion of a nerve graft (Brown et al., 1999; Brushart et al., 2002; Alrashdan et al.,
2010). However, many patients have incomplete recovery and experience long-term sequelae from the paralysis. Therefore, the third aim of this dissertation aimed to compare the effects of the combination therapy on functional recovery between the well-established sciatic crush injury and the transection(s) with repair(s) sciatic injury. Our findings demonstrated significant differences in recovery sciatic function among injury types and treatments.

B. MATERIALS AND METHODS

1. ANIMALS

Adult male Sprague-Dawley rats (~2 months old) were purchased from Harlan (Indianapolis, IN) and used for all experiments, as described in Chapter III. For sciatic nerve injuries, rats were anesthetized with isoflurane. The experimental design for this study is illustrated in Figure 20. Animals were divided into four experimental groups based on the type of sciatic nerve injury and therapy administered: 1) sciatic nerve crush, no treatment; 2) sciatic nerve crush, ES+TP; 3) sciatic nerve autograft (double transection and repair), no treatment; and, 4) sciatic nerve autograft, ES+TP.
Figure 20. Experimental Design for Aim 3. This timeline will be followed to examine the effects of the combination treatment on recovery from sciatic nerve crush and sciatic nerve autograft.
2. SCIATIC NERVE CRUSH INJURY

A gluteal incision was made, and the right sciatic nerve was exposed using an approach along the grain of the biceps femoris (refer to Chapter III for details). At approximately mid-thigh, before branching of the main trunk into common peroneal and tibial nerves, the sciatic nerve was crushed with jewelers’ forceps twice for 30 sec each on either side as with the other crushes. The standard crush performed ensured that all nerve fibers were crushed while the axonal sheath remained intact. In all animals, the left sciatic nerve was left intact to serve as an internal control. The post-operative survival time was 8 weeks for animals who received crush injury.

3. SCIATIC NERVE AUTOGRRAFT INJURY

The surgical procedure is described in detail in Chapter III. A 2 cm segment of the right sciatic nerve was exposed along its distal course. The nerve was transected at two points along the nerve, 7 mm apart, and then the epineurium was reattached with microsuture. In all animals, the left sciatic nerve was left intact to serve as an internal control. The post-operative survival time was 16 weeks for animals who received the transection and repair injury.
4. ELECTRICAL STIMULATION

A custom electrode apparatus constructed in our laboratory was used in all rats (refer to Chapter III). Two Teflon-coated wires, bared of insulation for 2-3mm, were soldered to two “male” connector pins in a connector strip. At the time of axotomy, the cathode wire was bent around the circumference of the right sciatic in place proximal to the injury site and the anode was sutured ~3-5mm away in the biceps femoris. The connector pins of rats were attached to leads of an isolated pulse stimulator, and rats were stimulated at a voltage at which they displayed twitching of the right hind limb. Immediately post-axotomy, rats were either stimulated with supramaximal pulses delivered at a frequency of 20 Hz or sham stimulated for 30 min.

5. HORMONE ADMINISTRATION

Immediately following injury, two Silastic capsules (0.062 in. id x 0.095 in. od; 10-mm length), equilibrated in physiological saline and containing 100% crystalline TP (Sigma), were subcutaneously implanted in rats receiving hormone treatment (refer to Chapter III). The dosage given has previously been shown to establish supraphysiological levels of systemic TP (Kujawa et al., 1989; Hetzler et al., 2008; Tanzer and Jones, 2004).

6. FUNCTIONAL RECOVERY ASSESSMENT – CATWALK GAIT ANALYSIS

Rats were trained once a day for two weeks prior to injury to traverse the lighted walkway of the CatWalk™ gait analysis apparatus at least three times in a row (refer to Chapter III). Recovery of sciatic function on the right side was compared weekly to the
intact function on the left side and the following parameters were analyzed: base of support, print length, print width, print area, step pattern preference, and regularity index. Each group contained an $n$ of 5-6.

7. STATISTICAL ANALYSIS

Significant differences among the four experimental groups were determined using a two-way ANOVA (factors = days post-axotomy and treatment), followed by a Holm-Sidak post-hoc test at $p < .05$.

D. RESULTS

1. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING TREATMENT WITH ES+TP COMBINATORIAL THERAPY AFTER SCIATIC CRUSH

Figure 21 demonstrates the effect of the combinatorial therapy of ES+TP on hind paw length. In A there is a trend towards increased paw length on the injured side among treated animals as compared to those that were untreated. Though these differences over time did not reach significance, they do suggest that the combination treatment may have a positive effect on repair. The print length is increased at the 2 week time point relative to the 4 and 8 week time points for both treated and untreated animals; this is due to heel walking, an adaptation rats will use when their paw will not support weight. Normally the heel does not impact the walking surface unless a rat is sitting. The use of this adaptation can make it difficult to detect treatment effects at the earlier time points as seen in Figure 21A. Absent heel-walking, the injured hind paw length generally
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<th>Axotomy (R)</th>
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<td>ES+TP</td>
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**Table 2. Symbol Legend for Aim 3.** For viewing consistency, the following labeling scheme has been used: open symbols indicate no treatment, closed symbols indicate treatment, triangles indicate the control side (also referred to as the unaxotomized or left side), and circles indicate the side undergoing axotomy (also referred to as the axotomized or right side). For parameters that take both control and axotomized sides into account, including base of support and step pattern frequency, squares are used.
Figure 21. Effects of brief ES and TP on hind paw length following a sciatic crush injury. Behavioral testing with the CatWalk™ apparatus was done to record paw print length during walking after injury. Shown are the print lengths of the injured right hind paw (A) and the contralateral uninjured left hind paw (B). Vertical lines represent standard error of the mean. $n = 6$/group.
increases over time, which is noted at weeks 4 and 8 post-operative. The uninjured paw length does not change appreciably in either treated or untreated animals over time.

Figure 22 shows the upward trajectory in injured paw width in animals over time. Animals that received ES+TP have increased measurements as compared to untreated animals at 2 and 8 weeks post-operative. There are no changes over time between untreated and treated animals in the uninjured paw; the line is relatively flat near 20 mm. By 8 weeks, the injured paw length of treated animals has increased to 17.4 ± .5 mm, nearly 85% of the uninjured side, whereas the untreated animals have a paw length of only 16.0 ± .8 mm (78%; p < .05 relative to no treatment). These differences are highlighted in Table 3.

Figure 23 illustrates the effect of treatment on hind paw area. The injured hind paw area in untreated animals increases over time from 109.0 ± 33.1 mm² to 161.1 ± 17.3 mm² as shown in A. This area in treated animals is starts off at a similar value to that of the untreated animals, 112.8 ± 12.4 mm², but increases to 218.9 ± 16.4 mm² by the end of 8 weeks. This final value is similar to the area of the uninjured side in untreated animals, 218.8 ± 15.2 mm². The increase in hind paw area with treatment (161.1 ± 17.3 mm² and 218.9 ± 16.4 mm²; p < .05) indicates that the combination therapy is accelerating recovery. In Figure 23B though the lines appear to be different at the 8 week time point, the overall ANOVA was not significant, indicating that there are no differences over time between the treated and untreated groups. Table 4 displays this data in tabular form.

There is a significant decline in hind paw base of support (BOS) at 2 wpo from 11.1 ± 2.3 mm to 4.6 ± 1.6 mm in untreated animals and 7.2 ± 2.2 mm in treated animals.
Figure 22. Effects of brief ES and TP on hind paw width following a sciatic crush injury. Behavioral testing with the CatWalk™ apparatus was done to record paw print width during walking after injury. Shown are the print widths of the injured right hind paw and the contralateral uninjured left hind paw. Vertical lines represent standard error of the mean. * = p < .05 comparing the treated and untreated animals’ injured paws. n = 6/group.
### Table 3. Comparison of Hind Paw Width at 2 and 8 wpo after Sciatic Crush Injury.

This table highlights the decrease from week 2 to week 8 in the differences between control and axotomized sides post-sciatic crush.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Axotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 wpo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>19.4</td>
<td>11.1</td>
</tr>
<tr>
<td>ES+TP</td>
<td>20.8</td>
<td>13.6</td>
</tr>
<tr>
<td><strong>8 wpo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>20.3</td>
<td>16.0</td>
</tr>
<tr>
<td>ES+TP</td>
<td>20.6</td>
<td>17.4</td>
</tr>
</tbody>
</table>
Figure 23. Effects of brief ES and TP on hind paw area following a sciatic crush injury. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the print areas of the injured right hind paw (A) and the contralateral uninjured left hind paw (B). Vertical lines represent standard error of the mean. * = p < .05; n = 6/group.
Table 4. Comparison of Hind Paw Area at 2 and 8 wpo after Sciatic Crush Injury. This table highlights the decrease from week 2 to week 8 in the differences between control and axotomized sides post-sciatic crush.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Axotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 wpo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>212.0</td>
<td>109.0</td>
</tr>
<tr>
<td>ES+TP</td>
<td>267.5</td>
<td>112.8</td>
</tr>
<tr>
<td><strong>8 wpo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>218.8</td>
<td>161.1</td>
</tr>
<tr>
<td>ES+TP</td>
<td>282.7</td>
<td>218.9</td>
</tr>
</tbody>
</table>
Figure 24. Effects of brief ES and TP on hind paw base of support following a sciatic crush injury. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the bases of support comparing treated and untreated animals. Vertical lines represent standard error of the mean. $n = 6$ / group.
as shown in Figure 24. This is due to the animals putting little to no weight on the injured paw and drawing it close to their body thus decreasing the BOS; essentially they are ambulating on three limbs at this early point during the time course. Though there were no significant differences between the ES+TP-treated and untreated animals over time; however, there does appear to be a trend towards increased BOS for treated animals versus untreated animals.

Figure 25 tracks the percentage of footfall patterns that are alternate (Aa and Ab combined) or are cruciate (Ca and Cb combined). The rotary patterns are rarely used, on average less than 5% of the time, and this does not change with injury or treatment. Before injury, the alternate patterns are clearly preferred, being used an average of 66.1 ± 4.9% of the time. The cruciate patterns are used an average of 32.3 ± 5.2% of the time. The change in footfall pattern usage over time for the alternate footfall patterns follows U-shaped curves; there are inverted U-shaped curves to describe the reciprocal changes in the use of the cruciate patterns. At 2 and 4 weeks post-operative, both treated and untreated animals decrease their usage of the alternate patterns and replace them with the cruciate patterns: % alternate for untreated animals at 2 wpo is 54.5 ± 9.4; % alternate for treated animals at 2 wpo is 49.5 ± 14.6; % cruciate for untreated animals at 2 wpo is 44.3 ± 10.5; % cruciate for treated animals at 2 wpo is 50.5 ± 14.6; % alternate for untreated animals at 4 wpo is 52.6 ± 7.5; % alternate for treated animals at 4 wpo is 42.9 ± 8.6; % cruciate for untreated animals at 4 wpo is 45.3 ± 8.5; % cruciate for treated animals at 4 wpo is 57.1 ± 8.6. At 8 weeks, though, footfall pattern preference has returned to nearly the same percentages as before injury. Though the differences at 2 and
Figure 25. Effects of brief ES and TP on footfall pattern following a sciatic crush injury. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the percentages of footfall pattern choices over time of alternate (A) and cruciate (B) patterns. Vertical lines represent standard error of the mean. n = 6/group.
4 weeks post-operative were not ultimately significant, this trend to decrease the percent of alternate patterns and increase the percent of cruciate patterns is interesting.

2. DIFFERENCES IN FUNCTIONAL RECOVERY FOLLOWING TREATMENT WITH ES+TP COMBINATORIAL THERAPY AFTER SCIATIC NERVE AUTOGRAPH

Figure 26 tracks hind paw length over time after injury and the effects of the combinatorial therapy of ES+TP on this parameter. In contrast to the sciatic crush injury, in Figure 26A paw length does not change appreciably on the injured side among untreated animals. The treated animals display a slight increase at 4 wpo, but this is non-significant. The uninjured paw increases in length over time from ~23 mm to ~34 mm similarly in treated and untreated groups.

Figure 27 demonstrates the differences in hind paw width over time due to treatment with ES+TP. At the 8 and 16 week time points hind paw width is increased in the injured paw when animals were treated with the combinatorial therapy as compared to the untreated animals (12.5 ± 1.1 mm, 11.3 ± 1.9 mm, 11.6 ± 1.0, and 10.2 ±1.1, respectively; p < .05). The combination treatment also stabilized the uninjured paw width over time. At 2, 4, and 8 weeks post-operative the animals’ uninjured paws are significantly narrower when untreated than the treated animals’ uninjured paws and take up to 16 weeks to reach that level (2 wpo: 19.4 ± 0.8 and 21.9 ± 0.5; 4 wpo: 18.8 ± 1.6 and 21.3 ± 0.7; 8 wpo: 19.9 ± 0.7 and 22.6 ± 0.9; p < .05 comparing treatment versus no treatment).
Figure 26. Effects of brief ES and TP on hind paw length following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw print length during walking after injury. Shown are the print lengths of the injured right hind paw (A) and the contralateral uninjured left hind paw (B). Vertical lines represent standard error of the mean. $n = 5$/group.
Figure 27. Effects of brief ES and TP on hind paw width following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw print width during walking after injury. Shown are the print widths of the injured right hind paw and the contralateral uninjured left hind paw. Vertical lines represent standard error of the mean. # = p < .05, comparing the uninjured left paw of untreated and treated animals. * = p < .05, comparing the injured right paw of untreated and treated animals. n = 5/group.
Figure 28 shows the effects of the combination therapy on recovery of hind paw area. The combinatorial treatment significantly accelerated the increase in the area of the injured hind paw as compared to no treatment (4 wpo:  81.5 ± 16.9 mm² and 48.0 ± 7.3 mm², respectively; p < .05) as well as that of the uninjured hind paw (2 wpo:  172.2 ± 6.6 mm² and 221.2 ± 13.4 mm², respectively; 4 wpo:  181.3 ± 31.4 mm² and 248.2 ± 23.2 mm²; p < .05 comparing treated to untreated animals). The area occupied by both left and right hind paws steadily increases over time (8 wpo data is shown in Table 5). In combination these indicate that both the calf muscles and intrinsic foot muscles are being reinnervated faster with treatment. Additionally, the combinatorial treatment also significantly accelerated the return of base of support as compared to no treatment (4.4 ± 0.9 mm and 9.9 ± 2.3 mm, respectively; p < .05; Figure 29). Treatment with ES+TP improved the base of support in animals receiving the autograft injury to nearly the same levels achieved by the crush injury level at 4 wpo (see Table 6); this is a substantial improvement considering that the autograft injury is much more severe.

Though the regularity index did not decrease significantly following the sciatic crush injury, there was a decline in this parameter following the autograft injury from the baseline value of 97.5 ± 0.6 % to 84.4 ± 3.4 % in untreated animals by the end of the study (Figure 30). Treatment with the ES+TP combination seemed to stall this decline, though the differences between treated and untreated animals were ultimately non-significant. Also in contrast to the crush injury, the footfall pattern preference did not seem to be altered immediately following the autograft injury, nor were there changes over time.
Figure 28. Effects of brief ES and TP on hind paw area following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the print areas of the injured right hind paw and the contralateral uninjured left hind paw. Vertical lines represent standard error of the mean. # = p < .05, comparing the uninjured left paw of untreated and treated animals. * = p < .05, comparing the injured right paw of untreated and treated animals. n = 5/group.
Table 5. Comparison of Hind Paw Area at 8 wpo between Sciatic Crush and Autograft Injuries. This table highlights the differences in hind paw area between the different injuries by week 8 and their responses to treatment with ES+TP.

<table>
<thead>
<tr>
<th>8 wpo</th>
<th>Crush</th>
<th>Autograft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Axotomy</td>
</tr>
<tr>
<td>Untreated</td>
<td>218.8 15.2</td>
<td>161.1 17.3</td>
</tr>
<tr>
<td>ES+TP</td>
<td>282.7 20.1</td>
<td>218.9 16.4</td>
</tr>
</tbody>
</table>
Figure 29. Effects of brief ES and TP on hind paw base of support following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the bases of support comparing treated and untreated animals. Vertical lines represent standard error of the mean. * = p < .05, comparing untreated and treated animals at a particular time point. n = 5/group.
Table 6. Comparison of Base of Support at 4 wpo in Crush versus Autograft Injury. This table highlights the differences in base of support between the different injuries by week 4 and their responses to treatment with ES+TP.

<table>
<thead>
<tr>
<th></th>
<th>Crush</th>
<th>Autograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8.7</td>
<td>4.4</td>
</tr>
<tr>
<td>ES+TP</td>
<td>10.0</td>
<td>9.9</td>
</tr>
</tbody>
</table>
Figure 30. Effects of brief ES and TP on regularity index following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown is the regularity index over time in treated and untreated animals. Vertical lines represent standard error of the mean. $n = 5$/group.
Figure 31. Effects of brief ES and TP on footfall pattern following autograft. Behavioral testing with the CatWalk™ apparatus was done to record paw prints during walking after injury. Shown are the percentages of footfall pattern choices over time of alternate (A) and cruciate (B) patterns. Vertical lines represent standard error of the mean. $n = 6$/group.
Figure 31 demonstrates the steady preference for the alternate step sequence pattern ~65% of the time and use of the cruciate step pattern ~35% of the time following autograft; this is unchanged with treatment.

E. DISCUSSION

Sciatic nerve paralysis is a common peripheral nerve injury that has significant functional impact on patients. Despite this, there are few available treatments for traumatic injury to the nerve and none are reliably effective in yielding functional success. The individual therapies of ES and TP have resulted in improvements but have not yet been combined (Alrashdan et al., 2010; Brown et al., 1999; Kujawa et al., 1993; Vivo et al., 2008; Walker et al., 1994). The current study found that administration of the combination of the two lead to significant improvements in some measures of functional recovery but not others and these varied depending on injury type.

The parameters hind paw width and hind paw area were both significantly improved by the combination treatment in both types of injury, indicating that they are useful functional recovery correlates to rely on in future studies. Since increasing paw width is correlated with reinnervation of the intrinsic foot muscles, the results suggest that regeneration of these axons is accelerated by the combinatorial treatment. Concomitantly, increases in paw length can be correlated to calf muscle reinnervation (Brown et al, 1999; de Medinaceli et al., 1982). Paw area is a composite parameter and thus encompasses the length and width parameters; hence, the accelerated recovery that occurs with treatment indicates that both are being positively affected by the combination of ES and TP. Even though there was only a trend towards accelerated recovery of print
length in the crush injury, the increased foot print area engendered by the ES+TP
treatment combined with this trend have positive implications for its treatment potential.
Additionally, since this combination appears to stabilize the print width of the uninjured
foot, while accelerating the return of this parameter on the injured side in the more severe
autograft injury, it suggests that the therapy is enhancing the function of the normal hind
limb to promote balance during the regeneration process. That this is occurring in the
more severe injury paradigm is encouraging for the combination’s potential in human
axonal injury.

As mentioned previously, sciatic injury causes an initial decrease in the hind paw
base of support because the injured paw cannot be used to bear weight and is thus
retracted in towards the belly with gradual increases in the parameter being observed over
time (Deumens et al., 2007). The combination of ES and TP significantly accelerated the
return of this function in the autografted animals, providing further optimism for
incorporating this treatment strategy into existing therapy regimens.

Interestingly enough, step pattern preference and regularity index differed
throughout the course of the experiment between the two different injuries studied:
among the crush-injured animals there was a trend toward a shift from clearly preferring
the alternate patterns prior to injury (2:1 choice of alternate patterns) to ambivalence (1:1
choice of alternate versus cruciate) at 2 and 4 weeks post-operative and a return to the
before-injury preference at 8 weeks post-operative. This could be a sort of protecting
phenomenon, in which the animal favors the injured limb and takes measures to protect it
from further injury, thus allowing it to heal faster. The cruciate patterns provide more
stability to the animal as both paws of the shoulder girdle are in contact with the ground
before the hind paws have to support any weight; it aids in balancing the animal’s center of gravity more towards midline. Utilizing these patterns may allow for undivided focus on repair by the neurons as opposed to splitting efforts between repair and generating unanswered action potentials. There was no such shift in the animals that had undergone an autograft. Adding ES+TP to the crush-injured animals further shifted their preference towards the cruciate step patterns. It is possible that the more time the animal spends ambulating by cruciate step patterns, the more unfettered time the neuronal cell bodies have to generate the necessary components to regrow their axons, resulting in accelerated functional recovery (corroborated by the trend towards maintainance of the regularity index with treatment).

Though the results of this study of the combination treatment of electrical stimulation and testosterone imply that it has utility in the human population, it is not a panacea for nerve injury be it axonotmesis nor neurotmesis. The importance of a combinatorial strategy cannot be overemphasized, however. Sharma et al., previously demonstrated the benefit of this combinatorial strategy (2010a). It is likely that for severe traumatic nerve injury cases, a variety of therapies will need to be applied to achieve maximal recovery.
CHAPTER VII
SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

A. SUMMARY

This dissertation investigated the effects of the ES plus TP combination therapy on peripheral nerve injury by studying the functional effects of treatment in three distinct, clinically relevant, peripheral nerves: the facial nerve, the recurrent laryngeal nerve, and the sciatic nerve. Aim 1 examined the effects of the combination therapy consisting of ES and TP in an intracranial facial nerve crush injury model, a model with limited spontaneous recovery potential. The results are summarized as follows: administration of the combination of ES and TP improved recovery of facial functional parameters as compared to no treatment; however, these improvements were only seen in parameters signifying partial function. Complete recovery was not achieved within the study time frame by any of the animals, with or without treatment. Aim 2 assessed the effects of ES and TP in a recurrent laryngeal nerve crush injury model, a model with robust spontaneous recovery potential. The results determined that administration of ES and TP had similar effects on functional recovery following a recurrent laryngeal crush as both treatments individually accelerated the return of vocal fold mobility; however, the combination of the two therapies did not provide any added benefit beyond the individual treatments. Aim 3 investigated the effects of the combination therapy including ES
and TP in two parallel models of sciatic nerve injury: sciatic nerve crush and sciatic nerve autograft. The results of aim 3 indicated that sciatic nerve crush injury altered the step pattern choice in rats, whereas a sciatic nerve autograft did not appear to influence step pattern. Functional recovery did not return to normal in untreated animals by the end of the study period of 8 wpo (crush) and 16 wpo (autograft); however, treatment with ES and TP improved functional recovery following both types of sciatic injuries.

B. CONCLUSIONS AND FUTURE DIRECTIONS

The overall results of the present study, as described above, have been presented and discussed in the corresponding chapters. The following topics now incorporate the results together in context of current peripheral nerve regeneration research.

1. INSIGHT INTO EXTENT OF ES+TP-MEDIATED THERAPEUTIC EFFECTS IN THE DIFFERENT PERIPHERAL NERVE INJURY MODELS

The experiments in the present investigation examined the broad efficacy of a novel combinatorial treatment strategy based on the underlying hypothesis that if the two treatments, ES and TP, work through different mechanisms, then together they might be used to obtain additive effects on regeneration. Previous studies have shown that this combination effectively accelerates recovery after both extratemporal and intratemporal facial nerve injuries (Hetzler et al., 2008; Sharma et al., 2010a).

The nerve injury site is a reliable factor for predicting recovery, as the more proximal the injury site is to the cell body, the more prolonged the recovery timeline. Crushing the facial nerve intracranially is the most proximal peripheral nerve crush injury
that has been studied to date; this injury shares similarities with ventral root avulsion in the spinal cord except no CNS trauma occurs with intracranial facial nerve crush. Applying the combination therapy to this severely proximal injury yields benefit, but not as striking as that seen in the intratemporal facial nerve crush model. This finding suggests that other therapies may need to be added to the ES+TP combination to achieve better recovery outcomes in the future. In contrast, the RLN is typically injured rather distal to the cell body, and as such spontaneous recovery is always exhibited in animal models. The application of the individual therapies appears to be sufficient to boost regeneration measurably by decreasing the time required to achieve complete functional recovery. This phenomenon may be advantageous for this particular nerve, as it is can be injured during cardiovascular surgeries, thyroidectomies, and anterior approaches to the cervical spine. In situations where TP administration would be contraindicated, such as pregnancy or prostate cancer, to have a potential treatment that works well enough on its own is promising. Unlike the RLN crush but similar to the proximal intracranial facial nerve injury, sciatic nerve injuries follow a protracted recovery timeline. The functional improvements seen following both sciatic nerve injuries indicate that though the combination therapy of ES and TP is helpful, there is still much work to be done in finding effective treatments for peripheral nerve injury.

2. INSIGHT INTO OTHER TREATMENTS THAT MAY BE COMBINED WITH ES AND TP

Prior research has demonstrated that ES mediates its positive effects by directly affecting the motoneuron cell body (Al-Majed et al., 2000a) to rapidly enhance the
expression of various regeneration-associated genes (Sharma et al., 2010b) which results in a decreased delay to sprout formation (Sharma et al., 2009b). Activation of neurotrophin signaling is most likely an important result of ES treatment and precedes upregulation of cytoskeletal proteins (Al-Majed et al., 2000b; Al-Majed et al., 2004). Another important source of neurotrophic factors are denervated Schwann cells and the distal nerve stumps; yet, the upregulation of BDNF and GDNF at these sites is delayed after axotomy (Hoke et al., 2006; Boyd and Gordon, 2003a; Boyd and Gordon, 2003b). The delayed availability of neurotrophic factors from these sources suggests that the neuronal source is of critical importance during earlier times of axonal outgrowth; furthermore, ES is likely enhancing regeneration via the immediate upregulation of neurotrophic molecules.

Previous studies have established that the androgen-induced augmentation of facial nerve regeneration is AR-dependent. Administration of flutamide, a potent AR blocker that prevents binding of TP, prevents the TP-induced increase in regeneration rate (Kujawa et al., 1995). Interestingly, axonal injury results in significant downregulation of AR in the FMN and Spinal Nucleus of the Bulbocavernosus models (Drengler et al., 1997; Lubischer and Arnold, 1995). Injury may induce transcription of cytoskeletal and growth-associated genes and shut down production of other less important genes such as AR. Therefore, TP may mediate a more long term effect by increasing the regeneration rate through the mobilization of preexisting steroid receptors.

Though the molecular regeneration program is important, other aspects of the regeneration process need to be explored to maximize the recovery potential. Electrical stimulation of the target musculature (transcutaneous and subcutaneous) has been
explored in humans after certain nerve injuries. Transcutaneous electrical nerve stimulation (TENS), though used more for pain relief due to its activation of the endorphins, enkephalins, and dynorphins (Solomon et al., 1980; Bushnell et al., 1991; Han et al., 1991), may have positive effects on motoneurons. Additionally, the application of a therapy that modulates pain sensitivity may indirectly affect motor function by removing the negative effect that pain has on voluntary movement. Subcutaneous stimulation of the target musculature may also keep it viable for longer, enhancing its potential for reinnervation. Voluntary movement in the form of exercise, as well as forced exercise, has been shown to improve functional recovery from traumatic peripheral nerve injury as well as spinal cord injury. This is thought to occur via increasing neurotrophin levels in the CNS near the neuronal cell bodies and altering the expression patterns of apoptosis-associated microRNAs following injury (English et al., 2011; Côté et al, 2011; Liu et al., 2010). Exercise and other vigorous forms of physical therapy may provide a bridge to further extending axonal regeneration to promote improved functional recovery outcomes. These therapies may aid the combination of ES and TP in accelerating regeneration and supporting recovery.

3. BENEFITS OF A COMBINATORIAL TREATMENT APPROACH

The need for combinatorial treatments is especially evident as neither ES nor TP alone are effective in improving complete functional recovery in rats following an intratemporal injury of the facial nerve (Sharma et al., 2010a). In addition, the current investigation demonstrated that this combination does not accelerate regeneration enough to achieve complete functional recovery in the intracranial crush injury within the time
limit of the experiment. The finding that the combinatorial treatment of ES plus TP substantially shortens the time to recover partial function may be due to ES recruiting more motoneurons to cross the crush zone early, while TP maintains neurotrophic factor and growth-associated gene expression to potentially promote target reinnervation. TP may also avert the muscle atrophy associated with prolonged target denervation. As ES has previously been reported to increase specificity of reinnervation and direct Schwann cell polarity, brief ES treatment may enhance the outcome of intracranial facial nerve injury by reducing deviant regeneration along the multiple branches of the motor nerve and steering axons in the correct direction. Therefore, a combinatorial treatment strategy would be especially beneficial in cases of prolonged recovery periods following nerve injury and attaching extra treatments that target other obstacles in the regeneration process would be additionally advantageous.

4. THERAPEUTIC NEED AND POTENTIAL FOR TRANSLATION OF THE ES+TP TREATMENT STRATEGY

Regeneration in humans is known to be at least 2 times slower in comparison to animal models of peripheral nerve regeneration (Gordon et al., 2008). Given the ~4 mm/d regeneration rate for the fastest growing axons in rats (Kujawa et al., 1993; Wang et al., 1997), the expected regeneration rate in humans would be ~1.7 mm/d, reported by Sunderland in 1947. Furthermore, the latency period for axons to traverse the injury site and enter distal nerve stumps is also longer in humans. In addition to an inherently slower regeneration rate, peripheral nerves in humans also have to regenerate over considerably longer distances post-injury as compared to rats. Therefore, suboptimal
functional recovery is often seen following peripheral nerve injuries. The past quarter of a century has focused on optimization of surgical repair of injured nerves, neglecting the opportunity to simultaneously target the molecular mechanisms of regeneration.

As ES and TP differentially enhance the intrinsic ability of neurons to regenerate, together they are an effective treatment strategy prospect. Several studies demonstrate successful translation of ES and TP treatments individually into the clinic. Brief ES of the median nerve in carpal tunnel syndrome patients has been shown to improve recovery of thenar function (Gordon et al., 2007). Direct ES of the sacral and hypoglossal nerves have also been conducted in patient trials for treatment of bladder dysfunction and obstructive sleep apnea, respectively (Sutherland et al., 2007; Schwartz et al., 2001). Therefore, ES, for a brief duration, represents a feasible approach as a treatment for peripheral nerve injuries especially when a nerve is injured throughout the course of a surgical procedure such as the RLN injury. Similarly, testosterone treatment has been tested in multiple patient trials. Intramuscular testosterone treatment in men diagnosed with Parkinson’s disease increased total testosterone levels by ~270% and improved non-motor symptoms of the disease (Okun et al., 2006; Okun et al., 2002). Sublingual administration of testosterone in healthy females also attenuates the integrated central stress response (Hermans et al., 2007). Clinical trials in male multiple sclerosis patients have demonstrated that application of testosterone gel to the upper arms increased circulating hormone levels by an average of 50% and improved cognitive function, slowed brain atrophy rate, and induced production of neurotrophic factors (Sicotte et al., 2002; Gold et al., 2008). Testosterone replacement therapy has also been successful in increasing muscle mass in AIDS patients with wasting (Sardar et al., 2010).
The application of testosterone treatment to human peripheral nerve injuries has not yet been attempted. As different doses and routes of delivery for testosterone treatment are currently being used in patient trials for other maladies, the most effective dosage and administration method would have to be determined for the various peripheral nerve injuries. Our studies have shown that supraphysiological levels (>3 times normal levels) are required for the effect of enhanced regeneration. Previous studies from our lab have demonstrated that following an extratemporal facial nerve crush in male hamsters, TP needs to be administered within 6 h of injury for a period of 6 h in order to have beneficial effects (Tanzer and Jones, 2004). Furthermore, a 6 h immediate TP exposure enhanced regeneration and functional recovery to the same extent as a 7 d treatment. These findings have important clinical implications and suggest that an immediate but short-term treatment with gonadal steroids may have significant therapeutic advantages for peripheral nerve injuries.

SIGNIFICANCE

In conclusion, the combination of ES and TP can be applied broadly to a variety of traumatic peripheral nerve injuries. Though the improvements seen in the models studied in this dissertation were varied, ranging from progress in partial recovery to accelerating the return of complete functional recovery, the overarching theme is that increases in function occurred in all of the nerve injury models following treatment with ES+TP. The treatments were effective in all four injury paradigms, indicating that the positive effects of ES+TP are not limited to injuries of the facial nerve, nor are they specific to cranial nerves. Thus, scientists and physicians should transition to
investigating this combination treatment in humans with traumatic peripheral nerve injuries.

The present study offers insight into the general effects of ES and TP on functional recovery in four peripheral nerve injury models. However, several questions remain to be elucidated and thus are presented as future directions below. These additional studies will further advance the translation of the proposed combinatorial treatment strategy (ES plus TP) into the clinic. These questions are as follows: what is the minimal dose and duration of TP treatment required to achieve therapeutic effects? Is there increased cell loss from the intracranial facial nerve crush injury that can explain the substantially delayed timeline for functional recovery in untreated animals? Does the combination of ES+TP rescue any cell loss in this model if it occurs? Finally, can other therapies, such as exercise and target muscle stimulation, further enhance recovery seen in the peripheral nerve injury models with prolonged recovery periods, the intracranial facial nerve crush, the sciatic nerve crush, and the sciatic nerve autograft?
REFERENCES


The author, Gina Nicole Monaco, was born to Ruth and Antonio Monaco in Chicago, IL on October 8, 1983. Growing up in Hyde Park, she completed her secondary education at The University of Chicago Laboratory Schools (Chicago, IL) and entered Princeton University (Princeton, NJ) in 2002. While at Princeton, Gina majored in chemical engineering and served as a resident advisor and peer health educator. During the summer of 2003 she worked in the laboratory of Dr. Nancy Schwartz under the direction of Dr. Miriam Domowicz at the University of Chicago investigating the effects of chondroitin sulfate proteoglycans on nervous system development. During the summer of 2004 she participated in an undergraduate research program sponsored by the University of Chicago; there in the laboratory of Dr. Gopal Thinakaran she began to study γ-secretase malfunction in relation to Alzheimer’s disease.

In 2005, Gina joined the molecular and behavioral neuroscience laboratory of Dr. Bartley Hoebel at Princeton, where she investigated brain neurotransmitter release linked to obesity for her senior thesis in chemical engineering and neuroscience. After graduating in 2006, Gina entered the MD/PhD program at Loyola University Stritch School of Medicine. For her PhD track, she decided to join the Cell Biology, Neurobiology, and Anatomy Graduate Program. During the summer of 2007, she
joined the laboratory of Dr. Kathryn Jones and became involved in peripheral nerve regeneration research in collaboration with the Department of Otolaryngology – Head and Neck Surgery at Loyola University Medical Center. Gina has presented her research at the national Society for Neuroscience meetings in 2009 and 2010 and received a travel award from the American Association of Anatomists to present at the Experimental Biology meeting in 2010 and 2011. She is currently a student member of the American Association of Anatomists and the Society for Neuroscience. After graduating from the MD/PhD program in 2013, Gina plans on pursuing a residency in neurosurgery and continuing to research therapies for nerve injuries.

Publications:

