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Axotomy and Regeneration of the Rat Facial Nerve: A Histomorphometric Study of the Facial Nucleus, Nerve and Orbicularis Oculi Muscle in an Experimental Model of Facial Paralysis

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AXOTOMY AND REGENERATION OF THE RAT FACIAL NERVE: A HISTOMORPHOMETRIC STUDY OF THE FACIAL NUCLEUS, NERVE AND ORBICULARIS OCULI MUSCLE IN AN EXPERIMENTAL MODEL OF FACIAL PARALYSIS

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ABSTRACT

AXOTOMY AND REGENERATION OF THE RAT FACIAL NERVE:
A HISTOMORPHOMETRIC STUDY OF THE RAT FACIAL NUCLEUS, NERVE AND ORBICULARIS OCULI MUSCLE IN AN EXPERIMENTAL MODEL OF FACIAL PARALYSIS

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Eastern Virginia Medical School and
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One of the most unsettling sequela of facial paralysis (FP) is the loss of eye sphincter function and the blink response, leading to functional, psychological and aesthetic deficits. A medley of restorative microsurgery approaches have been employed in treating these deficits, however full recovery of function remains elusive. The present research utilized the rat model of facial paralysis, and consisted of three stages. Stage I examined the facial motor neuron (FMN) pool of the eye sphincter (orbicularis oculi muscle=OOM) and identified the facial nerve branch which provides the majority of innervation to the OOM. II. Examined the efficacy of the cross-facial nerve graft (CFNG) treatment of FP (behaviorally and histomorphometrically). III. Evaluated the efficacy of a dual treatment approach, by combining the CFNG and local administration via osmotic pump of insulin-like growth factor-I (IGF-I). Results demonstrated that IGF-I (50 μg/ml) enhanced recovery of function, its time of onset, as well as the histomorphometric profile of the nerve graft. While results were encouraging, full recovery of function was not achieved with IGF-I. Further studies using different doses of IGF-I, as well as using multiple growth factors and nerve grafting are warranted.

This work is dedicated to my loving parents Konstantinos and Kyriaki Thanos to whom I owe all of my successes and happiness. Thank you for all your sacrifices! In addition, to my wonderful wife and english instructor extraordinaire, Renie B. Thanos who always gave much needed love and support despite her own arduous career. I would also like to thank my sisters Georgia and Vaya, for their advice and great humor, my brother Vittorio and my mother-in-law Fotini Blentsas. Finally, this work is dedicated to my hometown of Mesovouni, Karditsa, Greece.

Μεσοβουνι

Θελω να ανεβω στα Αγραφα
και πανω στα κατσαγραφα
στο Μεσοβουνι που αγαπω
να το ξεχασω δεν μπορω.

Στο πανηγυρι να βρεθω
και στην πλατεια τον χορο
που τα κλαρινα αντιλαλουν
και παλληκαρια τραγουδουν.

Στην σπηλια να ανεβω
απο την κρυα βρυση για να πιω
και τα παλια να θυμηθω
τον Κωτσο Θανο τον σοφο.

Παναγιωτης Κωνσταντινος Θανος (1997)

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CHAPTER I

INTRODUCTION

A. BACKGROUND AND SIGNIFICANCE

1. Incidence and Cost of Facial Paralysis

The face is a prominent feature of man and plays a critical role in an individual's physical, psychological, and emotional makeup. Facial disfigurement, such as Facial Paralysis (FP), can affect all of these components and can result in social and vocational handicaps, as well as psychiatric impairment. According to the American Medical Association, FP is listed as a 15% to 35% disability, depending on the severity of the symptoms. Determining the total annual health care cost of FP is a formidable task. Estimates, however, using the premise that the incidence of FP (in the US) is about 20-30/100,000 cases per year, suggest that the annual health care cost is approximately $1.09 billion dollars (Adour, 1996a; 1996b; Yanagihara, 1988).

Facial Paralysis (FP) is a devastating neurological disorder that gives rise to both functional and aesthetic deficits. Estimates indicate that over sixty thousand cases of FP occur in the United States each year (Schaumburg *et al.* 1992; Yanagihara, 1988), with no gender or geographical differences in incidence (Yanagihara, 1988). While there are many causes of FP, the ultimate goal of the health care provider is to restore function and

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The model journal used for this dissertation was The Journal of Comparative Neurology.

symmetry of the facial muscles. Discerning the complex series of neuromuscular events that are involved in recovery of function are fundamental to a more concrete understanding of FP. It is the purpose of this study to examine the restoration of function to denervated facial muscles and the role of growth factors in facilitating the reinnervation of these denervated muscles.

2. Embryology, Anatomy and Physiology of the Facial Nerve

The anatomy, embryology and physiology of the facial nerve makes it the most frequently injured nerve of the human body (Kartush, 1997). Understanding the anatomy, embryology and physiology may facilitate the reinnervation of denervated facial muscles and restoration of their function.

Development of the facial nerve and its related musculature is well underway by 90 days of gestation in humans (Sunderland and Bradley, 1962; Burt, 1993). By the first month of gestation, the acousticofacial primordium develops rostral to the otic placode, the precursor of the inner ear. The acousticofacial primordium gives rise to both the facial and acoustic nerves (Vidic 1978; Miglets et al., 1986).

In the second month of gestation, the acousticofacial primordium extends to the geniculate ganglion. Distal to this ganglion, the primordium differentiates into a caudal and a rostral trunk. The caudal trunk progresses into the mesenchyme of the second branchial arch and becomes the main trunk of the facial nerve (Gasser and May, 1987; Burt, 1993).

The rostral branch grows into the first arch and develops into the chorda tympani nerve. Facial nerve development consists of the separate development of its motor and sensory roots. The geniculate ganglion and nervus intermedius, arising from the second branchial arch, forms independently of the motor division of the seventh nerve (Gasser 1977). The facial nerve then develops within the temporal bone. The extratemporal portion of the facial nerve begins development during the second gestational month, with the more proximal branches forming first in all five branches. Facial muscles, derived from the second branchial arch, are formed at 8 weeks gestation and are rapidly innervated by the distal facial nerve branches. Facial muscles differentiate prior to being innervated by a motor fiber. The period of time that a developing facial muscle retains the ability to be innervated is unknown. After three month's gestation, all facial muscles can be identified and the majority are functional (Gasser 1967).

The facial nerve anatomy was largely a mystery until Gabrielle Fallopius identified the anatomy of the facial nerve within the temporal bone and recognized the chorda tympani nerve as a branch of the facial nerve in the 15th century (as referenced in Barr, 1993; Burt, 1993). It wasn't, however, until 1829, when the motor and sensory function of the face was first described by Charles Bell. Bell properly attributed motor innervation to the seventh cranial nerve and sensory innervation to the fifth cranial nerve (as referenced in Glascock et al., 1990).

The neural pathway involved in the voluntary responses of the facial musculature (such as smiling or tightly closing the eyes on command), begins with the neurons located in the cerebral cortex (May, 1986). The primary motor cortex, located in the precentral

gyrus (Broadmann areas 4, 6 and 8), provides projection neurons to the facial nucleus. The fibers project through the internal capsule and continue through the basal part of the pons via the corticobulbar tract. In the caudal portion of the pons, the majority of nerve fibers decussate to reach the facial nucleus of the opposite side, although some fibers innervate the ipsilateral facial nucleus. There is also extrapyramidal cortical input to the facial nucleus from frontal areas, hypothalamus and globus pallidus via the reticular formation. These inputs account for emotional control of facial expression (May, 1986; Portman, 1986). Other brainstem nuclei project to the facial nuclei, especially from sensory centers. Visual system afferents contribute to the blink reflex (Simpson, 1991); afferents from the trigeminal nerve and nuclei provide the basis for the corneal reflex; and those from the acoustic nuclei account for eye closure in response to loud noises (startle reflex).

The facial nerve is a mixed nerve carrying 1) motor axons, 2) autonomic efferent axons and 3) sensory axons (May, 1973). Somatic motor neurons in the facial motor nucleus supply the bulk of the axons of this nerve and innervate nearly all the muscles of the face. The facial nerve trunk prior to branching distal to the stylomastoid foramen is composed of approximately, 7000 myelinated axons in humans (May, 1986). Parasympathetic preganglionic neurons in the superior salivatory and lacrimal nuclei medial to the facial motor nucleus makes up a second efferent component of this nerve and supply secretory-motor fibers to the submaxillary gland, sublingual gland, and lacrimal glands (Fig. 1).

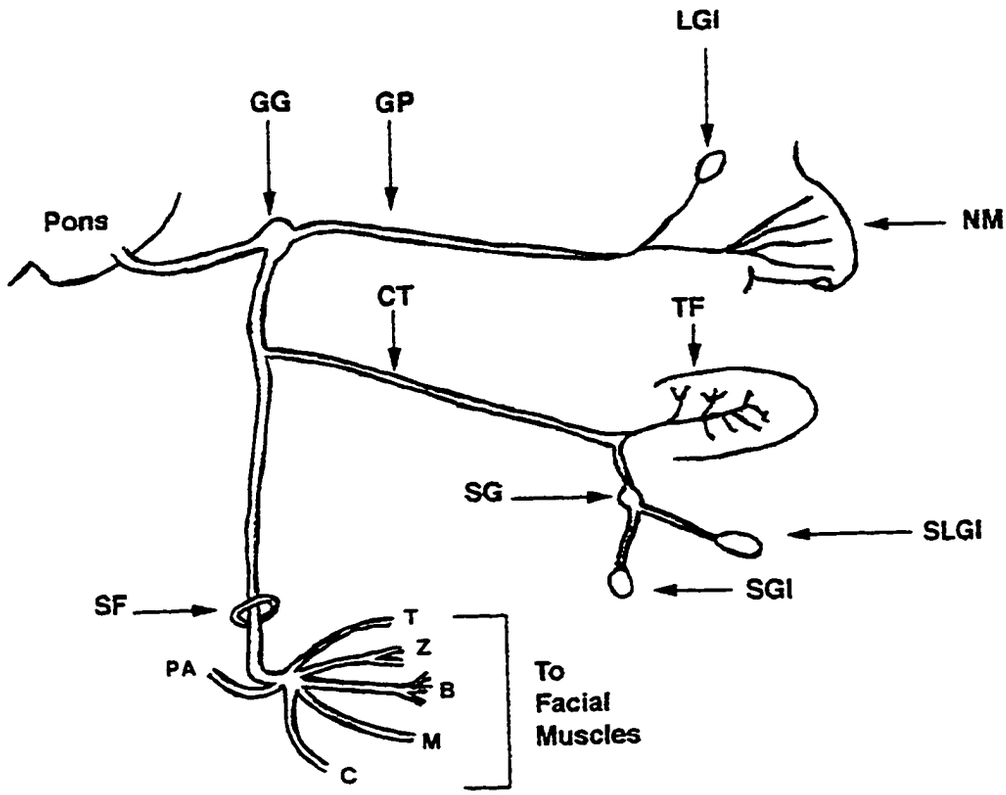


Fig. 1. Schematic representation of the human facial nerve (Adopted from Simpson, 1991; May, 1986). GG= Geniculate Ganglion; GP= Greater Petrosal nerve; LGl= Lacrimal Gland; NM= Nasal Mucosa; CT= Chorda Tympani nerve; TF= Taste Fibers; SG= Submandibular Ganglion; SLGl= Sublingual Gland; SGl= Submandibular Gland; SF= Stylomastoid Foramen; T= Temporal; Z= Zygomatic; B= Buccal; M= Mandibular; C= Cervical; PA= Posterior Auricular.

The majority of facial nerve branches contain only motor axons. However, afferent fibers innervate the mucous membranes of the nose, palate and lacrimal gland, through the greater petrosal nerve (May, 1986), as well as the submandibular, sublingual glands and taste buds in the anterior 2/3 of the tongue via the chorda tympani nerve (Role and Kelly, 1991). The cell bodies of these somatic and visceral afferents are found in the geniculate ganglion (Fig. 1). The extratemporal segment of the facial nerve that exits the stylomastoid foramen is composed almost entirely of motor fibers and enters the parotid gland after giving off its main branches that innervate the facial muscles.

The muscles that are responsible for facial expression and eye movements are among the most structurally and functionally diverse skeletal muscles. The eye muscle fibers extend the entire length of the muscle, are more rounded in transverse sections, and are smaller in diameter than most skeletal muscles. In addition, the orbicularis oculi muscle (OOM) and the extraocular muscles, controlling fine movement, have the smallest number (< 10) of muscle fibers per motor unit, whereas the larger skeletal limb muscles have a greater number of motor units, such as the gastrocnemius muscle with 2000 muscle fibers per motor unit (Bourne, 1973; May, 1986).

Human eye movements associated with the blink and voluntary eyelid closure can be described as consisting of a) transient relaxation with disappearance of tonic activity in muscles such as the levator palpebrae, which are responsible for keeping the lids open, followed by b) phasic activity in muscles such as the OOM, associated with eye closure (Terrell and Terzis, 1994). As in the human, the primary muscle responsible for eye closure in the rat is the OOM. In each case, the anatomic structure of the muscle shows

concentric loops around the eye with the fibers meeting at the medial canthus.

The many similarities between the human and rat facial nerve and its branches have been previously emphasized as amenable to experimental manipulation (Mattox and Felix, 1987) (Figs. 2, 3). Therefore, the rat was utilized as a model to study the facial nerve and FP. Previous studies have described the rat facial nerve with regards to the mean number of facial motor neurons, their diameter, and mean number of facial nerve axons (Martin *et al.* 1977; Anders *et al.* 1993). However, the specific organization and description of the FMN pool and the axons serving the OOM, have not yet been elucidated and are essential to the study of the OOM and the efferent aspect of the blink response.

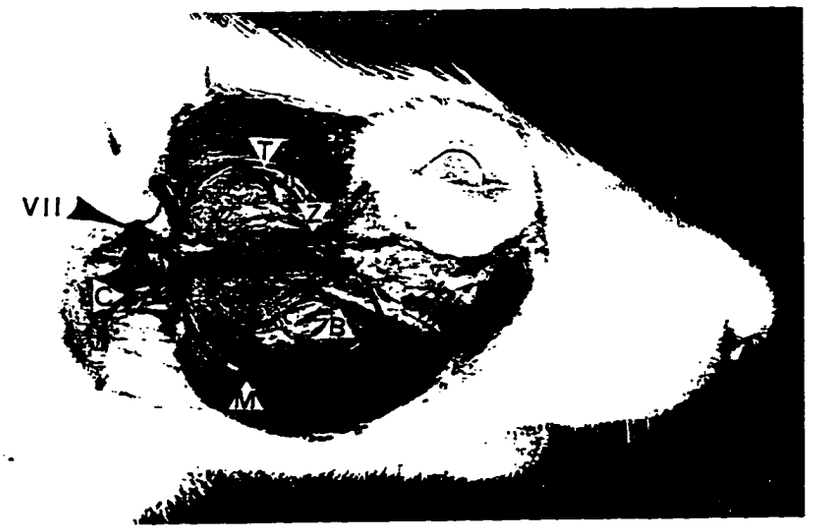


Fig. 2. Dissection of the facial nerve anatomy in the rat.

Fig. 3. A human dissection of the facial nerve and its branches (Terzis, 1989).

Furthermore, it has been reported that muscle branches of the facial nerve do not contain afferent fibers of geniculate ganglion origin (Martin and Mason, 1977; Martin and Biscoe, 1977). However, more recently it has been shown that about 30% of the total geniculate ganglion neurons contributed sensory fibers to the posterior auricular branch of the rat facial motor nerve to the external auricular muscles (Semba *et al.*,1984; Semba and Egger, 1986). The remaining facial trigeminal nucleus was found to send differential projections to various subdivisions of the VIIth nucleus depending on their origin with respect to the three cytoarchitectonically different subnuclei motor nerve branches contained few if any sensory fibers originating from geniculate ganglion cells. Thus, with the exception of the posterior auricular branch, all facial motor nerve branches are efferent.

3. The Facial Nucleus

The facial nerve motor nucleus is situated in the ventral pons, near the trigeminal nucleus (Role and Kelly, 1991; Barr, 1993; Burt, 1993). The rat facial nucleus is approximately 1.7 mm long and 1.5 mm wide and is composed of triangular and stellar shaped neurons with prominent Nissl substance (Watson et al., 1982). This general morphology of the facial motor neurons (FMNs) has been previously documented in both normal and injury models in cats (Papez, 1927; Nishimura et al., 1992; Terashima et al., 1993), mice (Chen and Bisby, 1993) and rats (Watson et al., 1982). Estimates of the number of motoneurons in the facial nucleus of the rat have been quite variable and range

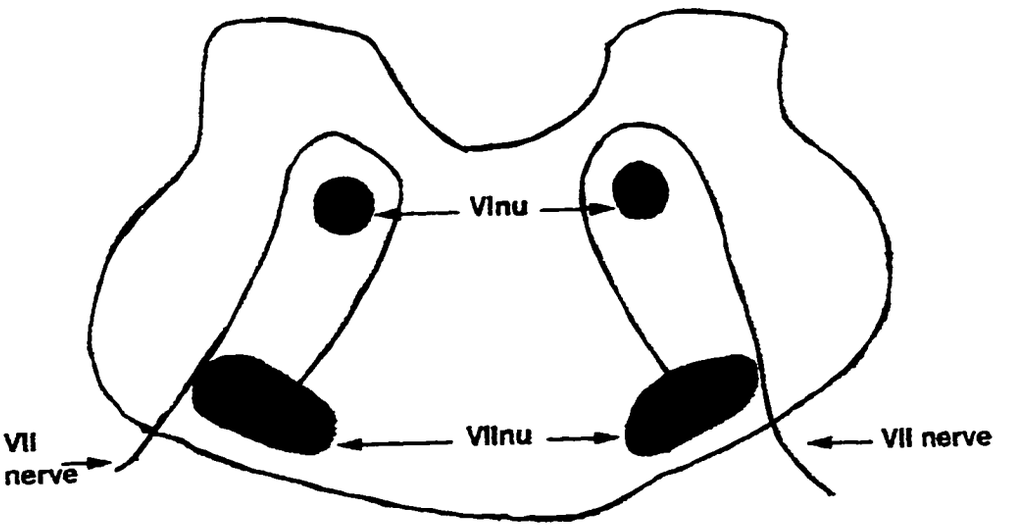


Fig. 4. Stereotaxic transverse section of the caudal pons illustrating the facial nucleus as well as the dorsolateral path of the facial nerve exiting the facial nucleus looping around the abducens nucleus and exiting the brainstem ventrallaterally. VII nu = facial nucleus; VI = abducens nucleus; VII= facial nerve. Brainstem shown at B= -10.8 mm, IA= -1.8mm (Adapted from Paxinos and Watson, 1982).

from 2100 (Streppel et al., 1997), 3000 (Anders et al, 1993), 4600 (Fernandez et al., 1995) to 5100 (Martin et al., 1977). This discrepancy can be attributed to the use of different section thickness, and counting methods. However, it is generally accepted that the diameter of FMNs ranges between 15-55 μm (Watson et al., 1988; Martin et al., 1977; Fernandez et al., 1995; Anders et al., 1993). In addition, it has been suggested that the FMNs within the rat facial nucleus are topographically organized. Specifically, retrograde labeling of the FMNs that innervate the facial muscles of the whisker pad demonstrated a topographic organization of the FMNs (Papez, 1927; Martin and Lodge, 1977; Hinrichsen and Watson, 1984).

The FMNs give rise to the facial nerve which exits the facial nucleus and forms a bundle that follows a circuitous course. The facial nerve then projects posteromedially, ascends medially to the abducens nucleus, and then as the genu of the facial nerve passes laterally around the abducens nucleus and then continues anterolaterally and caudally to emerge from the brainstem at the cerebellopontine angle (Fig. 4) before projecting to the facial muscles.

The afferent projections to the rat VIIth nucleus were widely studied by Erzurumlu and Killackey (1979) who demonstrated the ipsilateral projections of the trigeminal nucleus to the VIIth nucleus. This study along with Nord (1968), also demonstrated that the sensory surface of the rat face was topographically represented in the brainstem trigeminal complex. In addition, the trigeminal nucleus was found to send differential projections to various subdivisions of the VIIth nucleus depending on their origin with respect to the three cytoarchitectonically different subnuclei that compose the trigeminal nucleus. More

specifically, the magnocellular portion of the trigeminal nucleus (subnucleus caudalis) projects to the ipsilateral lateral subdivision of the VIIth nucleus; the interpolaris subnucleus projects to the ipsilateral dorsal and intermediate subdivision; and the oralis subnucleus projects bilaterally to the intermediate subdivision of the VIIth nucleus. In addition, the sensory axons in the facial nerve of the rat terminate in the lateral division of the nucleus solitarius before terminating in the trigeminal complex (Contreras *et al.*, 1982).

The FMNs are the final link of several neuronal pathways implicated in the facial expression of complex behaviors as different as emotions, speech, respiration, exploration of the animal's environment (sniffing, whisker movements), suppression of facial musculature tone and myoclonic twitches of whiskers, eyelids and ears during the paradoxical phase of sleep (Jouvet, 1962; Gassel *et al.* 1964). Most important to the present studies, the FMNs play a major role in the critical blink reflex.

In order to identify and describe the facial motor neuron pool of the temporal branch of the facial nerve and the OOM respectively, both biotin dextran histochemistry and fluorescence microscopy were utilized. This is critical in our analysis of eye sphincter function (blink), as well as understanding and establishing treatment strategies. Dextrans are hydrophilic polysaccharides that are characterized by their high molecular weight, good water solubility, low toxicity and relative inertness (Phelps et al., 1985). These properties make dextrans effective water soluble carriers for dyes. Moreover, their biologically uncommon α-1,6 polyglucose linkages are resistant to cleavage by most endogenous cellular glucosidases; thus, dextran conjugates are ideal long-term tracers for living cells as in this study (Phelps et al., 1985; Lang et al., 1986; Phelps et al., 1986).

The specific use of biotin dextran as a retrograde label is relatively new and first introduced, but not fully described, by Todorova and Rodziewicz (1995). This study presented evidence that biotin dextran (bd) provided retrograde tracing in the rat sciatic nerve. Using bd injected distal to a crush injury of either tibial or common peroneal nerves, spinal cord motoneuron counts after 48 h compare favorably with counts obtained using horseradish peroxidase. Advantages of bd include: fine staining of the soma and dendritic profile, as well as good staining of sciatic nerve axons 30 mm away from the crush site. Thus, biotin dextran, was demonstrated to be a good retrograde tracer in a peripheral nervous system model and was utilized in this study to morphologically describe the FMNs of the OOM and the temporal branch of VII. In addition, bd-labeled neurons could also be analyzed for the diameter of their soma using a light microscope interfaced with a digitizing tablet.

In addition to morphology, fluorescent tracers were utilized to address quantitatively the following objectives: 1) identify the facial motor neuron pool of the rat OOM and 2) identify with double labeling the specific facial nerve branch that provides the majority of innervation to the OOM.

Fluoro-Gold (FG), (hydroxystilbamidine-diisothionate) was first developed by Kuypers and his colleagues (1980) as a retrograde axonal tracer. FG is a retrograde tracer that is stable and remains fast within the cytoplasm and dendritic processes of retrogradely labeled neurons and is characterized by white-colored granules (Schmued and Fallon, 1986) visible under the fluorescent microscope using wide band ultraviolet excitation (emission max: 408 nm, excitation max: 323 nm). This study reported that FG had certain properties

such as: 1) very low molecular weight, 2) intense fluorescence, 3) filling of dendrites, 4) high resistance to fading, 5) no diffusion from labeled cells and 6) compatibility with other tracers such as the fluorochrome bisBenzimide (BB). Thus, the present study utilized FG for the quantitative analyses of FMN pools because of its very low molecular weight and thus could be injected into the OOM and undergo retrograde transport better than (the larger molecular weight) bd.

Bis-Benzimide is a nuclear and cytoplasmic tracer that labels the neuron light blue. Fluoro-Gold combined with bisBenzimide yields a double labeled cell following UV excitation, displaying a light blue nucleus, and cytoplasm and well defined dendritic processes (Schmued and Fallon, 1986). These observations can be made under the fluorescence microscope using wide band ultraviolet excitation (emission max: 408 nm, excitation max: 323 nm). More recently, a few studies have utilized fluoro-gold and bisBenzimide in double labeling studies; however, the double labeled cells were either not illustrated (Girman, 1994), or their methodology not adequately described (Horner and Kummel, 1993).

4. Classification and Etiology of Facial Paralysis

The second stage of this project examined the restoration of OOM function utilizing a cross-facial nerve graft. However, it is important to first provide some insight into the types of facial paralysis that exist and their etiology. This research addressed only FP due to

injury of the facial nerve distal to the stylomastoid foramen. There are several systems of classifying FP: One classification is based on topographic diagnosis-site of lesion causing the paralysis (Fig. 5). Another method classifies FP with respect to time of onset (Table 1). Bell's Palsy is one type of FP with a sudden onset not due to injury. Symptoms presented include: unilateral weakness of all facial muscles of sudden onset, recently associated with a viral insult, no evidence of CNS pathology, no evidence of lesion, no history of otologic disease.

Although the exact etiology of Bell's Palsy is still unclear, most clinicians now believe that herpes simplex infection is the most likely agent (Adour, 1996a; Mulkens and Schirm, 1997; Morgan, 1997). This belief was supported by an increase incidence of HSV antibodies in patients with Bell's Palsy when compared to age-matched controls (Adour, 1996a). Recent animal studies have also supported this theory (Sugita *et al.*, 1995; Murakami *et al.*, 1996). Furuta et al (1997) discovered a high level of HSV-1 shedding into the saliva of Bells Palsy patients within the first week after onset. In April of 1997, several reports from the VIIth International Symposium on Facial Nerve held in Matsuyama, Japan implicated the herpes simplex virus type 1 in the pathogenesis of Bell's Palsy (Kaga et al, 1997; Tanaka, 1997; Hato et al, 1997; Mirakami et al, 1997; Honda et al, 1997; Adour, 1997). Unlike trauma-induced FP; the prognosis for Bell's Palsy is generally good with 85-90% of patients recovering completely within one month (Adour, 1996a). The remaining progress to complete degeneration and will not usually show signs of recovery (Adour, 1996a).

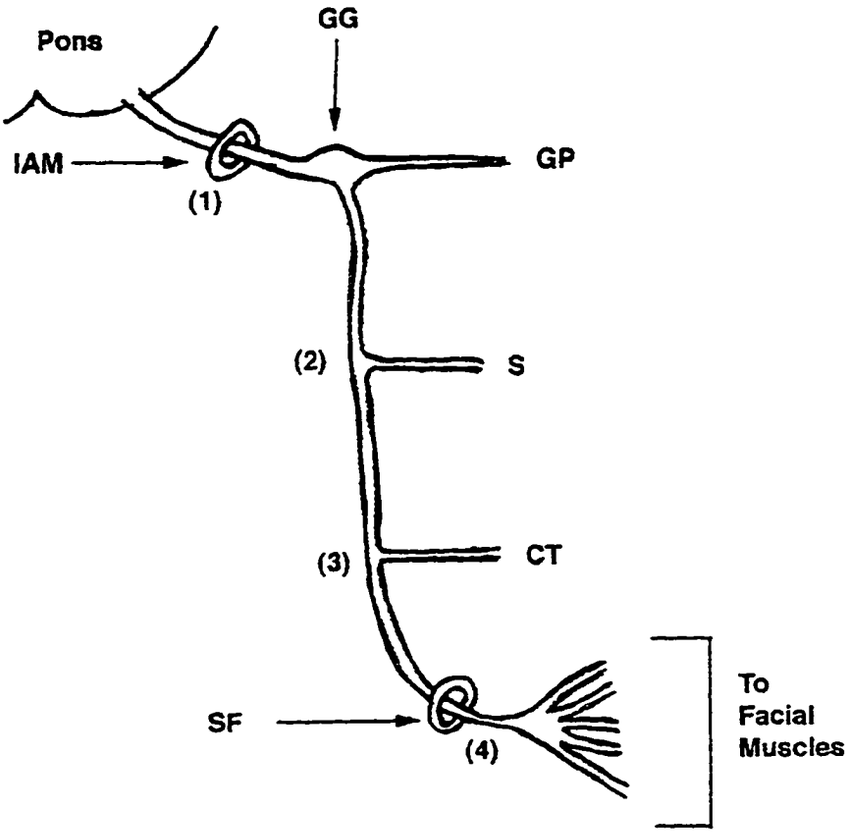


Fig. 5. Topographic diagnosis. Diagram of four possible lesion sites resulting in various symptoms. #1 impaired lacrimation, hyperacusis, impaired taste and FP. #2 hyperacusis, impaired taste and FP. #3 impaired taste and FP. #4 FP. (IAM= Internal Auditory Meatus; GG= Geniculate Ganglion; GP= Greater Petrosal nerve; S= stapedius nerve; CT= Chorda Tympani nerve; SF= Stylomastoid Foramen).

TABLE 1. Onset Classification of Facial Paralysis.

Acute	Progressive or Chronic
* Trauma	* Tumors
-Skull fracture, concussion	-parotid
-Iatrogenic	-metastatic
-Facial injury/ laceration	-Schwannoma
-Birth trauma	-neurofibroma
* Herpetic	-hemangioma
-Bell's Palsy/ herpes simplex	-Melkerson-Rosenthal Syndrome
-Ramsay-Hunt Syndrome herpes zoster	* Developmental (Unilateral or Bilateral)
* Otitis Media	-Mobius Syndrome
* Other	
-Guillian-Barre Syndrome	
-Lyme disease	

Facial nerve trauma, distal to the stylomastoid is the most debilitating and most difficult type of FP to treat and of very high prevalence among all types of facial nerve paralysis. Trauma-induced FP has generally a poor prognosis of recovery unless sophisticated microsurgical treatment is provided. It causes the greatest loss of productivity (days off work), and thus makes up the majority of health care costs in treating FP. This category of FP includes injury to the facial nerve from longitudinal temporal bone fractures, gunshot wounds of the temporal bone, iatrogenic injury during cosmetic, middle ear, mastoid surgery, facial nerve injury during birth or any other penetrating facial nerve injuries (Schaumberg *et al.*, 1992).

Characteristics of such paralysis due to injury at or distal to the stylomastoid foramen (Fig. 5) are severe. These symptoms can include, on the ipsilateral side, facial muscles sagging, including the corner of the mouth. This leads to dry mouth, oral infection and tooth disease, inability to properly eat, drink or speak and most critically, the inability to close the eyelid and loss of the blink reflex. If left untreated FP can result in drying and infection of the eye and eventual blindness (Salerno *et al.*, 1991). Each blink physically spreads the tear film over the ocular surface and allows for a continuous layer of moisture. The precorneal tear film continuity is also dependent on the volume of tears produced by the lacrimal gland. Lack of blinking and incomplete eyelid closure results in increased evaporation from the ocular surface, discontinuity of the precorneal tear film (exposure keratitis) and ulceration which ultimately can result in permanent visual loss (Chapman and Lamberty, 1989).

5. The Blink and Facial Paralysis

The blink is an important behavioral measure that reflects the patency of the facial nerve. However, it has been largely underutilized in both the evaluation of patients with FP, and in the basic science research of FP. Apart from the major functional deficit which was addressed in the current study, there is also a severe emotional and communication deficit (Ekman et al., 1972). Communication is impaired as the eyes convey emotional signals to the observer. These visual clues on the emotional state of the patient are not conveyed and detrimental to human communication and interaction. Loss of self expression results in stress and frustration which further impairs communication. Thus, the loss of the blink in facial paralysis patients has a significant impact on the emotional and psychological well being of the patient and supports the popular phrase "the eyes are the window of the soul". The emotional and psychological content we give to the blink is evident in various aspects of mythology, religion, literature and is portrayed in many popular phrases such as "she was so cold she didn't blink" or "I knew you were lying because you blinked".

The neuroanatomy of the blink reflex includes primarily the trigeminal nerve as the sensory limb from the cornea of the eye; and the facial nerve as the motor limb causing activation of the orbicularis oculi muscle to close the eyelid (Esteban and Salinero 1979; Cruccu *et al.*, 1986). There are other secondary afferents to the facial nucleus which include the dorsolateral pontine tegmentum, the medial tegmentum at the level of the hypoglossal nucleus and the red nucleus (Holstege et al., 1986a). In addition, blinking

involves to a lesser degree the inhibition of the levator palpebrae muscle and activation of the superior rectus muscle (Esteban and Salinero 1979).

The first to effectively study the blink was Eric Kugelberg (1952), utilizing electrophysiological methods of nerve conduction. In this study Kugelberg (1952) delineated the blink into its R-1 monosynaptic and the polysynaptic R-2 components. Specifically, electromyography of the OOM consists of two components; the early (9-12 msec latency) R1 which is ipsilateral and a later (15-25 msec) R2 component which is also ipsilateral (Kugelberg, 1952; Lindquist and Martensson, 1970; Hiaraoka and Shimamura, 1977) except in humans, in which R2 is bilateral (Kugelberg, 1952). While the literature contains many electrophysiological and neuroanatomical studies on the blink reflex in the normal state (Kugelburg, 1952; Rushworth, 1962; Kimura et al., 1969; Shahani and Young, 1972, 1973; Hiraoka and Shimamura, 1977; Tamai et al., 1986), reports addressing its absence in the paralytic face and restoration of the blink reflex via surgical manipulation are rare (Gyo *et al.*, 1981; Jankovic *et al.*, 1982).

The first attempt to clinically help the blinkless FP patient were simple and uncomplicated interventions to overcome the problems imposed by the paralyzed eye sphincter and the fact that the cornea was exposed. The application of an eye covering such as an eye patch to substitute for the lack of a closed eyelid and to act as a shield was an easy treatment that non-physicians could undertake. There is no report in the medical literature when such measures as an eye patch were first used, but we know that the eye patch was around at least since the days of the pirates in the 17th century (ie. Blackbeard). Although simply covering the eye prevents direct injury, the more complex problems of corneal

drying due to paralysis of the orbicularis muscle was not addressed. Probably the simplest and earliest surgical procedure first used was tarsorrhaphy which was refined by McLaughlin (1947), but was inadequate for the complete correction of lagopthalmia (the inability to close the eye). The first use of a dynamic surgical method to treat the patient with the paralyzed blink was the use of artificial material such as wires and magnets. Morel-Fatio and Laardrie (1964) first described the use of metal wires to close the eyelids and the functioning levator muscle to open the eyelids. Today, this technique has been refined and is still used and referred to as the eyespring procedure. Still other similar techniques exist that utilize gold weights, magnets, and silastic-silicone bands which all require a functioning levator muscle. The next phase of treatment (and most current) includes the reconstructive microsurgery techniques that are discussed in the next section.

6. Treatment of Facial Paralysis

The pioneering work of Bell (1821) was not only paramount for neurology as a whole but also for the study of facial paralysis. In 1814, he observed and described facial paralysis and the role of the facial nerve in facial expression when he sectioned the facial nerve of a monkey. Many successful techniques for treatment of facial nerve injury followed including: an anastomosis of the facial nerve to the spinal accessory nerve (Drobnik, 1896), the first facial nerve graft (Bunnel, 1927), and the first autogenous nerve grafts (Ballance and Duel, 1932). Extensive work followed by Lathrop (1953, 1956, 1963,

1964) and Conley (1955, 1957, 1961, 1975) that advocated immediate reconstruction and nerve grafting and popularized muscle transfers and nerve crossovers to restore function to the paralyzed face.

A variety of microsurgical approaches have been most recently employed to treat the deficits associated with trauma-induced FP (for review see, Terzis et al., 1997; Millesi, 1991). Initially, the use of cross-over procedures gained popularity. Nerve cross-over or nerve transfer involves transferring the proximal stump of a nerve to the distal stump of another nerve. The nerve transfer technique was first described at the turn of the century, and widely employed using the hypoglossal (Koerte 1903; Conley 1977, Kilmov and Linke 1978; Conley and Baker 1979; Drobnik 1896; Willer et al. 1992), accessory (Conley 1975; Delbeke and Thauvoy 1982; Baker 1987; Baker 1990), and phrenic nerves (Perret 1967) as motor donors. Unfortunately, while resting tonus was improved to all facial muscle groups, the sacrifice of a functioning cranial nerve and its target muscles was required. Other significant drawbacks with this approach remain, including uncontrollable mass movement, synkinesis of the eye sphincter with the lower face, absence of emotional expression, and unwanted contractures. Where the length of the nerve deficit precludes direct repair, restoration of eye-sphincter function is achieved through utilization of a nerve graft (Seddon 1947; Harii et al., 1976; Millesi 1977; Terzis et al. 1975; Terzis and Schnarrs 1992).

The cross-facial nerve graft (CFNG) technique is widely used today and involves the use of relatively long nerve grafts as conduits to carry axons from the normal to the paralyzed side of the face (Scaramella, 1970; Smith, 1971; Terzis, 1989; Schoeller et al,

1997; Werker et al, 1997; Terzis, 1997). CFNG's are clinically used in facial nerve injuries with a denervation time of six months or less where the proximal facial nerve stump is unavailable. Choice of the donor nerves in the past, varied between the buccal branch (Scaramella, 1979), buccal and zygomatic branch (Fisch, 1974), the cervical branch (Scaramella, 1979), or selected components of all the constituent branches of the facial nerve on the normal side by a technique of selective neurectomies and microcoaptation with the cross-facial nerve graft (Anderl, 1977).

Thus, the second stage of this study was to test the hypothesis that function to denervated facial muscles can be improved using a CFNG procedure without sacrificing a functional cranial nerve. More specifically, this component of the research project helped determine the efficacy of a CFNG procedure in restoring facial muscle function as indicated by behavioral and histomorphometric measures. Additional questions that were also addressed include: Is this reinnervation of paralyzed facial muscles using CFNG's complete or partial? Can this reinnervation of target muscle(s) be improved with neurotrophic factors?

7. The Rat Model of Facial Paralysis

The rat blink reflex was used as the model for studying FP and has been previously documented (Terrell and Terzis, 1994; Thanos and Terzis, 1995; Thanos and Terzis, 1996a, 1996b; Kalantarian et al., 1997). By using this animal model, we were able to

duplicate the conditions of trauma-induced FP, and allow study of behavioral, histochemical and morphological properties of the normal, denervated and reinnervated OOM, facial nerve and facial nucleus. It was essential to use rats for this study so as to properly describe and assess the complex cellular, behavioral, anatomical and morphological characteristics of the normal eye sphincter as well as the changes that occur with denervation and reinnervation.

To prepare the rat FP model, a unilateral transection of the VIIth nerve trunk was performed distal to the stylomastoid foramen. This cut produced a complete ipsilateral loss of function of the facial muscles, including (and most important to our model) paralysis to the OOM thus, producing total lack of eye closure and lagopthalmia. Subsequently, denervated animals were reinnervated with a saphenous nerve as the cross-facial nerve graft (CFNG). In the CFNG procedure, the saphenous nerve was coapted to one of two fascicles of the contralateral (normal) temporal branch of the VIIth nerve. The distal end of the saphenous nerve was coapted to the temporal branch on the injured side.

8. Growth Factors and Facial Paralysis

The third stage of this research challenged our rat model of FP with growth factors. Among several candidate growth factors, the neurotrophic factor Insulin-like Growth Factor-I (IGF-I) was employed for various reasons (to be discussed) and tested for its efficacy in restoring function to the denervated OOM, as well as the axonal profile of the

CFNG to which it was locally administered. These data will assist in understanding the role of neurotrophic factors on peripheral nerve regeneration and provide new insight into future treatment protocols of nerve injury.

Upon studying the restoration of function to the denervated OOM with a CFNG, it is equally important to study the possible role that specific neurotrophic factors may have on providing a stimulus or signal to the regenerating axons and promote neurite outgrowth into the nerve graft and facilitate restoration of function.

Following proximal nerve injury, axons regenerate at a rate of approximately 1mm per day and may require many months to reach their target organs, during which time atrophy and degeneration can lead to a virtual complete loss of muscle fibers resulting in permanent paralysis (Barker et al., 1986; Tower, 1932; Brooks, 1970; Drachman et al., 1967). Unfortunately, the molecular events that regulate the degree and rate of regeneration are not completely understood. It is thus of great interest to neuroscientists and clinicians to unlock the secrets of these endogenous factors that contribute substantially to the rate and degree of regeneration. It wasn't however, until the discovery of Nerve Growth Factor (NGF) and the formulation of the neurotrophic theory, which revolutionized our understanding of nerve regeneration. This theory proposed that there were specific diffusible soluble factors that were released from denervated tissues and that possessed different degrees of neurotrophic properties. Today, it is widely accepted that nerve regeneration is stimulated by soluble factors released from denervated nerves and end organs.

The role of trophic factors in nerve regeneration was first proposed by Forssman in 1898. Thirty years later, Cajal (1928) described that regenerating axons preferentially grew towards the distal stump of a transected nerve. This observation indicated that the deafferented nerves might release a soluble substance that could stimulate and direct regeneration. The significance of the distal nerve stump was later reported by the observation that peripheral nerve regeneration is attenuated upon removal of the distal stump (MacKinnon et al., 1985; Williams et al., 1984; Politis and Spencer, 1983; Politis et al., 1982).

Muscle also contains neurotrophic activity and is critical for the survival of neurons. Motor neurons have been shown to die when disconnected from their target muscles and their target derived neurotrophic factors (Hamburger, 1958; Oppenheim, 1981). Neurons compete for a limited amount of neurotrophic factor produced by target organs. Neurons receiving inadequate neurotrophic support die off and this phenomenon is called neuronal pruning which is involved in neural plasticity and development. In addition, it has been demonstrated that extracts from fetal or neonatal muscles contain greater neurotrophic activity than adult muscle extracts (Smith et al., 1985). Similarly, extracts from denervated muscle have been shown to be more potent in supporting neurite outgrowth in cultured motor neurons (Bennett et al., 1980; Henderson et al., 1983).

A plethora of potential candidate growth factors exist that could be considered for this research model in the treatment of facial paralysis (Table 2). Major neurotrophic factors are discussed below, along with their characteristics and applicability to the current model in the treatment of facial paralysis.

TABLE 2. Categories of Neurotrophic Factors (Reprinted from Thanos et al., 1997).

Class of Neurotrophic Factors	Examples
I. Neurotrophins	NGF, BDNF, NT-3, NT-4/5
II. Insulin Gene Family	IGF-I, IGF-II, Insulin
III. Neuropoietic Cytokines	CNTF, IL-6
IV. Fibroblast Growth Factors	aFGF, bFGF, FGF-5, FGF-6
V. Other	LIF, EGF, TGFα, TGFβ, GDNF

The first growth factor isolated (NGF), which was descovered by Rita Levi-Montcalcini and Victor Hamburger (1951) and later purified by Cohen (1959). This discovery launched research on nerve growth factors. Today, the list of nerve-growth-promoting compounds continues to grow, along with information on their receptor types, concentration, and localization in the CNS and PNS.

Neurotrophic factors today are defined on the basis of their receptors and can be classified into five major groups: 1) the neurotrophins, which include NGF, Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin 4/5 (NT 4/5); 2) insulin gene family, which includes IGF-I, II and Insulin; 3) neuropoietic cytokines which include Ciliary Neurotrophic Factor (CNTF) and Interleukin-6 (IL-6); 4) fibroblast growth

factors (aFGF, bFGF); 5) other growth factors include Leukemia Inhibiting Factor (LIF), Glial-Derived Neurotrophic Factor (GDNF) and Epidermal Growth Factor (EGF).

Since different neuronal populations respond to specific neurotrophic factors, injury or disease, a particular neuronal pool would best be treated by the administration of the appropriate factor. From this long list of neurotrophic factors there are many compounds that display neurotrophic properties in supporting, protecting and involved in the development of motoneurons (Terzis et al., 1997; Thanos et al., 1997). Some of the candidate factors are discussed.

Ciliary neurotrophic factor (CNTF), reduces cell death post axotomy in neonatal animals (Sendtner *et al,* 1990). CNTF is produced in small quantities by Schwann cells, but this production is significantly decreased in nerves undergoing Wallerian degeneration (Rende *et al,* 1992; Rabinovsky *et al,* 1992; Seniuk *et al,* 1992). It can be hypothesized then, that if this drop in CNTF levels (in injured peripheral nerve) is prevented or reversed by locally providing CNTF; a greater number of axon fibers will survive, regenerate and reinnervate the target. Thus, CNTF may be a good candidate to facilitate reinnervation of denervated facial muscles.

Of the neurotrophin class of growth factors, brain-derived neurotrophic factor (BDNF) has the greatest demonstrated therapeutic potential. It has been demonstrated that BDNF rescues motor neurons from natural cell death in chick embryos (Oppenheim et al, 1992) and prevents motor neuron axotomy induced cell death (Yan et al, 1996; Sendtner et al, 1992; Koliatsos et al, 1993; Clatterbuck et al. 1994). In addition, BDNF mRNA levels have been shown to increase in adult muscle by denervation (Koliatsos et al., 1993).

Furthermore, research has demonstrated that BDNF levels dramatically increase in Schwann cells, in the distal region of axotomized sciatic nerve (Meyer et al. 1992). Thus, the physiological relevance of BDNF as a trophic factor for motor neurons is supported by several findings. For instance, BDNF is expressed in the local environment of motor neurons throughout their postnatal life (Koliatsos et al., 1993). Another study demonstrated that alpha motor neurons express trk B, the receptor involved in BDNF signal transduction (Klein et al, 1991). BDNF has been shown to be transported rather selectively to motor neurons from skeletal muscles (Yan et al., 1996; Koliatsos et al., 1993) and BDNF significantly increased regeneration of axons towards their target (Meyer et al., 1992; Sawai et al., 1996).

Another candidate is fibroblast growth factor (FGF). It is produced by muscle cells, retrogradely transported to motoneuron cell bodies, and has been demonstrated to rescue hypoglossal neurons in neonatal rats from axotomy-induced injury (Grothe and Unsicker, 1992). However, the FGF decrease in cell bodies of axotomized motoneurons was transient, recovering by about 14 days and not dependent on target reinnervation. More recently, endogenous bFGF was characterized as facilitating both angiogenesis and nerve growth (Chen et al., 1997).

Recently, a great deal of attention has also focused on the importance of Glial-Derived Neurotrophic Factor (GDNF) on motor neuron injury. Several studies have suggested its involvement as a potent survival factor in Parkinson's disease (Lin et al., 1993; Beck et al., 1995; Tomac et al., 1995). Other research has suggested GDNF has a survival factor role in spinal motoneurons that degenerate in amyotrophic lateral sclerosis

(Henderson et al., 1994; Oppenheim et al., 1995; Yan et al, 1995). In addition, the list of diseases that GDNF may play a role in includes: affective disorders, sleep disorders, schizophrenia, and Alzheimer's disease (Arenas et al.,1995). More recently, Gundlach and Burazin (1997) reported that facial nerve crush in the mouse produced a significant upregulation of GDNF mRNA in the facial nucleus and may be an important trophic factor in facial nerve regeneration. Another study demonstrated that GDNF was expressed by astrocytes after facial nerve axotomy and may serve and support the survival and maintenance of facial motor neurons (Keller-Peck et al., 1997).

One other muscle-derived factor is choline acetyltransferase development factor (CDF). CDF is a skeletal muscle protein recently discovered, that has been shown to prevent cell death in axotomized neonatal rats (McManaman *et al,* 1991; Oppenheim *et al,* 1993; Yin *et al,* 1994). Unfortunately, the role of CDF in adult motoneurons has not yet been determined. CDF is a neuropoietic cytokine that acts as a trophic factor, that enhances neuronal survival and as a differentiation factor, altering neuronal gene expression. There is evidence that it also plays a role in the adult sympathetic and sensory neurons after injury (Banner and Patterson, 1994).

Other compounds that have been reported in treating motor nerve injury-induced cell death include polyamines (Gilad *et al.*, 1996) and acetyl-L-carnitine (ALC) (Fernandez *et al.*, 1995). The exogenous administration of polyamines or ALC to rats after motor nerve injury resulted in enhanced neuronal survival and accelerated the rate of axon regeneration.

Insulin-like Growth Factor-I (IGF-I) is a single chain polypeptide consisting of 70 aminoacids and three disulfide bridges and is a member of the Insulin gene family that also includes IGF-II and insulin. IGF-I and II and their receptors have been shown to be widely distributed in the CNS (Soares et al., 1985; Soares et al., 1986; Murphy et al., 1987; Shimatsu and Rotwein, 1987; Lund et al., 1986). IGFs, like NGF, undergo retrograde transport in axons (Hansson et al., 1987) and could possibly serve to signal the neural soma to synthesize cytoskeletal proteins such as α and β tubulins (Fernyhough et al., 1989; Ishii et al., 1989; Ishii et al., 1991) and neurofilament proteins (Wang et al., 1992) indicating a common biochemical pathway that when activated could lead to neurite formation.

Ishii and colleagues first demonstrated that physiological concentrations of highly purified IFG-I and II could enhance neurite outgrowth in human neuroblastoma cells (Recio-Pinto and Ishii, 1984; Ishii and Recio-Pinto, 1987). Later in vitro studies demonstrated that IGFs could induce neurite outgrowth in cultured sensory, sympathetic (Recio-Pinto et al., 1986), and motor (Caroni and Grandes, 1990) neurons.

Both IGF-I (Glazner et al., 1993) and IGF-II (Ishii et al., 1989) gene expression are increased in adult rat muscles after denervation. The IGF mRNA levels return to baseline following regeneration and reestablishment of synapses. Thus, these data demonstrated a feedback inhibition mechanism in adult muscle and that the increase in IGF mRNA levels is correlated to the biochemical state of muscle post denervation.

Immunohistochemistry studies have demonstrated that IGF-I immunoreactivity is increased in the sciatic nerve after lesion, particularly in Schwann cells, but little or no immunoreactivity was found in fibroblasts (Hansson et al., 1986; Muller et al., 1987;

Hansson et al., 1988). In addition, IGF-I immunoreactivity has also been reported in the myelinated axons of the sciatic nerve and in the motor and sensory neurons in the spinal chord (Hansson et al., 1987; Hansson et al., 1989). IGF-I has been described as critical to axonal regeneration by facilitating the proliferation of Schwann cells (Sjoberg et al., 1988). Further studies have also reported IGF-I receptors located on the growth cones and along the shaft of the axons (Caroni and Grandes, 1990; Hansson, 1993).

There is some data suggesting that IGFs are important in both sensory and motor fiber regeneration. Near et al. (1992) demonstrated that local infusion of IGF-II significantly enhanced the distance that evoked potentials could be detected in motor fibers. Unfortunately, no functional (behavior) or histomorphometric analysis was done. IGFs have also been shown to be important for sensory axon regeneration. Sensory axon regeneration was measured using a pinch test (Kanje et al., 1989; Glazner et al., 1993).

There is considerable in vivo evidence that supports the hypothesis that IGF-I is a neurotrophic factor critical to nerve regeneration. Hansson et al. (1986) reported that IGF-I accumulated in the rat sciatic nerve after transection and suggested that IGF-I could be involved in peripheral nerve regeneration. Nachemson et al., (1990) manipulated the microenvironment around the injured sciatic nerve and assessed the effects of IGF-I on regeneration. Following sciatic nerve section in the rat, the proximal nerve stump was inserted into a silicone block with three interconnected chambers (Y-tube). The opposing two chambers contained either IGF-I or vehicle. After 4 weeks the chambers were harvested and nerve regeneration assessed. Results demonstrated that IGF-I infusion increased the distance to which myelinated fibers regenerated.

In vitro, IGF-I was demonstrated to enhance neurite outgrowth from dorsal root ganglia (Fernyhough et al. 1993), increase myelination (Roth et al. 1995), as well as facilitate the survival of motoneurons (Caroni and Grandes, 1990; Hughes et al. 1993; Neff et al. 1993). In vivo, IGF-I systemically administered reversed or arrested diabetic neuropathy effects on hyperalgesia and nerve crush injury in rats (Zhuang et al. 1996) and enhanced the rate of regeneration after sciatic crush as measured by the number of nerve fibers per section of muscle (Lewis et al. 1993) and functional recovery as measured by gripping ability (Contreras et al. 1995).

By contrast, there have been no studies on the effects of IGF-I locally administered to a nerve graft. Some studies have examined IGF-I locally administered after a crush or freeze injury. In these studies, IGF-I locally administered around a sciatic nerve crush or freeze injury enhanced the rate of regeneration (Kanje et al. 1989; Sjoberg and Kanje, 1989). However, these studies failed to address the problem of ischemia and pressure injury to the nerve due to the silicone chamber around it. Kanje and colleagues (1991) also demonstrated that IGF-I locally administered to damaged neurons could overcome a cycloheximide-induced block of regeneration.

Most recently, local doses of IGF-I near the site of sciatic nerve crush or larger systemic doses protected against the impairment of sensory nerve regeneration in streptozotocin diabetic rats (Ishii and Lupien, 1995). Morphometric analyses of the nerve enables assessment of nerve regeneration since the number of myelinated axons forms a key issue as motor axons with few exceptions are always myelinated and are responsible

for critical functions (Hansson et al., 1986). The current study utilized both morphometric analysis as well as continuous behavior analysis to assess function.

Unfortunately, the limited availability of the above discussed growth factors restricted the scope of growth factors. A kind gift of IGF-I (Dr. Liuzzi and Genentech), along with the significant effects of IGF-I (just discussed) on motor neurons have promoted the consideration of this growth factor in treating nerve injury and paralysis such as FP.

Therefore, the present research also studied the effects of treating facial nerve axotomy with a dual treatment approach, that is a local administration of IGF-I to the CFNG. Efficacy of this dual treatment approach was determined in several ways: a) Behavioral testing of eye sphincter function (blink test) and b) Histomorphometric evaluation of the axons regenerating throughout the CFNG.

These data suggest that IGF-I serves a significant role in the neurotrophic milieu necessary for successful nerve regeneration. In addition, these data support the development of more effective therapeutic procedures used to treat FP patients, and more specifically the further study of IGF-I among other neurotrophic factors to be used in conjunction with microsurgical techniques, thus bringing us closer to dynamic human facial rehabilitation.

B. PURPOSE

Apart from the great emphasis that society places on facial expression and appearance, FP significantly hinders mastication, speech production and eye protection. As

previously mentioned, there are two major types of FP: a) an acute viral, inflammatory, autoimmune, demyelinating disease caused by the herpes simplex virus (Bell's Palsy), and b) trauma-induced. Facial paralysis caused by trauma is the focus of the current research. Although numerous restorative microsurgical approaches have been popularized to rectify this problem, complete restoration of function to denervated facial muscles remains elusive.

Among the pathological sequela of FP is a paralytic eye. A critical muscle that is often at risk is the orbicularis oculi muscle (OOM). Denervation of this muscle results in eye sphincter paralysis and, if left untreated, can result in infection of the eye and eventual blindness. Currently, the most popular method of treating this condition is the use of a cross-facial nerve graft (CFNG) to reinnervate the previously denervated muscle. The invasive nature of histochemical analysis of the OOM and its facial nerve branches prohibit us from seeking answers to basic science questions associated with facial nerve regeneration after axotomy, reinnervation of the eye sphincter and restoration of the blink reflex in FP patients. Thus, an animal model must be utilized.

The present research had three main stages and was conducted in the rat. This rat model allowed the investigation of the efferent circuit participating in eye sphincter function. Specifically, this study identified and described the FMN pool that serves the OOM and the blink reflex as well as identified the nerve branch that provides the majority of this innervation. Another component of this research described the (current and clinically popular) CFNG treatment procedure in reinnervating the paralyzed OOM and assessed its efficacy in restoring function both behaviorally and histomorphometrically. Finally, restoration of function to the denervated OOM was studied using a dual treatment

approach (IGF-I + CFNG). These data will help provide insight into the restoration of facial muscle function after trauma and assist in the future development of treatment techniques: their evaluation and comparison; thus, contributing to the successful management of this problem.

Hypotheses and Specific Aims

The first stage of the study consisted of three experiments which tested a couple of hypotheses a) that the facial motor neurons serving the Orbicularis Oculi Muscle (OOM) are topographically organized within the entire facial motor nucleus and b) that the motor neuron pool serving the temporal branch of the facial nerve provides the majority of the total innervation to the rat OOM. To test these hypotheses, retrograde labeling techniques were utilized to establish and characterized the FMN pool that innervates the rat OOM (experiment #1, specific aim #1) as well as the FMN pool that serves the temporal branch of the facial nerve (experiment #2, specific aim #2). The third experiment identified the facial nerve branch that provides the majority of innervation to the OOM, and their topographical localization, again utilizing fluorescent retrograde labeling (experiment #3, specific aim #3).

The second stage of the study consisted of two experiments which tested the hypothesis that function of denervated facial muscles in the rat can be significantly improved without sacrificing other cranial nerves by utilizing a cross-facial nerve graft from the normal side to the denervated side. Thus, these experiments investigated the efficacy of treating FP, with a CFNG in restoring function to the rat OOM, previously denervated

by unilateral facial nerve axotomy. To assess the efficacy of the CFNG procedure (specific aim # 4) several tests were employed. First, behavioral testing of the blink reflex, in response to an air puff stimulus 3 months post CFNG treatment was studied (experiment #4). Secondly, the motor endplates of the OOM reinnervated with a CFNG were analyzed (experiment #4). Finally, the nerve graft itself was morphometrically analyzed with respect to the number of axon fibers reaching the target, their axon diameter and myelin thickness (experiment #5).

The third stage of the study consisted of experiment six which tested the hypothesis that growth factors are vital to and will facilitate in the reinnervation of denervated muscle. Therefore, this final experiment challenged our model and the CFNG treatment by determining the effects of Insulin-like Growth Factor-I (IGF-I) in conjunction with a CFNG on the reinnervation of the OOM and recovery of function (specific aim #5). More specifically, this experiment examined both the CFNG histomorphometry and function over time (blink test) in both IGF-I and vehicle-treated FP rats.

CHAPTER II

MATERIALS AND METHODS

This research is divided into three stages and six experiments.

The first stage consisted of three experiments utilizing light and fluorescent microscopy. Experiment 1 addressed specific aim 1 with retrograde labeling of the FMNs supplying the OOM (FMN pool of OOM). Experiment 2 examined the FMN pool of the temporal branch of the facial nerve (specific aim 2). Experiment 3 utilized double labeling techniques to examined the FMN pool supplying the OOM via the temporal branch of the VIIth nerve (specific aim 3).

The second stage of this project consisted of experiment 4 and 5 which addressed the efficacy of a CFNG in restoring function to a denervated OOM (specific aim 4), utilizing histomorphometric analysis of the OOM endplates and the axons in the graft. This analysis of the CFNG and the OOM endplates was performed with light microscopy and computer image analysis.

Finally, the third stage of this research consisted of experiment 6 which investigated the effects of growth factors (in conjunction with the CFNG), on reinnervation of the paralyzed eye sphincter (specific aim 5), utilizing behavioral and histomorphometric measures. This analysis of the CFNG was performed with light microscopy and computer image analysis.

Ethical Treatment of Animals

All animals were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. These studies were approved by the Animal Care and Use Committee of Eastern Virginia Medical School and carried out according to NIH guidelines.

STAGE I:

Experiment 1

Determine the facial motor neuron (FMN) pool that innervates the rat OOM (specific aim #1).

This experiment analyzed the FMNs within the facial nucleus that innervate the rat OOM. Biotin dextran retrograde labeling was used for morphological and qualitative evaluation of the FMN pool of the rat OOM and retrograde fluorescent labeling the OOM with Fluoro Gold (FG), was utilized to provide a quantitative analysis of the FMN pool involved in OOM activation and eye closure in the rat blink. Analysis of these data helped answer the following questions:

1) How many motor neurons in the rat facial nucleus innervate the OOM?

2) Where are these neurons located within the facial nucleus?

3) Is there a topographic organization of these neurons in the facial nucleus?

STAGE I:

Experiment 2

Determine the FMN pool that serves the temporal branch of VII (VII-T) (specific aim # 2).

Biotin dextran retrograde labeling was again used for morphological evaluation of the FMN pool of the temporal branch to the OOM. This experiment examined quantitatively the innervation of the rat OOM via the temporal branch and thus its contribution to eye closure using FG retrograde labeling. These data provided a quantitative assessment on the "donor" FMN pool that is utilized in the CFNG procedure. This experiment also addressed the following questions:

1) How many FMNs serve the temporal branch of the facial nerve to the rat OOM?

2) Are these temporal FMNs topographically organized within the facial motor nucleus?

STAGE I:

Experiment 3

Determine the facial nerve branch that provides the majority of innervation to the OOM (specific aim #3).

By retrograde double labeling, the OOM with one tracer FG and labeling the temporal branch (VII-T) with another fluorescent tracer bisBenzimide (BB), this experiment described the topographical location of the double labeled FMNs. In addition,

using FG labeling total FMN counts were calculated for both OOM and VII-T. These data elucidated the significance of the temporal branch in the innervation of the rat OOM. This experiment also addressed the following questions:

1) Does the temporal branch of VIIth nerve provide the majority of the OOM innervation?
2) Is the temporal branch the best donor nerve that can be utilized in the cross-facial nerve graft treatment of the paralyzed eye sphincter?

STAGE I:

EXPERIMENTAL DESIGN

The main objectives of stage I were to analyze the total FMN innervation of the normal rat OOM, the VII-T, and to analyze if the majority of this innervation occurs by way of the temporal branch of the facial nerve (Fig 6). Qualitative and quantitative analyses of these labeled FMNs and their topographical localization within the facial nucleus were determined. In addition, the percentage of the OOM innervation via temporal branch was also studied. Thus, three experiments were conducted; (1) the FMN pool of the OOM (2) the FMN pool of the VII-T and (3) the topographical localization of the double labeled FMNs; and the facial nerve branch that provides the majority of the OOM innervation.

EXPERIMENT 1.

To examine the topography and number of FMNs serving the OOM, FG was used. FG was unilaterally microinjected into both the superior and inferior half of the OOM. This allowed the tracer compound to be retrogradely transported into the neuron cytoplasm. To examine the morphology of these FMNs, biotin dextran labeling was utilized. The design for this experiment was as follows:

A) Fluoro Gold

1) Quantity of animals. Twenty five adult male Sprague Dawley rats were used in this study and received a unilateral microinjection of 2.5% FG into the OOM. All animals were individually housed and provided with food and water ad libitum.

2) Surgical Procedure. Rats were anesthetized (100 mg/kg ketamine/ 10 mg/kg xylazine intraperitoneal injection) and operated on, in a sterile operating room and utilizing a Zeiss operating microscope. In addition, microsurgical instruments and sutures along with a 10 µl Hamilton glass microsyringe for the precise injection of the FG retrograde tracer was used. The animals face was shaved, treated with antiseptic solution and then a periopthalmic incision was made on the right side. The facial nerve and its branches were then explored, dissected and identified intraoperatively using a nerve stimulator (Vari-Stim 85-62010).

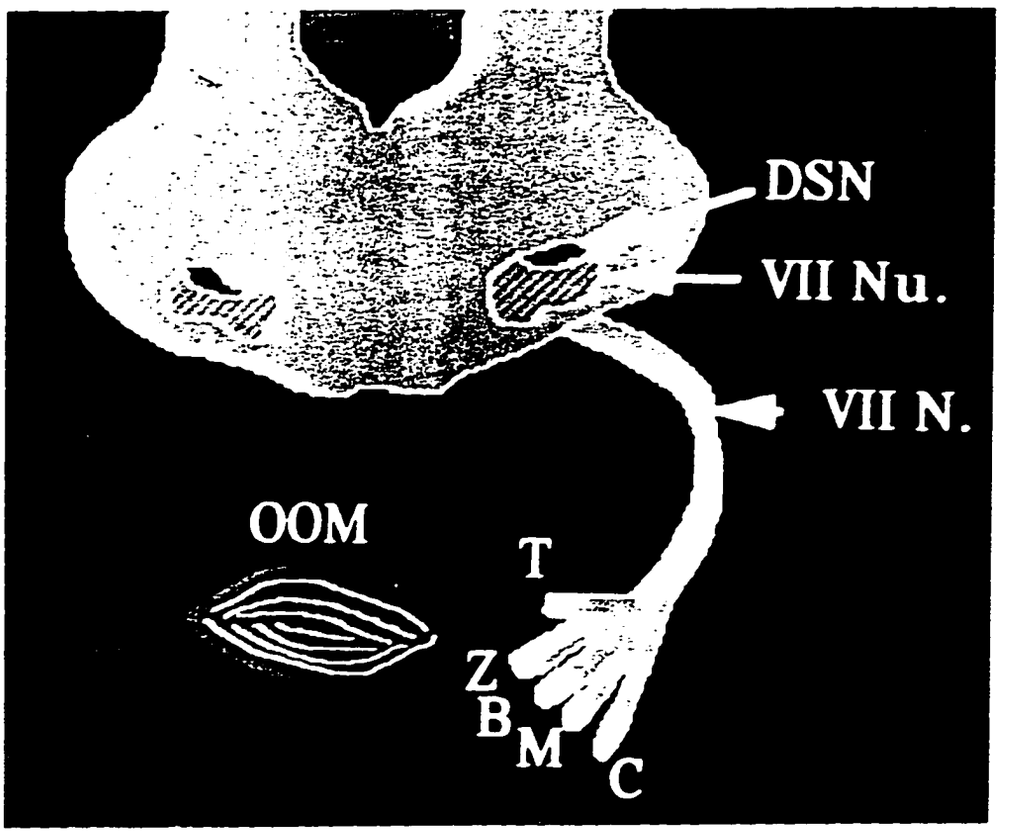


Fig. 6. Schematic representation depicting the facial nucleus, VIIth nerve, the OOM and sites of tracer injection: a) the OOM and b) the temporal branch (T) of the VIIth nerve.

Each animal then received a unilateral microinjection (20 μl) of 2.5% Fluoro-Gold (Fluorochrome) into superior and inferior halves of the orbicularis oculi muscle utilizing the Hamilton glass stainless steel tip microsyringe (Fig 7). Next, both temporal and superior zygomatic branches of the VIIth nerve were crushed for 30 sec using a pair of flat tipped microforceps (ASSI #1803) to stimulate retrograde transport. The incision was then closed and the animal returned to its cage.

Five days later, the animal was similarly anesthetized and transcardially perfused with 400 ml of 0.9% heparinized saline and then 1000 ml of 4^0 C fixative (4% paraformaldehyde, 1% picric acid in phosphate buffer saline, pH=7.4). Following this, the brain was harvested (Fig 8) and placed for 2h in the same fixative followed by a 15% sucrose in phosphate buffer solution overnight at 4^0 C. Next the brainstem was dissected using the operating microscope and cut transversly at 50 μm on a vibratome. Serial horizontal sections were then transferred to a 48-well plate with 0.1M Tris buffer (pH=7.4) and mounted on pretreated slides. Sections were then observed and photographed using a Nikon Microphot-FX microscope with epifluorescent attachments and the appropriate filter (λ = 350-450 nm).

Finally, sections were Nissl stained with 1% cresyl violet for brightfield microscopy in order to assess the total number of FMNs. All FMNs were counted using a digitizing tablet interfaced to a computer (with Sigmascan 3.1 software) and a microscope with a drawing tube attachment. The total number of FMNs was utilized to characterize the percentage of the total number of FMNs that innervated a) the OOM and b) the VII-T.

Fig. 7. Microinjection of tracer into the superior half of the OOM in a rat.

Fig. 8. Ventral view of the perfused rat brain illustrating the area of the facial nucleus in the caudal pons.

B) Biotin dextran

To examine the morphology and topography of FMNs, serving the OOM, biotin dextran (bd) tracer was utilized. The tracer was unilaterally microinjected into the OOM (Figure 7). By 48h, the majority of the tracer compound was retrogradely transported to the neuronal perikarya and dendrites. Therefore, by harvesting the brainstem at 48 h post-labeling, individual labeled FMNs were morphologically evaluated. The basic steps for the above procedure were as follows:

1) Quantity of animals. Ten adult male Sprague Dawley rats were used in this study and received a unilateral microinjection of bd into both halves of the OOM. All animals were individually housed and provided with food and water ad libitum.

2) Surgical Procedure. Rats were anesthetized (100 mg/kg ketamine/ 10 mg/kg xylazine intraperitoneal injection) and operated on, in a sterile operating room and utilizing a Zeiss operating microscope. In addition, microsurgical instruments and sutures along with a 10 μl Hamilton glass microsyringe for the precise injection of the bd retrograde tracer was used. The animal's face was shaved, treated with antiseptic solution and then a preauricular incision was made on the right side. The facial nerve and its branches were explored, dissected and identified intraoperatively with a nerve stimulator (Vari-Stim 85-62010). Using lysine-fixable low molecular weight biotin dextran, a 3% solution was made. Rats received a 20 μl microinjection of bd into the right OOM. Forty-eight hours

post-injection, the animals were similarly anesthetized, perfused with 4% paraformaldehyde and the brainstem harvested. Later, 50 μm transverse sections of the brainstem were cut using a vibratome. The sections were then processed for bd labeling.

3) Biotin dextran Histochemistry (Thanos & Terzis, 1996b).

After cutting, the sections were transferred to a 48-well plate with Tris buffer (0.1M, pH=7.4) and at 6°C. The sections were then blocked for endogenous peroxidase with 0.75% H_2O_2 diluted in Tris buffer (0.1M), 200 μl/well for 30 min. The sections were then rinsed twice with dH_2O, and then with 0.1M Tris buffer, and then incubated in streptavidin peroxidase (0.2 mg/ml, 250 μl/well, room temperature, 2 hr), which binds to biotin and amplifies the signal. To each well, a drop of 0.1% detergent (IGEPAL) was added. Then, the sections were rinsed twice with Tris buffer (0.1M), 5 minutes each. Next, sections were treated with True Blue substrate (KPL) for 30 min at RT. Following this, the sections were rinsed with dH_2O, counterstained with 5% contrast red, mounted on glass slides, dehydrated, cleared and coverslipped.

4) Analysis of Labeled FMNs

The qualitative and quantitative analyses of the labeled FMNs was performed using a Sigmascan image analysis computer system, and a Nikon light microscope. Statistical analysis was performed utilizing the Statview 3.5 software. Data consisted of a morphometrical description of the labeled FMNs including the shape, number, diameter, area of labeled FMNs, and localization of these neurons within the facial nucleus. The

soma of each labeled neuron was outlined using a digitizing tablet interfaced to a computer and a microscope with a drawing tube attachment. Neuronal diameter measurements were calculated using morphometric analysis software (Sigmascan 3.1). Experiments using cell counting methods may possess the possibility of double counting, or counting the same cell twice, once in each adjacent section. Typically, this is a concern when the size of the cell measured is greater than the thickness of each section. In experiments where this is a possibility a correction formula is usually applied to the cell counts (Abercrombie, 1946; Nadelhaft et al., 1980). Since in the current experiment the labeled OOM FMNs had a mean diameter of 28 $\pm$ 11 μm (see results), and each section was 50 μm thick, no such correction was applied. In contrast, the likelihood exists that some neurons may have been missed and thus these neuron counts may be low. In addition, the soma diameter of the OOM FMNs was compared to that of the VII-T FMNs using the t-test (McCall, 1986).

EXPERIMENT 2.

To examine the topography and number of FMNs, serving the temporal branch of the facial nerve (VII-T), a FG tracer was used. FG was unilaterally microinjected into the right VII-T (Fig 9). This allowed the tracer compound to be retrogradely transported into the neuron cytoplasm. To examine the morphology of these FMNs, Biotin Dextran labeling was utilized. The design for this experiment was as follows:

A) Fluoro Gold

1) Quantity of animals. Twenty five adult male Sprague Dawley rats were used in this study and received a unilateral microinjection of 2.5% FG into the VII-T. All animals were individually housed and provided with food and water ad libitum.

2) Surgical Procedure. As previously described rats were anesthetized and their face shaved, treated with antiseptic solution and then a periopthalmic incision was made on the right side. The facial nerve and its branches were explored, and identified intraoperatively using a nerve stimulator (Vari-Stim 85-62010). Next, the temporal branch of the VIIth nerve was isolated and crushed proximally for 30 sec using a pair of flat tipped microforceps (ASSI # 1803) to stimulate retrograde transport. The VII-T was then cut about 2 cm from the stylomastoid foramen and placed on a piece of sterile parafilm to prevent leaking of the tracer to surrounding sites. A piece of polyethylene tubing (0.5cm length and id=2mm), filled with sterile gelfoam was fitted over the proximal stump of VII-T and secured to the stump with a 9.0 microsuture (Fig. 9). The gelfoam was then injected with 5 µl of 2.5% FG. Both ends of the tube were then sealed with petroleum jelly to prevent leaking. The wound was then closed over the tube with 5.0 sutures and the animal returned to its cage.

Five days later, the animal was similarly anesthetized and transcardially perfused. Following this, the brain was harvested (Fig 8) and was dissected using the operating

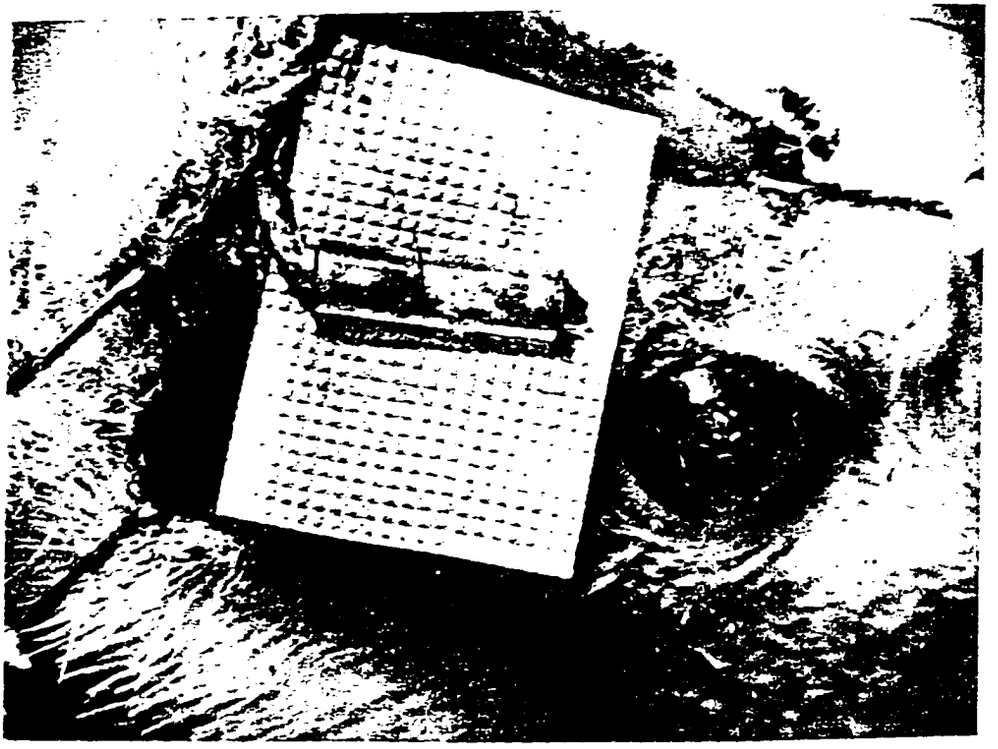


Fig. 9. Microinjection of tracer into the rat VII-T/ silastic tube.

microscope and cut transversly at 50 μm with a vibratome. Serial horizontal sections were transferred to a 48-well plate with 0.1M Tris buffer (pH=7.4) and mounted on pretreated slides. Sections were observed and photographed using a Nikon Microphot-FX microscope with epifluorescent attachments and then Nissl stained with 1% cresyl violet for light microscopy.

B) Biotin dextran

To examine the morphology and topography of FMNs, serving the VII-T, biotin dextran (bd) tracer was utilized. The tracer was unilaterally microinjected into the right VII-T (Figure 9). By 48h, the majority of the tracer compound was retrogradely transported to the neuronal perikarya and dendrites. Therefore, by harvesting the brainstem at 48 h post-labeling, individual labeled FMNs were morphologically evaluated. The general steps of bd labeling were the same as previously described in experiment 1 with the exception of the following:

Surgical Procedure, Biotin Dextran Histochemistry and Analysis.

Rats were similarly anesthetized and a lateral incision was made on the right side of the face. The facial nerve and its branches were then explored and identified intraoperatively with a nerve stimulator. Rats then had their right VII-T labeled with 3 % bd via the silastic tube and gelfoam (as previously described in experiment 1). Forty-eight hours post-labeling, the animals were similarly anesthetized, perfused with 4%

paraformaldehyde and the brainstem harvested. Later, the brainstem was mounted on a vibratome and 50 μm transverse sections made. The sections were then processed for bd labeling and analyzed as previously described (experiment 1: biotin dextran histochemistry, analysis of labeled FMNs). Similar to experiment 1, no correction formula was applied to the VII-T FMN counts because they had a mean diameter of 32 + 6 μm (see results) and the sections were 50 μm thick.

EXPERIMENT 3.

By retrograde double labeling, the OOM with one fluorescent tracer (FG) and the temporal branch (VII-T) with another tracer (BB), experiment 3 examined the topographical location of the double labeled FMNs. Utilizing the FG labeling quantitative data from experiments 1 and 2 the significance of the temporal branch in the innervation of the OOM was elucidated and the facial nerve branch that provides the majority of innervation determined. The design for this experiment was as follows:

1) Quantity of animals. Twenty five adult male Sprague Dawley rats were used in this study. Each rat received a unilateral microinjection of FG into the right OOM followed 4 days later with BB into the VII-T.

2) Surgical Procedure. Rats were anesthetized (100 mg/kg ketamine/ 10 mg/kg

xylazine intraperitoneal injection) and operated on in a sterile operating room, utilizing a Zeiss operating microscope. Similarly a 10 μl Hamilton glass microsyringe was used for the precise delivery of the fluorochrome tracers was used. Each animal's face was shaved, treated with antiseptic solution and then a periopthalmic incision was made on the right side. The facial nerve and its branches were then explored, dissected and identified by intraoperative nerve stimulation.

Each animal received a unilateral injection (20 μl) of 2.5% Fluoro-Gold (FG) into the orbicularis oculi muscle utilizing a 10 μl Hamilton glass stainless steel tip microsyringe (Fig 7). Next, both temporal and superior zygomatic branches of the VIIth nerve were crushed for 30 sec using a pair of flat tipped microforceps. The incision was closed and the animal returned to its cage. Four days later, each animal was reanesthetized and the previous incision opened. The VII-T was mobilized and cut about 2 cm from the stylomastoid foramen. A piece of polyethylene tubing (0.5cm length and id=2mm), loaded with sterile gelfoam was fitted over the proximal stump of VII-T and secured to the tube with a 9.0 microsuture (Fig 9). The gelfoam was then injected with 5 μl of 25% BB. Both ends of the tube were sealed with a non-soluble oil-based surgical lubricant. The wound was then closed over the tube with 5.0 sutures and the animal returned to its cage. Twenty six hours later, each animal was again anesthetized and transcardially perfused. Following this, the brain was harvested and the brainstem was cut transversly at 50 μm on a vibratome. Serial horizontal sections were then transferred to a 48-well plate with 0.1M Tris buffer (pH=7.4) and mounted on pretreated slides. Sections were then observed and

photographed using a Nikon Microphot-FX microscope as previously described and Nissl stained with 1 % cresyl violet for light microscopy observation.

STAGE II:

Experiments 4 and 5

Determine the efficacy of a Cross-Facial Nerve Graft (CFNG) in restoring function to the rat paralyzed OOM (specific aim #4).

After describing the FMN pool that innervates the normal eye sphincter, and the degree of this innervation to the OOM provided by way of the temporal branch, these experiments examined the effectiveness of the CFNG in reinnervating the denervated eye sphincter. Efficacy was determined several ways: Motor endplate profile of the OOM, function (blink reflex) and axonal morphometric analysis of the CFNG itself. From quantitative and qualitative analysis of these data, the following questions were addressed:

1) What are the long-term behavioral effects (3 months) on eye sphincter function when reinnervating the denervated muscles with a CFNG?
2) What are the morphological effects of facial nerve axotomy on the OOM? Is there a change in the number of motor endplates and in their distribution compared to a normal muscle? Is this motor endplate profile different in animals treated with a CFNG? and if so how?
3) What are the axonal morphometric characteristics of the CFNG three

months post reinnervation? How does the axonal profile of the temporal branch (proximal and distal) compare to the saphenous nerve graft? How do these data correlate with the motor endplate results?

STAGE II:

Experiment 4 and 5

EXPERIMENTAL DESIGN

The primary goal of this stage of research was to describe the efficacy of the CFNG in the treatment of the paralyzed OOM. More specifically, the following experiments were conducted: (1) The motor endplate characteristics of the normal, denervated and reinnervated facial nerve with a CFNG OOM. (2) The axonal morphometric properties of the CFNG from the proximal coaptation to the distal coaptation. The basic steps for the above design was as follows:

EXPERIMENT 4.

Evaluation of the behavioral and motor endplate characteristics of the normal, denervated and reinnervated with a CFNG OOM.

1) Quantity of animals. Twenty three adult male Sprague Dawley rats were used in this study and were divided into three groups. A) The control group will consisted of normal rats that had their OOM harvested and analyzed for motor endplates (N=3). B)

The denervated group which consisted of animals that had their right facial nerve transected (N=10). C) The reinnervated group received right facial nerve transection and were treated with a CFNG (N=10). Rats were procedures were anesthetized with 100 mg/kg Ketamine / 10 mg/kg Xylazine (intraperitoneal injection).

2) Surgical procedures.

Phase I. Surgery

Group B. The left facial nerve and its branches was explored and dissected via a preauricular incision. The temporal branch to the eye sphincter was then identified by intraoperative nerve stimulation (Vari-Stim 85-62010). The right facial nerve trunk was then explored, stimulated and transected just distal to the stylomastoid foramen producing a complete right facial paralysis (Fig 10).

Group C. The right facial nerve trunk and its branches were explored and dissected through a preauricular incision. The temporal branches to the eye sphincter were then identified using intraoperative nerve stimulation. The right facial nerve trunk was then transected producing a complete right facial paralysis (Fig 10). The left saphenous nerve (5 cm in length) was then be harvested as the graft and tunneled in the subcutaneous plane over the dorsum of the cranium to the right side through the incision (Fig. 11). Biopsies of the eye branches and both ends of the nerve graft were taken and coaptations of the left temporal branch to the saphenous nerve graft performed using 11-0 (Ethicon) sutures with a 30 micron needle. Skin closure was then accomplished with several 6-0 polypropelene (Prolene) sutures.

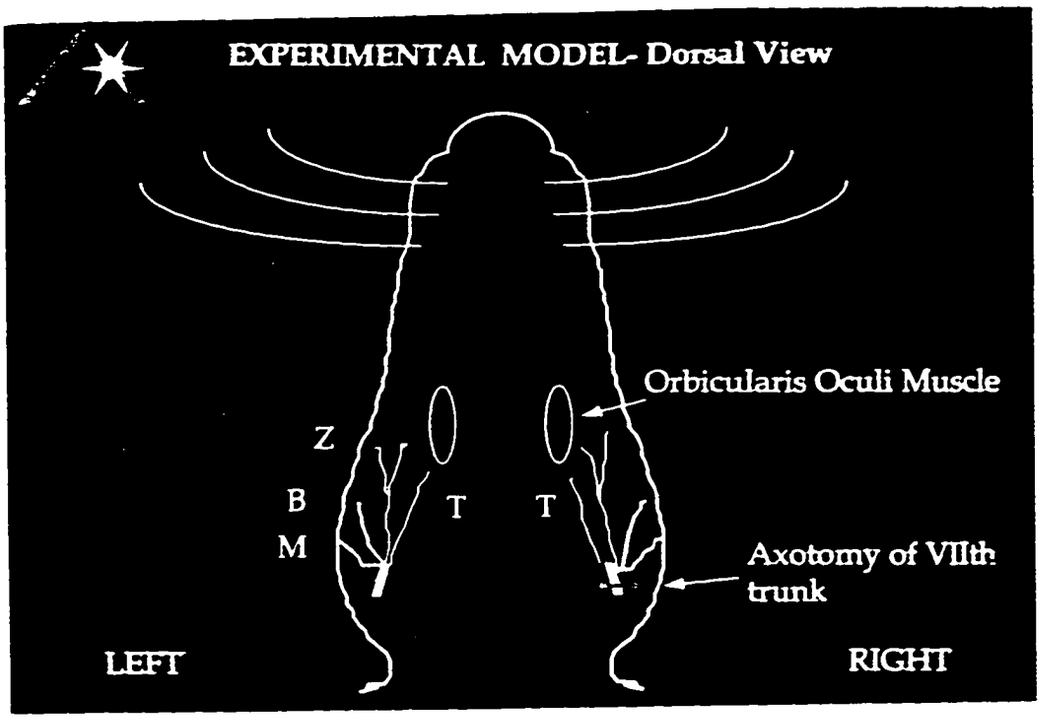
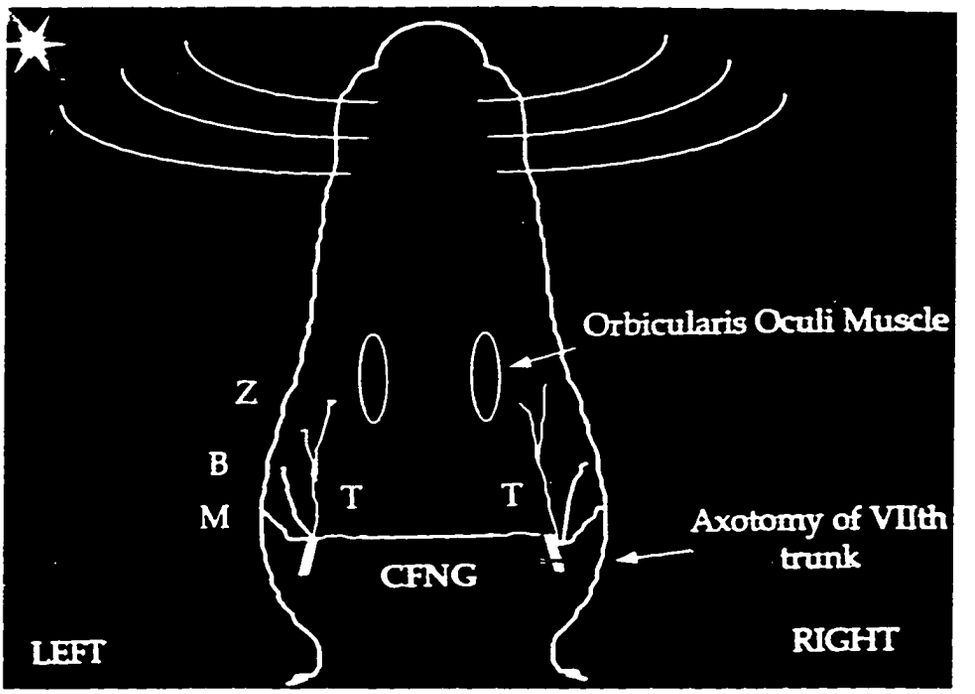


Fig. 10. Dorsal view diagram of the rat facial neuroanatomy illustrating the locus of the axotomy along with the major branches of the facial nerve (T= temporal; Z= zygomatic; B= buccal; M= mandibular) .

A.



B. Cross-Facial Nerve Graft

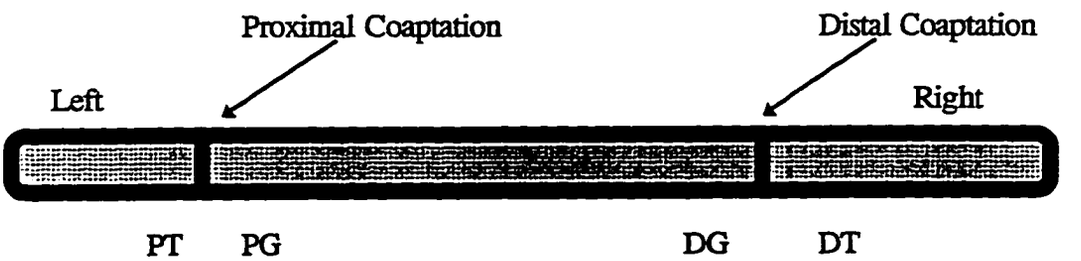


Fig. 11. A. Dorsal view diagram of the rat facial neuroanatomy illustrating the locus of the axotomy and the placement and coaptations of the CFNG. The major branches of the facial nerve are also illustrated (T= temporal; Z= zygomatic; B= buccal; M= mandibular). B. The cross-facial nerve graft divided into four specimens (PT=proximal temporal, PG=proximal graft, DG=distal graft and DT=distal temporal).

Phase II. Surgery

Group C. Three months following stage I, the right face was explored using a preauricular incision and the distal cross-facial nerve graft (CFNG) coapted to the right temporal branch (paralyzed side).

Phase III. Surgery

Groups B and C. Three months following stage II, the entire CFNG was explored, dissected and nerve stimulation performed proximal and distal to the coaptation site and in the midgraft region. Both OOM's were then harvested for motor endplate analysis.

3) Blink response

Each animal was photographed prior to any surgical procedure as well as at different intervals after treatment with a CFNG to document the status and symmetry of both eye sphincters. The blink response was evaluated prior to sacrifice (3 months post CFNG) utilizing a custom-designed apparatus that delivered a constant (20 ml) volume of air to the cornea and periorbital region at a distance of 2 cm. The air stimulus was delivered through a plastic microtubing system, connected to a volume reservoir located away from the animal. This apparatus provided an adequate stimulus for qualitative analysis of the blink and was a reliable method in determining the status of the blink.

4) Motor endplate histochemistry of the OOM (adapted from Gorio, 1983 and modified by Thanos and Terzis, 1995).

Upon completion of the experiment, the animals were deeply anesthetized with 10%

sodium pentobarbital, and the OOM specimens harvested. The harvested OOM's were pinned on a cork board to maintain their shape, frozen in a liquid nitrogen bath, and stored at -80° C and processed as follows:

a. The frozen orbicularis oculi muscles were positioned horizontally and sectioned in a serial fashion at 30-35 μm on a cryostat and mounted on 3% EDTA coated slides.

b. Slides were then soaked for 3 min. in 20% sodium sulphate, then rinsed in dH_2O.

c. Sections were then incubated at 37°C, for 30 min in an AChE substrate solution containing the following: 4.1 mg/ml 5-Bromoindoxyl Acetate, 0.8 mg/ml potassium ferrocyanide 1.7 mg/ml potassium ferricyanide, 1.1 mg/ml calcium chloride in dH_2O.

d. Rinse in dH_2O and place slides in fresh 0.5% $K_3Fe(CN)_6$ solution for 8 min. at RT.

e. Rinse in dH_2O and fix in formol-saline solution for 30 min. (18% formalin in dH_2O, 16 mg/ml NaCl, 7.3 mg/ml sodium phosphate monobasic, 11.8 mg/kg sodium phosphate anhydrous).

f. Wash in dH_2O for 20 min.

g. Incubate in fresh silver nitrate solution for 30 min at 37°C (20% silver nitrate, 1 mg/ml $CuSO_4$-$5H_2O$).

h. Rinse with dH_2O, and developer solution for 30 sec (5% Na_2SO_3 in dh_2O, 10 mg/ml hydroquione).

i. Rinse in dH_2O and fix in 5% sodium thiosulfate and dehydrate. The motor endplates thus stained blue.

5) Motor Endplate Analysis.

Quantitative assessment of the motor endplates, and their distribution was conducted on all OOM serial sections, using a Zeiss Universal light microscope, interfaced with a digitizer tablet (Kurta) and a Sigmascan (3.1) image analysis software. Motor endplate analysis consisted of both the total number of motor endplates per OOM sample, as well as endplate topographical distribution which was performed by dividing the OOM into quadrants and noting the number of endplates per quadrant (Fig. 12). Similar to the previous experiments, no correction formula for double counting was applied because the average motor endplate in mammalian skeletal muscles is approximately 33 μm (Engel, 1994; Coers, 1967) and each section was 30-35 μm thick. Statistical analysis of the number of endplates between groups was done using an analysis of variance (ANOVA). Similarly, an ANOVA was utilized to assess the difference in number of endplates between different regions of the OOM.

EXPERIMENT 5.

Analysis of the axonal morphometric properties of the CFNG from the proximal to the distal coaptation.

1) Quantity of animals. Ten adult male Sprague Dawley rats were used in this study and received a right facial nerve axotomy followed in three months by treatment with a

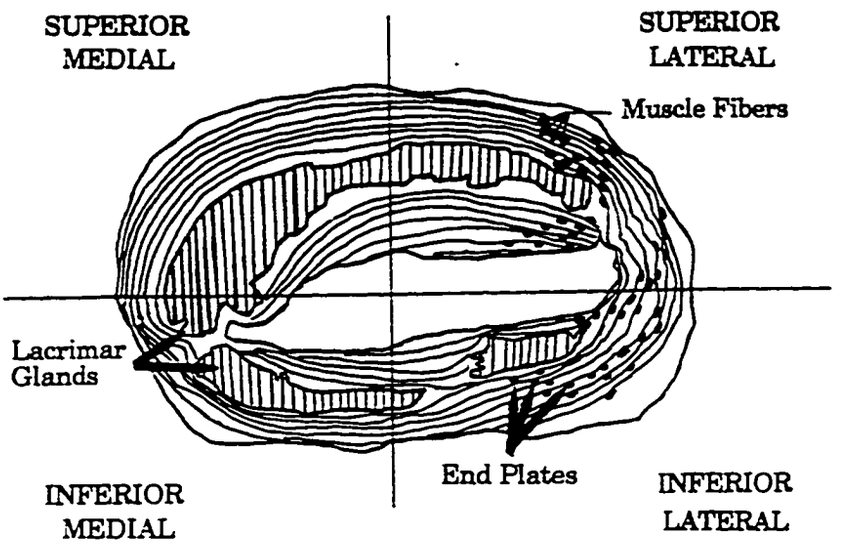


Fig. 12. Diagram of a longitudinal section the rat OOM divided into quadrants for topographical analysis of the endplates.

CFNG. Again rats were anesthetized with 100 mg/kg Ketamine / 10 mg/kg Xylazine (intraperitoneal injection) and operated on with the aid of a surgical operating microscope (Zeiss).

2) Surgical procedures.

Phase I. Surgery

The right facial nerve trunk and its branches was explored and dissected through a preauricular incision. The temporal branches to the eye sphincter were then identified using intraoperative nerve stimulation. The right facial nerve trunk was then transected just distal to the stylomastoid foramen producing a complete right facial paralysis (Fig. 10). The left saphenous nerve (5 cm in length) was then harvested as the graft and tunneled in the subcutaneous plane over the dorsum of the cranium to the right side through the incision (Fig. 11). Biopsies of the eye branches and both ends of the nerve graft were taken and coaptations of the left temporal branch to the saphenous nerve graft performed using 11-0 (Ethicon) sutures with a 30 micron needle. Skin closure was then accomplished with several 6-0 polypropelene (Prolene) sutures.

Phase II. Surgery

Three months following phase I, the right face was again explored using a preauricular incision and the distal cross-facial nerve graft (CFNG) coapted to the right temporal branch (paralyzed side).

Phase III. Surgery

Three months following phase II, the entire CFNG was explored, dissected and

nerve stimulation performed proximal and distal to the coaptation site and in the midgraft region. The entire graft was then harvested, for histomorphometric analysis.

3) Epon Processing of the CFNG (Thanos & Terzis, 1996a).

Once harvested, nerve specimens were pinned to cork strips with entomology (1 μm tip) micropins and fixed in a 4% para-formaldehyde, 0.2% picric acid solution for 48 hours. Furthermore, each graft was divided into four specimens (PT= proximal temporal branch-donor, PG= proximal nerve graft, DG= distal nerve graft and DT= distal temporal branch-target), rinsed in a 0.1M cacodylate buffer for 20 min. and post-fixed with 1% osmium tetroxide for 3 h. After dehydration in increasing concentrations of ethanol, the specimens were embedded in resin (Epon 812), cut at 1 micron thickness (using a Reichert-Jung Ultracut E microtome), mounted on glass slides and stained with 1% toluidine blue.

4) Axonal Morphometric Analysis of the CFNG.

Quantitative assessment of the CFNG axonal profile was studied using a Zeiss Universal light microscope, interfaced with a digitizer tablet (Kurta) and a Sigmascan (3.1) image analysis software. Axonal morphometric analyses of the CFNG was as follows: All nerve specimens (PT, PG, DG, DT and SN= saphenous nerve biopsy) were compared with respect to the following parameters: a) Mean number of axons in each specimen, b) Mean axon diameter (μm), c) Mean myelin thickness (μm). Quantitative assessment of all nerve specimens was based on complete axon counts without sampling. Each axon was traced

manually under high magnification microscope with a digitizing puck and the quantitative measurements were calculated by the computer. Statistical evaluation of these quantitative measurements was performed using paired t-test comparisons between each nerve specimen (McCall, 1986).

STAGE III:

Experiment 6.

Test the effects of a neurotrophic factor such as Insulin-like Growth Factor-I (IGF-I) in conjunction with a CFNG on the reinnervation of the OOM and recovery of function (specific aim #5).

As previously described (background) it appears likely that specific motor acting neurotrophic factors may have a critical role of providing a stimulus or signal to the regenerating axons to traverse into the nerve graft and assist restoration of function.

Experiment 6 therefore studied the effectiveness of one specific neurotrophic factors (Insulin-Like Growth Factor-I {IGF-I}) in conjunction with the CFNG. Efficacy of IGF-I in facilitating regeneration was determined several ways: 1) Behavioral testing of eye sphincter function by the blink test and 2) Axonal morphometric analysis of the nerve graft. From quantitative and qualitative analysis of these data, the following questions can be addressed:

1) What are the behavioral effects on eye sphincter function when reinnervating the OOM with a CFNG and local infusion of IGF-I in comparison to CFNG alone?

2) What are the effects of local administration of IGF-I at the proximal nerve graft coaptation of the CFNG on the axonal morphometric profile of the graft? How does this data compare with CFNG treatment alone?

STAGE III:

EXPERIMENTAL DESIGN

The primary goal of this study was to examine the role of the neurotrophic factor IGF-I in reinnervating a denervated OOM with a CFNG. Unilateral facial paralysis was similarly induced in rats (Fig 10) and subsequently treated with a CFNG (Fig 11) and local administration of IGF-I (Figure 13). Reinnervation was measured behaviorally (blink index) and histomorphometrically (axonal profile of the CFNG). Blink tests were performed on each rat prior to any surgery and then at least once a week after surgery.

EXPERIMENT 6

1) Quantity of animals.

Thirteen adult male Sprague Dawley rats were used in this study and were divided into two groups: A) The vehicle-treated group consisted of normal rats that received a unilateral

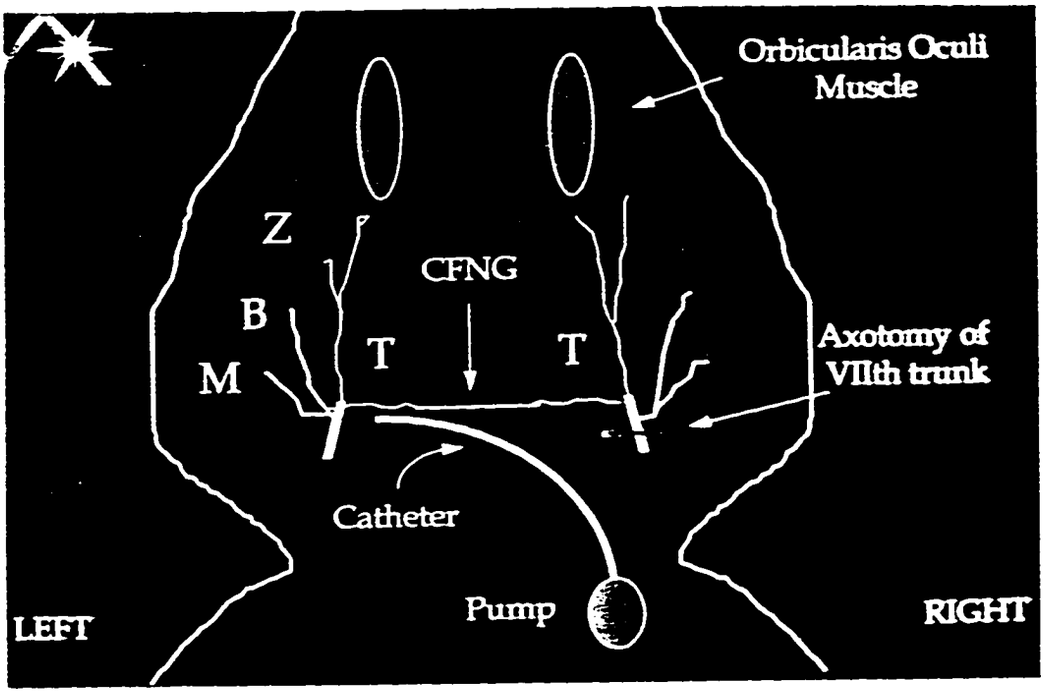


Fig. 13. Dorsal view diagram of the rat facial neuroanatomy illustrating the locus of the axotomy, placement and coaptations of the CFNG and placement of the IGF pump catheter.

facial nerve transection just distal to the stylomastoid foramen and treated with a CFNG and an ALZET osmotic pump (model # 2004) containing a vehicle (1mM acetic acid) solution (N=3).

B) The IGF-I-treated group consisted of animals that similarly received unilateral facial nerve axotomy, and subsequently were treated with a CFNG plus IGF-I (50 μg/ml) infused by similar pump (N=10).

2) Surgical procedures.

Again rats were anesthetized with 100 mg/kg Ketamine / 10 mg/kg Xylazine (intraperitoneal injection) and operated on with the aid of a surgical operating microscope (Zeiss).

The right facial nerve trunk and its branches were explored and identified through a preauricular incision. The temporal branch to the eye sphincter was identified using intraoperative nerve stimulation. The right facial nerve trunk was then transected producing a complete right facial paralysis (Fig 10). The left saphenous nerve (3 cm in length) was then harvested as a graft and tunneled in the subcutaneous plane over the dorsum of the cranium. The saphenous nerve graft was then coaptated to both the left and right VII-T using 11-0 (Ethicon) sutures with a 30 micron needle (Fig. 12). An ALZET (2004) osmotic pump fitted with a silastic tubing catheter (Fig. 14) was then fitted adjacent to the proximal coaptation of the CFNG by three 11-0 epineurial sutures (Fig. 13). Group A rats had their pump filled with a vehicle solution (1 mM acetic acid), while Group B animals

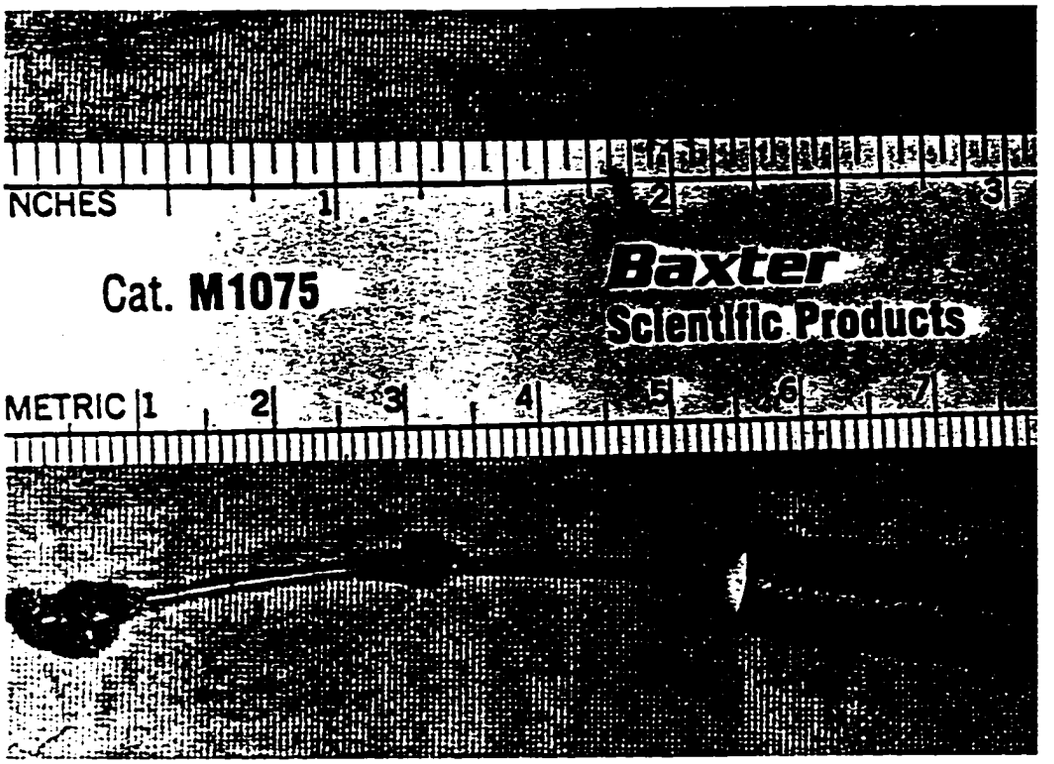


Fig. 14. An Alzet 2004 osmotic pump attached to a silastic catheter.

had their pumps filled with IGF-I (50 μg/ml). Skin closure was accomplished with several 6-0 polypropelene (Prolene) sutures.

Two months later, the entire CFNG was explored. The osmotic pump was then removed, along with the entire graft (CFNG) including the left and right temporal coaptations for histomorphometric analysis.

3. Behavioral evaluation of eye sphincter function- The Blink Test.

Each animal was videotaped for their blinking prior to any surgical procedure and at least once a week thereafter, to document the status of eye sphincter function in both eyes. The blink reflex was evaluated utilizing a custom-designed apparatus (Fig. 15) that delivers a constant 20 ml volume of an air puff to the cornea and periorbital region at a distance of 2 cm. The air stimulus was delivered through a plastic microtubing system, connected to a volume reservoir located away from the animal. Evaluation of the blink was performed by a volunteer who was unaware of each animal's treatment using a subjective 5 point blink index grading scale which has been previously used both clinically and experimentally (Yanagihara, 1984; Terzis, 1987; Kalantarian et al., 1997). Specifically, the blink index scores were as follows: 0 for no blink present- paralysis, 0.5 for slight contraction of the eye lid(s), 1.0 for observable minimal eye closure (1/3 closed), 1.5 for significant but inadequate eye closure (2/3 closed), and 2.0 for complete eye closure. This apparatus provided a behavioral analysis of the motor component of the blink and has been previously shown to be a reliable method in determining the patency of the facial nerve (Terrell and Terzis, 1994).

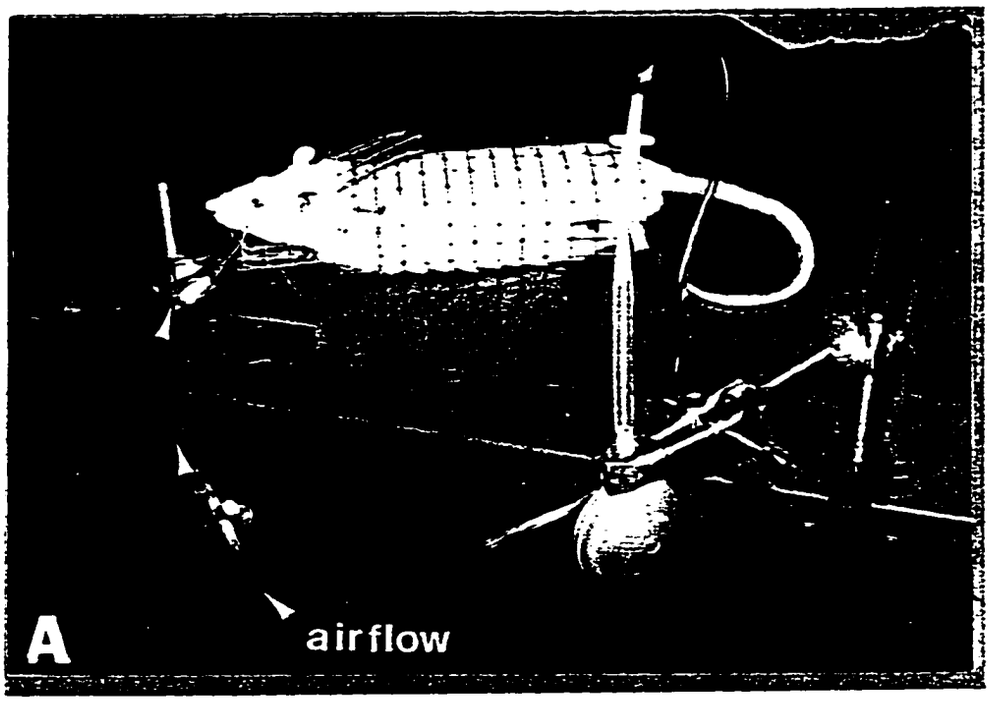


Fig. 15. The blink test apparatus.

3) Data Analysis.

Quantitative assessment of the CFNG of both vehicle-treated and IGF-I-treated animals was studied using a Zeiss Universal light microscope interfaced with a digitizer tablet (Kurta) and a Sigmascan (3.1) image analysis software. Axonal morphometric analyses of each CFNG was as follows: All nerve specimens (PT, PG, DG, DT and SN= saphenous nerve biopsy) were compared with respect to the following parameters:

a) Mean number of axons in each specimen, b) Mean nerve fiber diameter (μm),

c) Mean axon diameter (μm), d) Mean myelin thickness (μm). Quantitative assessment of all nerve specimens was based on complete axon counts without sampling. Each axon was traced manually under high magnification microscope with a digitizing puck and the quantitative measurements were calculated by the computer. Statistical evaluation of the blink test scores and the histomorphometric profile of each CFNG was performed using paired t-test comparisons (McCall, 1986).

CHAPTER III

RESULTS

Experiment 1.

Establish and characterize the neuroanatomy of the facial motor neuron (FMN) pool that innervates the rat OOM.

A. Qualitative Analysis

In this experiment, all histomorphometric evaluations of the facial nucleus and the facial motor neurons were made with reference to the stereotaxic atlas of Paxinos and Watson (1982). A diagram of the rat facial nucleus along with the facial nerve branches and the OOM is illustrated in Fig 16.

Biotin dextran-labeled FMNs serving the OOM were found to have a mutipolar stellate morphology with abundant and prominent dendritic processes typical of motoneurons (Fig 17). These FMNs were only found ipsilateral to the side of labeling and demonstrated a distinct topographical organization. The OOM FMN pool was found to comprise a dorsal subnucleus (DSN) within the facial nucleus (Fig 16, 18). Examination of the labeled FMNs showed no apparent grouping of these motoneurons by size or morphology within the DSN.

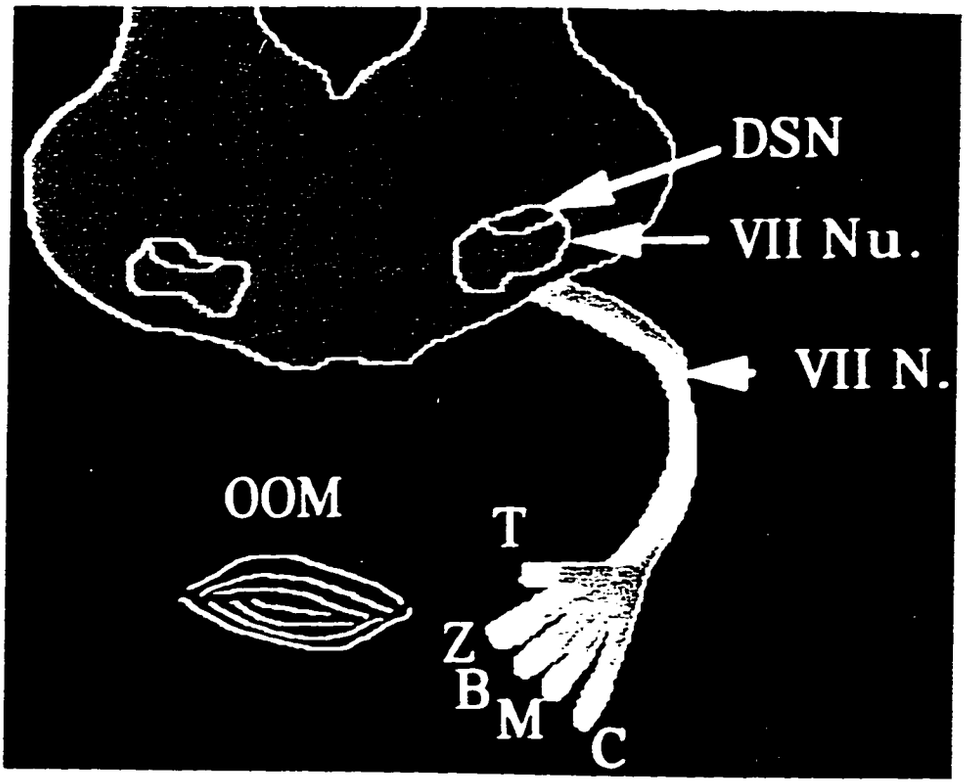


Fig. 16. Schematic depiction of a transverse section of the caudal pons illustrating the rat facial nucleus giving rise to the facial nerve, its branches (T= temporal, Z= zygomatic, B= buccal, M= mandibular, C= cervical) and the OOM. Brainstem shown at B= -10.8 mm, IA= -1.8mm (Adopted from Paxinos and Watson, 1982).

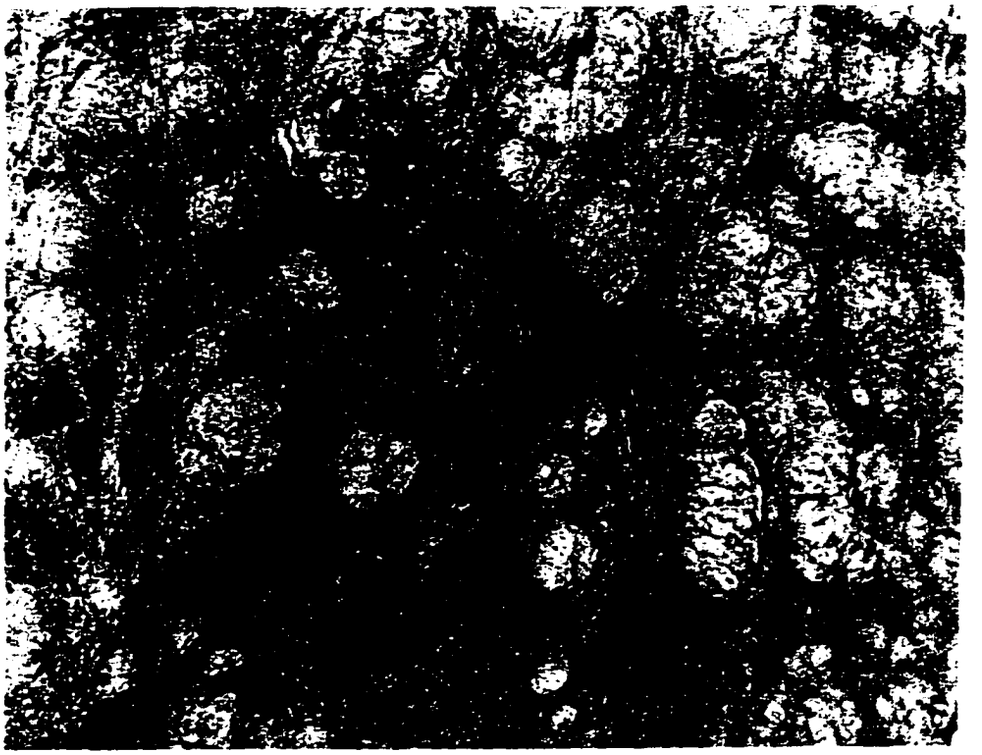


Fig. 17. Biotin dextran-labeled FMNs in the DSN of the rat facial nucleus and counterstained with Neutral Red (400X).

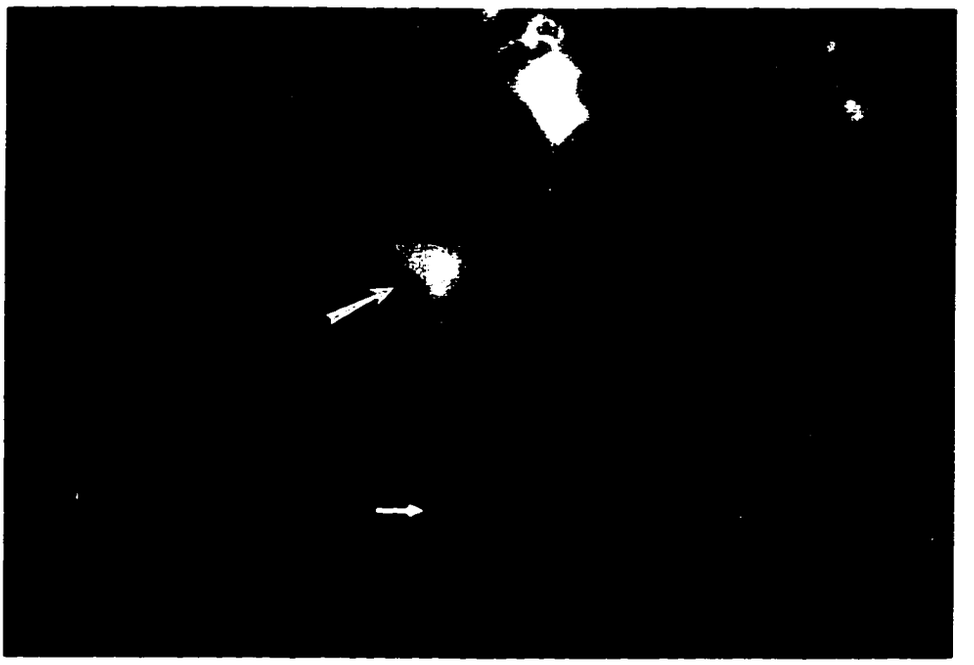


Fig. 18. FMNs in the facial nucleus of an animal injected with 2.5 % Fluoro Gold into the OOM (160X) Note the intensely stained cytoplasm along with clearly defined axon, and dendrites.

B. Quantitative Analysis

Quantitative analysis of the bd-labeled FMN pool of the OOM revealed that these neurons had a mean somal diameter of 28 $\pm$ 11 μm. Furthermore, the mean thickness of the DSN or motor neuron pool of the OOM was calculated by counting the number of 50 μm sections containing labeled neurons, and found to be approximately 2.56 mm. Nissl-stained FMN counts revealed that the facial nucleus contained 1680 $\pm$ 422 neurons. Of these neurons FG labeling revealed that the OOM FMN pool contained an average 481 $\pm$ 69 neurons that were within the DSN or 28.6% of the total FMNs (Fig. 19).

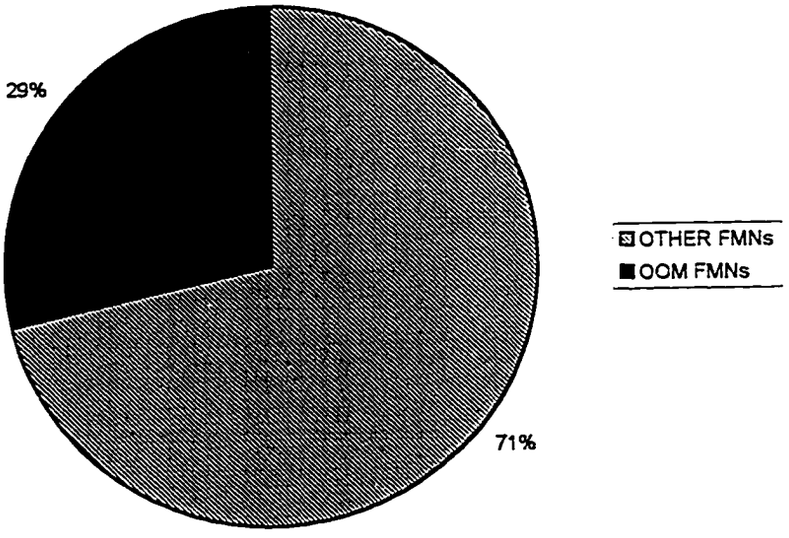


Fig. 19. The mean FMN pool of the rat OOM with respect to total FMNs.

Experiment 2.

Determine the FMN that serves the temporal branch of the facial nerve, which is utilized in our model.

A. Qualitative Analysis

Biotin dextran-labeled FMNs serving the temporal branch of the facial nerve to the OOM were found to have similar morphological characteristics as that of the OOM FMN pool described in experiment 1 (Fig. 17,18). These labeled neurons were also found to be located ipsilateral to the side of labeling and topographically located within the DSN of the facial nucleus (Fig. 16).

B. Quantitative Analysis

Quantitative image analysis of the bd-labeled FMN pool of the temporal branch of VII revealed that these neurons had a mean somal diameter of 32 $\pm$ 6 μm. There was no statistically significant difference in somal diameter of the labeled FMNs between the OOM and VII-T ($p > 0.05$). Furthermore, the mean thickness of the DSN or motor neuron pool of the VII-T was found to be approximately 1.32 mm. FG labeling revealed that the VII-T FMN pool contained an average 324 $\pm$ 54 neurons that were also within the DSN or 19.3% of the total FMNs (Fig. 20).

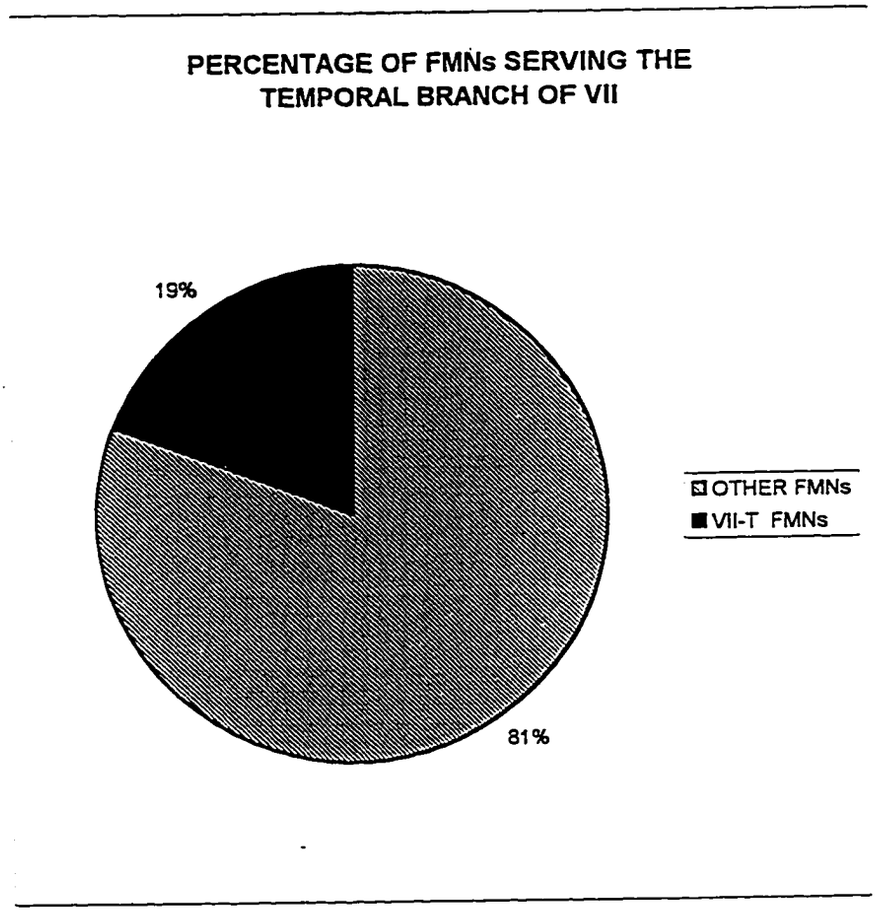


Fig. 20. The mean number of FMNs of the rat temporal branch of the facial nerve with respect to the total number of FMNs.

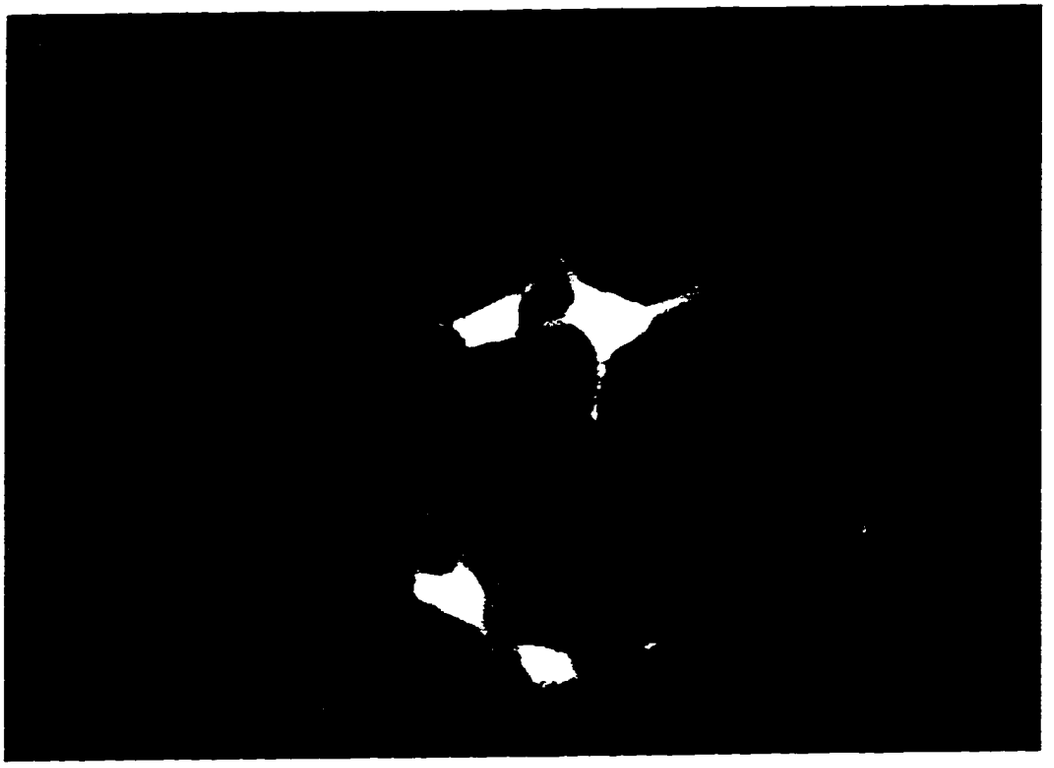


Fig. 21. FMNs in the facial nucleus of an animal injected with 2.5 % Fluoro Gold into the OOM (160X).

Experiment 3.

Determine the topographical localization of the double labeled FMNs and identify the specific facial nerve branch that provides the majority of innervation to the OOM.

A. Qualitative Analysis

After injecting FG into the orbicularis oculi muscle fluorescent microscopy revealed the FMN pool in the DSN of the facial nucleus (Fig. 21). All labeled neurons were found ipsilateral to the side of injection. In addition, bisBenzimide was used to label the temporal branch FMNs (Fig. 22). A light blue nucleus and cytoplasm characterized these labeled neurons. Double labeling of the FMNs that serve both the orbicularis oculi muscle -white cytoplasm and dendrites (FG), and the temporal branch of the VIIth nerve - light blue nucleus and cytoplasm revealed a population of neurons in the previously defined DSN but with no further topographical localization within the DSN. These double labeled cells also displayed some neighboring glial cell staining (Fig. 23).

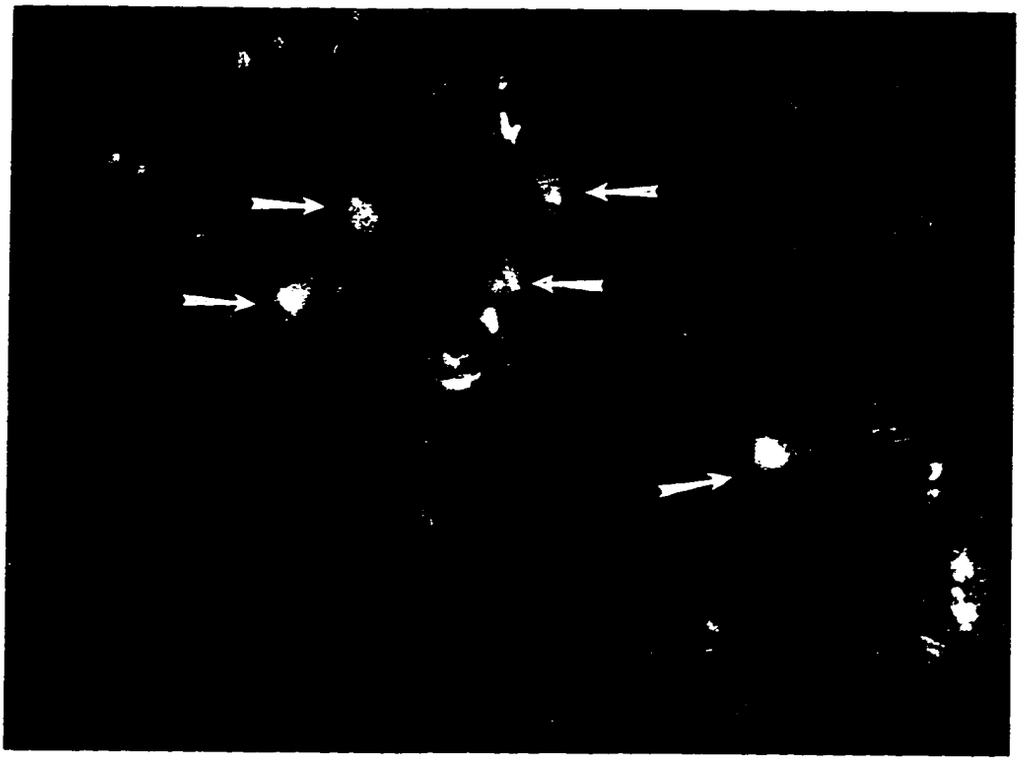


Fig. 22. FMNs in the facial nucleus of an animal injected with 20 % bisBenzimide into the VII-T (160X). Note the light blue stained nucleus and cytoplasm of these neurons.

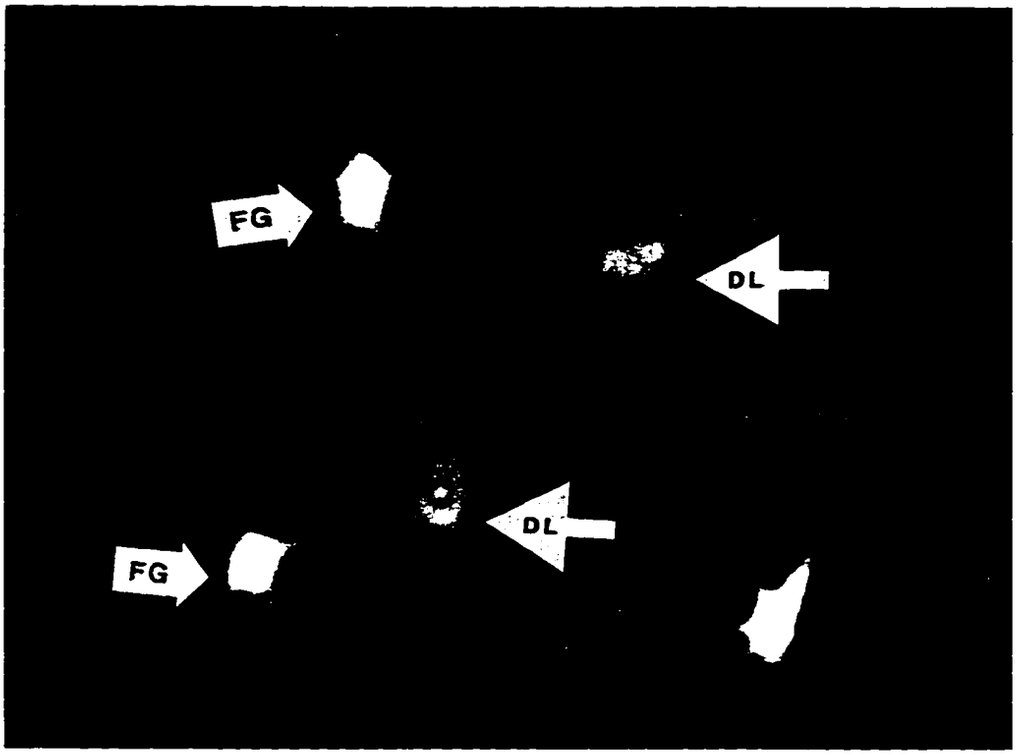


Fig. 23. FMNs in the facial nucleus of an animal injected with 2.5% Fluoro Gold into the OOM, and the VII-T labeled with 20% bisBenzimide (400X). DL= Double Labeled neuron, FG=Fluoro Gold labeled only neuron.

B. Quantitative Analysis

Quantitative analysis of the fluorescent-labeled mean number of FMNs of both the OOM and the temporal branch of VII with respect to the mean total number of FMNs revealed that the OOM FMN pool consisted of 481 + 69 OOM neurons, while the VII-T FMN pool consisted of 324 + 54 neurons (Fig 24). Since Nissl-stained counts of the entire facial nucleus revealed 1680 + 422 neurons (Fig 24) it can be summarized that approximately 29% of all FMNs innervate the rat OOM and that approximately, 19% of all FMNs serve the temporal branch of the facial nerve.

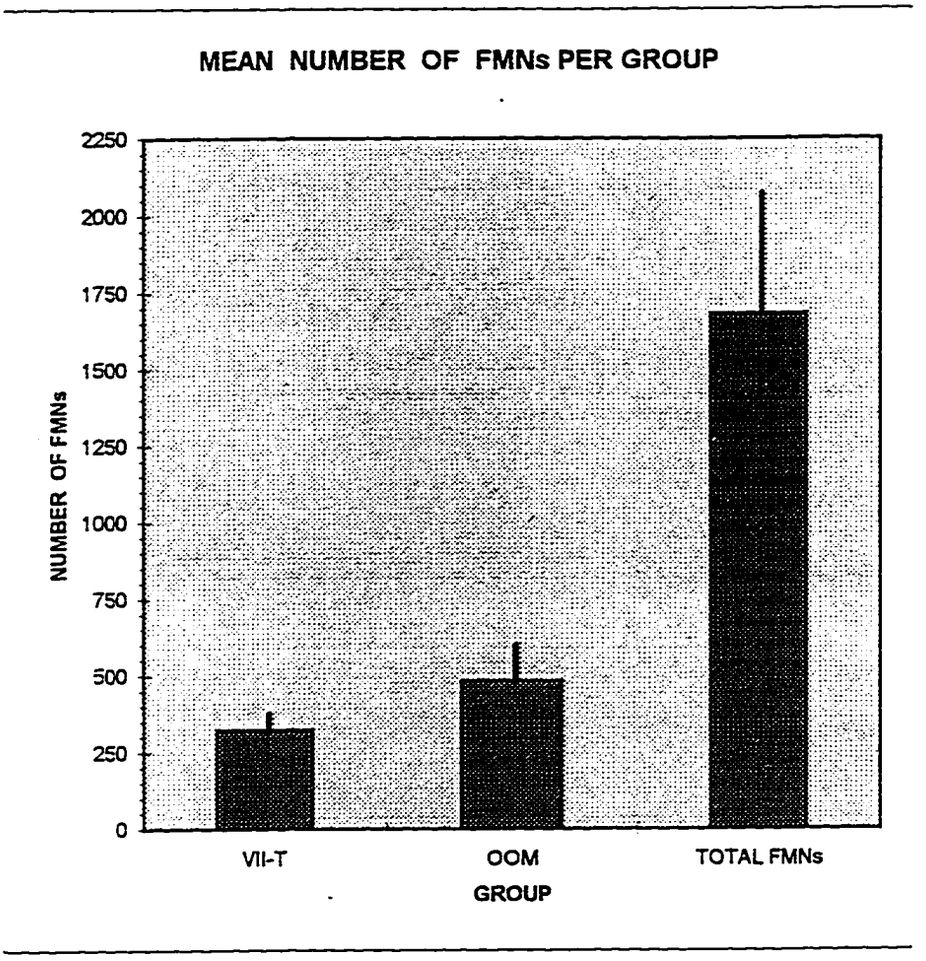


Fig. 24. The mean number of FMNs innervating the rat OOM and the temporal branch of the facial nerve with respect to the total number of FMNs.

Experiment 4.

Determine the histomorphometric (OOM motor endplates) and behavioral characteristics of FP due to facial nerve axotomy, and facial nerve axotomy followed by treatment with a CFNG.

A. Qualitative Analysis

Behavior analysis demonstrated that all animals receiving a unilateral facial nerve axotomy exhibited complete unilateral facial paralysis and loss of the blink (no response to air puff stimulus) and lack of symmetry (Fig. 25). However, animals treated with the cross-facial nerve graft procedure demonstrated some moderate recovery of the blink response three months post-axotomy.

Following acetylcholinesterase staining to reveal motor endplates, OOM sections were examined morphologically. Specifically, the distribution of the endplates was assessed and a quantitative analysis was carried out. An example of the OOM motor endplates in a normal rat eye sphincter is illustrated in Fig 26. In addition, the location of the motor endplates in the OOM was found predominantly in the lateral aspects of the OOM, as it receives its motor-nerve fibers in the superior and inferior lateral quadrants.

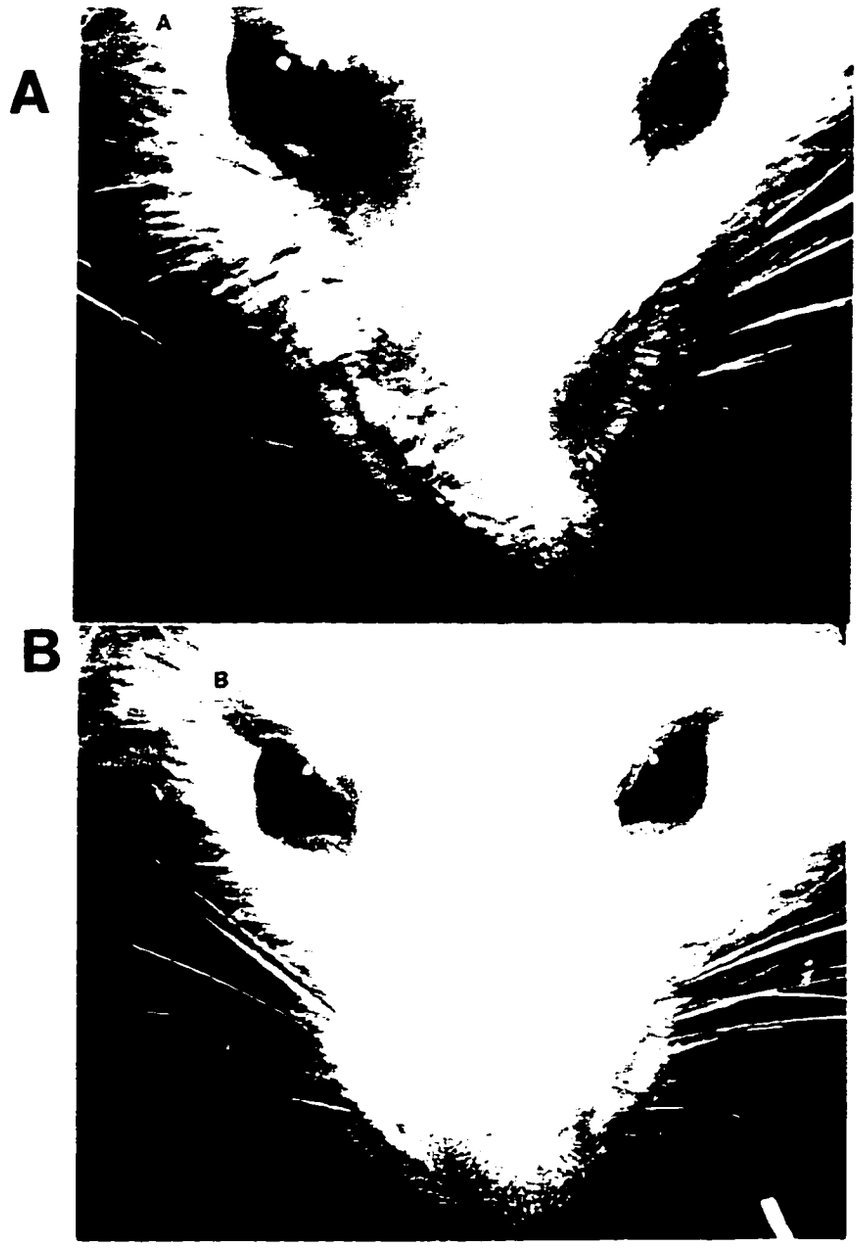


Fig. 25. A. Photograph of rat two weeks after receiving a right facial nerve axotomy thus inducing right facial paralysis. B. Photograph of a normal rat.

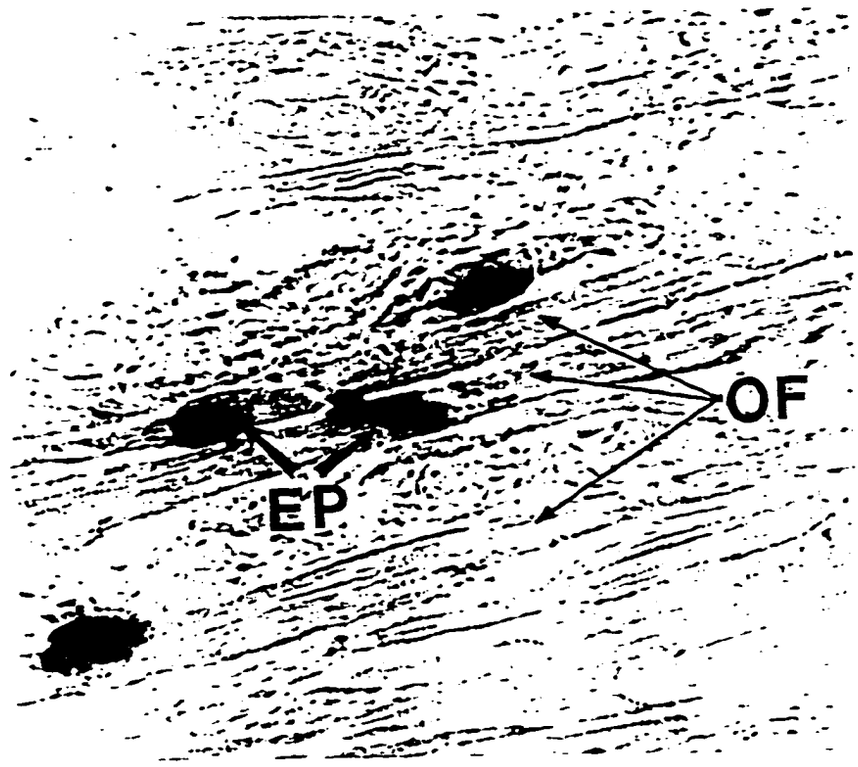


Fig. 26. Longitudinal section of a normal rat OOM stained for motor endplates (OF = Orbicularis Oculi muscle fibers, EP = endplates, 400X).

B. Quantitative Analysis

All OOM longitudinal sections were examined and all AChE-stained motor endplates counted. Assessment of the number of endplates between groups, using a one-way ANOVA showed a highly significant effect ($p<0.001$). Specifically, there was a significant decrease ($p<0.001$) in the mean number of endplates between the normal (94.07) and the denervated (7.62) OOM (Fig. 27). In addition, a significant increase was observed in the number of endplates between the denervated (7.62) and reinnervated with a CFNG (48.04) OOM (Fig. 27). A comparison of the number of endplates between regions of the OOM (Fig. 12) using a one way ANOVA demonstrated a strong effect ($p<0.001$; Fig 28). Further analysis showed a significantly greater number of endplates in the lateral regions of the OOM ($p<0.001$). Similarly, a comparison of the number of endplates per group of animals across regions of the OOM showed a significant interaction effect ($p<0.001$). This relationship (Fig 28) was consistent across all groups of animals, with endplates showing a strong predominance (80 %) in the lateral regions.

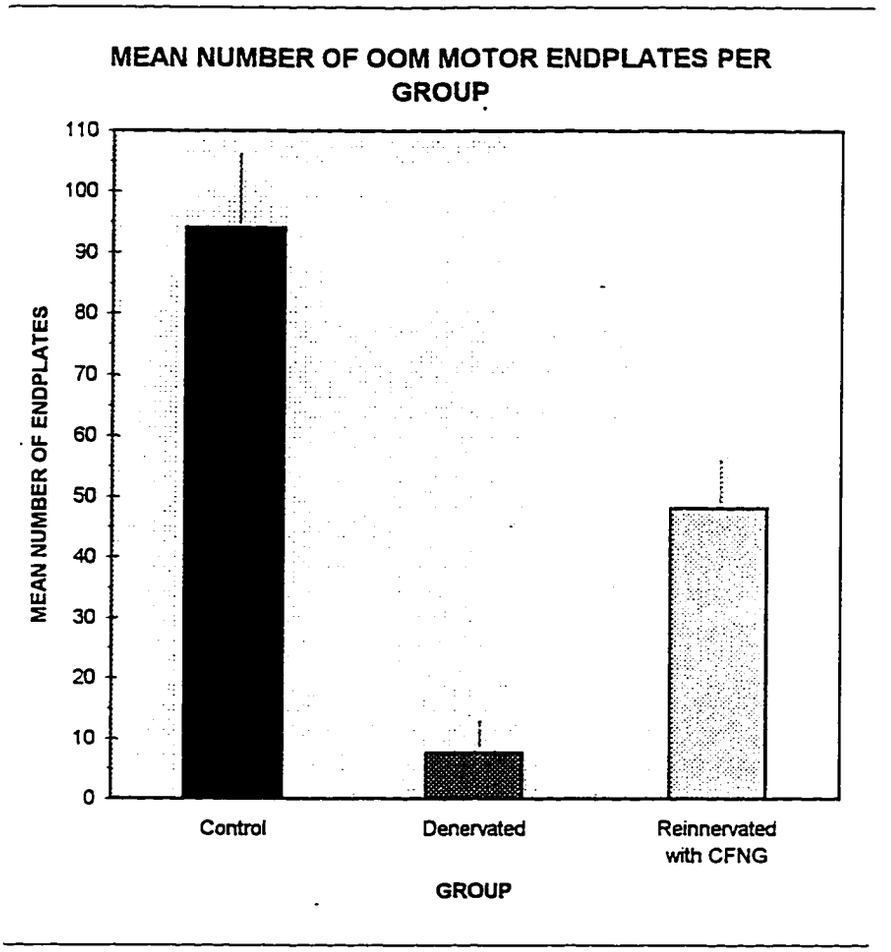


Fig. 27. Mean number of motor endplates in each group.

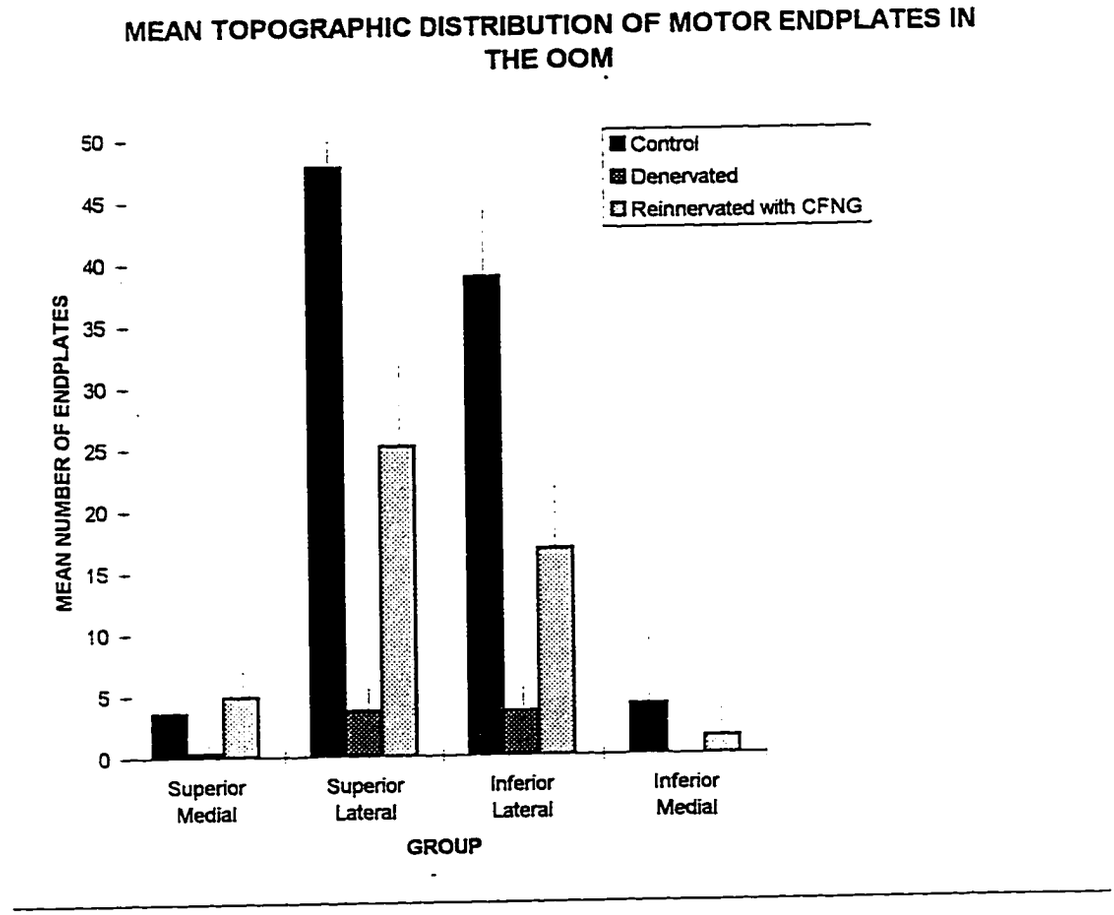


Fig. 28. Mean topographic distribution of motor endplates in each group per quadrant.

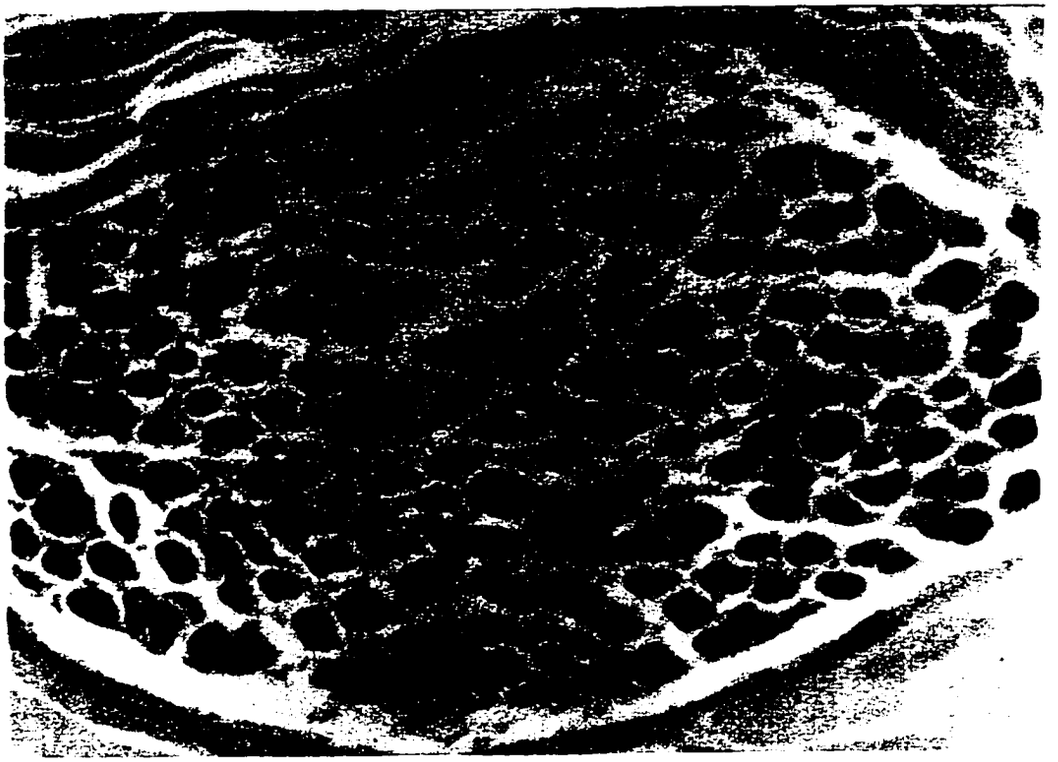


Fig. 29. Micrograph of a transverse 1 μm thick section from a proximal temporal (PT) specimen of a CFNG stained with 1% toluidine blue.

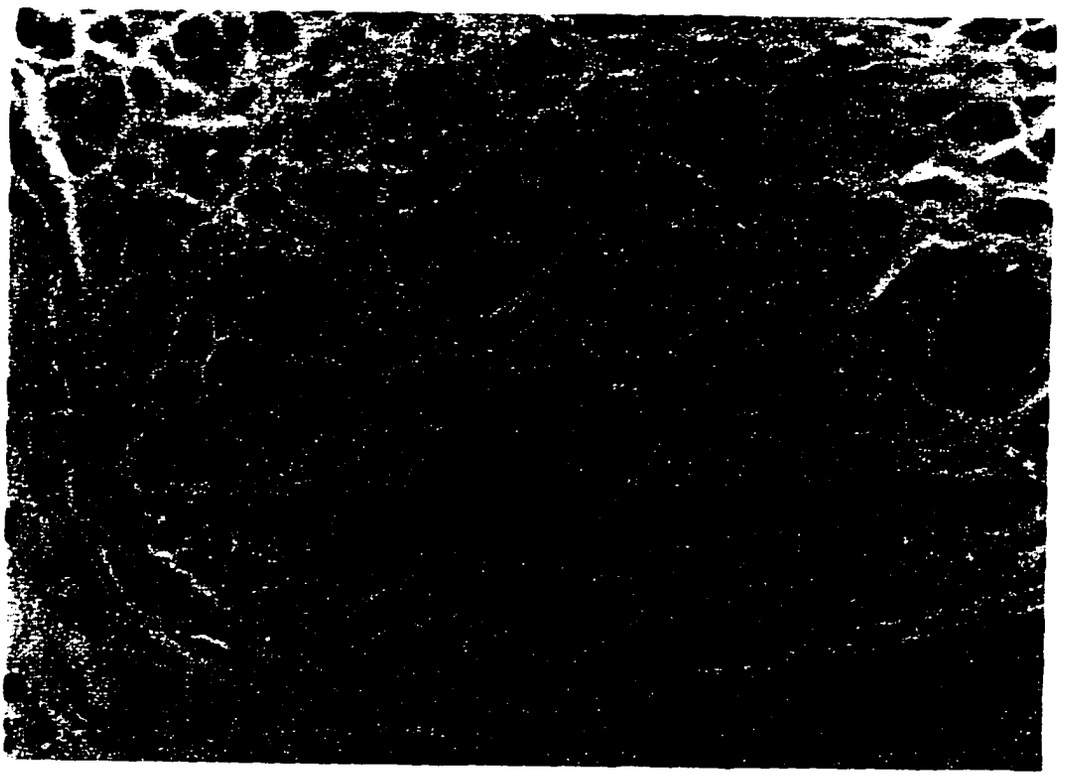


Fig. 30. Micrograph of a transverse 1 μm thick section from a proximal nerve graft (PG) specimen of a CFNG stained with 1 % toluidine blue.

Experiment 5.

Determine the histomorphometric characteristics of the CFNG used to reinnervate the paralyzed OOM of the FP rat.

A. Qualitative Analysis

Following toluidine blue staining of the CFNG specimens to reveal their axonal and myelin histomorphometry a quantitative analysis was carried out. Transverse 1 μm thin sections of the entire CFNG (PT,PG, DG, DT) is illustrated in Figs. 29-32 (see Fig. 11; page 56 for locus of nerve biopsy sites).

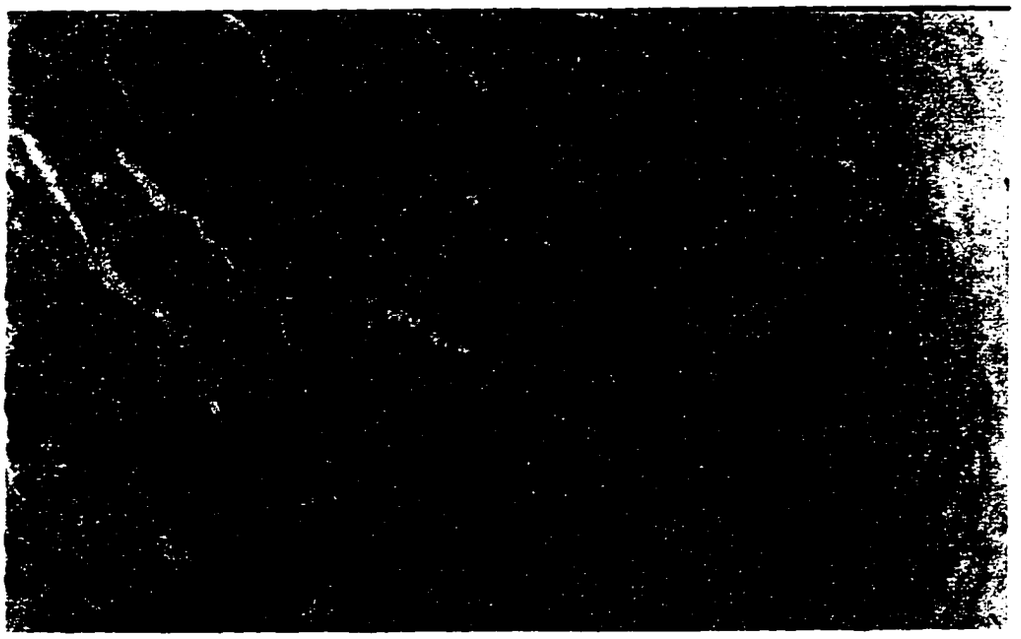


Fig. 31. Micrograph of a transverse 1 μm thick section from a distal nerve graft (DG) specimen of a CFNG stained with 1% toluidine blue.

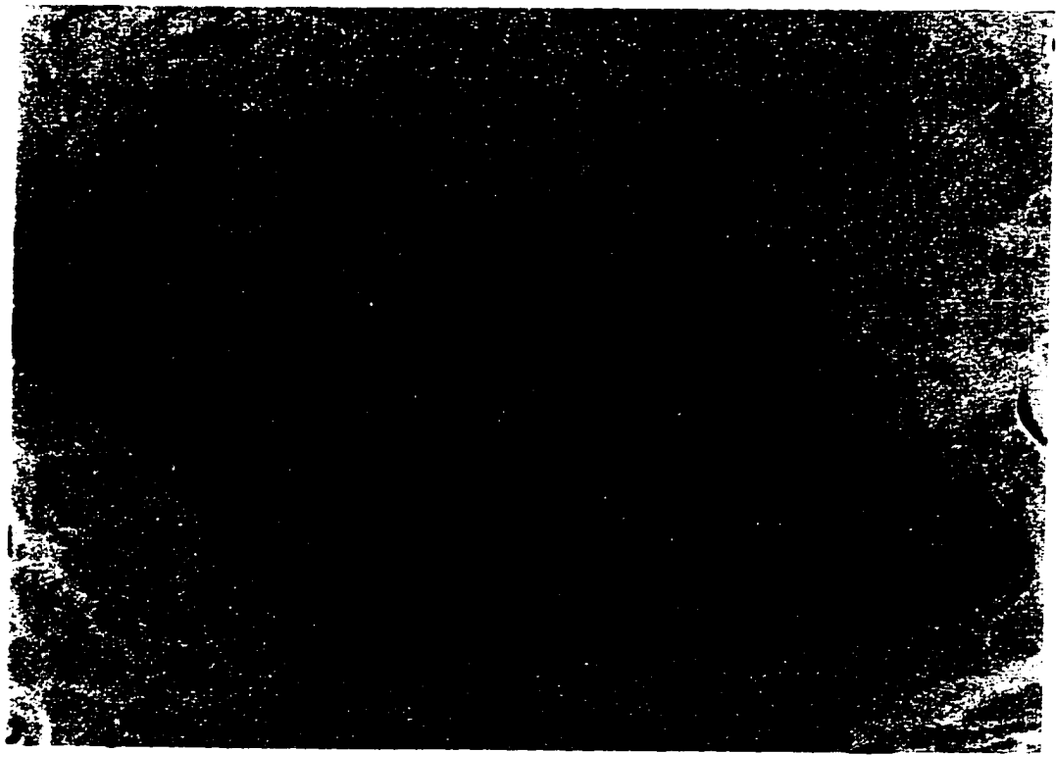


Fig. 32. Micrograph of a transverse 1 μm thick section from a distal temporal (DT) specimen of a CFNG stained with 1% toluidine blue.

B. Quantitative Analysis

The average number of axons found in each of the different CFNG specimens was as follows: 135 $\pm$ 35 (PT), 105 $\pm$ 43 (PG), 102 $\pm$ 31 (DG), and 32 $\pm$ 11 (DT). Assessment of the mean number of axons between the CFNG specimens using paired t-test comparisons, revealed the following significant differences (Fig. 33). The data revealed that the distal temporal (DT) specimens consisted of a significantly smaller number of axons than the other specimens; specifically compared to PT ($p < 0.02$), PG, DG, ($p < 0.05$). In addition, the number of axons in the PT did not significantly differ from the number of axons in the saphenous nerve ($p > 0.05$).

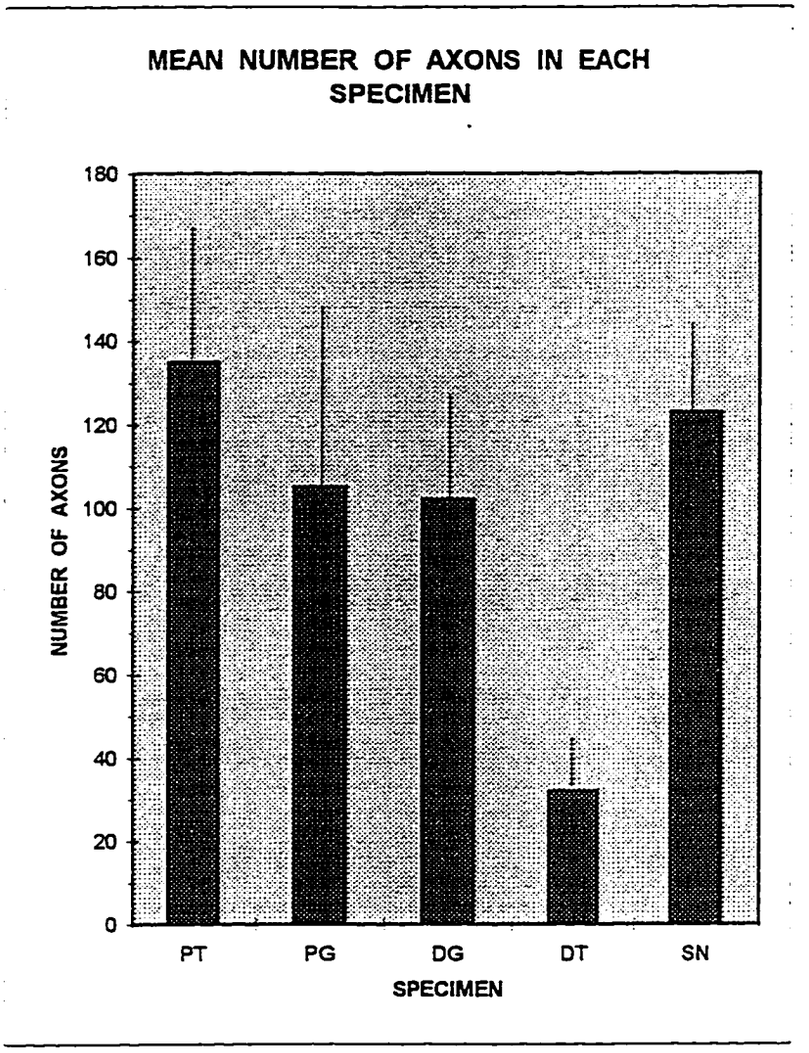


Fig. 33. Mean number of axons in each CFNG specimen (PT = proximal temporal - donor, PG = proximal nerve graft, DG = distal nerve graft, DT = distal temporal, SN = saphenous nerve).

The average axon diameter (μm) found in each of the different CFNG specimens was as follows: 3.18 ± 0.2 (PT), 2.47 ± 0.15 (PG), 2.54 ± 0.12 (DG), and 2.88 ± 0.21 (DT). Correspondingly, it was observed that the proximal temporal (PT) specimens showed a significantly greater mean axon diameter than the proximal nerve graft (PG; $p<0.01$), distal nerve graft (DG; $p<0.01$), and distal temporal specimens (DT; $p<0.05$, Fig. 34). However, the PT specimens did not significantly differ from the saphenous nerve specimens ($p>0.05$).

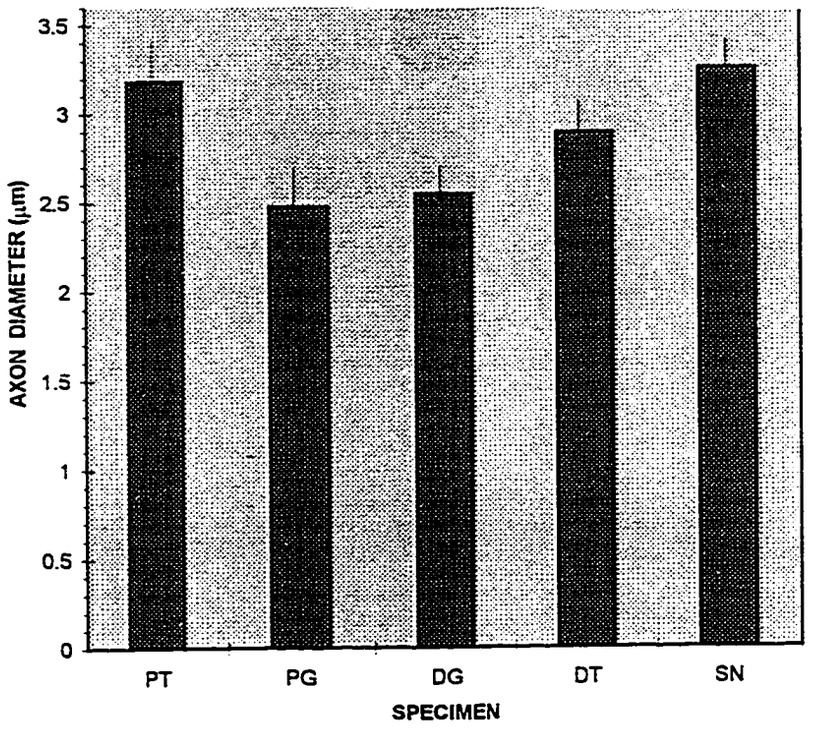


Fig. 34. Mean axon diameter in each CFNG specimen. (PT = proximal temporal - donor, PG = proximal nerve graft, DG = distal nerve graft, DT = distal temporal, SN = saphenous nerve).

The average myelin thickness (μm) found in each of the different CFNG specimens was as follows: 1.62 ± 0.3 (PT), 0.68 ± 0.1 (PG), 0.71 ± 0.08 (DG), and 0.92 ± 0.19 (DT). A comparison of myelin thickness between specimens using a paired t-test, demonstrated that the PT exhibited a significantly greater mean myelin thickness than the other specimens (Fig. 35). In particular, the PT revealed a greater myelin thickness than the PG ($p<0.01$), DG ($p<0.01$), and DT ($p<0.05$), although no difference was observed between the PT and SN ($p>0.05$).

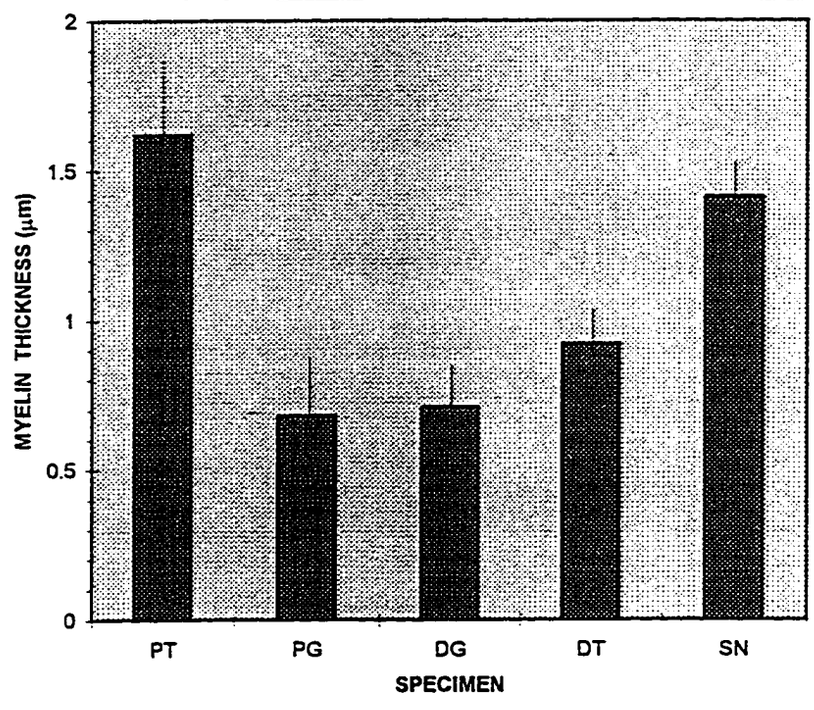
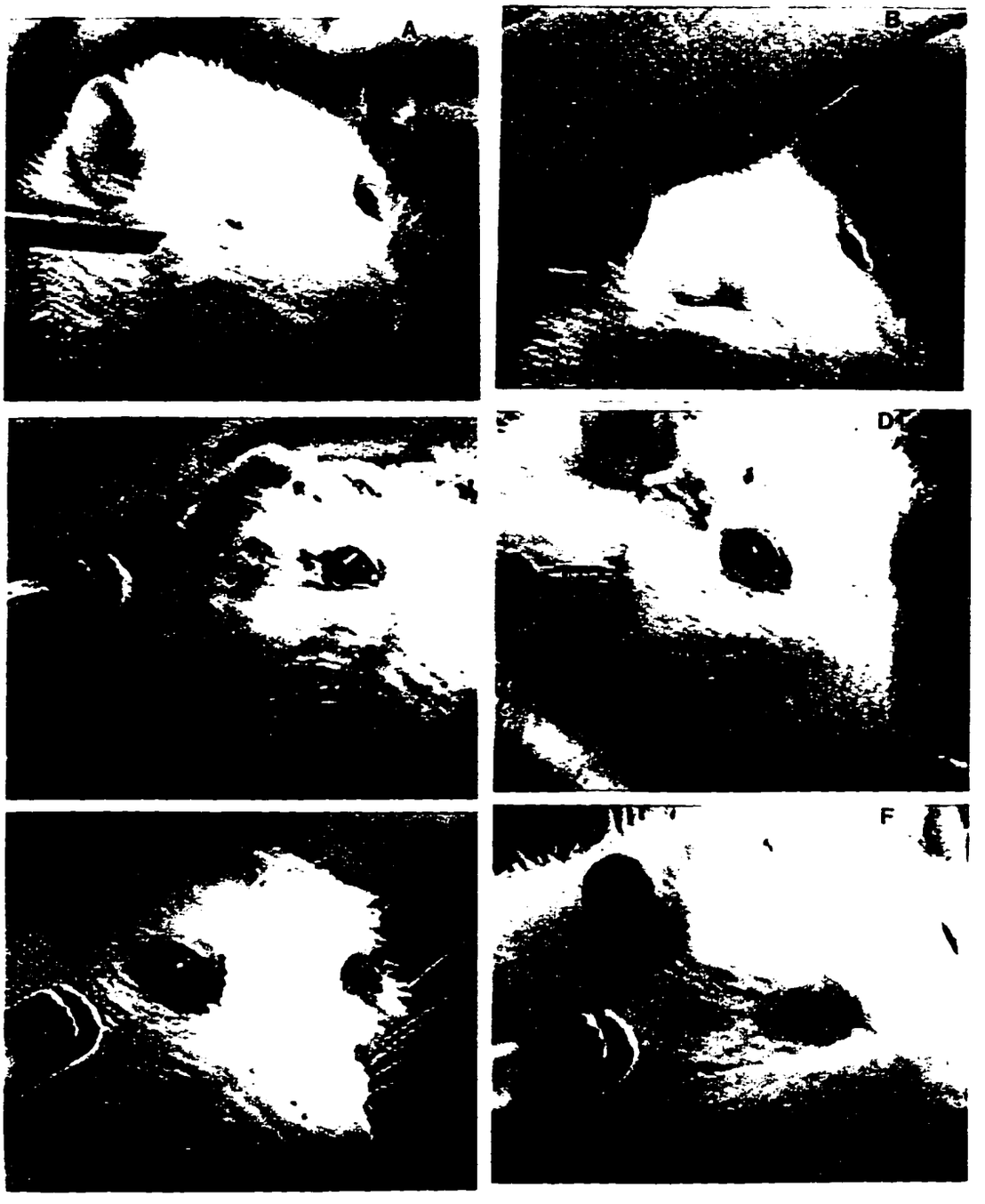


Fig. 35. Mean myelin thickness in each CFNG specimen. (PT = proximal temporal - donor, PG = proximal nerve graft, DG = distal nerve graft, DT = distal temporal, SN = saphenous nerve).

Correlation with motor endplate data

The motor endplate data of the OOM reinnervated by CFNG was correlated to the number of axons present in the distal end of the CFNG (DT). A Spearman correlation test (McCall, 1986) between the mean number of endplates in the reinnervated with a CFNG OOM (48) and the number of nerve fibers present in the distal nerve graft (32) revealed a positive correlation ($r = 0.64$).



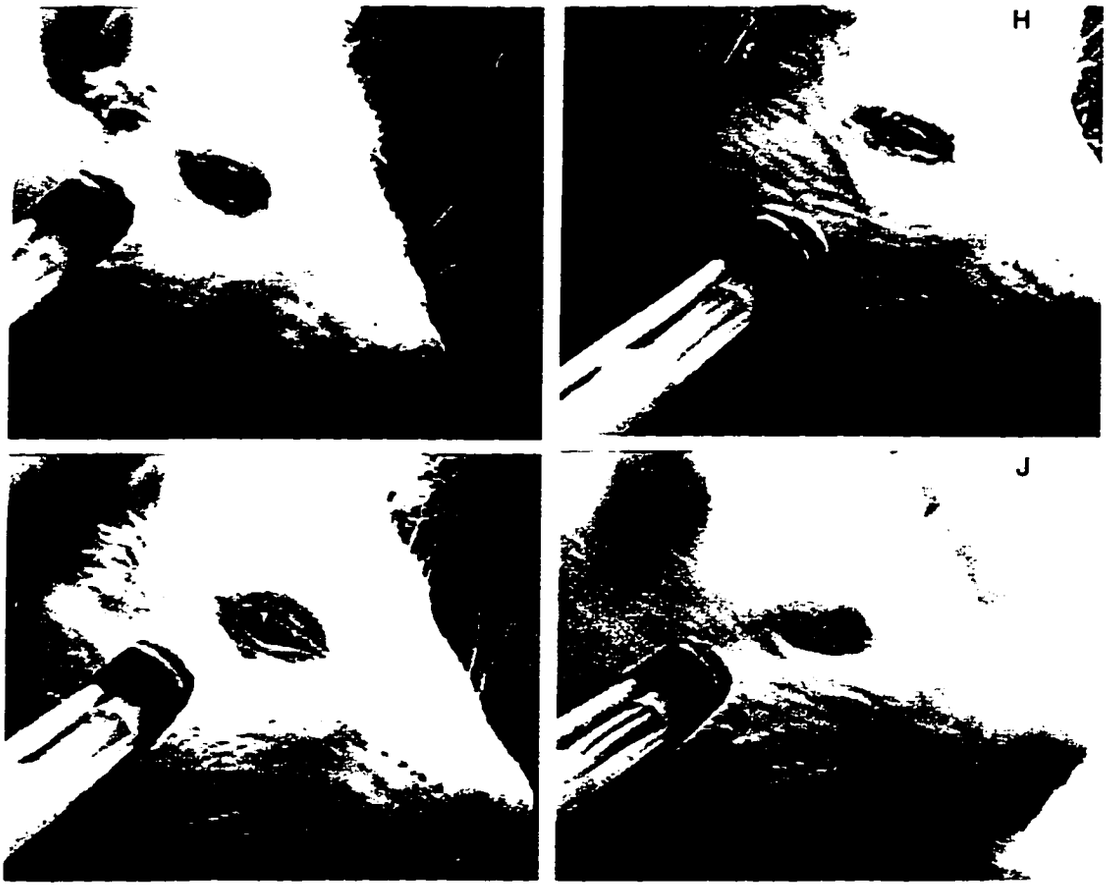


Fig. 36. Photographs of the blink response over time in a vehicle-treated (left) and IGF-I-treated (right) rat. A,B = normal preop blink; C,D = 2 weeks; E,F = 4 weeks; G,H = 6 weeks and I,J = 8 weeks post treatment.

Experiment 6.

Determine the effects of Insulin-like Growth Factor-I (IGF-I) in conjunction with a CFNG on the reinnervation of the OOM and recovery of function.

A. Behavior Analysis (The Blink Index)

A critical outcome of assessing the efficacy of any treatment of paralysis is function. In the rat model of facial paralysis, the blink test was utilized. Function was graded by a volunteer unfamiliar with the treatment group of each animal. The functional grading of the blink or the blink index (BI) was used as previously described (in methods). The blink test was given at least once a week and for a total of 17 sessions (Table 3). Qualitative assessment of the blink response in both a vehicle and an IGF-I treated animal over time is illustrated in Fig. 36. The mean blink index was calculated for each group in each session (Fig. 37). Assessment of the mean BI between the vehicle-treated rats (CFNG + vehicle) and the IGF-I-treated rats (CFNG + IGF-I), using paired t-test comparisons revealed that

TABLE 3. Number of Days Post Treatment to Which Each Session Corresponds.

Session	Post Treatment Day	Session	Post Treatment Day	Session	Post Treatment Day
1	4	7	31	13	48
2	10	8	35	14	53
3	17	9	37	15	58
4	20	10	39	16	63
5	24	11	41	17	67
6	27	12	45		

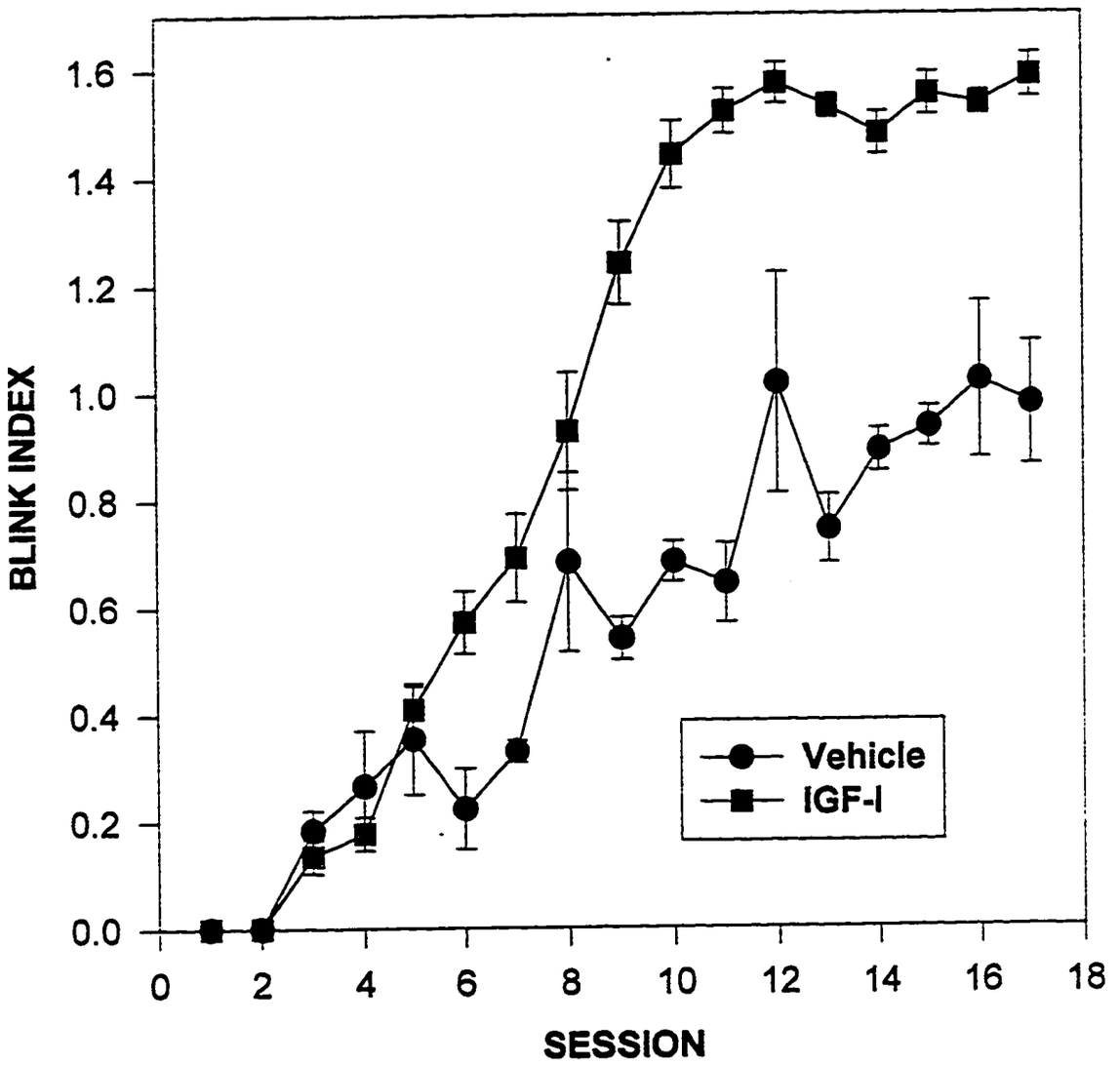


Fig. 37. The mean Blink Index in each group over time (Vehicle-treated = CFNG + vehicle, IGF-I-treated = CFNG + IGF-I).

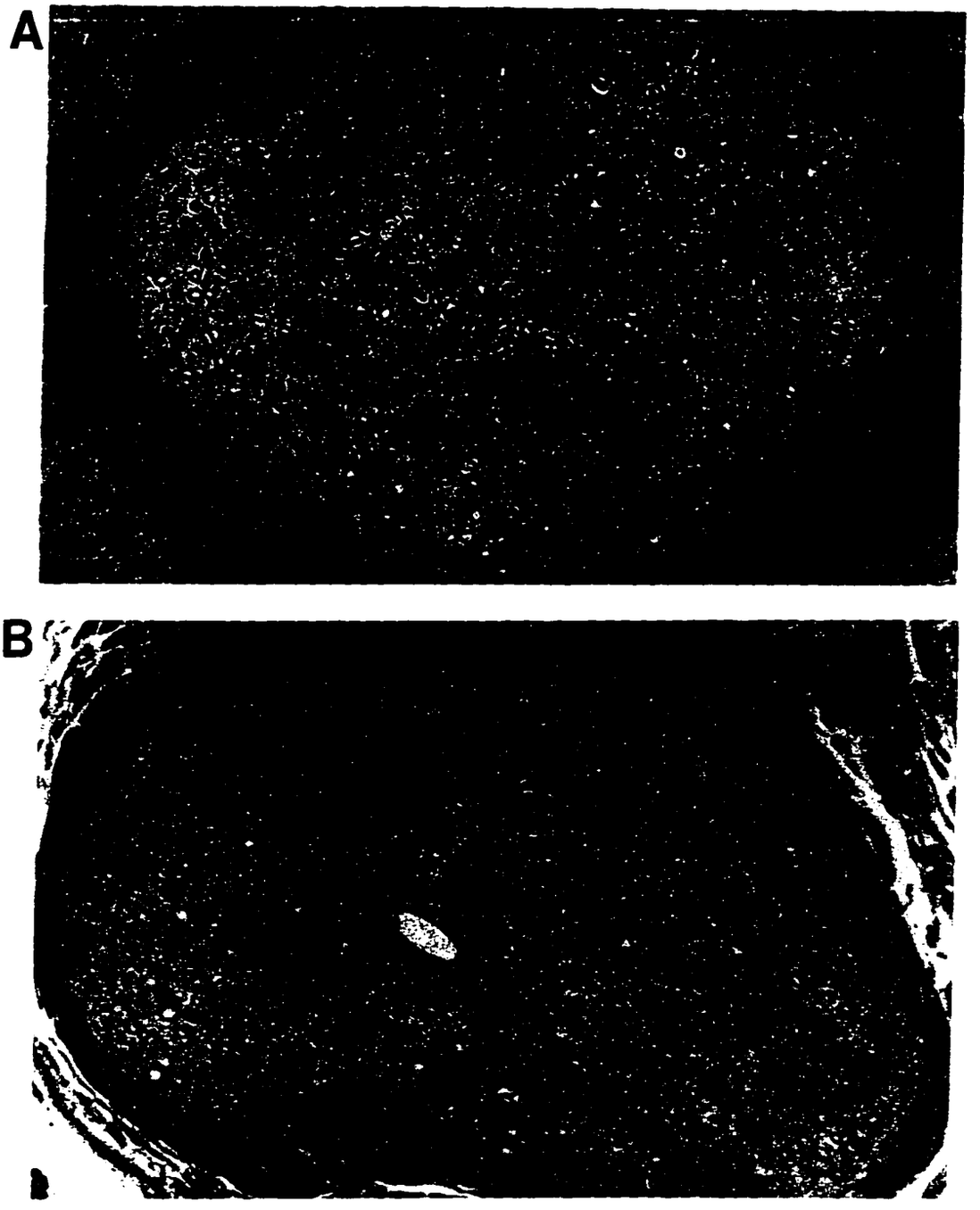


Fig. 38. Micrograph of a transverse 1 μm thick section from a proximal temporal (PT) CFNG specimen stained with 1% toluidine blue (A. Vehicle-treated, B. IGF-I-treated).

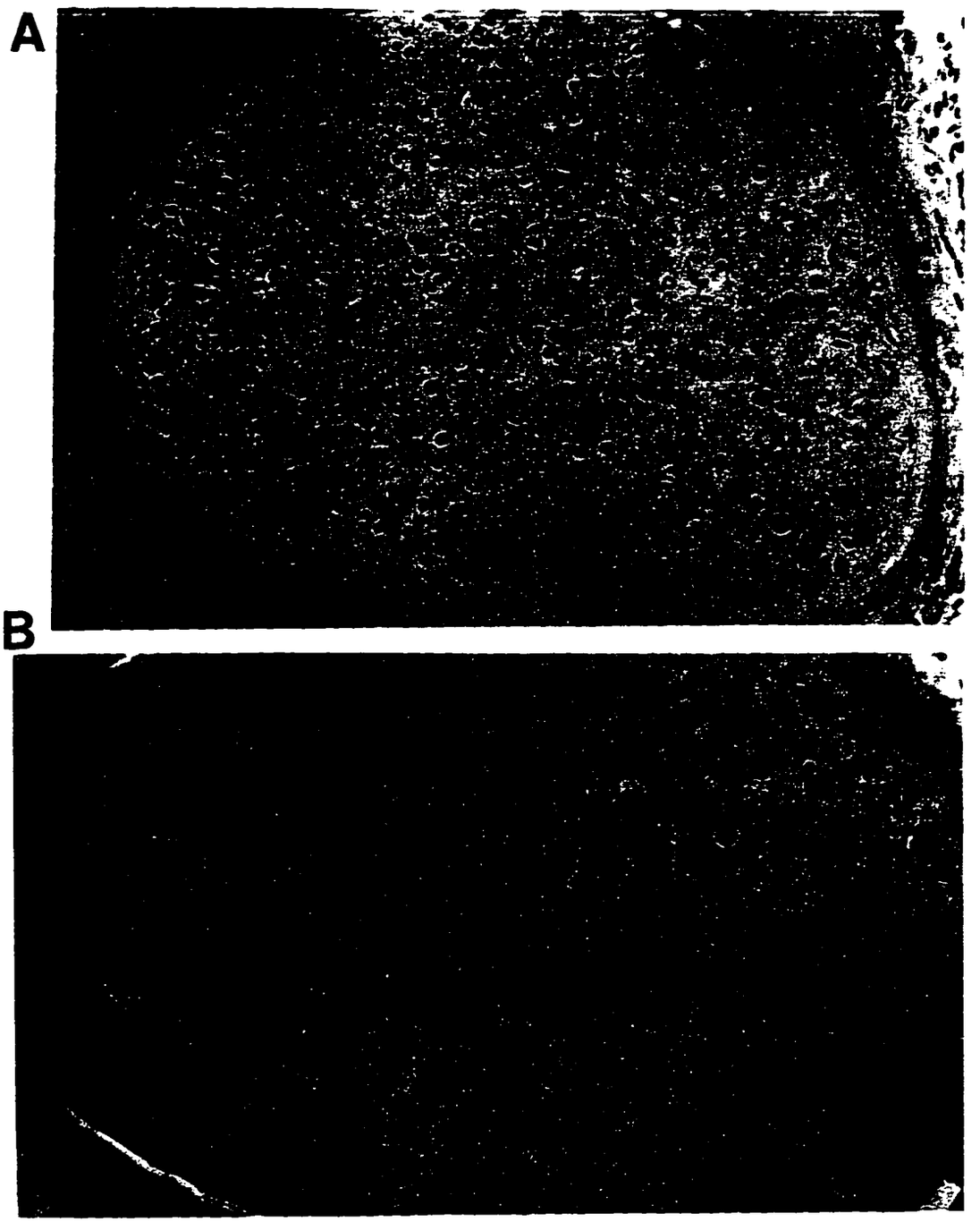


Fig. 39. Micrograph of a transverse 1 μm thick section from a proximal nerve graft (PG) CFNG specimen stained with 1% toluidine blue (A. Vehicle-treated , B. IGF-I-treated).

the IGF-I treated animals had a significantly greater BI than the vehicle-treated animals ($p<0.01$). More specifically, the BI was observed to significantly differ as early as session 9 (37 days after treatment).

B. Qualitative Analysis

CFNG specimens were stained with toluidine blue to reveal their axonal and myelin histomorphometry. Transverse 1 μm thick sections of the entire CFNG (PT,PG, DG, DT) is illustrated in Figs. 38-41 (see Fig. 11 for locus of nerve biopsy sites). IGF-I treated animals revealed in their CFNG specimens a greater abundance of both blood vessels and collagen. In addition, some IGF-I treated CFNG specimens revealed pockets of extrafascicular axons.

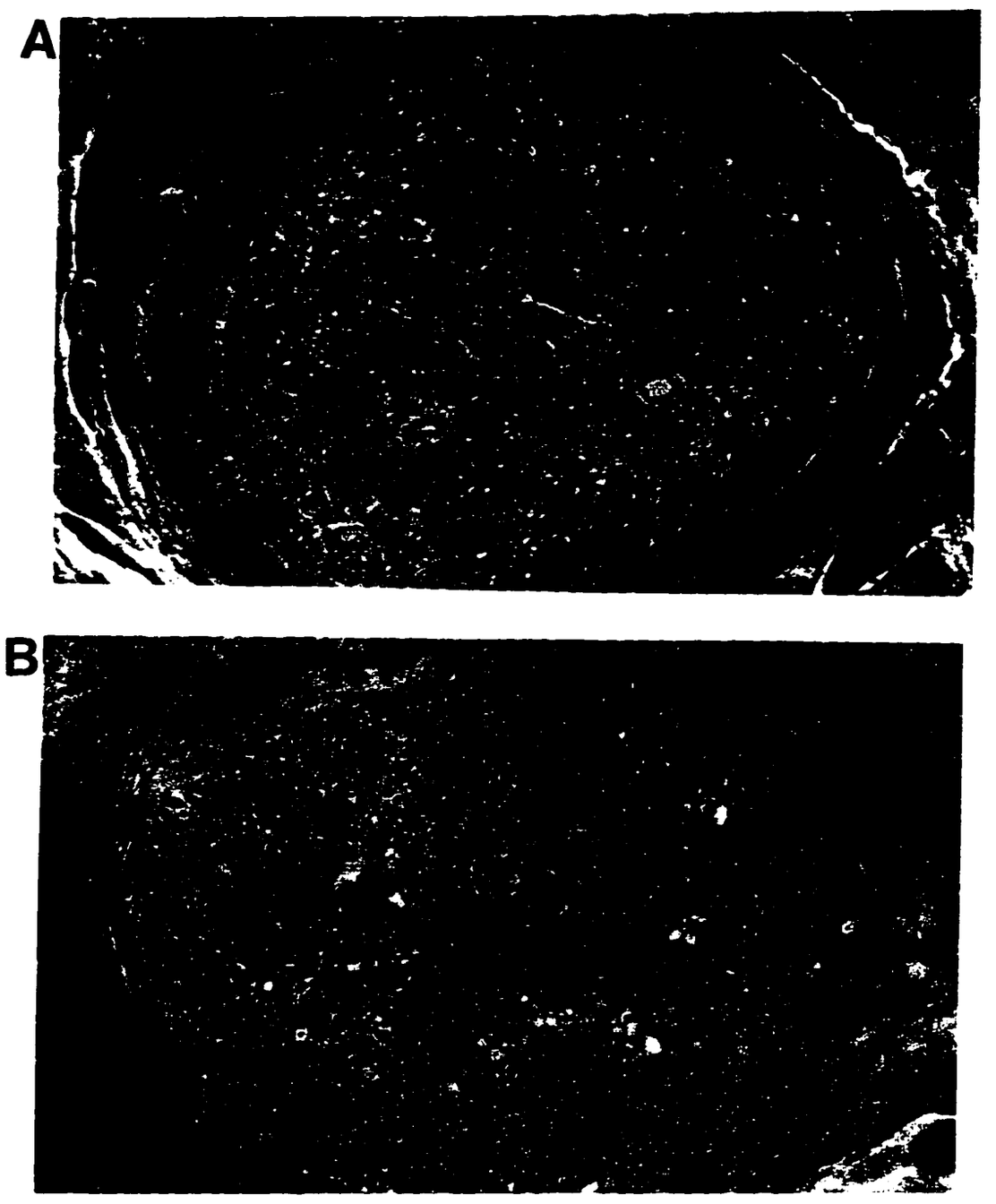


Fig. 40. Micrograph of a transverse 1 μm thick section from a distal nerve graft (DG) CFNG specimen stained with 1% toluidine blue (A. Vehicle-treated , B. IGF-I-treated).

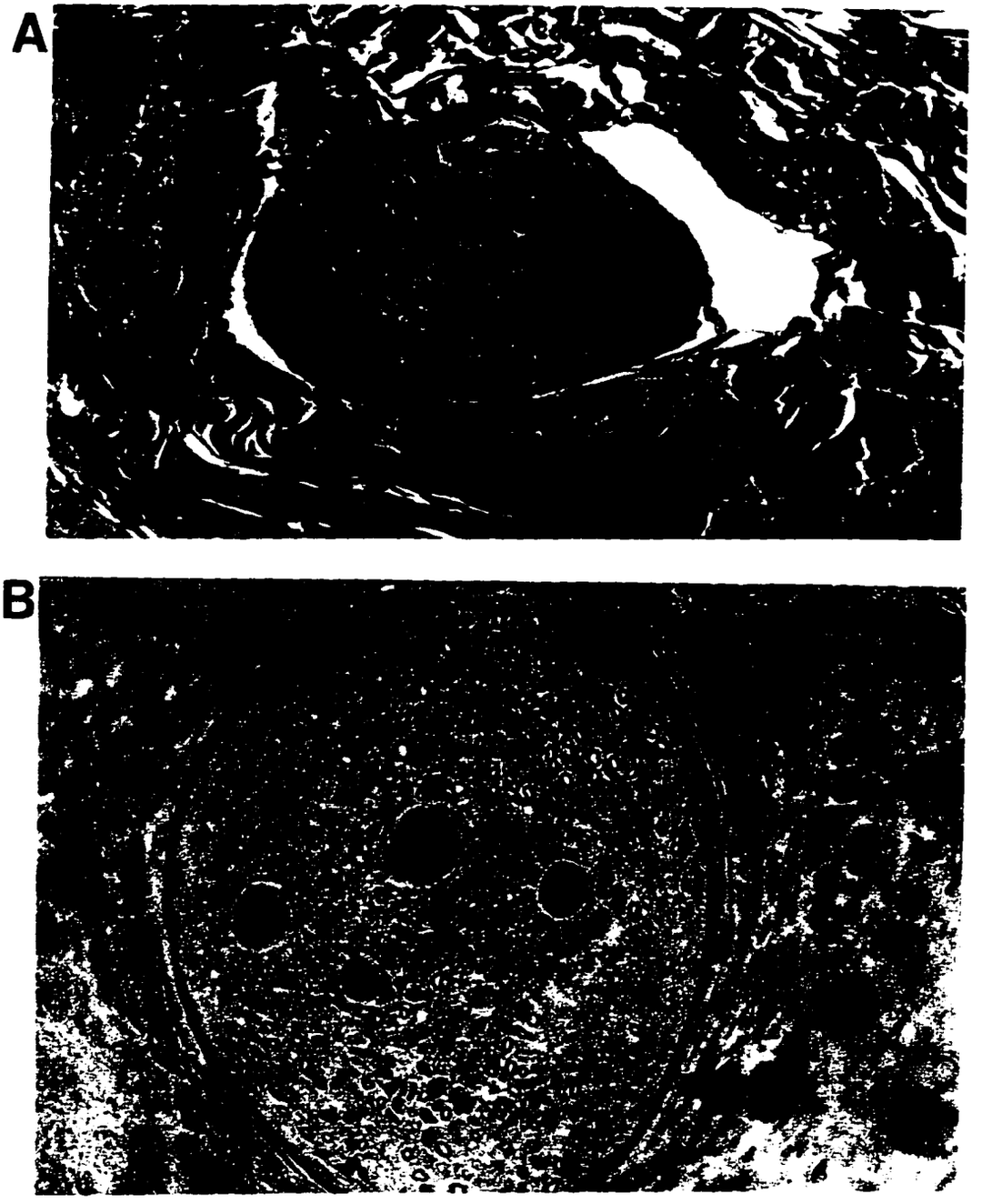


Fig. 41. Micrograph of a transverse 1 μm thick section from a distal temporal (DT) CFNG specimen stained with 1 % toluidine blue (A. Vehicle-treated , B. IGF-I-treated).

C. Quantitative Analysis

The average number of axons found in each of the IGF-I-treated CFNG specimens was as follows: 193 $\pm$ 9 (PT), 158 $\pm$ 8 (PG), 138 $\pm$ 9 (DG), and 115 $\pm$ 9 (DT). In addition, the average number of axons found in each of the vehicle-treated CFNG specimens was accordingly: 178 $\pm$ 5 (PT), 128 $\pm$ 23 (PG), 103 $\pm$ 11 (DG), and 67 $\pm$ 2 (DT).

Assessment of the mean number of axons between the vehicle and the IGF-I rats using paired t-test comparisons revealed that the IGF-I-treated animals showed a significantly greater number of axons versus vehicle-treated rats ($p < 0.001$; Fig. 42). Further analysis showed that the IGF-I treated rats contained significantly greater number of axons than vehicle treated rats in the proximal graft (PG; $p<0.01$), distal graft (DG; $p<0.01$) and the distal temporal (DT; $p<0.001$) specimens, but not in the proximal temporal (PT; $p>0.05$). More specifically, as illustrated in Fig. 42, IGF-I treated animals had 60% (115.1 versus 192.6 for PT) the number of axons reaching the distal temporal segment of the CFNG as opposed to 38% (67.3 versus 178 for PT) for the vehicle treated animals. Thus, the IGF-I-treated animals showed a 22% increase in the number of axons reaching the distal temporal segment compared to vehicle treated.

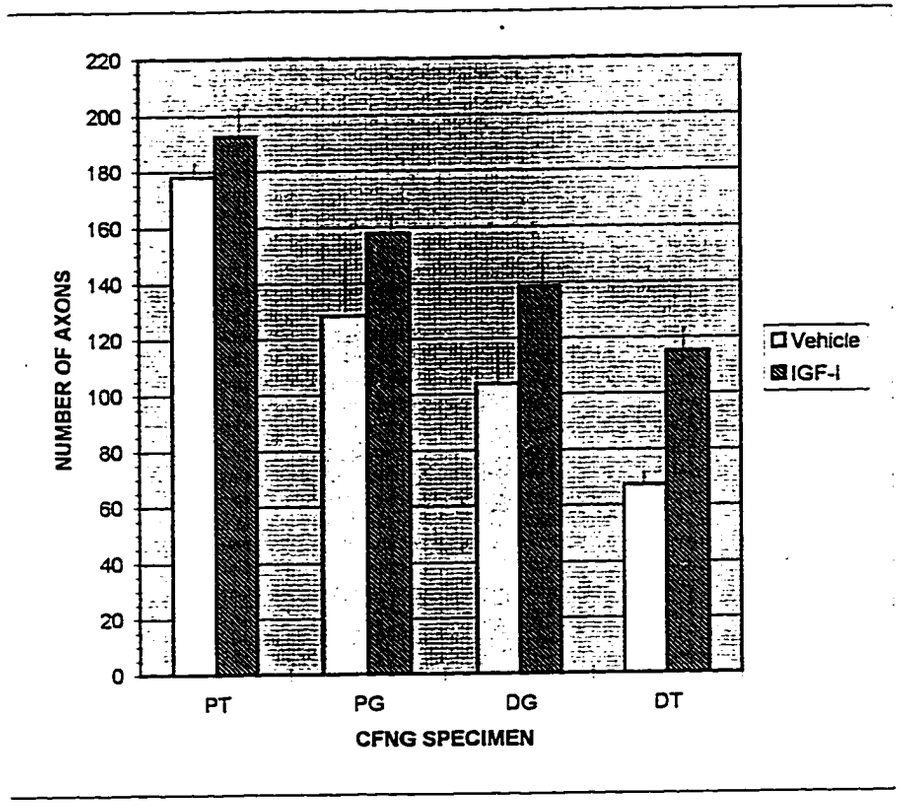


Fig. 42. Mean number of axons in each CFNG specimen versus treatment (PT = proximal temporal - donor, PG = proximal graft, DG = distal graft, DT = distal temporal, Vehicle-treated = CFNG + Vehicle, IGF-I-treated = CFNG + IGF-I).

The average nerve fiber diameter (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 5.32 $\pm$ 0.47 (PT), 4.94 $\pm$ 0.36 (PG), 4.56 $\pm$ 0.16 (DG), and 4.54 $\pm$ 0.20 (DT). In addition, the average number of axons found in each of the vehicle-treated CFNG specimens was accordingly: 4.21 $\pm$ 0.32 (PT), 4.32 $\pm$ 0.29 (PG), 4.15 $\pm$ 0.31 (DG), and 3.43 $\pm$ 0.09 (DT).

Assessment of the mean nerve fiber diameter in the CFNG between the vehicle and the IGF-I rats using paired t-test comparisons revealed that the IGF-I treated animals showed a significantly larger nerve fiber diameter versus vehicle treated rats ($p < 0.001$; Fig. 43). Further analysis showed that the IGF-I treated rats showed significantly larger nerve fiber diameter than vehicle treated rats in the proximal temporal (PT; $p<0.05$), distal graft (DG; $p<0.05$) and the distal temporal (DT; $p<0.001$) specimens but not in the proximal graft (PG; $p>0.05$).

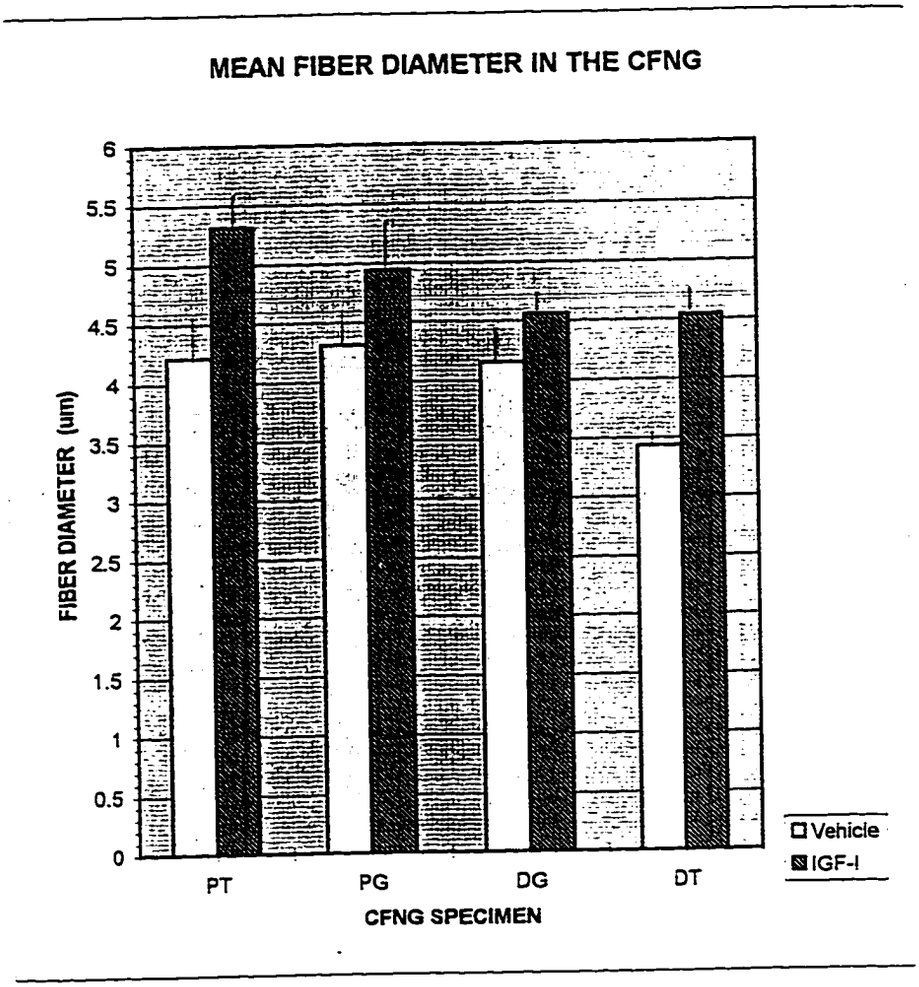


Fig. 43. Mean nerve fiber diameter in each CFNG specimen versus treatment (PT = proximal temporal - donor, PG = proximal graft, DG = distal graft, DT = distal temporal. Vehicle-treated = CFNG + Vehicle, IGF-I-treated = CFNG + IGF-I).

The average axonal diameter (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 3.09 $\pm$ 0.12 (PT), 2.99 $\pm$ 0.07 (PG), 2.90 $\pm$ 0.06 (DG), and 2.75 $\pm$ 0.14 (DT). In addition, the average number of axons found in each of the vehicle-treated CFNG specimens was accordingly: 2.54 $\pm$ 0.20 (PT), 2.83 $\pm$ 0.18 (PG), 2.76 $\pm$ 0.31 (DG), and 2.77 $\pm$ 0.26 (DT).

Assessment of the mean axonal diameter in the CFNG between the vehicle and the IGF-I rats using paired t-test comparisons revealed that the IGF-I treated animals showed a significantly larger axonal diameter versus vehicle treated rats ($p < 0.001$; Fig. 44). Further analysis showed that the IGF-I treated rats showed significantly larger axonal diameter than vehicle treated rats in the proximal temporal (PT; $p < 0.01$), and distal graft (DG; $p < 0.05$) specimens, but not in the proximal graft (PG; $p > 0.05$) and distal temporal (DT; $p > 0.05$).

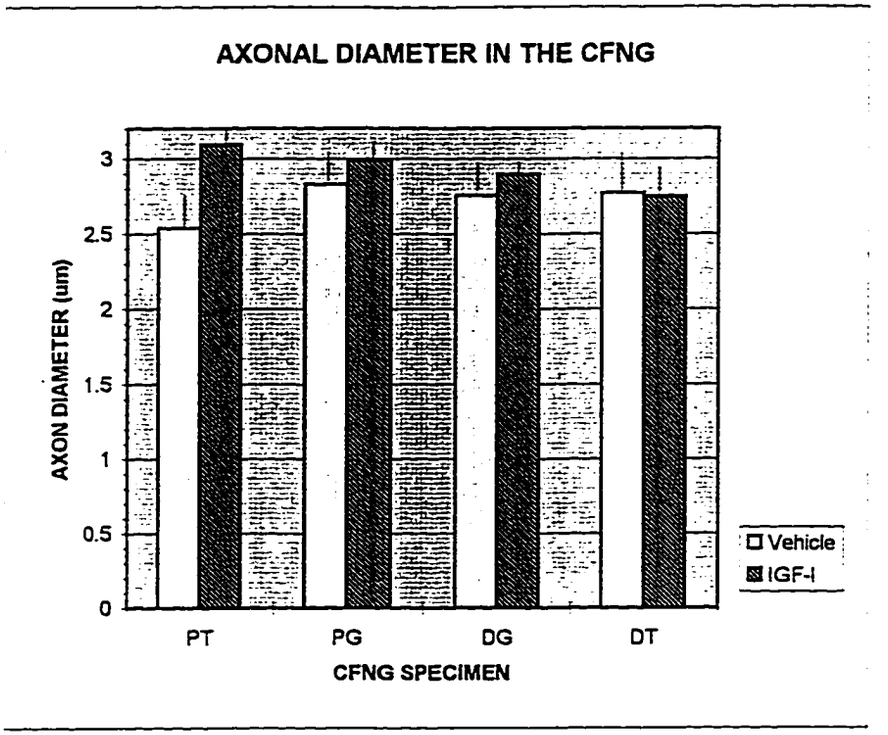


Fig. 44. Mean axonal diameter in each CFNG specimen versus treatment (PT = proximal temporal - donor, PG = proximal graft, DG = distal graft, DT = distal temporal. Vehicle-treated = CFNG + Vehicle, IGF-I-treated = CFNG + IGF-I).

The average myelin thickness (μm) found in each of the IGF-I-treated CFNG specimens was as follows: 0.96 ± 0.17 (PT), 0.88 ± 0.10 (PG), 0.86 ± 0.10 (DG), and 0.79 ± 0.11 (DT). In addition, the average number of axons found in each of the vehicle-treated CFNG specimens was accordingly: 0.82 ± 0.16 (PT), 0.77 ± 0.12 (PG), 0.78 ± 0.21 (DG), and 0.58 ± 0.09 (DT).

Assessment of the mean myelin thickness in the CFNG between the vehicle and the IGF-I rats using paired t-test comparisons revealed that the IGF-I treated animals showed a significantly larger myelin thickness versus vehicle treated rats ($p < 0.05$; Fig. 45). Further analysis however, failed to show any significant specimen specific myelin thickness differences ($p > 0.05$) between the IGF-I treated rats and the vehicle treated rats in the proximal temporal (PT), the proximal graft (PG), the distal graft (DG) and the distal temporal (DT) specimens.

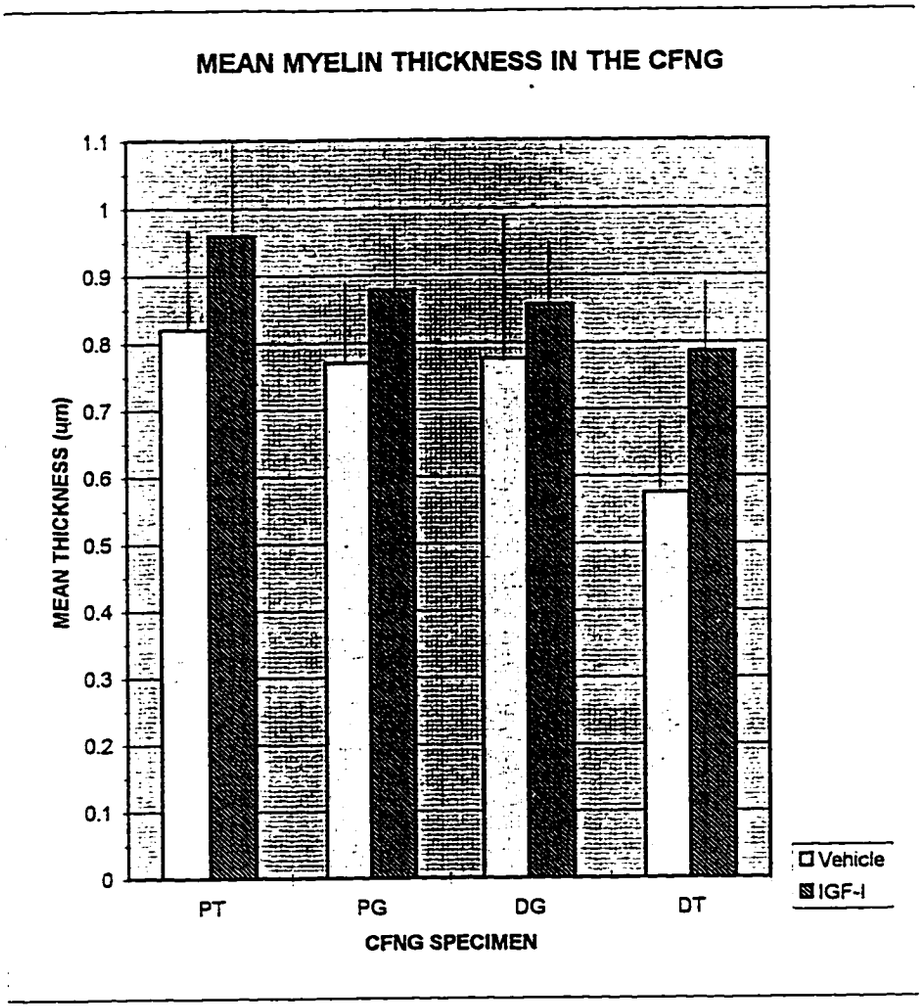


Fig. 45. Mean myelin thickness in each CFNG specimen versus treatment (PT = proximal temporal - donor, PG = proximal graft, DG = distal graft, DT = distal temporal. Vehicle-treated = CFNG + Vehicle, IGF-I-treated = CFNG + IGF-I.

CHAPTER IV

DISCUSSION

Experiments 1,2 and 3

In the present studies, it was demonstrated that the FMNs that project to the OOM were topographically located in a dorsal subnucleus (DSN) of the facial nucleus. It was interesting to note that the FMNs innervating the OOM via the temporal branch were also distributed throughout the same topographical location and showed no additional topographical localization within the DSN. Furthermore, the DSN topographical localization data of the FMNs corresponding to the OOM was in accord with previous research which reported the periorbital musculature to be innervated by FMNs in a dorsal segment of the facial nucleus (Watson et al., 1982). This qualitative data described the normal rat facial nucleus and the corresponding FMN pool of the OOM.

Several previous retrograde studies looking at the snout musculature (vibrissal area) also reported a topographic organization in both the rat and cat facial nucleus (Papez, 1927; Martin and Lodge, 1977; Watson et al., 1982; Hinrichsen and Watson, 1984; Nishimura et al., 1992; Chen and Bisby, 1993; Anders et al., 1993). In addition, it is interesting to point out that the sensory surface of the rat face has also been shown to be topographically organized (Erzurumlu and Killackey, 1979). In this study it was demonstrated that the sensory surface of the rat face was topographically organized within the trigeminal nucleus.

The morphology of the OOM FMNs was also evaluated with biotin dextran histochemistry. Biotin dextran-labeling revealed that these neurons had a multipolar stellate shape and a prolific dendritic arborization. A similar morphology was observed in the VII-T FMNs.

Fluoro Gold fluorescent retrograde labeling of the OOM produced intense marking of the FMNs that serve it. Fluoro Gold was found to have an intense fluorescent labeling of the dendritic processes, axons and the cytoplasm of the soma, but negatively staining the nucleus. However, the intensity of the FG-labeling bleached over into the nucleus. Combining FG labeling of the OOM FMNs with bisBenzimide labeling of the temporal branch of the facial nerve revealed double labeled neurons. Specifically, these neurons showed a FG-labeled white cytoplasm, and dendrites and a light blue bisBenzimide-labeled nucleus and cytoplasm. Thus a double-labeled FMN would have as a characteristic white dendrite labeling and a white with shades of light blue cytoplasm and nucleus. One of the characteristics of the bisBenzimide is that it is very transient and tends to leak out and stain surrounding glial cells if left longer than 26 hours post labeling of the facial nucleus. This leaking was also described in previous reports (Schmued and Fallon, 1986; Horner and Kummel, 1993; Girman, 1994). Despite some minor spread of label, the double labeled neurons were identifiable and corresponded to FMNs innervating the OOM via the temporal branch. However, these double labeled neurons showed no particular topographical organization within the DSN of the facial nucleus.

The quantitative analysis of the FMN pool using image analysis was also performed. In the present experiments neuronal counts were made by adding the counts from each

consecutive 50 μm section. No double counting correction formula was applied to these counts because the mean diameter of the FMNs was significantly smaller in size (28-32 μm) than the thickness of each section. This was in contrast to previous reports on the total number of FMNs counts that used 10 μm (Fernandez et al., 1995; Martin et al., 1977), and 15-30 μm (Watson et al., 1982; Streppel et al, 1997) sections without correction for double counting errors and thus resulting in higher total FMN counts.

Quantitative results demonstrated that a significant percentage of the entire FMN pool served the OOM (29%). In addition, it was demonstrated that of the entire FMN pool, 19% served the VII-T. Thus in summary, the majority (approximately 2/3) of the total innervation to the OOM occurred by way of the temporal branch of the facial nerve. By deduction, it was inferred that the remaining 1/3 of the OOM innervation occurred by way of the zygomatic branch. This finding is critical in establishing that the best donor branch that can be used in treatment strategies utilizing the rat model of facial paralysis and CFNG, is the temporal branch. This neuroanatomical evidence on the choice of the temporal branch for placement of the cross-facial nerve graft in this model complements the previous data (surgical availability within the same operating field and strength of OOM response to intraoperative nerve stimulation).

These findings are critical for future experimental studies on the CFNG and in general treatment strategies for facial paralysis. In this respect the significance of the temporal branch input to the OOM was delineated. Furthermore these data provided the quantitative and topographic identification of the specific FMNs that are involved in normal eye sphincter function in the rat. This will allow us to examine and compare reinnervation

strategies with respect to the FMN profile, and serve as a tool in developing and assessing microsurgical and neurotrophic models of facial paralysis treatment.

Today, the CFNG procedure is extensively utilized in humans with FP. As a matter of fact, it is the only procedure available that provides the anatomical and physiological substrate for emotional facial expression and voluntary animation (Thanos and Terzis, 1996a). The present morphometric analysis of the FMN pool of the rat OOM provided further insight into the neuronal dynamics of using the temporal branch as the donor in CFNG procedures for the restoration of the blink in our model. Furthermore, these data allow the future study of the long-term effects of the CFNG on the OOM FMN pool, and thus, help describe the phenomenon of bilateral reinnervation of OOMs originally each innervated by unilateral motoneurons. In addition, these data can help study the effects of a CFNG combined with specific neurotrophic factors on the FMN pool of the OOM.

Experiment 4

The muscles that are responsible for facial expression and eye movements are among the most structurally and functionally diverse skeletal muscles. The eye-muscle fibers extend through the entire length of the muscle, and are smaller in diameter than most skeletal muscles. In addition, the ocular muscles, along with the other facial muscles controlling fine movement, have the smallest number (< 10) of muscle fibers per motor

unit (Bourne, 1973), while the gross motor muscles of the limbs have a far greater number of muscle fibers per motor unit (as high as 2000).

Previous studies have demonstrated that the OOM receives nerve input from the temporal and zygomatic branches of the facial nerve (Warfel, 1985). Unfortunately, less is known concerning the OOM pattern of motor endplate innervation. Kakulas and Adams (1985) described three patterns of skeletal muscle innervation: 1) The motor endplates are scattered along the length of the muscle. 2) The endplates are predominantly located at the ends of the muscle. 3) The motor endplates are confined to the equator of the muscle. In the present study, it was demonstrated that the motor endplates of the OOM were confined to the lateral segments of the muscle, similar to Kakulas and Adams (1985) group 2. This was consistent with a lateral entry of the motor nerve fibers into the rat OOM and this pattern of innervation was congruous to the clinical situation (Terzis, 1984).

Apart from the motor endplate profile of the normal OOM, this experiment also demonstrated that unilateral facial nerve axotomy did not eliminate the motor endplates from the ipsilateral OOM, even though function of the OOM was absent. This suggests that the OOM may receive some minor input from another nerve (i.e. III, IV or VI).

Reinnervation of the OOM with a CFNG retained the pattern of motor endplate innervation (topographical localization of endplates was maintained) indicating strong trophic influences not lost during the long denervation-reinnervation period. Also, animals treated with a CFNG showed significantly greater number of motor endplates in the OOM, when compared to the denervated group. Although the CFNG-treated animals showed a significant increase in their OOM endplate profile, they were still significantly below

normal. Although some moderate recovery of function was achieved with the CFNG, it was not a full recovery of function. Clearly, the minimum number of endplates required to restore complete function is higher than that achieved by the CFNG treatment. Perhaps the introduction of a specific neurotrophic factor or a cocktail of several growth factors to the nerve graft would enhance the motor endplate profile of the reinnervated OOM and achieve full recovery of function.

Porter and colleagues (1989) examined the muscle typing of the primate OOM. This study reported that the primate OOM consisted of three distinct muscle fiber types. These were designated as slow-twitch, intermediate fast-twitch and pale fast-twitch. The slow-twitch fiber type exhibited skeletal muscle fiber type I fiber staining patterns, were smaller in diameter, and comprised about 10 percent of the total fiber count. In contrast, the intermediate fast twitch and pale fast twitch fibers exhibited skeletal muscle type II fiber staining patterns, were larger in diameter and comprised 90 percent of the monkey OOM fibers. This predominance of the two fast twitch fiber types in the OOM is consistent with the on/off function of the muscle in blinking.

The present study demonstrated that the utilization of a cross-facial nerve graft linking the paralyzed OOM to a contralateral normal temporal branch was able to generate a significant (50 percent) increase in the number of endplates in reinnervated rats, compared to denervated animals. This was achieved by the technique of sharing motor axons microsurgically without sacrificing a functioning cranial nerve (for a donor) and its muscles, as was the case in some previous studies (Delbeke and Thauvoy, 1982; Willer et al., 1992). In addition, it was demonstrated that the motor endplates were distributed similarly

to the normal distribution three months post reinnervation with a CFNG. Although there was a clearly significant increase in the number of OOM motor endplates when treated with a CFNG; the number of endplates remained significantly less than normal and this warranted further research onto the possible role that neurotrophic factors may play at restoring the number of endplates back to normal.

This study described the motor endplate profile of the reinnervated OOM in an experimental model of facial paralysis. Specifically these data provided critical information on motor endplate quantification in the normal and denervated states, as well as in a paralyzed OOM reinnervated with cross-facial nerve grafts. These findings thus assist in the future development and assessment of possible reinnervation techniques of the paralyzed OOM and possibly lead to a full recovery.

Furthermore, it is well known that a significant number of facial paralysis cases involve patients seeking treatment two or more years post injury, thus presenting a largely atrophic OOM. In such cases, a nerve graft alone would be ineffective choice of treatment. Future research in our laboratory will be evaluating the provision of new autologous muscle to substitute for an atrophic OOM. These studies will examine the quantitative and qualitative characteristics of a foreign target functioning as a new eye sphincter in animals with long-lasting paralysis.

Experiment 5

Quantitative assessment of the number of axons within the entire CFNG revealed a

gradual decrease in the number of axons from proximal to distal segments. There was also a significant decrease in the number of axons in the distal temporal (DT) branch specimens with approximately ¼ of the number of axons reaching the distal segments of the CFNG compared to the proximal segments. This was in contrast to the findings of Frey et al. (1991), who reported that, regardless of function, the same number of regenerated, thin, nerve fibers were found in the distal segments of a CFNG at time of clinical muscle transplantation.

When examining the CFNG specimens for their mean axon diameter, the PT segment revealed a superior axon diameter. Specifically, the PT mean axon diameter was greater than all of the three more distal nerve segments (PG, DG, DT) at 6 months following axotomy and 3 months post reinnervation with a CFNG. These results were in agreement with previous work on CFNG's (Beuche and Friede, 1985). Furthermore, the present results on the temporal branch (PT) of the VIIth nerve correspond very closely with the axon diameters reported on the facial nerve by Martin et al., (1977) and on the occulomotor nerve by Fraher (1989). However no difference was observed when comparing PT with a normal SN specimen. This indicated that, with respect to nerve fiber diameter, the saphenous nerve and the temporal branch were compatible, and thus that the SN was a suitable nerve graft.

Functionally, the myelin sheath serves as an insulator controlling leakage of current; its area would have to increase accordingly with axon diameter and nerve fiber diameter in general. The present results indicated that myelin thickness was greatest at the donor site (PT) and significantly smaller at the more distal locations (PG, DG, DT). In addition, no

statistically significant difference in myelin thickness was observed between the PT and the SN, and this provided further support for the suitability of the saphenous nerve as a graft to the facial nerve.

Collectively, the above results demonstrated a positive correlation between myelin thickness and nerve fiber diameter. Although conduction velocity is affected by myelin thickness, axon diameter and internode length, Brill et al. (1977) reported that internode length was of secondary importance. More specifically, the effect of internode length was shown to become manifest only for extremely short internodes. In general, axon diameter and fiber diameter is thought to be the most important parameter of conduction velocity (Arbuthnott et al., 1980; Beuche and Friede, 1985; 1986).

Previous research has suggested that regenerated myelinated nerve fibers have thinner sheaths than normal (Beuche and Friede, 1985; 1986; Schroder, 1972). Moreover, a comparatively thin sheath is widely accepted as a criterion for identifying a regenerating fiber. In contrast to the PT data, a state of "hypoplasia" of the myelin sheaths in the distal segments (PG, DG, DT) of the CFNG may be indicative of insufficient or unsuccessful regeneration. This may have been caused by difficulties associated with carrying out a perfect microsurgical coaptation, due to the tiny size of the VIIth nerve branches in this model. Furthermore, an impaired regenerative capacity of the Schwann cell for myelin formation, or an ineffective communication between the axon and the Schwann cell, may be involved (Hidalgo and Weller, 1992).

A critical element in studying nerve graft procedures is the morphometric analyses of the graft. Results illustrated a shift towards smaller axon diameter, and myelin thickness.

This pattern was in agreement with previous clinical and experimental CFNG studies (Frey et al., 1991).

Finally, the present results were compared to the OOM motor endplate data in the same animals (experiment 4). In particular, the relationship was studied between the number of endplates in the target (OOM) and the number of axons found in the distal temporal branch specimens of the CFNG. The comparison indicated a positive correlation. This suggests that recovery of eye sphincter function bears a linear relationship to the number of motor endplates and the number of axons successfully reaching the distal end of the CFNG. Therefore these findings suggest that future attempts at reanimating the paralyzed eye sphincter should emphasize CFNG continuity, with the provision of a well-vascularized and trophic environment.

Currently, the CFNG procedure is extensively utilized in humans with facial paralysis. It is the best procedure currently available that provides anatomic and physiologic substrates for emotional facial expression and voluntary animation. Estimates indicate thousands of cases per year of facial paralysis in the US alone could benefit from the use of CFNG techniques (Yanagihara, 1988). Although the CFNG may be the best current treatment for FP, full recovery of function is incomplete. In our experimental model, the number of axons reaching the OOM via the CFNG were significantly decreased compared to the proximal CFNG.

In conclusion, the present histomorphometric analysis of the CFNG in the rat model provides further insight into the neural dynamics for guiding microsurgical restoration of the

blink. More important, these data can be correlated with clinical results and can facilitate the development of methods better capable of restoring eye sphincter function. Of course, a problem in reestablishing facial symmetry with CFNG techniques is the possibility of an atrophic OOM. Current research in the present laboratory is addressing this problem by combining a CFNG in our model with provision of a new homologous muscle target.

Experiment 6

In order to gain further insight into the possible role of the neurotrophic factor IGF-I in reinnervating a denervated OOM with a CFNG, unilateral facial paralysis was similarly induced in rats and subsequently treated with a CFNG and local administration of IGF-I. Reinnervation was measured behaviorally (blink index) and histomorphometrically (axonal profile of the CFNG). Blink tests were performed on each rat prior to surgery and then at least once a week after surgery.

Behavioral results from this study showed that FP animals that were treated with a combination of a CFNG and IGF-I showed a significantly greater degree of function (blink index) two months after treatment than animals that received CFNG and vehicle. In addition, when examining the blink index over time this difference began to be significant as early as 37 days after treatment. These data were able to provide insight on function and reinnervation of the OOM target over time, as opposed to a difference at the endpoint of the experiment. Therefore, these data provided greater information on the rate of motor

regeneration with and without IGF-I in the CFNG. Thus far, the only behavioral data on IGF-I is on sensory fiber regeneration (mouse sciatic crush) by way of a pinch test (Kanje et al., 1989; Sjoberg and Kanje, 1989; Ishii and Lupien, 1995; Glazner et al., 1993). However, some of these studies had technical problems (previously discussed) with their IGF-I delivery mechanism (silicone chamber), and failed to adequately address the confounding effects of ischemia and pressure injury in their model.

Histomorphometric evaluation of the CFNG specimens, revealed that IGF-I treated CFNG specimens contained an overall greater abundance of fibroblasts, collagen and endothelial and extrafascicular blood vessels. This is in agreement with several reports on IGF-I and its effects on the cardiovascular system, nerve and muscle. Specifically, it has been widely reported that IGF-I plays a significant metabolic role in increasing glucose utilization, maintaining plasma glucose, and regulating protein and fat metabolism (Binoux, 1995; Frysburg, 1994; Lewitt, 1994) as well as critical for both growth and development of both somatic and sympathetic nervous tissue (Zackenfeis et al., 1995; Stewart and Rothwein, 1996). More recently, Duanmu et al. (1997) demonstrated that IGF-I is involved in modulating cardiovascular dynamics. The mechanism with which IGF-I exerts its effects on cardiovascular dynamics is by decreasing mean arterial pressure. IGF-I administration enhanced blood flow to the kidneys (Pete et al., 1996; Hu et al., 1996) and increased skeletal muscle blood flow (Hu et al., 1996).

Unlike most peptides IGFs circulate in association with specific IGFBP's (six have been thus far identified) which are thought to modulate the biological effect of IGFs on many tissues including muscle and nerve (Hickson et al., 1997). In addition, Hickson et al.

(1997) demonstrated that IGF-I infusion was capable of preventing glucocorticoid-induced muscle atrophy as does glutamine. IGF-I has also been shown to be involved in reduction of proteolysis in skeletal muscles (DiGiuli et al., 1997; Zdanowicz et al., 1995; Clemmons and Underwood, 1992; Clemmons et al., 1992; Umpleby et al., 1994). Zdanowicz et al. (1997) recently reported in a muscular dystrophy hamster model that IGF-I significantly decreased overall and myofibrillar protein degradation with minimal effects on protein synthesis. In addition, this study showed that IGF-I administration increased the size of muscle fibers and normalized their fiber distribution. Together, the above effects of IGF-I on increasing blood flow and possible angiogenic properties in the injured nerve, fulfills one of the cardinal criteria of a successful nerve graft which is to provide adequate vascularization to the nerve graft (Terzis, 1987; Terzis and Smith, 1990).

Histomorphometric analysis of the number of axons within the entire CFNG revealed an overall larger number of axons present in the IGF-I treated (CFNG + IGF-I) animals compared to the vehicle (CFNG + vehicle) treated animals. This significant difference in number of axons was found to be greatest in the distal temporal (DT) specimens where IGF-I-treated animals showed a 22% increase in number of axons, compared to vehicle-treated animals. Thus, IGF-I may have played a role in facilitating more axons in entering the nerve graft conduit and thus more of them making it to the distal segment of the graft and the target.

Similarly, when the nerve fiber diameter was examined, it was found that IGF-treated CFNG specimens contained significantly larger diameter nerve fibers than controls. Subsequently, in order to further isolate where in the nerve fiber, the IGF-I had its effects,

axon diameter and myelin thickness were examined. Results demonstrated that the IGF-I treated CFNG specimens had both a greater mean axonal diameter and myelin thickness than controls. However, the difference in axonal diameter was far greater than the myelin thickness as evident from their α levels of significance. In addition, the present axon diameter results on the temporal branch (PT) of the VIIth nerve corresponded very closely with the axon diameters reported on the facial nerve by Martin et al., (1977) and on the occulomotor nerve by Fraher (1989).

One explanation may be that the specimens were from a two month graft and that this was inadequate amount of time for the myelin maturation of these fibers and hence the less pronounced differences in myelin thickness. Recently, several studies have elucidated that IGF-I may play a role in myelination and Schwann cell proliferation. Feldman et al., (1997) studied the role of IGF-I in cultured DRG neurons. They reported that IGF-I promoted differentiation of Schwann cells and their migration to axons. In addition, this study reported that IGF-I promoted myelination independent of its effect on neuronal and Schwann cell survival. Cheng et al. (1997) described that IGF-I may enhance Schwann cell axonal contact by increasing cell-cell attachment and specifically increase Schwann cell expression of E-cadherin and other adhesion molecules which then leads to long term myelination.

These data have described the IGF-I treated nerve grafts to contain a significantly greater mean axonal diameter. Certainly the outgrowth rate of the IGF-I treated nerve grafts was similar to the slower phases of axoplasmic transport (<2 mm/day) and thus cytoskeletal proteins of microtubules and neurofilaments. Wang et al (1992) demonstrated

that neurite outgrowth induced by IGF-I activate and elevate the mRNA levels of the cytoskeletal 68 kDa and 170 kDa neurofilament and microtubule α and β - tubulin mRNA's. Neurite growth during regeneration requires assembly of the cytoskeleton and IGF-I affects the expression of genes which encode prominent structural proteins of axons. Neurofilaments are fibrillar structures of the axonal cytoskeleton that are assembled from 68, 170 and 200 kDa neurofilament proteins (Lazarides, 1980; Nixon and Logvinenko, 1986; Steinert and Parry, 1985).

Facial nerve axotomy initiates a plethora of events including: a) the expression of growth factors and their receptors (Kreutzberg, 1993; 1994; Raivich and Kreutzberg, 1993). b) microglial proliferation and activation and synthesis of various cytokines (Gehrmann and Kreutzberg, 1994) and c) astrocyte hypertrophy synthesis of glial fibrillary acidic protein (GFAP) mRNA and protein (Tetzlaff et al., 1988), followed by a surrounding of the regenerating facial motor neurons with GFAP-positive processes (Graeber et al., 1988). Recently it was reported that these GFAP-positive astrocytes produce IGF-I during toxin-induced demyelination (Komoly et al., 1992). Another study reported that facial nerve axotomy induced the gene expression of IGF-I and its receptors (Gehrmann et al., 1994) as well as the expression of other genes such as CGRP, c-jun and GAP-43 mRNA's (Mohri et al. 1997). Thus, a distant axonal injury is sufficient to induce this IGF-I astrocytic response. This process is associated with the proliferation of microglia. Their production of cytokines could have a role in the induction of this astrocytic IGF-I response (Graeber et al., 1988; Kiefer et al., 1993; Streit et al, 1988).

In addition, nerve injury causes invading macrophages to produce IGF-I and

Schwann cells express the IGF-I receptor, which facilitates axonal regeneration (Cheng et al., 1996). This regeneration can be further augmented by the ability of Schwann cells to secrete IGFBP's which in turn may increase local IGF-I bioavailability. However, it has been shown that Schwann cells are not the sole source of polypeptide growth factors in the PNS and trophic factors may also be produced by other cell types present in transected nerves including macrophages, endothelial cells, and fibroblasts (Blexrud et al., 1990). Cheng et al. (1996) found that macrophages which infiltrate injured nerves and remove tissue debris, serve as another source of IGF-I and also promote Schwann cell proliferation.

The present data on the role of IGF-I in motor regeneration through a graft is encouraging. Clearly further studies are warranted for the involvement and use of IGF-I with the CFNG in a treatment strategy for facial paralysis. It is also most probably not the full picture as far as the neurotrophic milieu critical to motor nerve regeneration. Instead, the combination of good microsurgical technique in the coaptation of a graft plus a 'cocktail' of several neurotrophic factors and their synergistic effects would provide the quintessential treatment approach.

Future studies will examine the effects of IGF-I on the OOM by way of motor endplate analysis (as previously described). as well as the ultrastructure of these IGF-I treated CFNG specimens using electron microscopy. Furthermore, it will be interesting to examine the facial nuclei of these animals for any possible evidence of IGF-I -induced astrocytic hypertrophy.

The severe functional, aesthetic, psychological and communication deficits associated with injury-induced facial paralysis in addition to the mounting constrains on our

health care system have been widely discussed. Apart from the role of neurotrophic factors and nerve graft continuity gene therapy techniques provide a new perspective of treatment. Gene therapy techniques, such as the use of a replication - deficient retrovirus carrying a desired specific gene, are useful tools for providing substances like neurotrophic factors. Gene therapies locally deliver genes important for nerve regeneration to nerve and muscle without tissue degeneration (Rabinovsky et al., 1995; Heberbrand et al., 1995). Presently, these techniques are becoming quite popular- for example, the intrastriatal implantation of fibroblasts (Leviver et al., 1995) or astrocytes (Yoshimoto et al., 1995) genetically engineered to produce BDNF and prevent degeneration of dopaminergic neurons in Parkinson's disease. These studies suggest that gene therapy with BDNF can ameliorate parkinsonian symptoms. Similarly, fibroblasts genetically modified to release CNTF have been shown to be a potential delivery system in treating ALS (Sagot et al., 1995). With the advancement of genetic engineering, gene therapy will find its proper place in the area of post-traumatic neurorrhaphy.

Gene manipulation and cloning are also existing technologies. A new discipline of tissue engineering is emerging, in which the principles of engineering and the life sciences are applied for the generation of biologic substitutes which are aimed at the creation, preservation or restoration of lost organ function. The problems of nerve grafting deficiency in the future may be alleviated by the genetic cloning of nerve fibers. With the aid of neurotrophins, it may soon be possible to approach nerve injury de novo via genetic engineering, with complete regeneration. Without the inherited problems associated with conventional nerve repair, perfect restoration of function may therefore be achieved.

Yesterday's science fiction can be today's reality. As we move toward a new millennium, we can believe that the amalgamation of modern technology, telecommunication systems, modern engineering, and medicinal breakthroughs will result in an explosion of knowledge that will enhance our understanding of developmental biology and will culminate in a new era in medicine and biomedical science, enabling us to restore lost tissue function. It has become more apparent that improved clinical results are likely to be realized from a greater understanding of the neurobiology of nerve repair. Intimate collaboration between modern technology and biomedical sciences is vital in the advancement toward this goal. The endpoint is limited only by the extent of our imaginations.

CHAPTER V

Summary

In summary the current experiments were able to:

1) Establish and characterize the neuroanatomy of the FMN pool that innervates the rat OOM, as approximately 29% of the total number of facial motor neurons. In addition, a distinct topographical organization of these neurons was described in the DSN of the facial nucleus.

2) Determine the FMN pool that serves the temporal branch of the facial nerve (which is used in the rat model of facial paralysis) as approximately 19% of the total number of FMNs. Again, topographical organization of these neurons was organized within the DSN.

3) Determine that the temporal branch of the facial nerve provides the majority of the innervation of the rat OOM (approximately 2/3). No particular subdivision within the DSN was noted for the neurons serving the OOM via the temporal branch.

4) Provide the motor endplate profile of the normal and denervated OOM. In addition, the effect on the number of endplates of treating the denervated OOM with a CFNG was significant (almost a 50% increase compared to the denervated). However, the number of endplates in this group was still significantly below the normal number of OOM endplates.

5) The blink response was partially restored three months post treatment with a CFNG.

6) Provide the axonal profile of the CFNG from its proximal to its distal coaptation.

A significant drop in the number of axons making it to the target was noted, along with a decrease in axon diameter and myelin thickness.

7) Insulin-like growth factor-I locally administered to the CFNG was found to enhance the onset of recovery of function as tested by the blink test, as early as 37 days post treatment and the degree of function. Furthermore, IGF-I treated animals showed a 22% increase in the number of axons reaching the distal end of the graft when compared to the vehicle treated animals. Similarly IGF-I treated animals showed an enhanced axon diameter and myelin profile in the distal segments of the graft.

Abbreviations

AChE	acetyl cholinesterase
ALC	acetyl-L-carnitine
ALS	amyotrophic lateral sclerosis
B	buccal branch
BB	bisBenzimide
bd	biotin dextran
BDNF	brain-derived neurotrophic factor
BI	blink index
C	cervical branch
CDF	choline acetyltransferase development factor
CFNG	cross-facial nerve graft
CGRP	calcitonin gene related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DG	distal nerve graft
DL	double labeled neurons
DRG	dorsal root ganglion
DSN	dorsal subnucleus
DT	distal temporal
EGF	epidermal growth factor
EP	endplates

FG	fluoro gold
FGF	fibroblast growth factor
FP	facial paralysis
FMN	facial motor neuron
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HSV	herpes simplex virus
id	internal diameter
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
kDa	kilo Dalton
LIF	leukemia inhibiting factor
M	mandibular branch
mRNA	messenger ribonucleic acid
MW	molecular weight
NGF	nerve growth factor
NIH	National Institute of Health
NT	neurotrophin
OO	orbicularis oculi muscle fiber
OOM	orbicularis oculi muscle
PG	proximal nerve graft

PT	proximal temporal
RT	room temperature
SN	saphenous nerve
T	temporal branch
TGF	transforming growth factor
VII-T	temporal branch of the facial nerve
UV	ultraviolet
Z	zygomatic branch

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