The effects of neurotrophic factors in facial nerve repair

Gerard Kelly

Submitted for the Degree of Doctor of Medicine, University of Edinburgh,

September 2001

Contents

Abstract

Transection of the facial nerve results in morbidity for the patient, which is both physical and psychological. Neurotrophic factors have been shown to support motor neurones *in vitro* and some studies have shown a benefit to nerve regeneration *in vivo*. The present work established a model for the electrophysiological assessment of the rat facial nerve and used the rat and the sheep as animal models for facial nerve repair. In the rat facial nerve, after transection and repair of the buccal division, there was an increase in the number of regenerated axons with 10 μg of both CNTF and BDNF administered exogenously near the site of nerve repair, over a period of 14 days ($p=0.003$). There was no increase in the number of fibres with similar administration of GDNF and NT 4/5. The administration of these neurotrophic factors failed to show any other improvement in electrophysiological or morphometric measurements in the rat nerve. The advantage of increasing the number of fibres after repair is dubious. In the sheep facial nerve three groups of animals were studied. Group 1 underwent nerve division and repair, group 2 underwent nerve division, repair and entubulation with a biodegradable glass tube and group 3 underwent nerve division and repair with administration of neurotrophic factors. In group 3 exogenous administration of 100 μg of CNTF and 50 μg of both BDNF and GDNF, over a period of 28 days, failed to show any improvement in electrophysiological or morphometric measurements. Nerve entubulation also failed to show any improvement in morphometric measurements but did show an increase in minimum conduction velocity. The reason for this was not clear and may be a spurious result. This study provides evidence for the use of CNTF and BDNF to increase the number of fibres after facial nerve repair on the rat. The functional advantage of this is uncertain. At present, the use of these neurotrophic factors cannot be supported in peripheral motor nerve repair.

Declaration

I, Gerard Kelly declare that this thesis has been composed totally by myself and that the work reported here is my own. Any technical assistance obtained has been acknowledged.

Acknowledgements

This research was supported by a grant from the Sir Jules Thorn Charitable Trust and without this funding this work would not have taken place. I would, above all, like to thank Mr M.A. Glasby for his help throughout every step of this project, from its planning to its completion, and for the laboratory facilities first in the Department of Anatomy, the University of Edinburgh and later in the Department of Clinical Neurosciences, the Western General Hospital, Edinburgh. I would also like to thank Ms G. Valler for her help in the operating theatre, Ms G. Valler and Mr R. Shields for their skilled guidance in the laboratory and Dr D. Lenihan for his help with electrophysiological testing.

I would also like to acknowledge Professor A.G.D. Maran and Dr A.I.G. Kerr for allowing me the opportunity to carry out this work during my time as a specialist registrar in Edinburgh.

Chapter 1

The facial nerve

Introduction

The facial nerve, the seventh cranial nerve, controls the muscles of facial expression. It is this nerve that gives us the ability, at least in part, to blink, smile, talk, sing, eat and play a variety of musical instruments. Loss of function of this nerve, in the human subject, results in considerable morbidity and a diminished quality of life. Morbidity is most often related to the inability to close the eye and this can result in keratitis and corneal ulceration. Speech can be impaired and the failure of the sphincter mechanism of the mouth results in loss of food and especially fluid while eating and drinking. Perhaps the most dramatic impact of facial paralysis, however, is the psychological effect on the patient. Sufferers are self-conscious and this can result in cases of isolation and fear of interaction with others. The cosmetic deformity of a complete facial paralysis is evident to everyone, even on cursory inspection. The accurate repair of a physical disruption to the nerve with no or minimal residual deficit is the goal of surgery when spontaneous resolution will not occur.

Anatomy and pathology of the facial nerve

Situated in the lower third of the pons, the facial motor nucleus contains approximately 7000 neurones. Fibres from the facial nucleus must loop around the adbucens nucleus (forming the

facial colliculus) before emerging from the brain stem. The nerve has a motor and a sensory root (the nervus intermedius). The two roots emerge from the caudal border of the pons, lateral to the recess between the inferior olive and inferior cerebellar peduncle, at the cerebellopontine angle. The motor root lies medial, and the vestibulocochlear nerve lies lateral, to the nervus intermedius. The two roots of the facial nerve and the vestibulocochlear nerve pass anterolaterally to the internal acoustic meatus. At the lateral end of the meatus the nerve enters the facial canal (Fallopian canal), lying above the vestibule; it then turns at the geniculum to produce the genicular ganglion and travels posteriorly through a bony canal on the medial wall of the middle ear. Here the facial nerve is unique. No other nerve in the body has such a long course (28-30 mm) in a bony canal. In the middle ear the facial canal lies behind the processus cochleariformis and tensor tympani tendon. The nerve travels horizontally above the oval window and the stapes. The facial nerve then turns downwards forming the second genu, the start of the mastoid segment of the nerve. It then runs vertically down the anterior wall of the mastoid process to emerge from the stylomastoid foramen. Near the foramen three branches arise. First, the posterior auricular branch, which ascends in front of the mastoid process, giving auricular and occipital branches to supply auricular muscles and the occipital belly of occipitofrontalis respectively. Second, the digastric branch which supplies the posterior belly of the digastric muscle and third, the stylohyoid branch which supplies the stylohyoid muscle. The main trunk of the facial nerve emerges from the stylomastoid foramen to run forwards in the parotid gland, crossing the styloid process, retromandibular vein and external carotid artery. Within the substance of the parotid gland the nerve divides into its terminal branches (temporal, zygomatic, buccal, mandibular and cervical). The temporal branch crosses the zygomatic arch supplying intrinsic muscles on the lateral surface of the auricle and the anterior and superior auricular muscles. The more anterior branches supply the frontal belly of occipitofrontalis, orbicularis oculi and

corrugator. The zygomatic branches cross the zygomatic bone to the lateral canthus, supplying orbicularis oculi. The buccal branch passes horizontally to a distribution below the orbit and around the mouth. This branch supplies procerus, the zygomaticus muscles, levator labii superioris, levator anguli oris, levator labii superioris alaeque nasi, buccinator and orbicularis oris. The mandibular branch runs forwards below the angle of the mandible, turning again above the mandible to supply the risorius and muscles of the lower lip and chin. The cervical branch runs anteroinferiorly to the front of the neck, under platysma, which it supplies (Gray's Anatomy 1989). Damage to the infra-nuclear facial nerve can happen anywhere alone its course.

The intracranial portion of the facial nerve can be affected by cerebellopontine angle tumours. The majority of these are vestibular schwannomas although other tumours include menigiomas, facial nerve schwannomas and congenital cholesteatomas. Any such pathology can cause facial paralysis although a more likely cause of facial paralysis is the surgical treatment of these tumours. The temporal portion of the facial nerve is at risk in fractures, osteitis and malignant tumours of the temporal bone. In advanced squamous carcinoma of the middle ear, subtotal petrosectomy may offer potential cure but will usually involve sacrifice of the facial nerve (Schaitkin & May 1997). Iatrogenous injury to the nerve can arise from mastoid and middle ear surgery, and this represents the greatest risk to the trunk of the facial nerve, potentially resulting in a permanent and total palsy. Chronic otitis media (mucosal and squamosal) can be responsible for paralysis of the nerve as can glomus jugulare and glomus tympanicum tumours. The extratemporal facial nerve is at risk in parotid surgery and from malignant parotid, oropharyngeal and nasopharyngeal tumours. Without the protection of the skull, the extratemporal facial nerve is vulnerable to penetrating trauma of the face. In infants, the mastoid process is undeveloped and the facial nerve lacks its protection, lying more

superficial and is thus more prone to injury by obstetric forceps or a mastoid exploration incision. The mandibular division of the facial nerve is at risk in anterior neck surgery such as submandibular gland excision for sialectasis or tumour. Thus, with many of the these disorders, or their treatment, paresis of the muscles supplied by the facial nerve is a potential outcome. Surgical repair offers, in many cases, the only possible hope for recovery. Yet even with the advances in microsurgery of the twentieth century, the results of nerve coaptation and grafting are disappointing. With meticulous technique of the best that microsurgery has to offer, recovery may be disappointing or even negligible. It is unlikely that further advancements in surgical technique offer real hope for the damaged peripheral nervous system. Much effort has thus been concentrated on 'neurotrophic factors' or nerve rescue agents in the hope that these may raise the plateau on which current surgical practice rests with respect to nerve coaptation.

Assessment of facial nerve function

The most widely known standard for assessment of facial nerve function and recovery is the House-Brackmann grading system (House & Brackmann 1985). Some journals require the use of this system when reporting results. The system is based on a six-point scale with I being normal and VI being total, flaccid paralysis (table 1.1). This system involves making measurements of the movement of the eyebrow and corner of the mouth and comparing the results with those on the unaffected side, which assists the categorization of the patient into one of the six grades.

Grade	Description	Characteristics
I	Normal	Normal function in all areas
II	Mild dysfunction	Gross: slight weakness noticeable on close inspection; may have very slight synkinesis At rest: normal symmetry and tone Motion Forehead: moderate to good function Eye: complete closure with minimum effort Mouth: slight asymmetry
III	Moderate dysfunction	Gross: obvious but not disfiguring difference between two sides; noticeable but not severe synkinesis, contracture, and / or hemifacial spasm At rest: normal symmetry and tone Motion Forehead: slight to moderate movement Eye: complete closure with effort Mouth: slight weakness with maximum effort
IV	Moderately severe dysfunction	Gross: obvious weakness and / or disfiguring asymmetry At rest: normal symmetry and tone Motion Forehead: none Eye: incomplete closure Mouth: asymmetric with maximum effort
V	Severe dysfunction	Gross: only barely perceptible motion At rest: asymmetry Motion Forehead: none Eye: incomplete closure Mouth: slight movement
VI	Total paralysis	No movement

Table 1.1 House-Brackmann facial nerve grading system.

The above is an invaluable grading system which has helped the consistent reporting of a patient's facial nerve function over time and helped to standardise reporting of facial nerve function between patients. This grading system however, still relies on subjectivity on the part of the physician. Electrophysiological assessment of the facial nerve is a common practice, indeed the seventh cranial nerve is the most common single nerve assessed by electrophysiology. This is because Bell's palsy is the most common mono-neuropathy, and where electrophysiological assessment is an accurate prognostic indicator of recovery.

There are several electrophysiological ways to assess nerve function. The ideal

electrophysiological test does not exist, and clinically, several tests have been used to assess the function of the facial nerve:

1. testing of nerve excitability
2. nerve latency
3. electromyography
4. compound muscle action potential
5. maximum stimulation test
6. blink reflex

Nerve excitability testing (NET) uses electrical stimulation of the facial nerve at the stylomastoid foramen. The NET measures the level of current required to evoke a minimally visible muscle contraction on one side of the face, comparing this to the opposite side. A clinically significant side-to-side difference varies between 2.0 and 3.5 mA or more (Alford 1967; Campbell *et al.* 1962; Grodon & Friedberg 1978), and is an accurate indicator of poor recovery in Bell's palsy.

Facial nerve latency testing uses similar electrical stimulation of the nerve at the stylomastoid foramen to NET. Instead of being observed, the muscle response is recorded electrically and the latency is measured from stimulus to the initial deflection of the motor response. A normal latency is 4 ms (Dumitru *et al* 1988). A normal latency would be expected even if only a small number of fast conducting axons remained. In contrast, if fast conducting axons were preferentially affected, a prolongation of the latency would be expected. Poor correlation between normal latencies and recovery has limited the usefulness of this test as a prognosticator of facial nerve recovery in acute palsies (Blumenthal & May 1986; Esslen

1977). It has, however, been useful in chronic diseases such as Charcot-Marie-Tooth, Guillain-Barré syndrome and other peripheral neuropathies (Johnson & Waylonis 1964; Kimura 1972; Mitz *et al.* 1980).

Electromyography (EMG) involves the use of a needle electrode inserted into the muscles of the face with the resulting electrical potentials displayed on an oscilloscope. Idiopathic facial paralysis usually results in a decreased number of voluntary motor units which is directly related to the number of nerve fibres involved in neurapraxia or axonal loss. After approximately 10 to 14 days, facial muscles deprived of their innervation develop unstable resting membrane potentials and begin to depolarise spontaneously. This is seen as positive sharp waves and fibrillation potentials. When reinnervation begins, small polyphasic action potentials may be seen. In recovery, these polyphasic potentials progress to become larger and more prolonged, eventually resulting in a voluntary activation pattern with the number of remaining fibrillation potentials reducing with time. Where recovery does not occur, fibrillation potentials remain. The maintenance of voluntary motor units 72 hours after the onset of idiopathic facial paralysis has been correlated with a good prognosis (Granger 1967; Granger 1976). The use of EMG in predicting recovery of idiopathic facial palsy has been criticised however. A few remaining motor units may suggest a favourable outcome when the majority of the nerve has been destroyed. Furthermore, a few severed axons may yield a large number of fibrillation potentials implying substantial denervation. May found that 75% of patients with no voluntary motor units in the affected facial muscles had an incomplete recovery at 6 months and that 38% of patients with no voluntary motor units in the affected facial muscles had an incomplete recovery at 6 months (May *et al* 1983). He concluded that EMG was an unreliable prognostic tool in idiopathic facial palsy. EMG remains, however, the only objective way to document reinnervation, especially in the early stages of recovery.

Compound muscle action potential recording of the facial nerve was popularised by Fisch for prognostic purposes in Bell's palsy (Fisch 1981), although he referred to it as electroneuronography (ENoG). This involves simulating the main trunk of the nerve at the stylomastoid foramen and recording from an active surface electrode lateral to the nasal ala and a reference surface electrode 18 mm inferior and lateral to the active electrode. With stimulation, a compound muscle action potential can be recorded. Each side of the face is stimulated and the peak-to-peak amplitudes are compared for both right and left sides. Fisch recommended that in Bell's palsy, if 90% degeneration had occurred within two weeks of onset of the paralysis (that is the evoked amplitude on the affected side was less than 10% of normal), surgical decompression of the nerve should be carried out (Fisch1981). These criteria have however been questioned (May *et al.* 1986) and in the United Kingdom, surgical decompression of the facial nerve in viral infections, is not generally practised. Comparing the recorded amplitude and area of the compound muscle action potential have not yielded a significant difference regarding the accuracy of predicting a functional outcome in idiopathic facial palsy (Thomander & Stålberg 1981). This study also evaluated surface and needle recording electrodes. Needle recording electrodes varied considerably and surface electrodes were deemed more appropriate for recording purposes.

Maximum stimulation testing of the facial nerve was popularised by May *et al* (May *et al.* 1971). This is similar to the compound action potential recording except that the muscle response is observed and not recorded electrically. This therefore does not require electrophysiology recorders or oscilloscopes and can be carried out using a hand held stimulator. This technique has been shown to be a good predictor of outcome in Bell's palsy.

The blink reflex is a contraction of the orbicularis oculi muscle resulting from electrical

activation of the supraorbital nerve. It consists of two temporally distinct responses, the first, an ipsilateral and the second, a bilateral response. The exact autonomic route is unknown. Kimura *et al* used this procedure in 81 patients with Bell's palsy (Kimura *et al* 1976). In two months all patients with an intact first, ipsilateral reflex recovered function whereas none of the patients with an absent first, ipsilateral reflex recovered function. The study did not continue after two months and the long term outcome of the patients with an absent first, ipsilateral reflex is unknown.

All of the above electrophysiological tests which rely on stimulation of the facial nerve at or distal to the stylomastoid foramen are only valid several days after the onset of Bell's palsy. Stimulation of a nerve, distal to the site of injury will result in a normal response until Wallerian degeneration (if it is going to occur) takes place. EMG does not rely on stimulation of the facial nerve and may give an earlier indication of outcome than tests that do rely on stimulation of the facial nerve directly. The blink reflex can also assess conduction across the intracranial portion of the facial nerve.

It must be noted that the majority of work studying these electrophysiological tests relates to idiopathic facial paralysis and is not necessarily applicable to the assessment of a surgically repaired facial nerve.

Nerve degeneration

When a peripheral nerve is divided (neurotmesis), Wallerian degeneration takes place distal to the site of injury and the proximal nerve undergoes degeneration at least to the nearest node of Ranvier, although greater degeneration can happen with more significant injury. The proximal end of the severed nerve swells and there is proliferation of schwann cells and fibroblasts. The distal segment of the nerve loses its connection with the cell body and cannot maintain its structural and functional integrity. Initially, the axon swells and myelin disintegrates into droplets, being phagocytised by schwann cells and macrophages. Wallerian degeneration is characterised initially by a granular disintegration of axoplasmic microtubules and neurofilaments, due to proteolysis. The collagen content of the distal segment is increased at the basement membrane of schwann cells. With collagen deposition in, and shrinkage of, the endoneurial tubes, their size becomes considerably reduced. Rapid mitosis in schwann cells results in their proliferation and formation of schwann cell columns (bands of Büngner). These columns are important potential regenerative pathways for new axons and contain the glycoprotein, laminin, on their basal laminae which promotes neurite growth. A critical phase in nerve regeneration after repair is axonal sprouting across the site of injury. Initially, many more axons will develop than previously existed in the healthy nerve. Many of these sprouts will fail to make peripheral connections and die. Axons that do establish peripheral connections will mature and enlarge, perhaps as a result of trophic substances from these targets. Thus the number of axons distal to a nerve repair may not vary proportionally with functional success of that repair, and indeed may indicate the opposite situation, with multiple branching due to scar tissue. Neurones are destined to be either myelinated or unmyelinated. If originally myelinated, axons will develop myelin, and similarly unmyelinated fibres will remain as such, even when regenerating into a graft which was originally myelinated. Rates of

growth vary after repair of neurotmeses. In the rat, a nerve will regenerate at approximately 3 mm per day (Forman *et al* 1979). In the human, repair occurs at approximately 1 - 2 mm per day (Seddon 1975).

Classification of nerve injury

In the early 1940's, Seddon produced a classification of nerve injuries (Seddon 1943) which has stood the test of time and is still used today.

Neurapraxia refers to a local conduction block in which the continuity of the axons, the excitability of the nerve distal to the lesion, and of the muscle are preserved. Such a conduction block is supposed to correspond to an acute local demyelinating block as demonstrated after compression injury (Denny-Brown & Brenner 1944). This conduction block persists until local myelin repair processes restore local excitability of the nerve fibre and conduction is restored over the damaged segment. This may take weeks or months to occur.

The next and more severe degree of nerve injury described by Seddon is axonotmesis, implying a loss of continuity of axons although the endoneurial tubes are still intact. Wallerian degeneration takes place. Recovery of function is good however as axons regenerate through intact endoneurial tubes and specificity for target organs is preserved. This injury corresponds to an advanced compression or traction injury.

Seddon's final classification of nerve injury is neurotmesis which he described as 'the nerve that has either been completely severed or is so disorganised by scar tissue that spontaneous

regeneration is out of the question' (Seddon 1975). This includes loss of continuity of some or all of the remaining elements of the nerve trunk, including endoneurial tubes, perineurium and epineurium. Surgical repair is required to improve chances of reinnervation.

In 1951, Sunderland introduced a further and more detailed classification of nerve injury (Sunderland 1951). Sunderland types I and II correspond to Seddon groups, neurapraxia and axonotmesis respectively, and neurotmesis corresponds to Sunderland type V. Sunderland types III and IV lie somewhere between axonotmesis and neurotmesis and cannot be classified under Seddon's system. A Sunderland type III injury implies loss of continuity of axons and endoneurial tubes while the perineurium is still intact. The continuity and orientation of endoneurial pathways are lost. The fibre disorganisation may be complicated by intrafascicular bleeding, oedema and ischaemia and this lesion might easily lead to the development of intrafascicular fibrosis. Sunderland type IV implies, in addition, loss of continuity of the perineurium with preservation of the epineurium, while Sunderland type V implies loss of continuity of the entire nerve trunk. The Seddon and Sunderland types of nerve injury are summarised in table 1.2.

Seddon classification	Sunderland classification	Pathology
Neurapraxia	I	Local myelin damage with no Wallerian degeneration
Axonotmesis	II	Wallerian degeneration with endoneurial tubes intact
	III	Loss of axons and endoneurial tubes Perineurium intact
	IV	Loss of axons, endoneurial tubes and perineurium Epineurium intact
Neurotmesis	V	Transection of the entire nerve trunk

Table 1.2 classification of nerve injury

Nerve repair

The first case of peripheral nerve repair is attributed to Ferrara in 1608 although it was not until the second world war that most of the pioneering work on this subject was recognised (Young & Medawar 1940) and notably in the middle of this century by Seddon (Medical Research Council 1954). The operating microscope transformed nerve repair into the technical skill of microsurgery and with it, evidence from experimental studies arose as to which surgical techniques gave the best results. The 'gold standard' of nerve repair is direct, tension-free suture of the proximal and distal nerve stumps. The classic method of suture is the epineurial suture, although in order, more correctly, to achieve orientation of nerve fasicles, 'fasicular repair' offers a theoretical advantage. The technique of fasicular repair inevitably requires resection of the epineurium and perineurial suturing which may lead to further trauma and could thus compromise healing. Direct suture of nerve stumps is not always possible. Damaged or lacerated nerve should be resected until healthy nerve can be defined and sutured. Again, tension should be avoided as should excessive mobilisation of nerve which can result in ischaemia. Thus autologous nerve grafting is an important technique in nerve repair and remains the ideal where there is insufficient length to suture proximal and distal nerve stumps directly. Nerve grafting, however, necessitates the sacrifice of a sensory nerve (often sural or greater auricular). Where the donor nerve is of insufficient diameter to support the injured nerve, 'cable' nerve grafting, where several strands of the nerve graft are arranged in parallel to make up the required cross section was devised (Kilvington 1908). A cable graft involves an epineurial repair, where as an interfasicular graft involves several donor nerve strands bridging a gap, and sutured in a fasicular repair. Recently the use of freeze-thawed muscle grafts have been evaluated in a large animal model (Glasby *et al.* 1995; Lawson & Glasby 1995) with good results over short distances.

Injury to the facial nerve in mastoid and middle ear surgery is uncommon, however mastoidectomy with or without tympanoplasty is the most common procedure resulting in iatrogenous facial nerve injury (Green *et al* 1995). Facial nerve injury is a devastating blow to both patient and otological surgeon. Injuries occur even in the hands of the most experienced surgeons. Contributing factors may be an aberrant facial nerve, granulation tissue, cholesteatoma, and revision surgery where anatomical landmarks may no longer exist. Most experts agree that immediate facial paralysis after otological surgery, which fails to recover in a few hours, should be surgically explored (Glasscock *et al.* 1979). The House Ear Clinic in California published a study of 22 patients undergoing surgery for a iatrogenous facial nerve injury (Green *et al* 1994), all but one patient having been a tertiary referral. All patients had complete paralysis (House-Brackmann grade VI). The most common location for injury to the nerve was in the lower tympanic segment (55% of patients). Four patients had multiple injured segments of the nerve and one patient had an injury extending from the stylomastoid foramen to near the geniculate ganglion. Ten patients underwent facial nerve grafting and three patients had a direct repair. Repair was either by fine monofilament suture or juxtaposition of the nerve ends within the Fallopian canal. The final result of nerve grafting (n=9) was a House-Brackmann grade of IV in 78% of patients (22% achieved grade III). With primary repair, all patients (n=3) achieved a House-Brackmann grade of III.

Neurotrophic factors

The term 'nerve growth-promoting substance' was introduced in 1954 to describe a substance isolated from mouse sarcomata 180 and 37 which enhanced growth and differentiation of sensory and sympathetic neurones (Cohen *et al* 1954). Shortly thereafter more potent sources of this factor were discovered in snake venom and in mouse salivary glands (Cohen & Levi-

Montalcini 1956; Levi-Montalcini & Cohen 1956). This factor, identified as a protein became known as 'nerve growth factor' or 'NGF' (Cohen *et al* 1954). In 1986, Levi-Montalcini and Cohen shared the Nobel Prize for physiology or medicine for the discovery. Since then many other neurotrophic factors have been identified with profound effects on the vertebrate nervous system. Brain-derived neurotrophic factor (BDNF) was identified in 1982 (Barde *et al* 1982) and ciliary neurotrophic factor (CNTF) two years later (Barbin *et al.* 1984). The key discovery that BDNF was structurally related to NGF led to the discovery of NT 3 and NT 4/5. This family of molecules is now termed the 'neurotrophins' (Hohn *et al.* 1990). Other previously identified growth factors were found to regulate neuronal survival *in vitro* and therefore can be considered neurotrophic. These include fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF). Another neurotrophic factor is glial-derived neurotrophic factor (GDNF). Despite the name 'growth factor' there is little evidence that these agents act as more than survival-promoting factors. The definition of trophic factors excludes membrane-bound molecules that affect adhesion and survival of neurones as well as non-protein compounds such as thyroid hormones, steroids and gangliosides. The definition of a neurotrophic factor as discussed further in this text will be 'a soluble protein regulating survival, growth, morphological plasticity, or synthesis of proteins for differentiated functions of neurones' (Loughlin & Fallon 1993). The experimental evidence for the effects of neurotrophic factors comes from various sources. Much work has concentrated on *in vitro* studies of cell cultures. Other work exists using embryonic and neonatal mammals as experimental models for neurotrophic action. Evidence can also be obtained from genetically modifying animals to be deficient in the gene responsible for a neurotrophic factor's production and thereafter assessing development. Most relevant to the human is experimental adult animal work.

Since its discovery in 1954, most experimental work on neurotrophism has involved NGF. The molecular structure of NGF has been determined with its amino acid sequence. The activity of NGF was initially established for neural crest-derived sympathetic and sensory neurones and the growth-cone of such a transected nerve on media enriched with NGF shows massive proliferation (figure 1.1)

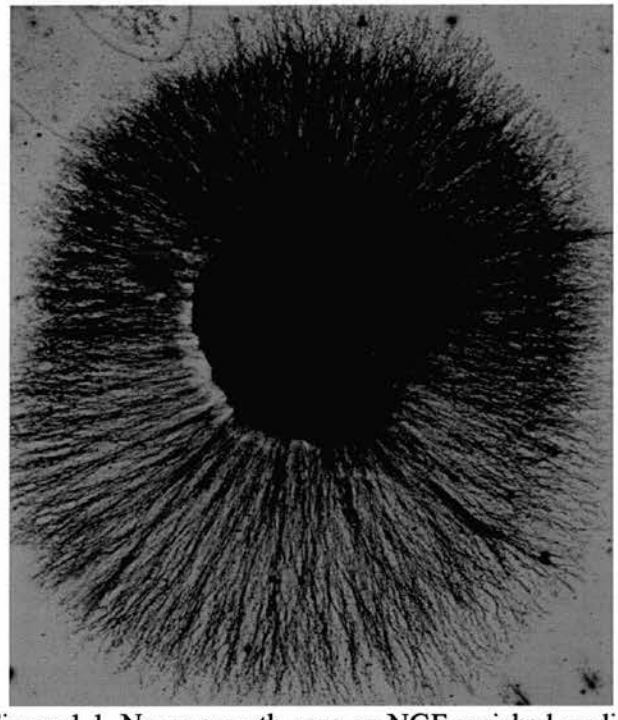


Figure 1.1. Nerve growth cone on NGF enriched media.

NGF supports neurite growth, but neither survival of ventral spinal cord neurones *in vitro* (Longo *et al* 1982; Wayne & Heaton 1990) nor *in vivo* (Oppenheim *et al.* 1988). Hughes *et al* showed that NGF failed to support cultured embryonic rat motor neurones above control levels, even at high concentrations (100ng ml^{-1}) (Hughes *et al* 1993). *In vivo*, after nerve transection in neonatal rats, NGF administration causes a greater loss of motor neurones, but an attenuated loss of sensory neurones (Miyata *et al.* 1997). NGF can also spare the death of adult sensory neurones (Rich *et al.* 1987).

BDNF shares about 50% similarity to NGF at the amino acid level in various mammalian species (Thoenen 1991). These amino acids are not randomly distributed, but are concentrated in conserved domains that include six cysteine residues, which form disulphide bridges which determine the molecule's three-dimensional shape. Despite the similarities between NGF and BDNF, these molecules show different neuronal specificities. BDNF is found in higher concentrations in the adult brain. *In vitro*, BDNF promotes the survival of embryonic retinal ganglion cells (Barde 1989) and mesencephalic dopaminergic neurones (Hyman *et al.* 1990), which are not supported by NGF. *In vitro* and *in vivo*, BDNF supports the survival of embryonic sensory neurones (Lindsay *et al* 1985). Neurotmesis-induced motor neurone death in neonatal mice is almost completely prevented by local application of BDNF, which occurs with NGF but to a lesser extent (Li *et al.* 1994) - 5 μg of growth factor applied to the proximal nerve stump. In neonatal rats, neurotmesis-induced facial motoneurone death can be partially prevented by administration of 5 μg of BDNF, whereas no effect was seen with NGF in a similar concentration (Sendtner *et al.* 1992). In adult mammals, transection of the hypoglossal nerve results in a dramatic loss of choline acetyltransferase (ChAT) in the hypoglossal motor nucleus. This injury-induced reduction in ChAT is prevented when 50 μl solution of BDNF is applied to the proximal end of the transected nerve (Chiu *et al* 1994). When CNTF is similarly applied, ChAT levels are not maintained. In mice genetically altered to lack the BDNF gene, no loss of motor neurones was found compared to a reduction in the number of sensory neurones (Liu *et al.* 1995). This suggested that BDNF was not essential for motor neurone development and survival although it does not rule out its role in other aspects of motor neurone function.

NT 3 was identified in 1990 by polymeraze chain reaction techniques (Hohn *et al* 1990) and shows strong similarities to NGF and BDNF. In contrast to NGF and BDNF however, NT 3

is not predominantly expressed in the central nervous system but in peripheral tissues such as skeletal muscle, liver and gut (Thoenen 1991). In the CNS, NT 3 is found mainly in the hippocampus and cerebellum (Maisonpierre *et al.* 1990). In contrast to BDNF it is found in highest concentrations during development. It can support embryonic sensory neurones *in vitro* (Lindsay *et al* 1985) and *in vivo* (Hofer & Barde 1988). NT 3 can also rescue virtually all neurotmesis-induced motor neurone cell death in the neonatal mouse, with effects similar to BDNF (Li *et al* 1994). In a similar study Sendtner *et al*, showed NT 3 could prevent the death of new-born rat facial nerve neurones although these effects were substantially less than the effects of BDNF (5 μg of NT 3 and BDNF was used) (Sendtner *et al* 1992).

NT 4/5 is another member of the neurotrophin family of molecules. NT 4/5 has been shown, like BDNF, to prevent injury-induced cell death in neonatal rats by its local application to the proximal nerve stump after neurotmesis, with 20μg (Koliatsos *et al.* 1994) and 10μg (Hughes *et al* 1993) applied to the nerve stump. Where the NT 4 gene was genetically deleted in mice, the facial motor nucleus and sympathetic neurones of the superior cervical ganglion were unaffected. In contrast there was a loss of sensory neurones, similar to results of genetically depleted BDNF mice (Liu *et al* 1995), again suggesting that NT 4 is required during the development for the survival of some peripheral sensory neurones but not sympathetic or motor neurones.

Ciliary neurotrophic factor was first identified and partially purified from embryonic chick eye tissues. Later, it was shown to be present in large amounts in sciatic nerves of adult rats and rabbits. This finding led to its final purification and cloning. Human CNTF shows 84% homology with rat CNTF and has no similarities to the neurotrophin family of molecules. CNTF is produced in the nerve and does not originate from retrograde transport. The target

organ is therefore not a source of CNTF in motor neurones innervating skeletal muscle. In new-born rats CNTF mRNA cannot be detected and high levels are not reached until the fourth postnatal week. This lack of CNTF mRNA parallels the differentiation of schwann cells in the nerve. CNTF is not secreted by the classical pathways of the endoplasmic reticulum and Golgi complex, but is present in high quantities within the cytoplasm of myelinating schwann cells and astrocytes (Sendtner *et al* 1994). It has been suggested that this would allow the molecule to act after cell damage (Barde 1994) although it may simply be that its secretion is facilitated by an, as yet, unknown mechanism. The regulation of CNTF in the peripheral and central nervous systems is very different. After a central lesion, CNTF mRNA is rapidly up-regulated and levels are maximal at three days after injury and remain elevated for at least three weeks. The CNTF seems to be produced by astrocytes around the wound cavity. Conversely, in the peripheral nervous system, CNTF mRNA levels drop rapidly to less than 5% within the first week after a nerve lesion. These levels slowly recover, with the first signs of CNTF mRNA being detectable by *in situ* hybridization 1 week after the lesion. Four weeks after the lesion there is then a significant rise in CNTF mRNA. CNTF has a broad spectrum of *in vitro* effects. These include the promotion of survival of ciliary, sympathetic, sensory, nodose, trigeminal and spinal motor embryonic chick neurones. Hughes *et al*, failed to show any significant survival benefits above the control group in cultured rat motor neurones (with concentrations of 100 ng ml^{-1}) (Hughes *et al* 1993). *In vivo* CNTF increases the number of spinal motoneurones in E5 and E9 (embryonic day 5 and 9) chicks, but does not alter the number of sympathetic, sensory and nodose neurones. Furthermore, Sendtner *et al* showed that more than 80% of new-born rat facial motor neurones die one week after neurotmesis (Sendtner *et al* 1990). When CNTF was administered (5 μg) locally to the transected facial nerve, death of the motor neurones in the brain stem was prevented. Newman *et al* applied CNTF (600 μg over 4 weeks) to repaired rat sciatic nerves in an osmotic pump

and used muscle mass and sciatic function index to assess recovery (Newman *et al.* 1996). They suggested a significant improvement with the CNTF treated group compared to the control group. This suggests that CNTF may be a 'lesion factor' for damaged motor neurones. In contrast to the apparent efficacy of CNTF, Winter *et al* reported results of a study where motor neurone degeneration mutant mice, which harboured a transgene resulting in the overproduction of CNTF, showed an increase in signs of motor neurone degeneration instead of any beneficial effects (Winter *et al.* 1996).

GDNF is a member of the transforming growth factor-ß superfamily of molecules and was initially identified as a neurotrophic factor for substantia nigra dopaminergic neurones (Lin *et al.* 1993). It is retrogradely transported by spinal cord motor neurones, but not sympathetic neurones, in neonatal rats and local application (approximately 20 μg) of GDNF to the transected facial nerve prevented neurotmesis-mediated cell death in the neonatal period. In adult rats locally administered GDNF was found to attenuate the lesion-induced decrease of ChAT in the facial nucleus (Yan *et al* 1995).

Bothwell first observed the response of neurite outgrowth to insulin-like growth factor (IGF) (Bothwell 1982). Low concentrations of IGF have been shown to enhance the survival of cultured embryonic sensory and sympathetic cells (Recio-Pinto *et al* 1986). *In vivo* experiments have shown IGF-1 infusion increased regeneration of sensory and motor axons in divided sciatic nerves (Kanje *et al.* 1989; Near *et al.* 1992). *In situ* hybridization has shown that IGF mRNA is up-regulated in the distal, but not the proximal nerve (Pu & Ishii 1993). It is possible that this creates an IGF concentration gradient greatest at the distal nerve segment and thus helps to stimulate and guide nerve regeneration. As well as neurotrophic factors acting on nerves, IGF has a role as a mitogen and a growth factor on target tissues and thus

differs from a classical neurotrophic factors such as NGF. This dual role of IGF may function in development to co-ordinate growth between the size of the nervous system and the size of the end organ which is to be innervated (Ishii *et al* 1997).

Less work has been concentrated on epidermal growth factor (EGF) and fibroblast growth factor (FGF), although FGF has been reported to promote motor neurone survival *in vitro* (Arakawa *et al* 1990; Henderson *et al.* 1993) although it was found to be ineffective in preventing mouse motor neurone cell death after neurotmesis (Li *et al* 1994).

From a consideration of the research into neurotrophic factors it can be seen that much effort has been concentrated on promoting the survival of cell bodies of transected neurones in embryonic and neonatal animals. Other research has concentrated on survival of tissue cultures of neurones. These situations are far removed from the reality of nerve repair and the ideal of potentiating the functional outcome of nerve coaptation or treatment of neurological disorders such as motor neurone disease. Many of the published studies end by suggesting that the effects of neurotrophic factors are *potentially* beneficial in these disorders. There is a paucity of research in the area of nerve coaptation with neurotrophic factors and what does exist places great emphasis on nerve morphology, even though this may not be the best indicator of functional outcome. Moreover, some studies are contradictory and no neurotrophic factor clearly stands out as superior to all others. NGF does appear, however, to support sensory neurones rather than motor neurones. It would appear therefore, that more research is needed into the use of neurotrophic factors in a surgical model of nerve repair. *In vitro* and embryonic and neonatal research is interesting and suggests avenues of study but extrapolation to adult mammalian models, let alone the human, is not necessarily valid. The assessment of outcome of nerve repair is paramount. It is likely that any benefit derived from

the use of neurotrophic factors in nerve repair will be small, although if present it may still represent clinically useful improvement. With the severe limitations of clinical testing of muscle function in the animal model, electrophysiology (by evoked electromyography) as well as nerve and muscle morphology offers the best assessment of reinnervation of the motor unit.

Conclusion

A normally functioning facial nerve is vital to a person's well being and self-esteem. Injury to the nerve is uncommon but devastating. Nerve repair is a well tried and accepted surgical procedure and offers a chance of recovery of some function, but even in the hands of super-specialists, results are far from good. Neurotrophic factors are powerful endogenous molecules and *in vitro* can influence growth and survival of neurones.

The question remains therefore: do the currently identified neurotrophic factors, if administered exogenously, offer any benefit in the outcome of surgical nerve repair? The experiments that follow are an attempt to answer that question in an animal model.

Chapter 2

Nerve entubulation

Introduction

It has long been believed that the repair of a defect in a length of nerve is best undertaken by autologous nerve grafting. However, much attention has been concentrated on entubulation of nerve ends as a method of repair. Similarly, although it is accepted that the repair of neurotmesis is best achieved by epineurial or fasicular suture of the nerve ends, some authors advocate entubulation in preference to direct suture repair and clinical studies have been carried out where substantial series of patients have undergone this technique with good functional results.

The problems with autologous nerve grafting have fuelled the search for other techniques of repairing nerve defects. The harvesting of donor nerve leaves a scar, a sensory deficit and occasionally, a painful neuroma. The idea of bridging the ends of a defect in a nerve by a non-neural tube is not a novel one. Experimental work with animals was first reported by Glück in 1880 and again in 1882 (Glück 1880; Vanlair 1882). Both of these reports used decalcified bone as a conduit. Since then, arteries (Foramitti 1904), veins (BenitoRuiz *et al.* 1994), amnion (Chao *et al* 1962), fascia (Lundborg & Hansson 1980) and a variety of synthetic materials (Giardino *et al.* 1995) have been employed in an attempt to find the ideal material. Apart from the absence of donor site problems, the use of entubulation over autologous nerve grafting offers other *theoretical* advantages. The avoidance of trauma to the nerve endings by

suture, and the tissue reaction this might provoke may be a beneficial effect of entubulation. The tube may also act to confine the nerve endings and stop neurites being lost to non-target-organ tissues. Last, the tube may prevent fibroblasts entering the site of nerve repair which could hamper regeneration and maintain an optimal microenvironment for nerve growth, perhaps with the concentration of neurotrophic factors around the site of repair. The cellular mechanisms for the establishment of nerve-to-nerve continuity within a silicone tube after neurotmesis has been studied by Schroder *et al* (Schroder *et al* 1993). At three days after surgery to create a 1 cm nerve defect in a rat sciatic nerve, a meshwork of fibrin had precipitated in the tube bridging the proximal and distal nerve stumps. Contained in this meshwork were erythrocytes, granulocytes, thrombocytes and some macrophages. Fibrin was mainly arranged longitudinally and there was a narrow cleft between the cord of tissue and the wall of the tube. This cleft had increased in size by seven days after surgery, owing to retraction of the fibrin clot, and there were bipolar cells arranged longitudinally on the outer part of the cord. Perineurial cells closed the gap between nerve stumps at 18 days and schwann cells, fibroblasts and blood vessels occupied the centre of the cord. By three weeks after creation of the neurotmesis and gap, axons from the proximal nerve stump had reached the distal stump although they were, as yet unmyelinated. It is thought that the perineurium serves as a diffusion barrier from the outside as well as from the inside of the nerve fascicle, thus providing an endoneurial environment for the developing nerve (Dyck *et al.* 1984; Klemm 1970).

Type of tube

Many types of tubing have been suggested for nerve repair. Lundborg has much experience of nerve repair and is an advocate of silicone as an ideal material for entubulation (Lundborg *et*

al 1991). Yet in clinical practice, two out of three of Lundborg's nerve repairs with silicone have required a 'second look' procedure for 'irritation' around the site of repair. Although the authors discount the importance of this, it would clearly be undesirable and cost ineffective for revision procedures to be required after nerve entubulation with silicone. Most experts now agree (Dellon 1994) that a biodegradable conduit is the method of choice if entubulation for nerve repair is to be considered.

Entubulation

In 1981 Lundborg *et al* showed that the proximal stump of a rat sciatic nerve would regenerate diagonally through the rectangular space of a silicone chamber to meet with a distal nerve stump (Lundborg *et al.* 1981). Since then many authors have published their success with entubulation using other materials and other nerves. Lundborg and Hansson successfully used pseudosynovial tubes, formed from the tissue reaction around a silicone rod, to bridge a nerve gap. A thin spiral of metal wire was used to prevent collapse of the tube (Lundborg & Hansson 1980).

Pham *et al* compared the standard sutured epineurial repair of the rat peroneal nerve to repair with a polyglycolic acid tube and collagen glue (Pham *et al.* 1991). Assessment of recovery was by nerve morphology and electrophysiology and significantly better conduction was found in the entubulation group compared with the sutured group. There was no difference in nerve morphology between groups. Hentz *et al* used polyglycolic acid tubes as a pseudoperineurium in an attempt to isolate the functional intrafasicular tissues from the extrafasicular fibroreactive process (Hentz *et al.* 1991). In this non-human primate study median and ulnar nerves were repaired by the three techniques of epineurial and fascicular repair and fascicular tubulization. This showed no significant difference between the three types of repair. Other

authors (Rosen *et al* 1989; Stevenson *et al* 1994) have similarly, failed to show a difference either physiologically or histologically between these three types of repair.

One of the most interesting concepts to be considered with nerve repair is whether entubulation with the deliberate creation of a small nerve defect of 3 to 5 mm, where epineurial suture would be possible, is the optimal management (Dellon 1994). This idea has the theoretical advantage of providing an optimal microenvironment for nerve regeneration and repair and potentially concentrating neurotrophic factors in the gap between the nerve stumps.

Properties of the tube

If the microenvironment at the site of nerve regeneration is indeed important, the permeability of the tube may also have an impact on functional outcome. In the assessment of repair of nerves with a 1 cm defect, macropore collagen conduits give greater functional recovery than either semipermeable or non-permeable collagen tubes (Kim *et al.* 1993). It is postulated that the macropore tube allows neurotrophic factors, being macromolecular proteins, to gain access to the nerve repair site. The internal diameter and wall thickness of the nerve conduit also appear to influence nerve regeneration (Den Dunnen *et al.* 1995). The tube must be large enough to accommodate any swelling of the nerve and not itself cause compression of the nerve by swelling when the tube degrades or the nerve grows.

From the above evidence it follows that the ideal nerve conduit should be biodegradable, provoking little or no inflammatory response and should not collapse or swell during degradation. It should be able to be manufactured in a range of sizes depending on the diameter of nerve to be repaired. A material that meets these requirements is a controlled

release glass (CRG). This is composed of inorganic polymers, normally based on phosphates of sodium and calcium, which have been converted to a glassy form by melting the constituents at about 1000 °C. They dissolve completely in water leaving no solid residue. The rate of solution can be selected by adjustment of the composition and physical form of the CRG and is constant for as long as any of the material remains. The product can be produced in many physical forms; as powder or fine granules, fibre or cloth, tubes or as cast blocks of various shapes. Elements other than sodium and calcium, including most metals and their oxides and a limited number of inorganic anions, can be included in the composition of the glass. These elements which may be biologically active can then be delivered at a constant rate into an ambient aqueous medium as the CRG dissolves. This has found application in veterinary medicine as a means of delivering such diverse substances as trace elements, anthelmintics and vaccines. In the course of development the biocompatibility and absence of toxicity of CRG based on Na_2O-(Ca,Mg)-O-P_2O_5 with and without other constituents have been investigated. In applications differing as widely as use in orthodontic devices (Savage 1982), in nutritional experiments with rats and in controlled supply of Cu, Co and Zn in cattle (Drake & Allen 1985), no ill effect were observed. When CRG pellets were implanted subcutaneously, intramuscularly and intraperitoneally in rats, sheep and cattle, reaction at the implant site was limited to a sterile fibrous encapsulation less well developed than that expected from biocompatible surgical materials (Allen *et al.* 1978). Other applications of CRG in the Na_2O-CaO-P_2O_5 system have been found as potential bone graft adjuncts or substitutes. No sign of cytotoxicity was observed in mouse fibroblasts (Docherty 1982) or after soft tissue implantation in sheep (Burnie *et al.* 1981). In further experiments with bone no ill effects nor bio-incompatibility could be detected (Burnie 1982; Burnie *et al.* 1983; Burnie & Gilchrist 1983; Duff *et al.* 1984).

Methods

A CRG tube was used in entubulation experiments. In the sheep experiments (chapter 4) the tube was manufactured 3 cm in length with an external diameter of 10 mm and an internal diameter of 7 mm. In the centre of the tube, a hole was drilled by a high speed dental burr to a diameter of 1 mm. A plastic catheter (Charles River UK Ltd, Kent, UK) was then inserted into the CRG tube and this was glued in position (Loctite 406). The tubes with catheters were sterilised by gamma irradiation (Figure 2.1) and they were stored, ready for entubulation of the buccal branch of the facial nerve (chapter 4) in the sheep. The plastic catheter was attached to a 2 ml Alzet osmotic pump (Charles River UK Ltd, Kent, UK) which was filled with saline as a control or neurotrophic factors. This osmotic pump is an implantable device in which a compressible reservoir is surrounded by a rigid semi-permeable membrane. Over a set period of time after implantation, the semi-permeable membrane allows water to diffuse through the outer casing of the pump compressing the reservoir and delivering the contents of the reservoir via a tube to the site of intended action. Pumps are available in different capacities and duration of delivery of their contents.

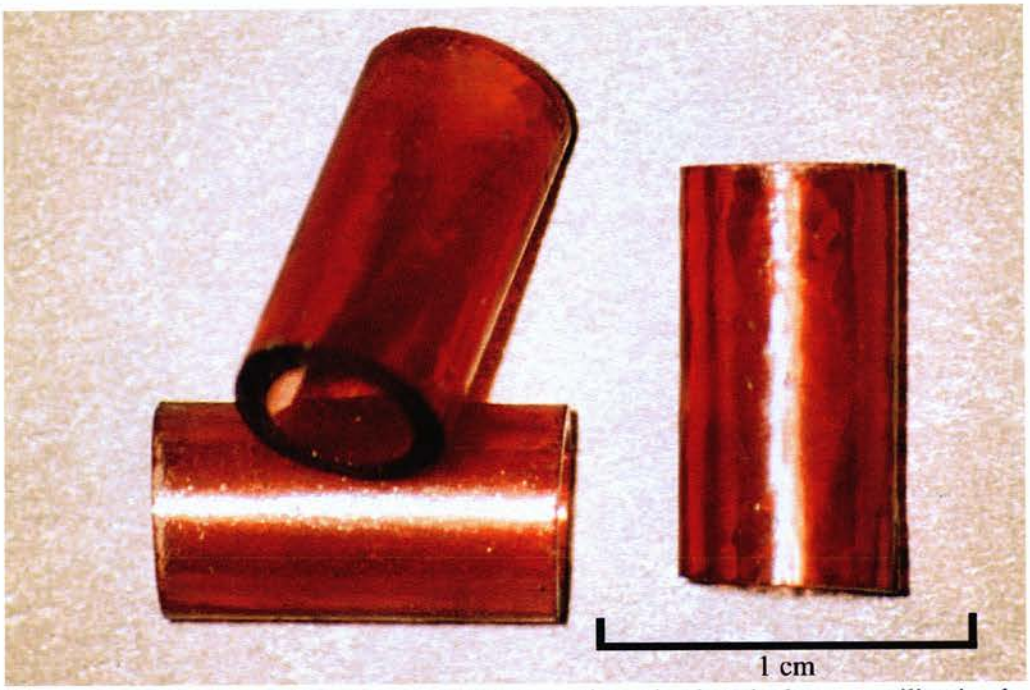


Figure 2.1. Three controlled release glass tubes. The red colour is due to sterilization by gamma irradiation.

Chapter 3

Rat experiments

Introduction
The anatomy of the rat facial nerve
Method of procedure

Introduction

The rat was used as one of the experimental animals for the investigation of the effects of nerve entubulation and neurotrophic factors on nerve repair. The rat is a commonly used laboratory animal and its use was within the financial resources of this study. Several groups of operations were performed, the purpose of which was to establish if entubulation nerve repair and entubulation repair and administration of neurotrophic factors had a measurable effect on nerve regeneration. There were six rats in each group. These groups are shown in table 3.1. After three months all rats from each group were re-operated on to obtain electromyography and nerve morphometry.

Group no.	Number of rats	Procedure	Time to assessment
1	6	N-N suture	3 months
2	6	N-N defect (4 mm glass tube)	3 months
3	6	N-N defect (4 mm glass tube, with 10 μg BDNF)	3 months
4	6	N-N defect (4 mm glass tube, with 10 μg CNTF)	3 months
5	6	N-N defect (4 mm glass tube, with 10 μg GDNF)	3 months
6	6	N-N defect (4 mm glass tube, with 10 μg NT 4)	3 months
7	6	N-N suture (10 μg BDNF and 10 μg CNTF to repair site)	3 months
8	6	N-N suture (10 μg GDNF and 10 μg NT4/5 to repair site)	3 months

Table 3.1. N-N = nerve-to-nerve

The anatomy of the rat facial nerve

The rat has been used as an animal model for surgical manipulation of the facial nerve (Mountain *et al.* 1993; Murray *et al* 1994). Mattox and Felix described the surgical anatomy of the rat facial nerve in detail, after microscopic dissection of ten animals (Mattox & Felix 1987). Previous texts on this subject were contradictory (Greene 1955; Hebel & Stromberg 1976). Mattox and Felix described that the nerve exits the stylomastoid foramen on the lateral side of the skull, posterosuperior to the external auditory canal (Mattox & Felix 1987). The posterior auricular branch diverges from the facial nerve as it leaves the stylomastoid foramen. The nerve trunk passes anteroinferiorly between the trapezius muscle and the external auditory canal. The first 2 mm of the nerve is covered by the cervical head of the trapezius muscle. Underlying the nerve trunk and separated by a thin fascia, the common trunk of the occipital and post-auricular arteries pass as they travel to the pina. The total length of the main trunk of the nerve (not including the posterior auricular branch) is 6 mm before its furcation.

The facial nerve divides into six branches. The furcation is obscured by the posterior facial branch of the external jugular vein (figure 3.1). This vein is a substantial vessel and drains the majority of the head, as the rat has no internal jugular vein. The external jugular vein passes lateral to all branches of the nerve except for the small, posterior cervical branch, which passes lateral to the vein. The internal maxillary and masseteric veins pass medial to the temporal and zygomatic branches of the nerve, as the veins converge to form the external jugular vein.

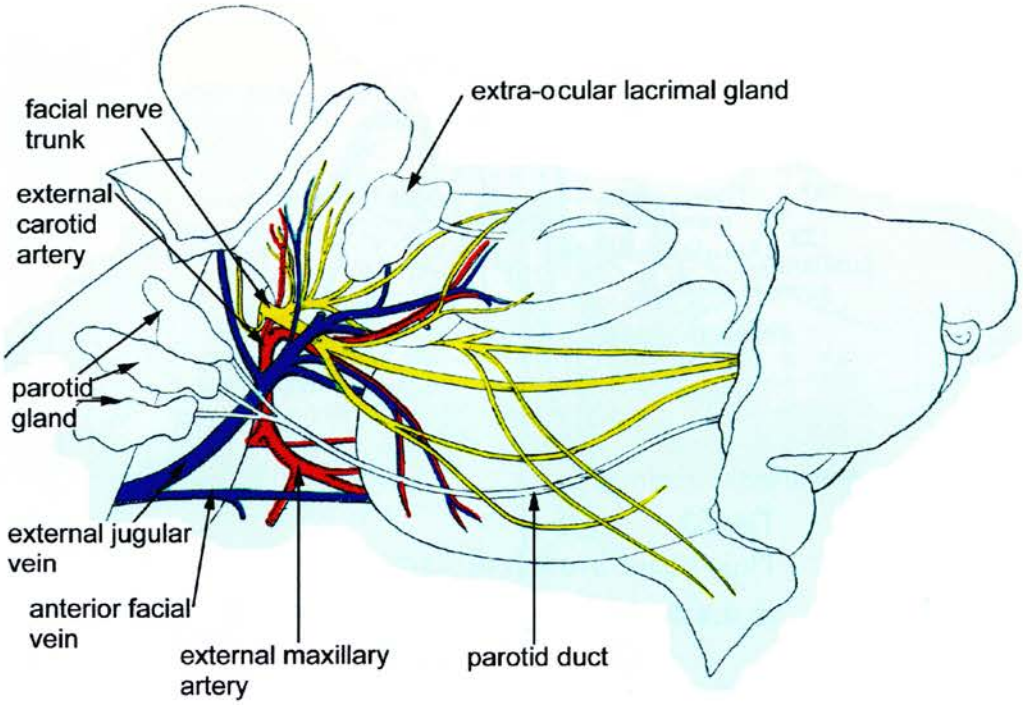


Figure 3.1. The facial nerve and associated blood vessels in the rat.

All peripheral branches of the facial nerve lie beneath the superficial fascia covering the facial musculature. As in other rodents, the nerve passes beneath, not within, the parotid gland. There are six branches of the facial nerve (figure 3.2):

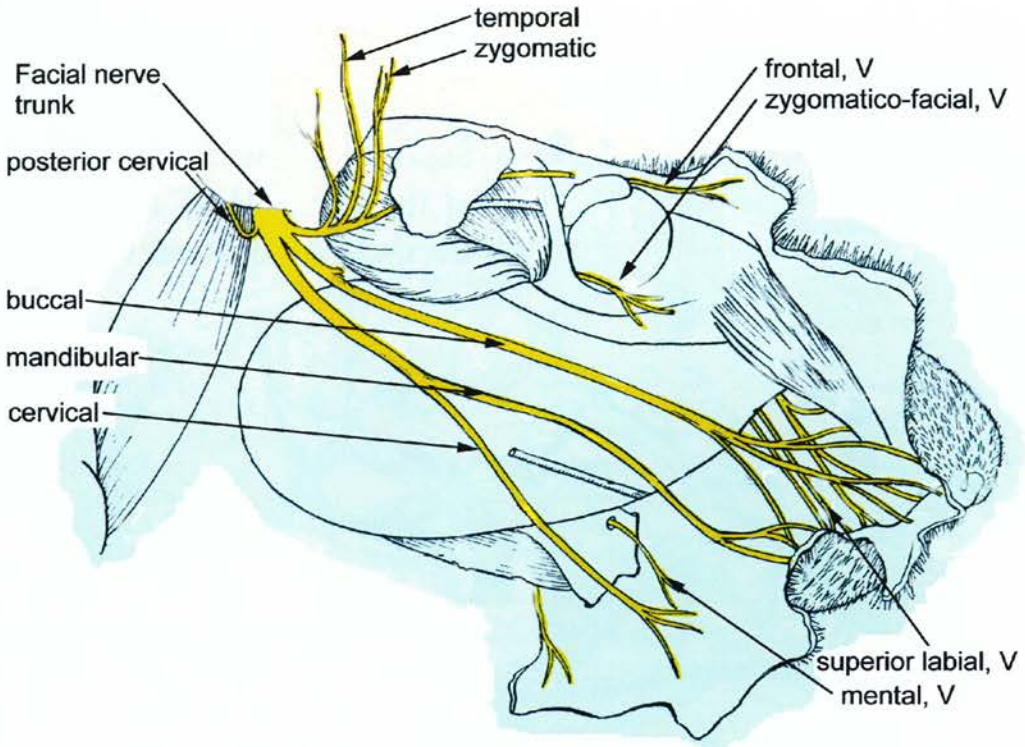


Figure 3.2. The branches of the facial and trigeminal (V) nerves in the rat.

The posterior auricular branch diverges from the facial nerve as it leaves the stylomastoid foramen and supplies the auricular muscles.

The posterior cervical branch is the first branch of the furcation and consistently passes lateral to the external jugular vein.

The cervical branch is the most posterior and inferior branch beneath the external jugular vein. It separates immediately from the mandibular branch, not midway as previously described (Greene 1955; Hebel & Stromberg 1976).

The mandibular branch is the largest branch of the facial nerve. It crosses the lower central portion of the masseter muscle and extends for 10 - 12 mm before branching into the musculature of the upper and lower lips.

The buccal branch of the nerve follows the grove between the masseter and the temporalis muscle. It passes beneath the eye and towards the nose and also sends branches to upper and lower lips. The auriculotemporal branch of the trigeminal nerve runs in close proximity to the buccal branch of the facial nerve 2 - 4 mm from the furcation. The two nerves can, however, be dissected apart with the aid of the operating microscope. Peripherally, the trigeminal fibres supply the skin in the area of the vibrissae.

The temporal and zygomatic branches may arise from the furcation or from the buccal branch. They have short and complex branching patterns. This, and their intimate relationship with the tributaries of the external jugular vein make them unsuitable for surgical manipulation.

Mattox and Felix also performed axon counts of the rat facial nerves under study (Mattox & Felix 1987). A mean value of 4650 axons were counted in the main trunk of the nerve. In the buccal division, a mean value of 1955 axons was obtained with a 13.7% mean difference between right and left sides.

Method of procedure

Anaesthesia

The rats were operated on under general anaesthesia achieved by halothane inhalation with air as a vehicle gas. The animals were placed in Perspex chamber and induction of anaesthesia was carried out with 4% halothane. Anaesthesia was achieved in 1 - 2 minutes and the rat was removed from the chamber and placed on an operating bench. Anaesthesia was maintained by

1.5% halothane administered by face mask, with a coaxial scavenging system (Hunter *et al* 1984) used to collect anaesthetic gases throughout surgery.

Surgery

The animal's fur on the left side of the face was shaved with electric clippers and the skin was cleaned with a povidone-iodine antiseptic solution (Betadine, Seton Health Care, England). Sterile drapes were used to mask the area not within the operative field and sterile instruments were used in the operation. An incision was made below the lower border of the mandible and the skin was retracted until the buccal division of the facial nerve was seen. The buccal division of the nerve was dissected from the groove in the muscle were it lay. Approximately 1 cm of nerve was dissected. The extra-orbital lacrimal gland was reflected on its upper pole to expose this length on nerve. It was not necessary to remove the gland.

GROUP 1

The nerve was transected using microscissors and a 2 mm length of the nerve was removed for histological analysis (chapter 6). Surgery was carried out using an operating microscope (Wild, Heerbrugg M690) with stepless variable magnification of 3.5 to 18 times. An epineurial repair was performed, without tension, with three 10/0 nylon (Ethilon, Ethicon) equally spaced, interrupted, circumferential sutures.

GROUP 2

The nerve was transected using microscissors and 4 mm of the nerve was removed for histological analysis (chapter 6). The cut nerve was then placed in a CRG tube, which had been sterilised by gamma irradiation. The nerve was held in position with a 10/0 Ethilon

suture transfixing the epineurium of each nerve stump. The suture was tied so that the gap between the stumps was 4 mm (as shown in figure 3.3). The middle of the tube was placed over the nerve defect.

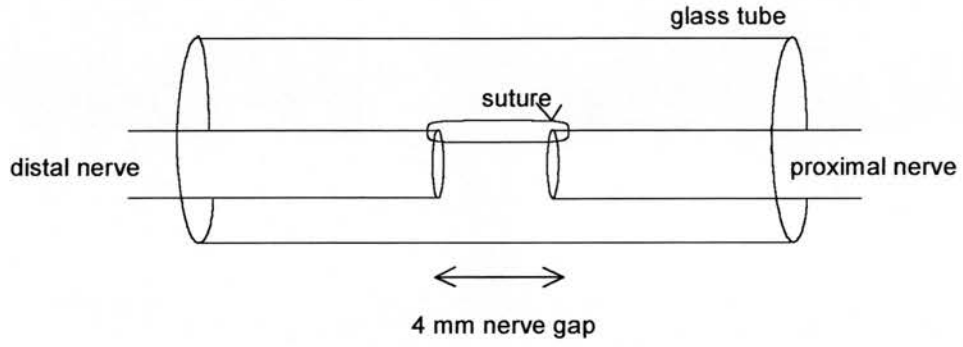


Figure 3.3 Entubulation repair of the buccal division of the facial nerve in the rat.

GROUP 3

An area between the scapulae of the rat was also shaved and prepared in a similar manner to the face. The buccal division of the facial nerve was transected using microscissors and a 4 mm length of the nerve was removed for histological analysis (chapter 6). The cut nerve was then placed in a CRG tube and held in position with a 10/0 Ethilon, epineurial suture holding each nerve stump. The suture was tied so that the gap between the stumps was 4 mm. The glass tube had a 1 mm central, drilled hole, through which a plastic catheter had been glued. Beforehand, the tube and catheter had been sterilised by gamma irradiation. The middle of the glass tube was then positioned over the site of the nerve defect. The glass tube was sutured to the underlying fascia with 4/0 Vicryl. An incision was made between the scapulae of the animal and a subcutaneous pocket was created in the rat's back. This pocket was extended rostrally in a subcutaneous tunnel to communicate with the facial incision. The plastic catheter, which was attached to the CRG tube was led through this tunnel. The catheter was

attached to a miniature osmotic pump (0.2 ml, Alzet, Charles River, Kent) which was filled with 10 μg of BDNF dissolved in sterile water. The osmotic pump was placed in the pocket in the rat's back and this was closed by interrupted 4/0 Vicryl sutures.

GROUP 4

The animals in this group were operated on in an identical manner to the animals in group 3 with the exception that 10 μg of CNTF was used in place of 10 μg of BDNF.

GROUP 5

The animals in this group were operated on in an identical manner to the animals in group 3 with the exception that 10 μg of GDNF was used in place of 10 μg of BDNF.

GROUP 6

The animals in this group were operated on in an identical manner to the animals in group 3 with the exception that 10 μg of NT4/5 was used in place of 10 μg of BDNF.

GROUP 7

The animals in this group were operated on in an identical manner to the animals in group 1 except that no glass tube was used and the nerve was repaired by circumferential epineurial sutures, as in group 1. The catheter was connected to an ultafine needle and this was place in the distal part of the nerve, 5mm from the repair site (figure 3.4). 10 μg of BDNF mixed with 10 μg of CNTF was used in the osmotic pump.

GROUP 8

The animals in this group were operated on in an identical manner to the animals in group 1 except that no glass tube was used and the nerve was repaired by circumferential epineurial sutures, as in group 1. The catheter was connected to an ultafine needle and this was placed in the distal part of the nerve, 5mm from the repair site (figure 3.4). 10 μg of GDNF mixed with 10 μg of NT4/5 was used in the osmotic pump.

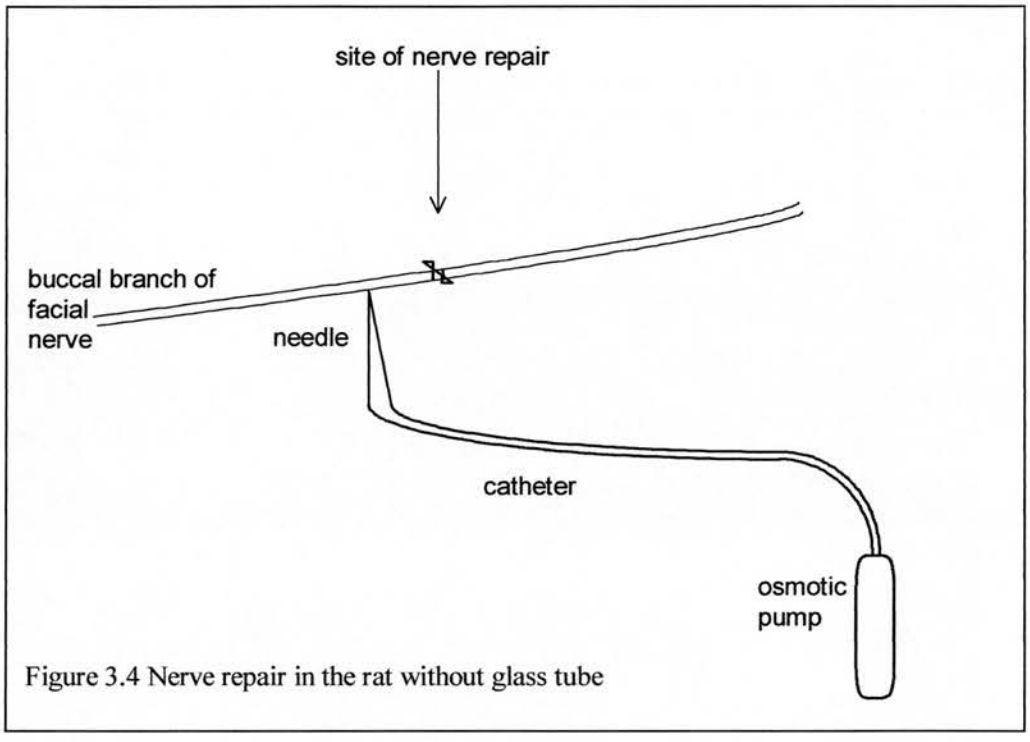


Figure 3.4 Nerve repair in the rat without glass tube

The facial incision in all groups was closed with 4/0 continuous Vicryl to the platysma muscle. 4/0 interrupted Vicryl was used to close the skin superficially with the knot buried. The closed incision was covered with an antiseptic vapour-permeable dressing spray (Opsite, Smith and Nephew, England). A single dose of subcutaneous enrofloxacin (0.1 ml) was injected as a prophylactic antibiotic. Each rat's ears were clipped to allow identification and this made possible a comparison of the nerve histology before and after nerve repair. Recovery took approximately ten minutes, after which the animal was returned to a cage alone and

observed for a further 30 minutes. Once complete recovery had occurred the animal could again be housed with other rats. The rats were checked daily for signs of wound infection although they had little difficulty establishing normal behaviour and eating patterns. Because the incision to expose the facial nerve was located below the mandible, and a musculo-cutaneous flap had been raised to expose the buccal division of the nerve, once the flap was replaced and the incision closed, the site of nerve repair or entubulation was well protected. This allowed nerve entubulation to be carried out with no extrusion of the CRG tube.

Three months after the original surgery each group of rats was re-operated on. General anaesthesia was carried out as above. After collection of the electrophysiological data (chapter 8) 5 mm of the buccal division of the left facial nerve (distal to the site of repair) was removed and processed for histology (chapter 6).

Chapter 4

Sheep experiments

Introduction
Method of procedure

Introduction

Large animals are more representative of the human than small animals such as rodents. The physiology of nerve repair is more similar to the human in the sheep than it is in the rat. Much research has therefore concentrated on the sheep as an animal model for nerve repair (Glasby *et al.* 1990; Hems *et al* 1994). The use of the sheep, however, brings with it complexities to experimentation that are not present in the rodent. Larger animals are more expensive to buy and maintain. Whereas small animals can be housed in an animal area for the duration of the study, sheep require to be transported to the animal area before and after surgery, with sufficient time given for the animal to acclimatise to a new environment. This time to allow the animal to 'settle in' reduces the stress on the animal and is required by law (Animals (Scientific Procedures) Act, 1986). Anaesthesia is more complicated and particularly difficult in ruminant animals because of gaseous build-up in the rumen and its associated problems. Anaesthesia in these animals also necessitates endotracheal intubation, intravenous access and, in many cases, intraarterial blood pressure monitoring. Because sheep are more representative of the human model, the sheep was used in this study to investigate the effect of various neurotrophic factors on nerve repair.

The anatomy of the extra-temporal facial nerve in the sheep is similar to the human. In the sheep the nerve exits from the stylomastoid foramen at the skull base and courses rostrally to enter the parotid gland where it divides into its terminal branches. The buccal branch of the

nerve lies on the masseter muscle approximately half way between the horizontal process of the mandible (which it runs parallel to) and the medial canthus of the eye. The distance from the origin of the nerve at the base of the skull and the distal buccal branch, before it enters its target muscles, is approximately fifteen centimetres and is thus suitable for the measurement of various electrophysiological variables. The buccal branch of the nerve supplies small muscles of the face of which the depressor labii maxillaris is most accessible to electromyographic study. This muscle acts as a guy-rope to anchor the upper lip and is uniquely supplied by the buccal branch of the nerve. The recording electrodes were placed over the tendon and the muscle belly. Three groups of sheep (five to six sheep per group) were studied. First, a group of sheep underwent neurotmesis of the buccal branch of the facial nerve with immediate, tension free repair by epineurial suture (Group 1). Secondly, a group underwent neurotmesis of the buccal branch of the facial nerve with immediate, tension free repair by epineurial suture and entubulation of the repair with a biodegradable glass tube attached to an osmotic pump which delivered 2 ml of saline over a period of four weeks (Group 2). Thirdly, a group underwent neurotmesis of the buccal branch of the facial nerve with immediate, tension free repair by epineurial suture and entubulation of the repair with a biodegradable glass tube attached to an osmotic pump which delivered 2 ml of neurotrophic factors over a period of four weeks (Group 3). 50 μg of BDNF and GDNF and 100 μg of CNTF were administered from the pump to this group. A mixture of factors, rather than three separate groups was chosen to reduce the number of animals, and experiments, in line with Home Office requirements (Animals (Scientific Procedures) Act, 1986). Because of the solubility of the neurotrophic factors these doses were the maximum which could be administered within a volume of 2 ml, the size of the osmotic pump. The groups of sheep are shown in table 4.1. The null hypothesis was '*Neurotrophic factors delivered by this route and in this concentration have no effect on nerve repair*'.

Group	Number of sheep	Procedure	Purpose
1	6	N-N suture	Control procedure
2	5	N-N suture with entubulation and saline pump	Control procedure
3	6	N-N suture with entubulation and neurotrophic pump	Neurotrophic experiment

Table 4.1. N-N = Nerve-to-nerve

Method of procedure

Anaesthesia

The sheep were transferred to the animal area one week before operation to allow the animals to become accustomed to their environment. Operations were carried out under general anaesthesia after a fast for solid foods of eighteen hours. Water was allowed up to the time of operation. Induction to anaesthesia was achieved by an intravenous injection of a mixture of etomidate and midazolam, (both 0.5 mg kg^{-1}) into the left internal jugular vein. The animal's trachea was intubated under direct vision with a cuffed endotracheal tube (Portex, Blueline) after which the sheep was positioned on an operating table in the left lateral position. An ear artery was cannulated and attached to an electronic blood pressure sensor. Venous access was by means of a lateral saphenous vein cannula, which was used for administration of a single dose of analgesic (flunixin, 2.2 mg kg^{-1} and buprenorphine, 10 μg kg^{-1}). Prophylactic cefuroxime (750 mg) was given intravenously immediately before surgery, in animals which were to recover. Muscle relaxation was achieved by intravenous mivacurium (200 μg kg^{-1}) after induction of anaesthesia or after electromyography, if carried out. Intravenous edrophonium (1 mg kg^{-1}) was used to reverse relaxation if the animal was to recover. Euthanasia was carried out by pentobarbitone (140 mg kg^{-1}). In recovery cases, twice daily

cefuroxime (750 mg) were administered by intramuscular injection for five days post-operatively.

Surgery

The animal's face was shaved with electric clippers on the left side. Recording electrodes were placed with the cathode over the muscle belly of levator labii maxillaris, just above and lateral to the mouth. The anode was placed over the nasal bones in the midline. The ground electrode was place on a shaved area of the back. The buccal division of the facial nerve was stimulated transcutaneously to map out the nerve. This made identification easier and less dissection was needed to locate the nerve. For the recovery groups of sheep, the skin of the animal was prepared with a povidone-iodine antiseptic solution (Betadine, Seton Health Care, England). Sterile drapes were used to mask the area not within the operative field and sterile instruments were used in the operation.

GROUP 1

An incision was made only over the identified buccal branch of the nerve. When this was found, the nerve was cut by means of a Meyer neurotome and repaired with three or four 10/0 polyamide (Ethilon, Ethicon, UK) circumferential epineurial sutures using an operating microscope (Wild, Heerbrugg M690). A tension-free repair was ensured. A 4/0 Vicryl (Ethicon) suture was used to close the subcutaneous tissues. 4/0 Vicryl was also used to close the skin by subcuticular suture with the knots buried to avoid the suture being removed by the sheep. The closed incision was covered with an antiseptic vapour permeable dressing spray (Opsite, Smith and Nephew, England) and the animal was allowed to recover from anaesthesia.

GROUP 2

Similarly to group 1, the buccal branch of the facial nerve was exposed and transected. Before suturing, the nerve was threaded through a biodegradable glass tube (Giltech, Ayr, Scotland, chapter 2). A tension free repair was ensured. The glass tube had a 1 mm central, drilled hole, through which a silastic catheter had been glued. Beforehand, the tube and catheter had been sterilised by gamma irradiation. The nerve was then sutured, as before, with epineurial nylon. The middle of the glass tube was then positioned over site of the nerve repair. The glass tube was sutured to the underlying fascia with 4/0 Vicryl and each end of the tube was plugged with a small piece of muscle taken from an adjacent site. This muscle was held in place by tissue glue (Tisseel, Immuno). Thus the glass tube ensheathed the nerve repair which was relatively isolated from the surrounding tissue (figure 4.1). A miniature osmotic pump (Alzet, Charles River, Kent) was filled with 2 ml of saline and the pump was connected to the plastic catheter. A 3 x 5 cm pocket was created by tunnelling subcutaneously under the lower skin flap into the neck and the pump, attached to the catheter, was placed in this pocket, in the neck.

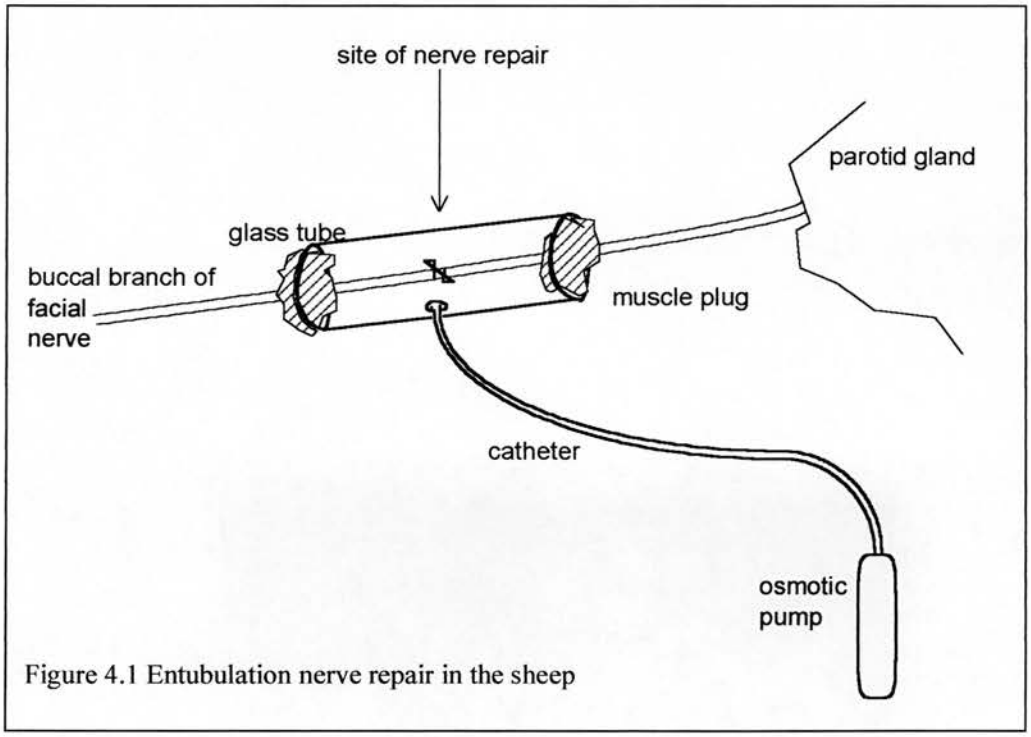


Figure 4.1 Entubulation nerve repair in the sheep

The wound was then closed with a 4/0 Vicryl suture to the subcutaneous tissues and 4/0 Vicryl was also used to close the skin by subcuticular suture. The closed incision was covered with an antiseptic spray (Nobecutane) and the animal was recovered from anaesthesia.

GROUP 3

This group underwent the same procedure as for group 2 except that a solution of neurotrophic factors (BDNF, CNTF, GDNF) filled the osmotic pump. Osmotic pumps are encased in a semipermeable membrane. Fluid moves by osmosis through the outer casing at a constant rate. Within the outer casing, the contents of the pump are contained in a compressible reservoir. Fluid diffuses through the casing, the reservoir is compressed and the contents of the reservoir are released. The contents of the pump were therefore released into the glass tube and around the nerve repair. The behaviour of recombinant proteins *in vivo* makes it difficult to demonstrate accurately their activity in animal models. Many proteins have extremely short half-lives of elimination *in vivo*. When administered by conventional delivery methods, such as systemic injection, these compounds are rapidly eliminated, resulting in wide variations in the level of recombinant protein in plasma and tissues. With the use of an osmotic pump, tissue levels of neurotrophic factors are maintained and unwanted side effects or systemic toxicity should be reduced because of protein delivery directly to the site of intended action. This pump has been used widely in research and has proved reliable (Mufson *et al.* 1996). Neurotrophic factors (BDNF, NT3 and NGF) have been shown to maintain their stability *in vivo* in these pumps (Altar *et al.* 1994; Gu *et al* 1994; Mufson *et al* 1996).

The animals were allowed to recover alone and observed by animal house staff until recovery from anaesthesia was complete and the animal was self-caring. They were housed with other animals and were monitored for signs of wound infection. The animals had little difficulty establishing normal eating patterns. In groups 2, 3 and 4, approximately eight months after the initial operation the animals were reoperated on, using the same techniques for general anaesthesia. Single fibre electromyographic jitter was assessed through the skin (chapter 7). The buccal branch of the facial nerve was identified by stimulation through the skin. The buccal branch was dissected proximally just after the nerve exited from the parotid gland and distally, near the angle of the mouth to expose the epineurium. Electrophysiological studies were carried out (chapter 7). A few millimetres of the buccal branch were then excised and processed for histological examination (chapter 6). The contralateral buccal branch of the facial nerve was similarly removed and processed, and the animal was killed.

Chapter 5

Statistical methods

Introduction

The application of statistical tests to data which have been derived from experimentation, is a means of simplifying large amounts of information. Without statistics, conclusions could often not be drawn from the data, or worse, inappropriate conclusions may be drawn which, without detailed analysis of the data may appear correct. There are two fundamentally different groups of statistical tests, parametric and non-parametric. Parametric tests involve the manipulation of quantitative data. Before parametric analysis can be carried out however, it should be shown that the data are normally distributed. Non-parametric tests involve placing variables in order of rank and thus some sensitivity is lost when using non-parametric statistics. For this reason, in the present work, parametric statistics were used whenever possible. Statistical testing was carried out using a computer and a statistical software package (Statistica, Statsoft, USA). A p-value of less than or equal to 0.05 was considered significant and the null hypothesis was rejected. The null hypothesis for this work was '*neurotrophic factors have no influence on regeneration of the facial nerve after division and repair*'.

The Normal Distribution

The Normal Distribution also known as the Gaussian Distribution is the fundamental distribution of probability in statistics. The word 'normal' in this context means 'conforming to a rule or pattern'. This distribution is symmetrical, with values more concentrated in the middle than in the tails, sometimes described as 'bell shaped'. The standard deviation of the

distribution shows the spread of data about the mean, and tends to be smaller for larger samples and larger for smaller samples. When the data in the set are not normally distributed, they may be converted to a normal distribution by the use of transformations (e.g. logarithmic or exponential).

The Half-Normal Plot

The half-normal plot was constructed by finding the mean of a measured set of variables. The residuals of each variable were found by subtracting the mean of all variables from each measured value of that variable. The sign of the residual (+ or -) was ignored. These residuals were then placed in order of rank. From these ranks the Z value was computed (i.e. standardised values of the normal distribution) based on the assumption that the data came from a normal distribution. These Z values were plotted on the y axis in the plot. If the observed residuals (plotted on the x axis) were normally distributed, then all values should have fallen onto a straight line. If the residuals were not normally distributed, then they would deviate from the line. Outliers became evident in this plot. When outliers were identified these were excluded from further statistical analysis.

The Kolmogorov-Smirnov plot

In this work, all data were tested for normality by plotting frequency distribution histograms and testing by the one-sample Kolmogorov-Smirnov categorised test. This statistical method plots the data as a histogram and constructs the graph of the equation of a normal distribution with the same mean and variance as this data. A one-tailed Kolmogorov-Smirnov test is then done to determine if there is a difference between the data and the calculated equation of the normal distribution. If the Kolmogorov-Smirnov test indicated statistical significance, the hypothesis that the observed data followed the normal distribution was rejected. If the

Kolmogorov-Smirnov test yielded a result which was not statistically significant, it was accepted that the data were normally distributed.

Statistical tests

The F test

The F test was carried out on all data which had been shown to be normally distributed. This test is a test of variance which can be applied to different groups simultaneously. A significant result from the F test does not indicate which of the groups is likely to come from a different population. In order to identify which of the groups differed from the others, the two-sample t test was used to detect differences between groups. The advantage of the F test is that only one test is required for all groups. If multiple t tests were carried out (accepting a significant result at $p<0.05$) then there would be a probability that 1 in 20 tests would yield a significant result. Multiple t testing therefore carries an increased risk of a type I error, when the null hypothesis is wrongly rejected. In this work, where the null hypothesis for the F test was accepted, t tests were not carried out.

The two-sided Student's t test.

The t-test is the most commonly used method to evaluate the differences in means between two groups. The groups can be independent (e.g., nerve conduction in animals that were administered neurotrophic factors compared to a control group that received a placebo) or dependent (e.g., fibre diameter in animals before compared to after nerve division and repair). The t-test can be used even if the sample sizes are very small as long as the data has been shown to be normally distributed.

Chapter 6

Histological methods

Anatomy of the peripheral nerve
Fixation of nerve
Cutting of nerve sections
Staining of nerve section
Assessment of nerve morphometry
Microscopy

Anatomy of the peripheral nerve

Erlanger and Gasser described the classification of peripheral nerves (A, B and C) according to conduction velocity, by three peaks identified on a compound action potential, recorded from a mixed nerve (table 6.1) (Erlanger & Gasser 1937). It is also known that there is a direct, proportional relationship, in a myelinated fibre, between fibre diameter and conduction velocity (Rushton 1951). Group A fibres are the largest, fastest conducting fibres. Group B are myelinated autonomic and preganglionic fibres. Group C are the smallest and slowest conducting unmyelinated fibres which function as somatic afferent fibres and postganglionic autonomic efferents. Group A can be subdivided into four groups by size. Aα are motor fibres, Aβ are associated with touch sensation, Aγ are motor to muscle spindles and Aδ are associated with sharp pain and temperature sensation (Lundborg 1988).

Fibre type	Function	Axon diameter (μm)	Myelinated / unmyelinated	Conduction velocity, ms^{-1}
Aα (I)	motor a – fibres	9-18	myelinated	70-120
	spindle afferents (Ia)			
	tendon organs (Ib)			
Aβ (II)	touch and pressure	5-12	Myelinated	30-75
Aγ (II)	motor to muscle spindles	3-6	Myelinated	18-36
Aδ (III)	pain, pressure, temperature	1-5	Unmyelinated	4-30
B (III)	Preganglionic	3	Unmyelinated	3-12
C (IV)	pain, touch, heat	1	Unmyelinated	1-2

Table 6.1. Classification of nerve fibres

The peripheral nerve is a composite of tissues which not only acts as a conduit for electrical conduction but also provides nutrition, protection and electrochemical insulation. Nerve fibres are closely packed in endoneurial connective tissue in fascicles or 'funiculi'. A perineurium surrounds these fascicles and these are in turn surrounded by an epineurium

In a motor nerve all motor fibres are myelinated. The myelin sheath of the axon is the cell membrane of the schwann cell which becomes wrapped around the axon multiple times, in a spiral with no intervening cytoplasm to separate the layers. This produces alternating layers of lipid and protein. It is here that exists the fundamental difference between myelinated and unmyelinated fibres. Unmyelinated fibres still possess schwann cells. In unmyelinated fibres however, many axons invaginate a single schwann cell and the schwann cell membrane does not spiral around the axons. In myelinated fibres one schwann cells surrounds only one axon longitudinally and at junctions between schwann cells, this covering is deficient (nodes of Ranvier).

The axon has a core of cytoplasm (axoplasm) surrounded by a plasma membrane (axolemma). The axolemma is bound to the myelin sheath of the schwann cell and to the schwann cell's basal lamina, which together with the reticular lamina forms the basement membrane. The 'endoneurial tube' or 'endoneurial sheath' (Millesi & Terzis 1984) consists of this basement membrane and the endoneurial reticular and collagen fibres providing the framework supporting the nerve fibre.

The endoneurium is a loose collagenous matrix with large extracellular spaces. The matrix contains schwann cells, endothelial cells, fibroblasts, mast cells and capillaries. The

endoneurial space thus allows diffusion of substances rapidly along the length of the nerve (Selander & Sjöstrand 1978).

The perineurium surrounds the endoneurial space, isolating it by its strength and density and protecting the contents of the space (the axons) from mechanical trauma and chemical changes (Thomas & Olsson 1984). The mechanical strength of the perineurium is impressive. The intrafascicular pressure can be increased to 750 mmHg before rupture of the perineurium (Selander & Sjöstrand 1978). The perineurium consists of a number of lamellae, of epithelium-like, flattened cells. These cells possess basement membranes on their outer and inner surfaces (Thomas & Olsson 1984). The number of lamellae vary with the diameter of the fascicle. In the mammalian nerve up to 15 lamellae may be present. The cells of the perineurium are joined by tight junctions, thus acting as a diffusion barrier. The lamellae of the perineurium contain capillaries within longitudinally and obliquely orientated collagen and elastic fibres. The perineurium is pierced by blood vessels throughout its length, taking with them a sleeve of perineurium. More importantly, the perineurium is deficient distally, near the motor end plate. This may be an important route for entry of substances, which could otherwise not penetrate the perineurium, into the endoneurial space.

It has been shown in many studies that the perineurium acts as a diffusion barrier e.g. to small-molecules proteins (Olsson & Reese 1969), ferritin (Waggener *et al* 1965) and exogenous proteins (Lundborg & Rydevik 1973). Perineural cells also show high activity of dephosphorylating and oxidative enzymes and this suggests that they are metabolically active. The perineurium is thought to play an important role in the regulation and maintenance of the 'internal milieu' of the endoneurial space. Nerves can maintain their function when surrounded by a pyogenic focus and this is probably due to the perineurium. This is perhaps best seen in

acute otitis media, where facial nerve function is invariably retained, despite the incidence of facial nerve dehiscence in the middle ear being as high as 56 or 60 % (Perez *et al* 1997; Moreano *et al.* 1994).

The epineurium is a loose, but strong, connective tissue which embeds and protects nerve fascicles. Epineurium is more abundant near joints where greater protection of fascicles is required. The epineurium contains a well developed vascular plexus with longitudinal vascular channels feeding endoneurial capillary plexuses. Deeper layers of epineurium separate fascicles while also keeping them loosely together. The connective tissue of the epineurium is condensed superficially to form an adventitia.

Fixation of nerve

Specimens of nerve tissue were processed by a standard method (Gschmeissner *et al* 1990) with minor modifications. This produced resin imbedded sections which were then cut using a microtome.

After the nerve had been removed from the animal the surrounding tissue and fat were trimmed grossly. The nerve specimen was placed on a piece of card and immersed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer at room temperature for between one and two hours. The specimen was then removed from the solution, placed on dental wax and viewed, at 25 times magnification, under a binocular, dissecting microscope (Wild, Heerbrugg). The cut ends of the nerve were irregular, and these were trimmed using a razor blade. The initial fixation increased the resistance of the tissue and enabled the nerve to be sectioned transversely, in 0.5 - 1 mm slices, resulting in smooth cylinders of tissue. These cut

cylinders of nerve were again placed in 2.5% gluteraldehyde in 0.1M sodium cocodylate buffer and left overnight in a tissue agitator (Rolamix, Bio-rad). The sections were then washed three times, each for 20 minutes, in 5% sucrose buffer. The tissue was then incubated in 1% cacodylate buffered osmium tetroxide at room temperature for three hours. Osmium preserves myelin and is essential for the identification of the myelin sheath of the nerve. The specimens were then washed three times, each time for 20 minutes in 10% alcohol and then washed three times, each time for 30 minutes in 100% alcohol. The tissue was then washed in propylene oxide for 30 minutes and embedded in Araldite overnight at room temperature. The sections were then removed from the Araldite which was not yet polymerised and reimbedded in fresh Araldite using the dissecting microscope to orientate the cylinders of nerve so that transverse section could be cut easily. The tissue was placed in an oven at 60 °C for 72 hours which allowed polymerisation of the Araldite to a hard mould.

Cutting of nerve sections

The fixed sections of nerve, once imbedded in Araldite, could be kept indefinitely and semi-thin sections (1 μm) were made at a convenient time. The Araldite mould with the section of nerve to be cut was mounted on a wooden dowel and a ultramicrotome (C. Reichert OMU3). A freshly cut glass knife was used to cut the sections. The sections were lifted by a needle and mounted on a glass slide over which a drop of chloroform was suspended to evaporate some Araldite. The slides were then dried on a hot plate.

Staining of nerve section

Two stains were used for nerve sections. First, toluidine blue and pyronin B stains all tissue structures to a varying degree and was used to check orientation and preservation of the block during sectioning (Figures 6.1 and 6.2).

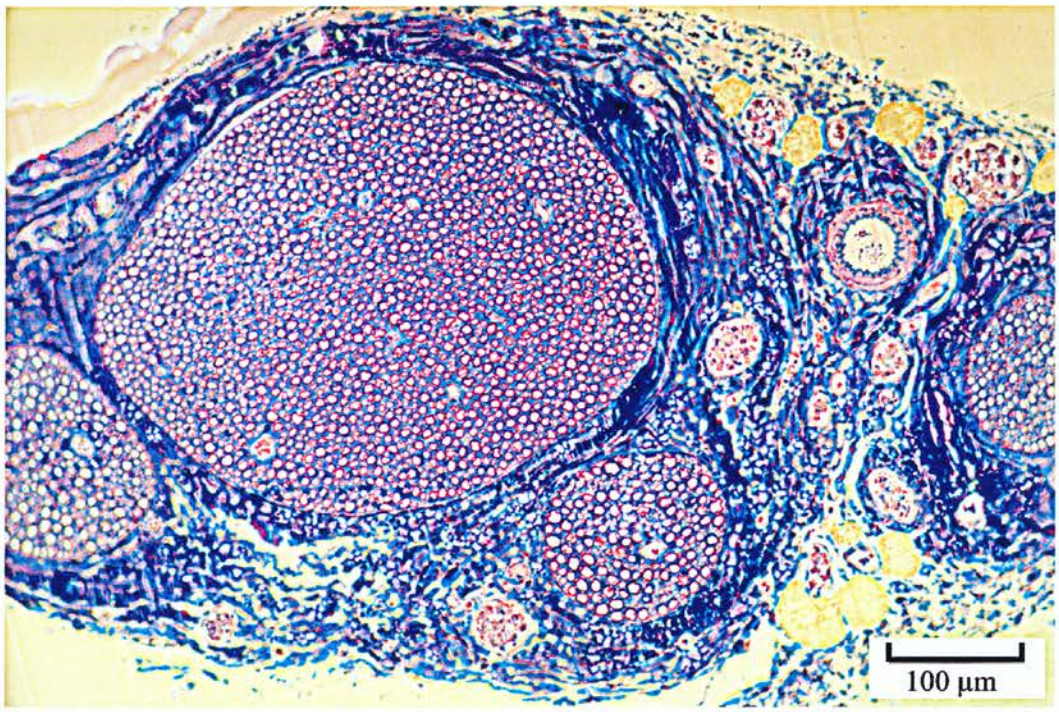


Figure 6.1. Toluidine blue and pyronin B stain of a normal buccal division of the facial nerve in the rat.

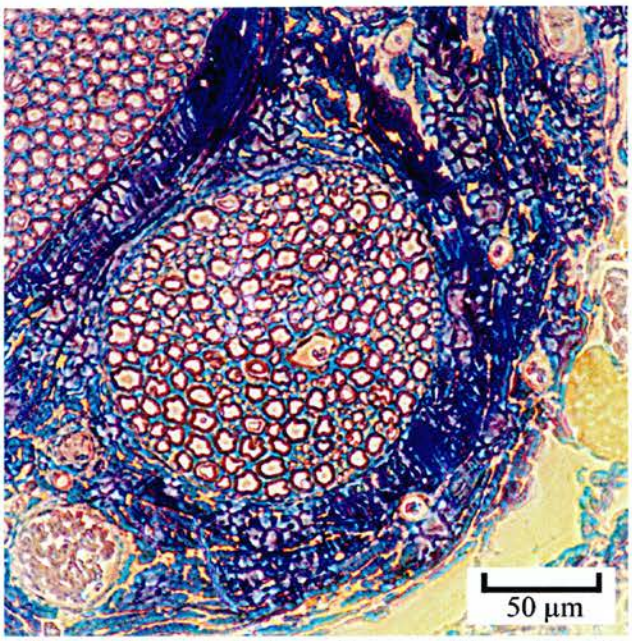


Figure 6.2. Toluidine blue and pyronin B stain of a normal buccal division of the facial nerve in the rat, high power view of figure 5.2.

Second, paraphenylenediamine is a specific stain for osmiophilic material and would thus preferentially stain the myelin sheath of nerve fibres (Figures 6.3 and 6.4). Paraphenylenediamine was used to advantage in that the nerve fibre could be clearly defined from surrounding tissue and computer recognition of axons was used in a commercial image recognition package (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada). 1% toluidine blue in 1% sodium tetraborate was dropped over the slide while the slide was still warm. When steam was seen to rise from the slide the stain was washed with cold water, taking care not to let the stream of water touch the tissue. For staining with paraphenylenediamine, slides bearing nerve sections were placed in a Coplin jar with a freshly filtered solution of 1% parapheneylinediamine for 1 hour. These were then washed with distilled water and 96% alcohol.

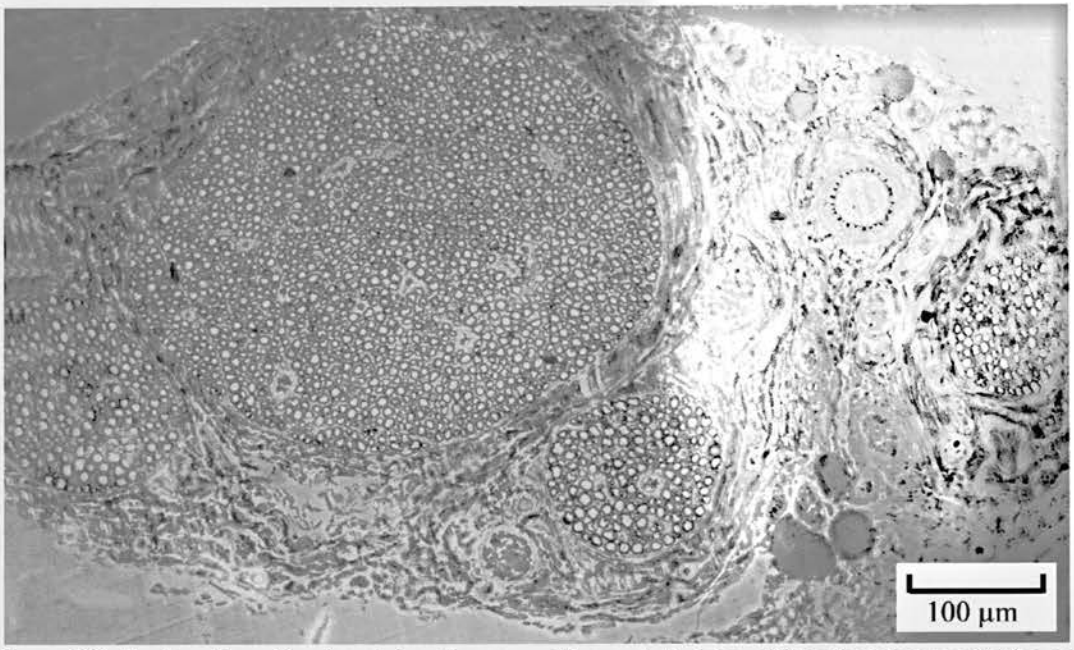


Figure 6.3. P-phenelenediamine stain of a normal buccal division of the facial nerve in the rat.

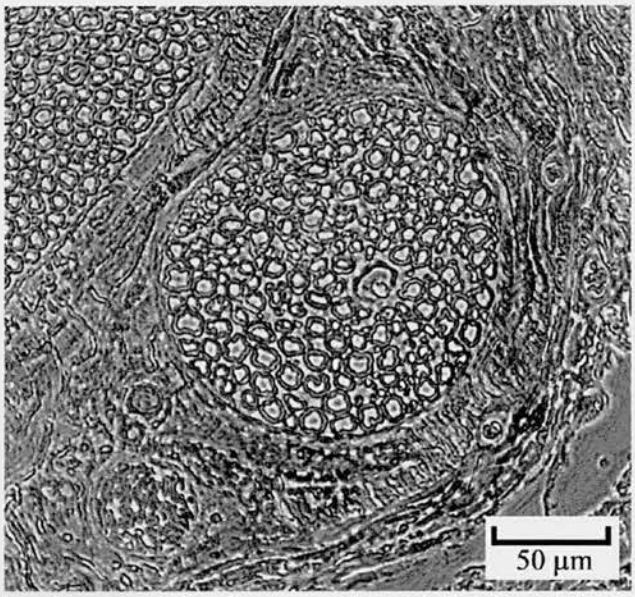


Figure 6.4. P-phenelenediamine stain of a normal buccal division of the facial nerve in the rat, high power view of figure 5.4.

Assessment of nerve morphometry

After neurotmesis and nerve repair, the recovery of function of a nerve depends on several variables which include: the number of fibres reaching the target organ, the maturation of the fibres, the speed with which the fibres reach the target organ and the appropriateness of the

connections made within the target organ. Many authors have concentrated on morphological measurements of the regenerated nerve. These have included:

Nerve fibre counts (Glasby *et al.* 1986b; Glasby *et al.* 1986a)

Axon diameter (Glasby *et al* 1990)

Fibre diameter (Glasby *et al* 1990)

Myelin sheath thickness (Glasby *et al* 1986b; Glasby *et al.* 1986d)

G-ratio (Guntinas-Lichius *et al.* 2000)

NERVE FIBRE COUNTS

After neurotmesis, the regenerating axon produces up to twenty neurites, explaining the large increase in the number of fibres found in regenerating nerves (Bray & Aguayo 1974). If these neurites fail to establish peripheral, end organ connections these neurites die and the total number of fibres decreases with time (Aitken *et al* 1947). Absolute fibre counts may therefore be misleading, not representing the final state of nerve morphology although neurotrophic factors can cause an increase in neurite sprouting after nerve transection (see chapter 1). Measurements of nerve fibre counts can be absolute (where all fibres are counted in the nerve) or derived (where the number of fibres in one area is counted and the total number of fibres calculated by scaling up to the total area of the nerve (Mayhew 1992)).

MEASUREMENT OF FIBRE DIAMETER

With time, after repair of a peripheral nerve, maturation of nerve fibres takes place and fibre diameter increases as smaller fibres, which fail to make peripheral connections, die. The eventual size of regenerating nerve fibres is dependant to some degree on the diameter of the parent fibre but mainly on whether contact is made with an appropriate end organ (Aitken & Thomas 1962; Bray & Aguayo 1974). Fibres which fail to make appropriate end organ

connections may become myelinated but may fail to reach normal size (Sanders & Young 1947). Fibre diameters were measured across the minor axes of elliptical profiles (Mayhew 1990) to avoid bias arising from sections cut obliquely. Mayhew suggested that at least 200 fibres should be counted to give a representative sample of the whole nerve (Mayhew 1990).

MYELIN SHEATH THICKNESS

In regenerating nerves the myelination of smaller fibres is less than in larger fibres. Myelin sheath thickness is therefore different from regenerating nerves and normal nerves. A better variable to measure is the g-ratio (axon diameter / fibre diameter). The g-ratio is an indicator of nerve fibre maturation (Glasby *et al* 1986a).

Microscopy

NEURONE COUNTS

To count the total number of neurones in a nerve it was decided to count the absolute number of fibres. This has the advantage of accuracy, in that no calculations are required to derive the total number of fibres, as in the scaling technique as described by Mayhew (Mayhew 1992). In order to identify single neurones, and thus allow a count of the total number of neurones in a nerve section, it was necessary to obtain an image using a 40 times magnifying lens. An image of lesser magnification failed to differentiate between small and closely spaced neurones. The resulting 40 times magnification image only encompassed a fraction of the total nerve section. Moving the microscope platform and counting sequential high power fields was prone to error by counting fibres twice, or not at all. A novel technique was devised where the nerve section was digitised by a camera attached to a microscope and stored on computer as a TIFF file (Tagged Image File Format). TIFF is a compressed and highly flexible, platform-

independent format which is supported by numerous image processing applications. The nerve section could be reconstructed from multiple TIFF images (at 40 times magnification). Each TIFF file was 'copied' and 'pasted' into a blank TIFF file in an image manipulation computer software package (Photoshop 5, Adobe, USA). Thus an image of the whole nerve was built up from between two and forty TIFF images (figure 6.5). This final image was saved on computer hard disc and loaded into an image analysis computer software package (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada). With the AIS software, each neurone on the reconstructed image was marked using a mechanical pointing device ('mouse'). The program automatically recorded the number of marks made over the image and thus the number of neurones in each nerve section was calculated.

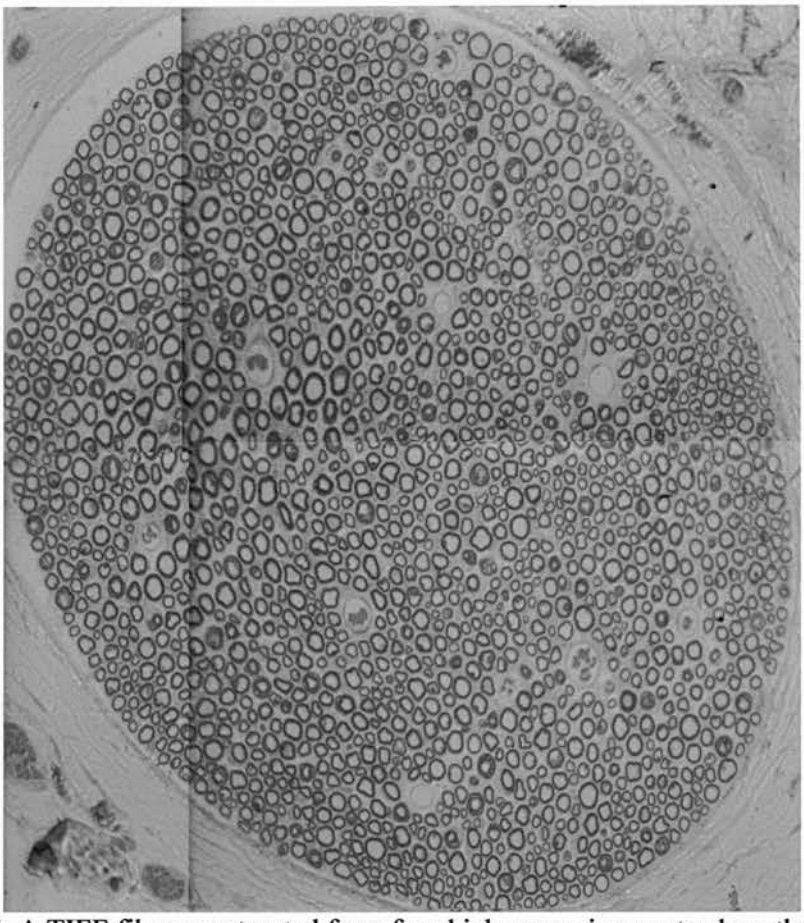


Figure 6.5. A TIFF file reconstructed from four high power images to show the entire nerve section.

This technique for counting nerve fibres was used in the rat facial nerve where the nerve section was small enough to enable the whole fibre to be reconstructed. In the sheep facial nerve, the nerve fibre was too large to allow reconstruction by this method. Also, in the sheep nerve, fixation of the sections was unreliable owing to their large size and not all fibres could readily be identified. In the rat, the repaired nerves (which tended to be larger than normal) were still small enough for their images to be reconstructed. Fixation of the smaller, rat nerve was also superior to the sheep facial nerve due to better penetration of fixative. In the rat, fibre counts were made on the nerve section removed at division and again on the nerve three months after repair. Thus it was possible to calculate a ratio of number of fibres after division and repair to number of fibres before division and repair (fibre ratio):

$$\text{Fibre ratio} = \frac{\text{number of fibres after division and repair}}{\text{number of fibres before division and repair}}$$

In the sheep and in the rat, morphological variables measured were:

Axon diameter

Fibre diameter

Myelin sheath thickness

G-ratio.

In addition to these measurements, fibre ratio was calculated in the rat experiments.

MEASUREMENTS OF SIZE

Nerve sections were viewed under 100 times magnification using an oil immersion technique. The dry side containing the semi-thin section of nerve embedded in araldite was placed on a microscope platform (Jenamed Variant, Carl Zeiss) and a drop of oil was placed over the section. No cover slip was used, resulting in a more focused image than if a cover slip was used. A beam splitter was used with the microscope, which allowed binocular vision of the section with 10 times magnifying eyepieces resulting in a effective magnification of 1000 times, and a camera (Kestril 25) image to be displayed. This camera image was input directly to a commercial image analysis computer software package (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada). Several images (figure 6.6) of the centre of the largest fasicle of each nerve were obtained and stored on hard disc as a TIFF file.

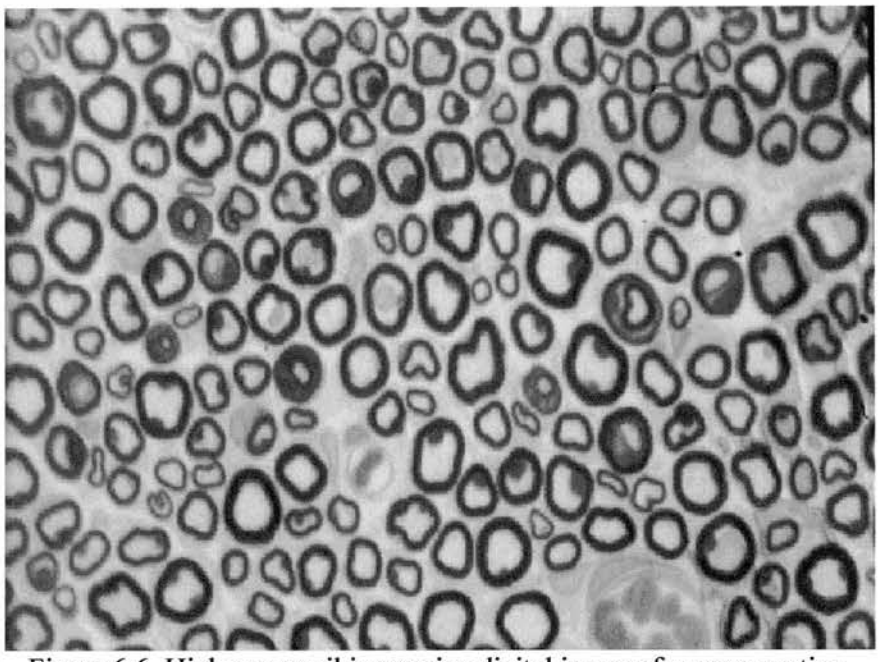


Figure 6.6. High power oil immersion digital image of a nerve section.

After images of all nerve sections had been digitised in this way they were later analysed by the image analysis program. This system required calibration, and the image of a 2mm graticule with 10μm divisions was similarly digitised using oil immersion microscopy. The computer program recorded this calibration and this was used for all measurements to be obtained. Measurement of the axon and fibre diameters were obtained on 300 neurones of each nerve section. Fibre diameters were measured across the minor axes of elliptical profiles to avoid bias arising from sections cut obliquely. The myelin sheath thickness was calculated by the formula:

$$\text{Myelin sheath thickness (μm)} = \frac{\text{Fibre diameter (μm)} - \text{Axon diameter (μm)}}{2}$$

The g-ratio was calculated by the formula:

$$\text{G-ratio} = \frac{\text{Axon diameter (μm)}}{\text{Fibre diameter (μm)}}$$

These measurements were exported from the AIS program to a spreadsheet (Excel, Microsoft, USA).

Chapter 7

Electrophysiology in the sheep

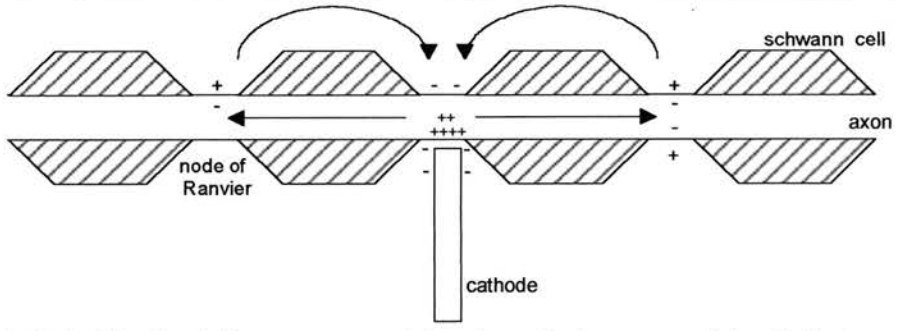
Introduction

Although electrical stimulation of nerve and muscle, as an aid to clinical diagnosis, has been used for more than one hundred years it has only been since the Second World War that these techniques achieved widespread use. Electrophysiology cannot replace the skill of clinical neurological examination, it can, however, provide more objective information on the function of the motor unit and sensory nerves. Electrophysiology can also be used in an animal model where clinical examination is unreliable and voluntary movement is unpredictable. The motor unit is an 'individual nerve cell and the bundle of muscle fibres it activates' (Sherrington 1929) and consists of the nerve cell, nerve fibre and its terminal branches, neuromuscular junction and the muscle fibres and constituent myofibrils. Each motor neurone innervates from less than twenty to more than one thousand muscle fibres, depending on how much fine motor control is required. A normal muscle fibre is innervated by only one neurone.

The mammalian peripheral nerve

Within the mammalian nerve cell there exists a very different microenvironment from that of the extracellular fluid. Within the cell there is an abundance of potassium ions and a dearth of

sodium ions. These cations together with anions maintain the osmolarity of the cell, however an electrical gradient exits, with the cell being negative relative to the extracellular fluid. This membrane potential is approximately -90 mV inside the axon and -70 mV inside the cell soma. An action potential is generated when the potential difference across the cell membrane reaches approximately -65 mV (that is, when the cell is depolarised to the threshold level). In normal function this occurs owing to influence by other neurones or end organs. Experimentally it can be induced by electrical stimulation of the axon. In a myelinated nerve, layers of phospholipid membrane from the ensheathing schwann cell insulates the axon. This layer is deficient at the node of Ranvier, where current can flow between intracellular and extracellular fluid. The application of a cathode to the nerve causes an accumulation of positive charges in the axon at this point. This depolarises the axon and when threshold is reached an action potential is generated, involving a complex series of ionic permeability changes in the cell membrane and the axon at this point becomes momentarily positively charged. In the cell, current flows from the depolarised area to the negatively charged areas both proximally and distally to the site of stimulation. An opposing current flows in the opposite direction through the extracellular fluid. With this flow of current bidirectionally, from the point of stimulation, the nodes of Ranvier on either side become depolarised and two action potentials are generated, one travelling orthodromically, the other travelling antidromically (figure 7.1). This 'saltatory' conduction propagates rapidly as current jumps from one node of Ranvier to the next. In a motor neurone, stimulated artificially in this way, conduction travels bidirectionally to the cell body and the neuromuscular junction.



1. Cathodal stimulation causes a positive charge in the axon and depolarisation
2. Action potential generated with large positive charge generated
3. Current spread to nodes of ranvier at each side
4. Further depolarisation at next node and cycle repeats with AP propagation

Figure 7.1 Saltatory conduction

Refractory period

For a short time (0.5 - 1 ms) after the initiation of an action potential in a neurone, the cell becomes unable to generate a further action potential regardless of the intensity of the stimulus delivered. This is the absolute refractory period. The cell then gradually recovers to be excitable only by a stimulus which is larger than that which would normally be required to generate an action potential. This period of reduced excitability is the relative refractory period (usually 3 - 5 ms). Underlying the refractory period is the inability of sodium channels in the cell membrane to respond immediately to repeated stimulation. The clinical value of refractory period measurement is uncertain (Kimura 1981). The refractory period is however, prolonged in low temperature (Paintal 1965), advanced age (Delbeke *et al* 1978) and after experimental demyelination (Smith & Hall 1980).

The neuromuscular junction

The neuromuscular junction is a synaptic structure consisting of the motor nerve terminal, junctional cleft and the muscle end plate. Within the motor nerve terminal, vesicles of acetylcholine are stored and released by a calcium-dependant depolarisation of the neurone. The acetylcholine released by the nerve terminal acts upon the motor end plate of the muscle cell to increase membrane permeability, mainly to sodium and potassium ions. As in nerve cells, potassium has a high, and sodium has a low intracellular concentration in muscle. This increase in membrane permeability results in sodium and potassium flux and depolarisation of the motor end plate. Again, as with neural tissue, if this depolarisation reaches a certain level (threshold) an action potential is generated. This propagates bidirectionally along the muscle fibre. There is therefore a delay in the activation of a muscle action potential by a nerve action potential, the neuromuscular delay. This represents the time taken for the release of acetylcholine, its time to cross the neuromuscular gap and trigger the ionic changes in membrane permeability.

The summated muscle action potential may be recorded by electrodes placed over the surface of the muscle, either directly or through skin. The cathode (negative electrode) is positioned over the muscle belly and the anode (positive electrode) over the muscle tendon with a galvanometer between the two electrodes. Initially there is no potential difference between the electrodes (figure 7.2a), with the muscle fibre being equally negative under both electrodes. On an oscilloscope, the trace is flat. When depolarisation of the muscle occurs, the cell becomes momentarily positively charged and this results in a negative deflection (by convention, upward) on the oscilloscope (figure 7.2b). When the action potential impulse reaches the midpoint of the electrodes there is again no potential difference between electrodes

and the trace crosses the baseline (figure 7.2c). When the action potential impulse reaches the anode a positive deflection occurs resulting (by convention) in a downward trace (figure 7.2d). The trace then returns to the baseline at the end of the action potential (figure 7.2e). The trace obtained from the muscle action potential is called the 'm-wave' or 'cMAP' (compound muscle action potential). The m-wave obtained in this way is biphasic.

Electrophysiology is an integral and routine part of neurological investigation. Nerve conduction studies are reliable for accurate characterisation of peripheral nerve function (Kimura 1989). These studies have been used clinically and experimentally (Fullarton & Glasby 1997; Glasby *et al* 1997; Glasby *et al* 1988) to assess nerve regeneration and repair.

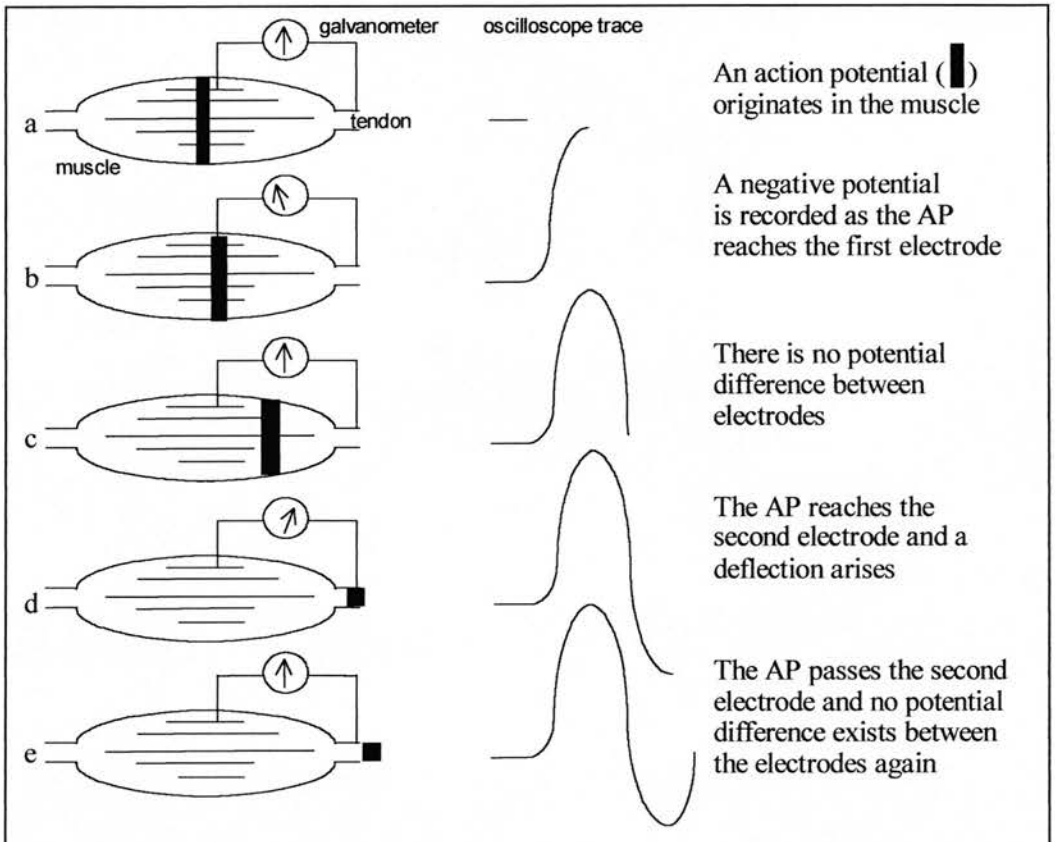


Figure 7.2 A muscle compound action potential

Measurement of conduction velocity

In 1944, Berry *et al* showed that the conduction velocity in a regenerating nerve fibre was slowed (Berry *et al* 1944). Since then this test has been used extensively in a clinical setting. It is not possible to calculate nerve conduction velocity by stimulating a nerve, a known distance from the muscle and recording a contraction after a measured time interval, because this time interval for muscle contraction consists of not only of the time taken for the impulse to travel along the nerve but also of the neuromuscular delay. To eliminate the neuromuscular delay, two points on the nerve are stimulated independently (S1 and S2 in figure 7.3). The time for muscle contraction is recorded from both points and the difference between the time to trigger

muscle contractions is known as the interstimulus interval. The maximum conduction velocity in the nerve is thus:

$$CV_{max} = \frac{d}{t}$$

Where CV_{max} = maximum conduction velocity (ms^{-1})

d = distance between stimulating electrodes (mm)

t = interstimulus interval (ms)

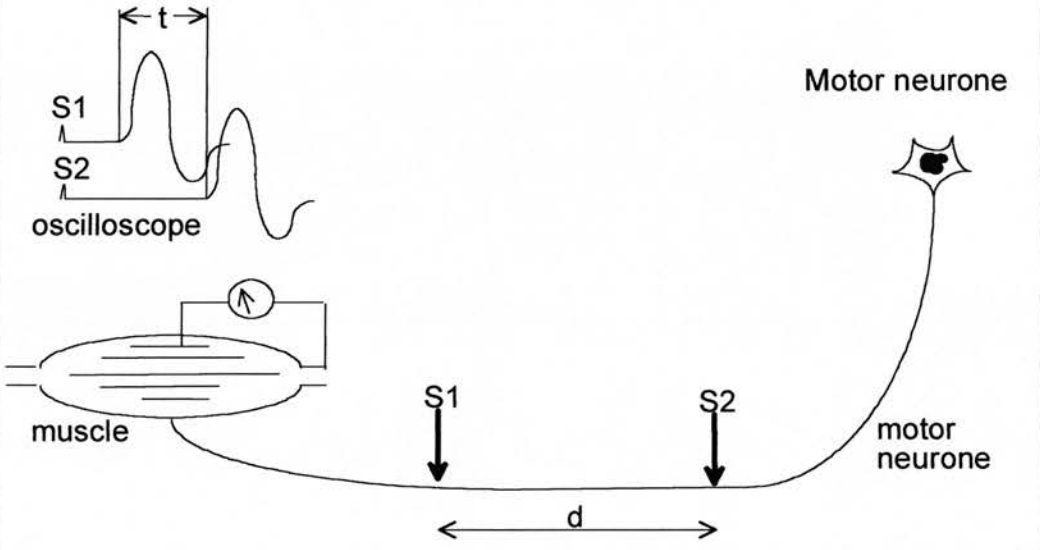


Figure 7.3 The measurement of nerve conduction velocity

As there is a range of conduction velocities in a nerve this only represents the fastest fibres which will first trigger a muscle contraction.

Measurement of refractory period

Measurement of refractory period has been investigated in experimental animals and in clinical practice. The 'paired shock technique' is used to stimulate a nerve at one point and apply a second stimulus after a time interval, *t* (figure 7.4). At the end of the absolute refractory period a small additional trace is seen on the first m-wave. This can be better identified by digital subtraction of the m-wave obtained from the first single stimulus. At the end of the relative refractory period a further m-wave, identical to the first is seen, and again by the use of digital subtraction of the initial wave this second m-wave is more easily identified. In figure 7.4, *t*1 represents the end of the absolute refractory period and *t*2 represents the end of the relative refractory period.

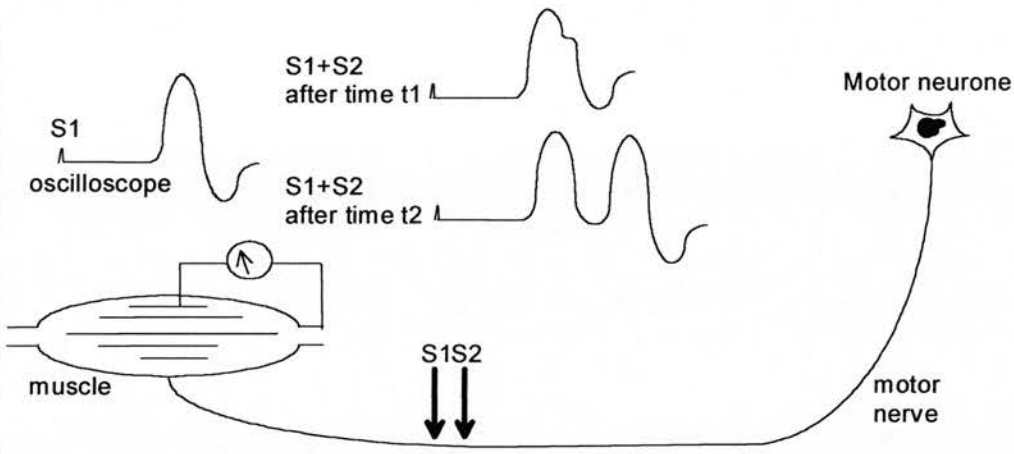


Figure 7.4 The paired shock technique to assess refractory period

Measurement of minimum conduction velocity

The measurement of conduction velocity above, only identifies the fastest nerve fibres. Using the technique of 'collision' (Thomas *et al* 1959) the velocity of the slowest conducting fibres

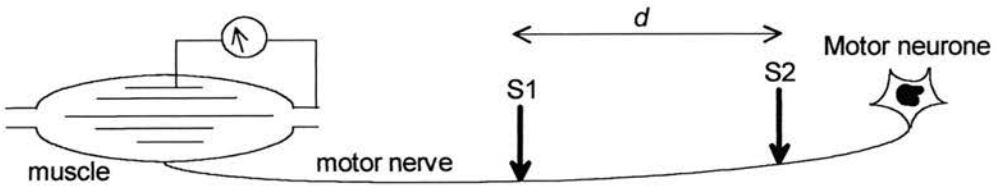
can be obtained. With this technique two points on the nerve are stimulated supramaximally (S1 and S2 on figure 7.5). When both stimuli occur together impulses travel orthodromically from S1 to the muscle producing a compound action potential. Impulses also travel from S1 antidromically towards S2. Impulses from S2 travel orthodromically and collide with the distal impulses cancelling each other out. No compound action potential is thus produced from the S2 stimulus (figure 7.5a). If S1 and S2 are separated by a time interval the antidromic impulses from S1 will travel towards S2 before the S2 stimulus is triggered. If the time interval is just long enough for impulses in the fastest conducting fibres to pass the point S2 before a stimulus occurs, these fastest conducting fibres will escape collision. The slower conducting fibres will still collide when the S2 stimulus is triggered and cancel each other out (figure 7.5b). Thus a second compound action potential is recorded from only the fastest conducting fibres. As with the technique of recording refractory period this can be better identified by digital subtraction of the m-wave obtained from the first stimulus. If the time interval between S1 and S2 is just long enough for the slowest conducting fibres to pass the S2 point before a stimulus occurs all fibres will escape collision and a second full compound action potential will be recorded (figure 7.5c). If the distance, d between electrodes, and the interstimulus interval which just produces a full second compound action potential, $t2$ are known the minimum conduction velocity can then be calculated from the formula:

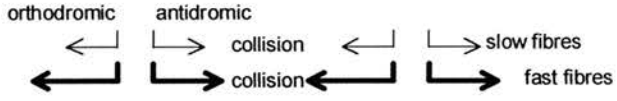
$$CV_{\min} = \frac{d}{t2}$$

where $CV_{\min}$ = minimum conduction velocity (ms^{-1})

d = distance between electrodes (mm)

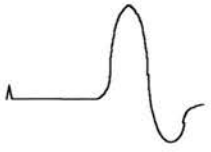
$t2$ = interstimulus interval (ms)

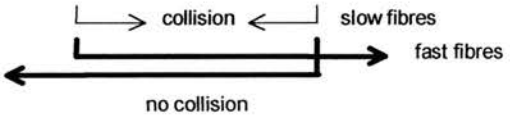




a) S1 and S2 simultaneously

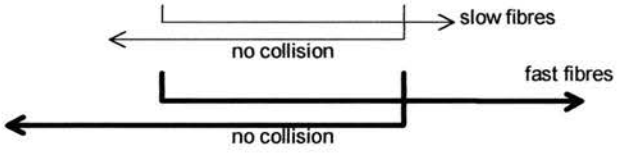
A cMAP is generated from stimulus, S1 but inpulses from S2 are cancelled by antidromic conduction from S1





b) S1 then S2 after time, *t*1

A cMAP is generated from S1 and a second cMAP is generated from impulses in the fast fibres from S2. Impulses from the slow fibres, stimulated by S2 collide with impulses from the slow fibres stimulated by S1. Thus, impulses from the slow fibres stimulated by S2 do not contribute to the second cMAP



c) S1 then S2 after time, *t*2

A cMAP from S1 and a second AP of equal size from S2 is generated from impulses from both stimuli as all fibres escape collision

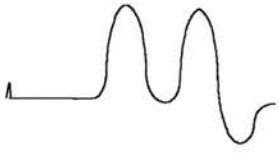


Figure 7.5 Technique of collision to calculate speed of the slowest conducting fibres

Electromyographic jitter

Single fibre electromyography (SFEMG) was developed (Stålberg & Trontelj 1979) to assess the microphysiology of the motor unit — terminal axon, neuromuscular junction and muscle fibre. Stimulated jitter, an SFEMG technique, is a means of measuring the variation in transmission at the neuromuscular junction. It involves the stimulating of a terminal motor axon and the recording of an associated muscle fibre's action potential.

Stimulating a nerve fibre repetitively results in almost, *but not exactly* the same latencies of a muscle fibre action potential (figure 7.6). This variability, in the order of tens of microseconds represents electromyographic jitter. It has been shown that there is normally little variability in the transmission of the impulse in the axon (Stålberg & Trontelj 1979; Trontelj *et al.* 1986) or muscle fibre (Trontelj *et al* 1990) during the jitter analysis. It is postulated therefore that the site of variability in neuromuscular transmission is at the end plate.

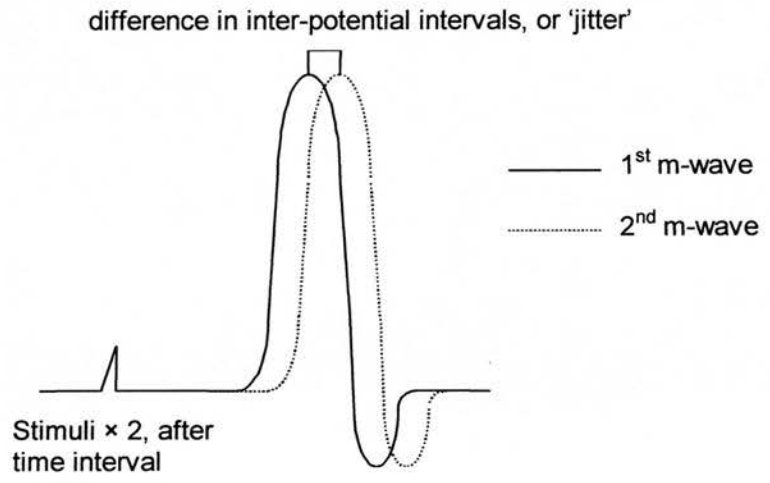


Figure 7.6 Electomyographic jitter

Transection of a peripheral motor nerve results in Wallerian degeneration of the distal nerve and degeneration of the neuromuscular junction. With a surgical repair, proximal axons grow to re-establish a neuromuscular junction. In the immature motor unit there is a large variability in the time for neuromuscular transmission due to lower and more variable threshold potentials at the motor end plate (Stålberg 1990; Stålberg & Trontelj 1979). As maturation of the motor end plate continues threshold potentials rise and stabilise. It has been shown in rats, that jitter values after transection and repair of a peripheral motor nerve were highest in the early stages after repair and declined to normal within 90 days of repair (Lenihan *et al.* 1997). SFEMG jitter may thus offer a means of assessment of the quality of nerve repair by assessing the state of the neuromuscular junction, which nerve conduction studies and assessment of refractory periods cannot do. SFEMG techniques such as jitter offer a more specific assessment of muscle action potentials. The SFEMG active electrode is a steel needle 0.5 mm in diameter with an insulated silver wire in its lumen. A 25 μm opening 3 mm from the tip of the needle contains the active electrode. This small area for recording increases the probability that the action potentials recorded will be from the same motor unit. Filtering

offers another method of increasing the selectivity of the recorded action potentials. Since muscles act as a low pass filter, they will remove high frequency signals originating from more distant muscle fibres. Thus, recordings from fibres situated near the active electrode will contain a relatively higher proportion of high frequency components. If the filter on the EMG amplifier is set at 500 Hz, impulses from distant muscle fibres will be removed while affecting muscle fibres adjacent to the active electrode only by a factor of 10% (Stålberg & Trontelj 1979). Parameters for jitter recordings have been defined. The stimulating pulse should be of 50 μs duration and of amplitude between 1 mA and 25 mA. The recorded amplitude should be >0.5 mA, with a rise time of <200 μs and a duration of 1-2 ms.

The latency between the stimulating pulse and the muscle fibre action potential is called the inter-potential interval (IPI). Each axon is stimulated at least 50 times and the IPI for each action potential is recorded. The jitter from the end plate which produces the action potential is calculated as the mean consecutive difference of the latencies (MCD) using:

$$MCD = \sum \left(\frac{|ipi_n - ipi_{n-1}|}{n-1} \right)$$

where:

MCD = mean consecutive differences (μs)

ipi = inter-potential interval

n = number of stimulations

Previous work has shown that for a jitter study to be meaningful, a population of at least 20 muscle fibres pairs should be assessed. The MCD for each recording is thus calculated and the mean of these values is the jitter value for that muscle. If the muscle was stimulated

directly, as evidenced by a jitter value of <5 μs (Stålberg *et al* 1992; Trontelj *et al* 1990), or if an adjacent motor axon was inadvertently stimulated to produce summation with the action potential under study, that reading was discarded.

Methods

Electrophysiological testing of the facial nerve in the sheep was carried out under general anaesthesia as described in chapter 4. The face of the animal was shaved with electric clippers from the midline over the nasal bones, under the eye and over the nose to the corner of the mouth. The skin of the sheep was then cleaned with chlorhexidine. A ground electrode was placed on the skin over the nasal bone.

Electromyographic jitter was recorded from the depressor labii maxillaris muscle. All recordings were made by a Medelec Sapphire 4 ME electrophysiology recorder. Two monopolar needles (Medelec MF37) were used as the stimulating anode and cathode. The cathode was inserted into the muscle midway between the external nares and the angle of the mouth. The anode was placed 0.5 cm rostral to the cathode. Stimulus pulses of 10 Hz frequency, 50 μs duration and 1 mA amplitude were used initially. The current was increased gradually until small twitches were observed in depressor labii maxillaris. At this stage an SFEMG needle recording electrode (Medelec SF25) was inserted into the muscle 2 cm caudal to the stimulating cathode. The position of the SFEMG needle electrode was adjusted until satisfactory recordings of muscle action potentials could be seen. The current was increased to 10% above maximum. The maximum current was defined as current at which no further increase in the amplitude of the m-wave would result from a further increase in the current. 20 values of jitter was then assessed as above. After each five recordings of jitter, the recording

needle electrode was moved by a few millimetres to assess another population of muscle fibres.

In animals which had undergone surgical repair of the nerve, the original incision was reopened and the buccal division of the facial nerve identified. The buccal division of the nerve was then dissected free from associated tissue as in chapter 4. As long a length as possible was exposed. The portion of the nerve surrounded by the parotid gland was not dissected. Previous experience had shown that if parotid secretions were allowed to come into contact with the nerve it would fail to stimulate electrically. The buccal division of the nerve was dissected from just distal to the parotid gland to the corner of the mouth. All stimulations and recordings were carried out using a Medelec Sapphire 4ME EMG recorder (Medelec, Old Woking, UK).

A 6 mm silver / silver chloride recording electrode (cathode) was placed over the motor point of the depressor labii maxillaris. A second 6 mm silver / silver chloride disc recording electrode (reference anode) was placed on the skin 1 cm superior to the insertion of the levator nasolabialis muscle over the nasal bone. A small amount of electrode cream (Grass Instruments Co., Maine, USA) was placed on each electrode to improve both electrode adhesion and m-wave recording.

A non-traumatic bipolar platinum wire stimulating electrode (S_1) was place on the buccal division of the facial nerve distal to the site of nerve repair, approximately 5 mm proximal to the zygomaticus muscle. A square wave of 50 μs duration of supra-maximal current intensity (30% above maximum stimulation) was then delivered to the nerve. The resulting m-wave recorded from depressor labii maxillaris was digitally filtered and amplified before being

displayed on the EMG recorder screen. The change in voltage from the isoelectric baseline to the peak (point of inflection) of the m-wave was taken as the m-wave amplitude. Where two or more peaks were present in the negative (upward) phase of the m-wave, the amplitude was taken as the distance from the baseline to the largest peak. Clinical electrophysiology conventions were followed so that the area of the m-wave was defined as the area of the negative phase of the m-wave.

A second bipolar stimulating electrode (S_2) was placed on the facial nerve proximal to the site of repair ensuring that the distance between both stimulating electrodes was greater than 5 cm. The nerve was then stimulated with the same intensity as above.

The above methods were then used to obtain the following four variables:

1. maximum conduction velocity
2. minimum conduction velocity
3. absolute refractory period
4. relative refractory period.

Chapter 8

The establishment of a model for the electrophysiological assessment of the facial nerve in the rat

Introduction
Methods
Results
Discussion
Conclusion

Introduction

There is no ideal animal model for the study of the facial nerve. Models used extensively include the cat (McGuirt & McCabe 1977; Yamamoto & Fisch 1975), rabbit (Spector *et al.* 1993) and sheep (Drew *et al.* 1995; Glasby *et al* 1995; Glasby *et al* 1993). While large animals may be more relevant to human neurosurgery, their cost can prohibit their use. If it is feasible to carry out studies on large animals, these must entail small numbers of animals (because of cost and legal requirements) and large animals are more prone to anaesthetic difficulties. Novel techniques of nerve repair, grafting and the use of neurotrophic factors must first be piloted on smaller laboratory animals to test the effectiveness of these techniques. The rat is a commonly used laboratory animal and within the financial resources of even small studies. The rat has been described as an excellent model for surgical experimentation on the facial nerve (Mattox & Felix 1987; Murray 1991). These studies have not however, been able to use electrophysiology in the assessment of the rat facial nerve, but have relied on morphometric analysis of the nerve. Electrophysiology is not only the gold standard in the assessment of nerve function, but is also the test used in the human when evaluating this function.

In the rat, the length of the facial nerve from the stylomastoid foramen to its distal branches is only 3 cm at most. It is therefore not possible to carry out nerve conduction testing with proximal and distal stimulating electrodes. The new generation of EMG recorders, however, are sensitive enough to allow meaningful and consistent recording from stimulating the rat facial nerve by a single electrode. In the present study, a model for the electrophysiological assessment of the rat facial nerve, was developed, similar to the clinical assessment of the facial nerve in humans, popularised by Fisch (Gantz *et al.* 1984). This technique will enable research to progress in nerve repair using this animal model.

Methods

Anaesthesia of the rats was achieved as described in chapter 3. Two groups of rats were studied. In one group, electrophysiological recordings were obtained from one, previously divided and repaired facial nerve and compared with the other, normal facial nerve in the same animal (GROUP 1). In another group, the electrophysiological recordings from one facial nerve were compared with the recordings from the other facial nerve in the same animal (GROUP 9).

GROUP 1

Anaesthesia

Anaesthesia was as described in chapter 3.

Surgery

Surgery was as described in chapter 3, Group 1. These animals were allowed to recover and kept for three months before electrophysiological assessment.

GROUPS 1 & 9

Anaesthesia

Anaesthesia was as described in chapter 3.

Surgery

The animal's fur on both sides of the face was shaved with electric clippers. An area on the nasal bones below the eyes and an area on the back of the animal were also shaved. Immac (Reckitt and Colman Ltd, Hull, UK) hair removal cream was applied to the area caudal to the mouth for about 1 cm^2, the shaved area on the nose and the shaved area on the back in order to produce a smooth surface for optimal electrical contact. The cream was left for 5 minutes then removed and the animal's skin was washed with chlorhexidine. An incision was made below the lower border of the mandible and the skin was retracted until the buccal division of the facial nerve was seen. Surgery was then carried out using an operating microscope (Wild, Heerbrugg M690) with stepless variable magnification of 3.5 to 18 times. The buccal division of the left facial nerve was dissected free of associated fascial tissue. This division was dissected in a proximal direction to the jugular vein, which was coagulated using bipolar diathermy, taking care to avoid current spread to the nerve. The furcation of the nerve was identified and the main trunk of the nerve exposed to the stylomastoid foramen. At the stylomastoid foramen the nerve trunk was transected with microscissors. All other branches of the facial nerve, except the buccal branch were exposed and transected close to the furcation. This left the buccal division of the facial nerve solely in continuity with the main trunk. The main trunk of the nerve was stimulated by a non-traumatic, bipolar, platinum wire electrode (figure 8.1) and stimulation and recording were performed using a Medelec Sapphire 4ME EMG recorder (Medelec, Old Woking, UK).

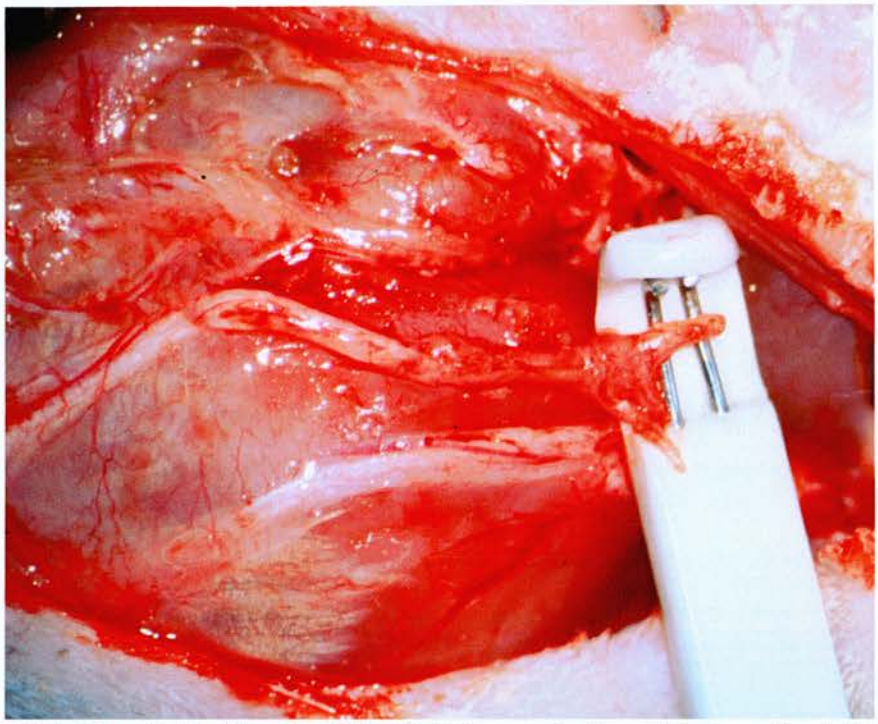


Figure 8.1. The divided trunk of the facial nerve in the rat being stimulated by a bipolar electrode.

Stimulation caused contraction of the small muscles of nose and upper lip. A 6 mm silver / silver chloride disc recording electrode (Medelec, Old Woking, UK) (the cathode) was placed just caudal to the angle of the mouth and the non-vibrissae-bearing skin. A second 6 mm silver / silver chloride electrode (the anode) was placed over the nasal bones in the midline. A ground electrode was placed over the back of the animal. A small amount of electrode cream was placed on each of the three electrodes to improve electrode adhesion and electrical contact. Placement of the cathode at this site gave the maximal amplitude for recording of the m-wave. A square wave of 50 μs duration of supra-maximal current intensity (130% of maximum) was then delivered to the facial nerve. This was repeated to give 10 m-wave recordings, which were digitally filtered (3 Hz low frequency filter, 10 kHz high frequency filter and sweep speed of 20 ms per division) and amplified before being displayed on the EMG recorder's screen. One such trace is shown in figure 8.2.

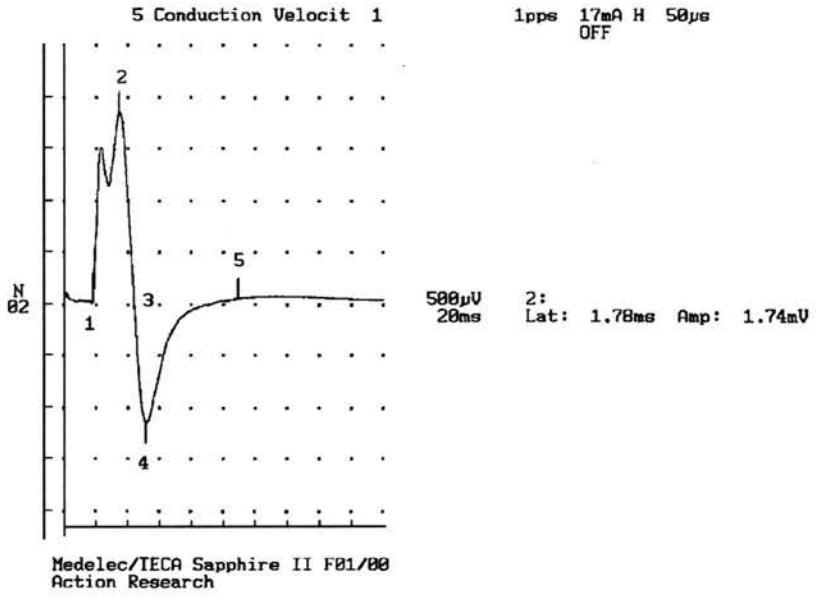


Figure 8.2. The m-wave recorded from the facial muscles in the rat from stimulation of the buccal division of the nerve.

The EMG recorder produced four variables:

1. latency to the start of the m-wave.
2. latency to the peak of the m-wave, where there was two or more peaks present in the negative phase of the m-wave the latency to the largest peak was recorded.
3. maximum amplitude of the m-wave, where there was two or more peaks present in the negative phase of the m-wave the amplitude was taken as the distance from baseline to the largest peak.
4. area under the upward (negative) part of the m-wave.

These variables are shown in figure 8.3.

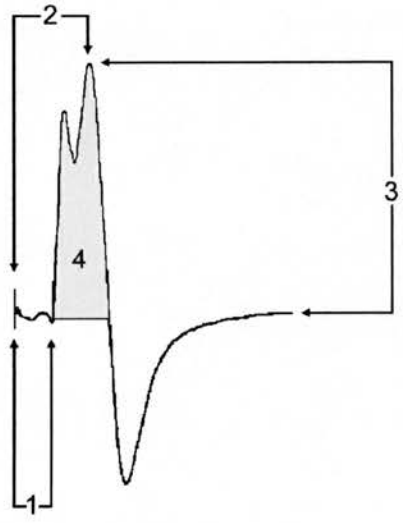


Figure 8.3. The variables from the m-wave, recorded in the rat.

Because there is no 'gold standard' to compare the results obtained on electrophysiological testing, it is necessary to compare one side with the other. It is assumed that these electrophysiological values are very similar, as it is assumed in clinical testing in the human. If these results are similar it suggests that the method of testing is accurate. The results were subjected to the Kolmogorov-Smiranov test for normality, to show that the data was normally distributed. The parametric t-test for dependant samples was then used to identify differences between groups. To decide if there was agreement between right and left sides of the same animal, these data were subjected to a Bland and Altman plot (Bland & Altman 1986). Data such as this are often analysed inappropriately by correlation coefficients (Bland & Altman 1986). The Bland and Altman plot was developed to compare two different methods of measuring the same variable, however is applicable, as in this case, to the measurement of variables from both right and left sides from one animal. This statistical technique is based on the graphical presentation of data and simple calculations.

Results

The latencies to the m-wave and latencies to the peak of the m-wave, including the standard error of the means, in each animal in group 9, are shown in table 8.1. Table 8.2 shows the amplitude of the m-wave and area under the negative peak of m-wave, including the standard error of the means, in each animal.

Rat	Mean left m-wave latency (ms) ± SEM	Mean right m-wave latency (ms) ± SEM	Mean left peak latency (ms) ± SEM	Mean right peak latency (ms) ± SEM
1	1.678 ± 0.009	1.722 ± 0.005	3.650 ± 0.004	3.664 ± 0.005
2	1.758 ± 0.013	1.644 ± 0.015	4.038 ± 0.019	3.944 ± 0.025
3	1.324 ± 0.005	1.362 ± 0.008	2.340 ± 0.148	3.550 ± 0.006
4	1.670 ± 0.007	1.668 ± 0.003	2.336 ± 0.007	2.890 ± 0.007
5	1.624 ± 0.005	1.682 ± 0.004	3.912 ± 0.009	4.290 ± 0.004
6	1.512 ± 0.005	1.544 ± 0.009	2.600 ± 0.006	2.560 ± 0.009
7	1.828 ± 0.003	1.762 ± 0.008	2.470 ± 0.003	2.778 ± 0.028
8	2.280 ± 0.003	2.130 ± 0.004	4.336 ± 0.008	4.650 ± 0.011
9	1.510 ± 0.015	1.608 ± 0.005	2.196 ± 0.007	2.792 ± 0.005
10	1.824 ± 0.012	1.828 ± 0.031	3.774 ± 0.008	4.308 ± 0.017
11	1.695 ± 0.012	1.735 ± 0.019	4.110 ± 0.039	3.417 ± 0.059
12	1.600 ± 0.000	1.733 ± 0.033	3.463 ± 0.246	3.573 ± 0.123

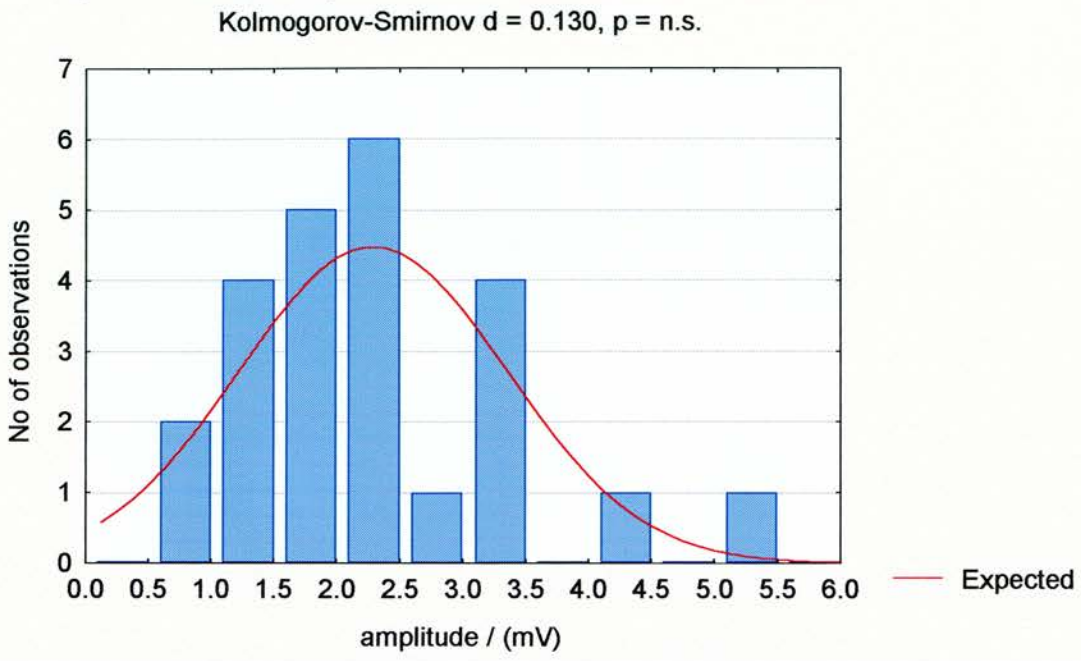
Table 8.1. The average latencies to the m-wave and the average latencies to peak of the m-wave (with standard error of the mean (SEM)) of the buccal branch of the facial nerve in 12 rats (group 9).

Rat	Mean left m-wave amplitude (mV) ± SEM	Mean right m-wave amplitude (mV) ± SEM	Mean left m-wave area (μVs) ± SEM	Mean right m-wave area (μVs) ± SEM
1	2.148 ± 0.028	1.746 ± 0.008	3.339 ± 0.035	2.926 ± 0.010
2	3.242 ± 0.015	1.333 ± 0.013	4.760 ± 0.033	2.334 ± 0.024
3	1.804 ± 0.016	1.373 ± 0.005	3.098 ± 0.015	2.540 ± 0.012
4	0.917 ± 0.005	1.224 ± 0.029	1.681 ± 0.023	2.396 ± 0.033
5	3.159 ± 0.008	3.374 ± 0.030	4.315 ± 0.009	5.551 ± 0.045
6	2.064 ± 0.005	2.449 ± 0.048	2.556 ± 0.009	2.320 ± 0.054
7	1.839 ± 0.011	1.878 ± 0.006	3.372 ± 0.011	3.650 ± 0.013
8	2.278 ± 0.013	3.125 ± 0.057	3.557 ± 0.014	5.825 ± 0.115
9	1.148 ± 0.013	0.964 ± 0.007	1.847 ± 0.025	1.882 ± 0.006
10	1.966 ± 0.010	2.128 ± 0.018	2.392 ± 0.011	3.534 ± 0.019
11	2.258 ± 0.132	5.381 ± 0.273	3.954 ± 0.222	7.451 ± 0.186
12	2.867 ± 0.128	4.253 ± 0.480	5.107 ± 0.205	5.657 ± 0.708

Table 8.2. The average amplitude of the m-wave and the average area under the negative peak of m-wave (with standard error of the mean (SEM)) of the buccal branch of the facial nerve in 12 rats (group 9).

The Kolmogorov-Smiranov test for normality was carried out on each of the four electrophysiological variables for the animals in group 9. One such test is shown in figure 8.4.

Figure 8.4 M-wave amplitude, R & L sides in normal rat facial nerves



The data in all four variable groups was normally distributed. Table 8.3 shows the results of t-tests for dependant variables carried out on each of the four electrophysiological variables between right and left sides.

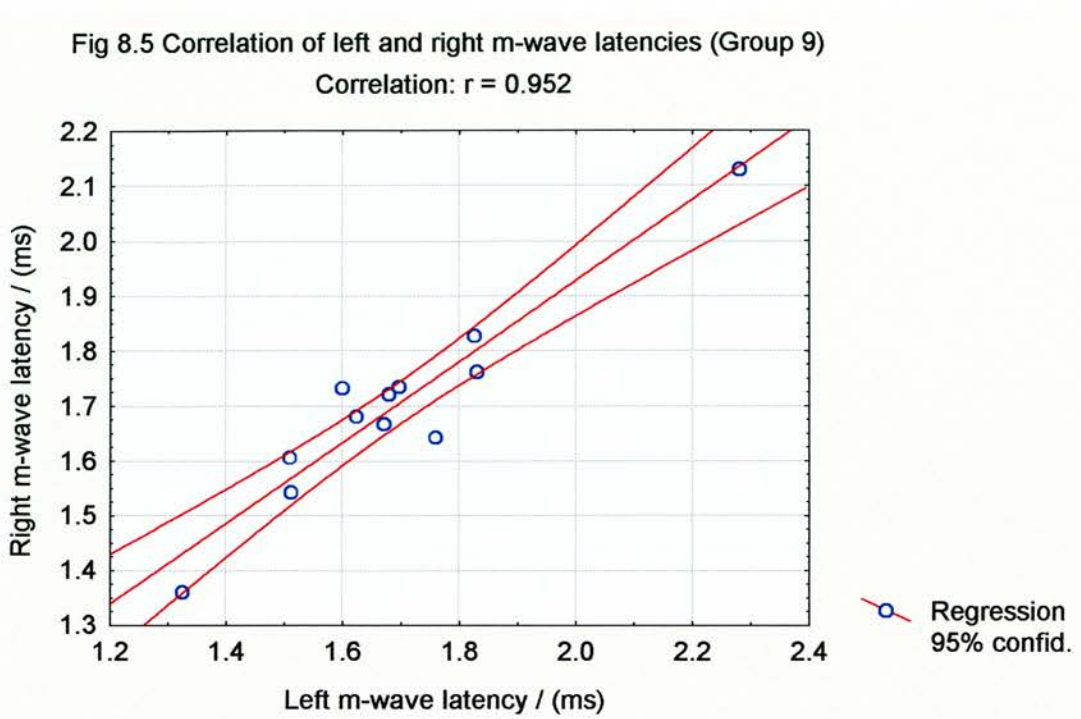
	Mean	Standard Deviation	n	t value	Degrees of freedom	p value
Left m-wave latency (ms)	1.692	0.234	12	-0.401	11	0.696
Right m-wave latency (ms)	1.702	0.181				
Left peak latency (ms)	3.269	0.812	12	-1.971	11	0.074
Right peak latency (ms)	3.535	0.681				
Left amplitude (mV)	2.141	0.709	12	-0.855	11	0.411
Right amplitude (mV)	2.436	1.355				
Left area (μVs)	3.331	1.090	12	-1.180	11	0.263
Right area (μVs)	3.839	1.818				

Table 8.3. t-tests for dependant variables carried out on each of the four electrophysiological variables between left and right sides (group 9).

There was no significant difference between the electrophysiological tests of the right and left nerves using a t-test for dependant variables. This does not, however, mean that there is good agreement.

The product-moment correlation coefficient (r) between the left and right m-wave latencies is 0.95 (figure 8.5). However, the correlation coefficient measures the *relation* between the two sides and not the *agreement*. Data which are in poor agreement may show high correlation coefficients.

Fig 8.5 Correlation of left and right m-wave latencies (Group 9)



Figures 8.6 - 8.9 show this data subjected to Bland and Altman plots. Here the zero line on the *y* axis indicates zero difference between the two sides. If the differences are normally distributed (which they are) 95% of these differences will lie within, 1.96 multiplied by the standard deviation, on either side of the mean difference. These are the 'limits of agreement'. Provided that differences within ± two standard deviations are not clinically important we can conclude that the measurements from right and left facial nerves are accurate enough to be used to assess nerve function.

Figure 8.6. Bland & Altman plot in normal rat facial nerves (Group 9)

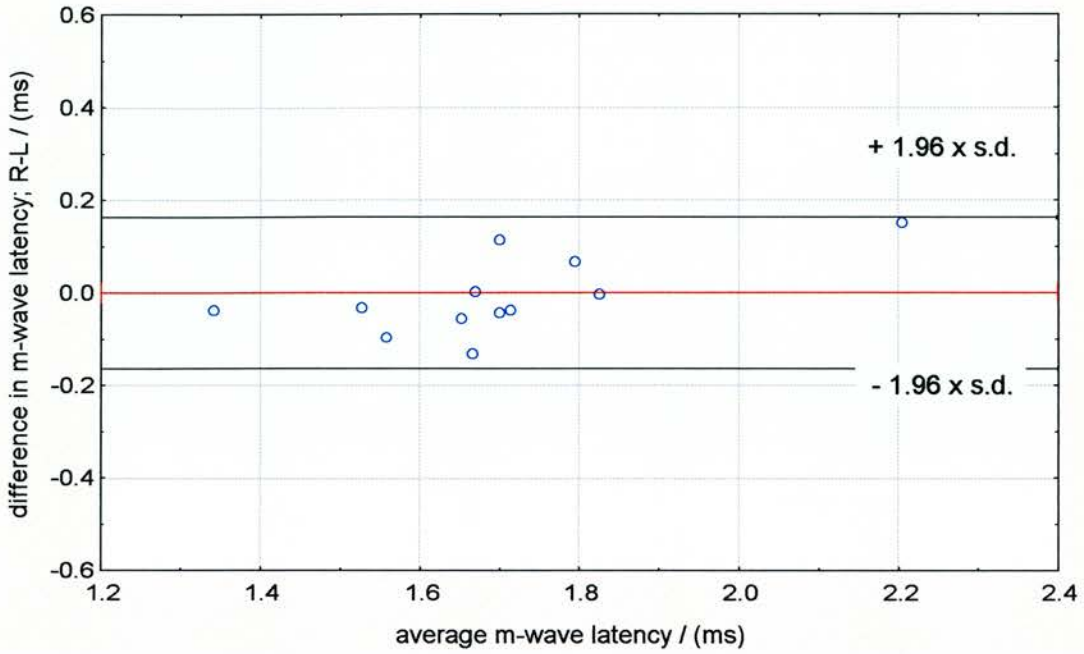


Figure 8.7. Bland & Altman plot in normal rat facial nerves (Group 9)

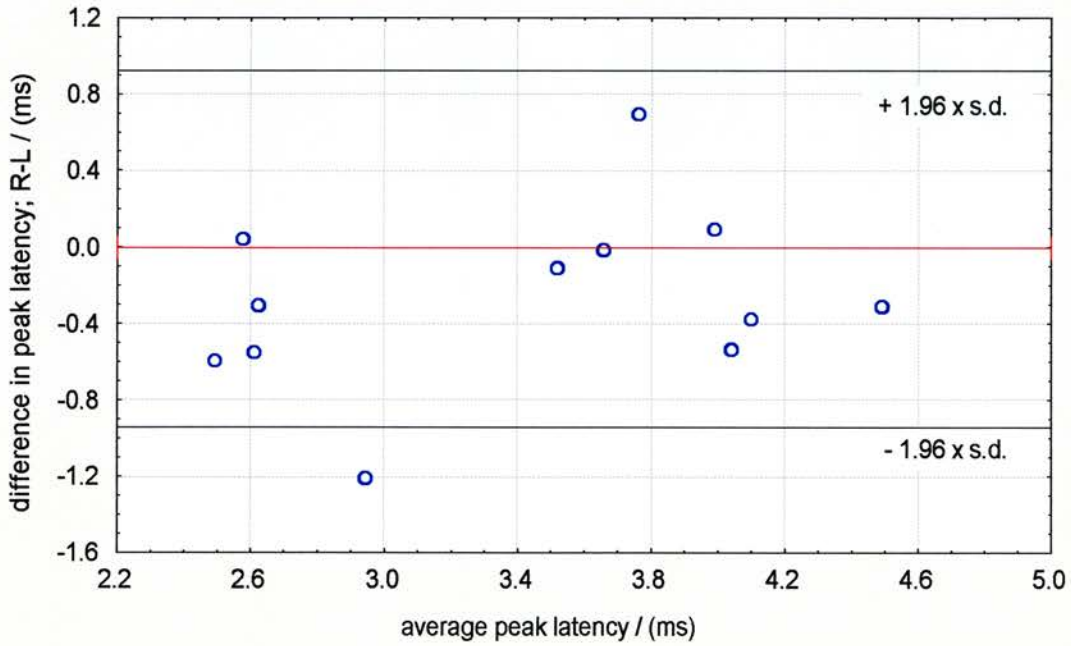


Figure 8.8. Bland & Altman plot in normal rat facial nerves (Group 9)

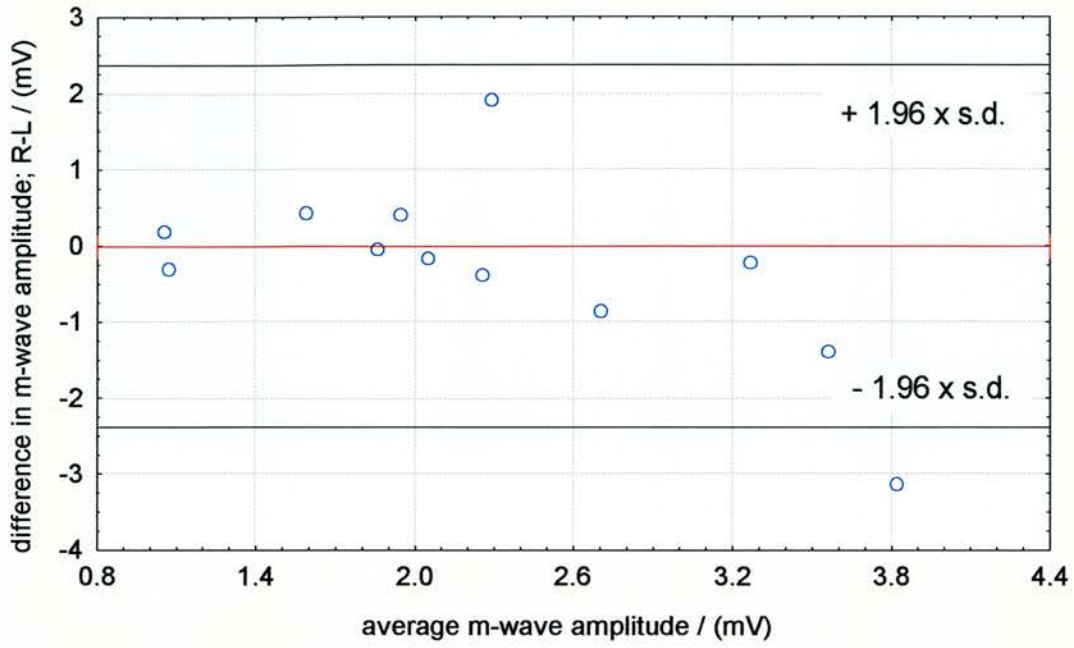
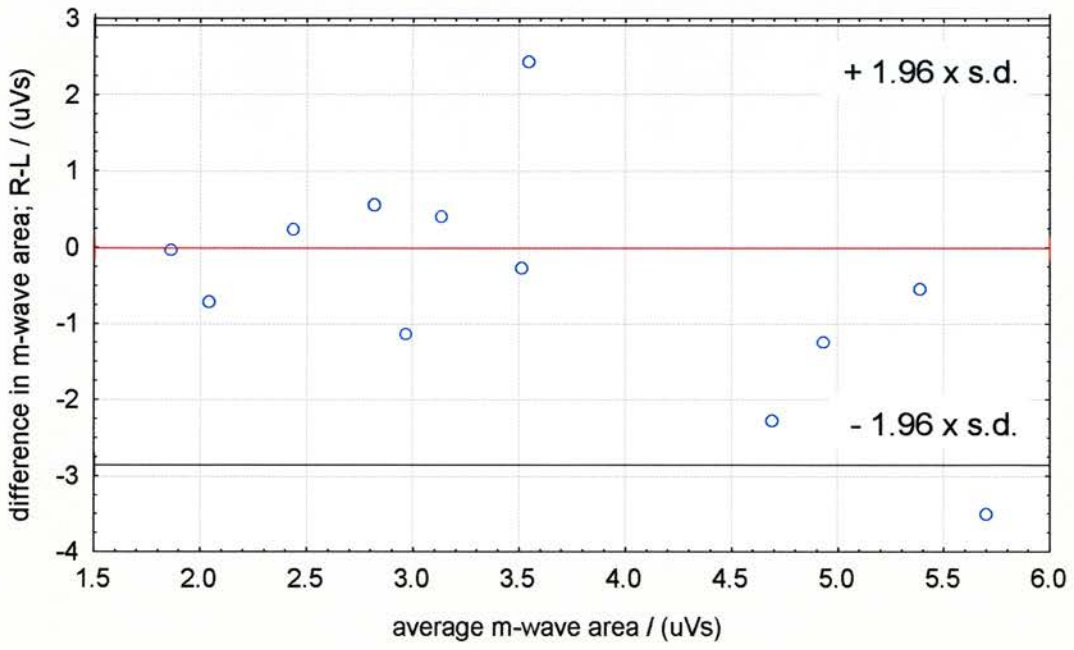


Figure 8.9. Bland & Altman plot in normal rat facial nerves (Group 9)



These 'limits of agreement', in other words, where 95% of electrophysiologically measured differences (95% confidence intervals) would lie are shown in table 8.4.

Electrophysiological variable	Limits of agreement
m-wave latency	0.162 ms
peak latency	0.916 ms
amplitude of m-wave	2.341 mV
area of m-wave	2.921 μVs

Table 8.4 Limits of agreement or 95% confidence intervals for the four electrophysiological tests.

It can be seen that the confidence intervals for the measured value of m-wave latency is ±0.162 ms. It is not known if measuring the m-wave latency to within ±0.162 ms is accurate enough to be used to detect differences between normal and repaired nerves. To determine this, it is necessary to compare these results in normal nerves to results in repaired nerves.

The latencies to the m-wave and latencies to the peak of the m-wave, including the standard error of the means, in each animal in group 1, are shown in table 8.5. Table 8.6 shows the amplitude of the m-wave and area under the negative peak of m-wave, including the standard error of the means, in each animal.

Rat	Mean left m-wave latency (ms) ± SEM	Mean right m-wave latency (ms) ± SEM	Mean left peak latency (ms) ± SEM	Mean right peak latency (ms) ± SEM
1	2.916 ± 0.050	1.756 ± 0.018	5.576 ± 0.126	5.236 ± 0.032
2	1.902 ± 0.007	1.574 ± 0.017	2.434 ± 0.004	4.858 ± 0.016
3	2.038 ± 0.013	1.650 ± 0.003	5.054 ± 0.012	4.322 ± 0.010
4	2.674 ± 0.034	1.956 ± 0.014	4.962 ± 0.076	3.672 ± 0.179
5	1.890 ± 0.018	1.650 ± 0.013	5.026 ± 0.020	4.082 ± 0.009
6	1.768 ± 0.014	1.506 ± 0.018	4.198 ± 0.024	3.328 ± 0.016

Table 8.5. The average latencies to the m-wave and the average latencies to peak of the m-wave (with standard error of the mean (SEM)) of the buccal branch of the facial nerve in 6 rats where the left nerve had been repaired 3 months before assessment (group 1).

Rat	Mean left m-wave amplitude (mV) ± SEM	Mean right m-wave amplitude (mV) ± SEM	Mean left m-wave area (μVs) ± SEM	Mean right m-wave area (μVs) ± SEM
1	0.184 ± 0.010	1.008 ± 0.010	0.849 ± 0.014	4.347 ± 0.024
2	0.108 ± 0.001	1.229 ± 0.016	0.082 ± 0.001	5.411 ± 0.020
3	0.203 ± 0.002	0.730 ± 0.004	0.520 ± 0.006	1.657 ± 0.009
4	0.083 ± 0.010	1.967 ± 0.051	0.109 ± 0.007	2.931 ± 0.036
5	0.281 ± 0.015	2.230 ± 0.016	0.563 ± 0.045	3.825 ± 0.019
6	0.447 ± 0.005	2.437 ± 0.012	0.868 ± 0.007	4.041 ± 0.024

Table 8.6. The average amplitude of the m-wave and the average area under the negative peak of m-wave (with standard error of the mean (SEM)) of the buccal branch of the facial nerve in 6 rats where the left nerve had been repair 3 months before assessment (group 1).

The Kolmogorov-Smirnov test for normality was carried out on each of the four electrophysiological variables in group 1. One such test is shown in figure 8.10. The data in all four variable groups was normally distributed.

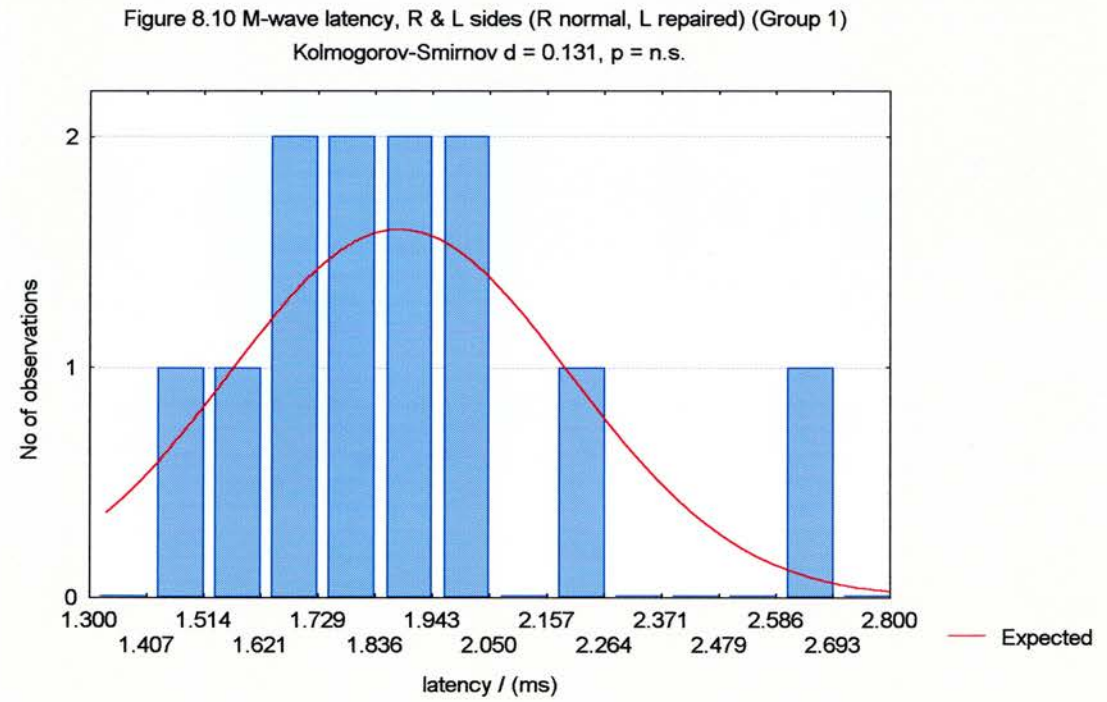


Table 8.7 shows the results of t-tests for dependant variables carried out on each of the four electrophysiological variables between right and left sides.

	Mean	Standard Deviation	n	t value	Degrees of freedom	p value
Left m-wave latency (ms)	2.078	0.327	6	5.552	5	0.003
Right m-wave latency (ms)	1.682	0.158				
Left peak latency (ms)	4.542	1.123	6	0.524	5	0.623
Right peak latency (ms)	4.250	0.715				
Left amplitude (mV)	0.218	0.133	6	-5.284	5	0.003
Right amplitude (mV)	1.600	0.704				
Left area (μVs)	0.498	0.343	6	-5.842	5	0.002
Right area (μVs)	3.702	1.284				

Table 8.7. t-tests for dependant variables carried out on each of the four electrophysiological variables between left and right sides (group 1).

There is a significant difference between the variables: m-wave latency, m-wave amplitude and m-wave area. No significant difference was found between the normal and repaired peak latency. Bland and Altman plots were carried out on the electrophysiological data obtained from the animals in group 1 (figures 8.11 - 8.14).

Figure 8.11 Bland & Altman plot in repaired rat facial nerves (Group 1)

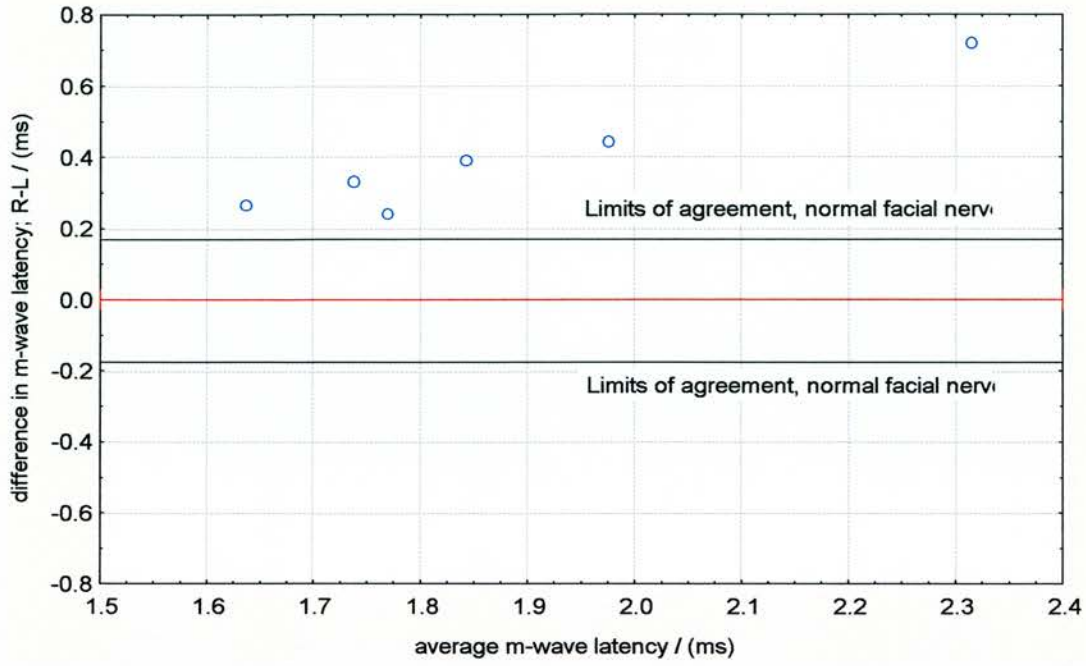


Figure 8.12 Bland & Altman plot in repaired rat facial nerves (Group 1)

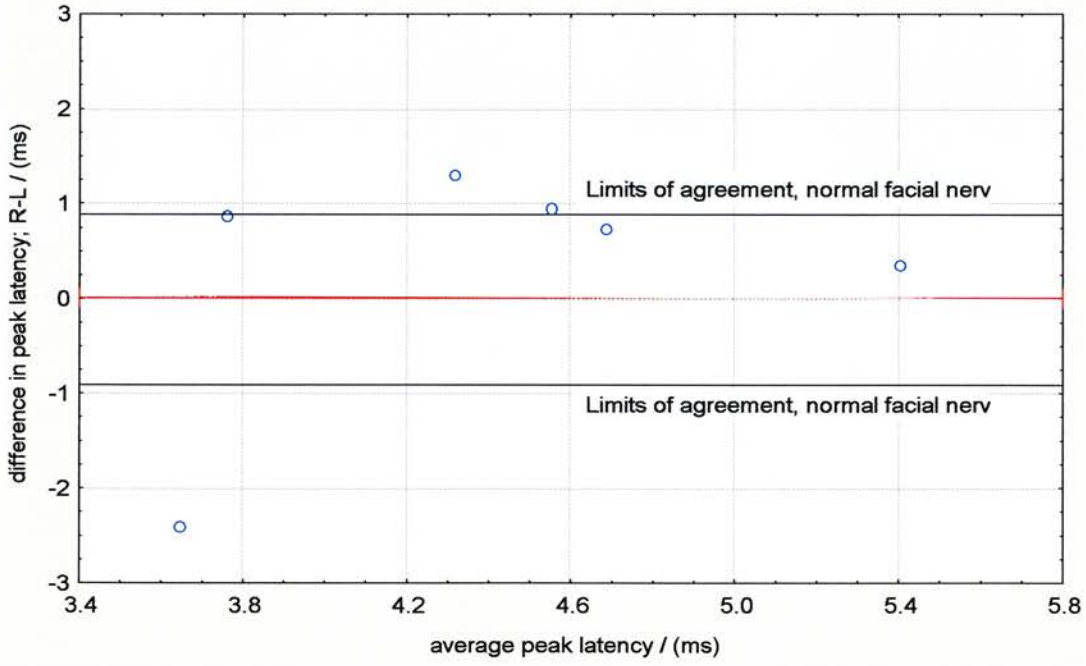


Figure 8.13 Bland & Altman plot in repaired rat facial nerves (Group 1)

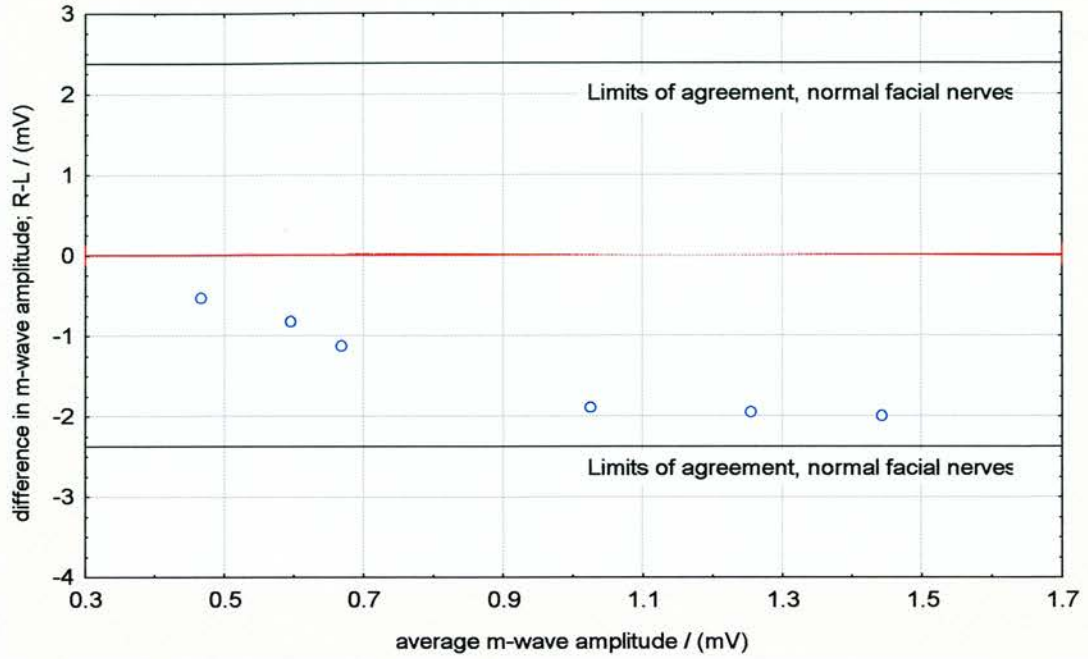
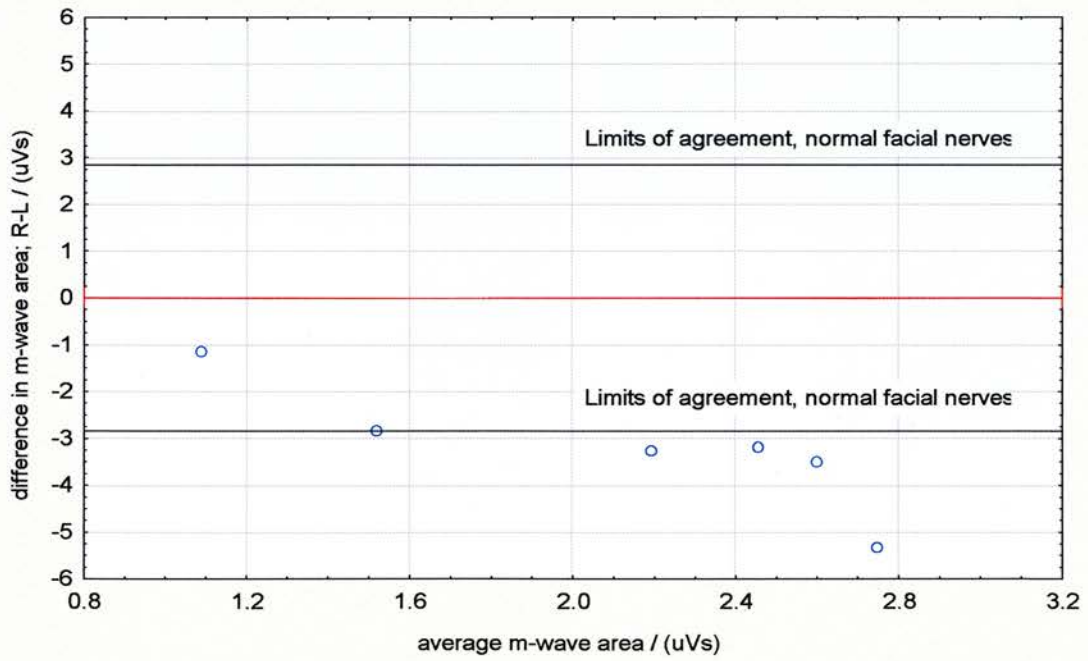


Figure 8.14 Bland & Altman plot in repaired rat facial nerves (Group 1)



This shows that all the differences between the m-wave latency in the repaired nerves and normal nerves (group 1) were out with the 'limits of agreement' for normal nerves (group 9). All but one measurement of the difference between m-wave area in the repaired nerves and

normal nerves (group 1) were out with the 'limits of agreement' for normal nerves (group 9). The difference in both, the measured amplitude and peak latency, between the normal and repaired nerves (group 1) were within the limits of agreement for normal nerves (group 9).

Conclusion

Modern electrophysiology recorders can record the muscle action potential produced by stimulation of the rat facial nerve. Values of m-wave latency and area of the m-wave are repeatable and reliable and show close agreement between right and left side of the same animal. Values of m-wave latency and area of the m-wave are measurably different between normal and repaired facial nerves in the rat. The m-wave latency in the repaired nerve is longer than in the normal nerve and the area of the m-wave in the repaired nerve is smaller than in the normal nerve. These two variables (m-wave latency and m-wave area) were used to assess outcomes of nerve repair.

Chapter 9

Results of rat experiments

Results
Discussion
Conclusion

Results

Results were obtained from 3 groups of rats, each with six rats per group. Table 9.1 shows the procedure carried out on the first group of rats studied.

Group no.	Number of rats	Procedure	Time to assessment
1	6	N-N suture	3 months
2	6	N-N defect (4 mm glass tube)	3 months
3	6	N-N defect (4 mm glass tube, with 10 μg BDNF)	3 months
4	6	N-N defect (4 mm glass tube, with 10 μg CNTF)	3 months
5	6	N-N defect (4 mm glass tube, with 10 μg GDNF)	3 months
6	6	N-N defect (4 mm glass tube, with 10 μg NT 4)	3 months
7	6	N-N suture (10 μg BDNF and 10 μg CNTF to repair site)	3 months
8	6	N-N suture (10 μg GDNF and 10 μg NT4/5 to repair site)	3 months

Table 9.1. Procedure carried out on the first set of rats studied.

In groups 2 – 6 above the glass tubes caused a severe fibrous reaction shown in figure 9.1. In the majority of animals, a neuroma at the proximal end of the buccal division of the facial nerve was found at re-operation and no electrophysiological data could be obtained. No meaningful data was therefore obtained from this first set of experiments.

Figure 9.1. Severe fibrous reaction to the glass tube in the face of the rat.

It was decided that the glass tube reaction was inconsistent with regeneration of the nerve in the rat facial nerve. It was therefore decided to abandon the use of biodegradable glass tubes in experiments on the rat facial nerve and a further set of experiments was devised using a suture repair of the nerve and delivery of neurotrophic factors by an osmotic pump to the distal limb of the repair site (groups 7 and 8).

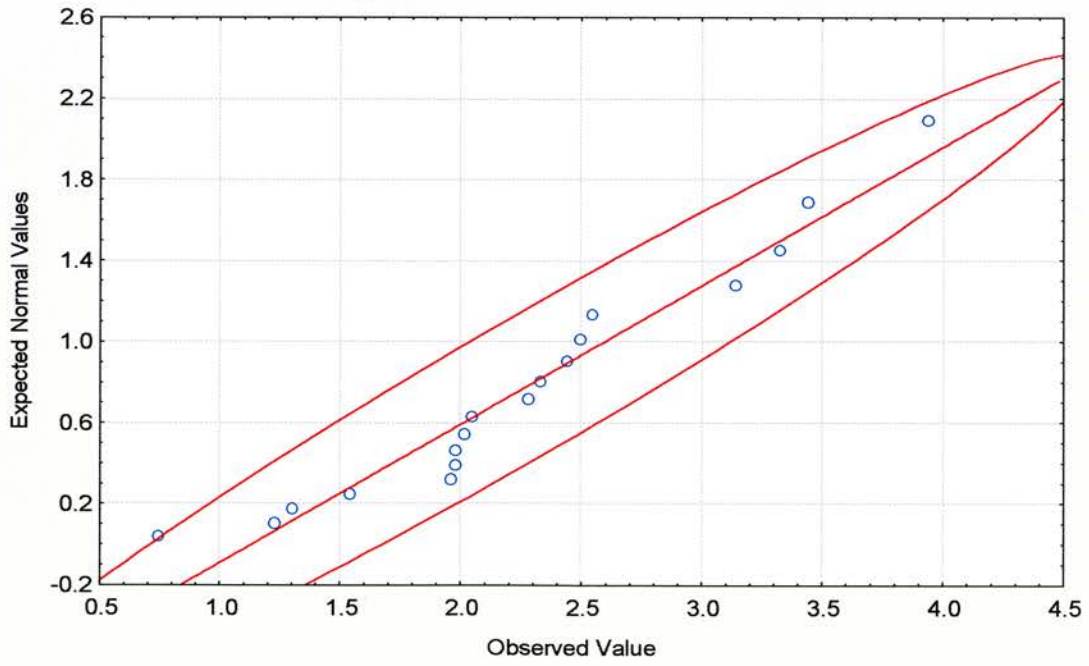
Table 9.2 shows the results obtained from the morphological and electrophysiological variables measured or calculated. All animals in these groups survived the initial surgical procedure and were able to be assessed after three months. Electrophysiological variables were obtained form all animals except the second animal in group 7. No recordings could be obtained from this animal.

Group	Fibre ratio	Axon diameter (μm)	Fibre diameter (μm)	Myelin thickness (μm)	g-ratio	M-wave latency ratio	M-wave area ratio
1	2.285	1.701	3.144	0.722	0.534	1.251	0.195
1	1.547	1.011	2.158	0.574	0.448	1.208	0.015
1	1.983	0.994	2.113	0.559	0.459	1.235	0.314
1	1.963	1.195	2.121	0.463	0.546	1.367	0.037
1	2.046	1.364	2.349	0.492	0.565	1.145	0.147
1	2.018	1.261	2.284	0.511	0.532	1.174	0.215
7	2.495	2.441	4.005	0.782	0.596	1.152	0.246
7	2.545	1.098	1.977	0.440	0.539	0.000*	0.000*
7	3.443	0.980	1.884	0.452	0.510	1.263	0.054
7	2.440	1.367	2.426	0.529	0.546	1.027	0.159
7	3.939	1.220	2.231	0.506	0.534	1.244	0.091
7	3.329	1.756	2.856	0.550	0.594	1.868	0.177
8	2.330	1.091	1.959	0.434	0.539	1.327	0.327
8	1.229	1.449	2.839	0.695	0.495	1.152	0.719
8	1.980	1.087	2.112	0.513	0.500	1.099	0.078
8	1.306	1.841	2.936	0.548	0.605	1.269	0.055
8	0.743	1.397	2.385	0.494	0.563	1.669	0.157
8	3.144	2.287	3.545	0.629	0.619	1.466	0.262

Table 9.2 Results obtained from the morphological and electrophysiological variables (* no electrophysiological recordings obtained)

To detect out-liers, half normal plots were constructed of all morphological and electrophysiological variables in table 8.1. An example of one of these plots is shown in figure 9.2. A 95% confidence line was plotted on the graph (shown as an ellipse) and outliers were taken as points out with this line.

Figure 9.2 Half-Normal Plot. Variable: Fibre ratio



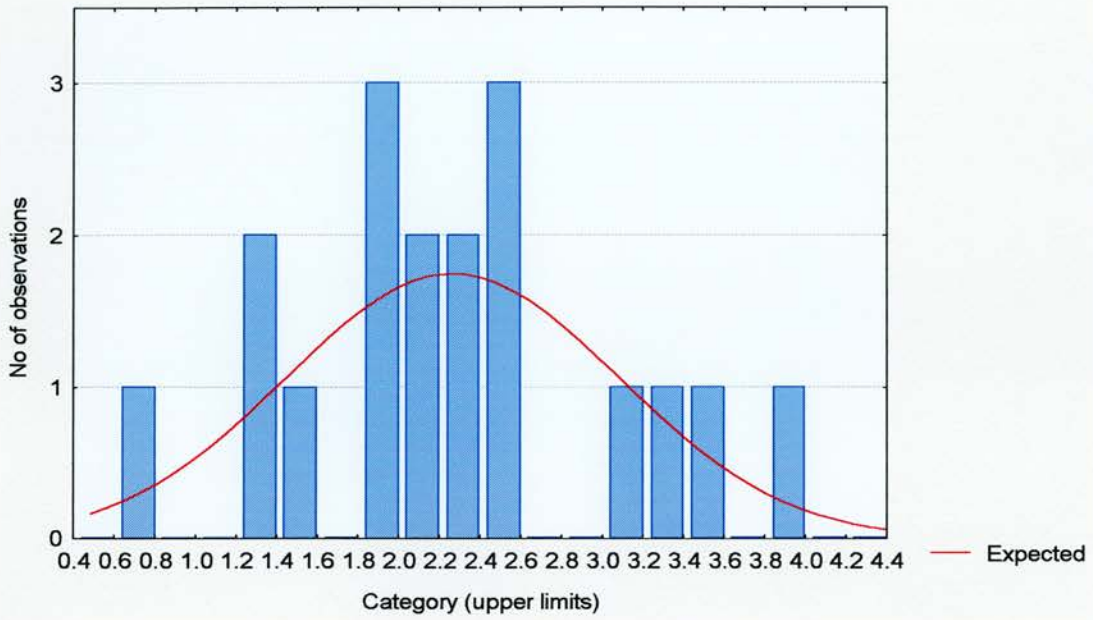
Outliers identified by half normal plots were removed from further statistical analysis. Table 9.3 shows the results obtained from the morphological and electrophysiological variables measured or calculated with outliers marked (*).

Group	Fibre ratio	Axon diameter (μm)	Fibre diameter (μm)	Myelin thickness (μm)	g-ratio	M-wave latency ratio	M-wave area ratio
1	2.285	1.701	3.144	0.722	0.534	1.251	0.195
1	1.547	1.011	2.158	0.574	0.448*	1.208	0.015
1	1.983	0.994	2.113	0.559	0.459	1.235	0.314
1	1.963	1.195	2.121	0.463	0.546	1.367	0.037
1	2.046	1.364	2.349	0.492	0.565	1.145	0.147
1	2.018	1.261	2.284	0.511	0.532	1.174	0.215
7	2.495	2.441	4.005*	0.782	0.596	1.152	0.246
7	2.545	1.098	1.977	0.440	0.539	0.000	0.000
7	3.443	0.980	1.884	0.452	0.510	1.263	0.054
7	2.440	1.367	2.426	0.529	0.546	1.027	0.159
7	3.939	1.220	2.231	0.506	0.534	1.244	0.091
7	3.329	1.756	2.856	0.550	0.594	1.868	0.177
8	2.330	1.091	1.959	0.434	0.539	1.327	0.327
8	1.229	1.449	2.839	0.695	0.495	1.152	0.719*
8	1.980	1.087	2.112	0.513	0.500	1.099	0.078
8	1.306	1.841	2.936	0.548	0.605	1.269	0.055
8	0.743*	1.397	2.385	0.494	0.563	1.669	0.157
8	3.144	2.287*	3.545	0.629	0.619*	1.466	0.262

Table 9.3 Results obtained from the morphological and electrophysiological variables with out-liers marked (*).

Kolmogorov-Smirnov plots were constructed of all morphological and electrophysiological variables in table 9.3, excluding out-liers. An example of one of these plots is shown in figure 9.3.

Fig 9.3 Variable: Fibre ratio
Kolmogorov-Smirnov d = .1192024, p = n.s.



For all variables, the Kolmogorov-Smirnov value was not significant and therefore the data could be regarded as normally distributed, as shown in table 9.4.

Variable	Kolmogorov-Smirnov value	p value
Fibre ratio	0.119	n.s.
Axon diameter	0.184	n.s.
Fibre diameter	0.191	n.s.
Myelin thickness	0.179	n.s.
G-ratio	0.067	n.s.
M-wave latency	0.221	n.s.
Area of m-wave	0.134	n.s.

Table 9.4 Kolmogorov-Smirnov values for morphological and electrophysiological variables.

Table 9.5 shows the mean results obtained in morphological and electrophysiological variables from groups 1, 7 and 8.

Group	Fibre ratio	Axon diameter (μm)	Fibre diameter (μm)	Myelin thickness (μm)	g-ratio	M-wave latency ratio	M-wave area ratio
1	1.974	1.254	2.362	0.554	0.527	1.230	0.154
7	3.032	1.477	2.275	0.543	0.553	1.092	0.121
8	1.998	1.373	2.629	0.552	0.540	1.330	0.176

Table 9.5 Mean results obtained from the morphological and electrophysiological variables excluding outlier in table 9.3.

An F test was carried out on all variables, excluding outliers. This is shown in table 9.6. The only significant difference between groups was with fibre diameter ratio. t tests were therefore carried out on the fibre ratio results in the three groups. This is shown in table 9.7. t testing of each group in respect to the variable of fibre diameter ratio yielded a significantly different result between groups 1 and 7 and groups 7 and 8. No difference was detected between groups 1 and 8. This is shown in a box and whisker plot in figure 9.4.

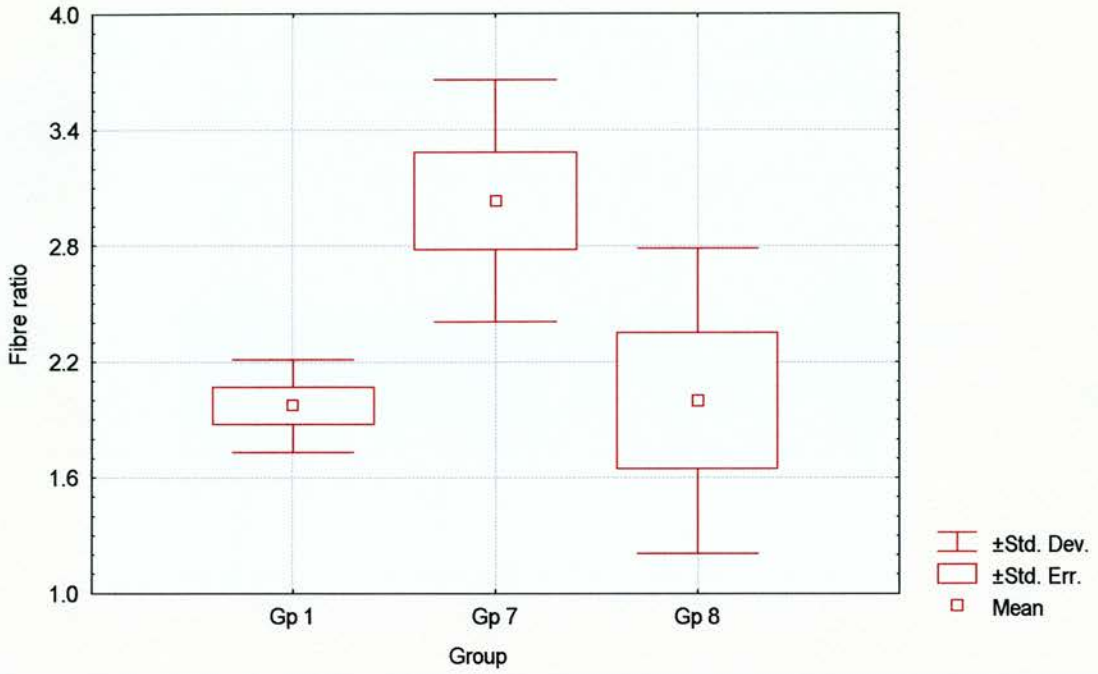
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Fibre ratio	4.260	2	2.130	4.737	14	0.338	6.296	0.011*
Axon diameter (μm)	0.149	2	0.075	2.205	14	0.157	0.474	0.632
Fibre diameter (μm)	0.387	2	0.193	3.137	14	0.224	0.863	0.443
Myelin thickness (μm)	0.002	2	0.001	0.663	15	0.044	0.018	0.983
G-ratio	0.002	2	0.001	0.021	13	0.002	0.569	0.579
M-wave latency ratio	0.171	2	0.086	2.108	15	0.141	0.609	0.557
M-wave area ratio	0.008	2	0.004	0.159	14	0.011	0.364	0.702

Table 9.6. F test of morphological and electrophysiological variables (*significant)

	Group 1	group 7	group 8
group 1	-	t = 3.872 p=0.003	t=0.072 p=0.944
group 7	-	-	t=2.428 p=0.04
group 8	-	-	-

Table 9.7 t tests for the fibre ratio of each group.

Figure 9.4 Box & Whisker Plot. Variable: Fibre ratio



There were therefore statistically significant differences in the number of fibres between groups 1 and 7 and groups 7 and 8. There was no other difference in any of the other parameters measured.

Discussion

The use of biodegradable glass for facial nerve repair by entubulation resulted in unexpected fibrosis at the site of operation. Many studies have shown the safety of CRG for nerve repair, but these studies have not involved the rat facial nerve. In small animals such as the rat, it is presumed that the tube is proportionally larger in size and volume of glass, resulting in slower breakdown and degradation. When this mass of CRG was inserted under the skin of the face of the rat it may have been that other factors existed which were unique to this site and caused fibrosis. It was not possible to manufacture glass tubes with a smaller wall thickness, which may have prevented at least some of the reactions seen in these experiments. Any benefit which may have arisen from nerve entubulation was overcome by the reaction to the glass and

the use of CRG in the rat had to be abandoned. Studies have used neurotrophic factors delivered to the site of nerve repair without nerve entubulation to some effect (Newman *et al* 1996). In the present study, neurotrophic factors were delivered just distal to the repair site with an ultra-fine needle inserted into the nerve sheath.

The 'trophic' actions of neurotrophic factors are well known. These actions include gene activation for cell growth and differentiation, neurite outgrowth, protein synthesis, alteration in membrane permeability and cytoskeletal rearrangement. But neurotrophic factors may also have a 'tropic' action where some guidance of the direction of growth occurs down a chemical gradient resulting in chemo-attraction of the growing proximal nerve stump. Thus, some authors have suggested this 'neurotropic' effect is responsible for selective regeneration into a distal nerve branch after neurotmesis (Maki *et al.* 1996).

If neurotrophic factors are truly 'trophic' delivery of these growth factors in this experiment may have stimulated growth and result in improved regeneration of the nerve. If neurotrophic factors are also 'tropic' then this delivery may guide regenerating axons down the distal nerve. There are thus two *potential* types of benefits for the use of neurotrophic factors when delivered in the method described in this experiment. The increase in fibre numbers may have resulted from increased sprouting from the nerve growth cone, induced by the combination of BDNF and CNTF, therefore producing more fibres in the distal nerve. A second possibility is that the tropic action of these growth factors caused a greater proportion of neurites (which were not increased in number), to enter the distal stump of the nerve.

This study shows that the local delivery of neurotrophic factors is technically feasible and does not result in side effects to the animal.

The finding that a combination of CNTF and BDNF increases the number of fibres in the regenerated facial nerve is an exciting discovery. It suggests that the regeneration of peripheral nerves can be influenced by exogenous chemicals. It has been known for many years that the number of fibres increases after nerve repair. This increase is presumably due to increased sprouting of neurites at the growth cone of the neurone or due to inhibition of apoptosis in neurites which do not reach their target organ.

The fate of regenerated nerve fibres has been described for decades. It has been shown that the size of fibres after repair, although partly influenced by the parent fibres is mainly dependant on factors peripheral to the point of injury (Hammond & Hinsey 1945; Simpson & Young 1945). Contact with the end organ is of paramount importance in achieving large diameter fibres (Sanders & Young 1945; Weiss *et al* 1945). In an elegant study, Aitken *et al* carried out various experiments on a motor nerve in the rabbit, the nerve to the medial head of gastrocnemius, which contains approximately 400 myelinated axons (Aitken *et al* 1947). When this nerve was left without a peripheral connection, by crushing it proximally and cutting it distally, turning the stump aside and fixing it to fascia, it formed a neuroma. The number of fibres was very much more in the nerve which formed a neuroma than in an equivalent nerve which had undergone transection and repair. The number of fibres in a regenerated nerve is therefore not a good indicator of success after nerve repair. Some studies have not appeared to accept this and have used the number of nerve fibres after repair to suggest a potential improvement in nerve regeneration. (Aebischer *et al.* 1987; Santos *et al.* 1998). The idea that, because there are more nerve fibres after regeneration, there will be a functional improvement is somewhat naïve, and may be contrary to what occurs in reality. Indeed, some authors suggest that the reduction in the number of nerve fibres to normal, or near normal values after nerve injury is an indicator of a good outcome (Aitken *et al* 1947).

The fibres identified and counted in this study, therefore (which were all identified by a myelin sheath and therefore myelinated) had not necessarily reached an end organ. Aitken *et al* demonstrated regeneration in a motor nerve co-apted to a cutaneous nerve, suggesting that motor fibres can be misdirected to supply non-muscular end-organs (Aitken *et al* 1947).

Despite the various morphological and electrophysiological measures used in this study, only fibre number was significantly affected by the use of neurotrophic factors. No other variable reached statistical significance and there appeared to be no *trend* for benefit in the growth factor treated group, which may have suggested a difference had there been larger numbers of animals in the groups.

In this study, while an increased number of nerve fibres where obtained with one group of neurotrophic factors, the end point of regeneration is not known. It is possible that after three months (when these experiments were assessed) 'die-back' of fibres which had not reached an end-organ may have resulted in an eventual return of fibre numbers to the level of the other two groups.

The time of assessment of the nerve repair is important in any experiment which involves the use of neurotrophic factors. If neurotrophic factors increase the number of regenerating fibres initially, but not the final number of fibres in the nerve no useful effect will be obtained. The ideal time to assess the effect of any treatment on nerve repair is when the number of fibres fails to increase or decrease after the initial neurotmesis. This could be achieved by having many groups of experiments (such as carried out above) and assessing the facial nerve at various time intervals (e.g. 30, 60, 90, 120, 150, 180 days). This would be a large undertaking

and if neurotrophic factors were used this would prove very expensive. It was decided therefore in this study to assess the rat nerve at 90 days only and other authors have used this time interval (Aebischer *et al* 1987; Newman *et al* 1996; Santos *et al* 1998). Although there may have been further changes after this time, the present work was a comparison of nerve repair with and without neurotrophic factors, assessed at the same time interval. It has also been shown that in the rat, where the facial nerve is divided at the stylomastoid foramen, there is recovery of function (as seen by movement of the vibrissae) as early as 17 days after neurotmesis and surgical repair (Kohmura *et al.* 1999). This would suggest that 90 days is certainly sufficient time for re-growth of axons to occur from the buccal division of the nerve and time to allow at least some maturation of these fibres. In the human, improvement of facial nerve grading can occur even two years after repair of a nerve injury (Malik *et al.* 2001). As the osmotic pump used in this study delivered its contents over two weeks, then at the very least, the fastest growing axons would have been supplied with neurotrophic factors during their re-growth.

Abandoning the use of CRG tubes for nerve repair in the rat allowed concentration on the effect of the two sets of neurotrophic factors (BDNF and CNTF, and NT4/5 and GDNF). Due to time and financial constraints of the study, it was not possible to examine the effect of four groups which would have shown which of the factors, BDNF or CNTF was responsible for the increase in numbers of regenerated nerve fibres (assuming that this effect is not due to synergism between the neurotrophic factors).

Conclusion

The null hypothesis, '*neurotrophic factors have no influence on regeneration of the facial nerve after division and repair*', cannot be rejected in these experiments in the rat.

Chapter 10

Results of sheep experiments

Results
Discussion
Conclusion

Results

Results were obtained from 3 groups of sheep. Table 10.1 shows the procedure carried out on each group.

Group	Number of sheep	Procedure	Time to assessment
1	6	N-N suture	9 months
2	5	N-N suture with entubulation and saline pump	9 months
3	6	N-N suture with entubulation and neurotrophic pump	9 months

Table 10.1. Procedure carried out on each group of sheep, N-N = Nerve-to-nerve

In these groups, the glass tubes did not cause a severe fibrous reaction and no animal developed an abscess or required removal of the tube. Neuromas were not identified at re-operation. Tables 10.2 and 10.3 shows the results obtained from the morphological and electrophysiological variables measured or calculated.

Group	Maximum conduction velocity (ms^{-1})	Minimum conduction velocity (ms^{-1})	CV-range (ms^{-1})	Absolute refractory period (ms)	Relative Refractory period (ms)	Amplitude (mV)
1	49.10	17.50	31.60	0.76	3.24	3.15
1	63.50	13.60	49.90	2.88	6.33	2.14
1	23.20	20.70	2.50	1.22	3.80	3.41
1	38.00	8.60	29.40	0.82	3.54	1.41
1	46.90	8.43	38.47	0.70	3.66	2.89
1	47.50	11.70	35.80	0.60	7.26	1.07
2	35.50	20.68	14.82	1.65	4.85	3.86
2	42.30	35.28	7.02	0.74	5.00	4.36
2	49.60	35.71	13.89	1.28	4.82	3.08
2	44.30	30.71	13.59	0.72	7.53	2.94
3	63.50	15.40	48.10	1.18	3.46	3.71
3	47.60	8.00	39.60	0.92	3.14	0.17
3	54.00	12.90	41.10	1.34	5.72	2.79
3	32.10	17.70	14.40	0.96	8.10	4.88
3	40.70	17.10	23.60	0.54	3.38	2.44

Table 10.2 Results obtained of electrophysiological measurements.

	Mean Jitter (µs)	Axon diameter (µm)	Fibre Diameter (µm)	Myelin Thickness (µm)	g-ratio
1	13.00	Failure of tissue processing			
1	14.00	2.06	4.43	1.19	0.46
1	10.00	1.92	3.78	0.93	0.49
1	10.00	2.46	4.40	0.97	0.54
1	18.00	2.40	4.75	1.17	0.49
1	9.00	2.44	3.97	0.77	0.59
2	Unavailable for technical reasons	1.44	3.03	0.80	0.45
2		2.46	4.96	1.25	0.46
2		2.50	4.34	0.92	0.54
2		1.41	2.56	0.57	0.54
3	14.00	2.01	3.41	0.70	0.58
3	19.00	2.26	3.74	0.74	0.57
3	17.00	Failure of tissue processing			
3	14.00	1.45	2.46	0.50	0.58
3	27.00	2.23	3.68	0.72	0.57

Table 10.3 Results obtained from the morphological and electrophysiological variables.

In group 2, no measurements of jitter were obtained due a fault with the electrophysiology equipment. This had the effect that there was no placebo limb for these experiments involving the measurement of jitter. It was still possible however, to compare the measurements of jitter in group 1 and in group 3.

In one animal in group 1 and in one animal in group 3 the process of tissue processing of the nerve failed and no histology was obtained for these animals. These histological results were excluded from the analysis although the electrophysiological results were analysed for these two animals.

To detect out-liers, half normal plots were constructed of all morphological and electrophysiological variables in tables 10.2 and 10.3. A 95% confidence line was plotted on the graph and outliers were taken as points out with this line.

Outliers identified by half normal plots were removed from further statistical analysis. Tables 10.4 and 10.5 show the results obtained from the morphological and electrophysiological variables measured or calculated with outliers marked (*).

Group	Maximum conduction velocity (ms^{-1})	Minimum conduction velocity (ms^{-1})	CV-range (ms^{-1})	Absolute refractory period (ms)	Relative Refractory period (ms)	Amplitude (mV)
1	49.10	17.50	31.60	0.76	3.24	3.15
1	63.50	13.60	49.90	2.88*	6.33	2.14
1	23.20	20.70	2.50	1.22	3.80	3.41
1	38.00	8.60	29.40	0.82	3.54	1.41
1	46.90	8.43	38.47	0.70	3.66	2.89
1	47.50	11.70	35.80	0.60	7.26	1.07
2	35.50	20.68	14.82	1.65	4.85	3.86
2	42.30	35.28	7.02	0.74	5.00	4.36
2	49.60	35.71	13.89	1.28	4.82	3.08
2	44.30	30.71	13.59	0.72	7.53	2.94
3	63.50	15.40	48.10	1.18	3.46	3.71
3	47.60	8.00	39.60	0.92	3.14	0.17
3	54.00	12.90	41.10	1.34	5.72	2.79
3	32.10	17.70	14.40	0.96	8.10	4.88
3	40.70	17.10	23.60	0.54	3.38	2.44

Table 10.4 Results obtained from the electrophysiological variables with outliers marked (*).

Group	Mean Jitter (μs)	Axon diameter (μm)	Fibre Diameter (μm)	Myelin Thickness (μm)	g-ratio
1	13.00	Failure of tissue processing			
1	14.00	2.06	4.43	1.19	0.46
1	10.00	1.92	3.78	0.93	0.49
1	10.00	2.46	4.40	0.97	0.54
1	18.00	2.40	4.75	1.17	0.49
1	9.00	2.44	3.97	0.77	0.59
2	Unavailable for technical reasons	1.44	3.03	0.80	0.45
2		2.46	4.96	1.25	0.46
2		2.50	4.34	0.92	0.54
2		1.41	2.56	0.57	0.54
3	14.00	2.01	3.41	0.70	0.58
3	19.00	2.26	3.74	0.74	0.57
3	17.00	Failure of tissue processing			
3	14.00	1.45	2.46	0.50	0.58
3	27.00	2.23	3.68	0.72	0.57

Table 10.5 Results obtained from the morphological and electrophysiological variables with outliers marked (*).

Kolmogorov-Smirnov plots were constructed of all morphological and electrophysiological variables in tables 10.4 and 10.5, excluding outliers. For all variables, the Kolmogorov-

Smirnov value was not significant (table10.6) and therefore the data was assumed to be normally distributed.

Variable	Kolmogorov-Smirnov value	p value
Axon diameter	0.149	n.s.
Fibre diameter	0.091	n.s.
Myelin thickness	0.146	n.s.
G-ratio	0.209	n.s.
Max conduction velocity	0.128	n.s.
Min conduction velocity	0.178	n.s.
CV range	0.184	n.s.
Absolute refractory period	0.164	n.s.
Relative refractory period	0.173	n.s.
Amplitude	0.065	n.s.
Jitter	0.213	n.s.

Table 10.6 Kolmogorov-Smirnov values for morphological and electrophysiological variables.

An F test was carried out on all variables, excluding outliers. This is shown in table 10.7. The only significant difference between groups was with minimum conduction velocity. T tests were therefore carried out on the minimum conduction velocity in the three groups. This is shown in table 10.8. T testing of each group in respect to the variable of minimum conduction velocity yielded a significant result between groups 1 and 2 and groups 2 and 3. No difference was detected between groups 1 and 3. This is shown in a box and whisker plot in figure 10.1.

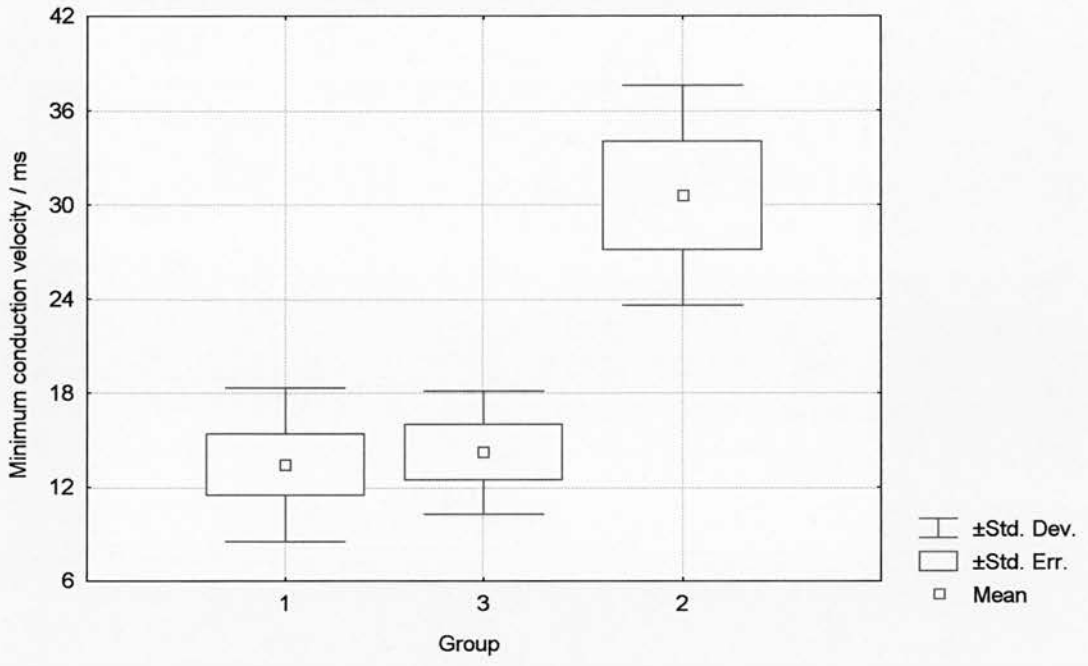
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Maximum conduction velocity (ms^{-1})	50.522	2	25.261	1576.216	12	131.351	0.192	0.828
Minimum conduction velocity (ms^{-1})*	830.667	2	415.333	329.534	12	27.461	15.124	0.001*
Conduction velocity range (ms^{-1})	1172.804	2	586.402	2059.984	12	171.665	3.416	0.067
Absolute refractory period (ms)	0.178	2	0.089	1.202	11	0.109	0.812	0.469
Relative refractory period (ms)	2.192	2	1.096	38.098	12	3.175	0.345	0.715
Amplitude (mV)	3.547	2	1.773	18.183	12	1.515	1.170	0.343
Mean jitter (μs)	93.867	1	93.867	172.133	9	19.126	4.908	0.054
Axon diameter (μm)	0.251	2	0.126	1.785	10	0.178	0.705	0.517
Fibre diameter (μm)	2.016	2	1.008	5.423	10	0.542	1.859	0.206
Myelin sheath thickness (μm)	0.255	2	0.128	0.403	10	0.040	3.170	0.086
G-ratio	0.013	2	0.007	0.019	10	0.002	3.504	0.070

Table 10.7. F test of morphological and electrophysiological variables (*significant)

Group	1	2	3
1	-	t = 4.603 p = 0.002	t = 0.292 p = 0.777
2	-	-	t = 4.470 p = 0.003
3	-	-	-

Table 10.8. t tests for the minimum conduction velocity of each group.

Figure 10.1 Box & Whisker Plot: Mimum conduction velocity



Discussion

In the sheep, the glass tubes did not seem to cause a severe inflammatory reaction, as was the case in the rat. This may have been due to the size of the tube compared to the animal. In the sheep the tube was proportionally of a much smaller volume.

The results obtained in these experiments show that there is no difference in the electrophysiological variables measured between the neurotrophic factor treated group of animals and in the nerve suture group. No variable reached statistical significance and there appeared to be no *trend* for benefit in the growth factor treated group, which may have suggested a difference had there been larger numbers of animals in the groups.

The only significant finding in this group was with minimum conduction velocity in the saline treated group which was higher than in the other two groups of animals (the sutured only group and the neurotrophic factor treated group). The reason for this difference, if genuine, is

difficult to explain. Examining the data it is evident that this result is not due to an outlier (which would have been removed from the analysis by the half-normal plot). There was however, no corresponding increase in any morphological characteristics in this group, which could corroborate this finding as genuine, and it is assumed that this result is spurious. This would suggest that there does not appear to be an advantage of entubulation as a method of nerve repair. The finding that the placebo group had a higher minimum conduction velocity than the other two groups does not change the finding that the neurotrophic factor group showed no such increase in minimum conduction velocity.

Unlike in the rat, the number of fibres in the sheep facial nerve was not counted. There were two reasons for this. The buccal division of the sheep facial nerve was of a larger diameter than in the rat nerve. Processing of the tissue was, to some extent, unreliable due its large size and undoubtedly fibres were 'lost' to microscopic analysis by inadequate fixation in some nerves. In two animals, tissue processing failed completely and these animals had to be excluded from morphological analysis. In other nerves the transverse section was so large that it would have been technically impossible to reassemble an image of the entire nerve to allow absolute fibre counts to be performed. Furthermore, as discussed in chapter 9, an increase in the absolute number of fibres does not necessarily suggest functional benefit.

The negative findings of this study in the sheep facial nerve are disappointing. Despite this, it is a powerful statement concerning neurotrophic factors in a large animal model. Trials in humans of neurotrophic factors have also proved disappointing and two trials of CNTF in the treatment of motor neurone disease have been discontinued because of adverse effects and failure to slow the progression of the disease (ALS-CNTF treatment study 1993; Longo 1994). With many outcome measures assessed, and none identified as showing benefit, we

must assume that the neurotrophic factors used here, delivered in this way and concentration have no effect on nerve recovery. The sheep is a more complex animal than the rat, and more akin to the human in terms of peripheral nerve repair, hence its use in these and other experiments on nerve repair. Although some studies of nerve repair using neurotrophic factors have shown a benefit in rodents none, as yet, have shown measured benefit in a large animal. This will be a prerequisite for human studies with neurotrophic factors and repair of peripheral nerves.

Conclusion

The null hypothesis, '*neurotrophic factors have no influence on regeneration of the facial nerve after division and repair*', cannot be rejected in these experiments in the sheep. Also, the use of nerve entubulation as a method of nerve repair cannot be supported.

Chapter 11

Conclusion

A combination of CNTF and BDNF increased the number of fibres in the rat facial nerve but did not change other morphological measurements. There was no perceived change in electrophysiological variables. There is some confusion in the literature as to whether an increase in fibre numbers is a useful indicator of improvement in nerve regeneration and some would suggest that an increase in fibre numbers is an indicator of poor outcome. The fact that there can be a dramatic increase in the number of fibres in a nerve which terminates in a neuroma suggests that fibre numbers is not a good indicator of nerve regeneration. The measurements of axon diameter, fibre diameter and myelin sheath thickness appear to be better indicators of recovery of nerve function.

A combination of BDNF, CNTF and GNDF had no effect on the morphology of the sheep facial nerve after repair. There was no perceived change in electrophysiological variables, with the exception of minimum conduction velocity in the placebo (saline) group which is likely to be a spurious result.

It is likely that recovery to normal will never be achieved after transection of the facial nerve, regardless of what treatment is given, and what advancements are made in nerve research. The facial muscles are represented by a large area on the motor cortex and control of these muscles is learned in the early years of life. A considerable problem in regeneration of a divided facial nerve is that these connections are haphazard and random, and clinically, synkinesis occurs. It is unlikely that neurotrophic factors will influence how proximal nerve fibres re-innervate their original muscle motor end plates. One criticism of using neurotrophic factors in an effort to improve upon current results is that even if nerve regeneration is

improved upon, synkinesis will be present to the same or even to a greater extent. However synkinesis can be treated effectively and safely by botulinum toxin injections (Armstrong *et al* 1996). Furthermore, synkinesis is only a problem when a lesion affects more than one division of the facial nerve and regeneration occurs. It has also been noted, that after facial nerve repair, movement is rarely restored to the forehead. There is therefore capacity for increased innervation to this area. It is also the case that even in the adult, there exists enough neuronal plasticity for facial muscle movements to be relearned after facial nerve reinnervation procedures (Sood *et al.* 2000). If more nerve fibres can be recruited to innervate facial muscles there is then more scope to improve upon cosmesis with other techniques, some of which use the selective removal of these unwanted muscle movements. The increase therefore, in movement of the face, even without fine muscle control is still a goal worth achieving. Unfortunately, it cannot be said from the present work that that step is any closer to being achieved.

If animal experiments with neurotrophic factors are to improve upon the functional outcome of facial nerve repair then it will be necessary to identify neurotrophic factors which influence the size of fibres innervating the facial musculature and the electrophysiological results after repair. The present work has established a useful model for facial nerve repair in two species and has established that neurotrophic factors can be delivered to some effect (on total number of fibres, in the rat), all-be-it without improvement in function.

The next step, if this research were to be continued would be to evaluate the effects of BDNF and CNTF alone in two groups of animals.

The osmotic pump delivery system is easy to use and did not result in problems in either the rat or the sheep. However an improvement in the delivery of neurotrophic factors could be achieved by the use of a slow-release biodegradable reservoir and such a method of delivery has been devised (Kohmura *et al* 1999).

There may be several explanations as to why there was no functional improvement in nerve repair in these experiments:

1. The delivery of the neurotrophic factors may have been inadequate
2. The dose may not have been optimal (either too high or too low)
3. The system of delivery may have interfered with functional improvement
4. Neurotrophic factors may have improved nerve regeneration at a level which could not be detected by the methods used
5. Neurotrophic factors may not have improved the functional outcome of nerve regeneration

The fact that there was an effect on fibre numbers in the rat facial nerve, suggests that the delivery system was effective, and that the neurotrophic factors were active when supplied to the regenerating nerve. It also suggests that these neurotrophic factors were active on the nerve at the concentrations used, although different concentrations may have caused different effects on nerve regeneration. It is possible that the delivery system including the catheter and needle, in the rat, and the catheter and CRG tube in the sheep interfered with improved regeneration. This does not seem to be the case however, as the animals in the saline control group of sheep experiments did not have a poorer functional outcome than either the animals in the sutured group or the neurotrophic factor treated groups. It is also possible that neurotrophic factors improved regeneration at a level which was too subtle to be detected by the methods used in these experiments. It is unlikely however, given the array of functional and morphometric tests

used here, that subtle differences *which would be clinically useful* exist with the use of neurotrophic factors. This leaves the depressing probability that exogenously administered neurotrophic factors do not influence that fate of facial nerve repair.

From its inception this was a very ambitious project. Many studies on the use of neurotrophic factors are contradictory and much work has concentrated on *in vitro* experiments which, although helpful, do not advance the treatment of patients with peripheral nerve injuries. This work has used many measures of outcome to investigate the effect of neurotrophic factors and has shown little benefit in the mammalian facial nerve for the use of the most likely candidates of growth factors. It is unlikely that in the human the delivery of these factors to the site of nerve repair will improve upon the current level of function to be expected after nerve repair. The exception to this is in the number of fibres found in the rat facial nerve. This however, did not appear to result in a better functional result. The number of nerve fibres may not, ultimately be relevant to the function of the nerve. Indeed it is known that the number of fibres in the human facial nerve may decrease to approximately 10% of the normal value (Axon & Ramsden 1999) in chronic conditions without a noticeable effect on the function of the face. More fibres do not mean more function in the face. It is more likely that the size of the fibres and myelination are important. These parameters affect the speed of conduction in the nerve. Furthermore, a nerve fibre which does not communicate with a muscle motor end plate is of no value to patient or laboratory animal.

The enhancement of peripheral nerve repair is still an elusive goal which will probably require many more negative studies before positive findings are uncovered.

References

1989, *Gray's Anatomy*, 37 edn, Churchill Livingstone, Edinburgh.

1993, "ALS-CNTF Treatment Study (ACTS): Recombinant human ciliary neurotrophic factor (rHCNTF) in amylotrophic lateral sclerosis (ALS) patients: phase I-II safety, tolerability and pharmacokinetic studies [Abstract]", *Neurology*, vol. 43, p. A416.

Aebischer, P., Valentini, R. F., Dario, P., Domenici, C., & Galletti, P. M. 1987, "Piezoelectric guidance channels enhance regeneration in the mouse sciatic nerve after axotomy", *Brain Research*, vol. 436, pp. 165-168.

Aitken, J. T., Sharman, M., & Young, J. Z. 1947, "Maturation of regenerating nerve fibres with various peripheral connexions", *Journal of Anatomy*, vol. 81, pp. 1-23.

Aitken, J. T. & Thomas, P. K. 1962, "Retrograde changes in fibre size following nerve section", *Journal of Anatomy*, vol. 96, pp. 121-129.

Alford, B. R. 1967, "Electrodiagnositic studies in facial paralysis", *Arch Otolaryngology Head Neck Surgery*, vol. 85, pp. 259-264.

Allen W.M., Sansom B.F., Drake C.F., & Davies D.C. 1978, "A new method for the prevention of trace-element deficiencies", *Veterinary Society Communications*, vol. 2, pp. 73-75.

Altar, C. A., Boylan, C. B., Fritsche, M., Jones, B. E., Jackson, C., Wiegand, S. J., Lindsay, R. M., & Hyman, C. 1994, "Efficacy of brain-derived neurotrophic factor and neurotrophin-3 on neurochemical and behavioral deficits associated with partial nigrostriatal dopamine lesions", *Journal of Neurochemistry*, vol. 63, pp. 1021-1032.

Arakawa, Y., Sendtner, M., & Thoenen, H. 1990, "Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurones in culture: comparison with other neurotrophic factors and cytokines", *Journal of Neurosurgery*, vol. 10, pp. 3507-3515.

Armstrong, M. W., Mountain, R. E., & Murray, J. A. 1996, "Treatment of facial synkinesis and facial asymmetry with botulinum toxin type A following facial nerve palsy", *Clin.Otolaryngol.*, vol. 21, pp. 15-20.

Axon, P. R. & Ramsden, R. T. 1999, "Facial nerve injury caused by vestibular Schwannoma compression: severity and adaptation to maintain normal clinical facial function", *Am.J.Otol.*, vol. 20, pp. 763-769.

Barbin, G., Selak, I., Manthrope, M., & Varon, S. 1984, "Use of central neuronal cultures for the detection of neuronotrophic agents", *Neuroscience*, vol. 12, pp. 33-43.

Barde, Y. A. 1989, "Trophic factors and neuronal survival", *Neuron*, vol. 2, pp. 1525-1534.

Barde, Y. A. 1994, "Neurotrophic factors: an evolutionary perspective", *Journal of Neurobiology*, vol. 25, no. 11, pp. 1329-1333.

Barde, Y. A., Edgar, D., & Thoenen, H. 1982, "Purification of a new neurotrophic factor from the mammalian brain", *EMBO j*, vol. 1, pp. 549-553.

BenitoRuiz, J., NavarroMonzonis, A., Piqueras, A., & BaenaMontilla, P. 1994, "Invaginated vein graft as nerve conduit: An experimental study", *Microsurgery*, vol. 15, pp. 105-115.

Berry, C. M., Grundfest, H., & Hinsey, J. C. 1944, "The electrical activity of regenerated nerve in the cat", *Journal of Neurophysiology*, vol. 7, pp. 103-116.

Bland, J. M. & Altman, D. G. 1986, "Statistical method s for assessing agreement between two methods of clinical measurement", *Lancet*, vol. i, pp. 307-310.

Blumenthal, F. & May, M. 1986, "Electrodiagnosis," in *The facial nerve*, M. May, ed., Thieme Inc, New York, pp. 241-263.

Bothwell, M. 1982, "Insulin and somatomedin MSA promote nerve growth factor-independent neurite formation by cultured chick dorsal root ganglionic sensory neurons", *Journal of Neuroscience Research*, vol. 8, pp. 225-231.

Bray, G. M. & Aguayo, A. J. 1974, "Regeneration of peripheral unmyelinated nerves. Fate of the axonal sprouts which develop after injury", *Journal of Anatomy*, vol. 117(3), pp. 517-529.

Burnie J. 1982, *Controlled release glass (CRG) a new biomaterial*, Strathclyde University Thesis.

Burnie J., Duff S.R.I., Drake C.F., Harding N.G.L., Malcolm A.J., & Gilchrist T. 1981, "Controlled release glasses (CRG) for biomedical uses", *Biomaterials*, vol. 2, pp. 244-246.

Burnie J., Duff S.R.I., Malcolm A.J., Doherty P.J., Drake C.F., & Gilchrist T. 1983, "Controlled release glass as a potential bone graft substitute", *Journal of Bone and Joint Surgery*, vol. 65B, no. 3, p. 169.

Burnie J. & Gilchrist T. 1983, "Controlled release glass a new biomaterial," in *Ceramics in Surgery*, 1 edn, Vincenzi P., ed., Elsevir Scientific, Amsterdam, pp. 169-176.

Campbell, E. D. R., Hickey, R. D., Nixon, K. H., & Richardson, A. T. 1962, "Value of nerve excitability measurements in prognosis in facial palsy", *British Medical Journal*, vol. 2, pp. 7-10.

Chao, Y., Tsang, Y., & Tsui, C. 1962, "Nerve regeneration through a gap; an experimental study", *Chin Med J*, vol. 81, pp. 740-748.

Chiu, A. Y., Chen, E. W., & Loera, S. 1994, "Distinct neurotrophic responses of axotomized motor neurons to BDNF and CNTF in adult rats", *NeuroReport*, vol. 5, pp. 693-696.

Cohen, S. & Levi-Montalcini, R. 1956, "A nerve growth stimulating factor isolated from snake venom", *Proc Natl Acad Sci USA*, vol. 42, pp. 571-574.

Cohen, S., Levi-Montalcini, R., & Hamburger, V. 1954, "A nerve stimulating factor isolated from sarcomas 37 and 180", *Proc Natl Acad Sci USA*, vol. 40, pp. 1014-1018.

Delbeke, J., Kopec, J., & McComas, A. J. 1978, "The effects of age, temperature, and disease on the refractoriness of human nerve and muscle", *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 41, pp. 65-71.

Dellon, A. L. 1994, "Use of a silicone tube for the reconstruction of a nerve injury [editorial; comment]", *Journal.of.Hand Surgery -.British.Volume.*, vol. 19, no. 3, pp. 271-272.

Den Dunnen, W. F. A., Van der Lei, B., Robinson, P. H., Holwerda, A., Pennings, A. J., & Schakenraad, J. M. 1995, "Biological performance of a degradable poly(lactic acid-epsilon -

caprolactone) nerve guide: Influence of tube dimensions", *Journal of Biomedical Materials Research*, vol. 29, pp. 757-766.

Denny-Brown, D. & Brenner, C. 1944, "Lesion in peripheral nerve resulting from compression by spring clip", *Archives of Neurology (Chic.)*, vol. 52, pp. 1-19.

Docherty P.J. 1982, *Development of a dynamic test method for in vitro biocompatibility studies*, Strathclyde University Thesis.

Drake C.F. & Allen W.M. 1985, "The use of controlled release glass for the controlled delivery of bioactive materials", *Biochemical Society Transactions*, vol. 13, pp. 516-520.

Drew, S. J., Fullarton, A. C., Glasby, M. A., Mountain, R. E., & Murray, J. A. M. 1995, "Reinnervation of facial nerve territory using a composite hypoglossal nerve - muscle autograft - facial nerve bridge. An experimental model in the sheep", *Clinical Otolaryngology*, vol. 20, pp. 109-117.

Duff S.R.I., Malcolm A.J., Drake C.F., Burnie J., Doherty P.J., Juhasz L., & Gilchrist T. 1984, "Biodegradeable glass material a potential bone graft adjunct/substitute," in *Strathclyde Bioengineering Seminars, Biomaterials in Artificial Organs*, 1 edn, Paul J.P. et al., eds., Macmillan, London, pp. 312-317.

Dumitru, D., Walsh, N. E., & Porter, L. D. 1988, "Electrophysiological evaluation of the facial nerve in Bell's palsy. A review", *American Journal of Physical medicine & Rehabilitation*, vol. 67, no. 4, pp. 137-144.

Dyck, P. J., Thomas, P. K., Lambert, E. H., & Bunge, R. 1984, in *Peripheral Neuropathy*, P. J. Dyck et al., eds., Saunders, Philidelphia, London, Toronto, p. 2323.

Erlanger, J. & Gasser, H. S. 1937, in *Electrical Signs of Nervous Activity*, University of Pennsylvania Press, Philidelphia, p. 221.

Esslen, E. 1977, *The acute facial palsies* Springer Verlag, New York.

Fisch, U. 1981, "Surgery for Bell's palsy", *Archives of Otolaryngology*, vol. 107, pp. 1-11.

Foramitti, C. 1904, "Zur Technik der nervenaht", *Arch Klin Chir*, vol. 73, pp. 643-648.

Forman, D. S., Wood, D. K., & Desilva, S. 1979, "Rate of regeneration of sensory axons in transected rat sciatic nerve repaired with epineural sutures", *Journal of Neurological Science*, vol. 44, pp. 55-59.

Fullarton A.C. & Glasby M.A. 1997, "An evaluation of different methods of peripheral nerve repair in the normal and diabetic rat.", *Journal of Hand Surgery*, vol. 22B, no. 4, pp. 486-491.

Gantz, B. J., Gmuer, A. A., Holliday, M., & Fisch, U. 1984, "Electroneurographic evaluation of the facial nerve. Method and technical problems.", *Ann Otol Rhinol Laryngol*, vol. 93, pp. 394-398.

Giardino, R., Aldini, N. N., Perego, G., Cella, G., Maltarello, M. C., Fini, M., Rocca, M., & Giavaresi, G. 1995, "Biological and synthetic conduits in peripheral nerve repair: A comparative experimental study", *International Journal of Artificial Organs*, vol. 18, pp. 225-230.

Gilchrist T., Glasby M.A., Healy D.M., Kelly G., Lenihan D.V., McDowall K.L., Miller I.A., & Myles L.M. 1998, "In vitro nerve repair - in vivo. The reconstruction of peripheral nerves by

entubulation with biodegradeable glass tubes - a preliminary report", *British Journal of Plastic Surgery*, vol. 51, pp. 231-237.

Glasby M.A., Fullarton A.C., & Lawson G.M. 1997, "Immediate and delayed nerve repair using freeze-thawed muscle autografts in complex nerve injuries. Cavitation, fibrosis and haematoma.", *Journal of Hand Surgery*, vol. 22B, no. 4, pp. 479-485.

Glasby, M. A., Clutton, R. W., Drew, S. J., O'Sullivan, M. G. J., & Whittle, I. R. 1995, "Repair of the facial nerve in the cerebellopontine angle using freeze-thawed skeletal muscle autografts. An experimental surgical study in the sheep", *Acta Neurochirurgica*, vol. 136, pp. 151-159.

Glasby, M. A., Gattuso, J. M., & Huang, C. L. H. 1988, "Recovery of peripheral nerves after surgical repair with treated muscle grafts. (1) Physiological assessment", *Neuro-Orthopaedics*, vol. 5, pp. 59-66.

Glasby, M. A., Gilmour, J. A., Gschmeissner, S. E., Hems, T. E. J., & Myles, L. M. 1990, "The repair of large peripheral nerves using skeletal muscle autografts. A comparison with cable grafts in the sheep femoral nerve", *British Journal of Plastic Surgery*, vol. 43, pp. 169-178.

Glasby, M. A., Gschmeissner, S. E., Hitchcock, R. J. I., & Huang, C. L. H. 1986a, "A Comparison of Nerve Regeneration through Nerve and Muscle Grafts in Rat Sciatic Nerve", *Neuro-Orthopedics*, vol. 2, pp. 21-28.

Glasby, M. A., Gschmeissner, S. E., Hitchcock, R. J. I., & Huang, C. L. H. 1986a, "Regeneration of the sciatic nerve in rats. The effect of muscle basement membrane", *Journal of Bone and Joint Surgery*, vol. 68B, pp. 829-833.

Glasby, M. A., Gschmeissner, S. E., Hitchcock, R. J. I., & Huang, C. L. H. 1986b, "The Dependence of Nerve Regeneration Through Muscle grafts in the rat on the Availability and Orientation of Basement Membrane", *Journal of Neurocytology*, vol. 15, pp. 497-510.

Glasby, M. A., Gschmeissner.S.E., Huang, C. L. H., & De Souza, B. A. 1986d, "Degenerated Muscle Grafts Used For Peripheral Nerve Repair in Primates", *Journal of Hand Surgery*, vol. 11B, pp. 347-351.

Glasby, M. A., Mountain, R. E., & Murray, J. A. M. 1993, "Repair of the facial nerve using freeze-thawed muscle autografts. A surgical model in the sheep", *Archives of Otolaryngology Head & Neck Surgery*, vol. 119, pp. 461-465.

Glasscock, M. E., Wiet, R. J., Jackson, C. G., & Dickens, J. R. 1979, "Rehabilitation of the face following traumatic injury to the facial nerve", *Laryngoscope*, vol. 89, pp. 1389-1404.

Glück, T. 1880, "Ueber Neuroplastik auf dem Wege der Transplantation", *Arch Klin Chir*, vol. 25, pp. 606-616.

Granger, C. 1967, "Toward an earlier forecast of recovery in Bell's palsy", *Archives of Physical Medicine and Rehabilitation* , vol. 48, pp. 273-278.

Granger, C. 1976, "Prognosis in Bell's palsy", *Archives of Physical Medicine and Rehabilitation*, vol. 57, pp. 33-35.

Green, J. D., Shelton, C., & Brackmann, D. E. 1994, "Surgical management of iatrogenic facial nerve injuries", *Otolaryngology- Head and Neck Surgery*, vol. 111, pp. 606-610.

Green, J. D., Shelton, C., & Brackmann, D. E. 1995, "Iatrogenic facial nerve injury during otologic surgery", *Laryngoscope*, vol. 105, no. 4 pt 1, pp. 444-445.

Greene, E. C. 1955, in *Anatomy of the rat*, Hafner Publishing co, New York.

Grodon, A. A. & Friedberg, M. D. 1978, "Current status of testing for seventh nerve lesions", *Otolaryngologic Clinics of North America*, vol. 11, pp. 301-324.

Gschmeissner, S. E., Gattuso, J. M., & Glasby, M. A. 1990, "Morphology of nerve fibres regenerating through freeze-thawed autogenous skeletal muscle grafts in rats", *Clinical Anatomy*, vol. 3, pp. 107-119.

Gu, Q., Liu, Y., & Cynader, M. S. 1994, "Nerve growth factor-induced ocular dominance plasticity in adult cat visual cortex", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, pp. 8408-8412.

Guntinas-Lichius, O., Effenberger, K., Angelov, D. N., Klein, J., Streppel, M., Stennert, E., & Neiss, W. F. 2000, "Delayed rat facial nerve repair leads to accelerated and enhanced muscle reinnervation with reduced collateral axonal sprouting durng a definite denervation period using a cross-anastomosis paradigm", *Experimental Neurology*, vol. 162, pp. 98-111.

Hammond, W. S. & Hinsey, J. C. 1945, "The diameters of the nerve fibres in normal and regenerating nerves", *Journal of Comparative Neurology*, vol. 83, pp. 79-89.

Hebel, R. & Stromberg, M. W. 1976, in *Anatomy of the laboratory rat*, Williams and Wilkins Co., Baltimore.

Hems, T. E. J., Clutton, R. W., & Glasby, M. A. 1994, "The repair of avulsed cervical nerve roots; an experimental study in sheep", *Journal of Bone and Joint Surgery*, vol. 76B, pp. 818-823.

Henderson, C. E., Camu, W., Mettling, C., & Gouin, A. 1993, "Neutrophins promote motor neuron survival and are present in embryonic limb bud", *Nature*, vol. 363, pp. 266-270.

Hentz, V. R., Rosen, J. M., Xiao, S. J., McGill, K. C., & Abraham, G. 1991, "A comparison of suture and tubulization nerve repair techniques in a primate", *Journal of Hand Surgery - American Volume*, vol. 16, pp. 251-261.

Hofer, M. M. & Barde, Y. A. 1988, "Brain-derived neurotrophic factor prevents neuronal death in vivo", *Nature*, vol. 331, pp. 261-262.

Hohn, A., Leibrock, J., Bailey, K., & Barde, Y. A. 1990, "Identification and characterization of a novel member of the nerve growth factor / brain-derived neurotrophic factor family", *Nature*, vol. 344, pp. 339-341.

House, W. H. & Brackmann, D. E. 1985, "Facial nerve grading system", *Otolaryngology- Head and Neck Surgery*, vol. 93, no. 2, pp. 146-147.

Hughes, R. A., Sendtner, M., & Thoenen, H. 1993, "Members of several gene families influence survival of rat motoneurons in vitro and in vivo", *Journal of Neuroscience Research*, vol. 36, pp. 663-671.

Hunter, S. C., Glen, J. B., & Butcher, C. J. 1984, "A modified anaesthetic vapor extraction system", *Laboratory animals*, vol. 18, pp. 42-44.

Hyman, C., Hofer, M., Barde, Y. A., & Lindsay, R. M. 1990, *Society for Neuroscience Abstracts*, vol. 16, p. 992.

Ishii, D. N., Glazner, G. W., & Pu, S. F. 1997, "Role of insulin-like growth factors in peripheral nerve regeneration", *Pharmac Ther*, vol. 62, pp. 125-144.

Johnson, E. W. & Waylonis, G. W. 1964, "Facial nerve conduction delay in patients with diabete mellitus", *Archives of Physical Medicine and Rehabilitation*, vol. 45, pp. 131-139.

Kanje, M., Skottner, A., Sjoberg, J., & Lundborg, G. 1989, "Insulin-like growth factor (IGF-1) stimulates regeneration of the rat sciatic nerve", *Brain Research*, vol. 486, pp. 396-398.

Kilvington, B. 1908, "An Investigation of the Regeneration of Nerves with regard to Surgical Treatment of Certain Paralysis (Part IV)", *British Medical Journal*, vol. i, pp. 1414-1419.

Kim, D. H., Connolly, S. E., Zhao, S., Beuerman, R. W., Voorhies, R. M., & Kline, D. G. 1993, "Comparison of macropore, semipermeable, and nonpermeable collagen conduits in nerve repair", *Journal of Reconstructive Microsurgery*, vol. 9, pp. 415-420.

Kimura, J. 1972, "An evaluation of the facial and trigeminal nerves in polyneuropathy; electrodiagnostic study in Charcot-Marie-Tooth disease, Guillian-Barré, and diabetic neuropathy", *Neurology*, vol. 21, pp. 745-752.

Kimura, J. 1981, "Refractory period measurement in the clinical domain," in *Demyelinating Disease: Basic and Clinical Electrophysiology*, S. A. Waxman & J. M. Ritchie, eds., Raven Press, New York, pp. 239-265.

Kimura, J. 1989, *Electrodiagnosis in diseases of nerve and muscle*, 2 edn, F.A.Davis, Philadelphia.

Kimura, J., Giron, L. T., & Young, S. M. 1976, "Electrophysiological study of Bell's palsy: Electrically elicited blink reflex in assessment of prognosis", *Archives of Otolaryngology*, vol. 102, pp. 140-143.

Klemm, H. 1970, "Das perineurium als Diffusionbarriere gegenüber Peroxydase bei epi- und endoneuraler Applikation", *Z Zellforsch*, vol. 108, pp. 431-445.

Kohmura, E., Yuguchi, T., Yoshimine, T., Fujinaka, T., Koseki, N., Sano, A., Kishino, A., Nakayama, C., Sakaki, T., Nonaka, M., Takemoto, O., & Hayakawa, T. 1999, "BDNF atelocollagen mini-pellet accelerates facial nerve regeneration", *Brain Research*, vol. 849, pp. 235-238.

Koliatsos, V. E., Cayouette, M. H., Berkmeier, L. R., Clatterbuck, R. E., Price, D. L., & Rosenthal, A. 1994, "Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons", *Proc Natl Acad Sci USA*, vol. 91, no. April, pp. 3304-3308.

Lawson, G. M. & Glasby, M. A. 1995, "A comparison of immediate and delayed nerve repair using autologous freeze-thawed muscle grafts in a large animal model; the simple injury", *Journal of Hand Surgery*, vol. 20B, no. 5, pp. 663-670.

Lenihan, D. V., Sojitra, N. M., Ikeda, M., Carter, A. C., & Glasby, M. A. 1997, "Stimulated jitter measurment in the assessment of recovery after peripheral nere repair", *Journal of Hand Surgery - British Volume* no. In Press.

Levi-Montalcini, R. & Cohen, S. 1956, "In-vitro and in-vivo effects of a nerve growth stimulating agent isolated from snake venom", *Proc Natl Acad Sci USA*, vol. 42, pp. 695-699.

Li, L., Oppenheim, R. W., Lei, M., & Houenou, L. J. 1994, "Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse", *Journal of Neurobiology*, vol. 25, no. 7, pp. 759-766.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., & Collins, F. 1993, "GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons", *Science*, vol. 260, pp. 1130-1132.

Lindsay, R. M., Thoenen, H., & Barde, Y. A. 1985, "Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor (BDNF)", *Developmental Biology*, vol. 112, pp. 319-328.

Liu, X., Ernfors, P., Wu, H., & Jaenisch, R. 1995, "Sensory but not motor neuron deficits in mice lacking NT4 and BDNF", *Nature*, vol. 375, no. 18 May, pp. 238-241.

Longo, F. M. 1994, "Will ciliary neurotrophic factor slow progression of motor neuron disease?", *Ann.Neurol.*, vol. 36, pp. 125-127.

Longo, F. M., Manthrope, M., & Varon, S. 1982, "Spinal cord neurotrophic factors (SCNTFs): I. Bioassay of Schwannoma and other conditioned media", *Developmental Brain Research*, vol. 3, pp. 277-294.

Loughlin, S. E. & Fallon, J. F. 1993, *Neurotrophic factors*, Academic Press, London.

Lundborg, G. 1988, *Nerve Injury and Repair* Churchill Livingstone, Edinburgh.

Lundborg, G., Dahlin, L. B., & Danielsen, N. 1991, "Ulnar nerve repair by the silicone chamber technique. Case report", *Scandinavian.Journal.of.Plastic.&.Reconstructive.Surgery &.Hand Surgery*, vol. 25, no. 1, pp. 79-82.

Lundborg, G., Dahlin, L. B., Danielsen, N. P., & et al 1981, "Reorganisation and orientation of regenerating nerve fibres, perineurium, and epineurium in pre-formed mesothelial tubes - An experimental study on the sciatic nerve of rats", *Journal of Neuroscience Research*, vol. 6, pp. 265-281.

Lundborg, G. & Hansson, H. A. 1980, "Nerve regeneration through preformed pseudosynovial tubes: A preliminary report of a new experimental model for studying the regeneration capacity of peripheral nerve tissue", *Journal of Hand Surgery (USA)*, vol. 5, pp. 35-38.

Lundborg, G. & Rydevik, B. 1973, "Effects of stretching the tibial nerve of the rabbit. A preliminary study of the intraneural circulation and the barrier function of the perineurium", *Journal of Bone and Joint Surgery*, vol. 55B, pp. 390-401.

Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., & Wiegand, S. J. 1990, *Neuron*, vol. 5, pp. 501-509.

Maki, Y., Yoshizu, T., Tajima, T., & Narisawa, H. 1996, "The selectivity of motor axons during regeneration with sensory axons", *Journal of Reconstructive Microsurgery*, vol. 12, no. 8, pp. 553-557.

Malik, T. H., Kelly, G., Ahmed, A., Saeed, S. R., & Ramsden, R. T. 2001, "Reanimation of total unilateral facial paralysis: a comparison of surgical techniques [Abstract]", *Journal of Skull Base Surgery*, vol. In press.

Mattox, D. E. & Felix, H. 1987, "Surgical anatomy of the rat facial nerve", *American Journal of Otolaryngology*, vol. 8, no. 1, pp. 43-47.

May, M., Blumenthal, F. S., & Klein, S. R. 1983, "Acute Bell's palsy: Prognostic value of evoked electromyography, maximal stimulation and other electrical tests", *American Journal of Otol*, vol. 5, pp. 1-7.

May, M., Harvey, J. E., Marovitz, W. F., & Stroud, M. 1971, "The prognostic accuracy of maximum stimulation test compared with that of the nerve excitability test in Bell's palsy", *Laryngoscope*, vol. 81, pp. 931-938.

May, M., Podvinec, M., Peiterson, E., & Klein, S. 1986, "Idiopathic (Bell's) palsy, herpes zoster cephalicus, and other facial nerve disorders of viral origin," in *The facial nerve*, M. May, ed., Thieme Inc., New York, pp. 365-399.

Mayhew, T. M. 1990, "Efficient and unbiased sampling of nerve fibres for estimating fibre number and size," in *Methods in Neurosciences*, vol. 3 P. M. Conn, ed., Academic Press, London, pp. 172-187.

Mayhew, T. M. 1992, "A review of recent advances in stereology for quantifying neural structure", *Journal of Neurocytology*, vol. 21, pp. 313-328.

McGuirt, W. F. & McCabe, B. F. 1977, "Effect of radiation therapy on facial nerve cable autographs", *Laryngoscope*, vol. 87, no. 415, p. 428.

Medical Research Council 1954, *Peripheral Nerve Injuries* H.M.S.O., London.

Millesi, H. & Terzis, J. 1984, "Nomenclature in peripheral nerve surgery. Committee Report of the International Society of Reconstructive Microsurgery", *Clinics in Plastic Surgery*, vol. 11, pp. 3-8.

Mitz, M., Prakash, A. S., Melvin, J., & Piering, W. 1980, "Motor nerve conduction indicators in uremic neuropathy", *Archives of Physical Medicine and Rehabilitation*, vol. 61, pp. 45-48.

Miyata, Y., Kashihara, Y., Homma, S., & Kuno, M. 1997, "Effects of nerve growth factor on the survival synaptic function of Ia sensory neurons in neonatal rats", *Journal of Neuroscience*, vol. 6, pp. 2012-2018.

Moreano, E. H., Paparella, M. M., Zelterman, D., & Goycoolea, M. V. 1994, "Prevalence of facial canal dehiscence and of persistent stapedial artery in the human middle ear: a report of 1000 temporal bones", *Laryngoscope*, vol. 104, no. 3 Pt 1, pp. 309-320.

Mountain, R. E., Glasby, M. A., Sharp, J. F., & Murray, J. A. M. 1993, "A morphological comparison of interposed freeze-thawed skeletal muscle autografts and interposed nerve autografts in the repair of the rat facial nerve", *Clinical Otolaryngology*, vol. 18, pp. 171-177.

Mufson, E. J., Kroin, J. S., Liu, Y. T., Sobreviela, T., Penn, R. D., Miller, J. A., & Kordower, J. H. 1996, "Intrastriatal and intraventricular infusion of brain-derived neurotrophic factor in the cynomologous monkey: Distribution, retrograde transport and co-localization with substantia nigra dopamine-containing neurons", *Neuroscience*, vol. 71, pp. 179-191.

Murray, J. A., Willins, M., & Mountain, R. E. 1994, "A comparison of glue and a tube as an anastomotic agent to repair the divided buccal branch of the rat facial nerve", *Clinical.Otolaryngology.*, vol. 19, no. 3, pp. 190-192.

Murray, J. A. M. 1991, *Facial nerve repair: A comparison of the anastomotic methods used to repair a divided facial nerve in the rat*, ChM, University of Edinburgh ChM thesis.

Near, S. L., Whalen, L. R., Miller, J. A., & Ishii, D. N. 1992, "Insulin-like growth factor II stimulates motor nerve regeneration", *Proceedings of the National Academy of Sciences, USA*, vol. 89, pp. 11716-11720.

Newman, J. P., Verity, A. N., Hawatmeh, S., Willard, E. F., & Terris, D. J. 1996, "Ciliary neurotrophic factor enhances peripheral nerve regeneration", *Arch Otolaryngology Head Neck Surgery*, vol. 122, pp. 399-403.

Olsson, Y. & Reese, T. S. 1969, "Inaccessibility of the endoneurium of the mouse sciatic nerve to exogenous proteins", *Anatomical Record*, vol. 63, pp. 722-728.

Oppenheim, R. W., Haverkamp, L. J., Prevette, D., McManaman, J. L., & Appel, S. H. 1988, "Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor", *Science*, vol. 240, pp. 919-922.

Paintal, A. S. 1965, "Block of conduction in mammalian myelinated nerve fibres by low temperatures", *Journal of Physiology*, vol. 180, pp. 1-19.

Perez, B., Campos, M.E., Rivero, J., Lopez-Campos, D., Lopez-Aguado, D. 1997, "Incidence of dehiscences in the fallopian canal", *International Journal of Pediatric Otorhinolaryngology*, vol. 40, no. 4, pp. 51-60.

Pham, H. N., Padilla, J. A., Nguyen, K. D., & Rosen, J. M. 1991, "Comparison of nerve repair techniques: suture vs. avitene- polyglycolic acid tube", *Journal of Reconstructive Microsurgery*, vol. 7, pp. 31-36.

Pu, S. F. & Ishii, D. N. 1993, "Distribution of insulin-like growth factor mRNA in rat nerve and muscle during nerve regeneration by *in situ* hybridization", *Society for Neuroscience Abstracts*, vol. 19, p. 253.

Recio-Pinto, E., Rechler, M. M., & Ishii, D. N. 1986, "Effects of insulin, insulin-like growth factor-II and nerve growth factor on neurite formation and survivial in cultured sympathetic and sensory neurones", *Journal of Neuroscience*, vol. 6, pp. 1211-1219.

Rich, K. M., Luszczynski, J. R., Osborne, P. A., & Johnson, E. M. 1987, "Nerve growth factor protects adult sensory neurones from cell death and atrophy caused by nerve injury", *Journal of Neurocytology*, vol. 16, pp. 261-268.

Rosen, J. M., Pham, H. N., & Hentz, V. R. 1989, "Fascicular tubulization: a comparison of experimental nerve repair techniques in the cat", *Annals.of.Plastic.Surgery*, vol. 22, no. 6, pp. 467-478.

Rushton, W. A. H. 1951, "A theory of the effects of fibre size in medullated nerve", *Journal of Physiology*, vol. 115, pp. 101-122.

Sanders, F. K. & Young, J. Z. 1945, "Effect of peripheral connection on the diameter of nerve fibres", *Nature*, vol. 155, pp. 237-238.

Sanders, F. K. & Young, J. Z. 1947, "Influence of peripheral connections on the diameter of regenerating nerve fibres", *Journal of Experimental Biology*, vol. 22, pp. 203-212.

Santos, X., Rodrigo, J., Hontanilla, B., & Bilbao, G. 1998, "Evaluation of peripheral nerve regeneration by nerve growth factor locally administered with a novel system", *Journal of Neuroscience Methods*, vol. 85, pp. 119-127.

Savage M. 1982, "A preliminary report into the development and use of soluble controlled-release glass timing discs implanted into orthodontic appliances", *British Journal of Orthodontics*, vol. 9, pp. 190-193.

Schaitkin, B. & May, M. 1997, *Scott-Brown's Otolaryngology*, 6th edn, Butterworth-Heinemann, Oxford.

Schroder, J. M., May, R., & Weis, J. 1993, "Perineurial cells are the first to traverse gaps of peripheral nerves in silicone tubes", *Clinical Neurology and Neurosurgery*, vol. 95, p. S78-S83.

Seddon, H. J. 1943, "Three types of nerve injury", *Brain*, vol. 66, p. 237.

Seddon, H. J. 1975, *Surgical disorders of the peripheral nerves*, 2 edn, Churchill Livingstone, Edinburgh.

Selander, D. & Sjöstrand, J. 1978, "Longitudinal spread of intraneurally injected local anaesthetics", *Acta Anaesthologica Scandinavica*, vol. 22, pp. 622-634.

Sendtner, M., Carroll, P., Holtman, B., Hughes, R. A., & Thoenen, H. 1994, "Ciliary neurotrophic factor", *Journal of Neurobiology*, vol. 25, no. 11, pp. 1436-1453.

Sendtner, M., Holtman, B., Kolbeck, R., Thoenen, H., & Barde, Y. A. 1992, "Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section", *Nature*, vol. 360, no. 24/31 December, pp. 757-759.

Sendtner, M., Kreutzberg, G. W., & Thonen, H. 1990, "Ciliary neurotrophic factor prevents the degeneration of motor axons after axotomy", *Nature*, vol. 345, pp. 440-441.

Sherrington 1929, "Some functional problems attaching to convergence", *Proc Roy Soc B*, vol. 105, pp. 332-362.

Simpson, S. A. & Young, J. Z. 1945, "Regeneration of fibre diameter after cross-union of visceral and somatic nerves", *Journal of Anatomy*, vol. 79, pp. 48-65.

Smith, K. J. & Hall, S. M. 1980, "Nerve conduction during peripheral demyelination and remyelination", *Journal of Neurological Science*, vol. 48, pp. 201-219.

Sood, S., Anthony, R., Homer, J. J., Van Hille, P., & Fenwick, J. D. 2000, "Hypoglossal-facial nerve anastomosis: assessment of clinical results and patient benefit for facial nerve palsy following acoustic neuroma excision", *Clin.Otolaryngol.2000.Jun.;25.(3):219.-26.*, vol. 25, pp. 219-226.

Spector, J. G., Lee, P., Derby, A., Frierdich, G. E., Neises, G., & Roufa, D. G. 1993, "Rabbit facial nerve regeneration in NGF-containing silastic tubes", *Laryngoscope*, vol. 103, pp. 548-558.

Stålberg, E. 1990, "Use of single fiber EMG and macro EMG in the study of reinnervation", *Muscle & Nerve*, vol. 13, pp. 804-813.

Stålberg, E., Trontelj, J., & Mihelin, M. 1992, "Electrical microstimulation with single-fiber electromyography: A useful method to study the physiology of hte motor unit", *Journal of Clinical Neurophysiology*, vol. 1, pp. 105-119.

Stålberg, E. & Trontelj, J. V. 1979, *Single Fibre Electromyography* The Mirvalle Press Limited, Old Woking, Surrey U.K.

Stevenson, T. R., Kadhiresan, V. A., & Faulkner, J. A. 1994, "Tubular nerve guide and epineural repair: comparison of techniques for neurorrhaphy", *Journal of Reconstructive Microsurgery*, vol. 10, no. 3, pp. 171-174.

Sunderland, S. 1951, "A classification of peripheral nerve injuries producing loss of function", *Brain*, vol. 74, pp. 491-516.

Thoenen, H. 1991, "The changing scene of neurotrophic factors", *Trends in Neurosciences*, vol. 14, no. 5, pp. 165-170.

Thomander, L. & Stålberg, E. 1981, "Electroneurography in the prognostication of Bell's palsy", *Acta Otolaryngolica*, vol. 92, pp. 221-237.

Thomas, P. K. & Olsson, Y. 1984, "Microscopic anatomy and junction of the connective tissue components of peripheral nerve," in *Peripheral neuropathy*, P. J. Dyck et al., eds., Saunders,W.B., Philadelphia, pp. 97-120.

Thomas, P. K., Sears, T. A., & Gilliatt, R. W. 1959, "The range of conduction velocity in the normal motor nerve fibres to the small muscles of the hand and foot", *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 22, pp. 175-181.

Trontelj, J. V., Mihelin, M., Fernandez, J. M., & Stålberg, E. 1986, "Axonal stimulation for end-plate jitter studies", *Journal of Neurology Neurosurgery and Psychiatry*, vol. 49, pp. 677-685.

Trontelj, J. V., Stålberg, E., & Mihelin, M. 1990, "Jitter in the muscle fibre", *Journal of Neurology Neurosurgery and Psychiatry*, vol. 53, pp. 49-54.

Vanlair, C. 1882, "De la regeneration des nerfs peripheriques par le procede de la suture tubulair", *CR Acad Sci (Paris)*, vol. 65, pp. 99-101.

Waggener, J. D., Bunn, S. M., & Beggs, J. 1965, "The diffusion of ferritin within the peripheral nerve sheath: an electron microscopy study", *Experimental Neurology*, vol. 24, pp. 430-443.

Wayne, D. B. & Heaton, M. B. 1990, "The response of cultured trigeminal and spinal cord motoneurons to nerve growth factor", *Developmental Biology*, vol. 138, pp. 473-483.

Weiss, P., Edds, M. V., & Cavanaugh, M. 1945, "The effect of terminal connections on the calibre of nerve fibres", *Anatomical Record*, vol. 92, pp. 215-235.

Winter, C. G., Saotome, Y., Saotome, I., & Hirsh, D. 1996, "CNTF overproduction hastens onset of symptoms in motor neuron degeneration (mnd) mice", *Journal of Neurobiology*, vol. 31, no. 3, pp. 370-378.

Yamamoto, E. & Fisch, U. 1975, "Facial nerve compression in cats", *Acta Oto-Laryngologica*, vol. 79, pp. 390-395.

Yan, Q., Matheson, C., & Lopez, O. T. 1995, "In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons", *Nature*, vol. 373, no. 26 January, pp. 341-344.

Young, J. Z. & Medawar, P. B. 1940, "Fibrin suture of peripheral nerves", *The Lancet*, vol. 2, pp. 126-128.

Appendix 1

Recipe for chemicals

P-phelenediamine

Take 3.000 g of 1% p-phenelenediamine powder and dissolve in 300 ml of distilled water, using a magnetic stirrer, for 10 minutes. P-phenelenediamine is light sensitive and all glassware used must be cover in aluminium foil to protect it from light. Filter the mixture and place in slide trays, again covered from the light.

2.5% gluteraldehyde in 0.1M sodium cacodylate buffer

Take 50 ml of 0.2M sodium cacodylate buffer, pH 7.4. Add 10 ml of 25% gluteraldehyde. pH to 7.4 again. Make up to 100 ml with distilled water.

0.2M sodium cacodylate buffer, pH 7.4

Take 50 ml of 0.4M sodium cacodylate, add 0.2M hydrochloric acid (about 8 ml) to pH the solution to 7.4. Make up to 100 ml with distilled water.

0.4M sodium cacodylate

Dissolve 8.5612 g sodium cacodylate in 50 ml of distilled water. Make up to 100 ml with more distilled water.

0.2M hydrochloric acid

Take 20 ml of 1N hydrochloric acid and make up to 100 ml, 0.2M hydrochloric acid.

5% sucrose buffer solution

Take 50 ml of 0.2M sodium cacodylate buffer and using a magnetic stirrer add 5.000 g of sucrose. pH to 7.4 and make up to 100 ml with distilled water.

0.1% osmium tetroxide in 0.1M sodium cacodylate buffer

Osmium tetroxide is dangerous and must only be handled in a fume cupboard. Take a 0.1 g ampoule of osmium tetroxide and remove its paper label, cleaning the ampoule with absolute alcohol to remove all trace of the paper. Place this in a universal bottle, with the cap tightly screwed closed, and shake to break the ampoule. Add 5 ml of 0.2M sodium cacodylate buffer, pH 7.4 and 5 ml of distilled water and shake to mix. Place the bottle overnight in the refrigerator to allow the crystals to dissolve.

Araldite epoxy resin

Araldite causes skin irritations and should be handled in a fume cupboard. There are four components of the resin which when mixed slowly polymerise:

1. Araldite CY212
2. Dodecenyl succinic anhydrite (hardener)
3. Dibutylphthalate (plasticiser)
4. Benzyldimethylamine (accelerator)

Mix 1 and 2 in equal proportions. Mix 3 and 4 in a 2:1 mix (two measures of dibutylphthalate with one measure of benzyldimethylamine). Fill a universal bottle to its 'shoulder' with the 1 + 2 mixture and add 0.5 ml of the 3 + 4 mixture. Leave overnight on the rotator and store in the refrigerator.

Pyronin B and Toluidine Blue stain

Mix 1 g of di-Sodium tetrahydoborate decahydrate (Borax) in 100 ml of distilled water (1% solution) with a magnetic stirrer for 1 hour). Then mix 1 g of toluidine blue ($C_{15}H_{16}N_3SCl$) and again stir for 1 hour (this makes 1% solution of toluidine blue in 1% borax).

Mix 1 g of Pyronin B in 100 ml of distilled water (1% solution, use magnetic stirrer for 1 hour).

Mix four parts of the 1% solution of toluidine blue in 1% borax with one part of the 1% Pyronin B solution. Filter the combined solution to remove any precipitate.

Appendix 2

Papers resulting from this work:

Gilchrist T., Glasby M.A., Healy D.M., Kelly G., Lenihan D.V., McDowall K.L., Miller I.A., Myles L.M., 1998 "In vitro nerve repair - in vivo. The reconstruction of peripheral nerves by entubulation with biodegradeable glass tubes - a preliminary report". *British Journal of Plastic Surgery* vol 51, pp 231-237.