MICROSURGICAL REPAIR
OF THE
INFERIOR ALVEOLAR NERVE
IN RATS USING SUTURING, NERVE
GRAFT, AND LASER SOLDER
TECHNIQUES

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DECLARATION

The work of this thesis is wholly that of Nigel Curtis, and was undertaken at the Microsearch Foundation and the Cellular and Molecular Pathology Research Unit, Department of Oral Pathology and Oral Medicine at Westmead Hospital within the University of Sydney. This work was carried out towards the degree of Doctor of Philosophy (University of Sydney), is original in nature and has not been published or presented elsewhere by others unless stated.
PUBLICATIONS OF WORK DESCRIBED IN THIS THESIS

Curtis N, Lauto A, Trickett R, Owen E, Walker M.
Preliminary study of the microsurgical repairs of the inferior alveolar nerve in rats.

Curtis N, Trickett R, Owen E, Lanzetta M.
Intraosseous repair of the inferior alveolar nerve in rats: An experimental model.

Curtis N, Lauto A, Trickett R, Owen E, Walker M
Laser activated solder weld repair of the inferior alveolar nerve.
SPIE International Conference BiOS’ 97.
Invited Paper at San Jose, USA Feb 1997.

Lauto A, Curtis N, Cameron A, Dawes J, Owen E.
Laser solder microsurgical technique to repair peripheral nerves.
IQEC; Invited Paper Sydney 1996.

Declarations, Dedications and Acknowledgements, Page D.2
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DEDICATION

I WOULD LIKE TO DEDICATE THIS THESIS TO THE EAST ANGLIAN KINGDOM OF LINDSAY WHERE MY FAMILY ORIGINATE.
SUMMARY

Damage to the inferior alveolar nerve is a well reported complication of oral surgical procedures involving the mandible (Hayward 1986). This phenomenon can occur in mandibular fractures and orthognathic surgery (Rutskii et al. 1987, Upton et al. 1987, Pratt et al. 1996) but it is in cases of elective dentoalveolar surgery that inferior alveolar nerve damage has attracted much attention (Robinson et al. 2004, Mozsary et al. 1985, Wessberg 1985). The resultant altered sensation or anaesthesia of the lower lip is an unpleasant feature, which is a not infrequent cause of litigation, thus causing an interest in the development of techniques to repair the nerve. This project investigated the effectiveness of repair of the intraosseous section of the inferior alveolar nerve. Four types of repair technique were analysed; primary microsuture, interpositional nerve graft, laser solder (liquid) welding, and laser strip (solid) welding, while the effect of delayed repair was also investigated.

Before the four above microsurgical repairs could be carried out a reliable experimental model for the exposure of the intraosseous segment of the inferior alveolar nerve in rats had to be established, since this had not previously been attempted or described. The development of this model involved establishment of anaesthetic and surgical technique which would allow consistent and reliable results of microsurgical repairs to be assessed. The development of liquid and solid strip solder in conjunction with laser welding was again a new technique, not previously used in repairs of the inferior alveolar nerve, developed in conjunction
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with the Microsearch Foundation of Australia where the surgical phase of this study was carried out.

Operating times for both laser (solid) strip and laser (liquid) solder technique were much shorter than that for the traditional techniques, while the laser techniques proved particularly suitable for the intraosseous repair. Histological examination of repaired nerves revealed foreign body reactions, axon deflection and connective tissue intrusion in microsuture based methods, while this was absent where laser based methods were used. In contrast, some perineurial necrosis was noted in laser repaired nerves, although this did not involve the underlying endoneurium and the vasa nervorum appeared intact. Good axonal regeneration was seen in all methods tested, although there appeared to be some disorientation of fibres in nerve graft repairs.

All repair methods tested increased nerve fibre number over sectioned without repair controls. An interesting observation was a shift towards smaller myelinated diameter fibres in laser and primary microsuture repaired nerves relative to unoperated controls, suggesting a mechanism for the symptom of hyperaesthesia following microsurgical repairs.

The trigeminal ganglion was assessed at the histological level for determination of neuron number, expression of nerve growth factor, nerve degeneration and uptake of peripherally applied horse-radish peroxidase tracer. Using this approach, the laser strip methodology was found least associated with pathological changes in

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the trigeminal ganglion. Where repair was delayed, however, the differences in outcome of repair with different surgical methods were reduced.

Although electrophysiological observations were not readily made with some animals, nerve conduction velocity and blink reflex peripheral testing as well as assessment of cortical evoked potentials revealed restoration of function with repair.

From the above, it was concluded that the laser strip repair technique is effective for intraosseous repair of nerves, while further developments of this methodology such as inclusion of neurotrophic factors may improve functional outcomes.
CHAPTER 1

NERVE STRUCTURE, FUNCTION,

INJURY AND REPAIR

RELATED TO THE INFERIOR

ALVEOLAR NERVE
1.1 The Structure of Nerves

Nerves are specifically adapted for the transmission of signals, and as such have an extended linear structure. Individual signals are transmitted by individual nerve fibres, usually termed axons. Axons are single cytoplasmic extensions of individual nerve cells or neurons and are dependent upon functional requirements, may extend in one, two or multiple directions from the nerve cell body to define unipolar, bi-polar and multi-polar nerve cells respectively.

The cytoplasm of the axon, or axoplasm, exists as a thin filament of viscous fluid containing mitochondria, endoplasmic reticulum and a cytoskeleton of microtubules and neurofilaments which are involved in bidirectional axoplasmic transport of vesicles (Sunderland 1978, Terzis et al. 1990). In unmyelinated fibres, the axons are ensheathed by thin cytoplasmic extensions from Schwann cells. Myelinated fibres on the other hand have axons surrounded by layers of myelin secreted by the Schwann cells. These have periodic interruptions of this myelin sheath approximately 1 mm apart termed the Nodes of Ranvier (Sunderland 1978). In this way, Schwann cells effectively define a "tube", within which individual nerve fibres reside.

In addition to the Nodes of Ranvier, myelinated fibres also have interruptions to the myelin sheath termed Schmidt-Lantermann clefts. These have been suggested as providing increased mechanical elasticity for nerves which otherwise would be subject to pressure damage (Lantermann 1874).
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External to the Schwann tubes are two layers of collagen fibres, macrophages and fibroblasts together forming the endoneurium. Fascicles are formed from several axons and endoneurium collected together and are the smallest unit which can undergo microsurgical repair (Mackinnon 1989). The fascicles, in turn, are invested in perineurium, which consists of a connective tissue sheath containing collagen, capillaries and macrophages. The endothelium of the capillaries together with the perineurial membrane act as a blood-nerve diffusion barrier (Lundborg et al. 1982). Depending on the size of the nerve, the fascicles are surrounded by a dense connective tissue layer called the epineurium. The epineurium consists of densely packed collagen and elastin fibres with fibroblasts and an intrinsic vascular plexus derived from the vasa nervorum (Best et al. 1994), (Fig. 1.1A). In small monofascicular nerves, such as the rat inferior alveolar nerve, the epineurium is indistinguishable from the perineurium and the intrinsic blood supply is closely related to the extrinsic blood supply from the inferior alveolar artery (Fig. 1.1B).

1.2. The Function of Nerves

Propagation of signals along nerve fibres occurs as action potentials, which are rapidly spreading waves of depolarization across the axon plasma membrane. Briefly, the balance in activity between sodium and potassium pumps and ion-channels of the normal resting axon produces a slight external positive charge relative to a negative interior. This polarized state can be reversed by changes in the membrane permeability to sodium and potassium, and this is the basis for the action potential. When an action potential is initiated, there is sufficient
depolarization of the membrane to activate voltage gated sodium channels with consequent flooding of the axon interior with sodium. This depolarization propagates along the nerve in large part because adjacent voltage gated sodium channels respond to depolarization. Repolarization is achieved by a combination of active sodium and potassium pump activity, passive movement of ions and a slower and more prolonged voltage gated potassium channel. The sodium channels are temporarily refractory to subsequent stimulation, ensuring that propagation of action potentials is in one direction only and not subject to reverberating waves of activation back and forth along the axon (Barker et al. 1987, Byers 1984). Similarly, because there is a threshold intensity for stimulation of action potentials and further increase in intensity produces no additional effect, nerve function is unambiguous and demonstrates an "All or None Rule" (Pogrel et al. 1992). Since myelin is a good insulator, in myelinated nerves the propagation of the impulse tends to jump between Nodes of Ranvier by so called Saltatory Conduction (Pratt et al. 1980). This dramatically affects the speed of impulse transmission which is up to fifty times faster in myelinated as compared with unmyelinated fibres. Also important in determining the speed of conduction, is the size of the nerve fibre, as larger fibres are able to effect more rapid changes in ion-flux and so mediate more rapid action potentials. The functional properties of nerve fibres according to size and myelination state are shown in Table 1.1.

Because action potentials have this essentially electrical basis, it is possible to assess nerve and cortical function by detecting the sum-total of action potentials in electrophysiological measurements of whole nerves or segments of the cortex.
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(Kocsis et al. 1982). Of interest for this thesis is that such experimental approaches have been used to assess function in the trigeminal nerve (Barker et al. 1987, Singh et al. 1982).

1.3. Nerve Injury

1.3.1. Classification of Nerve Injuries

The severity of nerve injuries has been classified by Seddon (1943) and Sunderland et al. (1950, 1951) with three basic categories defined.

In neuropraxia, there is pressure or shock injury causing contusions, but leaving the nerve intact with no disruption of axonal continuity. Sunderland (1978) describes this as a grade (i) injury.

In axonotmesis, the nerve sheaths intact but section of axons causes Wallerian degeneration of axons (usually to first node of Ranvier). Sunderland (1978) described three further grades of injury in this category. Grade (ii) axonotmesis involves section of axons but otherwise little internal disruption, with the endoneurium, perinuerium and epineurium all intact allowing effective regeneration following initial Wallerian degeneration. Grade (iii) axonotmesis is more severe, with section of axons and disruption of the endoneurium with associated contusions. Grade (iv) axonotmesis is the most severe, with section of
axons and disruption of endoneurium and perineurium, causing significant contusion of the nerve. In grades (iii) and (iv), there is an increasing chance of the regenerating axons entering wrong Schwann tubes and an increasing possibility of fibrosis within and around the nerve (Aebischer et al. 1989, Avellino et al. 1995, MacKinnon et al. 1998). The fibrotic scar tissue may be superficial, around the fascicles, or it may actually fill the endoneurial space thus preventing regenerating axons entering the distal Schwann tubes.

In neurotmesis, the third main pattern of injury, the nerve is completely severed with disruption of the epineurium and separation of the Schwann tubes making spontaneous regeneration and full recovery of sensation less likely. This is classified as grade (v) injury by Sunderland (1978) and again results in increased fibrotic scar tissue formation. On occasion, the regenerating fibres become enmeshed in a fibrous scarring nodule, defined clinically as a traumatic neuroma (Gregg 1990, Bora et al. 1976)

1.3.2. Functional Consequences of Nerve Injury

Although varying degrees of nerve injury are possible (Robinson 1983, Munger et al. 1989, Renahan et al. 1986), the physiological consequences are similar. Total interruption of nerve continuity results in complete loss of function, however, more subtle injuries are also of importance. The endoneurial space containing axonal fibres requires an unchanging environment free of tissue metabolites to

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maintain its impulse transmitting function. A “blood nerve” barrier (Azzam et al. 1991) protecting the space is formed by the perineurium externally and the endothelium of the endoneurial capillaries internally. Loss of this barrier function by injury results in derangement of nerve transmission function (Dyck et al. 1985).

If nerve injury causes contusions and fibrosis, there may be subsequent compression of the nerve trunk. Such compression can be caused by fibrous adhesion internal or external to the nerve and commonly is related to a neuropraxia. This microsurgical removal of such adhesions and nerve compression is termed Neurolysis and can be performed internally or externally. More severe injury to nerve trunks would require surgical restoration of continuity.

Sensory nerve neuropraxia may cause symptoms of paraesthesia, while axonotmesis commonly results in varied dysaesthesia, and neurotmesis produces anaesthesia with varied hyperaesthesia in the repair phase (Klein et al. 1991). In traumatic neuromas, a painful condition may occur termed anaesthesia dolorosa or hyperpathia (Gentle 1986). Gregg (1990) states that these painful phenomena are due to the Aβ mechanosensitive and C fibre chemosensitive neuroma units.
1.3.3. Peripheral Changes After Nerve Injury

When axons are sectioned in a nerve injury, the distal segments undergo Wallerian degeneration (Brown et al. 1994, Stoll et al. 1989, Perry et al. 1993). This involves the proliferation of macrophages and Schwann cells with degradation and phagocytosis of myelin/axon debris. Axon sprouting then occurs from the proximal segments, and these regenerating fibres may enter the empty Schwann tubes in the distal segments, but only if there is no obstruction. Once the axon sprouts have entered empty Schwann tubes, the regenerating fibres follow the basal lamina right up to their target sites with a regenerative rate of anything up to 1mm a day reported (Chiaia et al. 1988, Angelov et al. 1996, Bray et al. 1974). Importantly for this thesis, this has been observed following injury to the trigeminal nerve (Klein et al. 1998, Gregg 1990).

As noted above, the axoplasm is not involved in impulse propagation but does allow transport of neurotrophic substances essential for metabolism and growth (Raivich et al. 1991). Survival of neurons and their regenerative capacity requires a continuous supply of neurotrophic factors (Fukada et al. 1991).

1.3.4. Central Changes After Nerve Injury

Central degeneration also occurs in more severe injuries and these changes have been well documented in relation to the trigeminal ganglion (Arvidsson et al.)
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1990, Zuniga et al. 1990, Waite 1984, Rhoades et al. 1987, Aldoskogius et al. 1978). These studies have shown that in adult rats, as apart from neonatal rats, unrepaird injury to peripheral branches of the trigeminal nerve result in transganglionic degeneration involving degeneration argyrophilia, chromatolysis and primary neuron loss.

The continuity of central nervous tissues with peripheral sites via potentially interrupted nerves can be demonstrated using peripherally injected horseradish peroxidase (HRP) tracer. HRP with a molecular weight of 44,100 was initially crystalized and reported by Theorell in 1942. Extra cellular HRP gains entry into neurons through a process of endocytosis and the endocytotic vesicles containing the enzyme are transported along neural processes emanating from the site of administration. Subsequent staining for HRP tracer uses benzidine type products such as diaminobenzidene (DAB) to produce precipitates at the site of HRP activity, visible by light microscopy (Gobel et al. 1984, Brucell et al. 1987). The injected tracer moves centrally across any injured or repaired site and can be reliably demonstrated at the level of the primary neuron in the trigeminal ganglion by DAB histochemical staining (Vacca et al. 1975, Zuniga et al. 1987, Halprin et al. 1975, Rhoades et al. 1989).

Cresyl violet (CV) staining has also been used to detect central degenerative changes after peripheral nerve injury, in addition to the use of HRP tracer (Aldoskogius et al. 1985, Zuniga et al. 1990, Holland et al. 1990). Importantly,
these central changes appear reversible by microsurgical repair of the peripheral injury (Zuniga et al. 1990).

1.3.5. Electrophysiological Changes After Nerve Injury

Electrophysiological changes after nerve injuries are discernible at both the peripheral (Kocsis et al. 1982) and cortical levels (Turnbull et al. 1991). These changes have previously been demonstrated by the use of sensory evoked potentials and cortical evoked potentials respectively. A common method to assess the peripheral electrophysiological changes involves the measurement of nerve conduction velocity (NCV) and sensory action potential (SAP) (Nocini et al. 1999). At a peripheral level, nerve injury affects the action potential, and the use of sensory evoked potentials can demonstrate this at both a peripheral and cortical level (Stockard et al. 1981, Vriens et al. 1994). Relatively short latency evoked potentials with frequencies from 10-100 Hz (Stockard et al. 1979, Pratt et al. 1980) demonstrate changes in nerve conduction velocity and amplitude of conduction.

The equipment involved in analysis of evoked potentials involves a stimulating unit, amplifier and a display unit. Somatosensory evoked potentials at a cortical level produce a classical display as demonstrated in Fig. 1.2 (Williamson et al. 1987). The receptors are placed over the brain to measure cortical evoked potentials and, as discussed by Kosar et al. (1986) and Zarzecki et al. (1993),
following peripheral nerve injury the so-called cortical map of sensory areas is changed.

In peripheral analysis of somatosensory evoked potential following injury or nerve block, there is reduction in both nerve conduction velocity (NCV) and sensory action potential (SAP) (Narita et al. 1997, Nocini et al. 1999, Vriens et al. 1994). The size of the sensory action potential (SAP) appears to be proportional to the number of excitable fibres present, and there also appears to be some evidence that larger myelinated $A\alpha$ and $A\beta$ fibres appear to recover slower and to a lesser degree than small myelinated or unmyelinated fibres, as demonstrated by reduced somatosensory evoked potentials (Fridrich et al. 1995). In summary a decreased NCV and appearance of low amplitude response indicates injured or demyelinated fibres and thus disturbance of conduction.

Nakayama et al. (1998) used electrophysiological techniques to investigate conduction velocities and the sizes of myelinated and unmyelinated fibres of wistar rats of various ages. The pelvic nerves were stimulated with square waves of 0.5 to 15 volts and action potentials were amplified by a 0.33 secs Highcut 10 kHz amplifier and observed on an oscilloscope. Conduction velocities of less than 2m/sec were classified as due to unmyelinated fibres.
Peripheral nerve injuries and their microsurgical repair can also be assessed at a cortical level by mapping of the somatosensory cortex as described by Chau et al. (1991) and Goyal et al. (1992). Chau describes peripheral nerve injury in kittens and demonstrated a changed somatosensory cortex following spinal nerve injury. Cortical evoked potentials in relation to peripheral nerve injury have indeed been investigated by several authors (Kosar et al. 1986, Turnbull et al. 1991, Kawakami et al. 1989), but these have generally used larger animals such as cats or raccoons and also concentrate on larger areas of the represented cortex rather than the relatively small area responsible for trigeminal nerve stimuli.

Both peripheral and cortical somatosensory evoked potentials were investigated in this thesis to assess inferior alveolar nerve function after repair.

Finally, there has been some work assessing nerve injury by magnetic source imaging (Sutherling et al. 1988, Roberts et al. 1995). The advantage of magnetic source imaging is that images are produced of organ function rather than anatomy, and the challenge of the technology using a biomagnetometer as stated, is to produce meaningful recordings compared to the magnitude of ambient noise. The magnetic field typically recorded from the brain in response to somatosensory stimulation has a peak amplitude of approximately 50fT. Since this response is sensitive to highly sensitive equipment involving the use of super conducting coils is required and these are continuously digitized at a rate of 4 kHz/channel. McDonald et al. (1996) have performed an introductory trial of non invasive
somatosensory monitoring of injured inferior alveolar nerves in humans utilising magnetic source imaging. Though the results appear promising initially it must be remembered that the background noise in such procedures is usually of the order of 1 Tesla compared to the pick-up signal of approximately 50fT and so the accuracy of recordings must be questioned.

1.4. Nerve Repair Methods

1.4.1. Primary Suture

Primary suture repair involves accurate placement of epineurial microsutures with minimal intrusion into the underlying endoneurium. In this method, the goal is accurate approximation of sectioned nerve endings in a tension free environment (Sunderland 1978, Hausamen et al. 1996) (Fig. 1.3).

1.4.2. Free Nerve Grafts

Nerve grafting has been extensively used and is described by Seddon (1985), Sunderland (1978) and more recently by Evans et al. (1994). The free nerve autograft is essentially a method of producing a conduit for regeneration of axons in a sectioned nerve. The graft is sutured to the sectioned ends of the nerve and allows tension free anastomosis in instances where there has been relatively large tissue loss or when a neuroma, tumor or other blockages are excised (Fig. 1.3).
Simple epineural sutures are generally used in these cases as it is almost impossible to anastomose individual fasciculi in such cable grafts. Edinger et al. (1986) compared free autogenous nerve graft techniques utilising non-absorbable and absorbable sutures in rabbits. Comparisons including the electrical stimulus threshold, conduction velocity and histological examination showed little difference in these microsurgical parameters, though low grade foreign body reactions persisted around the ethicon sutures.

1.4.3. Vascular Nerve Grafts

The vascularised nerve graft has been described by Schultes et al. (2000) and using the long thoracic nerve this appears to have produced a good clinical result. Vascularised grafts may be most appropriate for future autogenous grafts, and this has been discussed in a paper by Hobson (2002).

1.4.4. Laser Repair Methods

1.4.4.a. Direct and Laser Solder Repair

The direct laser welding of sectioned nerves has been attempted in a variety of ways, but often the techniques have produced an inadequate anastomosis or thermal damage to the underlying axons (Korff et al. 1992, Huang et al. 1992, Menovsky et al. 1994).
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An alternative method of laser based microsurgical nerve repair using a specialized solder material has recently been studied by several authors (Oz et al. 1990, Lauto et al. 1995). Towards this, the Microsurgical Foundation of Australia has developed a laser solder method of approximating severed nerve endings (Trickett et al. 1997), and this process is specifically investigated in this thesis (Fig. 1.3).

One of the first laser solder techniques to repair nerves was described by Lauto et al. (1995) and then followed by a further report by Trickett et al. (1997). In these papers, a technique of direct laser fusion of nerves utilising a YAG laser was compared with a laser solder fusion technique, utilising an albumin based solder and a diode laser. Trickett et al. (1997) found that the laser solder technique produced successful welds with stronger tensile strength than the direct laser fusion technique, and also with much less thermal damage to underlying tissue through selective absorption of laser energy in the dye within the solder. Trickett et al. (1997) noted that with the addition of a dye such as indocyanine green, they were able to use a simple continuous wave (CW) diode laser to achieve satisfactory nerve welds.

Mean tensile strengths of 15 g have been recorded in in-vitro experiments conducted on laser-welded nerves with the bovine serum albumin based solder. In a further paper by Lauto et al. (1997) the use of a concentrated bovine serum albumin solder with indocyanine green dye in conjunction with a GaAl diode laser
Operating at a 90 milliwatt output with a wavelength of 800 nanometres is described.

1.4.4.b. Laser Strip Solid Solder Methods

In the course of performing experimental work for this thesis, a novel laser solder technique was developed using solid bands of albumin solder, and this appeared to be a marked improvement in the laser solder fusion technique (Fig. 1.3). This was because these bands could be accurately placed across sectioned ends of the nerve without dripping into the defect, prior to fusion with the laser. The effect of this appeared to be more accurate approximation of nerve ends. Both Lauto et al. (1998) and McNally et al. (1999) describe various advantages of solid band protein strips, including higher initial tensile strength of repair, reduced foreign body reaction, reduced entry of fibrous tissue into repair site, reduced levels of thermal damage, and also easier approximation of sectioned ends during the repair.

Lauto et al. 1998 and McNally et al. (1999) describe improvements in a solid band albumin solder, and use of a GaAlAs diode laser with a wavelength of 810 nanometres and output of 90 milliwatts. Protection from thermal damage to the endoneurium has been markedly improved and the tensile strength of the repairs are increased to a level that approaches the traditional microsuture techniques.
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Because this novel repair method raised the possibility of improved outcomes, it was also studied in this thesis.

1.4.4.c. Composition of Liquid and Solid Laser Solders

The Microsearch Foundation has recently developed a reliable laser weld technique which uses a liquid bovine serum albumin solder, containing indocyanine cardiogreen (ICG), and an infrared (IR) diode laser of 810 nm wavelength. The ICG dye in the solder selectively absorbs the IR radiation which then fuses the albumin solder with the epineurium of the severed nerve thus joining the sectioned edges. Purified bovine serum albumin, was preferred for use as the laser solder in this study for reasons of its availability in highly pure form, low cost, biocompatibility and physical properties. The albumin solder also has the advantage of high solubility in water, allowing easy handling, and can be heated to 60°C for 10 hours thus inactivating hepatitis and human immunodeficiency virus without coagulation, should human albumin be used.

The solid protein bands are produced by using an indocyanine green dye with 60% bovine serum albumin mixed in deionised water. This mixture is pressed by a small micrometer controlled device to a thickness of 0.15mm and then cut into strips measuring 3 mm x 0.5 mm. These solid band protein solder strips are then stored in a light proof container prior to use.
1.5. Requirements for Successful Nerve Repair

1.5.1. Axonal Sprouting, Regeneration and the Response of Schwann Cells

Microsurgical repair of nerves has been carried out by several approaches dating back to the 14th century, when Guider Shauliac reported reconstruction of severed nerves. Waller in the 1850s started to experiment on degeneration and regeneration of nerves following injury and established the basics for microsurgical repair of nerves for the future. Waller clearly described regenerating fibres growing from the proximal to the distal stump following nerve section, and it became clear in following years that accurate approximation of the nerve stumps was mandatory for successful nerve repair (Langley et al. 1904). The process of axonal sprouting in the case of a severed nerve was further demonstrated by comprehensive studies by Harrison in 1910 and this phenomenon was crucial in the development of techniques for microsurgical repair of peripheral nerves.

The spatial arrangements of Schwann tubes is of importance when an axon is severed and the regenerating unit has to find its way down the appropriate Schwann tube to reinnervate its target site (MacKinnon et al. 1991, Brown et al. 1990). In myelinated fibres, once a regenerating unit has entered a denervated Schwann, tube it should have an unimpeded pathway to the target. A regenerating unmyelinated axon, however, may have to follow a more complex pathway (Kinnman et al. 1988), crossing between different tubes surrounded by basal laminae as described by Perry et al. (1993). The ratio of myelinated to
unmyelinated fibres in a nerve is therefore an important factor in relation to regenerative capacity (Kwan et al. 1999), and will be discussed later with specific reference to the inferior alveolar nerve.

The Schwann cell reaction also appears important in nerve graft success (Carey et al. 1986, Clemence et al. 1989, Davis et al. 1990). There appears to be an inverse relationship between the number of migrating Schwann cells and number of regenerating axons, while Schwann cells are only generally seen to migrate where there is a gap or lack of continuity in the regenerating nerve (Gulati et al. 1988, Hall 1986, Anderson et al. 1991).

Silver stain histochemistry can be used to determine levels of myelination in individual nerve fibres, while several authors have reported recovery of fibre numbers following microsurgical repair of nerves in animal experiments (Gutmann et al. 1943, MacKinnon et al. 1991). Despite this, others have observed depletion of nerve fibres following microsurgical repair (Kwan et al. 1999 and Jenq et al. 1985). In this thesis, a computerized image analysis system was used to quantitate changes in the number of and level of myelination of fibres following different methods of surgical repair.
1.5.2. Maintained Vascularity

The necessity of maintaining a vascular supply to regenerating and or repaired nerves has been discussed by several authors, such that reduced axonal regeneration occurs if vascular patency of vasa nervorum is compromised. This is also illustrated by reduced axonal regeneration when autologous nerve graft techniques are used (Azzam et al. 1991, Hobson 2002).

Hobson (2002) approached the issue of vascularisation in axonal regeneration from a molecular biology standpoint, and describes an increase in vascular endothelial growth factor with nerve regeneration.

Preliminary experiments in this thesis demonstrated the efficacy of Masson's Trichrome (MT) (Masson 1929) staining for visualizing vessels in histological sections of nerves. This staining method imparts a strongly red colour to muscle fibres and erythrocytes, while collagen appears strongly green or blue. This was found superior to otherwise more widely accepted vascular staining methods such as Banderia simplicifolia lectin histochemistry or alkaline phosphatase histochemistry, due to unacceptably high background levels of labelling.
1.5.3. Surgical Factors

1.5.3.a. Suture Technique

Sir Sydney Sunderland (1978) established benchmarks for the successful repair of peripheral nerve injuries, and these have been revised by Hausamen et al. (1996). The major factor involved for successful repair concerns the accurate approximation of severed axons in a tension free environment. Independent fascicular repair does have some advantages in large multi fascicular nerves and epineurial repair has more advantages in smaller nerves with one fascicule present thus reducing the possibility of surgical trauma in the approximation.

It has been long noted that microsurgical techniques should avoid passing microsuture material into the endoneurium (Holmes et al. 1942, Sunderland 1978, MacKinnon 1989).

1.5.3.b. Delayed Surgical Repair

The effect of delayed microsurgical repair of peripheral nerves after injury has been a contentious issue for some time. For example, Brunetti et al. (1985) report that moderate time delays cause absolutely no difference to axonal regeneration, while some studies report a marked difference. For example, Fu and Gordon in their two papers in 1995 studied the reinnervation of motor fibres following section and delayed repair in rats, and demonstrated a marked reduction in viable
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axons and motor fibres following delay and subsequent microsurgical repair. More recently the studies by Brenner et al. (2004) have found that there is improved neuroenhancement by the immuno-suppressive FK506, following a delayed type of repair indicating that delay significantly reduces axonal regeneration. Further studies by Eckardt et al. (1990) demonstrated regeneration in the inferior alveolar nerve of rabbits following surgical injury and delayed repair, and the authors conclude that regeneration is possible after a delay following section. Also, there appears to be a curious reduction in small diameter fibres or an increase in the larger diameter fibres in this study by Eckardt et al. (1990). It may indeed be that the effect of delayed repair alters the fibre composition within regenerated nerves, and it may be worth comparing this effect with that produced by an interpositional nerve graft. The effect of the various described repairs and delay is assessed in relation to fibre counts and diameters within the nerves distal to injury in this thesis.

1.5.3.c. Repair Using Tubing Materials

The use of tubing as a conduit of regeneration of injured nerves in the instance of an inferior alveolar nerve injury has been described by Pogrel et al. (1998) and Bu et al. (1999). Bu et al. (1999) also used NGF in an attempt to induce regeneration through these conduits. Miloro et al.(2000) conducted further animal experiments but neither of the techniques using gortex or silicone tubing appear to have improved clinical outcome significantly. An autologous repair may be considered.
preferable as described by Pitta et al. (2001), who reports extremely poor clinical results for this synthetic tube technique.

1.5.3.d. Nerve Growth Factor

Nerve Growth Factor (NGF) was first discovered in sarcomas by Levi-Montalolini in 1953. NGF is readily purified from mouse salivary glands and reacts with two kinetic types of NGF receptors in peripheral neurons: the high affinity neuroblastoma type 1 receptor, and the low affinity PC 12 type 2 receptor. It appears that the beta subunit only has neurotrophic activity (Rush et al. 1984).

In humans NGF is a protein consisting of five subunits which stimulates the growth of neurons and could have a role in nerve regeneration, though this is yet to be completely explained (Chan et al. 1987). In the rat, NGF is a protein complex consisting of 3 dissimilar subunits of which the beta subunit appears to be the only neuroactive component (Thoenen et al. 1988). NGF influences are thought to be initiated by the formation of NGF-receptor complexes on the cell surface and subsequent translocation of the complex into the cytoplasm. NGF binds selectively to receptors at the axonal terminals, where it is internalised and transported retrogradely along the axon to the cell body (Daniloff et al. 1986).

Following binding of NGF to the receptors there appears to be an increased formation of cyclic AMP, hydrolysis of phosphoinositides, induction of Na+...
influx, and membrane ruffling. This then leads to rapid changes in protein phosphorylation and increased gene transcription.

The distribution of NGF throughout tissues, as well as its possible role in regeneration of neurons has previously been described by several authors including Olson et al. in (1987), Rich et al. (1986), Crockett et al. (2000). Ramón y Cajal proposed his chemotropic theory of axon guidance in the 19th century and the subsequent discovery of NGF appears to support his original suggestion (Messier-Lavigne et al. 1991). The physiological effects of NGF have been summarised by (Black et al. 1984) as: (1) an essential neutrophic or nourishing influence during early development resulting in selective neuronal survival; (2) a potent influence on neurone differentiation; and (3) a strong neurotropic or guiding influence on direction of neurite growth. The axoplasm of nerves has a bidirectional flow and this allows the movement of NGF along the axon, supporting the idea that this factor may contribute to nerve regeneration after injury (Siegel et al. 1993).

Finn (1987) demonstrated NGF by immuno-histochemistry in growing nerves of the embryonic mouse while localization of NGF to regenerating nerves has been well described by Olson et al. in (1987). These authors used an antibody to mouse salivary gland NGF and peroxidase immuno-histochemistry, similar to the approach used in this thesis, to localize NGF in tissues. In this thesis, expression
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of NGF in the trigeminal ganglia of rats following nerve injury and repair is described.

1.5.3.e. Application of NGF and Other Agents to Aid Nerve Regeneration

The role of neurotrophic factors such as NGF in peripheral nerve regeneration has previously been investigated with inconsistent results. There is some evidence that there is an increase in nerve growth receptor sites when peripheral nerves are regenerating, and recently the use of exogenous NGF to increase the rate of nerve regeneration has yielded some encouraging results, but unfortunately these have been inconsistent in nature (Bu 1999).

FK506 is an immunosuppressive immunophilin ligand, which mediates action through binding to protein 12 and also stimulates axon regeneration (Chunasmuwankul et al. 2002, Gold et al. 1998, Gold et al. 1995, Doolabh et al. 1999). This agent has been applied successfully at the time of injury and microsurgical repair to stimulate axonal regeneration and it appears to increase mRNA levels in nerve cells. FK506 appears to promote neuroregenerative growth following transection or crush type injury and while initial results are promising further study is required before clinical application.
1.6. Nerves and Nerve Repairs in Rats

Although there are comparatively few publications characterizing nerve fibre size and conduction velocity in rats, Nakayama et al. (1998) and Pogatzki et al. (2002) have published detailed studies. Nakayama et al. (1998) looked at the fibre size and conduction velocities of myelinated and unmyelinated fibres in the pelvic nerve of rats, and also assessed the effect of aging. Pogatzki et al. (2002) analysed Aδ and C fibres innervating the plantar rat region and the effect of incision in this area. Prior to work published in this thesis, specific studies have been carried out on fibre size or conduction velocity on the inferior alveolar nerve of rats. Nonetheless, the papers by Nakayama et al. (1998) and Pogatzki et al. (2002) do demonstrate general similarities between rat fibres as compared with humans. In rats, myelinated Aδ fibres have an average diameter of 2.5 μm and a conduction velocity of 10.5 m/s, while unmyelinated or C fibres have an average diameter of 0.65 μm and a conduction velocity of 1.5 m/s.

Nerve injury appears to affect the unmyelinated C fibres less, with little reduction in the number of C fibres, while there does appear to be some spontaneous activity in both Aδ and C fibres following section in rats. Byers (1984) describes the predominance of the Aδ, Aβ and C fibres in the terminal branches of the trigeminal nerve in rats but no details of any alterations of fibre content during injury are mentioned.
1.7. The Inferior Alveolar Nerve

1.7.1. Structure of the inferior alveolar nerve

The inferior alveolar nerve is a branch of the mandibular division of the trigeminal nerve and is closely associated with the inferior alveolar artery in a neurovascular bundle within the mandible. Of particular importance for this thesis, is that it has an extensive intraosseous component, entering the mandible at the mandibular foramen before coursing through much of the length of the lower jaw and exiting through the mental foramen to become the mental nerve (Fig. 1.4). This nerve is primarily sensory, containing both myelinated and non-myelinated nerve fibres with primary neurons in the trigeminal ganglion (Seddon 1985) (Fig 1.4).

The primary neurons for the afferent fibres of the inferior alveolar nerve are located in the trigeminal ganglion, particularly on the postero-lateral aspect of the mandibular division of the ganglion (Fig. 1.5). Cells are of the bipolar type III or IV type (Anil et al. 2003), with central connections to the spinal or brain stem nuclei in the Pons. (Afshar et al. 1983). Central projections follow spinal and cortical pathways thus allowing trigeminal reflex activity and somatic sensory representation respectively (Yamamoto et al. 1999, Matsushima et al. 1989). The so called “Cortical Picture”, is the representation of somatic sensory input to the cerebral cortex and is disproportionately large for the distribution of the inferior alveolar nerve (Fig. 1.2) in the rat. The inferior alveolar nerve is predominantly sensory while the familiar somatotropic organisation of the trigeminal ganglion
allows dependable analysis of nerve function as described by Aldskogius et al. (1985) and Zuniga et al. (1990).

1.7.2. Function of the Inferior Alveolar Nerve

The inferior alveolar nerve supplies sensation to the lower lip and chin, mandibular teeth, mandibular bone, mandibular mucoperiosteum and gingivae. This is a primarily sensory nerve, with only autonomic motor function. Impulse conduction in health has a biphasic action potential with both positive and negative deflections.

If the nerve is damaged and the axollema disrupted, the membrane becomes relatively negative and the action potential only demonstrates a positive deflection (ie Monophasic) (Godfrey et al. 1987).

Heasman et al. (1987) described the specific axon-myelin relationships in the inferior alveolar nerve and demonstrated a wide distribution of axon diameter, myelin content and showed that the larger Aβ fibres had relatively less myelin covering their axons than the smaller pain-transmitting Aδ fibres. Heasman et al. (1987) also concluded that the Aβ fibres are more susceptible to surgical and traumatic damage than the Aδ fibres, since they are relatively less protected. The study of axon diameter in the inferior alveolar nerve showed a mean value of 6.17 um with a range of 2.93-10.81 um thus indicating that there are relatively few unmyelinated C fibres in the human non injured nerve.
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Heasman et al. (1987) also described the presence of Schmidt-Lantermann clefts in the inferior alveolar nerve, which would seem to protect axons from tractional and compressive forces during surgery or trauma and limit subsequent segmental demyelination (Ochoa et al. 1971).

1.7.3. The Effect of Trigeminal Nerve Transection

As indicated in 1.3.4. and 1.3.3., both central and peripheral changes have been consistently reported following trigeminal nerve resection. The peripheral changes in the sectioned nerve involve initial loss of myelinated axons due to wallerian degeneration, with recovery of numbers and axon function if axonal continuity is restored (Gutmann et al. 1943, Jenq et al. 1985).

Aldskogius and Arvidsson (1978) described the central reaction of primary sensory neurons in the trigeminal ganglion following peripheral nerve injury. Peripheral axotomy produces changes in the somatotopic organisation of first order neurons of the trigeminal ganglion, which may influence the results of any subsequent peripheral microsurgical repair (Aldskogius et al. 1985). Analysis of the organisation of first order neurons in the trigeminal ganglion following peripheral axotomy has also been assessed by several other authors, and has usually involved HRP tracer uptake to assess neuronal continuity. Zuniger et al. (1990) reports that in the rat, actual loss of first order neurons following unrepaired peripheral axotomy occurs at a very slow rate of 8% per 100 days. A study by Arvidsson et al. (1990) looked at transganglionic degeneration in young
rats of various ages, and described degeneration argyrophilia of the primary neurons in the trigeminal ganglion. The degeneration of neurons following peripheral nerve transection seems more prominent in neonatal rats, while in mature rats this appears less prominent. Waite (1984) describes survival of myelinated fibres proximal to peripheral nerve section in the trigeminal system of the adult rat, and also reports a slow rate of transganglionic degeneration. Both Arvidsson and Waite agree that there is a much greater neuron loss, in excess of 20%, and transganglionic degeneration, when nerve section is performed in neonatal rats rather than adult rats which generally show a very small absolute loss in neurons and a low rate of degeneration argyrophilia. These changes appear to occur after 60 days following nerve transection.

For these reasons, it was considered that adult rats were most appropriate for the current study, and this also informed choice of appropriate time intervals for delayed repairs and the sacrifice of animals prior to analysis.

Further, Chiaia et al. (1988) reports reduced transganglionic HRP uptake following peripheral injection after infraorbital nerve transection and spontaneous regeneration in rats. These studies have been repeated and similar results obtained by Rhoades et al. in (1987) and Takemura et al. (1990) following transection of the infraorbital and inferior alveolar nerve in rats.
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Similar results were obtained by Zuniga et al. (1990), who describe a double fluorescent labelling technique with fast blue and diamidino yellow, to assess the regenerative organisation in the trigeminal ganglion following mental nerve section. These authors found an orderly somatotopic reorganisation of trigeminal neurons occurring up to 90 days after injury and repair of the mental nerve. Zuniga et al. (1990) clearly identified the neurons of the mandibular division of the trigeminal ganglion in this study. Zuniga et al. (1987) also performed a study of the microsurgical repair of the mental nerve in rats, and found uptake of HRP tracer in the trigeminal ganglion cells confirming axonal continuity following both primary and secondary repair.

Several studies have investigated NGF expression in the trigeminal ganglion of rats following inferior alveolar nerve injury and Fristad et al. (1996) relates this specifically to analysis of neuropeptide Y expression, indicating that this factor is involved in the regenerative process.

Khullar et al. (1998) also report the presence of neuropeptide Y following inferior alveolar nerve injury with growth associated protein 43 in regenerating neurons. Some authors, such as Bu et al. (1999) and Spector et al. (1993), have applied exogenous NGF to inferior alveolar nerve regeneration in conjunction with silicone tube pathways. Bu et al. claim that exogenous NGF increases myelinated fibre regeneration after injury to the inferior alveolar nerve of adult white rabbits. Silicone tube implants, however, appear to have mixed reports so that the results
of additional NGF have to be considered against this inconsistency (Diamond et al. 1992).

As indicated in 1.3.5. electrophysiological changes occur after nerve injuries at both the peripheral (Kocis et al. 1982) and cortical levels (Turnbull et al. 1991), and several studies have been conducted specifically in relation to inferior alveolar nerve injuries. Noccini et al. (1999) used an electrophysiological approach to investigate the effect of transposition of the inferior alveolar nerve as part of dental implant surgery, upon nerve function. NCV and sensory action potential (SAP) were determined by stimulating the region of the mental foramen with 50 microsecond square wave pulses at a 1Hz frequency and at three times the sensory threshold. Signals were recorded in the region of the mandibular foramen with a conventional electromicrograph set at a 50-5000 Hz band-pass. The difficulty of establishing a recording probe in the mandibular foramen is described, and the inconsistent results of NCV and SAP recordings may be attributable to this (Nocini et al. 1999). The authors also describe the high sensitivity of neurographic measurement in this region, and compensation mechanisms from the central nervous system related to this.

Jaaskelainen et al. (1996) examined the blink reflex as related to the stimulation of the mental and inferior alveolar nerves in an attempt to objectively analyse peripheral injuries in humans. It was found that Aβ fibres of the mandibular division form the afferent arc of an elicited blink reflex, and the facial nerve...
serves at the efferent pathway for this reflex. Blink reflex responses were recorded with EMG equipment using 100 Hz low and 1KHz high cut-offs in conjunction with an evoking stimulus. Jaaskelainen et al. (1996) report reasonably consistent responses with the blink reflex in this study, but technical problems may have interfered with results in animals.

Vriens and Pasman (1994) assessed human trigeminal nerve function using short latency somatosensory evoked potentials with or without repair of the inferior alveolar nerve following tumour surgery. Evoked potential testing of these nerves proved a successful method of evaluating nerve function. Narita et al. in 1997 further supported this by evaluating changes within the trigeminal somatosensory system using evoked potentials applied to the inferior alveolar nerve with and without local anaesthetic nerve blocks. The results suggested that the trigeminal somatosensory evoked potential was a reliable method to confirm whether conduction anaesthesia had been achieved.

1.7.4. Techniques For Exposure Of The Inferior Alveolar Nerve

There have been several descriptions of exposure and microsurgical repair of the inferior alveolar nerve in humans and these have been described by a transoral or transcutaneous approach. Probably the most common description is that of exposure of the inferior alveolar nerve by an extended sagittal split of the
mandible as in the traditional mandibular osteotomy approach described extensively by Wessberg and Epker (1982). This relatively extensive approach has a high likelihood of complication and morbidity, and this technique has been further analysed by both Mozsary et al. 1985 and Donnoff (1995), who also described a transcunaneous approach. Jones (1992) described the microsurgical repair of the inferior alveolar nerve injured during third molar surgery and reported that it is sometimes possible to directly visualise the nerve during this form of surgery through the empty extraction socket. Le Blanc et al. (1992) have given a thorough account of transoral and transcunaneous approaches to the inferior alveolar, while more recently Milloro (1995) gave a thorough description of a proposed sagittal buccal plate osteotomy technique. All these techniques are relatively extensive and require internal fixation to replace the osteotomised bone segment at the end of the procedure.

1.7.5. Repair of the Inferior Alveolar Nerve

With regard to the microsurgical repair of the inferior alveolar nerve specifically, there has been considerable interest in the oral and maxillofacial surgery literature. Mozsary et al. (1989), Wessberg (1985) and Donnoff (1995) have all shown interest in the development of techniques for the microsurgical repair of the inferior alveolar nerve after injury in humans. These techniques have involved the use of microsutures with nerve grafts if required, and have exposed the intraosseous segment of the nerve during the procedure.
Some relatively novel approaches following unilateral injury to the inferior alveolar nerve have been described such as a cross mental nerve graft (Kaban et al. 1986), but the potential complications and success rate of this technique excluded it from mainstream management.

There has been some debate (Mozsary 1987) as to whether microsurgical repair of inferior alveolar nerve injuries should be provided in humans. Several studies (Libersa et al. 2003, Takemura et al. 1990) stress that there is early regeneration after injury of the intraosseous segment of the inferior alveolar nerve, but this does not appear to approach completion in cases of neurotmesis (Choukas et al. 1974, Robinson 1988). Animal studies in relation to delayed repair of the inferior alveolar nerve (Eckardt et al. 1990, Zuniga et al. 1990) indicate little effect of delay between injury and repair, or the use of interpositional nerve grafts, although the literature appears inconsistent in relation to this matter (Fu et al. 1995).

The intraosseous nature of injuries to the inferior alveolar nerve has undoubtedly caused difficulty for both the microsurgery and assessment of repairs (Tutskii et al. 1987), and this may have contributed considerably to variability in assessments and views regarding microsurgical repair of the intraosseous segment. With this in mind, the general purpose of this thesis was to implement and evaluate up-to-date
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microsurgical techniques for the repair of the intraosseous inferior alveolar nerve using a rat model system.

1.8. The Absence of Intra-Bony Nerve Repair Studies

Numerous animal experimental studies involving traditional suture techniques have already been conducted, but these have concentrated on the mental nerve, which is the superficial segment of the nerve already emerged out of the bony channel. This is despite the fact that most clinically significant injuries occur within the bony segment of the inferior alveolar nerve. Importantly, the superficial location of the mental nerve affords considerably easier repair, while the fibre content is markedly different to that in the intraosseous segment of the inferior alveolar nerve (Heasman et al. 1987). In the rat, exposure of intraosseous segment of the inferior alveolar nerve has not been described and it was necessary to establish a reliable and reproducible method for this as part of the current thesis. It must be noted that rats were selected for this study in part because the mental and inferior alveolar nerves in this species tend to be large relative to body size, and this was considered potentially helpful for the operative technique in these animals (Emmers 1988).
Figure 1.1. Diagram demonstrating the structure of multifascicular nerves (A) and a surgical photomicrograph of the rat inferior alveolar nerve entering the mandible through the mandibular foramen (B). (A) Fascicles are collections of nerve fibres, together with intrafascicular vessels, enclosed within perineurial connective tissue sheaths. Fascicles are themselves bundled together within a further epineurial sheath, within which larger vessels of the vasa nervorum may be found. (B) The rat inferior alveolar nerve is sufficiently small to consist of a single fascicle, and is seen as a neurovascular bundle (NVB) together with the inferior alveolar artery and vein, which course across the surface of the nerve proper. This nerve courses towards the anterior aspect of the jaw (Black arrow) as it enters the medial surface of the mandible (M) through the mandibular foramen (MF).

Bar = 1mm.
Figure 1.2. Diagram representing the cortical picture of the somatic sensory distribution of the rat (A), as well as a typical somatosensory evoked potential as measured from the cortex after peripheral stimulation (B). (A) Although the inferior alveolar nerve supplies only the lower jaw, lip and chin, the cortical representation of this nerve is disproportionately large as compared with most other body sites (Green = Intraoral distribution, Red = Both Intraoral and Extraoral distribution). This may relate to the importance of foraging habits for the animal. The cortical map is constructed by direct electrophysiological measurement of the exposed rat cortex in response to stimulation of rat tissues. (B) A typical evoked cortical sensory potential recording is shown, demonstrating the cortical response to stimulation over time. P2 and P3 represent localised and generalised repolarisation. N1 and P1 are specific and localised at the cortical reception area while N2 and N3 are generally found diffusely over the cortex and thus do not contribute to construction of the cortical map.
Figure 1.3. Diagram illustrating the four microsurgical repair techniques studied in this thesis including: (A) primary microsuture, (B) interpositional nerve graft, (C) laser (liquid) solder, and (D) laser (solid) strip solder methods. Both primary microsuture and interpositional graft techniques involve the placement of microsutures through the epineurium (Small arrows) (A,B), while in interpositional nerve grafts, sutures are placed from the direction of the graft (Large arrow) towards the continuous in-situ nerve (B). (C) When liquid solder is applied, a laser probe is used to coagulate the solder, tethering the sectioned nerve ends together. (D) Solid solder strips are used in the same way, with the advantage, however, of easier placement as compared with liquid solder.
Figure 1.4. Diagram illustrating the human trigeminal nerve and passage of the inferior alveolar nerve through the mandible. The trigeminal ganglion resides in the base of the skull and is the origin of ophthalmic (V1), maxillary (V2) and mandibular (V3) nerves, each of which exit the skull through separate foramina. The mandibular nerve branches into three separate major divisions, being the buccal nerve, lingual nerve and inferior alveolar nerve (Green). The inferior alveolar nerve passes into the mandible on the medial side of the bone through the mandibular foramen, and then courses forwards through the bone in the inferior alveolar canal together with the artery and vein of the same name. Within the canal, numerous branches supply the teeth and adjacent bone and soft tissues, while the nerve eventually emerges through the mental foramen on the facial surface of the bone to become the mental nerve supplying the lip and chin.
Figure 1.5. Diagram illustrating the distribution of primary sensory neurons for the inferior alveolar nerve within the trigeminal ganglion. The trigeminal ganglion of the rat is slightly different as compared with that of the human, in that the ophthalmic and maxillary divisions are largely fused and anterior to that of the mandibular division. The primary neurons of the inferior alveolar nerve occupy a crescent shaped volume in the posterior portion of the ganglion and are marked IA.
**Table 1.1.** The functional properties of nerve fibres with regard to size and myelination.

<table>
<thead>
<tr>
<th>Fibre Type</th>
<th>Function</th>
<th>Myelination</th>
<th>Fibre Diameter (µm)</th>
<th>Conduction Velocity (m/sec)</th>
<th>Spike Duration (m secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A α</td>
<td>Proprioception</td>
<td>+</td>
<td>12-20</td>
<td>70-120</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>A β</td>
<td>Touch, Pressure</td>
<td>+</td>
<td>6-12</td>
<td>35-70</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>A γ</td>
<td>Motor</td>
<td>+</td>
<td>3-6</td>
<td>12-38</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>A δ</td>
<td>Pain, Temp, Touch</td>
<td>+</td>
<td>2-5</td>
<td>5-35</td>
<td>0.4-0.5</td>
</tr>
<tr>
<td>B</td>
<td>Autonomic</td>
<td>+</td>
<td>&lt;3</td>
<td>3-15</td>
<td>1.2</td>
</tr>
<tr>
<td>C</td>
<td>Pain, Reflexes</td>
<td>-</td>
<td>0.4-1.2</td>
<td>0.5-2</td>
<td>2.0</td>
</tr>
<tr>
<td>C</td>
<td>Autonomic</td>
<td>-</td>
<td>0.3-1.3</td>
<td>0.7-1.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Conduction velocity is greater with increasing diameter and myelination of fibres. Spike duration is a measure of the time during which action potentials pass individual points on nerve fibres, and is greater in slower unmyelinated fibres.
CHAPTER 2

MATERIALS AND METHODS
2.1. Establishment of the Surgical Experimental Model

This project necessitated development of a new surgical procedure for exposure of the inferior alveolar nerve in rats, as well as methods for the intraosseous microsurgical repair of this nerve following transection. It is important to note that this thesis describes the first such study in rats. Consequently, numerous preliminary experiments were performed, using a variety of approaches and varying a number of parameters. Amongst the most critical factors, was the anaesthetic technique, which required significant modification and was based upon the widely accepted use of Pentobarbitone, while hypothermia proved to be an important cause of animal mortality. An anaesthetic technique using Ketamine and Fentanyl was trialed but found to result in an excessive rat mortality. These difficulties were eventually recognized and overcome by development of an inhalational anaesthetic technique based on Halothane plus methods for maintaining body temperature. Additional factors found critical for this study in early experiments, were establishment of a novel bone window approach to the rat inferior alveolar nerve, establishment of methods for accessing and removing both the inferior alveolar nerve and trigeminal ganglion after sacrifice, as well as tissue fixation protocols via intracardiac perfusion.

The numerous but unsuccessful approaches tried in early experiments are only very briefly described below, as these would be both tedious to read and potentially confuse interpretation of experiments. Instead, complete details of only the finally established surgical methods used in experiments yielding interpretable data are provided.
2.2. Anaesthetic Technique

All surgical experiments were conducted following approval by the Microsearch Animal Ethics Review Committee. Wistar rats weighing 250 to 350 g were used throughout, while anaesthesia was induced with 4% halothane with 96% oxygen (Airliquide Pty Ltd, Alexandria NSW, Australia) and subsequently maintained with halothane at 2% with oxygen (98%) applied via a small anaesthetic mask especially constructed from a 20ml syringe (Becton Dickinson Medical Pty Ltd, Singapore) in order to allow access to the mandible (see enclosed Compact Disc).

Active ventilation with the oxygenated anaesthetic agent was maintained throughout surgery, and this appeared to benefit the animals’ respiratory status when compared with the traditional Pentobarbitone type anaesthesia in which the animal self ventilates with air. This approach was found to improve cardiovascular stability during the peri-operative and recovery phases of surgery. Bleeding was controlled by local application of a 20% solution of the vasopressor PRO8 (Ornipressin, Sandoz Australia Pty Ltd) and this also limited cardiovascular fluctuations by reducing blood loss. A 2% Lignocaine local anaesthetic agent (Astra Zeneca, Auckland, New Zealand) was injected locally into the wound, and this powerful analgesic both reduced the depth of inhalational anaesthetic required and improved post-surgical analgesia.
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Throughout surgery and during initial recovery, animals were gently warmed using a heat pad (Valley Lab, Boulder Col, USA) and a cotton wool blanket, and this was found to improve survival by preventing hypothermia.

2.3. Surgical Exposure of the Intraosseous Portion of the Inferior Alveolar Nerve

2.3.1. Early Approaches to Exposure of the Nerve

The technique of intraosseous exposure of the inferior alveolar nerve in the mandible of the rats required development of a new surgical technique, and this incorporated aspects of several different approaches initially tried.

The traditional technique of sagittal split osteotomy of the mandible was not viable in rats due to restrictions of access and potential blood loss. The technique evolved was a cortical window approach, and this required several alterations in technique utilising burs and chisels, as well as the application of local vasopressor PRO8 and bone wax.

2.3.2. The Bone Window Method for Exposing the Rat Inferior Alveolar Nerve

Surgical exposure of an intraosseous segment of the inferior alveolar nerve was consistently achieved by forming a bone window in the buccal plate of the mandible.
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A submandibular approach allowed both excellent access to the mandible and visible isolation of the mental nerve as it leaves the bony foramen (Fig. 2.1A). Briefly, the skin of the submandibular region was shaved and disinfected with betadine (Faulding, Salisbury, South Australia). A No. 15 scalpel (Swann Morton, Sheffield, England) was then used to access the mandible via a 1 to 2 cm incision along the inferior border of the mandible. A self-retaining retractor was used to allow unimpeded access to the underlying mandible, and soft-tissue dissection was carried out with a No. 15 scalpel and Vickers microdissectors (Beckett Instruments, Sunderland, England). Local bleeding control in the soft tissues was achieved by the use of cotton wool pledgets, gauze, and limited bipolar diathermy (Valley Lab, Boulder Col, USA).

The bone window (Fig. 2.2. and enclosed Compact Disk) was formed using an electric drill with a bur and chisels (Satelec, Merignac, France), while intermittent saline irrigation was used to remove bone chips and blood.

Haemorrhage control was aided by applying bone wax (Johnson & Johnson, Brussels, Belgium) and injection of at most 1ml of PRO8 (20%).

Once exposed, the inferior alveolar nerve was isolated by placing a small sheet of expanded gortex (PTFE) 0.1 mm thickness surgical membrane (W.L. Gore Pty Ltd, Flagstaff Az, USA) below the neurovascular bundle (Fig. 2.2. and enclosed Compact Disk). In order to mimic clinical injury, the inferior alveolar nerve was then sectioned and subsequently burned for 3 seconds.
The microsurgical repairs were then performed under an OPMI 7 operating microscope (Oberhokn, Germany) at magnifications ranging from 10 to 12. Once complete, soft tissues including the periosteum, muscle and skin of the submandibular region were closed in layers with 4:0 PDS resorbable sutures (Ethicon, Somerville NJ, USA). The skin was closed with a subcuticular continuous suture to prevent damage to the wounds by the animal. The treated animals were allowed to recover from anaesthesia on a protective sheet of paper in their own cages at room temperature and subsequently maintained on a standard diet of laboratory chow and water.

2.5. Surgical Application of Horse Radish Peroxidase

To confirm patency of nerves in repaired and unreppaired sites, HRP tracer was applied to both left and right mental nerves of animals before harvesting tissues. Briefly, 48 Hrs prior to sacrifice, anaesthesia was obtained as indicated in 2.2. and the submandibular regions of both previously operated and unoperated sides of the animals opened as indicated in 2.3. 100µl of HRP (0.4 mg/ml) (Biosource International, Camarillo CA, USA) in saline was then injected on each side of the animal using a sharp micro-pipette into the soft tissues adjacent to the terminal branches of the exposed and visualized mental nerves (Fig. 2.2F).
2.6. Sacrifice and Harvesting Procedures

2.6.1. Perfusion Fixation

Animals were sacrificed 12 months following the repairs by intracardiac perfusion with 10% buffered formaldehyde as described by Renahan et al. (1986). Briefly, anaesthesia was induced as indicated in 2.2, and once deep anaesthesia was achieved the thorax was opened and a cannula inserted into the left ventricle of the heart during which time the chest cavity was irrigated with Ringer's solution.

Formaldehyde (10%) was run in through this cannula from an elevated burette, allowing immediate fixation of tissues, while femoral vein was sectioned to allow the circulating blood to leave the animal (Aldskogius et al. 1992).

2.6.2. Removal of the Trigeminal Ganglion, Mental Nerve and Inferior Alveolar Nerve

The trigeminal ganglia from both operated and unoperated sites were removed, together with specimens of the mental nerves and repair sites of the inferior alveolar nerves.

Removal of the trigeminal ganglia from the base of skull followed an intra cranial dissection, where the cranial vault was penetrated with bone nibblers and burs before removal of the brain from the cranial vault. The position of the trigeminal ganglia are seen in Fig. 2.1B and the accompanying structures in the base of skull are also demonstrated in this photograph.
Microsurgical Repair of The Rat Inferior Alveolar Nerve

The inferior alveolar nerve was accessed by a submandibular incision and bone window identical to that initially used to access the nerve for injury (2.3.2). The mental nerve was also harvested at this time via the same incision.

2.7. Methods for Microsurgical Repair of the Inferior Alveolar Nerve

2.7.1. Preparation of Nerve Ends for Repair

Prior to repair, the cut ends of the bur sectioned nerve were prepared by trimming the epineurium and any protruding axoplasm (enclosed Compact Disk).

2.5.2. Primary Microsuture Repairs

Microsuture repairs were performed by carefully passing three separate 10:0 nylon sutures (Ethicon, Somerville NJ, USA) through the epineurium of the sectioned ends of nerves with micro-instruments. The sutures were placed so that the sectioned ends of the nerve were closely approximated, but not put under tension (Fig. 2.2A, Fig. 2.2C, and enclosed Compact Disk).

2.7.3. Nerve Graft Technique

2.7.3.a. Harvesting the Femoral Nerve Graft

Free interpositional nerve grafts from femoral nerve donor sites were used. The femoral nerve was accessed via an incision through skin of the rat thigh which had been shaved and disinfected with betadine, similar to 2.3.2. Soft tissues were blunt dissected to expose the femoral nerve, from which 3mm to 5mm of length were removed prior to primary microsuture reanastamosis identical to that
Microsurgical Repair of The Rat Inferior Alveolar Nerve

described in 2.7.2 for the inferior alveolar nerve. Muscle was then closed in layers with 4:0 PDS suture material, while the same material was used to close the skin with a continuous subcuticular suture (enclosed Compact Disk).

2.7.3.b. Placing Interpositional Nerve Grafts

Grafts measuring from 3mm to 5 mm were inserted to replace small segments of the inferior alveolar nerve using a microsuture technique with three sutures per anastamotic site, similar to that described for primary microsuture repairs in 2.7.2. (Fig. 2.2B, Fig.2.2D, and enclosed Compact Disk). Nerve grafts were placed in a reverse direction to reduce the possibility of inappropriate regeneration through side branches.

2.7.4. Laser Weld Technique using Liquid Solder

The laser-weld repairs were performed with an IR diode laser and an albumin-based solder containing ICG dye (kindly provided by Dr Lauto, Microsearch Foundation, Sydney). The GaAlAs laser used to denature the solder produced a continuous wave (CW) at a power output of 80mW± 5 mW and a wavelength of 810 nm. Laser light was applied via a multimode optical fiber, with a 100 μm core diameter, to produce a light surgical tip.

In order to produce the laser weld, the sectioned ends of the nerve were first approximated with microforceps, and a 2-mm strip of liquid solder was then painted in a longitudinal direction across the sectioned ends, using a 30-G cannula (Becton Dickinson Medical Pty Ltd, Singapore) freshly coated with the solder.

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With the surgeon wearing protective goggles, the laser irradiation from the surgical tip was then used to denature the liquid solder into a coagulated strip weld which held the sectioned nerve ends together. The ICG dye changed color from green to light brown, indicating that the solder was denatured. Three such liquid solder strips were sequentially denatured to effect welding of the sectioned nerve ends (Fig 2.2C and Fig 2.2E). The liquid laser solder repairs are referred to as Laser Solder repairs in the text and figures.

2.7.5. Laser Weld Technique using Solid Strip Solder

Solid strip solder became available in the course of the project described in this thesis, and so was included in the study at a later time relative to experiments with liquid solder.

Solid strip solder bands were suggested by Lauto et al. (1997) and McNally et al. (1999) increasing the initial tensile strength of repair following anastomosis, increasing the ease of tissue alignment, and reducing the possible thermal damage to tissue. The solid protein bands were aligned as with the liquid solder bands in parallel across the sectioned ends of the nerve. These were then denatured with a laser as described for liquid solder (2.7.4), (Fig. 2.2D and enclosed Compact Disk).

The solid strip laser solder repairs are referred to as Laser Strip repairs in the text and figures.
2.7.6. *Delayed Microsurgical Repairs*

Delayed repairs were performed 1 month after the initial nerve injury was inflicted. To facilitate later access to injured sites, bone windows were covered with PTFE gortex membrane prior to closure of wounds after nerve injury. Delayed repairs were then performed by uncovering the wound sites, removing the PTFE membrane, and repairing the severed nerves as indicated in 2.7.2 to 2.7.5.

2.7.7. *Controls Consisted of Unoperated animals and Nerve Section Without Repair*

Two types of control were used for surgical repair. Firstly, in each operated case, the unoperated side acted as a control. Secondly, additional experiments were performed in which the inferior alveolar nerve was sectioned as indicated in 2.3.2, but with no repair.

2.7.8. *Use of Animals for Direct and Delayed Repair*

Primary microsuture repair of the inferior alveolar nerve utilising 10:0 nylon suture was studied in 7 cases while 3 additional cases were carried out as delayed repairs one month after section of the nerve.

Microsurgical repair using femoral nerve fascicular grafts with 10:0 nylon suture material was performed on a total of cases 7, with an additional 3 cases performed on a delayed basis one month after section of the nerve.
Repair of the inferior alveolar nerve with liquid solder and laser repair was carried out with 7 rats, and 3 additional cases were performed on a delayed basis one month after section of the nerve.

Microsurgical repair with solid strip solder and laser repair was performed on 7 animals, with an additional 3 cases performed on a delayed basis one month after section of the nerve.

Microsurgical section of the inferior alveolar nerve with no repair performed on 7 animals.

2.8. Histological Methodology

2.8.1. Fixation and Tissue Processing
All tissues removed were fixed with a paraformaldehyde (1%) and glutaraldehyde (1.25%) in a 0.1 M phosphate buffer with a pH of 7.4 at 4°C. Mental and inferior alveolar nerves were then dehydrated with graded alcohols before infiltration and embedding with paraffin. 8 µm paraffin sections were prepared for subsequent histological and histochemical analysis.

After initial fixation for 8 hrs, trigeminal nerve specimens were transferred to a 0.1 M phosphate buffer (Ph 7.4) containing a 10% sucrose solution at 40°C and left overnight. Impregnation with sucrose minimized ice crystal damage upon
subsequent snap freezing in liquid nitrogen, while frozen tissues were embedded in cryoform for frozen sectioning at 10 μm and mounting on adhesive slides. After drying overnight onto slides, the mounted sections were then rehydrated in distilled water.

2.8.2. Histochemical Method to Demonstrate Neurons, Neuron Degeneration and HRP Uptake in Trigeminal Ganglia

Tracer HRP activity was detected by staining with DAB (Ajax Chemicals, Auburn NSW, Australia) (0.75 mgs/ml) in 0.1 M phosphate buffered solution containing 0.003% hydrogen peroxide for 15 mins in a darkened area. The specimens were then again rinsed in distilled water three times for 5 mins each.

Counterstaining was for 5 min with CV (Merck, Darmstadt, Germany) (0.5 g/100 ml) in a 20% ethanol solution, with 10 drops of glacial acetic acid. This solution was warmed and filtered before use.

Sections were then dehydrated with graded alcohols and passed through histoclear prior to cover slipping.

2.8.3. Immuno Histochemical Method to Demonstrate NGF in Trigeminal Neurons

Endogenous red cells and HRP tracer peroxidase was inactivated with 3% hydrogen peroxide followed by washing with water. Frozen sections of trigeminal ganglia were treated with a 1:2,000 dilution of rabbit anti NGF (Sigma NGF 2.5S,
St Louis, USA) which had been reconstituted with 0.1ml of de-ionised water prior to use. 5 drops of the reconstituted anti NGF solution were applied to each section and left for 1 hour at 4°C in humidified chambers. Following this, sections were treated with 3% hydrogen peroxide solution for 5 mins followed by washing with neutral 0.1 M phosphate buffered solution for 2 mins. Goat anti-rabbit antibody peroxidase conjugate (Biosource International, Camarillo CA, USA) was then applied in 1% bovine serum albumin, 0.1 M phosphate buffered saline solution, 5% normal goat serum solution, and a 0.1% bronidox solution. The secondary antibody solution was left in situ again for one hour and then the process of counterstaining with DAB and CV was followed as described previously. CV was again used as the counterstain, similar to 2.8.2.

2.8.4. Masson's Trichrome to Demonstrate Blood Vessels in Inferior Alveolar Nerves

Paraffin sections of the inferior alveolar nerve removed were stained with MT in order to facilitate assessment of the degree of vascularity. MT staining of the sections of the inferior alveolar nerve and accompanying vascular tissue, involved the use of 1% aqueous phosphomolybdic and ponceau-acid fuchsin solution, and 2% light green in 2% acetic acid solution (Masson 1929). The sections of the neurovascular bundle were initially stained with iron haematoxylin solution followed by differentiation and washing in distilled water. The sections were then treated with the ponceau-acid fuchsin solution for three minutes and then washed again in distilled water. This was followed by treatment with phosphomolybdic acid solution for 15 mins and again followed by a further wash in distilled water.
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Counter staining was then performed with 2% light green in 2% acetic acid in distilled water and left for one min. Following this, sections were washed, dehydrated, cleared and mounted on slides.

2.8.5. Silver Staining to Characterize Myelination in Mental Nerve Specimens

The silver staining was with the Holmes Silver technique in borate buffer based on the method described by Bielschowsky (1904), and utilised 1% aqueous silver nitrate and a 1% pyridine solution. Briefly, paraffin sections were soaked in pyridine for two days and then washed in distilled for a further 24 hours. The sections were then exposed to the 1% aqueous silver nitrate (Ajax Chemicals, Auburn NSW, Australia) solution in the dark for four days and again washed in distilled water prior to exposure to the pyridine solution in borate buffer for 4 hours. Sections were again washed in distilled water and then further exposed to a developer (including hydroquinine, sodium sulphite, 0.2% gold chloride, 1% oxidic acid and sodium thiosulphate solution) for a further 12 hours. This allowed reduction and was followed with dehydration, clearing and cover-sliping.

2.8.6. Central Analysis of Sections of the Trigeminal Ganglia

Counts of neurons in the mandibular division of the trigeminal ganglion with degenerate morphology, as well as of cells expressing NGF and HRP tracer uptake were performed using a 0.5mm graticule at x40 magnification under blind conditions. The method of accumulative means was used to determine the number of ganglion sections required to obtain statistically reliable results. Analysis of accumulative means showed that following Neuron and HRP counts in 3 to 5
Microsurgical Repair of The Rat Inferior Alveolar Nerve

microscopic fields, the variation fell below +/- 5% (Fig. 3.4). 5 microscopic fields were therefore used for the analytical counts in this study.

2.8.7. Peripheral Analysis of Sections of the Mental Nerves

Silver stained sections of mental nerves distal to the point of injury or repair were analysed at the Key Centre for Microscopy and Microanalysis on the campus of the University of Sydney. Silver stained sections were examined under oil at x100 magnification with a Zeiss (Munich, Germany) Axioplan Light Microscope and analysed with a 3 CCD colour camera (Sony, Tokyo, Japan) and Zeiss K400 image analysis software. Nerve axons were differentiated by grey scale thresholding and fibre counts, areas and diameters were determined by this image analysis system which produced objective analysis of the silver stained images on a black/white (yes/no) basis.

2.8.8. Peripheral Analysis of Blood Vessels at the Site of Injury/Repair

MT stained sections of the inferior alveolar nerve were used to assess vascularity using a 0.5mm graticule at x 20 magnification. Vessel profiles were quantitated per graticule area and the method of accumulative means was again used to confirm that five fields were required for reliable determination in this analysis.

2.8.9. Statistical Methods

Accumulative means analysis demonstrated a likely normal distribution of data and supported the use of Students T Test for most statistical analyses performed. In some cases where data appeared to be non-parametric in nature, Wilcoxon's
Microsurgical Repair of The Rat Inferior Alveolar Nerve

Ranked Sign test was used where samples were paired, or alternatively, the Mann Whitney U-Test was applied where samples were not directly related. A p value of < 0.05 was considered statistically significant in two tailed tests.

2.9. Electrophysiological Methodology

2.9.1. Assessment of Nerve Conduction Velocity

Prior to sacrifice, 12 months following the microsurgical repairs, a Cadwell-Sierra evoked potential device (Cadwell Laboratories, Kennewick WA, USA) was used to determine nerve conduction velocity and sensory action potential. Briefly, after establishment of anaesthesia, a 25 gauge insulated stimulating electrode was placed in the deep area of the mandibular foramen on the lingual surface of the mandible, and a recording electrode of a similar gauge was placed at the position of the mental foramen where the inferior alveolar nerve emerges at the anterior part of the mandible (Nocini et al. 1999). The inferior alveolar nerve was stimulated with a 30 mA rectangular pulse at a frequency of 1-10 Hz, and the resulting trace observed with an oscilloscope (Bennett et al. 1983, Singh et al. 1982).

2.9.2. Assessment of Blink Reflex

A blink reflex (H-Reflex) following stimulation in the region of the mental foramen was also assessed using the Cadwell-Sierra equipment. In this technique, as described by Jaaskelainen et al (1996), the A\(\beta\) fibres of the mandibular division of the trigeminal nerve act as the afferent fibres and these link with the motor arc served by the facial nerve supplying the periorbital muscles. A 25 gauge
stimulating electrode was placed again at the site of emergence of the mental nerve from the mental foramen, and the recording electrode was placed in the region of the lower eyelid. A stimulating pulse of 30 mA was provided, with a high cut off of 1000 Hz and a low cut off of 10 Hz established. Results were recorded using the Cadwell-Sierra oscilloscope and were described as H-Reflex analysis.

2.9.3. Assessment of Cortical Evoked Potentials

Animals were anaesthetised as indicated in 2.2 and following careful surgical exposure of the cerebral cortex using a bone drill and burrs, direct stimulation of the mental nerve was achieved via a 25 gauge electrode. The stimulating electrode delivered square pulses of 1-2 mA with a high cut off 10 kHz amplifier and the recording electrode was placed on potentially receptive areas of the cortex (Williamson et al. 1987). Recordings were made using an oscilloscope to determine the most receptive areas of the cortex, and any marked changes in the cortical picture noted (Turnbull et al. 1991).
Figure 2.1. Surgical photographs of a submandibular exposure of the rat mandible and mental nerve (A), as well as the trigeminal ganglia in the base of the skull (B).

(A) The facial surface of the mandibular bone appeared white against the adjacent masseter (M), mylohyoid (Mh) and anterior digastric (ADg) muscles. The mental nerve (MN) exited through the mental foramen in the anterior aspect of the mandible (Black arrow), and was recognized as the terminal portion of the inferior alveolar nerve. (B) The floor of the calvarium was viewed from above after first removing the brain by opening the calvarium (C) and sectioning the brain stem (BS). From this vantage point, the trigeminal ganglia (TG) were readily seen as white neural tissue against the slightly darker vascular bone of the base of skull.

The major portion of each ganglion comprised the ophthalmic-maxillary division (OMD), while the mandibular division (MD) was readily identified. Both the ophthalmic-maxillary and mandibular divisions exit the skull via separate foramina (White arrow heads). The olfactory nerves (ON) were seen at the most anterior portion of the skull (Black arrow).

Bars = 5 mm for (A) and 7 mm for (B).
Figure 2.2. Surgical photographs illustrating the mandibular bone window approach to the rat intraosseous inferior alveolar nerve (A-B) for primary microsuture (C), interpositional nerve graft (D), and laser solder (E) repairs, as well as the injection of HRP into the surgically exposed mental nerve region (F). The bone of the mandible was readily identified while definition of the anterior direction was important for orientation (Arrows). The mental nerve (MN) was seen emerging from the anterior superior portion of the mandible, while surrounding anterior digastraic (ADg), mylohyoid (Mh) and masseter (M) muscles were readily deflected to provide access to surgical sites via a submandibular incision. Bone windows (W) were prepared using burs and chisels to define the peripheral borders of windows in the cortical mandibular bone (A), and then removing the cortical window to expose the underlying intraosseous portion of the inferior alveolar nerve (IAN) (B). Fine vessels were often seen coursing over the surface of the exposed inferior alveolar nerve (White arrow heads), comprising the main vascular supply to the nerve. Confirming the continuity of nerves studied, was movement of the mental nerve upon traction of the inferior alveolar nerve. Gortex membrane material (G) was used to help isolate the inferior alveolar nerve for surgery. Microsutures (MS) were effective in drawing sectioned nerve ends together in both primary microsuture (C) and interpositional nerve graft (ING) (D) repairs. Laser solder (LS) was also effective in joining nerve segments (E). (F) Application of HRP tracer to the mental nerve (MN) required a separate surgical approach, in which the mental nerve and its branches (Br) were exposed by deflection of the overlying orbicularis oris (OrO) muscle with dissection of muscle attachments (At) away from the underlying mandible. HRP was then injected into the soft tissues adjacent to the mental foramen via a micropipette (P). Bar = 4 mm.
CHAPTER 3

SURGICAL AND MORPHOLOGICAL OBSERVATIONS OF INFERIOR ALVEOLAR NERVE REPAIRS IN RATS
3.1. **Introduction**

Before microsurgical techniques could be assessed, a reliable and reproducible method for exposure of the intraosseous segment of the inferior alveolar nerve in rats had to be established.

A number of parameters were considered important including: minimal morbidity and complications for the animal; prevention of unnecessary iatrogenic damage to the intraosseous inferior alveolar nerve during exposure; ready access to repaired nerves for inspection and tissue harvesting at different times after operation; minimization of intraosseous bleeding which may interfere with nerve repair; and closure of wounds in a way minimizing interference by animals.

With regard to development of the microsurgical repair methods, a number of specific issues were of particular interest for study. Firstly, it desired to determine the performance of functional microsurgical repair of the intraosseous segment of the inferior alveolar nerve using traditional microsuture techniques, nerve autografts and the new laser nerve welding techniques. Importantly, it was desired to compare the traditional microsuture and nerve graft approaches with the new laser nerve welding techniques in the specific environment of the intraosseous segment of the inferior alveolar nerve. Further, since most surgical repair of the intraosseous nerve injuries occurs after some delay, the effect of delayed repair was of interest. In addition, the possibility that laser based methods may cause significant thermal damage to the underlying endoneurium or vasa nervorum was considered important.
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With the above aims in mind, an experimental model for the surgical exposure of the intraosseous segment of the inferior alveolar nerve was established.

3.2. Results

3.2.1. The Method for Surgical Exposure of the Inferior Alveolar Nerve Was Reliable With Minimal Morbidity and Mortality

The anaesthetic and surgical procedures detailed in 2.2 and 2.3 were found to have a very low morbidity and mortality for animals. Once the methodology was established, out of 50 rats operated on, there were only 3 anaesthetic deaths while tissues harvested from 47 animals were of sufficient quality for inclusion in this study. No problems in post-operative recovery, wound healing, wound interference by rats or infection were encountered, greatly facilitating subsequent analysis.

3.2.2. Laser Solder Strip Repairs Required Less Operating Time Than Other Microsurgical Repair Methods Studied While Nerve Grafts Were Most Time Consuming

Comparison of operating times for each of the surgical repair methods tested are shown in Fig. 3.1. Nerve grafts required significantly more time to perform as compared with any other method tested ($p < 0.001$). The fastest repair method was that using laser strips ($p < 0.001$).
3.2.3. Complications of Traditional Microsuture Repairs Were Seen

As seen in Fig. 3.2 showing nerves one year after primary repair using microsutures, suture material elicited a mild foreign body giant cell reaction. In addition, axons appeared deflected around sutures penetrating into the endoneurium while there was also penetration of fibrous connective tissues into the body of the nerve around suture materials (Fig. 3.2).

Nonetheless, nerve regeneration did appear to occur, as evidenced by continuity of axons across previously injured sites (Fig. 3.3). A wavy course was often noted for regenerated axons in nerve grafts (Fig. 3.3), and this was attributed to being either due to less ordered axon regeneration or alternatively internal folding of nerve grafts slightly longer than the repair defect.

3.2.4. Nerve Regeneration in Laser Solder and Laser Strip Repairs Without Thermal Damage to Endoneurium or Vessels

Histological examination of paraffin sections of inferior alveolar nerves harvested immediately after laser solder repair revealed close apposition of nerve ends (Fig. 3.2). Importantly, although there was some apparent heat induced necrosis of the epineurium in immediate contact with the solder material, the underlying endoneurium appeared unaffected. Similarly, vessels within the nerve appeared normal in sections (Fig. 3.2 and 3.3).
3.2.5. **Limited Quantitative Analysis Demonstrated the Suitability of the Experimental Model for Further Quantitative Studies and Confirmed HRP Uptake**

To determine the suitability of the experimental model system for further detailed quantitative analysis at the histological level, a limited analysis was performed comparing the number of neurons present in trigeminal ganglia, as well as HRP tracer labeling in nerves treated by either primary microsuture repair or liquid laser solder repair, as compared with untreated controls.

Groups contained 7 rats each, and after tissue processing, histochemical staining and nerve cell quantification as described in Chapter 2, data were subjected to a method of accumulative means. This determined the number of ganglion sections required to obtain statistically reliable results as 5 since at this point, variation fell below +/- 5% (Fig. 3.4).

The mean neuron counts for the microsuture and laser solder were 24.11 ± 2.02 and 22.91±1.17, respectively, which was comparable to the unoperated controls (25.91 ± 1.47 and 24.82 ±1.54, respectively counting controls as contralateral unoperated nerves in each group). The mean number of neurons with HRP tracer uptake for the microsuture and laser solder repairs was 3.68 ± 0.80 and 3.77 ± 0.43, respectively, which were comparable to the unoperated controls (4.37 ± 0.40 and 4.41 ± 0.41, respectively counting controls as contralateral unoperated nerves in each group). In this preliminary analysis, neuron counts and HRP tracer uptake in the microsuture and laser solder groups compared well with unoperated

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controls, suggesting restoration of nerve function after repair with both the microsuture and laser solder techniques.

3.3. Discussion

Despite early difficulties, the anaesthetic and surgical procedures developed in this thesis for accessing the inferior alveolar nerve via a mandibular bone window proved to be both effective and minimally traumatic for animals. Surgical recovery was generally uneventful, while it was also noted that recovery of the graft donor site was uneventful in terms of healing and function during this study. In addition, all of the surgical repair methods for sectioned inferior alveolar nerve were found to establish mechanical continuity of the nerve, without unduly extended surgical times. Interpositional nerve grafting was found to require the longest surgical time, while laser strip soldering was the quickest repair technique studied. It should be borne in mind, however, that although laser strip repairs were found very quick as compared with other methods, savings in time due to the actual nerve repair procedure are limited by the large amount of time required to first expose the nerve and then close the wound.

In the laser weld group, the need for protective eye wear did not cause undue difficulty for procedures under the operating microscope. In fact the time to perform both the laser solder and laser strip repairs was markedly shorter than for primary microsuture repairs.

Some evidence of axon deflection and disorientated regeneration in nerve grafts was seen. However, there was no sign of neuroma formation where microsuture techniques were used, as previously described by Bora et al. (1976).
Surgical repairs appeared satisfactory at the histological level, in that axon regeneration occurred using all methods studied. However, foreign body giant cell reactions, axon deflection and connective tissue intrusion were noted around sutures, supporting the need to develop alternative methods. Laser solder and laser strip repairs appeared to offer an alternative surgical approach without these difficulties. The initial concern that these methods may result in vascular damage or extensive thermal necrosis of the nerve appeared unfounded. This was because the vasculature and endoneurium appeared normal immediately after laser treatment, and laser treated nerves appeared to recover well over the year following repair.

HRP tracer was detected in trigeminal ganglia, suggesting that this experimental approach to confirming continuity of peripheral nerves with the central ganglion was effective. Similarly, the CV staining method appeared effective for assessing neuron degeneration in the ganglion as expected from the literature (Aldskogius et al. 1992).

The need for quantitative analysis was apparent, and the limited analysis of neuron number and HRP tracer uptake performed was sufficient to provide confidence that a more detailed quantitative histological study was justified. This further histological analysis is described in Chapter 4.
Figure 3.1. The mean operating time required for surgical exposure and repair of the inferior alveolar nerve using primary microsuture, interpositional nerve graft, laser (liquid) solder and laser (solid) strip solder microsurgical techniques. Nerve grafting required the most time ($p < 0.001$), reflecting the need to harvest nerve tissue from a separate donor site. Primary microsuture repair required longer than either laser solder techniques studied, consistent with the greater time required to place individual sutures as compared with placement and activation of solder ($p < 0.001$).
Figure 3.2. Photomicrographs of paraffin sections of rat inferior alveolar nerves one year after primary microsuture repair (A,B,C), or immediately after injury and laser (liquid) solder repair (D,E,F). Axons were readily identified in paraffin sections stained using Masson's trichrome as red-brown-purple linear and or circular structures (Ax), often with accompanying Schwann cell nucleii. Collagen fibres contrasted strongly with these, staining blue to green with Masson's trichrome. Microsutures (MS) were seen in sections of repaired nerves, and a mild foreign body giant cell (GC) response was noted around these in some sections (A), as was axon deflection (AxD) (B) and connective tissue intrusion (CTI) (C). This contrasted with laser solder repaired nerves where no such changes were seen. The possibility was considered that laser based techniques may cause significant thermal damage to the endoneurium or vessels. When laser liquid solder repaired nerves were processed for histology immediately after repair, however, endoneurium and blood vessels appeared normal (D,E,F). At low magnification, necrotic perineurium (nP) was seen only in association with laser solder (LS), while adjacent perineurium (P) appeared normal. Very close approximation of cut ends (arrow) was seen, while examination at higher magnification (E,F) revealed both intact and apparently normal endoneurial vessels (Red arrow heads), and the accumulation of a small amount of proteinaceous material and occasional erythrocytes (E) in the gap between sectioned nerve ends (E,F). These observations suggested that laser solder based repair methods may be superior as compared with microsuture based repairs. Masson's Trichrome, Bars for: A&B = 20 μm; C = 50 μm; D = 400μm; E = 200 μm; F = 50 μm.
Figure 3.3. Photomicrographs of paraffin sections of rat inferior alveolar nerves one year after repair by primary microsuture (A), interpositional nerve graft (B), laser (liquid) solder (C), and laser (solid) strip solder (D) techniques. Axon regeneration (AR) was seen in all repair techniques studied, as evidenced by the presence of axons (Small black arrows) throughout repaired nerves, and continuity of nerves across previously interrupted sites. The perineurium appeared normal in all repairs, while perineurial (Red arrow heads) and endoneurial (Black arrow heads) blood vessels were seen. Occasional peripheral fasciclar bundles (PFB) were seen in repairs, indicating that small nerve branches were retained and or restored after repair. A wavy course was noted for axons in some regions of interpositional nerve grafts (Large black arrows) (B), and this was attributed to either disorientated regeneration or alternatively, folding of nerve grafts within the repaired site.

Masson's Trichrome, Bars for: A, C & D = 50 μm; B = 200 μm.
Figure 3.4. The effect of accumulating means upon determination of the mean number of neurons per 0.5mm graticule area, at a x40 magnification, in trigeminal ganglia of unoperated cases. To establish confidence in the quantitative approach planned for determination of neuron number in trigeminal ganglia, a method of accumulative means was applied to determine the minimum number of observations to obtain reliable results. For these counts the variation about the mean needed to be less than the difference between the mean of the experimental group and the mean of the control group. A < 5% variation about the mean was consistently obtained following 5 observations and this number of counts was subsequently used for analysis in this study.
CHAPTER 4

QUANTITATIVE HISTOLOGICAL COMPARISON OF MICROSURGICAL REPAIR METHODS FOR THE INFERIOR ALVEOLAR NERVE
4.1. Introduction

Chapter 3 describes the establishment of procedures for exposure, injury and repair of the rat inferior alveolar nerve. Also, a preliminary and limited histological assessment of neuron number and HRP uptake, indicated that a more detailed quantitative histological analysis of tissues obtained from this experimental model was justified.

Chapter 4 extends these preliminary histological studies, to compare the effect of the different repair methods used. In particular, changes in the trigeminal ganglion, inferior alveolar nerve and the mental nerve are described.

In the course of conducting these experiments, the laser strip method for nerve repair became available for study. This provided the opportunity to extend the project past the laser (liquid) solder method originally investigated in assessment of mental nerve changes. Because of this, subsequent characterization of vascular changes in the inferior alveolar nerve as well as events in the trigeminal ganglion included laser strip repair specimens.
4.2. Results

4.2.1. Changes in Myelination Size of Fibres in the Mental Nerve With Surgical Repair

4.2.1.a. Myelinated Fibres Were Readily Recognized in the Mental Nerve by Positive Silver Staining

Silver stained paraffin sections of the mental nerve permitted clear identification of both myelinated and unmyelinated fibres. Myelin appeared as an intense brown stain, while unmyelinated fibres were recognized as distinct linear or circular structures lacking brown stain. In places, myelin sheaths appeared incomplete and this was interpreted as sites where sections passed obliquely through Nodes of Ranvier (Fig. 4.1).

4.2.1.b. Sectioning of the Inferior Alveolar Nerve Reduced Myelination in the Mental Nerve, and This Was Reduced By Nerve Repair

The intense positive stain for myelin permitted application of an image analysis system for characterization of changes in nerve fibre myelination in the mental nerve. Image analysis involved the conversion of photomicrographic images of silver stained mental nerve sections from color to black and white, and further quantitation of the relative proportion of sections occupied by myelin. Fig. 4.2A shows a typical image analysed by the image analysis software, while in Fig. 4.3, sectioning of the inferior alveolar nerve is seen to significantly reduce levels of mental nerve myelination (p < 0.001) when assessed with the student t-test).

Demyelination of the mental nerve was reduced by surgical repair, while micro-
suture and laser solder methods appeared more effective than nerve grafts in maintaining myelination (p < 0.05).

4.2.1.c. Sectioning of the Inferior Alveolar Nerve Reduced The Number of Nerve Fibres in the Mental Nerve, While Surgical Repair Protected From This

Image analysis of silver stained paraffin sections of the mental nerve indicated a significant reduction in the number of nerve fibres after injury (p < 0.001), while surgical repair significantly increased the number of fibres preserved (p < 0.05) (Fig. 4.4). Of the three methods examined in this part of the study, nerve grafting resulted in the most pronounced reduction in nerve fibre numbers (p < 0.05).

4.2.1.d. Sectioning of the Inferior Alveolar Nerve Altered The Nerve Fibre Size Distribution of the Mental Nerve, While Fibre Size was Reduced in Surgical Repairs Relative to Untreated Controls

The diameter of nerve fibres also changed following nerve sectioning (p < 0.05), while fibres in nerves which had been repaired by laser solder and microsuture techniques were smaller as compared with untreated controls (p < 0.05) (Fig. 4.4). This contrasted with the effect of nerve grafting, where despite a significant reduction in nerve fibre number relative to control specimens, there was only a modest reduction in nerve fibre size (p < 0.2) with the majority of fibres having diameters comparable to those in controls (Fig. 4.4).
4.2.2. Vascularity of the Inferior Alveolar Nerve Was Reduced by Sectioning, And Restored by Surgical Repair

Because vessels were readily recognized in MT stained sections (Fig. 4.2B), it was possible to investigate levels of vascularity in nerves following repair. Fig. 4.5 demonstrates the effect of nerve sectioning in significantly reducing vascularity of the inferior alveolar nerve (p < 0.001). Surgical repair restored levels of vascularity to those of control tissues (p < 0.1) in all cases other than where nerve grafts were applied, where only a more modest improvement in vascularity was noted (p < 0.05).

4.2.3. Changes in the Trigeminal Ganglion With Surgical Repair

4.2.3.a. Cresyl Violet Staining Distinguished Between Intact and Degenerate Neurons

Fig. 4.6 shows the characteristic appearance of the trigeminal ganglion stained with CV. Intact neurons demonstrated the characteristic intense CV staining expected with this histochemical method. On the other hand, some cells had a pale and highly vacuolated appearance, and this was interpreted as evidence for neuronal degeneration (Fig. 4.6).

4.2.3.b. Surgical Repair Increased Total Neuron Number Relative to Unrepaired Nerves, and This Was Most Pronounced in Laser Strip Repairs

Surgical section without repair caused a significant reduction in trigeminal neuron number (p < 0.001) (Fig. 4.7). All surgical repair methods tried maintained neuron number control levels (p < 0.05), although this did not achieve untreated control...
levels (p < 0.001), with the exception of laser strip repairs. Also, laser strip repair appeared more effective in this as compared with primary microsuture (p < 0.05), laser (liquid) solder (p < 0.05) and nerve grafting (p < 0.001) (Fig. 4.7).

4.2.3.c. *Surgical Repair Reduced the Number of Degenerate Neurons Relative to Unrepaired Nerves, and Laser Strip Repairs Were Similar to Control Specimens*  
The number of degenerate neurons in the trigeminal ganglion per graticule area increased significantly with section of the inferior alveolar nerve (p < 0.001) (Fig. 4.8). Nerve grafting was associated with a similar number of degenerate neurons while primary microsuture, laser (liquid) solder and laser (solid) strip repair methods were associated with a significant reduction in the number of degenerate neurons found (p < 0.05). Of these, nerves repaired with laser (solid) solder strips appeared to result in the lowest number of degenerate neurons as compared with the other repair methods tested (p < 0.001), while there was no statistically significant difference between these and unoperated control sites (Fig. 4.8).

4.2.3.d. *Surgical Repair Increased HRP Transport to the Trigeminal Ganglion from the Mental Nerve Relative to Unrepaired Nerves, and Laser Strip Repairs Were Similar to Control Specimens*  
HRP tracer was readily detected in the trigeminal ganglion (Fig. 4.9). Nerve section reduced uptake of HPR tracer to negligible levels (p < 0.001), while all surgical repair methods greatly increased HRP transfer from the mental nerve to the trigeminal ganglion (p < 0.05) (Fig. 4.10). HRP uptake was comparable between laser strip repaired nerves and unoperated control specimens, with HRP.
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levels being higher as compared with other repair methods studied (p < 0.05) (Fig. 4.10).

4.2.3.e. Nerve Growth Factor Levels Increased with Injury, and Were Further Increased by Surgical Repair

NFG was readily detected by immunoperoxidase histochemistry in the trigeminal nerve (Fig. 4.9). Only few neurons had detectable labeling with NGF in control specimens. However, sectioning of the inferior alveolar nerve increased the number of cells expressing NGF in the ganglion (p < 0.05), and surgical repair significantly increased this again (p < 0.001), (Fig. 4.11).

4.2.3.f. Delay of Surgical Repair Reduced Differences in Outcome of Surgical Techniques Tested

Where surgical repair was delayed for one month after injury the three groups of delayed repairs analysed primary microsuture, laser strip and nerve graft all show significantly different values (p < 0.001) for neuron counts, HRP uptake and neuronal degeneration when compared to the unoperated controls. The details are shown in table 4.1. The values for the laser strip repairs appear slightly better than for primary microsuture and nerve graft techniques in all the parameters of analysis including neuron counts, HRP uptake and neuronal degeneration but statistical analysis demonstrates no significant difference between the techniques. There does appear to be a leveling out of values following delayed repair compared with the primary repairs.
4.3. Discussion

The histological analysis produces a comprehensive assessment of the reliability of the microsurgical repair techniques used at both an intracranial and peripheral level.

Within the peripheral analysis the silver stained sections of the mental nerve were assessed as a relative percentage of area of myelin in the sections (Fig. 4.3.) and also by counts of fibre numbers plus diameter (Fig. 4.4.). As far as the percentage area of myelin in the sections assessed by the confocal system is concerned it does appear that primary microsuture repair is most comparable to the unoperated controls but in fact the larger standard deviation in this group makes it not significantly different from the laser repair group. However the relative percentage of myelinated fibres is significantly reduced in the nerve graft group and certainly is even more reduced in the group of section without repair. Persistence of myelinated fibres in the sections is relevant since regenerating axons will indeed grow into the unoccupied swan tubes following Wallerian degeneration which is, of course, a consequence of nerve injury. The counts of fibre numbers and diameters is demonstrated in Fig. 4.4 as a distribution curve of the different microsurgical repairs. In this graph there appears to be a marked reduction of fibre numbers within the group section without repair and an increase in the smaller (< 1.5 micron) diameter fibres which are generally recognised as unmyelinated fibres. This increase in unmyelinated fibres is consistent with previous theories postulated by Gregg (1990) who suggests that an increase in C
fibres as a consequence of such an untreated injury may occur and are probably related to the formation of neuroma units. Both the primary microsuture and laser repair groups have the peak of their fibre diameter distribution curve deflected slightly to the left compared to the unoperated controls indicating a relative increase in the percentage of slightly smaller fibres in the range 1.5-3 microns of diameter. This indeed may be a reflection of the sensitivity of the larger Aβ fibres to trauma when compared to the Aδ fibres which have relatively more protection with their myelin sheaths as described by Heasman et al. 1987. This phenomena would explain the unpleasant feature of hyperaesthesia complained of by patients following microsurgical repair of sensory nerves and may well be related to the rapidly conducting Aδ fibres. Fig. 4.4. also shows that the nerve graft technique appears to demonstrate a similar distribution to the unoperated controls but with significantly less fibres present than either the primary microsuture or laser repairs previously noted.

The peripheral assessment of blood vessels detected in the segments of inferior alveolar nerve removed demonstrates that the laser solder and laser strip techniques are comparable to both the unoperated controls and the microsuture techniques as seen in Fig. 4.5. This is confirmation that the laser technique does not cause undue thermal damage and also is evidence that an intact vasa nervorum is coincidental with an effectively regenerating nerve as suggested by Hobson (2002). It is also noted that the level of blood vessel growth in the region of the inferior alveolar nerve is markedly reduced in the group of section without repair further indicating that reduced vascular supply is coincidental with poor
regeneration and the vascular response in the nerve graft technique group is also reduced compared to the other microsurgical techniques. As noted in 1.5.2, vascularity is thought important for the successful repair of nerves (Azzam et al. 1991). It is possible that the comparatively poor response of nerve grafts as compared with other methods for repair was due to inadequate vascularity of these free grafts, and this is consistent with the low levels of vascularity seen in inferior alveolar nerves restored by nerve grafts in the current study. Vessels are thought to undergo apoptotic degeneration if poorly perfused, and it seems possible that the period during which vascular perfusion is lost following nerve grafting results in reduced graft vascularity which is not restored once vessel anastomosis occurs. This, together with the reported dependence upon vascularity for good surgical repair may account for the more extreme changes seen in most parameters studied for nerve grafts.

Central changes at the level of the trigeminal ganglion were generally less pronounced when nerves were repaired with the laser strip method. This may reflect improved continuity and function, as well as the relative speed with which nerve repairs can be made using the laser strip method.

Within the intracranial analysis the neuron counts (Fig. 4.7.) demonstrate that the laser strip repairs appear most comparable to the unoperated controls as far as maintaining numbers of primary neurons in the trigeminal ganglion is concerned. The category of nerve section without repair indeed is seen to manifest a large reduction in primary neurons within the trigeminal ganglion whereas primary
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microsuture and liquid laser solder techniques are somewhat better than the interpositional nerve graft techniques within this segment of the results. As previously stated the loss of neurons within the ganglion and the increase in percentage of degeneration following nerve injury does demonstrate a loss of innervation within the inferior alveolar nerve. The percentage of degenerating neurons within the sections analysed also indicate a greater percentage of degenerate neurons within the group nerve section without repair (Fig. 4.8.). Again the laser strip repair group appears to be most comparable to the unoperated control group showing a small percentage of degenerate neurons within the sections analysed. The percentage of degenerate neurons is also considerably elevated in the nerve graft technique group when compared to the other groups such as primary microsuture and liquid laser solder techniques.

Within the analysis of uptake of HRP tracer in the neurons of the trigeminal ganglion the laser strip repair group again is most comparable to the unoperated control group as see in Fig.4.10. There again is a marked reduction in HRP tracer uptake in the group nerve section without repair and again the groups primary microsuture plus liquid laser solder have improved uptake when compared with the interpositional nerve graft technique. Uptake of HRP tracer is an indication of patency of repair in these techniques and is a reflection of the competency of the peripheral repair.

Within the delayed groups of repair performed one month after initial injury there appears to be a levelling off between the microsurgical techniques of laser strip, primary microsuture, laser (liquid) solder and nerve graft groups involving
neurons counts, percentage of degenerated neurons plus HRP tracer uptake. In all these parameters primary microsuture, laser strip and laser solder all become close to the values of the interpositional nerve graft but the HRP tracer uptake within the nerve graft technique does appear still to be somewhat reduced. The effects of delay of repair in this analysis does appear to be counter productive to the primary repair techniques but the laser strip technique does appear to maintain better values than the other repairs in all the parameters assessed.

The assessment of nerve growth factor antibody reaction within the neurons of the trigeminal ganglia following repair is demonstrated in Fig. 4.11. and it shows increased reactivity within the nerve graft group when compared to the other repair groups including primary microsuture and laser strip repairs. This may be a reflection of the relatively slow process of regeneration due to a longer and more tortuous pathway for axonal regeneration. It is noted that the unoperated controls have a very low component in this analysis and the section without repair group has some ongoing NGF complex identified due presumably to prolonged attempts and ineffective regeneration. Since NGF is thought to have potent neurotrophic activities (Bu 1999), the high levels of NGF expression seen in the ganglia of repaired nerves may reflect an ongoing neurotrophic role for this growth factor after repair. Elevated expression of NGF in ganglia supplying nerve grafts and other repair methods may reflect a stronger stimulus and need for neurotrophic signals.
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Since most clinical repairs are some time after initial injury, it is of particular interest that delayed surgical repair reduced differences in outcome between the different surgical repair methods studied.
Figure 4.1. Photomicrographs of a paraffin section of a normal mental nerve stained with silver for myelin at low \((A)\) and high \((B)\) magnification. The perineurium \((P)\) enclosing this monofascicular nerve was noted, as was the brown stain marking myelin around nerves. Large (Black arrows) and small (Red arrows) myelinated fibres were clearly identified, as were large (Blue arrows) and small (Orange arrows) unmyelinated fibres. In some myelinated fibres, the myelin sheath appeared incomplete (Black arrow heads), and these sites were interpreted as either Nodes of Ranvier or Schmidt-Lantermann clefts. Silver Stain; Bar for \(A = 30 \mu m\), Bar for \(B = 10 \mu m\).
Figure 4.2. A typical photomicrographic image used for computerised quantitation of levels of myelination in the rat mental nerve (A), and a photomicrograph of blood vessels in an untreated rat inferior alveolar nerve (B). (A) Image analysis for determination of nerve fibre myelination and diameter required conversion of images from colour to black and white. This allowed definition of an "all or none" descriptor for each nerve fibre assessed as either myelinated or unmyelinated. The size of fibres varied considerably, with myelinated fibres ranging from small (Red arrows), to large (Orange arrows), and unmyelinated fibres varying in the same way (Blue arrows). (B) Blood vessels (BV) were readily identified as endothelial lined linear and ovoid spaces in Masson's trichrome stained sections of the inferior alveolar nerve. This permitted use of such sections for quantitative assessment of nerve vascularity. Silver and Masson's Trichrome, Bars = 30 μm.
Figure 4.3. Histogram showing the relative percentage of area occupied by myelin in paraffin sections of the mental nerve one year after section of the inferior alveolar nerve with or without repair by primary microsuture, laser liquid solder, or interpositional nerve graft as compared with untreated nerves. Sectioning of the inferior alveolar nerve significantly reduced levels of mental nerve myelination (p < 0.001), while each of the nerve repair methods tested reduced this effect (p < 0.001). Nerve grafts appeared less effective in retaining myelination of the mental nerve as compared with the other methods studied (p < 0.05).
Relative Percentage of Nerve Section Area Occupied by Myelin

Control  Section  Micro Suture  Laser Solder  Nerve Graft
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Figure 4.4. Illustration of the effect of inferior alveolar nerve section and repair by primary microsuture, laser solder and interpositional nerve graft, upon mental nerve fibre number and diameter. Sectioning of the inferior alveolar nerve reduced the number of nerve fibres in the mental nerve (p < 0.001), while surgical repair protected from this (p < 0.001), although nerve grafts were less effective as compared with laser solder and primary microsuture techniques (p < 0.05). The diameter of nerve fibres also changed with sectioning (p < 0.001), while fibres in nerves which had been repaired by laser solder and microsuture techniques were smaller as compared with unoperated controls (p < 0.05). This contrasted with the effect of nerve grafting, where despite a significant reduction in nerve fibre number relative to control specimens, there was only a modest reduction in nerve fibre size (p < 0.2) with the majority of fibres having diameters comparable to those in controls.
Figure 4.5. Histogram showing the mean number of blood vessels per graticule area in paraffin sections of the rat inferior alveolar nerve one year after sectioning, with or without repair by primary microsuture, laser (liquid) solder, laser (solid) strip, or interpositional nerve graft as compared with untreated nerves. Vascularity of the inferior alveolar nerve was reduced by sectioning (p < 0.001), and restored by surgical repair. In nerve grafts, however, vascularity was significantly lower as compared with either unoperated controls or any of the three other repair techniques studied (p < 0.05).
Mean Number of Blood Vessels detected in Graticule Area

- Control
- Section
- Micro Suture
- Laser Solder
- Laser Strips
- Nerve Graft
Figure 4.6. Photomicrographs of cryostat sections of a rat trigeminal ganglion, one year after sectioning and repair by primary microsuture, stained with cresyl violet and viewed at low (A) and high (B) magnification. The trigeminal ganglion (TG) was seen to divide into the ophthalmo-maxillary (OMD) and mandibular (MD) divisions. Primary neurons supplying the inferior alveolar nerve occupied a posterior domain of the mandibular division and were easily identified on the basis of their intense labelling with cresyl violet. Intact neurons (iN) stained deeply with cresyl violet, while degenerate neurons (dN) were highly vacuolated in appearance.

Cresyl violet, Bar for A = 200 μm, Bar for B = 50 μm.
Figure 4.7. Histogram showing the mean number of neurons per graticule area in cryostat sections of the rat trigeminal ganglion one year after sectioning of the inferior alveolar nerve, with or without repair by primary microsuture, laser (liquid) solder, laser (solid) strip, or interpositional nerve graft as compared with untreated nerves. Surgical section without repair caused a significant reduction in trigeminal neuron number (p < 0.001) while all surgical repair methods studied were associated with improved neuron number (p < 0.05). Laser strip repair appeared to increase neuron numbers more than the nerve graft method (p < 0.001).
Figure 4.8. Scattergram showing the mean number of degenerate neurons per graticule area in cryostat sections of the rat trigeminal ganglion one year after sectioning of the inferior alveolar nerve, with or without repair by primary microsuture, laser (liquid) solder, laser (solid) strip solder, or interpositional nerve graft as compared with untreated nerves. The number of degenerate neurons increased significantly with section of the inferior alveolar nerve (p < 0.001). Nerve grafting was associated with a similar number of degenerate neurons while primary suture, laser solder and laser strip repair methods were associated with a significant reduction in the number of degenerate neurons found (p < 0.05). Of these, nerves repaired with laser strips appeared to have the lowest number of degenerate neurons as compared with the other repair methods tested (p < 0.001), while there was no statistically significant difference between these and untreated control sites.
Figure 4.9. Photomicrographs of cryostat sections of the rat trigeminal ganglion one year after section of the inferior alveolar nerve and repair by the primary microsuture technique, stained with cresyl violet (A), for HRP tracer uptake (B) or NGF (C). (A) Degenerate primary neurons (dN) were identified as highly vacuolated cells in cresyl violet stained sections. (B) When sections were stained with DAB for HRP uptake, isolated primary neurons labelled intensely for this label (N), while the peripheral granular deposit expected from the literature was also seen (Arrows). (C) NGF was present in some neurons and also presented as strongly positive cells.

Cresyl violet, DAB histochemistry and NGF immunohistochemistry; Bars = 30 μm
Figure 4.10. Scattergram showing the mean number of neurons per graticule area with HRP tracer in cryostat sections of the rat trigeminal ganglion one year after sectioning of the inferior alveolar nerve, with or without repair by primary microsuture, laser (liquid) solder, laser (solid) strip solder, or interpositional nerve graft as compared with unoperated nerves. Nerve section reduced uptake of HRP tracer to negligible levels (p < 0.001), while all surgical repair methods greatly increased HRP transfer from the mental nerve to the trigeminal ganglion (p < 0.001). HRP uptake was comparable between laser strip repaired nerves and unoperated control specimens, with HRP levels being higher as compared with other repair methods studied (p < 0.05).
Figure 4.11. Histogram showing the mean number of neurons positive for NGF per graticule area in cryostat sections of the rat trigeminal ganglion one year after sectioning of the inferior alveolar nerve, with or without repair by primary microsuture, laser (solid) strip solder, or interpositional nerve graft as compared with untreated nerves. Although very few neurons had detectable labelling with NGF in control specimens, sectioning of the inferior alveolar nerve increased the number of cells expressing NGF (p < 0.05), and surgical repair significantly increased this again (p < 0.001).
Mean Number of Neurons with NGF per Graticule Area

- Control
- Section
- Micro Suture
- Laser Strips
- Nerve Graft
**Table 4.1. Table comparing the effects of delayed as compared with immediate repair of the inferior alveolar nerve, on trigeminal ganglion neurons, as well as results for unoperated control and sectioned/unrepaired nerves.**

<table>
<thead>
<tr>
<th></th>
<th>Neuron Counts (Cells/Graticule Area)</th>
<th>Degenerate Neurons (Cells / Graticule Area)</th>
<th>HRP Tracer Uptake (Cells / Graticule Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unoperated Control</strong></td>
<td>NA</td>
<td>28.71 ± 1.35</td>
<td>5.61 ± 1.18</td>
</tr>
<tr>
<td>Delayed Repair</td>
<td>NA</td>
<td>0.72 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>Immediate Repair</td>
<td>21.07 ± 0.76</td>
<td>2.33 ± 0.30</td>
<td>2.40 ± 0.20</td>
</tr>
<tr>
<td><strong>Primary Microsuture</strong></td>
<td>24.11 ± 2.02</td>
<td>1.12 ± 0.42</td>
<td>3.68 ± 0.80</td>
</tr>
<tr>
<td>Delayed Repair</td>
<td>20.13 ± 0.81</td>
<td>2.13 ± 0.42</td>
<td>1.8 ± 0.53</td>
</tr>
<tr>
<td>Immediate Repair</td>
<td>21.41 ± 1.57</td>
<td>4.37 ± 1.79</td>
<td>3.17 ± 0.58</td>
</tr>
<tr>
<td><strong>Nerve Graft</strong></td>
<td>20.95 ± 0.85</td>
<td>2.32 ± 0.48</td>
<td>2.43 ± 0.35</td>
</tr>
<tr>
<td>Delayed Repair</td>
<td>22.91 ± 1.71</td>
<td>1.4 ± 0.28</td>
<td>3.77 ± 0.43</td>
</tr>
<tr>
<td>Immediate Repair</td>
<td>22.50 ± 0.58</td>
<td>2.00 ± 0.40</td>
<td>2.67 ± 0.32</td>
</tr>
<tr>
<td><strong>Laser Solder</strong></td>
<td>27.20 ± 0.84</td>
<td>0.57 ± 0.18</td>
<td>5.60 ± 0.65</td>
</tr>
<tr>
<td>Delayed Repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate Repair</td>
<td>13.80 ± 1.90</td>
<td>6.71 ± 2.62</td>
<td>0.40 ± 0.16</td>
</tr>
<tr>
<td><strong>Laser Strip</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed Repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate Repair</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the method of repair affected the outcome, with regard to neurons of the trigeminal ganglion, delay of repair reduced these differences.

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CHAPTER 5

ELECTROPHYSIOLOGICAL STUDIES
5.1. Introduction

In Chapter 4, continuity of the mental nerve with the trigeminal ganglion was demonstrated past earlier repairs of the inferior alveolar nerve by HRP tracer uptake. It was also of interest to know if this anatomical continuity reflected restored function at the cortical level, beyond the trigeminal ganglion. Also, it was felt important to try and ascertain any changes in inferior alveolar nerve conduction following repair.

This Chapter describes electrophysiological experiments aimed at confirming that peripheral repair restored activity at the cortical level, as well as investigating peripheral nerve function after repair.

5.2. Results

5.2.1. Stimulus Evoked Cortical Potentials Were Detected After Nerve Repair

Although convincing cortical evoked potentials were detected in only one out of four unoperated control inferior alveolar nerves, none of the four sectioned and untreated inferior alveolar nerves demonstrated any cortical response (Fig. 5.1).

When the response past nerves was studied in quadruplicate groups, again only one case from each quadruplicate set of animals repaired by primary microsuture, laser (liquid) solder, laser (solid) strip repair, or interpositional nerve graft, demonstrated a convincing cortical evoked response. Figures 5.2 and 5.3 illustrate the responses of the two animals with positive cortical responses repaired by primary microsuture and interpositional nerve graft methods respectively. Despite
the disappointing variability of data with evoked potentials ranging from 100 to 400 microvolts, the few positive results obtained following nerve repair indicated that cortical function could be restored across repaired nerves in the model system established by this thesis.

5.2.2. Sensory Action Potentials Were Detected After Repair of the Inferior Alveolar Nerve

Variability in data made interpretation of these experiments difficult, with many recordings being negative and amplitudes of those action potentials detected ranging from 47.82 mV to 419.2 mV.

However, although two out of four unoperated control cases displayed action potentials of over 20 mV, none of the four cases in which the nerve had been sectioned and not repaired demonstrated any detectable action potential. This suggested that although the methodology for detecting action potentials was unreliable, that when action potentials could be identified, they reflected nerve function rather than random noise.

Two out of four of the laser (solid) strip repaired inferior alveolar nerves produced action potentials of over 20 millivolts (Fig. 5.4), while two of the four laser (liquid) solder and primary microsuture repairs also elicited convincing action potentials across earlier repairs.

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There was great variation in nerve condition velocity, ranging from nerve conduction velocity from 23 m/sec to 78 m/sec, while the fastest responses seen were with laser (solid) strip solder repairs.

5.2.3. Blink Reflex Data Were Largely Overwhelmed by Background Signals

A positive blink reflex mediated by the facial nerve after stimulation of the mental nerve was detected in only 1 out of 4 unoperated control sites (Fig. 5.5), with data from all remaining sites and animals tested overwhelmed by background interference.

5.3. DISCUSSION

Despite the extreme variability of electrophysiological data described, it was apparent that in at least some of the animals tested, a functional cortical response was mediated past inferior alveolar nerve repairs and the trigeminal ganglion. Confidence in this conclusion was increased by the absence of any response in all cases where the nerve was sectioned and not repaired, while equivalent variability was seen between unoperated control sites and nerves which had been repaired.

Where action potentials were detected in repaired nerves following mental nerve stimulation, there was a change in the form of the potential consistent with changes described for regenerating neurons described by Kocsis et al. (1982). Although the fastest conduction velocities were measured in laser (solid) strip repaired nerves, the extremely limited sample available precludes conclusion that
the type of repair affects the speed of condution. Nonetheless, this data did confirm that action potentials are able to pass repair sites.

Variability in data may reflect the very small size of animals used, relative to those more often studied by these methods, as well as the intraosseous location of the nerve. In addition, background noise overwhelmed recordings in some cases due to the short distance between stimulation and detection sites, and this was a particular problem in attempting to assess the blink reflex.
Figure 5.1. Mental nerve stimulus and cortical evoked potential tracing from a rat in which the inferior alveolar nerve was sectioned without repair one year previously. A distinct stimulus trace is seen, as is a simultaneous small cortical trace, interpreted as noise from the initial stimulus. The absence of a cortical response is consistent with total loss of inferior alveolar nerve function after nerve sectioning.
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Figure 5.2. Mental nerve stimulus and cortical evoked potential tracing from a rat in which the inferior alveolar nerve was sectioned and repaired by the primary microsuture method one year previously. A strong slightly delayed and prolonged cortical response is seen, demonstrating functional continuity between mental nerve and the cerebral cortex in this animal.
Figure 5.3. Mental nerve stimulus and cortical evoked potential tracing from a rat in which the inferior alveolar nerve was sectioned and repaired by the interpositional nerve graft method one year previously. A slightly delayed and prolonged cortical response is seen, demonstrating functional continuity between mental nerve and the cerebral cortex in this animal.
Figure 5.4. Action potential measured from the inferior alveolar nerve at the mandibular foramen after stimulation of the mental nerve following repair of the inferior alveolar nerve with the laser strip technique. The time of stimulation is marked at S, while the action potential hyper-polarization (P) and subsequent depolarization (T) is seen from 1 to 2 ms after stimulation.
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Figure 5.5. A single blink reflex trace of mental nerve stimulation followed by a facial nerve response (H Reflex). This was seen in only one animal, being an unoperated control, while traces from other animals were uninterpretable due to overlying background noise.
CHAPTER 6

GENERAL DISCUSSION
6.1. Conclusions

This thesis established for the first time, a reliable experimental model for exposure of the intraosseous inferior alveolar nerve in rats. This surgical technique developed differed significantly from the mandibular osteotomy approach used by earlier workers (Wessberg et al. 1982), and instead relied upon creation of a bone window with direct access to the nerve. Importantly, the model has been found sufficiently reproducible for detailed quantitative histological analysis to be performed. Despite this, limitations for electrophysiological studies were identified, and likely reflect the technical difficulty of performing such studies in animals of this size.

An important finding of this thesis is that it is possible to effect reliable nerve repairs in bony canals without interference of bony healing, space filling haematomas or proliferative periosteum which could compromise the nerve repairs. Although such repairs are frequently performed in humans (Donoff 1995, Mozsary 1989), the absence of an experimental model system for repeated study of intraosseous nerve repairs has cast a shadow over the reliability of intraosseous nerve repair. Findings of the current thesis at least in part, suggest that excessive concern regarding the possible compromise of nerve repair in bone may be unnecessary.

Microsurgical repairs of the inferior alveolar nerve were performed with successful outcomes at surgical, histological and functional levels. The primary
Microsurgical Repair of The Rat Inferior Alveolar Nerve

Microsuture method with epineurial microsutures resulted in effective microsurgical repairs, but was associated with a variety of difficulties related to insertion of sutures into the nerve including foreign body cell reactions, deflection of axons and connective tissue intrusion. Similar difficulties were noted in interpositional nerve grafts while laser solder based methods provided the opportunity to potentially overcome these.

Initial concerns of possible thermal injury to the nerve were resolved by histological assessment of nerves immediately after laser solder repair, in which no significant vascular or thermal damage was seen. In the course of performing this study, a solid laser solder strip method became available, so that later experiments included this technique for comparison with the other methods of repair tested. Solid solder strips were found to be more convenient for use, while liquid laser solder was also comparatively more simple to use than either primary microsuture or interpositional nerve graft methods.

The laser (liquid) solder and laser (solid) strip repairs resulted in a much shorter operating time for the microsurgical repair element of surgery as compared with methods requiring suturing. The laser strip repair generally appeared a more refined way of using the albumin based solder, with less potential leakage of solder between sectioned ends of the injured nerve. However it appears that good torsional strengths have been achieved using both the liquid and solid strip solder techniques (Lauto et al. 1995, McNally et al. 1999). It should be noted that the current thesis describes the first use of the laser solder technique for the
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microsurgical repair of the inferior alveolar nerve, and this technique appeared very suitable for the repair of the nerve in this intraosseous pathway.

With regard to histological changes following repair, there seemed little if any difference between primary microsuture and laser based repair techniques with regard to parameters assessed in peripheral nerves. However, laser solder and particularly laser strips repairs appeared to restore parameters measured in the trigeminal ganglion more effectively towards untreated controls than the other repair methods tested. In almost all parameters tested, interpositional nerve grafts were less effective in restoring nerves and ganglia towards the uninjured state.

Generally, laser (solid) strip repairs produced the best results as compared to the other repair techniques. Neuron counts, tracer uptake, level of neuron degeneration and relative nerve growth factor activity all demonstrated that the laser strip technique was comparable and occasionally better than the traditional microsuture techniques.

The liquid laser solder technique did not appear to perform as well as the more refined solid strip technique, and this indeed may be related to more difficulty of approximation of the sectioned nerve ends and possible leakage of solder between the nerve endings.

The interpositional nerve graft technique certainly performed more poorly than the primary microsuture laser (solid) strip and laser (liquid) solder techniques.
though it did perform much better than the nerve section without repair group, which was universally poor across all categories.

The vascularity of repairs appeared to correlate with effective nerve regeneration, consistent with earlier work by Hobson (2002). As noted above, the vasa nervorum appeared not to be affected by the laser techniques.

Both laser and microsuture approaches resulted in good axonal regeneration, but there did appear to be a shift to smaller myelinated fibres in the Aδ class, when compared to the unoperated control. This in fact contrasted with the response to the interpositional nerve graft group which maintained a similar fibre distribution to the unoperated control group, but with a much reduced fibre number content. The shift of the primary microsuture and laser repair technique to contain proportionately more smaller myelinated fibres could indicate the susceptibility of the larger myelinated fibres to injury, and may explain the symptom of hyperaesthesia complained of by some patients following such microsurgical repair.

Unfortunately, it was not possible to confirm improved function at the electrophysiological level for any particular microsurgical repair method tested. However, it was possible to confirm that the repair methods used established functional continuity between mental nerve and the cerebral cortex.
Despite the apparent improvement in some parameters with laser solid strip and liquid solder techniques, it was sobering to note that when repair was delayed, differences in outcome between repair methods were markedly reduced though the laser strip technique did still appear to perform better across all assessed parameters. This feature may reflect the effect of extensive Wallerian degeneration which could progress before the delayed repairs were performed. From a clinical standpoint, this suggests that there may be little difference for the patient receiving a delayed repair, irrespective of the repair method used. Nonetheless, if this were the case, the comparative ease with which laser solid strip solder can be used to repair nerves would suggest this as the method of choice, all else being equal.

6.2. Directions of Future Work

Because laser solid solder strips became available only at a later time during this project, while the computer aided mental nerve analysis was performed at an earlier stage, there is an absence of data from the mental nerve for this type of repair. Future work should address this deficiency, and also expand observations of the inferior alveolar nerve to assess myelination and fibre size distribution.

Further, the effect of reduced and increased periods of delayed repair upon peripheral and central recovery should be assessed, to probe the possible basis for
the loss of responsiveness to the type of surgical repair performed as seen in this study.

Improved methodology for electrophysiological assessment could be developed, and this would be valuable for confirming the functional status of nerves repaired in future studies. Studies aimed particularly at defining the relationship between fibre size distribution and electrophysiological responses would be most interesting from the perspective of understanding and potentially improving upon the symptom of hyperaesthesia following repair.

The current thesis was limited to an essentially surgical and histological study, while it would be interesting to exploit the established model system for more detailed molecular analysis of nerve regeneration events. In particular, it would be interesting to investigate the expression of specific neurotrophic and Schwann Cell growth factors, as well as the differentiation of Schwann cells during regeneration. The precise role of Schwann cells in defining the fate of individual fibres seems important to determine, particularly with regard to determination of fibre size and myelination status.

The enclosed and physically stable environment of the intraosseous location in this model may provide an ideal opportunity to study the effect of exogenous agents upon nerve regeneration, without the confounding effects of altering tissue and nerve tension inevitable for nerve repairs in soft tissue environments.
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It may also be possible to impregnate solid or liquid solder materials with neurotrophic agents, such as FK506 or NGF, and the effect of this upon repair would be interesting to determine.

Progression towards clinical studies in humans with laser solid strip repairs may be justified, in view of the successful demonstration of nerve repairs in rats.
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