Effect of the Peripheral Target on Motoneuron Survival, Emphasising the Role of a Locally Acting Splice Variant of IGF-1

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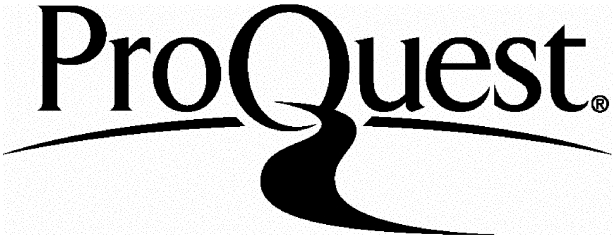

ABSTRACT

While it is clear that target tissue and the periphery in general play an important role for the survival and maintenance of motoneurons, the underlying mechanisms remain unclear. The experiments described in this thesis use a model of axotomy-induced motoneuron death to study the effect of insulin-like growth factor-1 (IGF-1) isoforms, diet and muscle damage on the survival of adult facial motoneurons. Particular attention is paid to the effects on motoneurons of the autocrine/paracrine-acting splice variant of IGF-1 (MGF), which has been isolated recently from active skeletal muscle.

Using stereological methods, it was found that facial nerve avulsion of *ad libitum*-fed adult rats resulted in approximately 80% loss of motoneurons. However, diet restriction, which is known to alter IGF-1 levels, of rats from the age of 6 months reduced the number of facial motoneurons by about 50% at 24 months-of-age but prevented any further loss of motoneurons following facial nerve avulsion. These results challenge the common view that diet restriction is universally beneficial.

MGF gene transfer prior to nerve avulsion conferred marked neuroprotection of facial motoneurons at 1-month post injury. Such neuroprotection was approximately two-fold greater than that obtained with the liver-type IGF-1 (endocrine) splice variant. MGF E-domain peptide delivered to avulsed motoneurons also protected motoneurons at 1 month.

In addition to MGF gene transfer, muscle denervation was also associated with perturbations of MGF mRNAs. Direct damage to target-muscle prior to nerve avulsion rendered motoneurons more resistant to death. Specific markers for motoneuron survival, death, regeneration and neuroglial activation were used throughout the study to characterise the response of motoneurons to nerve avulsion.

The results are discussed in the context of current theories of motoneuron target-dependence and the possible neurotrophic effects of MGF.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter-1

General Introduction

While it is clear that the periphery plays an important role for the survival and maintenance of motoneurons, the underlying mechanisms remain unclear. Examining changes in the response of motoneurons to injury after defined alterations of their environment may provide a better understanding of the survival mechanisms operating. The present thesis uses a model of axotomy-induced motoneuron death to study the role of the peripheral target in pre-determining motoneuronal survival, paying particular attention to the effects of age, diet, muscle damage and IGF-1 isoforms.

On the experimental basis of target-dissociation in neonatal animals, numerous muscle-derived trophic factors have been identified (Oppenheim 1996). However, the degree to which information derived from studies of the developing neuromuscular system can be applied to the adult and ageing animal is unclear. One obstacle to the study of neurotrophic rescue is the lack of a suitable animal model in which the same lesion produces motoneuron death across the lifespan of the animal. In this study a nerve avulsion model is used to investigate how prior perturbation of conditions in the periphery may alter the level of survival of motoneurons after lethal nerve trauma. Using the present model the present thesis examines the roles of age, dietary restriction (DR), muscle damage and IGF-1 on motoneuron survival. The present thesis falls into three parts (experimental chapters: 3-5). The first part examines the effect of age, and indirect perturbations of IGF-1 (DR and muscle damage) on motoneuron survival. The second part looks at the effects of IGF-1 perturbation directly following the over-expression of the gene for different IGF-1 isoforms. The final part investigates the mechanisms by which neuroprotection is brought about. A brief rationale for undertaking these studies follows in this chapter. More detailed descriptions are found in the relevant 'introduction' sections of experimental chapters 3, 4 and 5 (sections 3.1, 4.1 and 5.1 respectively).

Chapter 3: DR is commonly used to extend the lifespan of laboratory rodents in studies of ageing (Weindruch et al. 1986). It is generally regarded, therefore, as beneficial to all cell types, providing a means of reducing age-related cellular damage such as oxidative stress (Mattson et al. 2002). Moreover, it is known that DR can reduce the age-related loss of some

populations of neurons (Cowen et al. 2000). Whether this observation can be generalised to other parts of the ageing nervous system is unclear as age-related neural loss varies widely across neural systems. Experimental studies of age-related neural death are also complicated by incomplete data on how neurons of the same system respond to potentially lethal trauma across the lifespan of the animal. Therefore, the relationship between DR, age and the response of motoneurons to injury remains unclear. One aim of the present study is to investigate the role of DR in motoneuron survival.

Muscle-derived neurotrophic factors play an important role in the target-dependence of motoneurons. Amongst the first to be defined under this title was insulin-like growth factor-1 (IGF-1). The pleiotropic actions of this growth factor are, in part, a direct result from its secretion from the liver into the general circulation facilitating an endocrine mode of action. However, skeletal muscle is known also to be a major site of IGF-1 synthesis post-natally, and promotes the regeneration of motor axons of peripheral nerves *in vivo* (Ishii et al. 1994). Damaged muscle is able to regenerate itself usually by up-regulating genes for myotrophic proteins such as IGF-1 (Goldspink 1999). It should be possible; therefore, to use muscle damage to expose and pre-condition motoneurons to elevated trophic conditions such as that in regenerating muscle before the nerve is subjected to injury. Another aim of this thesis is to examine the effect of prior muscle damage on motoneuron survival. Together with the work on the effect of age and DR, this forms the basis for chapter-3 of the present thesis.

Chapter 4: Neuroprotection is afforded experimentally by over-expressing the gene of a neurotrophic factor in motoneurons (Baumgartner & Shine 1998; Hottinger et al. 2000). The proposed rescue of injured motoneurons by IGF-1 delivered in this manner has so far been studied only with respect to the mature hormone found in the general circulation and has shown inconsistent levels of neuroprotection (Hughes, R.A. et al. 1993; Li, L. et al. 1994; Vergani, et al. 1998; Yuan et al. 2000). Recent work in this laboratory however, has identified and isolated cDNA encoding the IGF-1 Eb splice variant or MGF, which was localised and up-regulated specifically in active/damaged skeletal muscle (Yang, H. et al. 1995; Yang, S. et al. 1996). It is unknown whether MGF is able to promote the survival of motoneurons. A further aim, and the work comprising chapter-4 of this thesis, is to

determine if MGF gene transfer to either motoneurons and/or skeletal muscle affects motoneuron survival after nerve avulsion in both adult and neonatal rats.

Chapter 5: Following investigation in to the various treatments described above, chapter 5 aims to understand the mechanisms underlying neuroprotection. Injection of a plasmid into the snout muscle prior to disruption of nerve-muscle interaction potentially allows not only expression of the transgene in the target muscle but also retrograde transport of the vector and/or protein product to the cell bodies of facial motoneurons. Knowledge about transport of the vector and protein would help interpret the mechanism of MGF with respect to its potentially neurotrophic qualities and clarify further the significance of such gene expression endogenously under conditions of muscle activity for determining motoneuron survival. This is done by mRNA expression analysis in the relevant tissues. In addition, by applying the putative active component of MGF, the E-domain peptide (Siegfried et al. 1992; Yang, S. & Goldspink 2002) to avulsed motoneurons, it may be seen whether MGF is important for protecting neurons after lethal trauma.

The second part of chapter 5 is concerned with understanding further the role of MGF as a component of the signal generated by active or damaged muscle in relation to conferring endogenous protection to facial motoneurons prior to lethal nerve trauma. Qualitative RT-PCR is used firstly to reveal the relative expression of a multitude of possible mRNA transcripts following denervation of the snout muscle, which will help define further a basis for localised MGF protein expression in the neuromuscular system. Histololgical techniques are then applied to investigate the mechanism(s) by which direct muscle damage pre-conditions motoneurons, attempting to correlate motoneuron survival firstly, with a role for IGF-1 isoforms, and secondly, with the level of inflammation in the peripheral target. Finally, identifying correlates of retrograde reaction to nerve avulsion is aimed specifically to characterise and supplement potentially neuroprotective mechanisms operating centrally. The materials and methods used in all parts of the present study are described in chapter 2.

Chapter-2

Materials and Methods

2.1 Animals

Sprague-Dawley rats aged 7 days (neonate), 6 months (adult) or 24 months (ageing) were used for the present study. Male rats were used for all adult and ageing groups. Whole litters, comprising male and female rats, were used for all the neonatal groups. All animals were housed and maintained in the Royal Free and University College Medical School Comparative Biology Unit. All experimental procedures were carried out in accordance with Home Office and local ethical committee approval and appropriate steps were taken to minimise the number of animals used. Neonatal animals were produced in-house from breeding stock. Seven day-old rats rather than 1 day-old rats were used to study the neonatal nervous system, as this avoided the post-natal period of natural motoneuron death that has been described previously (Sendtner et al. 2000). Additionally, preliminary investigations showed that the facial nerve in newborn rats was too fragile to perform nerve avulsion. 6 month-old *ad libitum*-fed rats were purchased (B.K. Universal, Hull, UK) and were used following an initial climatisation period of approximately 2 weeks. 6 months was chosen to study adult rats because this is consistent with previous definitions of the term 'adult' in rats (i.e. of weight of approximately 250-300g) (Buck et al. 2000; Chai et al. 1999; Chen et al. 2001). Other 6 month-old rats were maintained with unlimited access to food until the age of 24 months. It is reported that 50% of *ad libitum*-fed, Sprague-Dawley rats die at 24 months (Vanden Noven et al. 1996). Therefore, 24 months was taken to be representative of the senescent period in these animals and was chosen to study the ageing nervous system in the present study. Diet restricted (DR) rats, which had been raised on 15g of standard rat chow per day from the age of 6 months, were purchased (B.K. Universal, Hull, UK) aged 22 months. These animals were maintained on this diet until they were 24 months old. DR animals were kept in cages of 4 animals from the onset of the diet regimen and then transferred to cages of 6 animals post-operatively.

2.2 The Facial Nerve

This study is concerned with lower motoneurons of the facial nerve of the rat, innervating the superficial muscles of the head and neck. The cell bodies are located in the facial nucleus, found within the lower pons. It may be subdivided into several cellular groups: four major (medial, intermediate, lateral and dorsal) and two minor (ventromedial and ventrolateral) subdivisions, each of which innervate subsets of superficial musculature. In the context of this study, where substances are injected intramuscularly into the snout region, it is important to observe that in rats, the whisker pad or snout muscle is innervated by motoneurons whose cell bodies are located in the lateral subnucleus.

The facial nerve was chosen for this study as a model system for the following reasons. It is of reasonable size (~1mm in diameter) thus aiding in the performance of axotomy. The facial nucleus is large and compact, and, therefore, located easily in sections of the brainstem. In the current study, this aids the rapid examination of multiple animals to evaluate the effect of nerve injury and to assess levels of neuroprotection. Cell bodies of the facial motor pool are distributed in a discrete fashion aiding cutting and analysis relative to motoneurons of the sciatic nerve. Spinal motor pools, in contrast, are distributed over several cord segments and, in the absence of retrograde tracers, qualitative and quantitative judgments would be hampered by the presence of non-injured motoneurons and interneurons. In addition, facial nerve avulsion is preferable to ventral root avulsion of spinal nerves since monosynaptic spinal afferents may have trophic influence on avulsed spinal motoneurons (Okado & Oppenheim 1984; Peyronnard & Charron 1983) whereas no monosynaptic afferents have been identified for the facial nerve.

2.3 Injection of the snout muscle

Rats were injected under anaesthesia (4% halothane in 2L/min oxygen). Intramuscular injection of substances into the facial (snout) muscle of adult and ageing rats was achieved by three separate punctures of a 0.5ml insulin syringe (Micro-fine, BD, France) in the area of the right whisker pad (approximately 1.5cm^2). The three areas injected were in the

following positions: the proximal part of the lowest row of vibrissae, the middle of the middle row and the distal part of the top row of vibrissae (**Fig.2-1**)

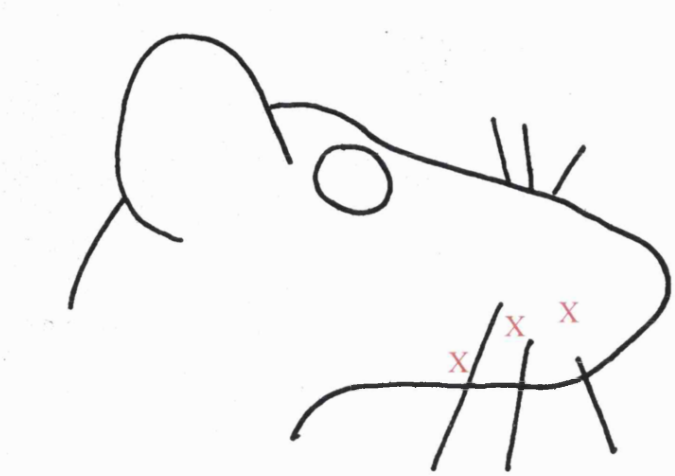


Figure 2-1 Illustration to show the approximate injection sites of all substances into the snout muscle. X marks the sites.

Each injection was 20μl, giving a total volume of 60μl of plasmid (2μg/μl) or human MGF E-peptide or L.IGF-1 (1mg/ml) dissolved in physiological saline (8.8g NaCl per 1L of sterile/distilled water). For neonatal rats, a maximum volume of 10μl was delivered by a single injection into the snout muscle. A period of 7 days after injection of either plasmid vector, or MGF E-domain peptide, or muscle-damaging treatment was allowed prior to nerve injury. Such an interval was determined empirically in cell culture by transfecting C2C12 mouse myoblasts with the same plasmid encoding MGF as that used for the present study. Increased myoblast proliferation was observed after 3 days (Yang, S. unpublished data). It was assumed that 7 days would allow for uptake of the plasmid, gene expression, retrograde transport of plasmid and/or protein and the accummulation of sufficient levels to bring about a biological affect on facial motoneurons.

Injections of 1% Fluoro-Gold retrograde tracer (Flurochrome, Colorado, USA) were made into the facial whisker pad 24hours prior to tissue collection. This was done to confirm the

location of the facial nucleus. Labeled motoneurons were visualised under ultra-violet (UV) light.

2.4 Surgery

Rats were anaesthetised with 2% halothane carried by 2L/min oxygen. The right facial nerve was exposed (**Fig. 2-2, A**) and freed from periosteal attachments around the stylomastoid foramen (**B**). The nerve was then subject to gentle sustained traction until a length of nerve of approximately 3mm was delivered from the foramen (**C**) along with a small quantity of cerebrospinal fluid. The segment of nerve pulled from the brainstem as well as an additional 1-2mm of nerve distally was removed to avoid the possibility of the distal stump re-inserting into the foramen.

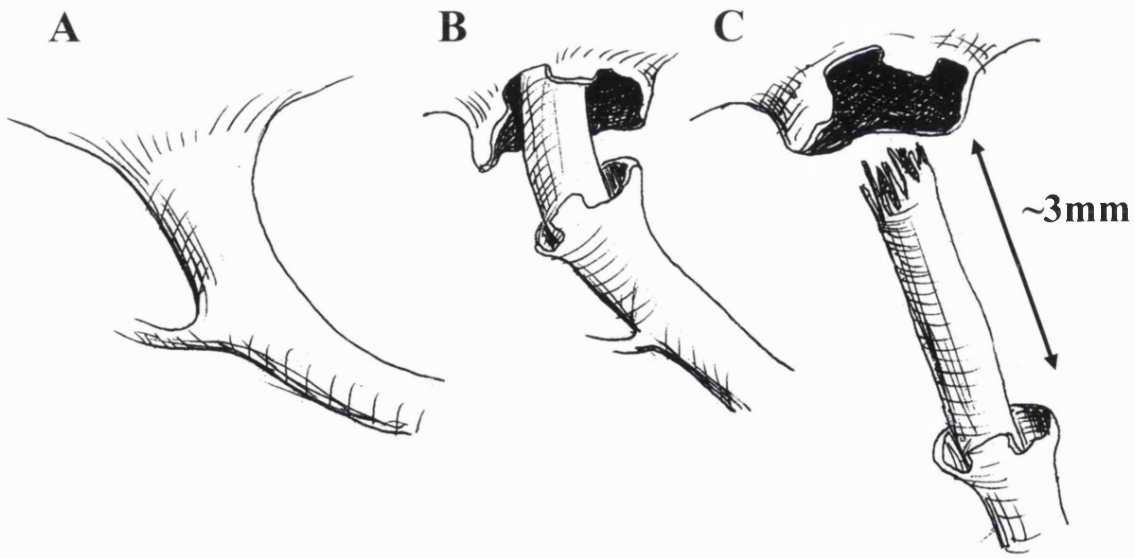


Figure 2-2 An illustration (drawn not to scale) summarising the exposure (A), freeing (B), and traction (C) of the facial nerve resulting in avulsion of facial motoneurons.

Preliminary analysis of brainstem cryosections immediately after avulsion of adult rats revealed that this procedure had created axotomy at the rootlet/brainstem junction. Nerve transection on the other hand, was achieved simply by cutting with surgical scissors across the trunk of the combined branches of the nerve distal to its exit from the stylomastoid foramen. Subsequent to lesion, 3-4 sutures were placed to close the wound. All animals were given a single injection of Temgesic (Schering-Plough, NJ, USA) subcutaneously and fed *ad libitum* post surgery until termination.

2.5 Tissue collection

Animals were deeply anaesthetised with sodium pentobarbitone (45mg/Kg). Tissue was either removed fresh and frozen immediately in liquid nitrogen (for RNA and selected histochemical analysis only) and stored at –70°C for later use, or animals were perfused via the left ventricle with physiological saline (8.8g/L sterile distilled water) followed by 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS: 0.1M solution of NaH_2PO_4 was added slowly to a solution of 0.1M Na_2HPO_4 until a pH 7.4 was achieved, NaCl was added to the final mixture to give a final concentration of 140mM) and taken for stereology and histochemical analysis only. Saline and PBS solutions were made up using RNase-free reagents in diethylpyrocarbonate (DEPC)-treated water. Perfused brains of all the animals were removed and immersed in fresh fixative at 4°C for 24 hours.

2.5.1 Preparation of fixed tissue

Perfusion-fixed brainstems were dissected from whole brains by firstly transecting mid-way through the pons, as viewed from the anterior aspect, then removing the cerebellar hemispheres and finally transecting approximately 5mm below the lower limit of the pons (**Fig. 2-3**).

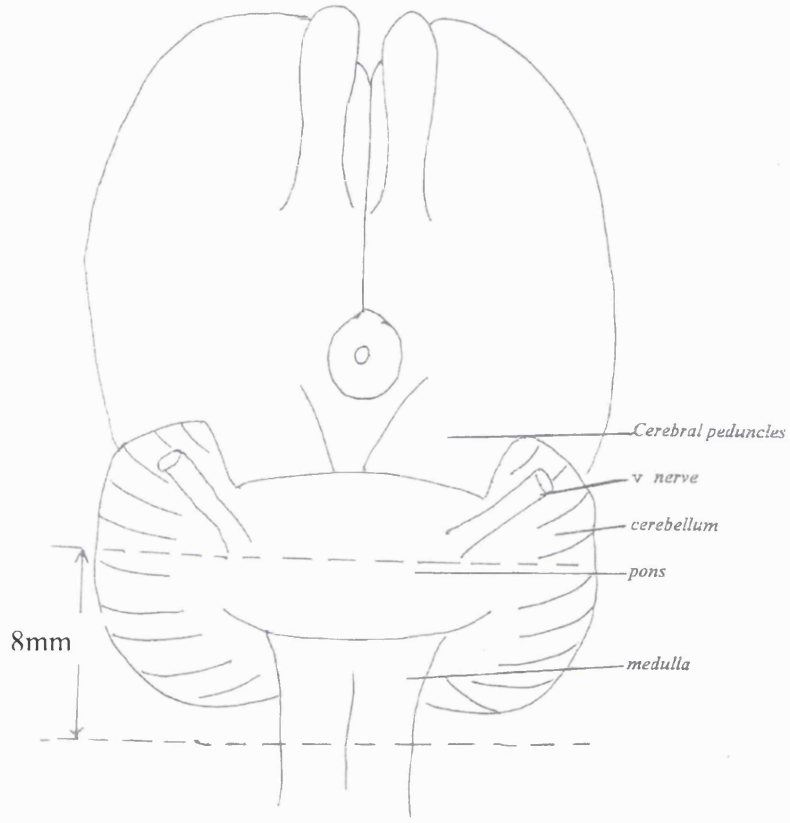


Figure 2-3 Illustration of the anterior aspect of the rat bran to show the upper and lower limits of transection in order to isolate the region containing the facial nuclei.

Surface fixative was removed with a piece of filter paper. Into adult and ageing brainstems a small 'V'-shaped groove was cut using a razor blade along the posterior rostro-caudal axis on the contralateral side to enable orientation (**Fig. 2-4**). The rostral surface of each brainstem was fixed to the specimen stage of a vibrating blade tissue sectioner (Vibratome, Oxford Instruments Ltd., UK) using cyanoacrylate glue. With the specimen submerged in phosphate buffer, 70μm serial transverse sections were then cut through the entire length of the brainstem, stopping with approximately 1mm of specimen remaining attached to the platform. After perfusion-fixation, neonate brainstems were difficult to section using the Vibratome. Instead, they were immersed for a further 24 hours in 20% sucrose/PBS. The rostral surface of the brainstem was mounted in OCT (BDH, Poole, UK) on a cork disc and

frozen rapidly in isopentane chilled in liquid nitrogen. For orientation, a rostro-caudal hole was bored into the contralateral side of the frozen brainstem using a fine hypodermic needle of an insulin syringe (Micro-fine, BD, France) taking care to avoid the region containing the facial nucleus. 50μm cryosections were required for stereology. These were cut and allowed to thaw on the end of a fine brush. For all ages, adjacent wet sections were submerged individually in the sequential wells of a transparent multi-well dish filled with 0.1M PBS.

Some staining techniques required the use of immobilised tissue sections. For these procedures, 10μm cryosections were thawed on to poly-L lysine-treated, DNase- and RNase-free slides (Superfrost-Plus, BDH, Poole, UK), and allowed to dry over night at room temperature. Slides were either used immediately for staining procedures or stored under RNase-free conditions at $-70^{\circ}C$. For use after storage, sections were allowed to dry over night at room temperature.

2.5.2 Location of Section Series Containing the Facial Nucleus

Each section series containing the facial nucleus was determined by observing free-floating wet sections in multi-well dishes under a light microscope (**Fig.2-4**). Rostral and caudal poles of each facial nucleus were easy to identify because of the abrupt nature with which the nucleus tapers, transforming from a large unequivocally identified structure to nothing in the space of 1-2 sections. In this study there were approximately 23-24 sections through each facial nucleus in adult and ageing rats and 21-22 sections in 2 week-old rats (neonatal). With a section thickness of 70μm and 50μm in adult/ageing and neonatal rats respectively, the estimated error in determining the total number of sections through the facial nucleus is therefore 70-140μm and 50-100μm respectively, or less than 10%.

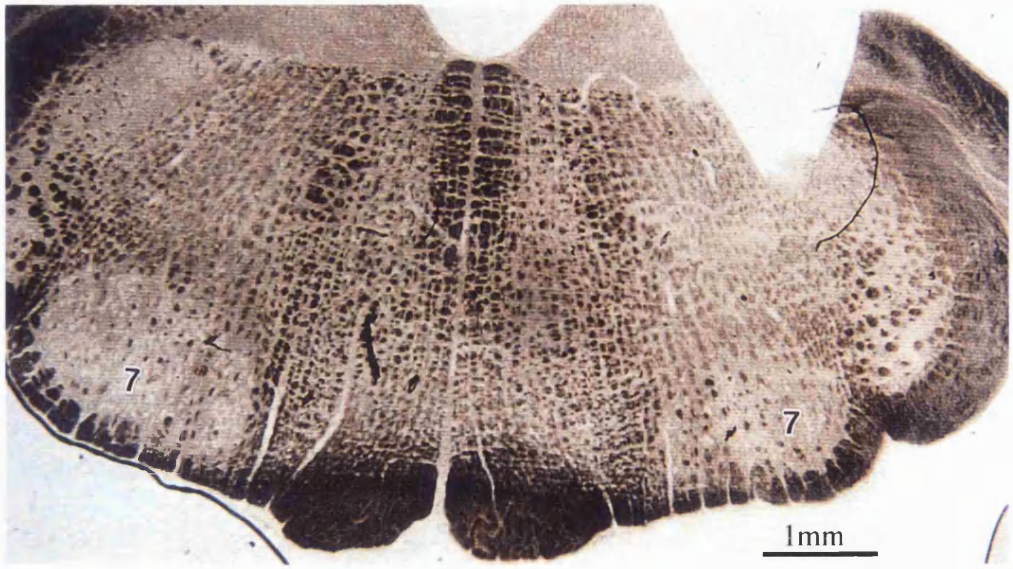


Figure 2-4 Micrograph of a wet vibratome section through the brainstem at the level of the facial nucleus. 7 marks the motor pools of the 7th cranial (facial) nerve.

2.6 Systematic Random Sampling

Every third (neonates) or fifth section (adult and ageing) was randomly and systematically selected for stereological analysis of total facial motoneuron number. Selecting, without bias, a number from 1 to 3 for neonatal rats and 1 to 5 for adult/ageing rats, randomised the starting section in each systematically chosen sample. When the sampling start point was altered, it was found that values for the estimated total volume of the facial nucleus differed by no greater than 10% from the actual (calculated) total volume. Additionally, this sampling interval had previously been found to give estimates of total facial motoneuron number, which varied by less than 10% between replicate experiments on groups of normal adult rats (Johnson, I.P. 2001).

2.7 Stereology

2.7.1 Fluorescence labeling of motoneurons

Sections intended for stereological analysis (approximately 5-6 per rat) were removed from the multi-well dishes using a fine brush and aligned on a glass slide. After removing excess buffer, sections were covered with a few drops of 1:1000 dilution of a fluorescent basophilic stain (YOYO-1 iodide; Molecular Probes, Oregan, PA) in 0.1M phosphate buffer (PBS without the addition of NaCl) for approximately 10min. After removing excess dye, sections were covered minimally with aqueous mountant (Citifluor, Agar Scientific, UK) and a glass coverslip put in place. Slides, 1 per rat, were left in the dark overnight at room temperature and the edges of the coverslip then sealed with nail varnish.

2.7.2 Justification of the Optical Disector

Facial motoneurons were counted using a modified optical disector method adapted for the confocal microscope (Johnson, I.P. 2001). In combination with the Cavalieri method (Gundersen & Jensen 1987), an unbiased and three-dimensional estimate of total neuron number in the facial motor nucleus may be obtained. This method was chosen over others as it made no prior assumption about motoneuron shape or size and since it relied on sampling, it was much faster than methods involving serial reconstruction. The current method was developed specifically for estimating the total number of motoneurons in the facial nucleus (Johnson, I.P. 2001). As a fractionator or disector approach, it is regarded as unbiased or non-assumption-based (Pakkenberg & Gundersen 1995; Sterio 1984), that is, free from bias in determining the number of neurons in a known fraction of the volume of the facial nucleus (Gundersen 1986; West 1999). As a result, the counts should be evenly scattered around a true mean with no requirement for any correction (Guillery 2002).

The total number of motoneurons in the facial nucleus (Nv) was estimated using the following formula:

Nv =(Vref * Q-)/Vdis

Where: Vref = total volume of the facial nucleus, Q- = mean number of motoneurons per disector volume, and Vdis = disector volume

2.7.3 Calculation of Vref

The area of the facial nucleus was measured using the Cavalieri method. Each section was viewed under trans-illumination. Using the X10 objective to encompass a single facial nucleus, the image was captured using a charge coupled device (CCD) camera on a visual display unit (VDU). An interactive image analysis system (Kontron KS400) was then used to draw manually on screen around the boundaries of the nucleus. The boundaries of the facial nucleus were taken to be the point where a marked qualitative decrease in the numerical density of large neuronal cell bodies was seen and an imaginary line drawn between nearest cell bodies on the perimeter of the nucleus. This boundary usually correlated with the point of transition between the more heavily stained neuropil of the nucleus and the less heavily stained neuropil surrounding it. The computer software calculated the area within the boundary (*a*). This value was multiplied by the total number of sections through the facial nucleus (*s*) and then multipled by the section thickness (*t*) (or *a*s*t*) to give an estimate of the volume of the facial nucleus (Vref).

Given that the accuracy of optical disector methods depend strongly on neurons having a spatially random distribution (Benes & Lange 2001), it was calculated further, using constants for both mean number of motoneurons per optical disector and mean number of sections per facial nucleus, that 1% error in the estimated total volume of the facial nucleus resulted in a proportional 1% shift in estimate of the total number of facial motoneurons per nucleus.

2.7.4 Optical Disector (calculation of Vdis and Q-)

Sections were viewed under a confocal laser-scanning microscope (CLSM) (Bio-Rad MRC 600), using 488nm laser illumination, a 3-4mm pinhole aperture and a X20, 0.7 numerical aperture dry objective, resulting in an optical section thickness of less than 5μm. This value was less than half of the Z plane distance of the optical sections used for the disector counts, ensuring no overlap between optical disectors. Optical sections were visualized on a VDU. A schematic representation of the optical disector sampling method is illustrated in **Fig 2-5**.

Up to four X20 objective fields of each facial nucleus were chosen, roughly corresponding to the anterior, posterior, medial and lateral aspects usually allowing slight overlap in the x-y plane. The number of fields were modified to three, two or even one to account for the decrease in facial nucleus area observed at the poles of the nucleus. Such sampling allowed for most of the area of each facial nucleus (at the chosen Z 'window') to be analysed thus increasing the probability that all the motoneurons in the sample had an equal chance of being counted.

The top of the section was brought into focus using 5μm step increments in the Z plane. Finer increments were made (1-2μm) to identify the exact point at which images started to appear on the VDU. This served as the reference point from which the middle 10μm could be observed without altering the x/y coordinates. Two optical scans were recorded 10μm apart in the middle of the Z plane of the section, *i.e.* the optical section whose upper and lower borders comprise the scans at 30μm and 40μm respectively in the Z plane axis. Thus for each optical disector, pairs of optical images were collected and recorded onto disc for subsequent analysis of motoneuron number.

Vdis was calculated by multiplying the disector area (200934μm^2) by the optical disector thickness (10μm). This gave a constant value of 2009340μm^3.

2.7.5 Counting Motoneurons

Motoneurons, defined as multipolar cell bodies with diameters greater than 20μm containing nuclei and nucleoli, were only counted if they appeared in the upper of the two scans. This was achieved by superimposing the 30μm scan over the 40μm scan, and assigning them as green and red images respectively. Thus the number of green 'tops', which were not in contact with the forbidden left and lower borders of the screen, was recorded into a Microsoft Excel spreadsheet. The numbers of motoneurons ('tops') in each of the disector volume areas of the facial nucleus (anterior, posterior, lateral, medial) were determined and a mean for the entire area of the facial nucleus (Q-) calculated.

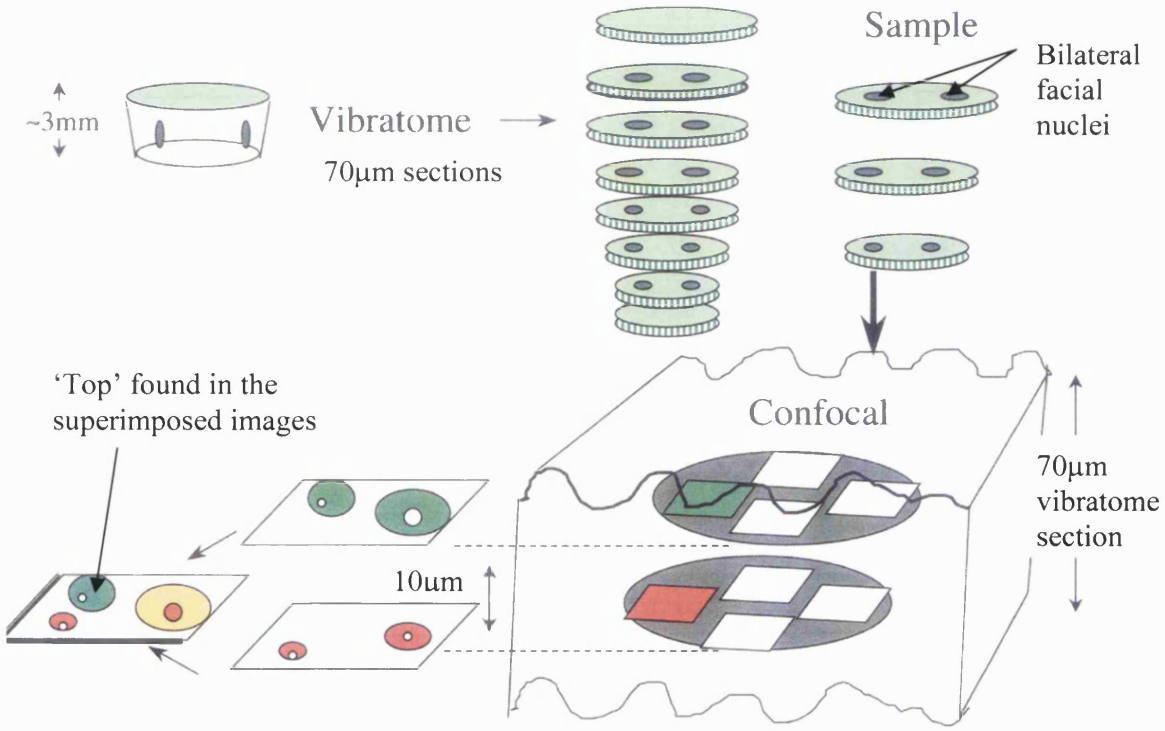


Figure 2-5 Schematic representation of the basic protocol for sectioning and examination under the confocal microscope. 23-24 sections of 70μm (adult and ageing animals) are produced (21-22 sections of 50μm in neonates) within the section series containing the bilateral facial nuclei. Sections are taken randomly and

systematically for analysis in the confocal microscope. Estimation of the number of 'tops' was done by superimposing two optical sections each 10μm apart from the middle 10μm of the section. The two confocal scans are stored as separate colours and superimposed on screen. Of the three neurons (defined here as having diameters >20μm) containing a nucleus which appear when the two optical sections are superimposed, one is identified by its yellow colour as being present in both optical sections, one is identified by its red colour as being present in the lower optical section only and one is identified by its green colour as being present in the upper section only. In the present protocol only the green neuron is counted. The left and lower borders of the counting frame were taken as 'forbidden lines'. Any profile which crossed these lines, or their extensions above and to the right of the counting frame, were not counted. Adapted from Johnson, I.P. (2001).

2.7.6 Statistical Analysis of Motoneuron Counts

Advice on appropriate statistical analysis was given by Dr. Richard Morris (Senior Lecturer of Medical Statistics, Department of Primary Care and Population Studies, Royal Free and University College Medical School). When estimates of either the total number of motoneurons per facial nucleus for all rats irrespective of treatment, or total numbers of normal motoneurones were plotted on a frequency distribution histogram, the data was not normally distributed. This was confirmed by analyzing the same data through the Kolmogorov-Smirnov test for verification of Gaussian distribution (Campbell 1989).

A normal distribution is a stringent assumption required to perform parametric statistics (Broughton Pipkin 1986), whereas two tests of the present data showed that it was not normally distributed. While it is possible to transform data so as to render it normally distributed, it was difficult to determine the extent to which such a transformation would affect the validity of any significant differences between experimental groups that may be obtained. Therefore, because the data was not normally distributed and the effect of artificially transforming it to a normal distribution was unclear, non-parametric alternatives to parametric ANOVA and t-tests were used to test for significance. The tests chosen (see below) are based on the comparison of mean ranks between groups. The assumptions of

these analyses, both of which are satisfied by the present data, are as follows (Fowler, Cohen, et al. 1998):

(i) samples are randomly and independently drawn

(ii) the measures within each of the samples have the properties of at least an ordinal scale of measurement, so that it is meaningful to speak of "greater than," "less than," and "equal to."

In the current study, a combination of the following tests was used: Kruskal-Wallis test for detecting significant differences between all groups simultaneously; and the Mann-Whitney unpaired (U) test. A brief description of the tests and how they were used in this study follows.

In all cases, the Kruskal-Wallis test was used, which is the non-parametric alternative to analysis of variance (ANOVA). Multiple group data are presented in the present thesis, i.e. there is more than one treatment group per experiment exclusive to and in comparison with normal animals (neither subjected to treatment nor nerve injury). Therefore, there are at least three experimental groups that require analysis, meaning in practice, that there is a need to examine the differences between the mean ranks of more than two samples. In order to examine the significance of any one treatment, therefore, it would be statistically inappropriate not to consider the complete range of data inclusive of other treatments. Any measure of significant difference between means of just two groups would lead to an additive effect of the probability of making a Type 1 error (i.e. concluding that the two population means are different when, in fact, they are equal) when all the between-group analyses are combined.

Such analysis across all data groups however, fails to identify from which source variability arises. Therefore, the Mann-Whitney U-test was used as a post hoc analysis for detecting significant changes between individual pairs of groups but only in instances where the mean

ranks of all groups differed significantly (i.e. shown by Kruskal-Wallis test). The chosen level of significance for all tests was P=0.05.

The statistical package used was 'StatMate' (Prism 2 Software, San Diego, USA) for the PC. To verify that the statistical correlates were calculated correctly, the tests were repeated via manual calculation on selected data on formulae published on-line at http://faculty.vassar.edu/lowry/webtext.html

Approximately 4-5 sections in (18-23% of the total volume of the nucleus in animals of all ages) were analysed stereologically. The remaining brainstem sections through the facial nucleus were randomly and systematically allocated for analysis using immunohistochemistry and *in situ* hybridisation. Sections that were not used immediately for these purposes could be dehydrated in graded methanols to absolute methanol and stored at –20°C. Sections could then be re-hydrated for use no longer than 7 days after initial storage (immunohistochemistry) or 3 months (*in situ* hybridisation) (Aperghis et al. 2000). These staining procedures were used to indicate at least two markers of the following: (i) motoneuron survival, (ii) motoneuron regeneration, (iii) the neuroglial response to injury in the facial nucleus, and (ii) motoneuron death by an apoptotic-like process.

2.8 Histochemistry

2.8.1 Immunohistochemistry

2.8.1.1 Floating Sections

Floating sections were processed for immunocytochemistry suspended in 5ml Sterilin tubes (Bibby Sterilin, Staffordshire, UK). Sections were permeabilised in 0.1% Triton X-100 in 0.1M tris-buffered saline (TBST) for immunocytochemistry and blocked for endogenous

peroxidase activity in a solution of 1-3% hydrogen peroxide in 75% methanol for 20min. Non-specific antibody binding was blocked with 5% bovine serum albumin (BSA) plus 5% normal secondary antibody host serum in TBST and washed briefly in TTBS. Sections were incubated overnight at 4°C with primary antibody and then washed for 20min.

All antibody binding was detected using the avidin-biotin complex (ABC) method. For antibody-treated sections only, the appropriate biotinylated secondary antibody (ABC kit, Vector Laboratories, Peterborough, UK) was added, diluted 1:200 in TBST including 5% of secondary antibody host serum, then washed for 2 x 20min in TBST. Biotin was subsequently detected in primary antibody-treated sections using streptavidin conjugated to horseradish peroxidase (HRP) (Vector Laboratories, Peterborough, UK), diluted according to manufacturers' guidelines in TBST, then washed for 2 x 20min in tris-buffered saline (TBS), or until there was no evidence of bubbles in the Sterilin tubes. Sites of peroxidase activity were visualised using diaminobenzidine (DAB) (Sigma, Dorset, UK) as substrate. Chromophore development was quenched in distilled water for 5min. placed on slides using a fine brush, covered with xylene until sections become transparent and mounted in DPX (BDH), or mounted in Citfluor (Agar Scientific, UK) as wet sections (neonatal sections only).

2.8.1.2 Immobilised Sections

Fresh frozen sections were fixed on the slide in 4% paraformaldehyde made up in 0.1M PBS for approximately 5-10min. All sections, including those that were perfusion-fixed were then washed for 2 x 5min in buffer. All washes throughout the procedure were carried out in 50ml glass coplain jars placed on a shaking platform mixer. Endogenous peroxidase was blocked by the addition of 1% hydrogen peroxide in 75% methanol for 30min then washed for 3 x 5min in distilled water. Non-specific binding was blocked by dropping approximately 100µl of a solution containing 5% bovine serum albumin (BSA), 5% secondary antibody host serum in TBST on to each section for 40min. Endogenous avidin and biotin was blocked with ready-made dropping solutions of avidin D blocker (Vector

Laboratories) for 20min followed by 1 x 5min wash in TBST and then treated with biotin blocking solution (Vector Laboratories) for 20min. After a further 5min wash in TBST, solutions of appropriately diluted primary antibody (**Table 2.1**) in TBS were dropped over individual sections and incubated over night in a humidity chamber at 4°C.

Following 3 x 5min washes in TBST, sections were incubated for 1½ hours with an appropriate secondary antibody (Vector Laboratories) diluted 1:200 in TBS including 5% of secondary antibody host serum, prior to washing for 3 x 5min in TBS. Biotin was detected with streptavidin conjugated to HRP (Vector Laboratories) placed over sections for 30min and washed for a further 3 x 5min in TBS. Sites of peroxidase activity were visualised using DAB as substrate. Chromophore development was quenched in distilled water for 5min. When appropriate, sections were counterstained with haematoxylin by submersing slides for 10-30 seconds and then left to wash out under tap water for 5-10min. Sections were dehydrated through graded ethanols to absolute ethanol, then xylene and finally mounted in DPX (BDH). **Table 2.1** shows relevant information regarding the use of all the primary antibodies intended for immunohistochemistry in the current thesis.

Table 2.1

Details regarding use of primary antibodies in immunohistochemistry

Antibody	*Manufacturer*	*Poly/monoclonal*	*Dilution/buffer*	*Mounting*
Cleaved Capase-3	New England Biolabs	Polyclonal	1:200 TBS	Citifluor
CGRP	Penninsula	Polyclonal	1:1,000 TBS	DPX
GFAP	Dako	Polyclonal	1:10,000 TBS	DPX

For controls, an equal volume of the appropriate buffer replaced primary or secondary antibodies. Immuno-absorbed antibodies were also used as a control whenever suitable binding peptides could be obtained.

2.8.2 Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL)

Fresh frozen sections were processed according to the NeuroTACS II protocol (R&D Systems, Mineapolis, USA). All washes were carried out in glass 50ml coplain jars. Sections were permeabilised on slide with 0.5% (vol/vol) proteinase-K ('neuropore reagent'; R&D Systems) for 30min at 37°C, washed in DNase-free water (supplied with kit). Endogenous peroxidase activity was quenched with 3% H_2O_2 for no longer than 5min. After a brief wash in PBS, samples were immersed in 1X terminal deoxynucleoside transferase (TdT) labeling buffer (100mM cacodylic acid pH 6.8, 0.1mM DTT, 1mM $CoCl_2$) for 5min at 18-24°C. Reaction mix containing 0.25mM biotinylated deoxynucleotide phosphates, Mn^{2+}, labeling buffer and TdT in the ratios recommended in the protocol supplied with the kit, was added to the sections. On separate slides, reaction mix with TdT omitted was added, which served as an unlabeled sample control. The samples were left inside a humidity chamber for 1 hour at 37°C. The reaction was stopped by immersing the sections in 10mM EDTA, pH 8.0, for 5min at 18-24°C and then washed for 2 x 2min in PBS. Biotinylated nucleotides were detected by the addition of streptavidin conjugated to HRP (Vector Laboratories), covered with a coverslip and incubated for 10min at 18-24°C. After a brief wash in PBS, peroxidase activity was detected using DAB as a substrate. The reaction was quenched in distilled water for 5min and sections were counterstained in haematoxylin. Sections were dehydrated through graded ethanols prior to mounting in DPX.

2.8.3 *In Situ* Hybridisation

Stringent precautions were observed to maintain RNase-free conditions at all times. All glassware was baked in an oven over night at 200°C prior to use and solutions were made up using DEPC-treated water wherever appropriate. Separate glassware was reserved for post-hybridisation procedures.

2.8.3.1 Free Floating Sections

Free-floating Vibratome sections were permeabilised with 0.1% Tween 20 in 0.1M phosphate buffered saline (PBST). Sections were then treated with proteinase-K (50µl/ml in PBST) (Roche Molecular Biochemicals, West Sussex, UK) for 10min before re-fixation (4% paraformaldehyde/0.2% glutaraldehyde in PBST). After a brief wash, sections were transferred to a solution containing 0.1M triethanolamine (pH 8.0) to which 2.5µl/ml acetic anhydride had been added, for 10min, washed for 10min, then pre-hybridised for 1 hour at 65°C in buffer consisting of (final concentration): 50% formamide, 5x saline sodium citrate (SSC), 0.1M Tween 20, 50µg/ml heparin (Sigma) and 10µg/ml yeast tRNA (Sigma), all in DEPC-treated water. Having been transferred to fresh buffer, 5µl of purified DIG-labeled antisense RNA probe was added and sections were left over night at 65°C in a swinging-platform hybridisation chamber. Sense probe was used as a hybridisation control.

Five stringency washes were carried out: firstly, in a solution containing 50% formamide and 2x SSC (in PBST) for 1 hour at 65°C then four washes in 2x SSC/PBST at 55°C, 10min each. Unhybridised RNA was digested with 20µg/ml RNase A1 and 100µg/ml RNase T1 in PBST (Roche) for 1 hour at 37°C. After a series of washes in 2x SSC at 37°C then 55°C, 0.2x SSC at 55°C and PBST at room temperature, sections were incubated with pre-absorbed anti-DIG alkaline phosphatase Fab fragments (Roche) at a dilution of 1/4000 in solution-B (0.1M PBS, 0.2% Tween 20, 0.2% Triton X-100 and 2% sheep serum) at 4°C over night. After three 30min washes in solution-B, endogenous alkaline phosphatase was blocked with 1mM levamisol (Sigma) in solution B at room temperature for a further 30min. For detection, sections were initially washed in detection buffer (100mM tris-HCl pH 9.5, 50mM $MgCl_2.6H_2O$, 100mM NaCl, 0.1% Tween 20 and 1mM levamisol) for a series of three 10min washes, then in detection mix consisting of detection buffer with the addition of 4.5µl/ml nitroblue tetrazolium chloride (NBT) (Roche) and 3.5µl/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche). Chromagen was allowed to develop in darkness at room temperature for approximately 2 hours. Sections were mounted (hydrated) in Citifluor on to glass slides and viewed under a light microscope.

2.8.3.2 Immobilised Sections

These two protocols are based on and further optimised from those published in the Roche Molecular Biochemicals non-radioactive *in situ* hybridisation application manual pages 126-130 (for fresh frozen tissue sections) and pages 136-138 (for frozen and perfused-fixed CNS tissue).

Fresh Frozen Tissue

Unless specified otherwise, sections were treated in 50ml coplain jars. Sections were washed for 2 x 5min in PBS, treated for 15min in PBS containing 0.3% Triton X-100, washed briefly in PBS and permeabilised on slide in TE buffer (100mM tris-HCl and 50mM EDTA, pH 8.0) containing 1μg/ml Proteinase-K at 37°C for 30min. Post-fixation was carried out for 5min in 4% paraformaldehyde at 4°C, followed by washing in PBS and acetylation by immersing slides in freshly made 0.1M triethanolamine (TEA) buffer (pH 8.0.) containing 0.25% (v/v) acetic anhydride (Sigma) for 2 x 5min on a rocking platform. Sections were then incubated at 37°C for at least 10min with pre-hybridisation buffer (4 x SSC containing 50% (v/v) deionised formamide). Pre-hybridisation buffer was drained and the sections were covered with hybridisation buffer containing 40% formamide, 10% dextran sulphate, 1 x Denhardt's solution (Sigma), 4 x SSC, 10mM DTT, 1mg/ml yeast t-RNA (Sigma), 1mg/ml denatured/sheared salmon sperm DNA and 5μl of purified DIG-labeled RNA probe. Coverslips were placed over sections, which were then incubated overnight at 42°C in a humidity chamber. In a shaking water bath at 37°C, sections were washed for 2 x 15min in 2 x SSC and then 2 x 15min in 1 x SSC.

Unbound (single-stranded) RNA probe was digested by immersing slides in a NTE buffer (500mM NaCl, 10mM tris, 1mM EDTA, pH 8.0 containing 20μg/ml RNase A). Sections were then washed at 37°C for 2 x 30min in 0.1 x SSC. For immunological detection of DIG, sections were washed for 2 x 10min in buffer-1 (100mM tris-HCl, pH 7.5, 150mM NaCl),

covered with blocking solution (buffer-1 containing 0.1% Triton X-100 and 2% normal sheep serum) and then incubated overnight at 4°C in a humid chamber with covered with buffer-1 containing 0.1% Triton X-100, 1% normal sheep serum and a 1:1,000 dilution of alkaline phosphatase-conjugated, anti-DIG Fab fragments. Sections were then washed in buffer-1 for 2 x 10min and incubated in buffer 2 (100mM tris-HCl, pH 9.5, 100mM NaCl, 50mmM $MgCl_2$). Alkaline phosphatase activity was detected by immersing slides in detection buffer (buffer 2 containing 4.5µl/ml NBT, 3.5µl/ml BCIP, 1mM levamisol). The reaction was stopped in distilled water at 4°C. Sections were mounted in Citifluor.

Perfusion-fixed CNS tissue

After an initial pre-treatment with 4% paraformaldehyde for 20min sections were rinsed 3-5 times in TBS, treated for 10min with 200mM HCl to denature proteins, washed in TBS, acetylated in freshly prepared 100 mM tris (pH 8.0) containing 0.5% acetic anhydride, washed again in TBS and incubated in 100µg/ml Proteinase-K in TBS for 20min at 37°C. Digestion was quenched in 4°C TBS for 5min. Sections were dehydrated in graded ethanols and rinsed briefly in chloroform. Re-hydration of sections was carried out in a humid chamber at 55°C for 30min. Hybridisation buffer (2 x SSC, 10% dextran sulphate, 0.01% sheared salmon sperm, 0.02% SDS and 50% formamide) containing 10ng of DIG-labeled RNA probe per 30µl of hybridisation buffer was added to each section. Coverslips were put in place and slides were incubated in a humid chamber for 4-6 hours at 55-65°C. Coverslips were removed in 2 x SSC at room temperature, then sections were stringency-washed in 50% formamide in 1 x SSC for 3 x 20min at 55°C and then in 1 x SSC for 2 x 15min at room temperature. At this point, sections were processed analogously to those in the protocol for fresh frozen sections, commencing with RNase treatment.

2.9 Molecular Biology

All stock reagents were of molecular biology grade. Where fresh extracted tissue was to be used for RNA analysis, stringent precautions were taken to avoid the contamination of tissue with RNases. These included the use of disposable gloves at all times in combination with

RNase Erase spray (ICN Biochemicals, Ohio, USA); instruments were autoclaved, washed in DEPC-treated water and sprayed with RNase Erase prior to use; and Eppendorf tubes were autoclaved and baked overnight at 80°C. Precautions were also made to avoid cross-contamination of plasmid-injected samples into non-injected samples from the same animal. Such stringency was of the utmost importance for down-stream PCR analysis.

2.9.1 Expression Plasmids

cDNA sequences for the full-length coding regions of rabbit MGF and L.IGF-1 were isolated from stretched skeletal muscle (Yang, S. et al. 1996) using Kpn 1- and Xba 1-restriction endonuclease site-tagged primers and inserted in-frame into pcDNA3.0 plasmid vectors (Invitrogen), seen in **Fig. 2.6**.

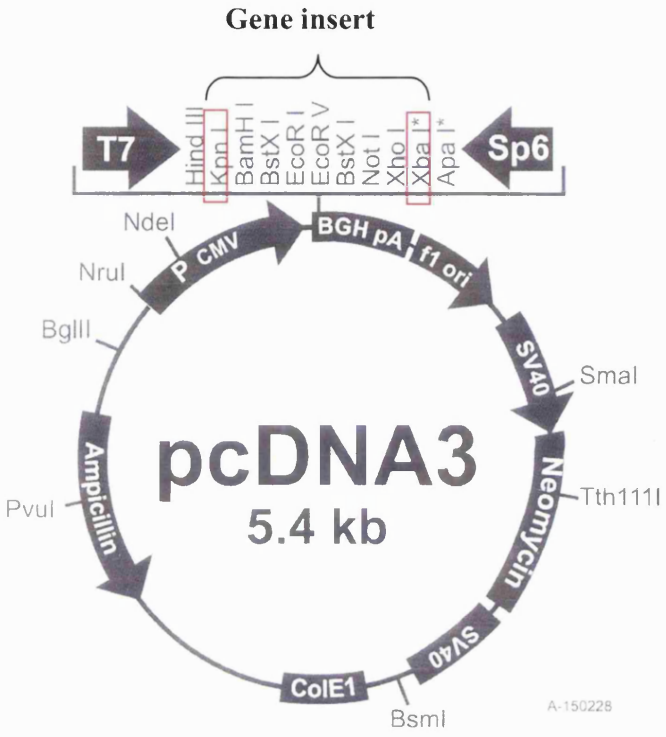


Figure 2-6 Schematic representation of the plasmid vector used for gene transfer. cDNA for the coding regions of MGF and L.IGF-1 were inserted by replacing the Kpn 1-Xba 1 fragment of the multiple-cloning site. The plasmid contains a CMV promoter up-stream of the gene and a myosin light-chain enhancer (not shown) down-stream of the cDNA sequence. (Adapt from Invitrogen product data sheet)

2.9.2 Extraction and Preparation of Total RNA

The single-step-based method of RNA isolation using acid guanidium thiocyanate-phenol-chloroform (Chomczynski & Sacchi 1987) was used in the present study.

Frozen samples of tissue, weighing approximately 100-400mg, were homogenised in Tri-Reagent (Sigma) (1ml per 50-100mg of tissue), using an Ultra-Turrax T25 homogeniser (Janke-Kunkel, Germany) in a 50ml Falcon tube. Homogenised samples were incubated at room temperature for 5min prior to the addition of of chloroform (0.2ml per 1ml of Tri-

Reagent). After thorough mixing and a further incubation at room temperature for 15min, the samples were centrifuged at 12,000 x *g* for 30min at 4°C. The upper (aqueous) phase was transferred to a 15ml Falcon tube taking care not to disturb the interphase (containing DNA). After the addition of of isopropanol (0.5ml per 1ml of Tri-Reagent), the RNA was allowed to precipitate at room temperature for 10min and then centrifuged at 12,000 x *g* for 10min at 4°C. The supernatant was removed and the pellet was washed with 75% ethanol followed by centrifugation at 7,500 x g for 5min at 4°C. This step was repeated another two times. Pellets were air-dried prior to re-suspension in deionised formamide to give a final concentration of approximately 1-3µg/µl. RNA concentration was measured using a spectrophotometer measuring optical density (OD) at 260nm and 280nm. RNA/formamide samples were stored at -70°C for periods of up to 6 months. To verify RNA quality, formamide-dissolved RNA samples were separated by electrophoresis on a gel containing: 1X MOPS pH 7.0, 20mM 3-N-morpholine propanesulphonic acid, 5mM sodium acetate, 1mM EDTA, 10mg/ml ethidium bromide and 1% agarose for 2 hours in 1X MOPS running buffer. Viewed under ultra-violet light, well-defined bands relating to the 18S and 28S ribosomal RNA subunits, and with the absence of any smearing, signified samples of total RNA of satisfactory quality.

Prior to use in cDNA synthesis, 1-15µg of stock RNA was precipitated in 1.5ml Eppendorf tubes by the addition of 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 x volume 100% ethanol and incubation of samples at –20°C for 2 hours. RNA was pelleted by centrifugation at 12,000 x g for 20min at 4°C, washed twice with 75% ethanol, air-dried for approximately 5min and re-suspended in 20µl of DEPC-treated water.

2.9.3 DNase Digestion of Total RNA

Contaminant genomic DNA was removed from purified total RNA preparations with the use of RQ1 RNase-free DNase (Promega, Wisconsin, USA). This was especially important for down-stream analysis using the reverse transcriptase polymerase chain reaction (RT-PCR). To each reaction mix in a 1.5ml Eppendorf tube on ice, 1-8µl of RNA (of known amount),

1µl of 10X buffer (containing final concentrations: 40mM tris-HCl pH 8.0, 10mM $MgSO_4$, 1mM $CaCl_2$), 1µl RQ1 RNase-free DNase (1µl/µg RNA) and nuclease-free water to a final reaction volume of 10µl. Reaction volumes of multiples of 10µl were used where concentrations of sample RNA and requisite volume of enzyme resulted in final reaction volumes exceeding 10µl. The reaction was incubated at 37°C for 30min, quenched by adding 1µl of DNase stop solution (final concentration: 1mM EGTA, pH 8.0) and incubated at 65°C for 10min to inactivate the enzyme. RNA was purified by phenol-chloroform extraction followed by ethanol precipitation. 0.5 x volume of phenol (pH 4.3) and 0.5 x volume chloroform isoamyl alcohol (24:1) were added to each reaction tube. After thorough vortexing, tubes were spun at 14,000 rpm in a micro-centrifuge for 1min and the upper phase was transferred to a new Eppendorf tube. 1 x reaction volume of chloroform was added to the upper phase, vortexed, and spun. The upper phase was transferred to a new tube, to which was added 1/10 x reaction volume of 3M sodium acetate (pH 5.2) and 2.5 x reaction volume of 100% ethanol. After thorough mixing, RNA was precipitated at –20°C for 2 hours, pelleted by spinning in a microcentrifuge for 20min at 4°C. After 2 washes in 75% ethanol. pellets were air-dried for no longer than 5min and re-suspended in 20µl DEPC-treated water.

2.9.4 First Strand cDNA Synthesis

To a 1.5ml Eppendorf tube, 25pmol of RoRi-dT_{17} (oligo-dT) primer or 5pmol gene-specific primer (for analysis of endogenous MGF/L.IGF-1 mRNA expression only, chapter-5) (for sequences, see below), 2µg of total RNA was added along with DEPC-treated water to a total volume of 11µl.

RoRi-dT_{17}

5' TCGATGGTCGACGCATGCGGATCCAAA

GCTTGAATTCGAGCTTTTTTTTTTTTTTTT **3'**

EX6-RT (IGF-1 gene-specific)

5' TTGCAAGGTTGCT **3'**

The mixture was heated at 70°C for 10min to denature RNA and chilled quickly on ice for a further 5min. To make a final reaction volume of 20µl, first strand buffer (final concentration: 10mM Tris-HCl pH 8.3, 15mM KCl, 0.6 mM MgCl), 10mM DTT, 1mM each dATP, dCTP, dGTP, dTTP (Roche), 1µl RNase H- reverse transcriptase (SuperScript II, Invitrogen, Carlsbad, USA) and 1µl recombinant ribonuclease inhibitor (RNaseOUT, Invitrogen) were added to the mixture and then incubated at 42°C (for RoRi primer) or 50-55°C (for gene-specific primer) for 50min. Heating the tubes to 70°C for 15min inactivated the reaction. cDNA samples were stored at –20°C until use.

2.9.5 Polymerase Chain Reaction (PCR) Amplification of cDNA

Reactions were performed in a 50µl volume containing reaction buffer (final concentrations: 20mM Tris-HCl pH 8.4, 50mM KCl), 2.0mM $MgCl_2$ (determined empirically), 0.2mM each of dATP, dCTP, dGTP, dTTP (Roche), 0.2µM each of forward and reverse primers (**Table 2.2 B and C**), 2µl (equivalent to 200ng of total RNA from which cDNA was synthesised) of first strand cDNA and 0.4µl antibody-complexed Taq DNA Polymerase (Platinum Taq, Invitrogen).

The reaction components were mixed gently and then centrifuged briefly prior to loading into thermal-cycler (Hybaid). All samples were heat-denatured for 10min at 94°C prior to commencing the cycling program. This also provided the automatic 'hot start' by denaturing the complex between antibody and enzyme, thus activating Taq polymerase. Hot-starts are typically used in PCR to increase sensitivity, specificity, and yield while allowing assembly of reactions at ambient temperatures. The extra time, effort and contamination risks associated with manual hot-start procedures are minimised with Platinum Taq Polymerase (Invitrogen). Amplification was carried out by cycles of denaturation at 94°C for 30sec,

annealing for 30sec at empirically determined temperatures (see below), and elongation at 72°C for 1min. To maximise amplification (for vector gene expression analysis and cDNA cloning purposes only), 35 cycles were programmed. A final elongation step at 72°C for 10min was included after completion of both cycling programs to ensure all PCR products had 3' A overhangs. PCR products were analysed by electrophoresis of an aliquot of the reaction on a 1% agarose gel containing ethidium bromide (10mg/ml).

Primer melting temperatures were calculated using the following formula:

$T_M = 2(A+T) + 4(G+C)$ where A = adenine, T = thymine, G = guanine, C = cytosine

Table 2.2

Nomenclature and nucleotide sequence of PCR primers in the current thesis

Forward primers		*Reverse primers*	
Name	*Sequence (5'-3')*	*Name*	*Sequence (5'-3')*
A: Endogenous expression of IGF-1 splice variants[1]			
5UTR-F	CCAGCTGTTTCCTGTCTACAGT	EX5-R	TCCTTCTCCTTTGAGCTTC
UEX1-F	ATCTGCCTCTGTGACTTCTTGA	L64-R	AAATGTACTTCCTTCTGGGTCT
EX2-F	CCACTCTGACCTGCTGTGTAA	EX6-R	TTTTGCAGGTTGCTCAAGC
RtEX3-F	GCTTGCTCACCTTTACCAGC		
EX5-F	CCTATCGACACACAAGAAAAGG		
PE186-F	GCTTGCTAAATCTCACTGTCT		
B: Vector derived (rabbit) MGF mRNA expression			
RbEX3-F	ATTTCAACAAGCCCACAGG	PLD-R	TAGAAGGCACAGTCGTGGC
C: cDNA cloning			
EX3-F (IGF-1)	GCTTGCTCACCTTTACCAGC	EX6-R (IGF-1)	TTTTGCAGGTTGCTCAAGC
GAP43-F	GGCTCATAAGGCTGCAACCA	GAP43-R	GGCTTGTTTAGGCTCCTCCTT
AKT-F	GGCTGGACTGCTCAAGAAGG	AKT-R	GGGAACGCAAACCAAAGG
D: Direct generation of riboprobe template for MGF and L.IGF-1[2]			
IGFSP6-F	TGATTTAGGTGACACTATAGAATCTAGA ACACTGACATGCCCAAGA	IGFT7-R	TGTAATACGACTCACTATAGGGAAGCTT TGCACTTCCTCTACTTGTGT
MGFSP6-F	TGATTTAGGTGACACTATAGAATCTAGA TCCCAGCCCCTATCGACACA	MGFT7-R	TGTAATACGACTCACTATAGGGAAGCTT CTTTCCTTCTCCTTTGCAGC

[1] indicates that forward and reverse primers are not shown in specific pairs because all three of the reverse primers in the right-hand column were used in combination with more than one (singularly) of the six forward primers shown in the left-hand column. [2] Sequences in RED encode the SP6 or T7 (as indicated) RNA polymerase recognition sites so as to produce PCR products ready suitable for riboprobe synthesis. Sequences in BLUE refer to IGF-1 or MGF sequences only (For use of these primers, see **Fig. 2-8**). All primers supplied by Sigma Genosys.

For RT-PCR study in chapter-5 where endogenous IGF-1 mRNA expression of splice variants were investigated, amplification was quenched during the exponential phase of amplification. This has been reported previously to occur after approximately 22-23 cycles of PCR for the amplification of GAP-43 (Hager et al. 1999). However, in order to visualise amplified products on an agarose gel and make subjective comparisons of relative band intensity, a greater amount of DNA is required than that which would exist after such low-level cycle numbers. To overcome this problem, and to increase the likelihood of making meaningful comparisons of relative levels of amplification between samples, a pre-amplification step for all cDNA samples was introduced (Hager et al. 1999) . In theory, this method raises proportionally the baseline 'starting' amount of cDNA, allowing a second round of PCR to be quenched and visualised easily at approximately 22-23 cycles and, therefore, in the exponential phase of amplification. From each of 12 cDNA samples, six identical PCR reactions were made-up (*i.e.* for 12 tissue samples, there were a total of 72 individual PCR reactions), each to a final volume of 25µl (*i.e.* proportionally half of each of the components in a 50µl reaction described ealier). For primer sequences, see **Table 2.2 A.** Pre-amplification comprised 12 cycles of PCR (steps as defined earlier). Afterwards, each of the six identical reactions from each sample were combined and then purified using the Wizard DNA purification system (Promega). PCR products were purified by mixing the combined PCR reaction volumes with 100µl of direct-purification buffer (containing: 50mM KCl, 10mM Tris-HCl pH 8.0, 1.5mM $MgCl_2$, 0.1% Triton X-100). Samples were then incubated with 1ml Wizard DNA purification resin (5.4M guanidine thiocyanate) at 60°C for 5min, passed through Wizard purification columns by use of a manifold vacuum pump and washed with 2ml isopropanol. DNA was eluted from columns with 50µl nuclease-free water. The second round of PCR was performed, applying 23 cycles with 2µl of the pre-amplified DNA in a total volume of 25 µl. A schematic diagram of this procedure is illustrated in **Fig. 2-7**.

Certain limitations of this present method require discussion in order to make useful evaluations of the data obtained. It is not possible to compare qualitatively relative levels of amplification from one primer pair to another because these represent different PCR

experiments and thus subject to spurious variability in conditions that alter their efficiency of amplification. Another source of error is likely to arise from inconsistent differences between melting temperatures (Tm) of the various primer pair combinations. Optimal primer sequences were chosen for each reaction but it was not always possible to find sequences with close Tm values.

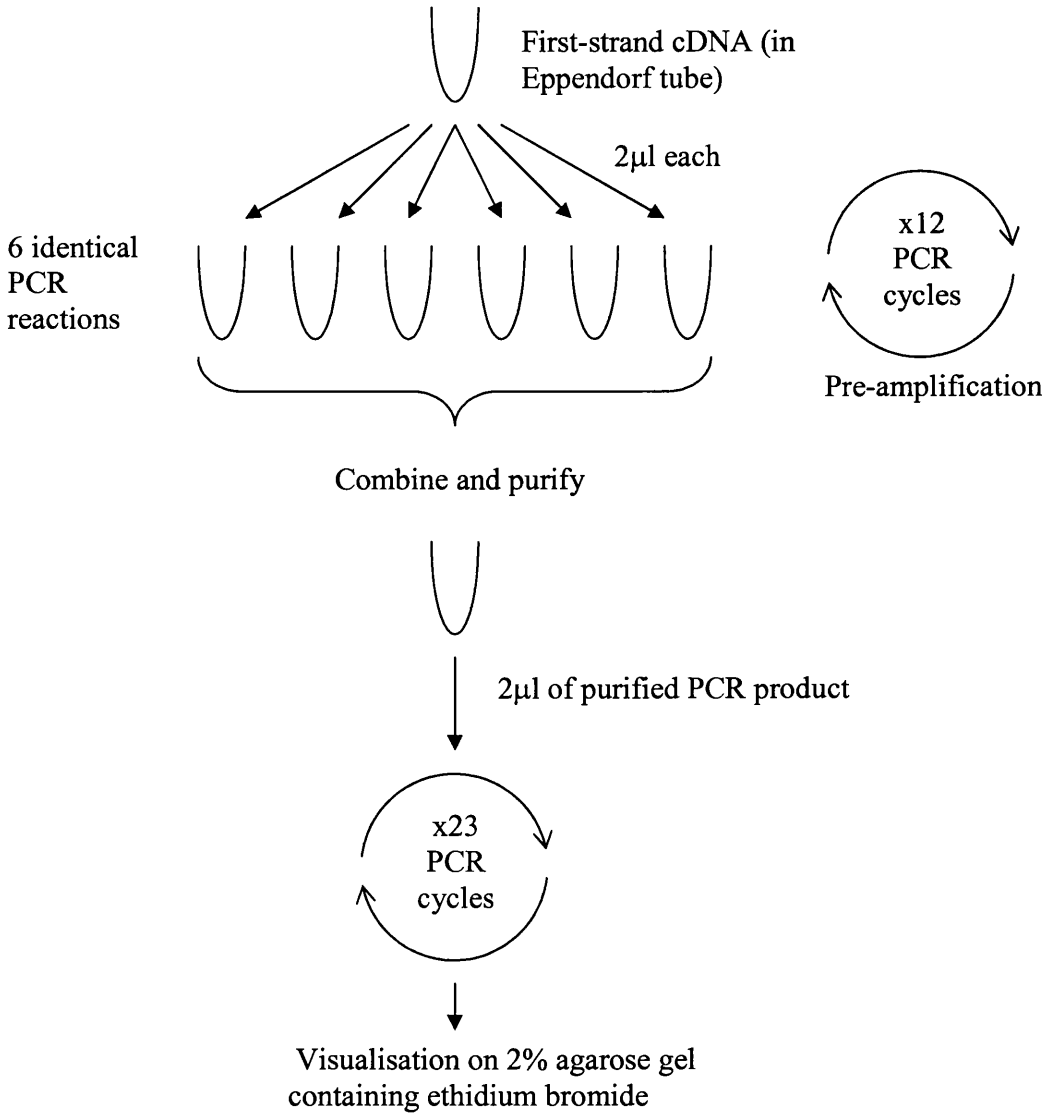


Figure 2-7 Schematic representation of PCR strategy to visualise PCR-amplified cDNA quenched during the exponential phase of amplification.

2.9.6 cDNA Cloning

cDNA sequences of MGF, L.IGF-1, GAP-43 and Akt were cloned into plasmid vectors so as to facilitate the down-stream generation of riboprobes for detecting mRNA expression of these genes *in situ* (**Section 2.9.7**). PCR-amplified products can be cloned efficiently into specifically designed vectors using the TA cloning method (Mead et al. 1991). Since Taq DNA polmerase has a non-template-dependent activity which adds a single adenine residue to the 3' ends of PCR products (Clark 1988), it is possible to ligate PCR products into linearised vectors that have a single thymidine residue added to their 3' ends. The pGEM-T Easy vector system (Promega) was used to incorporate PCR products from cDNA amplifications. Denervated rat snout muscle tissue was used to clone the partial cDNA sequences for MGF and L.IGF-1. Using the primer pair RtEX3-F and EX6-R (**Table 2.2**), two amplification products were obtained relating to the presence (MGF) or absence (L.IGF-1) of the 52bp insert of exon-5 (McKoy et al. 1999; Yang, S. et al. 1996). As a result, products had to be separated on a 1.5% agarose gel followed by gel extraction. Brainstem tissue from rat was used as the source of RNA from which cDNA sequences relating to GAP-43 and Akt were cloned. All PCR products were purified with use of the Wizard DNA purification system (Promega), as performed for purification for pre-amplified PCR products in **Section 2.9.5**. DNA concentration was measured on a spectrophotometer reading absorbance at 260nm and 280nm.

To each ligation reaction the following was added: 50ng linearised pGEM DNA, reaction buffer (final concentrations: 30mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 5% polyethylene glycol), PCR purified product (so as to result in 1:3 to 1:5 molar ratio of vector:insert), 1µl T4 DNA ligase (Promega) and deionised water to a final reaction volume of 10µl. Ligation was performed overnight at 4°C. For a background control, PCR product was omitted from the reaction mix and replaced with an equal volume of deionised water.

Transformation of JM109 competent cells (Promega) was carried out as follows: 50µl of competent cells were added to 2µl of ligation product in a 1.5ml Eppendorf tube. The tubes

were incubated on ice for 20min and then heat-shocked in a water bath for 45sec at 42°C, followed by incubation on ice for 2min. 950µl of room temperature LB medium (containing per litre: 10g tryptone, 10g NaCl, 5g yeast) was added to each tube, and they were then incubated at 37°C on a rotary shaker for 1.5 hours. 100µl of cells were spread on LB agar plates containing 50µg/ml ampicillin, which had been spread with 40µl of 100mM IPTG and 20µl of 40mg/ml X-gal. The plates were incubated overnight at 37°C. Positive transformants were initially identified by blue/white colony selection (Yanisch-Perron et al. 1985). Where multiple PCR products were ligated in a single reaction, which were non-separable by gel extraction either due to similar product size or poor yield, many colonies needed to be analysed to increase the chances of identifying the required insert. This was done rapidly by colony PCR analysis. Sterile cocktail sticks dabbed carefully on to individual colonies and then dipped into a PCR reaction mixes, analogous to those used for initial amplification. Running PCR products on a 1% agarose gel containing ethidium bromide identified positive transformants. Positive identification of amplification products of appropriate size meant that a single small-scale plasmid preparation was required, carried out from overnight cultures using the Qiaprep Miniprep kit (Qiagen). Where a single PCR product was ligated, the colony PCR step was omitted. In all cases, the presence of PCR product insert was further verified by restriction endonuclease digestion of the new plasmid constructs and electrophoresis on a 1% agarose gel. (see section 2.9.7.1 below: digestion was performed using both *Not* 1 and *Spe* 1 enzymes in the same digestion reaction, on 5µl of plasmid, for 1 hour). Final confirmation of the correct dNTP sequence of cloned PCR products was achieved by ethanol precipitating approximately 5µg of plasmid, spinning at 12,000 rpm and drying the resultant pellet. Samples were sent to MWG Biotech (Ebersberg, Germany) for custom DNA sequencing analysis. Working amounts of plasmid DNA for riboprobe synthesis (pGEM) or for gene expression (pcDNA3.0) were produced by larger-scale plasmid preparation using the GenElute endotoxin-free plasmid maxi-prep kit (Sigma).

2.9.7 Riboprobe Synthesis

2.9.7.1 Template synthesis

Non-radioactive, digoxygenin (DIG)-labeled cRNA probes were synthesised using PCR amplified cDNA as a template. Templates for GAP-43 and Akt probes were generated by linearisation of pGEM plasmids into which PCR-amplified cDNAs were subcloned. pGEM contains T7 and SP6 RNA polymerase binding sites either side of the multiple-cloning region allowing cRNA probes (antisense and sense) to be run-off during *in vitro* transcription. Plasmids were linearised by setting up the following analogous endonuclease digestion reactions, differing only in the enzyme used, to generate templates for both sense and antisense probes: (in 1.5ml Eppendorf tubes) 4μg of plasmid DNA, digestion buffer (final concentrations: Tris-HCl pH 7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT), 4μl of either *Not* I or *Spe* I and nuclease-free water up to a final reaction volume of 40μl. Digestion was performed to completion by incubating the tubes at 37°C for 3 hours. 1μl of digested DNA was run on a 1% agarose gel to verify successful linearisation and absence of uncut plasmid. Templates were purified by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 20μl nuclease free water containing 1μl RNase inhibitor (RNaseOUT, Gibco).

MGF and L.IGF-1 cDNAs (exons 3, 4, 5 and 6 for MGF; exons 3, 4 and 6 for L.IGF-1) were sub-cloned previously into separate pGEM plasmids using PCR primers EX3-F and EX6-R. Each plasmid, in turn, was used as a secondary PCR template in reactions containing specific primers tagged with the nucleotide-binding sequences for T7 and SP6 RNA polymerase (**Table 2.2 D**). PCR products resulting from this secondary PCR included sequences for T7 polymerase at the 5' end of the sense strand and SP6 polymerase at the 3' end of the antisense strand (**Fig. 2-8**). The prior sub-cloning of these IGF-1 cDNA fragments into separate plasmids, described ealier, meant that L.IGF-1 cDNA templates could be generated without the co-production of MGF cDNA, thus avoiding the need to gel-purify the resulting templates for subsequent *in vitro* transcription.

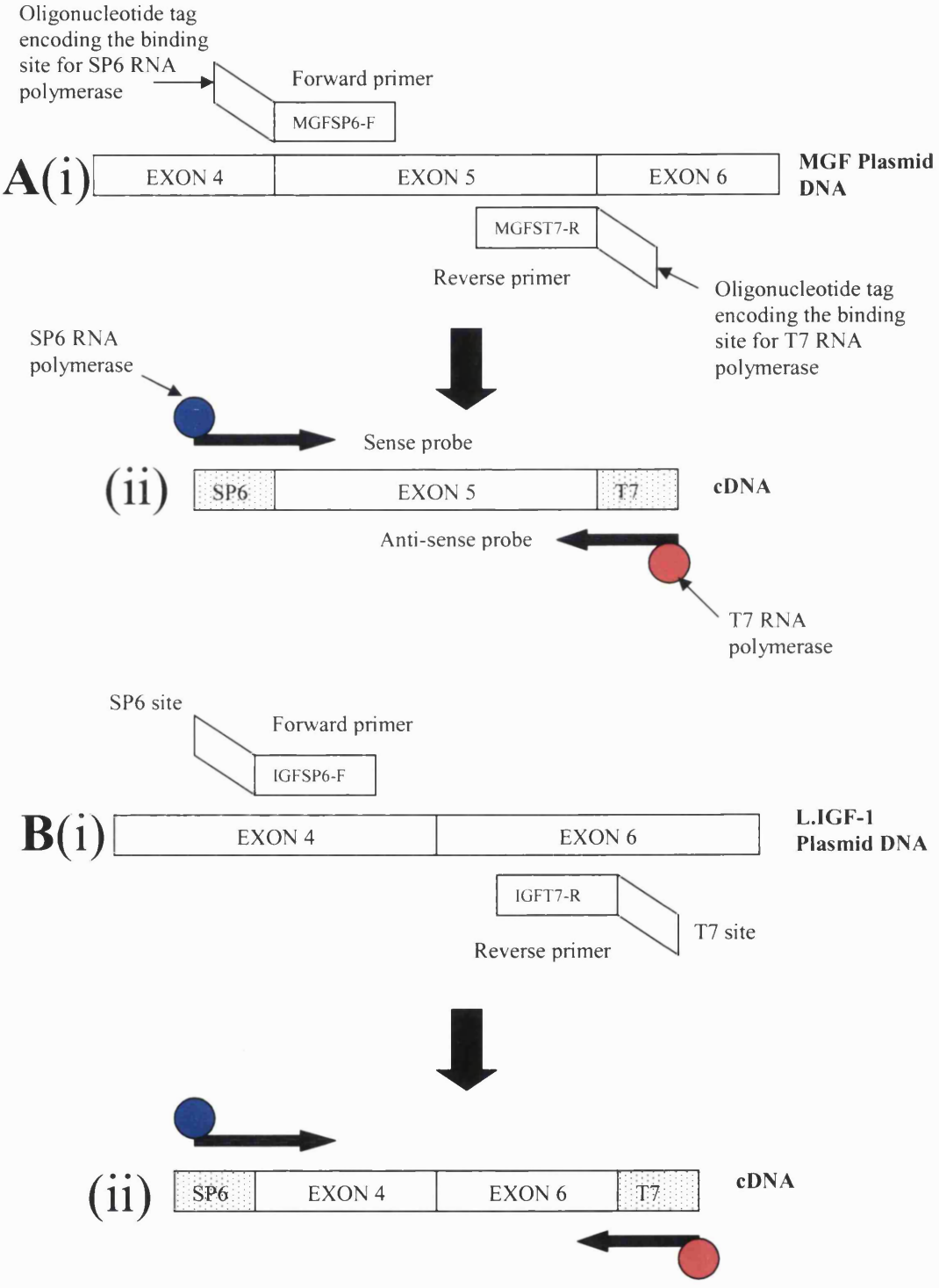

Oligonucleotide tag encoding the binding site for SP6 RNA polymerase
Forward primer
MGFSP6-F
A(i)
EXON 4
EXON 5
EXON 6
MGF Plasmid DNA
MGFST7-R
Reverse primer
Oligonucleotide tag encoding the binding site for T7 RNA polymerase
SP6 RNA polymerase
Sense probe
(ii)
SP6
EXON 5
T7
cDNA
Anti-sense probe
T7 RNA polymerase
SP6 site
Forward primer
IGFSP6-F
B(i)
EXON 4
EXON 6
L.IGF-1 Plasmid DNA
IGFT7-R
T7 site
Reverse primer
(ii)
SP6
EXON 4
EXON 6
T7
cDNA

Figure 2-8 Schematic representation of the strategy used to generate MGF- and L.IGF-1-specific cDNA templates for subsequent *in vitro* transcription-labeling with DIG. A(i): MGF exon 3, 4, 5 and 6 fragment (previously sub-cloned into pGEM vector, exon 3 not shown). Specific PCR primers (SP6MGF-F and T7MGF-R, see **Table 2.2 D**) were designed within exon 5 (specific to MGF) that contained 5' extension oligonucleotides encoding the recognition sites for either T7 or SP6 RNA polymerases. A(ii): cDNA resulting from PCR, therefore, could be used as a template for *in vitro* transcription labeling. B(i) and (ii): L.IGF-1 exon 3, 4 and 6 fragment (previous subcloned into a separate pGEM plasmid, exon 3 not shown). Specific PCR primers (SP6IGF-F and T7IGF-R) were designed so as to generate a short cDNA sequence bridging the excision of exon 5. Since template DNA was a plasmid encoding a fragment only from L.IGF-1, PCR products containing exon 5 were not produced.

2.9.7.2 In vitro transcription labeling

For each *in vitro* transcription reaction, the following was added to a 1.5ml Eppendorf tube: 1μg of linearised plasmid DNA (GAP-43 or Akt) or 200ng of PCR product (MGF or L.IGF-1), DIG RNA labeling mix (Roche) (final concentrations: 1mM of each of ATP, CTP, GTP, 6.5mM UTP, 3.5mM DIG-11-UTP pH 7.5), 2μl transcription buffer (final concentration: 4mM Tris-HCl pH 8.0, 6mM $MgCl_2$, 10mM DTT, 2mM spermidine), 1μl RNase inhibitor, nuclease-free water to a total volume of 18μl, then 2μl of either T7 or SP6 RNA polymerase. Both sense and antisense probes were generated. The reactions were incubated at 37°C for 2 hours. The reaction was stopped by adding 2μl of 0.2M EDTA (pH 8.0). Ethanol precipitation was performed by adding 1/10 x reaction volume LiCl and 2.5 x reaction volume of ice-cold 100% ethanol followed by chilling at –20°C for 2 hours, centrifugation at 13,000 x *g* for 15min at 4°C and washing the pellet twice in 70% ethanol. After air-drying for no longer than 5min the pellet was dissolved in 49μl nuclease-free water and 1μl RNase inhibitor. The amount of probe required for satisfactory *in situ* labeling was determined empirically. It was found that 5μl of the purified probe was required for all *in situ* hybridisation protocols. 5μl aliquots were stored at –70°C until use.

Chapter-3

The effect of age, diet and muscle damage on motoneuron survival following injury

3.1 Introduction

The maintenance and survival of peripheral motoneurons, whose axons lie outside the central nervous system, is determined to a large extent by interaction with their peripheral targets. It is of special interest to the present thesis that peripheral motoneurons provide a means by which muscles can retrogradely influence the neuron cell body. The nature of this interaction changes with age (Navarrette & Vrbova 1993), with immature motoneurons being more dependent for survival on peripheral target contact than mature motoneurons (Lowrie & Vrbova 1992). As a result, neonatal animals are used commonly to study target-derived mechanisms underlying motoneuron survival, while such information for mature animals is lacking.

Typically, motor axon disruption (axotomy) is used to perturb motoneuron-target interactions and potentially neuroprotective substances are applied to the injured nerve to determine if this reduces the amount of motoneuron loss. This provides information only on mechanisms underlying the maintenance and survival of immature motoneurons.

There are two significant and recurring problems that appear to have arisen on review of the considerable amount of data that has accumulated:

(i) It is uncertain whether information derived from young animals may be extrapolated reliably to predict the survival or death of motoneurons in adult and ageing animals.

(ii) The rescue of motoneurons with treatments applied after nerve injury is not consistent with age- and disease-related neurodegeneration, where motoneurons degenerate with nerve and target-muscle intact.

This study aims to overcome the first of these problems by utilising an injury model that results in significant motoneuron loss in animals of all ages. Using this model, particular attention will be paid in chapters 4 and 5 to potentially neuroprotective properties of a newly

cloned splice variant of IGF-1, mechano-growth factor (MGF). The second problem is addressed by applying neuroprotective treatments to the nerve *prior* to injury.

3.1.1 Axotomy as a Model of Experimental Neuronal Injury and Cell Death

Axonal disruption provides a useful paradigm to study cellular responses to injury, to include mechanisms of regeneration and plasticity, and processes that lead to nerve cell degeneration (Dekkers & Navarrete 1998; Fawcett & Keynes 1990). As a result, such *in vivo* models have become an essential experimental tool in the development of potential therapies for age-related motoneuron degeneration. Lesions can be made with precision, depending on the chosen neural system, location of the lesion, and age of the animal (Soreide 1981a; 1981b). These models allow the opportunity to examine a range of neuronal responses (Koliatsos & Price 1996; Kreutzberg 1996).

Responses to lesion can range from rapid cell death, common to motoneurons of newborn animals reacting to nerve axotomy (Houenou et al. 1994), or nerve regeneration followed by re-establishment of neuromuscular contact in adult rats (Soreide 1981a). In either scenario, studies at the cellular and molecular level have provided clues as to how to exploit and/or enhance the mechanisms controlling the continued survival of motoneurons (Dreyfus et al. 1999; Griesbeck et al. 1995; Kreutzberg et al. 1989; Sendtner et al. 1992b).

3.1.2 Target-derived neurotrophism

The earliest attempts to understand the interactions between motoneurons and their targets observed the changes brought about by the disruption of the wingbud of 3 day-old chick embryos. M. L. Shorey (1909) reported what appeared to be hypoplasia of both the brachial motor column and spinal ganglia a few days after removal of the forelimb bud prior to the period of peripheral axon outgrowth. It was not for another twenty years that the significance of these findings was grasped quantitatively. Subsequent investigators confirmed the massive neuron loss following limb bud removal but concluded ultimately

that the loss was, in fact, owing to cellular degeneration, not faulty differentiation (Hamburger 1934; Oppenheim 1981). However, the first systematic account of both degenerating and healthy neurons undergoing normal death during development was made for the cervical and thoracic dorsal root ganglia in the chick which contained the first suggestion that neuronal survival might be contingent on the supply of a trophic substance or 'factor' by the targets of the neurons (Hamburger & Levi-Montalcini 1949). This was reinforced by prior observations that when sarcomas were implanted in the hind limb fields of embryonic chicks, there was an invasion of the tumours by the processes of peripheral ganglia accompanied by neuron cell hyperplasia, but not of motor axons (Bueker 1948). The discoveries described above set an important conceptual framework, *i.e.* a neurotrophic hypothesis, for the eventual discovery and characterisation of the first neurotrophic factor, nerve growth factor (NGF) (Levi-Montalcini & Cohen 1956). This was later to be isolated to molecular homogeneity via biochemical fractionation techniques, albeit not from neural tissue (Cohen 1959; 1960). Neurotrophic factors may be defined as endogenous soluble proteins that regulate the survival and growth of differentiated neurons (Purves et al. 1988). A basic neurotrophic theory was devised whereby neurotrophic factors derived from the peripheral target exert a retrograde trophic influence over the neurons that innervate them (Davies 1996). This view was promoted further by experiments which showed that NGF is retrogradely transported from the synaptic terminal in smooth and cardiac muscle (Hendry et al. 1974), which are the principle source of the factor (Korsching & Thoenen 1983). Despite the growing wealth of information on the multiple modes of action and sources of neurotrophic factors and, therefore, the prevailance of alternative views on trophic support, for example the trophic influence of afferents (Okado & Oppenheim 1984; Peyronnard & Charron 1983) and glia (Clatterbuck et al. 1996), the neurotrophic hypothesis has remained the best explanation for how neuronal target fields, in the developing peripheral nervous system at least, regulate the number of innervating cells by which they are supplied.

3.1.3 Neurotrophic Factors and Motoneurons

Much of the earlier work failed to characterise as fully the trophic regulation of peripheral targets specific to motoneurons, compared to that which was apparent for other types of

neurons. It is reported that approximately 50% of embryonic chick spinal motoneurons die during the course of normal development and that limb bud removal enhances this process (Hamburger 1958; 1975). Furthermore, it was shown that transplantation of additional limb bud tissue during the period of developmental cell death reduced the extent of motoneuron loss (Hollyday & Hamburger 1976). Therefore, experimental evidence indicated the trophic influence also of motoneuron target cells during development. Since it was found that motoneurons were unresponsive to NGF, it was suspected that another protein derived from skeletal muscle was responsible for motoneuron survival. The biochemical fractionation technique, which had yielded NGF, also resulted in purification of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) through their ability to promote neuronal survival *in vitro* (reviewed in Nishi 1994) but failed to produce a motoneuron factor. Nontheless, crude and partially purified extracts from embryonic hind limbs applied to chick embryos *in ovo*, during the period of normal cell death, rescued a significant number of motoneurons from degeneration. In addition, kidney or lung extracts and heat-inactivated hind limb extracts were ineffective (Oppenheim et al. 1988; Oppenheim & Haverkamp 1988). The initial failure of BDNF and CNTF however, to promote motoneuron survival has been ascribed to the use of out-dated *in vitro* screening assays. With the advent of an appropriate *in vitro* assay, which utilised novel techniques to enrich and purify motoneurons (Arakawa et al. 1990; Bloch-Gallego et al. 1991; Camu & Henderson 1992), muscle extract was then shown conclusively to contain as yet unidentified proteins that maintained motoneurons in culture (Kaal et al. 1997). Subsequently, over 20 different growth factors purified from different sources, including BDNF and CNTF, have now been shown to promote the survival of cultured embryonic motoneurons to various degrees and potencies when added to the medium. The exact composition of muscle extracts is not known but has been shown to contain many of the neurotrophic substances shown in **Table 3.1**.

Table 3.1

A partial list of purified substances shown to promote the survival of motoneurons *in vitro* or *in vivo* (in alphabetical order). Adapted from Oppenheim (1996).

Brain-derived neurotrophic factor (BDNF)	Interleukin-6 (IL-6)
Ciliary neurotrophic factor (CNTF)	Insulin
Cardiotrophin-1 (CT-1)	Insulin-like growth factor-1 (IGF-1)
Fibroblast growth factor-1 (FGF-1)	Insulin-like growth factor-2 (IGF-2)
Fibroblast growth factor-2 (FGF-2)	Leukaemia inhibitory factor (LIF)
Fibroblast growth factor-5 (FGF-5)	Neurotrophin-3 (NT-3)
Fibroblast growth factor-9 (FGF-9)	Neurotrophin-4/5 (NT-4/5)
Glial cell-line derived neurotrophic factor (GDNF)	Platelet-derived growth factor (PDGF)
Glial growth factor (GGF)	Transforming growth factor-β (TGF-β)
Hepatocyte growth factor (HGF)	Vasoactive intestinal peptide (VIP)

The neurotrophic factors listed above fall into families of proteins based on functional or structural similarities. For example; the neurotrophins BDNF, NT-3 and NT-4/5 are structurally related to NGF. CNTF, LIF and IL-6 have cytokine-like properties that are mediated through a common receptor complex. Of particular interest to the present study are the insulin-like growth factors, whose expression during skeletal muscle damage and activity (McKoy et al. 1999; Yang, S. et al. 1996) make them ideally suited to understanding the physiological influence of target muscle on intact motoneurons. Since the present chapter is not concerned with establishing directly any link between IGF-1 over-expression and motoneuron survival, detailed background information on this growth factor may be found later in **Section 4.1** (chapter 4).

3.1.4 Experimental Neuroprotection *in vivo*

It is unclear whether all of the neurotrophic factors listed above have an endogenous role in the survival of motoneurons. Many of them have been tested and characterised *in vivo* on models of injury-induced motoneuron death with the greatest emphasis on those models, which use axotomy to cause significant motoneuron death in neonatal animals. This information with respect to the most widely studied neurotrophic factors is summarised in **Table 3.2**.

Table 3.2

Neurotrophic factors promoting motoneuron survival *in vivo*

Ligand	Receptor	Source	Survival *in vitro*	Protection/Effect *in vivo*
NT-3	TrkB /TrkC	g,m,n	+++	Neonatal axotomy: survival Efficacy in *pmn* mice (Hughes, R.A. et al. 1993; Li, L. et al. 19
NT-4/5	TrkB	g,m,n	++++	Neonatal axotomy: survival Adult axotomy: maintain ChAT phenotyp (Hughes, R.A. et al. 1993; Alderson et al. 1996)
BDNF	TrkB	g,m,n	++++	Neonatal axotomy: survival Efficacy in *pmn* mice Adult axotomy: survival (Hughes, R.A. et al. 1993; Novikov et al. 1995)
CNTF	CNTF α/ LIF-Rβ/ gp130 (complex)	g,m,s	+++	Neonatal axotomy: survival Efficacy in *pmn* and *Wobbler* mice (Sendtner et al. 1990; Andersson et al. 1988; Mitsumoto et al. 1994)
LIF	CNTF α/ LIF-Rβ/ gp130 (complex)	m	+++	Neonatal axotomy: survival (Hughes, R.A. et al. 1993)
IL-6	CNTF α/ LIF-	g	Not known	Neonatal axotomy: survival (Ikeda et al. 1996a)

	Rβ/ gp130 (complex)			
CT-1	CNTF α/ LIF-Rβ/ gp130 (complex)	m	++	Neonatal axotomy: survival Efficacy in *pmn* mice (Pennica et al. 1996; Bordet et al. 1999)
TGF-β	TGF-βR	g,m,s	++	Neonatal axotomy: survival Adult axotomy: survival (Jiang et al. 2000; Iwasaki et al. 1997b)
GDNF	GFRα1, c-ret	m,s	++++	Neonatal axotomy: survival Adult axotomy: survival (Hottinger et al. 2000; Matheson et al. 1997)
FGF-1	FGF-R	n	+(+)	Neonatal axotomy: survival (Cuevas et al. 1995)
FGF-2	FGF-R	n	+	Neonatal axotomy: survival Efficacy in *Wobbler* mice (Ikeda et al. 1996b; Iwasaki et al. 1997a)
IGF-1	IGF-1R	b,g,m,s	++(+)	Neonatal axotomy: survival Adult (normal): terminal sprouting Efficacy in *Wobbler* mice (Hughes, R.A. et al. 1993; Vergani et al. 1998; Hantai et al. 1995)
IGF-2	IGF-1R, mannose-6P receptor	m	++	Neonatal axotomy: survival (Pu et al. 1999b)
PDGF	PDGF-Rα	g,n,s	Not known	Neonatal axotomy: survival (Iwasaki et al. 1997a)

b=blood, g=CNS glial cells, m=muscle, n=nerve and s=Schwann cells. In vitro efficacy ranges from + (very low effect) to ++++ (almost complete survival). Neurotrophic factors that have been tested in adult models of motoneuron survival are highlighted in red.

It is clear that some neurotrophic factors are more potent than others at protecting motoneurons from injury-induced cell death *in vivo*, and that no single neurotrophic factor is capable of affording protection to all motoneurons in a given population. The identification

of many of these factors in muscle extracts as well as studies applying combinations of the above (Arakawa et al. 1990; Kato & Lindsay 1994; Nishi 1994; Vejsada et al. 1998) suggest that a mixture of neurotrophic factors are responsible for regulating the growth and survival of neonatal motoneurons *in vivo*. Nonetheless it is crucial to understand how the availability of any single neurotrophic factor may promote motoneuron survival in order to ascertain its physiological relevance. Methods by which the role of an individual neurotrophic factor may be characterised adopt either one of two approaches: firstly, to expose dying motoneurons to elevated levels of neurotrophic factor; or secondly, to block their endogenous activity. The latter commonly involves the use of specific antibodies raised against neurotrophic factors, which are costly and may not be relied upon fully to neutralise endogenous activity. One way in which to make certain of the absence of function of a particular neurotrophic factor would be to use a gene knockout mouse. It cannot be certain however, whether changes in motoneuron phenotype or response to injury as a result of gene deletion would be due to lack of neurotrophic factor function in muscle targets or to the effect on other cell types including motoneurons themselves. The most common method is to elevate the level of neurotrophic factors available to normal or injured motoneurons. In the present study exogenous supply of the gene (chapter 4) or protein (chapter 5) of a single neurotrophic factor is made available to fatally injured adult motoneurons.

3.1.5 Adult motoneuron survival following peripheral nerve injury

A reliable indicator of motoneuron survival is a measure of the total number of morphologically similar motoneurons in a given population (West 1999). In this study, the promotion of motoneuron survival is investigated across the lifespan of the animal. It is vital to this study, therefore, to use an injury model that causes quantifiable loss of motoneurons in animals of all ages. The majority of data assessing the rescue of dying motoneurons with exogenous neurotrophic factors has been acquired by use of neonatal axotomy models. This is because motoneuron death can be achieved easily and rapidly by nerve transection or crush, resulting in significant cell loss within a week (Soreide 1981b). Few studies exist that have attempted to evaluate motoneuron rescue in adult animals because the same type of

nerve injury in these animals usually results in almost complete recovery, i.e. negligible cell loss and extensive axonal regeneration (Soreide 1981a; 1981b). Although, it has been reported that axotomy in some adult mouse models does result in motoneuron loss (de Bilbao et al. 1999, motoneuron regeneration is highly indicative of the decline of target-dependence in adult animals, a phenomenon that has been reported previously (Pollin et al. 1991). However, alternative sources of neurotrophic support associated only with mature motoneurons, *e.g.* mature Schwann cells in the periphery (Hansson et al. 1986) and more abundant neuroglia centrally (Dreyfus et al. 1999; Major et al. 1997), are likely to be important in this response, supplying axons and cell bodies respectively with neurotrophic factors and guiding regenerating axons to their peripheral target and helping to promote wound regeneration.

Nerve avulsion represents an experimental model of death of adult motoneurons *in vivo*. Its first application experimentally was on the ventral roots of the adult lumbar spinal cord (Koliatsos et al. 1994). The concept underlying the use of nerve avulsion is to remove a greater proportion of trophic influences derived from the periphery compared to nerve trasection, *i.e.* Schwann cells as well as that from the target musculature. Avulsion should result ideally, therefore, in transection as close as possible to motoneuron exit points for spinal motoneurons in ventral roots. Avulsion leads to massive retrograde cell death in adult rats. Using this injury, relatively few studies reporting trophic factor-induced neuroprotection have been done on adult animals (Chai et al. 1999; Kishino et al. 1997; Novikov et al. 1995; 1997). The equivalent injury has also been developed for cranial motoenurons (Mattsson et al. 1999; Ruan et al. 1995; Soreide 1981b) in which the rescue of facial motoneurons with exogenous neurotrophic factors has not, until now, been studied.

So on balance, there is reason to believe that the trophic relationship between muscle and motoneuron may continue into adulthood. It is not clear, however, whether skeletal muscle continues to be a valuable source of neurotrophic support necessary for survival of adult motoneurons, and how preservation of neuromuscular interaction facilitates this influence.

3.1.6 Neurotrophic factors and ageing

Neurotrophic theory is based on the premise that failure of muscle cells to release the appropriate neurotrophic support would result in impaired function of anterior horn cells (Appel 1981). It is not understood how the relative requirements for target-derived neurotrophic factors changes from the adult to the ageing animals. It has been reported previously that neurotrophin receptors in motoneurons are less abundant in ageing compared to adult animals (Johnson, H. et al. 1996). One theory of age-related motoneuron degeneration is based on the decline in availability of target-derived neurotrophic factors. Central to this hypothesis is the principle that decreased neurotrophic support renders motoneurons more vulnerable to injury. It was shown previously that injury-induced loss of facial motoneurons, caused by nerve transection, was greater in ageing Fischer 344 and Wistar rats, compared to that reported for adult rats (Johnson, I.P. & Duberley 1998). Furthermore, the increased mRNA expression of BDNF and its receptor TrkB reported in adult rats (Kobayashi et al. 1996) following sciatic nerve axotomy was observed in less than 50% of aged rats subjected to a similar injury of the facial nerve (Johnson, H. et al. 1999). This study aims to shed further light on this by comparing avulsion-induced motoneuron loss in rats across the postnatal lifespan of Sprague-Dawley rats.

3.1.7 Diet restriction

Dietary restriction (DR) can prolong the life of the entire organism (Weindruch et al. 1986). However, it is not clear to what extent DR affects post-mitotic cells such as neurons. Such information is necessary if current theories of ageing, which are based primarily on studies of mitotic cells, are to be extrapolated in a reasoned manner to the nervous system, and in particular, to mechanisms of age- and disease-related motoneuron degeneration. This part of the present study examines the effect of DR on the survival of facial motoneurons after nerve avulsion.

Since the discovery by C. M. McCay (1935) that dietary or caloric restriction dramatically increased the lifespan of rats, numerous laboratories with a variety of strains of rats and mice have confirmed this initial observation and have shown that reducing calorie intake significantly increases both the mean and maximum survival of rodents (Weindruch et al. 1986; van Remmen et al. 2001). Currently, DR is the only experimental manipulation that has been shown to retard ageing of mammals. Mechanisms by which DR is known to operate remain speculative but much of the emerging data from short-term studies suggest that animals become more resistant to stress and have an enhanced ability to protect cells against insult. Some of these data are reviewed in **Table 3.3**.

Table 3.3

Acute DR studies using an alternate day feeding regimen for a 3-month period.

Animals	*Conclusion*	*Reference*
6 week-old mice	Diet decreases oxidative stress damage in hippocampal slices caused by presenillin-1 death-promoting mutation	(Zhu et al. 1999)
2 month-old mice	Increased BDNF expression and decreased vulnerability to kainite damage	(Duan et al. 2001a)
3 month-old rats	Increased BDNF expression and decreased vulnerability to seizure damage	(Duan et al. 2001b)
1 month-old rats	Ameliorates focal ischaemia	(Yu & Mattson 1999)
3 month-old rats	Cortical synaptosomes protected from oxidative stress by increasing glucose and glutamate uptake	(Guo et al. 2000)
3 month-old rats	Increased survival of new hippocampal cells and NT-3 expression	(Lee et al. 2002)

3 month-old rats	Increased number of new cells and BDNF expression in dentate gyrus	(Lee et al. 2000)

Comparing life-long-treated DR animals with age-matched, *ad libitum*-fed controls has identified putative mechanisms that mediate the effect of DR on longevity.

Table 3.4

Ageing studies of DR-fed rodents

Duration of feeding	*Method of DR*	*Conclusion*	*Reference*
12-17 months (3-6 month-old rats)	60% of *ad libitum*	Decreases age-related increments in oxidative stress	(Dubey et al. 1996)
18 months (3- and 10-month old rats)	40% of *ad libitum*	Increases fibronectin levels in brain	(Dorner et al. 1996)
2-21 months (4 month-old mice)	NIA* mice	Attenuates age-related membrane fluidity. No effect on oxy-radical production	(Gabbita et al. 1997)
24 months (6 week-old rats)	50% of *ad libitum*	Attenuates GFAP expression in aged rats	(Major et al. 1997)
32 months (3 month-old rats)	No information	Decreases number of age-related mtDNA species	(Melov et al. 1995)
21 months	NIA* mice	Decreases glial hyperactivity in aged rats	(Morgan et al. 1999)
22 months (6 month-old rats)	50% of *ad libitum*	Increase in DNA polymerase activity	(Prapurna & Rao 1996)

22 months (6 month-old rats)	60% of *ad libitum*	Increased protein synthetic activity	(Sonntag, Lynch, et al. 1999)
22 months (6 month-old rats)	60% of *ad libitum*	Attenuates age-related decline in muscle levels of IGF-1	(Sonntag et al. 1999)
6-16 months (6 week-old rats)	70% of *ad libitum*	Increased IGF-1 mRNA in muscle compared to age-matched AL	(Tomita et al. 2001)

* National Institute of Ageing: (30-60%) exact level of diet restriction not stated

Information such as that in **Table 3.4** has subsequently aided the formulation of general theories of cellular ageing (Kowald & Kirkwood 1996), which correlate increased cellular survival with longevity of the whole organism. However, a distinction between the effects of ageing on mitotic and post-mitotic cells is often not made, so such theories may not be applicable to all cell types. Neurons, for example, are exposed to potential cell stressors cumulatively during the entire lifespan of the organism, not just during the lifetime of the cell. The factors promoting their survival, therefore, may differ from those of mitotic cells. The level of diet restriction generally used in experimentation ranges from 40% to 60% (Diao et al. 1997; Dubey et al. 1996; Major et al. 1997). The present study uses a restricted diet comprising just 40% of that of *ad libitum*-fed rats so as to maximise the effect of DR in the present model.

Of particular interest to the current thesis are the observations related to protein synthesis and levels of IGF-1 in muscle. IGF-1 is one of the most important myotrophic factors responsible for regulating muscle mass (Adams 1998; Goldspink & Yang 2001). Additionally, IGF-1 has been linked extensively with mechanisms regulating lifespan in rodents (Sonntag et al. 1999). Age-related changes in muscle mass, therefore, may be important in understanding further the effect of age on motoneurons.

3.1.8 Age-related neuronal loss

There are reports of DR reducing the age-related loss of enteric neurons (Cowen et al. 2000) and of spiral ganglion neurons in mice with familial prepubertal deafness (Park et al. 1990). However, it is unclear whether these observations can be generalised to other parts of the ageing nervous system since age-related neural loss varies widely across neural systems (Curcio & Coleman 1982; Flood & Coleman 1988; (Sturrock 1987, 1988c). In the ageing mouse, for example, neuron number remains constant in the trigeminal motor nucleus (Sturrock 1988b), the hypoglossal nucleus (Sturrock 1991a), the occulomotor nucleus and the trochlear nucleus (Sturrock 1991b), yet neurons are lost from the trigeminal mesencephalic nucleus (Sturrock 1987) and facial nucleus (Sturrock 1988a; 1988c). Experimental studies of age-related neural death are also complicated by incomplete data on how neurons of the same system respond to potentially lethal trauma across the lifespan of the animal. Adult rats, unlike their neonatal counterparts, fail to lose a significant number of motoneurons after peripheral nerve crush or transection (Johnson, I.P. et al. 2000; Johnson, I.P. & Duberley 1998). As a result most studies of motoneuron death have been done on neonatal rats where peripheral nerve injury provokes marked and rapid neuronal death (Li, L et al. 1998; Sendtner et al. 2000).

In this study, facial nerve avulsion was used to produce potentially lethal trauma to motoneurons in animals of all ages. Numbers of facial motoneurons were quantified in *ad libitum*-fed rats aged 1 week (neonatal), 6 months (adult) and 24 months (ageing) and were compared to numbers of motoneurons in 24 month-old rats raised from the age of 6 months on a diet restricted regimen. The effects of facial nerve avulsion on motoneuronal survival in these groups are also compared.

3.1.9 Muscle damage

Exposing neural systems to moderate insult has the effect of increasing the resistance to damage of the corresponding neurons to subsequent lethal trauma (Barone et al. 1998; Matsushima & Hakim 1995; Wada et al. 1996). In particular, it has been shown that alterations in neurotrophic factors induced by the initial challenge increases the propensity of neurons to survive secondary insult (Mattson 1997). Drawing upon this evidence and in keeping with the theme of the present thesis, it seems reasonable to predict that such 'pre-conditioning' of motoneurons may be achievable by damaging peripheral targets.

3.1.10 Retrograde reaction and pre-conditioning motoneurons

The influence of the periphery on motoneurons becomes most prominent after axonal lesions that lead to reaction of the motoneuron perikarya and the surrounding glial cells (Lieberman 1971; Fawcett & Keynes 1990). Sub-lethal injuries to motoneurons such as distal axon transection and nerve crush are ineffective at producing significant neuron loss despite causing retrograde cell reactions. This is in contrast to nerve avulsion, which also results in retrograde cell response but produces massive motoneuron loss 1 month after injury. This suggests that some aspects of the retrograde reaction, elicited only under mild injury, may serve to protect motoneurons from cell death (Fernandez et al. 1981). For example, proliferating Schwann cells, in response to distal nerve crush, are a rich source of neurotrophic factors (Bolin et al. 1995). Enzyme changes also in the injured facial nucleus following a conditioning lesion suggest a non-linear amplification of response compared to single-lesioned motoneurons (Tetzlaff & Kreutzberg 1984) indicating a form of memory.

It is possible to initiate an axonal reaction in the absence of lesion made directly to the nerve (Mariotti et al. 2001). This may be achieved by eliciting an inflammatory response in muscle target. Experimentally, this is preferable since the accessibility of target muscles to manipulation is much greater than the motor axons themselves. In addition, such information may be useful in understanding further the relationship between motoneurons and target muscles.

3.1.11 Responses of skeletal muscle to injury

Skeletal muscle is a highly plastic tissue. It is able to retain and adapt its structure, function and metabolism during normal growth and following a variety of mechanical stimuli and trauma to which it is subjected. Regeneration of skeletal muscle after different levels of damage is testimony to their great potential for repair. Following direct mechanical (Carlson 1986) or chemical (Foster & Carlson 1980; Couteaux et al. 1988) trauma, after severe exercise (Irintchev & Wernig 1987) or during the course of myopathic or neuromuscular disease, skeletal muscle fibres degenerate partially or completely and then regenerate in a stereotyped manner which restores some of their structure and function (Carlson & Faulkner 1983). Muscle growth/regeneration is associated with the production of numerous neurotrophic factors and cytokines, including IGF-1 (Marsh et al. 1997; McKoy et al. 1999; Yang et al. 1996), LIF (Kurek et al. 1996b), CNTF (Helgren et al. 1994). These may be synthesised by damaged muscle fibres and possibly other cell types.

It is clear; therefore, that damage of skeletal muscle may perturb the trophic conditions to which motoneurons are subjected in physiological conditions. This present study examines whether prior muscle damage, which is known to stimulate synthesis of neurotrophic factors such as IGF-1, inflicted to the snout muscle in adult rats, can protect facial motoneurons against avulsion-induced cell death.

3.1.12 Damage of the snout muscle

Although the snout muscle is comprised of striated muscle fibres, it is irregular in morphology compared to skeletal muscle of the limbs in that it lacks the larger and clearer foci of insertion and origin characteristic of limb muscles. With no obvious point of leverage, it is difficult to manipulate mechanically via established methods of elongation (Goldspink et al. 1992; Goldspink 1999) and forced activity with minimal invasiveness to tissue. Injecting large volumes of liquid, however, can cause significant mechanical damage

to muscle (Jarvinen & Sorvari 1975; Oshida et al. 1979). Alternatively, the osmotic potential of injectable liquids is crucial to prevent cellular lysis. Water serves as a perfect tool to create the steepest possible osmotic gradient. Water permeability of lipid membranes has been described extensively (Fettiplace & Haydon 1980) and may serve as a useful tool to elicit damage of the snout muscle. In the absence of osmotic potential, physiological saline may be expected still to cause mechanical damage. Bupivacaine is a local anaesthetic that produces an immediate and massive myonecrosis followed by phagocytosis of necrotic debris and a rapid and apparently complete regeneration of muscle fibres 3-4 weeks after injection (Hall-Craggs 1974). Regeneration is known to involve increased levels of IGF-1 (Marsh et al. 1997). Thus, three strategies have been outlined above and will be used in the present study to cause muscle degeneration with the expectation of causing subsequent alteration in the trophic environment of the periphery.

3.1.13 Specific aims

To determine the effect of age and DR on the number of facial motoneurons

To establish the effect of facial nerve avulsion on motoneurons across the lifespan of rats

To determine the effect of DR and prior muscle damage on motoneuron survival 1 month following nerve avulsion

3.2 Results

The materials and methods for these experiments can be found in chapter 2.

3.2.1 Retrograde tracing of facial motoneurons

To confirm the location of the facial nucleus within the brainstem and to ensure the connectivity of motoneurons within this structure to muscle located in the snout, adult rat motoneurons were labeled with Fluoro-Gold retrograde tracer. Retrograde tracer was applied to the transected proximal stump of the right facial nerve and injected into the left snout muscle. After 24 hours, Vibratome sections of perfusion-fixed brainstem were cut then stained with YOYO-1 and viewed either under UV or epifluorescence. The number and distribution of retrogradely labeled motoneurons was similar after intramuscular Fluoro-Gold or when it was applied to the proximal stump of the facial nerve. Double labeling of motoneurons with YOYO-1 and Fluoro-Gold within the facial nucleus confirmed the presence of facial motoneurons and the absence of any other neuronal phenotype, which might interfere with neuronal counts (**Fig. 3-1**).

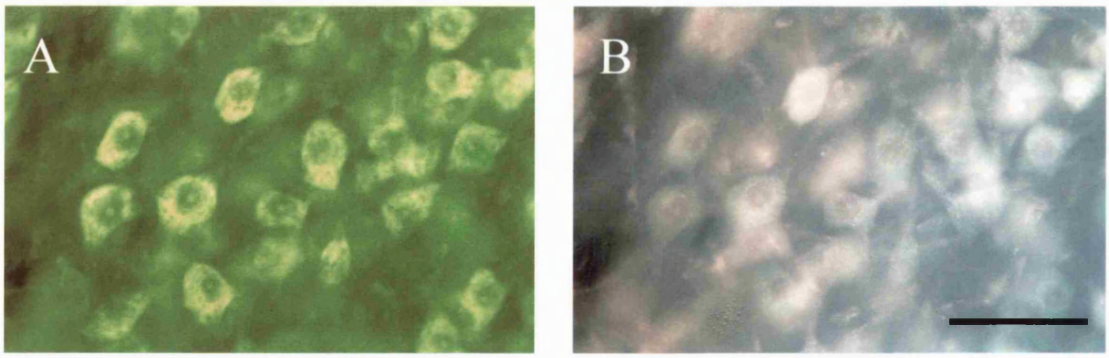


Figure 3-1 Identical fields of the facial nucleus showing the same motoneurons labeled by (A) YOYO-1, and (B) Fluoro-Gold retrograde tracing. Scale bar = 100μm

3.2.2 Motoneuron number in rats of different ages and the effect of dietary restriction

In normal *ad libitum*-fed animals there are similar numbers of motoneurons per facial nucleus across the age span, albeit with the right nucleus consistently having slightly more on average than the left nucleus. Whilst there is a tendency of loss of motoneurons by 24 months in *ad libitum*-fed animals, when the average number of motoneurons for both sides is calculated and compared across age groups, the number of motoneurons per facial nucleus in ageing *ad libitum*-fed animals (2531 motoneurons) is not significantly different from those of neonatal (3322 motoneurons) and adult animals (3099 motoneurons). In contrast, 24 month-old DR rats had only 1400-1600 motoneurons in each nucleus (**Table 3.5**). This represented a loss of approximately 50% of motoneurons (P=0.05) from the onset of DR at 6 months of age (**Fig. 3-2**). For an example of YOYO-stained sections, see **Fig. 4-4** (ch 4).

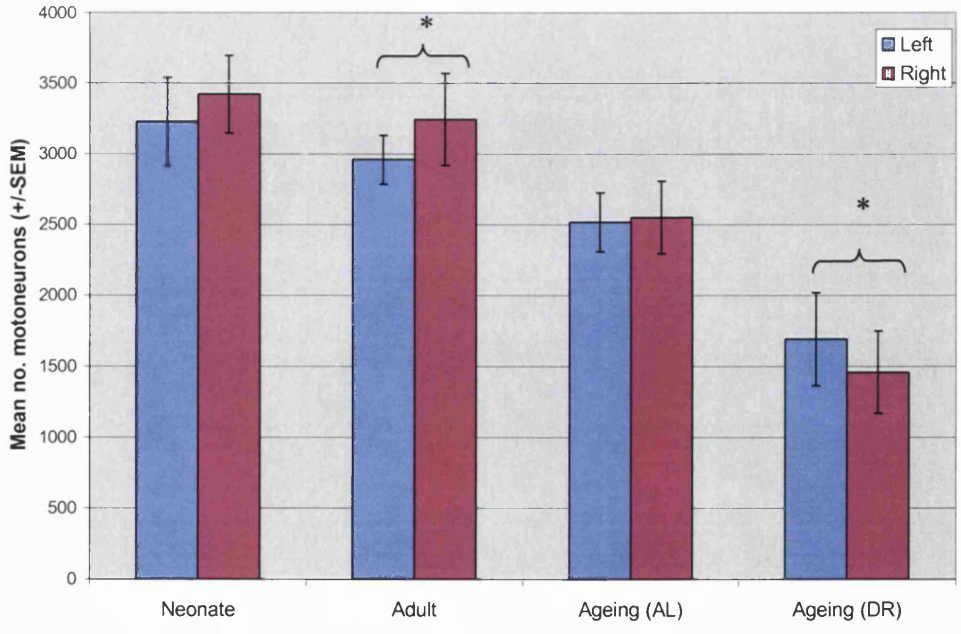


Figure 3-2 Effect of age and diet restriction on the total number of motoneurons in the facial nucleus. (AL=*ad libitum*-fed; DR=diet restricted). Means are given plus and minus the standard error of the mean. * indicates a significant decrease in the number of motoneurons of ageing (DR) compared to adult rats.

General observations of 24 month-old rats showed that those maintained on a DR regimen were approximately 2/3 of the weight of those fed ad libitum (approximately 400-450g versus 650-700g respectively). Additionally, DR rats were more active in response to movement of the cage and maintained better standards of grooming.

3.2.3 Effect of facial nerve avulsion

Estimates of the mean total number of motoneurons per facial nucleus after nerve avulsion at different ages are given in **Table 3.5**.

Table 3.5

Mean total of motoneurons (± SEM) in each facial nucleus of all age groups and feeding regimens

	Total of numbers of facial motoneurons (mean ± S.E.M.)			
	Normal (uninjured)		Injured	
Experimental Group	Left	Right	Contralateral (left)	Ipsilateral (right)
1 week (neonate)	3225 ± 313 (n=8)	3419 ± 276	3584 ± 538 (n=8)	554 ± 76 *
6 month (adult)	2957 ± 172 (n=6)	3242 ± 325	3278 ± 150 (n= 6)	806 ± 80 *
24 month/Ad Lib (aging)	2514 ± 291 †‡ (n=6)	2548 ± 325	2359 ± 633 (n=3)	1387 ± 371 *
24 month/DR (aging)	1689 ± 358 † (n =6)	1457 ± 317	1775 ± 154 (n=6)	1775 ± 189

* indicates that values are significantly decreased (P = 0.05) on the ipsilateral side compared to contralateral side after injury. † indicates that the total number of motoneurons is significantly less (P = 0.05) compared to the corresponding facial nucleus of uninjured 1 week- and 6 month-old rats. ‡ indicates that when left and right nuclei are combined, no significant difference is found between 6m and 24m/*ad libitum*- fed animals whereas, a significant loss is found for 24m/*ad libitum* versus neonate rats. (All between-group analyses were assessed by Kruskal-Wallis and Mann-Whitney U-test.)

After nerve avulsion a significant number of motoneurons were lost from the ipsilateral facial nucleus in animals of all ages. 1 month after injury, this loss is equal to 74% and 45% in adult (n=6) and ageing (n=3) animals respectively, compared to the combined mean total number motoneurons for both facial nuclei in normal animals (3099 and 2531 motoneurons respectively) (**Fig. 3-3**). In neonatal animals, 16% of motoneurons had been lost from the ipsilateral facial nucleus 1 day after injury (n=7), rising to 45% by 3 days (n=8), and 86% by 7 days (n=8), compared to normal animals. This loss with time is shown in **Fig. 4-6** and **Table 4.3** (chapter-4). Preliminary stereological analysis of adult rats 7 days after avulsion showed no significant loss of motoneurons from the ipsilateral facial nucleus (data not shown). No stereological data for motoneuron number was available for ageing rats 1 week after avulsion. The number of motoneurons per facial nucleus in normal animals at all ages is similar to those of the nucleus contralateral to nerve injury.

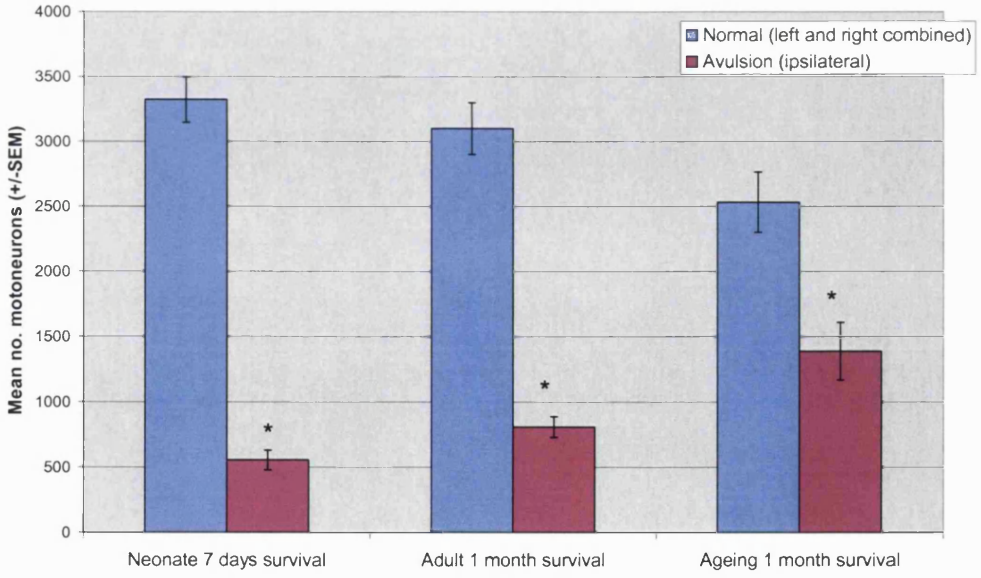


Figure 3-3 Effect of age on the survival of facial motoneurons 1 month after nerve avulsion. Means are given plus and minus the standard error of the mean. * indicates mean value for the total number of motoneurons ipsilaterally is statistically different to the combined mean in both left and right nuclei of normal rats.

In contrast, 1 month following avulsion of 24 month-old DR rats (**Table 3.5**), no significant loss of motoneurons was quantified (**Fig. 3-4**). A notable further observation was the

increased variability of estimates of individual rats in ageing *ad libitum*-fed animals 1 month after injury, compared to other groups.

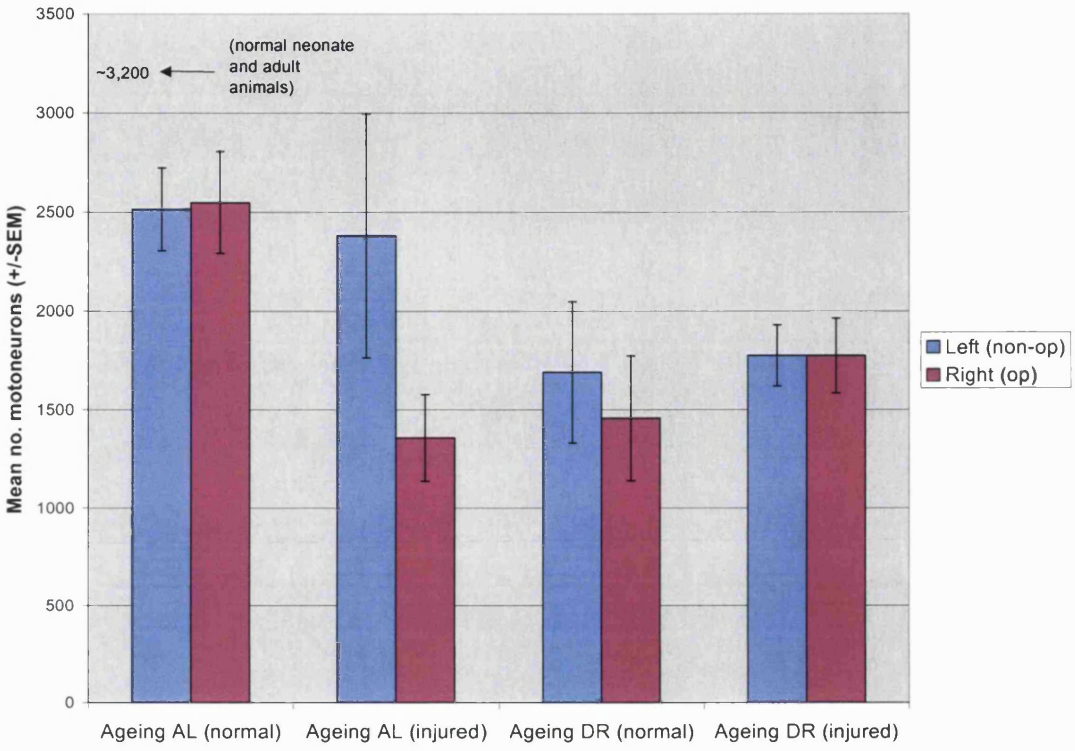


Figure 3-4 Effect of diet restriction from the age of 6 months on the survival of avulsed facial motoneurons in ageing animals, 1 month after injury. Means are given plus and minus the standard error of the mean. There is considerably greater standard error with respect to ageing AL rats (injured) compared to the corresponding DR group.

To verify whether ageing and diet restriction had resulted in tissue shrinkage, total volumes of each facial nucleus (Vref) in both *ad libitum*-fed and diet restricted animals were compared to values for adult rats. Vref for all adult and aging groups was similar (mean 1.13-1.31 $x10^9$ μm^3), with no significant difference between the right and left facial nuclei. For neonatal rats, avulsion-induced motoneuronal loss was associated with a reduction of the volume of the facial nucleus by 30% ipsilaterally (mean 4.65 $x10^8$ μm^3) compared to the contralateral nucleus (mean 6.58 $x10^8$ μm^3).

3.2.4 Effect of muscle damage

Rats were injected into their right snout muscle with either physiological saline (mechanical damage), the myotoxin bupivacaine (mechanical and toxic damage) or distilled water (mechanical and osmotic damage). 7 days later the right facial nerve was avulsed and animals were allowed to survive for a period of 1 month. After tissue processing, sections through the facial nucleus were examined for stereological or immunocytochemical analysis, for estimating the total number of motoneurons in each facial nucleus, or assessing correlates of retrograde response to injury (see chapter 5) respectively. Findings were compared to normal (untreated and uninjured) animals and rats subjected to avulsion only.

3.2.4.1 Motoneuron numbers after prior muscle damage

Values of the mean total number of motoneurons per facial nucleus in each group are given in **Table 3.6** and expressed graphically in **Fig. 3-5**.

Table 3.6

Effect of prior muscle damage on the survival of avulsed motoneurons 1 month after injury. Mean totals of motoneurons per facial nucleus (± SEM). All animals were adult (~300-350g)

	Experimental group				
	Normal	*Avulsion only*	*Avulsion + saline*	*Avulsion + bupivacaine*	*Avulsion + water*
Left (Contalateral)	2957 ± 172° (n=6)	3278 ± 61° (n=6)	3253 ± 135 (n=6)	3210 ± 106 (n=6)	3082 ± 111 (n=6)
Right (Ipsilateral)	3242 ± 325°	806 ± 32*°	1380 ± 318*	1595 ± 512*	2114 ± 214*#

* indicates that values are significantly decreased (P=0.05) on the ipsilateral side compared to the combined (left and right) mean of normal animals. # indicates that the number of motoneurons ipsilaterally in water-injected animals is significantly increased compared to that of animals subjected to avulsion alone. ° indicates that values are taken from **Table 3.5**. (All between-group analyses were assessed by Kruskal-Wallis and Mann-Whitney U-test.)

In all treatment groups, significant loss of motoneurons (P=0.05) from the ipsilateral facial nucleus was observed compared to the combined mean totals of both nuclei in normal animals. Loss of motoneurons was reduced from 74% in animals subjected to avulsion alone, to either 55% when animals were injected with physiological saline, or 49% with bupivacaine 1 month after injury. However, animals that were injected with distilled water showed a significant increase (P=0.05) in survival of motoneurons ipsilaterally. In these animals, avulsion-induced motoneuron loss was reduced to 31%, which is equal to 51% neuroprotection.

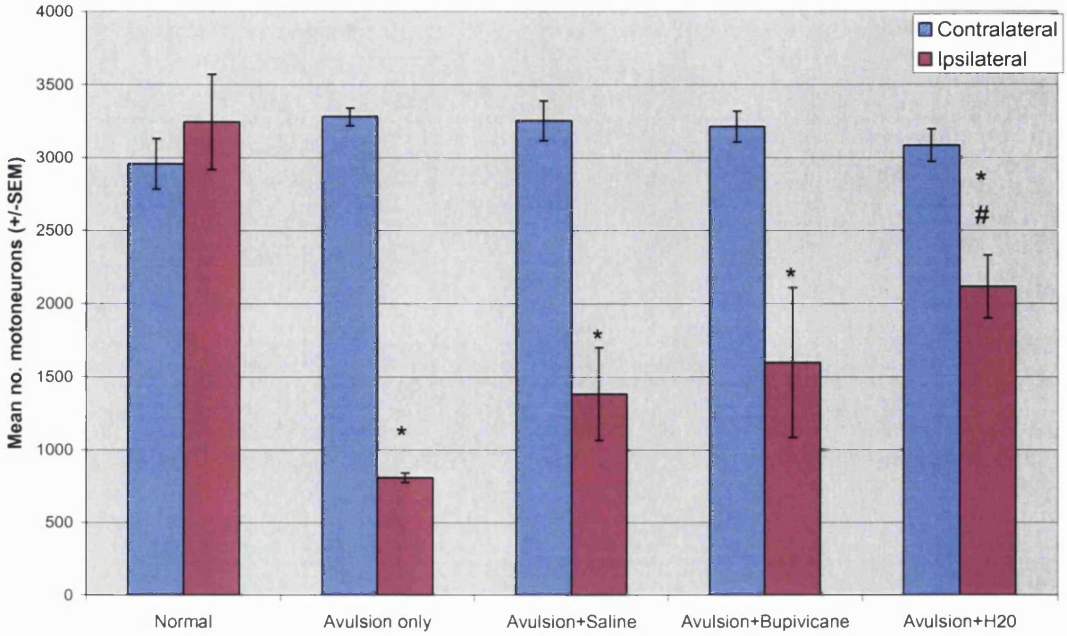


Figure 3-5 Graphical representation of the effect of prior muscle damage on the survival of avulsed facial motoneurons 1 month after injury. Means are given plus and minus the standard error of the mean. For * and #, see **Table 3.6**.

3.3 Discussion

Experiments of this chapter have revealed three key findings. Firstly, diet restriction of adult rats from the age of 6 months results in increased age-related motoneuron loss by 24 months compared to age-matched, *ad libitum*-fed rats. Secondly, DR prevented any further loss of motoneurons in response to nerve avulsion. Thirdly, injection of distilled water conferred significant protection to facial motoneurons 7 days prior to nerve avulsion. Injection of identical volumes of bupivacaine and physiological saline also were effective at rescuing a proportion of motoneurons. Before highlighting their significance, the following section attempts to discuss in detail methodological errors that could distort the data and which relate to all stereological investigation throughout the thesis.

3.3.1 Discussion of methodology

One likely source of error in the present data, and in other studies that rely heavily on stereological data, is the method adopted for counting motoneurons. The method used in the present study is in contrast to the majority of other studies that use an assumption-based approach when estimating the total number of neurons in the motor pool (Hottinger et al. 2000; Li, L. et al. 1995; Sakamoto et al. 2001; Watabe et al. 2000), notwithstanding the use in most cases of neonatal nerve axotomy models (Ikeda et al. 1996a; Li, L. et al. 1994; Matheson et al. 1997; Sendtner et al. 1990, 1992a; Schmalbruch & Rosenthal 1995; Vejsada et al. 1995; 1998; Yan, Matheson, et al. 1995). Such methods are inherently prone to over-counting and rely heavily on accurate measurement of the caliper diameter of the objects counted normal to the plane of sectioning in order to calculate appropriate levels of correction such as that used by Abercrombie (Abercrombie 1943). Corrections are made on the assumption that the objects being counted do not change in size or shape. The typically multi-polar profiles of neurons, however, are likely to give highly variable and therefore inaccurate measurements of such parameter. Furthermore, motoneurons responding to nerve lesion are prone to change in both size and shape compared to uninjured motoneurons (Li, L. Wu, et al. 1995; Novikov et al. 1995). Such variation is likely to affect motoneuron cell

counts, usually by over counting. The term "unbiased" designated to the present method may be contentious since observer bias, which cannot be corrected after it has occurred, is not included in this consideration. With respect to the present method, the typically multi-polar morphology of motoneurons causes difficulty in discriminating between dendritic profiles and glial cells. Dendrite-like cross-sectional profiles are distinguished by their lack of nuclei. Their appearance on the lower confocal scan beneath or in close proximity to motoneuron profiles on the upper scan subsequent to superimposition, is usually interpreted as belonging to the neuron with which it is associated, thus resulting in the elimination of that neuron from the optical disector. Glial cells however, also become associated with neurons, especially following nerve lesion (Kreutzberg 1995). Although glia generally have a high nucleus-to-cytoplasm ratio, and thus easily identifiable, occasional profiles were observed which showed close resemblance to dendrite-like structures yet may have been neuroglial cells cut in an optical plane that did not include the nucleus. Such structures were often a source of contention amongst independent observers in the current study and were always interpreted as dendrites. No retrogradely labeled motoneurons were observed with cell body diameters in the size range of neuroglial cells (<20μm) so it is unlikely that neuroglia would have been included in the counts.

With regard to sampling there are three important issues that require addressing: (1) the size of the sample volume should be of reasonable magnitude; (2) samples should be distributed randomly; (3) neurons need to be distributed homogeneously so as to have equal chance of being counted. Adequate measures were taken to satisfy each of these criteria. Whereas smaller samples of area would have allowed more time to conduct stereology in even more experimental groups, controls groups and larger group sizes, there is the risk of introducing error due to capture of neurons within an optical disector with non-uniform distribution. This may increase or lower neuron estimates for the whole facial nucleus artificially. On the other hand, it is possible that smaller sample areas may have given comparable results but this was not investigated here.

Based on a rank scoring system, the Mann Whitney U-test avoided assumptions that the population data was normally distributed when assessing the significance of a given

treatment compared to a control group. The use of this test is consistent with other studies of neurotrophic factor-mediated neuroprotection (Watabe et al. 2000). The majority of other studies have employed Student's T-test, which is dependent on mean and standard deviations in each of the experimental groups. They assume, therefore, that irrespective of treatment or injury, individual animals within each experimental group are normally distributed around the true mean with respect to numbers of neurons within the motor pool. This is a notion that is difficult to prove but it is appreciated that analysis of significance incorporating related numerical values serves as a more powerful statistical tool. It is potentially erroneous, therefore, to assume that a given treatment, which is hypothesised to promote motoneuron survival, does not target a particular population of motoneurons within the facial motor pool. Individual rats within a group may have subsets of motoneurons of variable proportion projecting to each muscle group. Individuals with a proportionally larger subset of neurons innervating the snout muscle may be more likely to receive survival-promoting signals from the periphery. As a result, animals of this phenotype would show a greater level of resistance to avulsion-induced motoneuron death because the motoneurons of the snout muscle would make up a greater proportion of the total population of the facial nucleus relative to other animals.

The technique employed in the current study to produce facial nerve avulsion gave similar levels of motoneuron loss to other studies. Previous studies resulted in 90% (Mattsson et al. 1999) and 80% loss (Ruan et al. 1995) of neuronal profiles ipsilaterally compared to the contralateral side 4-weeks after injury. Therefore, an acceptable level of sensitivity was achieved. In these previous studies, facial nerve avulsion was achieved either by transecting the nerve proximally in the facial canal after partial and temporal craniotomy, or at the site immediate to the nerve's exit from the stylomastoid foramen. It is unlikely that traction of the facial nerve would result in physical removal of cell bodies from the facial nucleus since the axons project into the facial canal from the facial nucleus towards the posterior aspect of the brainstem before looping back on themselves to exit the brainstem finally from the anterior-lateral aspect. It is possible, however, that such traction may cause tearing of nerve fibres, giving irregular sites of axotomy for individual or bundles of axons. Nonetheless, removal of a segment of nerve up to 3mm in length from within the cranium, in addition to a

further 3mm segment externally from the distal stump, would ensure that the present technique would result in axotomy further proximal than that used by Ruan et al. (1995). The observation of further motoneuron loss ipsilaterally at 3 months (chapter-4) compared to 1 month after injury would oppose the argument that some regenerative capacity is retained after injury due to the presence of Schwann cells remaining associated with lesioned axons.

3.3.2 Motoneuron number and the effect of diet

In the present study, diet restriction of rats was associated with the loss of facial motoneurons by 24 months of age, whereas *ad libitum*-fed rats aged 24 months showed no significant loss. In addition, those motoneurons remaining at 24 months of age in DR rats were resistant to nerve avulsion-induced death, while this type of nerve injury provokes motoneuronal loss in *ad libitum*-fed rats aged 7 days, 6 months and 24 months. These results are surprising, as diet restriction increases longevity in rodents and reduces the age-related loss of other subpopulations of neurons (Cowen et al. 2000; Park et al. 1990). These findings indicate that age and diet affect facial motoneurons differently to other cell types and call into question the view that DR is universally beneficial.

3.3.2.1 Age-related motoneuronal loss and the effect of diet restriction

There was no significant age-related loss of motoneurons in *ad libitum*-fed rats. Although post-mitotic cell loss is predicted by many theories of ageing where cumulative cellular damage is assumed (Harman 1965; Miquel 1992; Wallace 1992), it is also clear that age-related neuronal loss varies markedly across the nervous system. Thus, approximately 50% of rat enteric neurons are lost during ageing (Cowen et al. 2000) and a slight (~18%) loss found with extreme old age in the mouse motor trigeminal (Sturrock 1987; 1988b) and facial nuclei (Sturrock 1988c). In contrast, no loss of neurons is found in the motor nuclei of the hypoglossal (Sturrock 1991a), trochlear and oculomotor (Sturrock 1991b) nuclei of ageing

mice. The mechanisms underlying the differential vulnerability of these neural systems to ageing are unclear. Limited evidence indicates that muscular activity in old age may be a important factor in regulating age-related motoneuron loss (Kanda & Hashizume 1998; Samorajski & Rolsten 1975) or maintaining neuronal phenotype centrally (Churchill et al. 2002). It has been reported in neonatal rats that muscle activity is linked directly with post-natal maturation of motoneurons (Pastor et al. 2003; Navarrette & Vrbova 1993). Muscle groups, which remain relatively active throughout the lifespan, may be expected to accommodate a well-maintained innervating neural system. Vibrissae movement may be expected to persist into extreme old age relative to activity of limb muscles because despite becoming less mobile, whisker and general movement of muscles in the facial region are likely to remain important for feeding. This is consistent with loss of facial motoneurons only in extreme old age in the mouse (Sturrock 1988a; 1988b), correlating perhaps, with the point at which such animals are no longer able to feed themselves.

In contrast to the survival of facial motoneurons in ageing *ad libitum*-fed rats, DR was associated with the loss of approximately 50% of motoneurons. This contrasts with the protection afforded by DR for ageing neurons in the enteric and auditory systems (Cowen et al. 2000; Park et al. 1990). Notwithstanding differences in the counting methods used in these studies, these results may simply reflect intrinsic differences in the response of different neural systems to DR during ageing. It is also possible that different levels of DR may be responsible. The present study employed a 60% reduction in diet from *ad libitum*-fed rats. In another study, utilising the same level of DR, anti-ageing effects were seen in dopaminergic neurons (Diao et al. 1997), whereas others have used a 40% reduction (Cowen et al. 2000; Sonntag et al. 1999), also with protective effect. Muscle atrophy is often associated with ageing (Ansved & Larsson 1990; Borst & Lowenthal 1997; Carlson 1997) and studies of the developing neuromuscular system have demonstrated that perturbation of neuromuscular contact leads to motoneuronal death (Houenou et al. 1994; Lowrie & Vrbova 1992).

It is possible therefore, that additional atrophy of the peripheral targets of motoneurons due to severe DR in this study may have reduced the target-derived neurotrophic support of

motoneurons over a prolonged period of time leading to motoneuronal death. There is no data from the current study revealing the effects of this DR regime on muscle, although moderate (40%) caloric restriction is associated with the maintenance of high levels of protein synthesis in muscle (Sonntag et al. 1992), preservation of its anabolic response to IGF-1 (Sonntag et al. 1999), an important regulator of muscle mass, and increased IGF-1 mRNA expression in muscle (Tomita et al. 2001), to compensate, perhaps, for the reduction of IGF-1 levels in plasma (Breese et al. 1991). On account of this latter observation of systemic IGF-1 levels, it is interesting that the protective effects of physical exercise against brain insults of different aetiology is mediated, in part, by circulating IGF-1. It is conceivable, therefore, that reduced plasma IGF-1 may be important for regulating long-term motoneuron survival (Carro et al. 2000; Carro et al. 2001). This is in argument however, with the idea that target-derived neurotrophic support is the key determining factor of motoneuron survival. Although neuronal loss has not yet been associated with moderate caloric reduction, a recent report that approximately 30% of cortical synapses are lost by 29 months compared to *ad libitum*-fed rats indicates that some neuropathology nevertheless results (Shi et al. 2002).

It is possible that cell atrophy due to severe diet restriction may also extend to motoneurons in a manner similar to that seen following nerve avulsion (Li, L. et al. 1995; Novikov et al. 1995). If this were true, atrophic motoneurons may no longer have met the criteria (mulitipolar morphology and cell body diameters greater than 20μm) for inclusion in the cell counts. This is unlikely, as multipolar cells less than 20μm in diameter were not observed in any of the groups. It is also unlikely that motoneurons in DR rats had shrunk and also lost their multipolar morphology, as many ageing motoneurons contained lipofuscin-like pigment that is characteristic of senescent neurons (Brody 1980; Goyal 1982), yet cells less than 20μm in diameter and containing this pigment, were observed rarely. The use of motoneuron-specific markers and retrograde labeling in combination with unbiased stereological counting methods would help resolve these issues.

3.3.3 Avulsion-induced motoneuron ioss and diet restriction

Facial nerve avulsion resulted in motoneuron loss in *ad libitum*-fed rats operated at 1 week-, 6 months- and 24 months-of-age. This appears to be the first description of a peripheral nerve injury model, which provokes motoneuron death across the lifespan of rats. To date, most hypotheses on the mechanisms underlying age-related motoneuronal death have been derived principally from the study of the developing neuromuscular system (Appel 1981; Beck, M. et al. 2001; Johnson, I.P. 1996; McComas et al. 1988). The present model will allow some of these hypotheses to be tested in the ageing animal.

Diet restriction of ageing rats in this study prevented loss of facial motoneurons 1 month after nerve avulsion. It is possible that avulsion-induced neuronal atrophy (Kishino et al. 1997; Li, L. et al. 1998) may have contributed to the reduced total number of motoneurons in the ipsilateral facial nucleus in adult rats. This is unlikely, as motoneuron-like multipolar neurons below the size threshold for counting were not observed in the facial nuclei of any of the groups. This surprising result suggests that during the process of motoneuron loss over the course of 18 months DR has served to select those with the capacity to resist degeneration induced by mechanical injury. Whether this is due to some constitutive change in the remaining motoneurons, or whether DR results in a different response to injury, perhaps by a mechanism similar to a 'conditioning lesion' (Prolla & Mattson 2001; Reich et al. 1990; Yu & Mattson 1999), is unknown. It is noteworthy that DR has been shown previously to induce heat-shock protein 70 (HSP70) in spinal sensory neurons (Houenou et al. 1996) and striatal cells *in vivo* (Yu & Mattson 1999). Furthermore, temporal and spatial expression of its gene suggests strongly that it is important for ischaemic pre-conditioning in the rat brain (Truettner et al. 2002). HSP70 is known as a 'stress protein', which forms part of a non-specific, intrinsic response to insult for protecting cells in the brain (Lowenstein et al. 1991; Sharp et al. 1999). If it were accepted, as discussed earlier, that diet restriction from young adulthood results in muscle atrophy, a situation would arise in theory, whereby motoneuron loss would be limited to those with greatest target-dependence. Remaining motoneurons would be selected for survival by their lower target-dependence and their ability to withstand the metabolic stress associated with dietary restriction (Yu & Mattson

1999). Ultimately, a shift in trophic dependence from the periphery to that of the CNS and intrinsic protective mechanisms may dictate the propensity of motoneurons to resist the effect of nerve avulsion. It is intriguing, therefore, that there have been previous reports of DR causing an increase in BDNF expression and activity in the brain (Lee et al. 2000) and that its increased production is necessary for DR's neuroprotective effect in a brain seizure model (Duan et al. 2001b). Another selective influence on motoneuron survival is steric hindrance (**Fig. 3-7**). In a facial nucleus crowded with motoneurons and dendritic processes, some motoneurons will potentially become less well endowed with glial-derived trophic support than others, pre-determining, therefore, levels of target-dependence of individual motoneurons under physiological conditions.

Interpreting the effect of DR further, it is tempting to speculate that those motoneurons lost due to diet restriction are from the same subpopulation that is lost after nerve avulsion in *ad libitum*-fed rats. Such a concept suggests that motoneurons deficient of target-derived neurotrophic support are more vulnerable to metabolic or oxidative stress, linked putatively, with feeding *ad libitum* (Mattson et al. 2002). Such a relationship has been implied through numerous studies that reveal the various abilities of neurotrophic factors to protect motoneurons from degeneration, invoked by the over-expression of the mutated human gene encoding Cu/Zn superoxide dismutase-1 (SOD-1) in mice (Bordet et al. 2001; Manabe et al. 2002). Normal (wild-type) SOD-1 is an anti-oxidant enzyme, which is also induced as part of the protective response of the brain and may also be considered as a 'stress protein' (Maier & Chan 2002). The exact mechanisms linking neurotrophic factor signaling and oxidative stress in motoneurons remains uncertain. It would be of general interest however, regarding selective vulnerability of motoneurons to age- and disease-related degeneration, to investigate whether DR can prevent or delay onset of motoneuron loss associated with this mutation. Since DR can increase the lifespan of Sprague Dawley rats by up to 42% (Merry & Holehan 1979), it would be interesting also to determine if the protection against avulsion-induced motoneuronal death that was observed at 24 months in the present study is permanent (Jacob & McQuarrie 1993).

The illustration in **Fig. 3-7** describes a possible mechanism whereby DR generates an environment of decreased trophic support from the periphery followed by loss of target-dependent motoneurons possibly facilitated by the differential availability of central support. As a result there is increased age-related motoneuron loss.

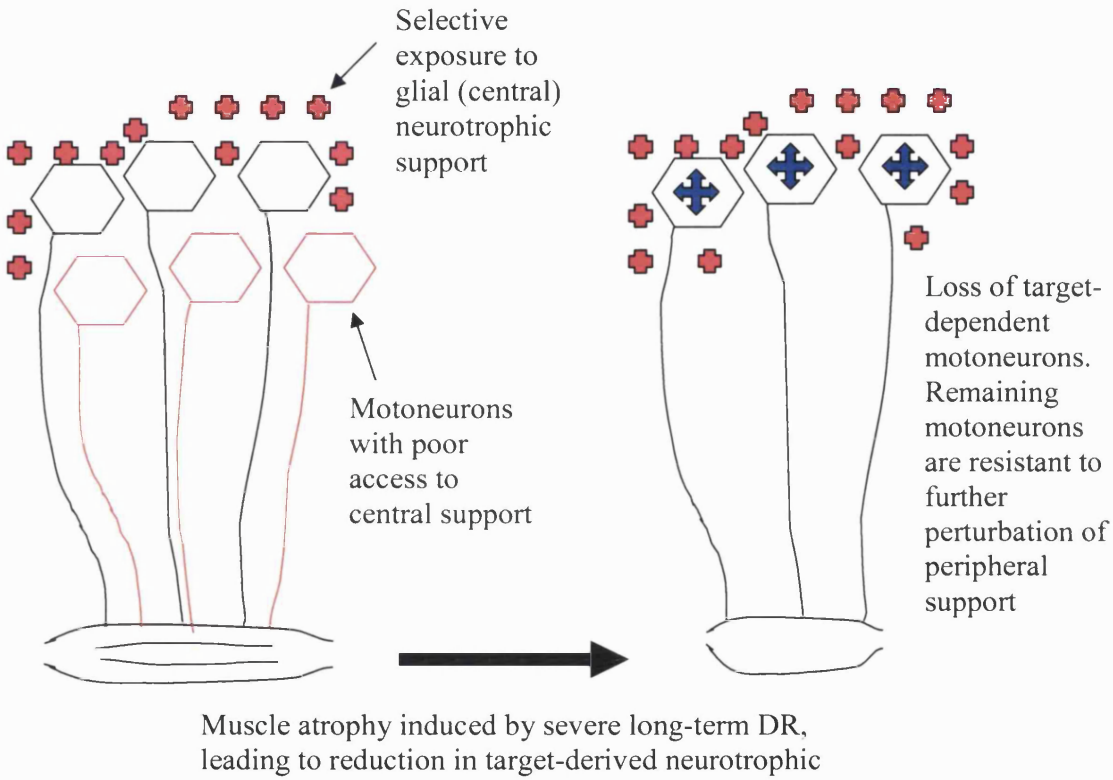


Table 3.7 Schematic representation of a possible mechanism of switch from peripheral to central neurotrophic support resulting in accelerated age-related motoneuron loss and resistance of remaining motoneurons to avulsion injury. Outward-directing blue arrows within surviving motoneurons represent intrinsic mechanisms of cellular protection induced by the effects of DR.

Because it can extend the lifespan of animals, DR is generally regarded as beneficial to all cells, providing a means of reducing age-related cellular damage through decreased oxidative stress (van Remmen et al. 2001; van Remmen & Richardson 2001); decreased susceptibility to chemical toxicity (Hart et al. 1992); increased protein synthetic activity

(Sonntag et al. 1999) or increased DNA repair (Prapurna & Rao 1996). An alternative view which, is forced by the present results, is that DR is not beneficial to all cell types. Instead, DR may act as an additional perturbation of some cells, such as neurons, that can combine with the general cell stress of ageing to produce cell death. General age-related neuronal stressors may include the loss of neuromuscular synapses (Frey et al. 2000), reduced nerve-sprouting activity (Pestronk et al. 1980), reduced neurotrophic factor availability (Appel 1981; Cowen 2002) and reduced responsiveness to neurotrophic factors (Horton et al. 1998). In the context of the present study, it is tempting to speculate that ageing and DR have combined to produce loss of vulnerable motoneurons, leaving the remaining motoneurons resistant to further trauma. Caution is needed therefore, when general theories of cellular ageing are extrapolated to particular cell types and cell populations.

Given that the current thesis aims to emphasise the role in motoneuron survival of IGF-1 isoforms, it is tempting to speculate further about the mechanisms underlying increased age-related motoneuron loss paying particular attention to this target-derived neurotrophic factor. If it is accepted that muscle atrophy is a direct consequence of severe DR then there are grounds for speculating that prolonged deprivation of target-derived IGF-1 isoforms, which are perhaps one of the most important proteins with respect to maintenance and generation of muscle mass (Goldspink 1999; Loughna et al. 1992), may be a key factor in accelerating loss of motoneurons with age. If the converse of this is true, then it follows that subjecting animals to prolonged muscle activity and/or overload, or causing a response in skeletal muscle so as to mimick muscle growth such as direct muscle damage, motoneurons would become exposed to increased IGFs as well as other neurotrophic factors (Marsh et al. 1997; Sakuma et al. 2001). This was tested in the present study by injecting the snout muscle with potentially damaging substances.

The present results have shown various degrees of protection afforded to facial motoneurons in adult rats, from avulsion-induced neuronal death, by damaging the target muscle with different modes of insult prior to nerve injury. Injection of distilled water (mechanical and osmotic damage) into the snout muscle 7 days prior to nerve avulsion conferred significant protection to facial motoneurons 1 month after injury. Injection of identical volumes of

bupivacaine (mechanical and toxic damage) and physiological saline (mechanical damage) also rescued a proportion of motoneurons but neither was significant. It is possible that increased availability of IGF-1 isoforms in muscle fibres induced by muscle damage (McKoy et al. 1999; Yang, S. et al. 1996), in turn, leads to retrograde transport of gene product or increased retrograde signal transduction from the periphery resulting in increased motoneuron survival. Other studies have reported that an inflammatory response induced in the periphery may also affect motoneurons retrogradely (Mariotti et al. 2001; 2002b). The exact nature of the retrograde signal however, is not known. These present results indicate that perturbation of peripheral targets can lead to increased motoneuron survival and that muscle damage results in the production of some factor that influences motoneuron survival and/or repair. It is not clear how direct muscle damage relates to long-term muscle atrophy induced by DR with respect to availability of target-derived neurotrophic support. Mechanisms of neuroprotection regarding altered IGF-1 levels are explored in chapter-5.

3.3.4 Conclusions

It is clear that protective mechanisms within neural systems may be manipulated. The present thesis has shown so far that specific trophic interactions of motoneurons may be enhanced through perturbation of peripheral targets, and that motoneurons are affected by non-specific, 'whole-body' alteration, such as DR. Specifically, the results show that DR increases age-related motoneuron loss from the facial nucleus compared to age-matched *ad libitum*-fed controls. Facial nerve avulsion of DR rats resulted with no further loss of motoneurons ipsilaterally. It seems possible that those motoneurons lost by DR may be the same population that would have been lost a result of nerve avulsion. It seems reasonable to predict, therefore, that induction of a stress or trophic response, specific to motoneurons, could confer a state of heightened protection against mechanical injury. Indeed, facial motoneurons can be rescued from avulsion-induced death by prior muscle damage. Putative osmotic and mechanical damage following injection of distilled water resulted in significant protection of facial motoneurons, followed by bupivacaine, and then physiological saline.

The next chapter focuses on a particular component of the target-derived signal that may influence motoneuron survival in adult rats.

Chapter-4

Effect of IGF-1 isoforms on motoneuron survival

4.1 Introduction

The majority of single-dose studies have evaluated only short-term rescue of motoneurons with neurotrophic substances, which is in contrast to the prolonged nature of degeneration associated with ageing and disease. Transferring a gene encoding a neuroprotective peptide to the neuromuscular system may enhance longer-term protection of motoneurons. This study aims to investigate the effect of prior gene transfer of IGF-1 isoforms on motoneuron survival in adult and neonatal rats following facial nerve avulsion.

4.1.1 Gene transfer and long-term neuroprotection

To date, single-dose delivery of recombinant neurotrophic factors close to the cut nerve in neonatal rats and mice has allowed mostly transient protection of axotomised motoneurons against cell death (Henderson et al. 1993; Koliatsos et al. 1993; Li, L. et al. 1994; Pennica et al. 1996; Schmalbruch & Rosenthal 1995; Sendtner et al. 1990; 1992a; Yan et al. 1992). Furthermore, additional supplement of locally applied factor alone or combined with subcutaneous injections failed to further increase motoneuron survival significantly (Vejsada, Sagot, et al. 1994; 1995). Long-term protection has been afforded in selected studies whereby sustained exposure to neurotrophic factors was achieved by use of gelfoam implants soaked with neurotrophic factor protein or injection of surrounding muscles with a high concentration of protein (Henderson et al. 1994; Hughes, R.A. et al. 1993; Li, L. et al. 1994; Matheson et al. 1997; Schmalbruch & Rosenthal 1995; Sendtner et al. 1992a; Yan, Matheson, et al. 1995), or by gene transfer (Baumgartner & Shine 1998; Finiels et al. 1995; Gravel et al. 1997; Hottinger et al. 2000; Ribotta et al. 1997). Gelfoam is able to sustain delivery of proteins to injured motoneurons for a prolonged period of time. However, many neurotrophic factors are known to degrade rapidly with loss of bioactivity following subcutaneous injection and/or implantation of gelfoam (Clatterbuck et al. 1994; Melov et al. 2000). Such limitation in protein half-life may be overcome using a mini-pump to infuse the lesion site with fresh protein but this may be costly. Gene transfer, on the other hand, offers long-term *de novo* synthesis of neurotrophic factors resulting from a single injection of an appropriate vector. Furthermore, the gene may be delivered locally to intact motoneurons

intramuscularly, allowing potentially the uptake of vector and/or gene product to motoneurons and making available an elevated supply of neurotrophic factors prior to neurological insult.

Viral vectors are commonly used to deliver genes to the neuromuscular system. The first-generation of adenovial vectors in particular, are reported to provoke reaction from the immune system (Kass-Eisler et al. 1996) and this has hampered their progression into clinical trials. A safer and more practical alternative is non-viral based vectors such as DNA plasmids. Plasmids are easily constructed and can also be designed to express cell-specifically the proteins that they encode. Intramuscular expression of plasmid-encoded neurotrophic factors has been shown to affect motoneurons (Alila et al. 1997; Flint et al. 1999). There is only limited evidence that plasmids may be transported axonally to the cell body (Johnson, I.P. et al. 1998; Sahenk et al. 1993). In this study, a plasmid encoding IGF-1 and a previously untested IGF-1 splice variant called mechano-growth factor (MGF) will be delivered to the facial muscle to expose motoneurons to enhanced levels of these factors prior to nerve avulsion.

The present thesis examines the promotion of motoneuron survival in adult rats with an emphasis on the role played by the target muscle. IGF-1 was chosen to demonstrate such a role for the following reasons: (i) IGF-1 promotes motoneuron survival, (ii) IGF-1 is a myotrophic factor; (iii) levels of IGF-1 and its associated actions decline with age; (v) levels of IGF-1 alter following DR, and (vi) levels of IGF-1 alter after muscle damage.

4.1.2 IGF system

The IGFs (IGF-1 and IGF-2) are trophic factors with both growth-promoting (anabolic) and insulin-like metabolic activities (Feldman et al. 1997). Each is the product of a single, large and complex gene (~95 and ~35 kilobases respectively). The growth-promoting actions of both IGF-1 and IGF-2 are mediated via the type-1 IGF-1 receptor (IGF-1R), which is a tyrosine kinase receptor, and also by insulin-like growth factor binding-proteins (IGF-BPs),

which are responsible for regulating the availability of IGF's interaction with these receptors (Feldman et al. 1997; LeRoith et al. 1992). Both are expressed in most tissues from early on in embryological development, with IGF-2 being more abundantly expressed *in utero.* IGF-1 is more abundant in post-natal life and is more commonly reported as having a role in the nervous system, and in particular, an effect on peripheral motoneurons (Ishii et al. 1994). IGF-1 has been chosen, therefore, as the gene of interest in this study with special interest in the locally expressed (autocrine/paracrine) splice variant named mechano-growth factor (MGF) whose cDNA has been isolated recently (Yang, S. et al. 1996).

The rat IGF-1 gene consists of at least six exons separated by five introns and spanning more than 80 kilobases. All mammalian mature IGF-1 protein molecules characterised to date are about 70 amino acid residues and contain four domain structures, termed, B, C, A and D, based on the homology to B, C, and A domains of insulin (**Fig. 4-2**) (Arkins et al. 1994). In addition, protein precursors contain a carboxy-terminal extension or E-domain peptide, which is not found in mature circulating IGF-1. This may be important with respect to IGF-1 activity (Lowe et al. 1988; Rotwein 1986; Siegfried et al. 1992; Tian et al. 1999; Yang, S. & Goldspink 2002) and is described later on in more detail. Activity from at least two promoters and alternative RNA splicing gives rise to extensive coding sequence mRNA heterogeneity (**Fig. 4-1**).

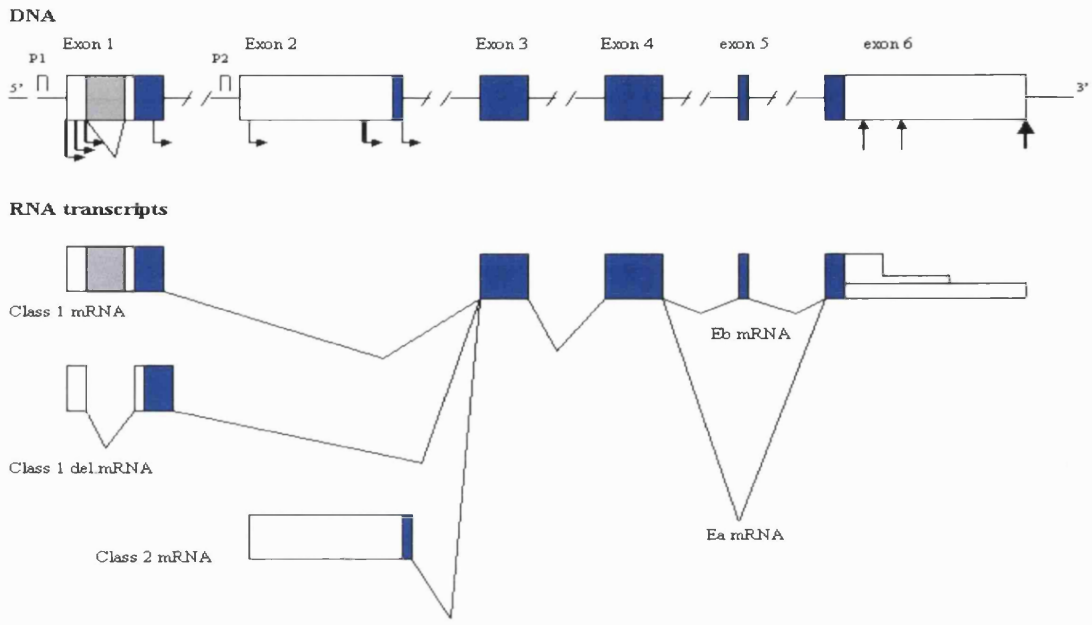


Figure 4-1 Schematic representation of the rat IGF-I gene, and different classes of mRNA. Boxed regions represent exons and blue areas represent open reading frames. The arrows under exons 1 and 2 indicate multiple transcription start sites. Promoter elements 5' to exons 1 and 2 are shown as P1 and P2 respectively. The arrows in exon 6 indicate the polyadenylation sites. Exon 1 or 2 is spliced to exon 3 to give rise to class 1 or class 2 mRNA respectively. Splicing out a 186-nucleotide sequence in exon 1 (dark grey) generates an additional class 1 variant, the class 1 deletion. (Taken from Arkins *et al.*, 1994).

While all the IGF-1 mRNAs encode the same mature IGF-1, they may differ in coding sequences for amino- or carboxy-terminal precursor peptides that flank the mature IGF-1 sequence (Daughaday & Rotwein 1989; Hepler & Lund 1990). Mature rat IGF-1 is encoded by parts of exons 3 and 4, which, along with exon 6, are found in all rat IGF-1mRNAs. Two different IGF-1 precursor peptides, designated pre-pro-IGF-1Ea and pre-pro-IGF-1Eb, arise *via* alternative splicing at the 3' end. In the rodent, exon 4 encodes the proximal proportion of the E-domain and exons 5 and/or 6 contain the distal portions of the E-domains of Eb and Ea respectively, together with 3' untranslated sequences (Adamo et al. 1993b). Various signal peptides are also generated by alternate splicing of either of the upstream exons (1 or

2) to exon 3 (Adamo et al. 1993a), generating class-1 and class-2 IGF-1 mRNAs respectively. The alternative transcription units and the predicted protein precursors of IGF-1 mRNA are summarized in **Fig. 4-2**.

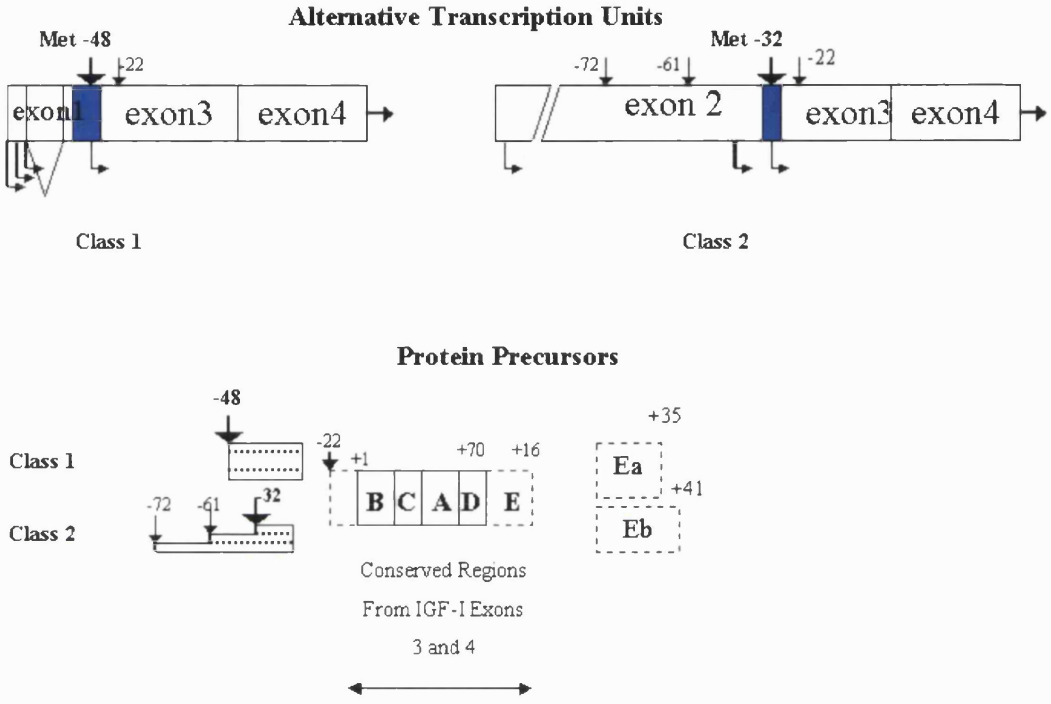


Figure 4-2 Schematic representations of alternative transcription units and the predicted protein precursors of IGF-I mRNA. Boxed regions represent exons and blue areas represent open reading frames. Transcription initiates from a disperse series of sites within exon 1 and less within exon 2. The resulting 5' sequences may encode different IGF-I pre-peptides. The most common pre-peptides arising from class 1 and 2 mRNAs contain 48 and 32aa signal peptides, respectively. Pre-peptides arising from the use of other in-frame AUG sequences are possible, depending on the site of transcription initiation and the minor arrows above exons 1, 2, and 3 in the transcription units indicate these. The conserved regions of the mature IGF-I peptide are designated B, C, A and D. (taken from Arkins *et al.*, 1994)

The physiological significance of IGF-1 mRNA heterogeneity is not clear but the tissue-selective manner in which these transcripts are expressed would suggest differential functional importance.

4.1.3 IGF receptors and signal transduction

The IGFs act on cells by binding with high affinity to a membrane receptor. Two cell surface receptors, the IGF-1 (or type-1 receptor) and the IGF-II (or type-II) receptor, are known. The type-II receptor is identical to the cation-dependent mannose-6 phosphate receptor and it functions in the trafficking of lysosomal enzymes but has no known IGF-1 signaling function in contrast to the type-1 receptor (Feldman et al. 1997). The IGF-1 receptor is a heterotetrameric transmembrane protein containing paired, disulphide-linked α- and β-subunits (Siddle 1992). The extracellular α-subunits binds IGFs, whereas the β-subunits span the cellular membrane and possess tyrosine kinase domains. Binding of IGF-1 to the α-subunit induces a conformational change in the receptor that results in autophosphorylation of the β-subunit, and sets into motion signaling cascades that involve phosphorylation of a series of intracellular messengers (Siddle et al. 2001). Tyrosine kinase activity results in phosphorylation of docking molecules insulin-receptor substrate-1 (IRS-1) and/or IRS-2. The IRS proteins contain tyrosine phosphorylation sites, which serve as binding sites for the p85α subunit of phosphatidylinositol-3 kinase (PI-3K) and growth factor receptor bound-2 protein (Grb2), two src homology-2 (SH2) domain-containing proteins (Myers & White 1993). The p85α subunit of PI-3K activates the 110-kDa catalytic subunit, which phosphorylates phosphoinositides, generating PI-3-phosphate, PI-1,4-bisphosphate, and PI-3,4,5-trisphosphate (Feldman et al. 1997). These phosphatidylinositides, in turn, activate protein kinases such as protein kinase B (PKB/Akt) and other less well-characterised downstream molecules. Activated Akt has been shown to promote neuronal survival (Dudek et al. 1997). Akt phosphorylation of Bad, an up-stream component of the apoptotic pathway, therefore, couples cell survival signals with the cell-intrinsic death machinery (Datta et al. 1997). Interestingly, IGF-1 has been shown to prevent

apoptosis in sympathetic neurons (Russell et al. 1998) and sensory neurons after neurotrophic factor withdrawal (Russell & Feldman 1999). Other downstream targets may be activated *via* the ras/raf pathway. Phosphorylation of c-*raf* activates the downstream protein kinase, MAP kinase kinase 1 (MKK1) or MKK2. MKK1 and MKK2 activate two members of the MAP kinase family ERK1 and ERK2. Once activated, either or both of these are translocated to the nucleus where they phosphorylate a variety of transcription factors including *c*-fos and *c*-jun (early-response genes). These stimulate the transcription of late-response genes encoding effectors of growth factor stimulation. This pathway is common to the actions of many growth factors. Although only some of the above data was deciphered in neuronal cells, many of the second messengers, downstream signaling molecules and transcription factors have been identified experimentally in motoneurons *in vivo* (Allsopp 2000; Brunet et al. 2001; Dudek et al. 1997; Patapoutian & Reichardt 2001; Segal & Greenberg 1996).

4.1.4 IGF binding proteins

As a strongly mitogenic protein, the action of IGF-1 needs to be regulated, particularly in tissue with high cellular turnover (*e.g.* intestines) and with a rich blood supply, as it has powerful tumour promoting properties, especially in adult and ageing animals (Moschos & Mantzoros 2002; Wetterau et al. 1999). IGF-1 circulates at high level in the blood but the availability of free IGF-1 in the serum and in target tissues is severely limited. In human adults for example, as much as 75-90% of total IGF-1 in serum is protein-bound (Kostecka & Blahovec 1999). This is due mainly to its binding with insulin-like growth factor binding proteins (IGF-BPs), of which six have been identified so far (IGF-BP1-6). They comprise a family of proteins that share a common cysteine motif in their amino- and carboxyl-termini, which accounts for their capacity to bind IGFs (Clemmons 1993). By regulation of free IGF-1 levels in circulation they also modulate IGF activity, being capable of both inhibiting and augmenting IGF-1's interactions with cell-surface receptors. IGF's effects may be potentiated by phosphorylation (IGF-BP1), its association with both the cell surface (IGF-BP3) and extracellular matrix (IGF-BP5), and by proteoloysis (IGF-BP3 and -BP5). The

binding of any IGF-BPs to IGF-1 can also withhold IGF's association with target receptors. Parallel to IGFs, IGF-BPs are expressed in many, if not all, tissues but each IGF-BP is cell-specific and often developmentally regulated. Such coordinate expression indicates that IGF-BPs also regulate IGF-1 activity in the localised environment as well as general availability in blood (Kostecka & Blahovec 1999).

4.1.5 Local *versus* Systemic Action of IGF-1

4.1.5.1 Liver-derived IGF-1

The "Somatomedin Hypothesis" originally proposed that circulating IGF-1 acting in various tissues mediate the effects of growth hormone (GH). GH binds with high affinity to the GH receptor, which is found in tissues throughout the body; activation of its receptor stimulates the synthesis and secretion of hepatic IGF-1. IGF-1 circulates in the blood at high concentrations and stimulates DNA, RNA, and protein synthesis by binding and activating the type-1 IGF-1 receptor found with similar ubiquity and abundance (particularly during development) amongst many tissue types (Le Roith et al. 2001).

4.1.5.2 Extra-hepatic IGF-1 expression

With the discovery that most if not all tissues produce IGF-I, the role of autocrine/paracrine IGF-I *versus* the circulating form has been hotly debated. Recent experiments using transgenic and gene-deletion technologies have attempted to answer these questions. It was observed that after Cre/loxP-induced conditional knockout of liver-specific IGF-1 synthesis/secretion in mice, circulating levels of IGF-1 decrease by approximately 75% (Liu, J.L. et al. 2000; Sjogren et al. 1999; Yakar et al. 2001). Interestingly however, these mice exhibited no defect in growth or development. When administered exogenously, GH stimulated IGF-1 production in several extra-hepatic tissues as well as body growth. It is apparent, therefore, that tissue (autocrine/paracrine) specific IGF-1 is able to compensate functionally for hepatic (endocrine) IGF-1. Following from this, in hypophysectomised rats

in which GH secretion is stemmed, the regenerative capacity of skeletal muscle is preserved suggesting that endogenous production of IGF-1 can occur independent of GH (Jennische & Matejka 1992; Sommerland et al. 1989). Extra-hepatic synthesis of IGF-1 is of great significance because it raises the possibility of autocrine/paracrine activity, independent of its endocrine actions. In other words, it can be classified either as a local growth factor or a broad-acting hormone respectively. IGF-1 is expressed locally during development and maturity in many other tissues, most notably in skeletal muscle (Alexandrides et al. 1989; Beck, F. et al. 1988). This is important because wherever IGF-1 deficiency may be apparent, local levels of IGF-1 may be altered either directly by exogenous supply to the organ, or indirectly by enhancing its autocrine/paracrine production.

Whereas the hormonal action has been characterised extensively, the nature of paracrine/autocrine action is less well understood. The expression of IGF-1 in skeletal muscle, and concomitant sensitivity to this growth factor of peripheral nerves, provides an excellent example of how locally produced IGF-1 can regulate the growth of the tissue in which it is synthesised (autocrine regulation), and that of a neighbouring dependent tissues (paracrine regulation). This study is particularly concerned with locally produced IGF-1 because this is in parallel to the mode of action of other target-derived neurotrophic factors (Thoenen et al. 1993).

4.1.6 IGF-1 in the Nervous System

4.1.6.1 IGF-1 in Neural Development and Post-Natal Growth

In the nervous system, IGF-1 and IGF-1 receptor expression is high during early foetal development. During rat embryogenesis, IGF-1 mRNA is detected in regions of active nerve sprouting, spinal ganglia, and facial target regions of the trigeminal nerve. IGF-1 mRNA is present in the human foetal brain, as well as protein in the cerebrospinal fluid (Bondy et al. 1990). Additionally, the mRNA expression profiles of IGFBPs 2, 4 and 5 in the normal developing CNS is well characterised and are shown to have distinctive, non-overlapping

distributions (Arnold et al. 2000; Brar & Chernausek 1993). IGF-1 is required for Schwann cell mitogenesis and differentiation (Pu et al. 1995; Stewart et al. 1996) and developing oligodendrocyte progenitor cells require IGF-1 to progress to mature, myelin-forming phenotypes (Goddard et al. 1999). Transgenic (Tg) mice over-expressing IGF-1 postnatally exhibit brain overgrowth characterised by increased neuron and oligodendrocyte number, as well as marked increases in myelination. Mutant mice with ablated IGF-I and IGF-1 receptor expression, as well as those with over-expression of IGFBPs capable of inhibiting IGF actions exhibit brain growth retardation with a variety of growth deficits (D'Ercole et al. 2002). Evidence from experiments in these mouse models also indicates that IGF-1 has a role in recovery from neural injury.

4.1.6.2 Alterations of IGF-1 After Injury in the nervous system

Changes in the IGF axis indicate autocrine/paracrine actions of IGF-1 within wounds of the brain (Walter et al. 1997). After hypoxic-ischaemic injury of the rat brain, IGF-1 accumulates in blood vessels and IGF-1 mRNA in microglia of the damaged hemisphere. This is in parallel with cell-selective changes in IGFBPs 2, 3, 5 and 6 (Beilharz et al. 1998). Furthermore, the level of ischaemic brain injury can be reduced by topical application of IGF-1 after transient middle cerebral artery occlusion in rats (Wang et al. 2000). Region-specific IGF-1 expression is also observed in other models of brain injury such as prolonged seizure activity and focal brain injury (Hughes, P.E. et al. 1999) and penetrating injury (Li, X.S. et al. 1998). Selective IGF-1 and IGF-BP mRNA and protein expression has been reported following axonal injuries to peripheral nerve as well as to spinal root avulsion injury. The injured sciatic nerve expressed IGF-1 as well as IGFBP-4 and IGFBP-5. Following avulsion injury, CNS scar tissue expressed IGF-1, IGFBP-2, and IGFBP-5. IGFBP-6 mRNA was strongly up-regulated in spinal motoneurons after both types of lesions. IGFBP-6-like immuno-reactivity was present in motoneuron cell bodies, dendrites in the ventral horn, and axons in the sciatic nerve. In line with *in vivo* findings, cultured Schwann cells expressed IGF-1, IGFBP-4, and IGFBP-5 mRNAs, whereas cultured astrocytes expressed IGF-1, IGFBP-2, and IGFBP-5 mRNAs. These findings show that IGF-

1 is available to lesioned motoneurons both after peripheral and central axonal lesions (Gehrmann et al. 1994; Hammarberg et al. 1998).

4.1.7 IGF-1 in Motoneuron Regeneration and Degeneration

4.1.7.1 IGF-1 in Axon Regeneration

Neurite outgrowth in cultured human neuroblastoma cells (Recio-Pinto et al. 1986) and embryonic chick sensory and sympathetic neurons first indicated the potential neuro-regenerative action of purified IGFs. The first *in vivo* studies showed that local infusion of IGF-1 increased the distance of regeneration of sensory axons in lesioned sciatic nerves (Kanje et al. 1989; Sjoberg & Kanje 1989). It was shown later to have corresponding action in sciatic motor axons (Near et al. 1992). IGF-1 (and IGF-2) emerged as the first example of an identified soluble neurotrophic substance produced in nerves that supports both sensory and motor nerve axon regeneration (Ishii et al. 1994). Various studies have elaborated further the role of endogenous IGF-1 in motoneuron regeneration by employing different nerve lesions, such as nerve crush, transection or ventral root avulsion and recording the changes in spatial and temporal expression of IGF-1, IGF-1R and various IGFBPs in the nerves and spinal cord (Glazner et al. 1993; Glazner & Ishii 1995; Hammarberg et al. 1998 ; Pu et al. 1999a; Suliman et al. 1999; 2001). IGF mRNA levels may be seen to down-regulate to more normal levels only after nerve crush but remaining high after transection (Glazner et al. 1993). *In situ* hybridisation shows that IGF mRNA is up-regulated in the distal but not proximal nerve during regeneration (Pu & Ishii 1993), supporting the hypothesis that regenerating axons grow towards the vicinity of higher IGF concentration (Ishii et al. 1994). Hansson et al. (1986) showed that IGF-1 immuno-reactivity increases in injured nerves and this was associated only with Schwann cells. Further observations of Schwann cell activity after lesions, such as proliferation in the basement membrane tubes of the formerly myelinated nerve fibres (Johnson, P.C. & Asbury 1980) and the proximo-distal spread of labeled Schwann cells (Oaklander et al. 1987) strongly supports IGF-1 as a neurotrophic factor that helps regulate axon regeneration.

4.1.8 Intramuscular nerve sprouting

The number of muscle fibres per motor unit may vary from as few as four or as many as several hundred. To achieve this, motoneurons must branch as each motor axon enters into the muscle. When muscle fibres are lost, motor axons react by sending out intramuscular nerve sprouts in a manner similar to synapse formation during development (Murphy, R.A. 1993). Experimental evidence indicates that IGF-1 has a crucial role to play in this activity in both of these situations.

Development: IGF-1 induces neurite outgrowth *in vitro* (Recio-Pinto et al. 1986) and *in vivo* (D'Costa et al. 1998) as well as increasing branching and synapse formation in chick embryos. Activity blockade during programmed cell death (PCD) results in increased expression of endogenous IGF-1 mRNA in muscle (D'Costa et al. 1998) and increased number of surviving motoneurons (Greensmith & Vrbova 1991). Additions of IGFBPs to paralysed embryos, which interfere with IGF-1's action, reduce motoneuron number, axon branching and synapse formation after normal PCD. In normal embryos, IGF-1 treatment prior to PCD increases subsequent synapse formation and motoneuron survival, and this potentiates the survival-promoting activity of BDNF and GDNF in this same period (D'Costa et al. 1998). During the period of synapse elimination, muscle fibres are unable to accept further innervation and this has been shown to proceed in parallel with decline in levels of muscle IGF-1 mRNA (Caroni & Becker 1992).

Maturity: IGFs mRNA levels become elevated in muscles that are denervated (Caroni et al. 1994) or paralysed with Botulinum toxin (Caroni & Schneider 1994). This is concomitant with increased intramuscular nerve sprouting in the vicinity of inactivated muscle fibres, which was prevented by the addition of local delivery of IGFBP-4 (Caroni & Schneider 1994) and inhibited by the presence of an anti-IGF-1 antibody (Streppel et al. 2002). Application of IGF-1 to muscle leads directly to increased expression of growth-associated proteins, *e.g.* GAP-43, in growth cones of neuronal sprouts (Flint et al. 1999).

These observations are consistent with the proposed mechanism of IGF-1 in promoting motoneuron survival by increasing access, via multiple nerve sprouts, to muscle-derived neurotrophic factors (D'Costa et al. 1998). IGF-1 may in addition act as a neurotrophic factor by disrupting apoptotic signaling (Dudek et al. 1997; Parrizas et al. 1997). It is such activity in skeletal muscle that makes IGF-1 a particularly attractive candidate for potential therapy for age- or disease-related neurodegeneration.

4.1.9 Experimental Motoneuron Rescue with IGF-1

Table 4.1 reviews the information that has accumulated regarding neuroprotection by exogenous application of IGF-1.

Table 4.1

Experimental rescue of motoneurons with IGF-1

Species	*Model*	*Effect of IGF-1*	*Reference*
Chick embryo	PCD	Dose-dependent increase in number of motoneurons. 25% more lumbar motoneurons than control animals with optimum dose	(D'Costa et al. 1998; Neff et al. 1993)
	PCD	IGF-1 rescues 20% trochlear and 30% oculomotor neurons	(Rind & von Bartheld 2002)
	Deafferentation	73% rescue of lumbar motoneurons (only 20-30% of motoneurons die in this model)	(Neff et al. 1993)
	Limb amputation	50% rescue of motoneurons (50% motoneurons die in this model)	(Neff et al. 1993)
Rat embryo	*in vitro*	Supports survival of 50% of isolated motoneurons	(Hughes, R.A. et al. 1993)

Mouse embryo	*in vitro*	2-fold increase in E15 motoneurons surviving up to 8 days only in the presence of astrocytes	(Ang et al. 1992)
Mouse adult	C7 root avulsion	Maintains survival of <50% of motoneurons (~66% motoneurons die in this model)	(Li, L. et al. 1995)
Neonatal rat	Facial nerve transection	31% facial motoneurons rescued by IGF-1	(Hughes, R.A. et al. 1993)
	Sciatic nerve transection	Systemic IGF-1 rescues 42% of motoneurons 6 weeks after injury (50% motoneurons die in this model)	(Vergani et al. 1998)
		intraperitoneal IGF-1 and riluzole in combination rescued lumbar motoneurons	(Iwasaki & Ikeda 1999)
	Spinal root avulsion	IGF-1 failed to rescue any motoneurons (100% motoneurons die in this model)	(Yuan et al. 2000)
Neonatal mouse	Sciatic nerve transection	~100% rescue of lumbar motoneurons 7 days after injury (66% motoneurons die in this model)	(Li, L. et al. 1994)
Transgenic mice	*wobbler*	Prevention of further motoneuron loss after co-treatment with IGF-1 and glucosaminoglycans	(Di Giulio et al. 2000)
	wobbler	Number of motoneurons unchanged compared to controls after 6 weeks of treatment with IGF-1	(Hantai et al. 1995)

Studies investigating IGF-1-mediated protection of motoneurons are limited mostly to the embryonic and neonatal stages. There have been few studies on IGF-1-mediated neuroprotection in adult animals and none on ageing animals.

4.1.10 IGF-1 myotrophic action

Development: IGF-1 is expressed in primary muscle cells within developing muscle tissue (Gerrard et al. 1998), where it acts to regulate the number of primary myofibers, and, therefore, size of skeletal muscles (Mitchell et al. 2002). *In vitro* studies have identified IGF-1 as an important regulator of myoblast proliferation and myogenic differentiation (Tollefsen et al. 1989; Yang, S. & Goldspink 2002).

Maturity: Mature muscle is a highly adaptable dynamic tissue with great plasticity, whose complement of muscle bulk must equilibrate with the mechanical and metabolic demands with which it is utilised. To obtain this, a functionally reciprocal relationship exists between motoneurons and muscle. Motoneurons supply electrical stimulation, required for muscle contraction. Muscle fibres adapt accordingly in response to mechanical and metabolic demands placed upon them by changes in the size, duration and frequency of nervous impulse (Murphy, R.A. 1993). Damaging muscle either mechanically by stretch (McKoy, Ashley, et al. 1999; Yang, S. Alnaqeeb, et al. 1996) metabolically by glycogen depletion/ischaemia (Jennische et al. 1987), or causing atrophy by denervation (Shiotani & Flint 1998) or Botulinum toxin-induced paralysis (Caroni & Schneider 1994) upsets this equilibrium. Skeletal muscle regenerates its tissue mass following damage/injury by rapidly up-regulating IGF-1 mRNA transcripts (Jennische & Hansson 1987). A response to muscle inactivity is increased intramuscular nerve sprouting, which is mediated in part by IGF-1 (Caroni et al. 1994). It is believed; therefore, that IGF-1 derived from damaged muscle is utilised to re-establish its own viability by stimulating growth of the tissues, *i.e.* motoneurons, upon which it is dependent. It is noteworthy then, that senescent muscle becomes less responsive to IGF-1 (Dardevet et al. 1994; Willis et al. 1997), and it is believed that this underlies, in part, the impaired regenerative capacity of this tissue in

ageing animals (Barton-Davis et al. 1998; Grounds 1998; Musaro et al. 2001; Owino et al. 2001). However, it has not been shown directly that such alteration contributes to age-related motoneuron degeneration and functional motor deficit.

There appears to be good evidence therefore that IGF-1, via its myotrophic activities, lays central to the developmental and functional equilibria between nerve and muscle of developing and mature animals respectively. This project aims to understand more fully how target muscle influences the survival of motoneurons, therefore it is of particular interest to study the effect of IGF-1.

4.1.11 IGF-1 and ageing

It is postulated that brain ageing and the associated neurodegenerative processes associated with ageing result from a deterioration of mechanisms that regulate the maintenance of basic cellular processes. In adult neurons, this involves maintaining cell phenotype, supporting synaptic plasticity and providing neuroprotective and neuroregenerative mechanisms (Cowen 1993; Hof 1997), all of which requires sustained protein synthetic activity. The decline of growth hormone secretion and resultant plasma IGF-1 concentration is one of the most reliable and best characterised endocrine events that occur with age in humans and rats (Sonntag et al. 1999). Given that IGF-1 is essential for the growth of many tissues during normal development, there appears to be good argument for a central role for IGF-1 in general age-related decline in tissue function.

It is likely that decreased availability and/or diminished responsiveness of tissues to growth factors such as IGF-1 may be partly responsible for decreases in total protein synthesis previously observed in ageing animals (D'Costa et al. 1993). In the ageing rat brain, IGF-1 stimulation of protein synthesis is attenuated in cerebral cortex of ageing rats (D'Costa et al. 1995), reduced IGF-1 mRNA expression is observed in the hippocampus (Lai et al. 2000), decreases in brain concentrations of IGF-1 and IGF-1R are associated with the age-related decline in memory function (Lynch et al. 2001) and delayed changes in IGF-1 gene

expression are associated with slow remyelination in the CNS of aged rats (Hinks & Franklin 2000).

In the context of the neuromuscular system, a decrease in protein-synthesis could have a profound effect. Motoneurons in some neural systems are lost in ageing animals (Johnson, I.P. & Duberley 1998; Sturrock 1987; Sturrock 1988b; Zhang et al. 1996). Such loss could be a result of age-related vulnerability of motoneurons to a decline in neurotrophic support (Johnson, H. et al. 1996; Johnson, I.P. & Duberley 1998; Ming et al. 1999) but there is no evidence as yet to show that age-related motoneuron loss and/or degeneration is a direct result of decline in IGF-1 signaling. However, it is reported that deficiencies in IGF-1 signaling underlie the decline in protein-synthetic activities (Willis et al. 1998) and function (Wang et al. 2002) of ageing skeletal muscle and that this has a knock-on effect of degrading its regenerative capabilities. For example, localised IGF-1 transgene expression can sustain hypertrophy, regeneration (Barton-Davis et al. 1998; Shiotani et al. 2001) and strength (Musaro et al. 2001) in senescent muscle. Given that muscle-derived neurotrophic factors act in a paracrine fashion, it is of particular interest to this study to observe that ageing skeletal muscle fails to up-regulate a locally acting splice variant of IGF-1 known as MGF in response to muscle overload (Owino et al. 2001).

The general precedence of IGF-1 over other neurotrophic factors, as the key to determining age-related degeneration of motoneurons, is based on the compounding effect of two factors: (i) the marked decline of intrinsic IGF-1 signaling, not uncommon to other neurotrophic factors but not to the same extent; and, (ii) the general decline of tissue viability, with an emphasis on muscle mass and the putative knock-on effect that this has on the availability of muscle-derived neurotrophic factors.

4.1.12 Mechano-Growth Factor (MGF)

4.1.12.1 Splicing at the 3' end generating alternate E-domain peptides

IGF-1 mRNA transcripts differ in their 3' sequences encoding the E-domain peptide of the immature protein. This has been reported independently in rat (Lowe et al. 1988; Roberts et al. 1987), mouse (Bell et al. 1986) and human (Rotwein 1986). These changes arise from alternate splicing interplay between exons 4, 5 and 6. The insertion of part or all of exon 5 (in rat and mouse this is a cassette of 52 nucleotides), or the splicing of exon 4 to exon 6 and thus excision of exon 5, results in a shift of reading frame and stop codon. This generates different peptides that differ in their carboxy-terminal sequence (**Fig. 4-3**).

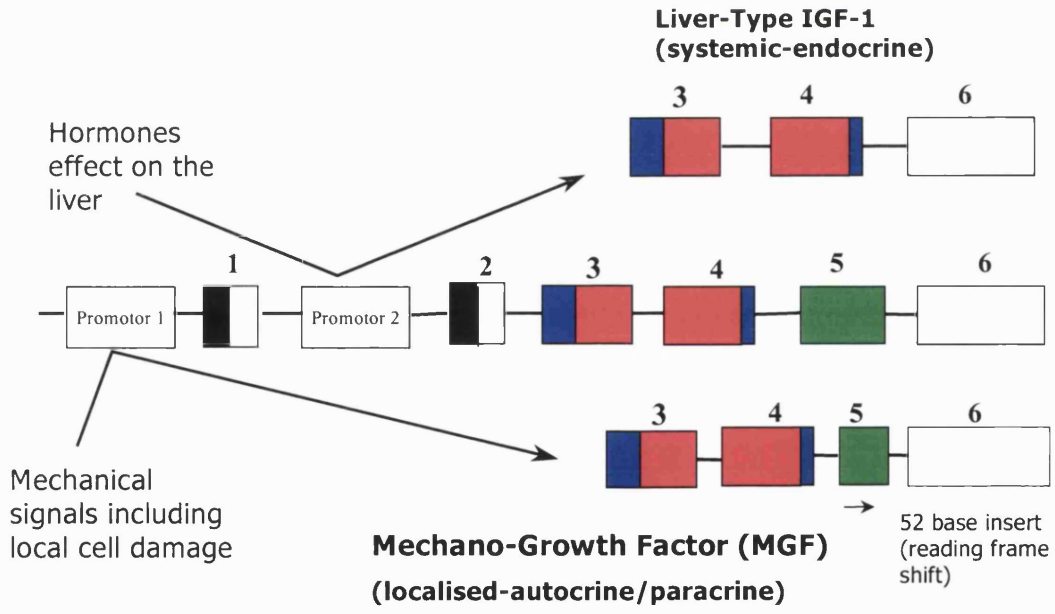


Figure 4-3 Alternative splicing of the IGF-1 gene to produce either MGF or Liver-type IGF-1. Insertion of 52 base-pairs from exon 5 (green) results in a reading frame shift causing a change in amino acid sequence at the C-terminus. The coding region of the mature IGF-1 protein is coloured red. MGF mRNA tends to be

transcribed under the control of local mechanical signals, whereas liver-type IGF-1 tends to be under hormonal control.

Until recently, the biological roles of the E-peptides have been overlooked. It was generally believed that they were biologically inert. The Ea type E-domain contains two potential N-linked glycosylation sites and is glycosylated *in vitro*. The Eb E-domain, found only in MGF, is not glycosylated *in vitro* and is highly basic (Bach et al. 1990). These structural characteristics suggest different biological roles for each of these peptides. The Ea type E-domain is highly conserved among vertebrates (Shamblott & Chen 1993) whereas the Eb types are conserved in the first 15 amino acids and differ thereafter among humans, rats and mice. The conservation of the E-peptide sequence implies that a selection pressure exists against random mutations in these and puts forward a possibility for functionality in E peptides. The classification of different IGF-1 isoforms was based originally on their expression in the liver tissue. When applied to IGF-1 splice variants from non-hepatic cells and also across species, IGF-1Eb in the rat is equivalent to IGF-1Ec in the human. Comparison between the human and rat cDNA and deduced peptide sequences suggests that the exon 4-5-6 splice variants in both species are equivalent (Roberts et al. 1987; Shimatsu & Rotwein 1987b).

This study is concerned with the IGF-Eb splice variant, homologous between rats and rabbits or IGF-1Ec in humans. Pre-pro-IGF-1Eb is the pro-hormone for whose mRNA was found to be specifically 'up-regulated' in active skeletal muscle (McKoy et al. 1999; Yang, S. et al. 1996). The protein for which it encodes is known as MGF. Liver-type IGF-1 will be referred to as L.IGF-1.

4.1.12.2 Tissue-Specific Expression

Under normal conditions IGF-1Ea (encoding L.IGF-1) is the major transcript in all tissues (LeRoith & Roberts 1991). However, MGF (IGF-1Eb) is specifically up-regulated in exercising and/or stretched skeletal and damaged muscle (McKoy et al. 1999; Yang, S. et al.

1996) and neural tissue (Owino et al., unpublished observations). Furthermore, when a muscle is exercised, it is that muscle which undergoes hypertrophy and not all the skeletal muscles of the body. MGF therefore, as well as being mechano-sensitive, is regarded as a local growth/repair factor. The alternative mRNA transcripts of the IGF-1 gene and their peptides, which have different biological functions in muscle (Yang, S. & Goldspink 2002), provide the molecular basis for autocrine/paracrine *versus* endocrine action of IGF-1.

Characterisation of MGF mRNA expression and regulation in skeletal muscle has provided a candidate protein to link localised mechanical stimulus with altered gene expression of structural genes in this tissue (McKoy et al. 1999). This protein is distinguished from the IGF-1 receptor ligand-domain, or mature IGF-1 protein, from which the E-domain peptide is cleaved. All the IGF-1 isoforms possess the IGF-1 ligand-binding domain or liver-type IGF-1 (L.IGF-1), including MGF. However, the E-domain of MGF has been shown also to act as a separate growth factor, which in muscle activates the satellite (muscle stem) cells (Yang, S. & Goldspink 2002). Muscle and neural tissue are post-mitotic tissue in which there is no appreciable cell replacement. It is imperative; therefore, that there is an efficient local tissue repair mechanism. The purpose of this study is to determine if MGF has a role in protecting motoneurons. Given that muscle-derived neurotrophic factors influence motoneurons in a paracrine fashion MGF, the autocrine/paracrine splice variant of IGF-1, would be ideally suited to mediate the reciprocal trophic interactions between nerve and muscle. Previous studies indicate that L.IGF-1 promotes only moderate survival of motoneurons (Hughes, R.A. et al. 1993; Li, L. et al. 1994; Li, L. et al. 1995). MGF has never been tested on neural tissue. Therefore, it is of particular interest to this study to see whether MGF is the preferred isoform of IGF-1 with respect to protection of motoneurons in young and adult animals. MGF also satisfies the encompassing aim of the present thesis, which is to investigate the influence of skeletal muscle in promoting the survival of mature motoneurons.

4.1.13 Specific aim

To determine whether intramuscular injection of a plasmid vector containing the gene for either L.IGF-1 or MGF prior to nerve injury can increase the survival of facial motoneurons in adult and neonatal rats.

4.2 Results

The materials and methods for these experiments can be found in chapter 2

4.2.1 Effect of MGF and L.IGF-1 plasmid on injured motoneurons in neonatal rats

Mean total estimates of the number of motoneurons per facial nucleus are shown in **Table 4.2.**

Table 4.2

Mean total number of motoneurons (± SEM) per facial nucleus after facial nerve avulsion in adult animals (300-350g) 1- and 3-months post injury, examining the effect of prior gene transfer of MGF and L.IGF-1.

	Adult (1 month survival)		*Adult (3 month survival)*	
Experimental group	*Non-operated*	*Operated*	*Non-operated*	*Operated*
Normal (untreated/unoperated)	2957 ± 172* (n=6)	3242 ± 325*	3068 ± 130 (n=6)	3272 ± 198
Avulsion only	3278 ± 150* (n=6)	806 ± 80*	3252 ± 285 (n=6)	586 ± 255
Control plasmid +avulsion	3572 ± 98 (n=6)	809 ± 42	3343 ± 220 (n=6)	373 ± 330
L.IGF-1 + avulsion	3473 ± 106 (n=6)	1638 ± 52	3246 ± 236 (n=6)	1128 ± 455
MGF + avulsion	3549 ± 72 (n=6)	2807 ± 92	3505 ± 199 (n=6)	972 ± 180

* indicates that data is taken from **Table 3.5**

Adult animals injected with MGF plasmid (n=6) showed a significantly greater number of motoneurons in the ipsilateral facial nucleus 1 month post-avulsion compared to the non-injured side as well as to normal (untreated/uninjured) and control plasmid groups (n=6) (**Fig. 4-5**). The number of motoneurons surviving nerve avulsion increased from 806 ± 80 motoneurons (25% of the value for normal animals, left and right nuclei combined), and 809

± 42 motoneurons (control plasmid, ipsilateral side), to 2807 ± 92 in the MGF-treated group (91% of the value for normal animals). Therefore neuroprotection is conferred to 88% of the motoneurons that were lost due to nerve avulsion compared to normal animals. Micrographs depicting avulsion-induced motoneurons loss and its prevention by injection of MGF plasmid are shown in **Fig. 4-4**. In animals injected with the plasmid encoding L.IGF-1, it was observed that the number of motoneurons in the ipsilateral facial nucleus, which had survived nerve avulsion had increased to 1638 ± 52 (53% of the value for normal animals). Such an increase corresponds to 37% of motoneurons that were lost due to nerve avulsion alone, which is just 42% of the increase associated with treatment with MGF plasmid.

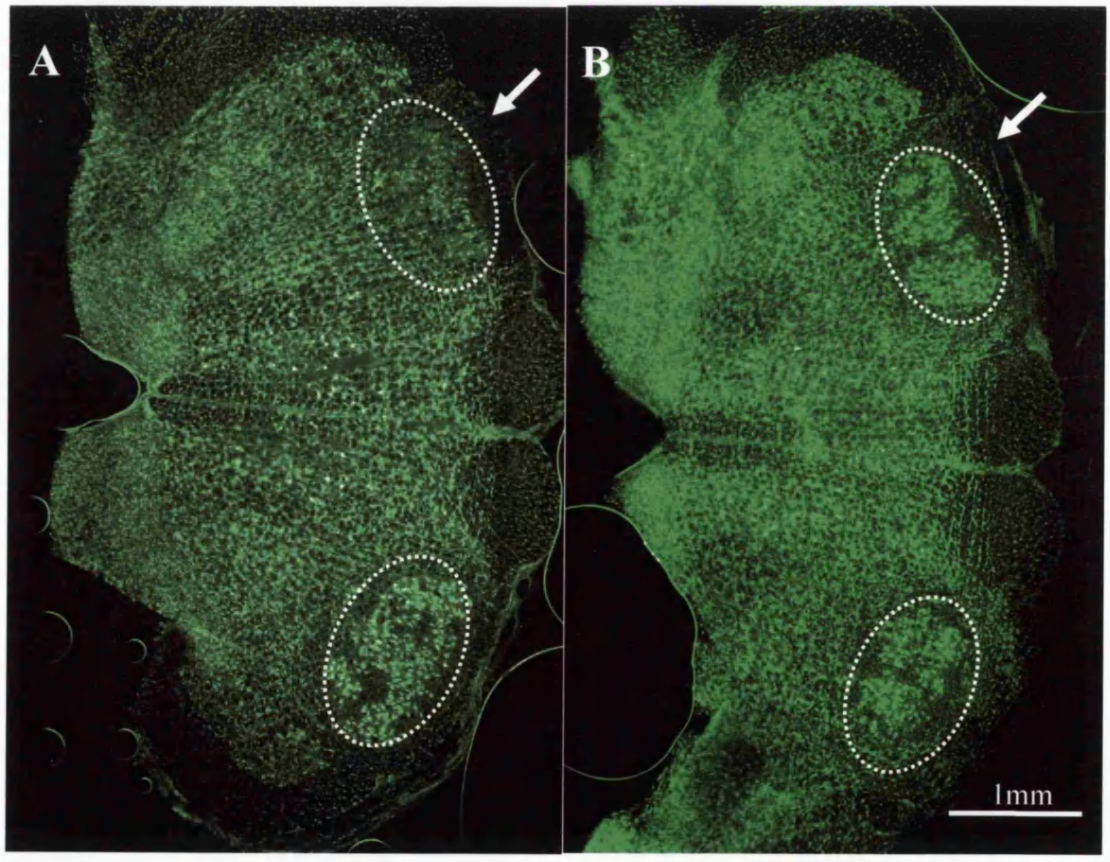


Figure 4-4 Effect of nerve avulsion alone (A), and avulsion with prior MGF gene transfer (B), on motoneurons in the facial nucleus in an adult (6 month-old) rat. 70μm vibratome sections through the brainstem at the level of the facial nucleus, stained with a fluorescent basophilic dye (YOYO-1) viewed under epifluorescence. Arrow indicates operated side. Facial nuclei are indicated with dashed oval boundaries.

To determine whether injection of MGF plasmid was able to enhance survival of motoneurons for longer than 1 month, an analogous experiment was set up where 6 month-old rats were allowed to survive for 3 months. The total number of motoneurons per facial nucleus is shown in **Fig. 4-5** adjacent to the previous results for 1 month survival.

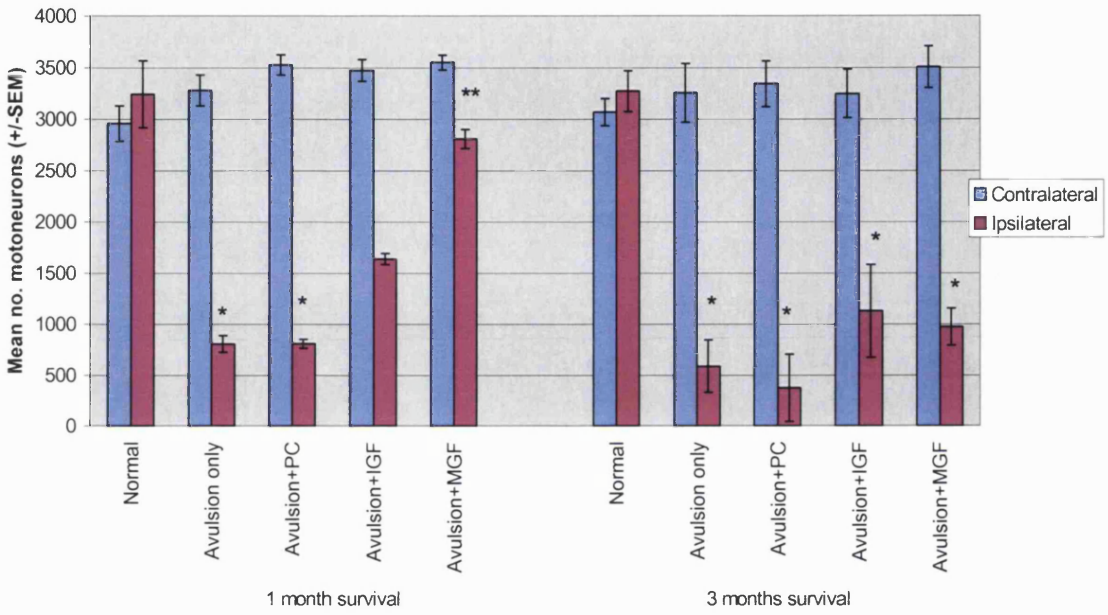


Figure 4-5 Effect of MGF plasmid on the survival of avulsed motoneurons in 6 month-old rats, 1 and 3 months after injury. Means are given plus and minus the standard error of the mean. * indicates significant motoneuron loss ipsilaterally compared to the mean value for the combined estimates of for left and right nuclei of normal rats. ** indicates significant increase in motoneuron survival ipsilaterally compared to the ipsilateral nucleus of rats subjected to avulsion only and avulsion with L.IGF-1 groups (Kruskal-Wallis and Mann-Whitney U-test, P=0.05).

When adult animals were allowed to survive for 3 months after avulsion, there was a further loss of motoneurons in the ipsilateral facial nucleus from 74% (806 ± 80 motoneurons) to 83% (586 ± 255 motoneurons, n=6) of the combined mean total for left and right nuclei in normal animals. Rats injected with either a control plasmid (n=6) or L.IGF-1 plasmid (n=6) also showed further loss from 74% to 88% (373 ± 330 motoneurons) and from 47% to 64%

(1128 ± 420 motoneurons) in the ipsilateral facial nucleus, neither of which is significantly different from the 1 month survival counterparts. However, rats treated with MGF plasmid (n=6) showed a significant reduction in the number of surviving motoneurons from 91% at 1-month, to 69% (972 ± 180 motoneurons) at 3 months after injury compared to the mean of combined left and right nuclei normal animals.

4.2.2 Effect of MGF and L.IGF-1 plasmid on injured motoneurons in neonatal rats

Injection of MGF plasmid 7 days prior to nerve injury had no effect on the number of surviving motoneurons after avulsion in neonatal animals compared to rats subjected to avulsion only. In fact, at 3 days post-injury the number of surviving motoneurons in the MGF-treated group was significantly less than those surviving avulsion alone (774 ± 211 *versus* 1842 ± 223 motoneurons respectively). The effect of L.IGF-1 plasmid is similar to MGF in that it had no effect on the number of motoneurons surviving avulsion 7 days post-operatively and, at 3 days, more motoneurons are lost compared to rats subjected to avulsion alone. Motoneuron estimates are tabulated in **Table 4.3** and represented graphically in **Fig. 4-6**.

Table 4.3

Mean total number of motoneurons (± SEM) per facial nucleus after facial nerve avulsion in neonatal animals 1, 3 and 7 days post injury, examining the effect of prior gene transfer of MGF and L.IGF-1.

	Neonate (1-day)		*Neonate (3-day)*		*Neonate (7-day)*	
Experimental group	*Non-op*	*Operated*	*Non-op*	*Operated*	*Non-op*	*Operated*
Normal (untreated/unoperated)	3447 ± 296 (n=5)	2902 ± 310	3075 ± 216 (n=5)	2975 ± 120	3225± 313 (n=8)*	3419 ± 276*
Avulsion only	3752 ± 349 (n=7)	3104 ± 369	3801 ± 232 (n=8)	1842 ± 223	3584± 538 (n=8)*	554 ± 76*
L.IGF-1 + avulsion	No data	No data	3381 ± 104 (n=4)	1107 ± 149	3423 ± 98 (n=6)	589 ± 28
MGF + avulsion	3938 ± 308 (n=6)	2831 ± 262	2888 ± 299 (n=5)	774 ± 211	3200 ± 119 (n=5)	811 ± 147

* indicates that data is taken from **Table3.5**

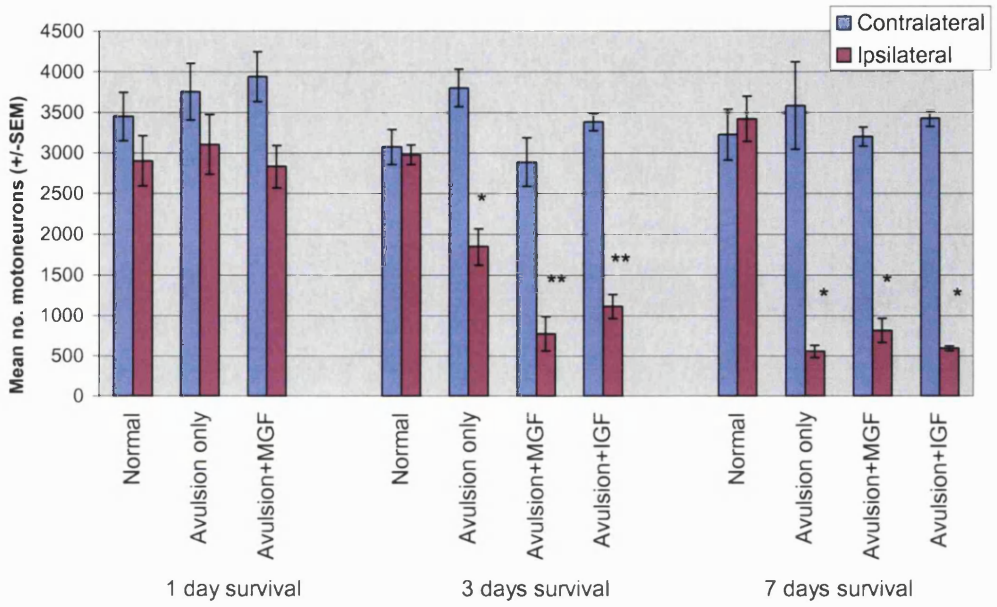


Figure 4-6 Effect of MGF plasmid on the survival of avulsed motoneurons in 7 day-old rats, 1, 3 and 7 days after injury. Means are given plus and minus the standard error of the mean. * indicates significant motoneuron loss ipsilaterally compared to the mean value for the combined estimates of for left and right nuclei of normal rats. ** indicates significant motoneuron loss compared to the ipsilateral nucleus of rats subjected to avulsion only at 3-days post avulsion (Kruskal-Wallis and Mann-Whitney U-test).

4.3 Discussion

The results of this study have shown that intramuscular injection of a plasmid encoding MGF, prior to injury, confers significant protection to facial motoneurons in adult rats for up to 1 month post-injury. Additionally, the level of neuroprotection observed with MGF gene transfer is greater than twice that afforded by L.IGF-1. These are significant findings because this is the first time that neuroprotective properties of MGF have been investigated following the initial implication of its significance in active/damaged skeletal muscle (McKoy et al. 1999; Yang, S. et al. 1996) and the first time that motoneuron survival in adult rats has been promoted by IGF-1 gene transfer irrespective of isoform. Additionally, this data supports the notion that MGF, as the putative autocrine/paracrine-acting splice variant of IGF-1, is more important for survival of lethally challenged adult motoneurons.

4.3.1 Significant neuroprotection at 1 month

The present study indicates that muscle gene transfer is able to expose motoneurons to elevated levels of MGF, which significantly increases the capacity of these motoneurons to survive lethal nerve trauma. Not withstanding differences in methods of gene transfer and counting of motoneurons, survival induced by MGF gene transfer measures favourably with the increase in motoneuron survival observed in other studies of neurotrophic factor gene transfer. Adenovirus encoding GDNF delivered to avulsed C7 motoneurons by gelfoam resulted in 69% survival at 8 weeks (Watabe et al. 2000). This is 10% fewer surviving motoneurons than MGF gene transfer shown in this study at 4 weeks post surgery. It is possible, therefore, that greater survival would have been apparent were the animals in that study terminated after 4 weeks. The present work did not assess motoneuron survival at 8 weeks but examination at 3 months showed loss of neuroprotection compared to that shown at 1 month. A linear decline in motoneuron number between the two time intervals would give rise to a figure less than 69% at 8 weeks but such decline cannot be concluded without repeating the experiment and examining motoneuron loss at regular time intervals between 1 and 3 months post injury. Motoneuron counts may be stable for some time after 1-month

survival, possibly dependent on vector availability, after which motoneuron numbers decline rapidly. In another study, prior gene transfer via intrathecal injection into the facial nucleus with a lentiviral vector encoding GDNF resulted in almost 100% survival of facial motoneurons up to 3 months after injury (Hottinger et al. 2000). Axotomy at the stylomastoid foramen however, was used to induce motoneuron loss in adult mice, which caused less than 60% loss of facial motoneurons compared to the non-operated facial nucleus at 30 days, showing no further loss by 90-days. This is in contrast to the present study where 75% of motoneurons were lost ipsilaterally, increasing further by 90 days. The extent to which GDNF promotes survival in the cited experiment is questionable since motoneurons would be exposed to a greater degree of trophic interaction with remnant Schwann cells, which are a rich source of neurotrophic factors, especially subsequent to peripheral injury (Fu & Gordon 1997). Although no true comparison can be made with the present study, it seems reasonable to propose that motoneuron survival promoted by MGF gene transfer is similar in extent to that afforded by GDNF gene transfer in previous studies. Importantly however, no other study has sought to expose nerve terminals in target muscle to increased levels of neurotrophic factors prior to nerve injury in adult rats. Because of the array of experimental conditions that have been used in these other studies, it is impossible to give an absolute rank score of efficacy of MGF against other neurotrophic factors. GDNF however, is accepted generally as the most potent neurotrophic factor for motoneuron survival to date (Henderson et al. 1994), in models similar to that used presently.

Gene transfer of MGF was found to be significantly more effective than L.IGF at promoting motoneuron survival. It would seem that MGF fulfills a role in promoting neuronal survival, which L.IGF-1 is only partially capable of matching. The sequence of events caused by the presence of MGF stimulation must be different from and, therefore, more effective than, or additive to those caused by L.IGF-1. Due to the presence of putative cleavage sites, mature IGF-1 may still yield from proteolytic processing of MGF (Nakayama 1997; Siegfried et al. 1992). A possibility arises, therefore, that MGF-induced motoneuron survival is additive to that caused by L.IGF-1 since interpretation of results would suggest that it is responsible for approximately half of the survival of motoneurons supported by MGF. It is essential; therefore, that activity of L.IGF-1 is discussed with regards to motoneuron survival in the

present study. Previous studies investigating motoneuron survival with IGF-1 *in vivo* have concentrated solely on the effects of the mature protein, equivalent to that found at high level in the circulation Vergani et al. 1998). This is the first time that IGF-1 has been shown to promote significantly the survival of adult motoneurons in the rat. Others have yielded only moderate protection of motoneurons in neonatal nerve axotomy models or adult mouse, similar to the level of protection afforded by L.IGF-1 in the present study. This may be significant because it shows the possibility that analogous mechanisms of motoneuron survival are present in both young and adult animals. IGF-1 signaling is associated with nerve sprouting accompanied by the expression of GAP-43 in adult animals (Caroni & Grandes 1990; Flint et al. 1999). Increased nerve sprouting induced by IGF-1 in developing chick embryos increased access of immature motoneurons to target-derived neurotrophic factors (D'Costa et al. 1998). It is possible; therefore, that exposure of adult motoneurons to increased levels of L.IGF-1 in the present study may have caused a brief period where motoneurons had greater access to neurotrophic factors from the periphery prior to nerve avulsion. This contrasts however, with observations that deprivation of target-derived neurotrophic factors in adult rats, caused by nerve avulsion, does not result in such, seemingly, rapid change of signaling so as to lead to motoneuron death at 1 week post injury (equal to the interval between injection of plasmid and nerve injury). This has not been qualified directly by experimentation. Instead, it is assumed that because mature motoneurons do not die as rapidly as those in immature animals, they are less sensitive to neurotrophic factors. This underlies the contention of extrapolating information derived from young animals, to adult animals (Aperghis et al. 2003). Alternatively, nerve-sprouting activity may have caused new connections with other targets, perhaps centrally, branching to areas of rich neurotrophic support, remaining out-of reach from nerve avulsion (**Fig. 4-7**).

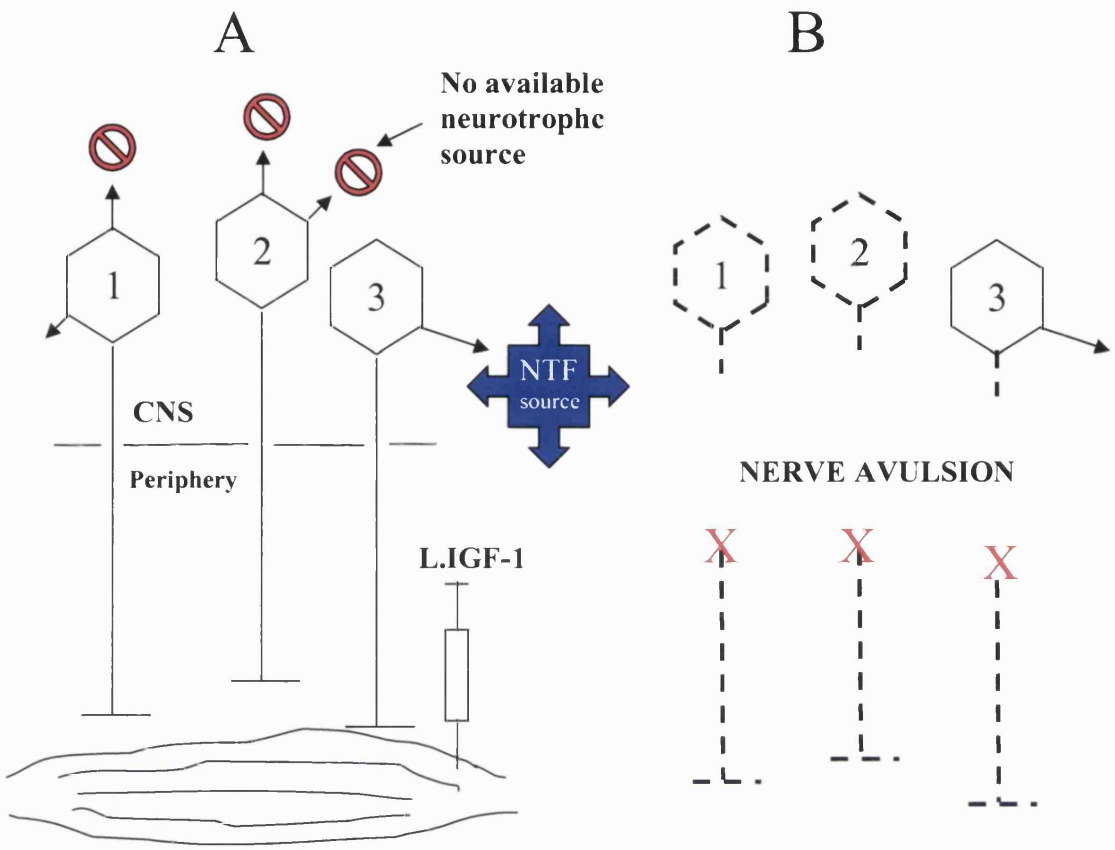


Figure 4-7 Diagram to illustrate how a mechanism of L.IGF-1-induced nerve sprouting may confer selective protection to a population of motoneurons. At 7 days following injection of L.IGF-1 plasmid, all motoneurons exposed to the effects of the transgene have branched extensively (A). Only one motoneuron (motoneuron 3), however, has located a source of trophic support. Following nerve avulsion (B), motoneurons, which failed to establish new interactions, are lost selectively. X marks avulsed motoneurons.

Cell biology studies have deciphered a number of possible signaling pathways, more than one of which may be operative in adult motoneurons, and which promote motoneuron survival, growth or differentiation in cultured cells. Notably, activation of the type-1 IGF-1 receptor activates the PI-3K/Akt pathway *in vitro* (Datta et al. 1997) and has been shown indirectly *in vivo* (Kermer et al. 2000). While it is possible that this is one up-stream signal for GAP-43 synthesis, although this has not been proven, the serine/threonine kinase Akt is

a well-characterised down-stream mediator of IGF-1-induced cellular activation. Akt has been shown to bind directly to Bad, a member of the Bcl-2 family of proteins. Dimerisation with and phosphorylation of Bad inhibits the pro-apoptotic activity of this molecule by sequestering it from and de-regulating its interaction with other down-stream components of this pathway, whose role is to promote cytochrome-c release (Kennedy et al. 1999) through channels (formed by Bcl-2) on the mitochondrial membrane. Cytochrome-c release, under some circumstances, is considered to be associated with the point at which neurons are committed to die (Fletcher et al. 2000). Interestingly, IGF-1 has been shown to prevent apoptosis in sympathetic neurons (Russell et al. 1998) and sensory neurons after neurotrophic factor withdrawal (Russell & Feldman 1999). There is clearly however, only limited effect on motoneuron survival, identifying a subpopulation of motoneurons, which are responsive to L.IGF, defined possibly by the presence of the type-1 IGF-1 receptor. Such a population may also be defined by the limitation of bystander effect of L.IGF-1 gene transfer on adjacent motoneurons in the facial nucleus (discussed further in chapter 5), given that the snout muscle is innervated by only a subset of facial motoneurons.

Signaling activated by MGF is not known. The presence of a separate ligand-binding site and/or signaling pathway has been inferred from studies on cultured mouse myoblasts, where a synthesised protein based on the sequence of the human MGF E-domain (also used in this study, chapter 5) produced activity unhindered by the presence of a function-blocking antibody of the type-1 IGF-1 receptor (Yang, S. & Goldspink 2002). This indicates that the full-length MGF is not required for activity in these cells. *De novo* synthesis of MGF, therefore, may give rise to two proteins each with different activities, which are not required in unison to promote function associated with MGF. It is not known currently whether this is also the case in motoneurons. GAP-43 mRNA expression would have been a useful marker to verify IGF-1 activity (Flint et al. 1999) derived from cleavage of vector-derived MGF but used in this study only as a marker to check for motoneuron regeneration after nerve avulsion (see chapter 5). To add further complexity, it is possible that MGF can activate the type-1 IGF-1 receptor prior to cleavage of the E-domain, which may also be correlated with intramuscular nerve sprouting (Caroni & Grandes 1990) and increased GAP-43 in motoneurons. According to a receptor-mediated model, therefore, MGF activity would be

regulated by either the presence of the IGF-1 receptor and its subsequent effect inside motoneurons additive to that of L.IGF-1, or on the presence of separate MGF receptors with their own unique activity, independent of the presence of IGF-1 receptors. This may have implications for MGF access/availability to motoneurons following its expression in target tissue.

4.3.2 Loss of neuroprotection after 1 month

When rats subjected to MGF gene transfer were allowed to survive for up to 3 months, the neuroprotection observed at 1-month post injury had disappeared completely, reverting to levels of motoneuron survival similar to that of rats subjected to avulsion only. There are two key factors that influence this situation. Firstly, limited survival beyond 1 month post injury may be non-specific to the effect of MGF and may be related to insufficiencies associated with the method of gene transfer, which are exposed only after this time point. Alternatively, in the absence of limitation of gene transfer, failure of long-term survival by MGF may expose different mechanisms of action between MGF and L.IGF-1.

Irrespective of vector transport to either motoneurons or blood (see chapter 5), vector stability would be important for determining the availability and potential for exposing motoneurons to elevated levels of MGF. Failure of long-term motoneuron survival (over 1 month) may reflect insufficient transfection of either muscle fibres or motoneurons. As a foreign substance, it is expected that plasmid DNA would be subject to immune clearance (Niidome & Huang 2002) for as long as the vector remains outside cellular membranes. It is advantageous, therefore, for cells to be tranfected as soon as possible, both to facilitate transgene expression as well as to avoid degradation of vector by the immune system. It has been shown, for example, that transfected muscle fibres retain DNA vector and express mRNA for up to 1 month following intramuscular injection (Alila et al. 1997; Levy et al. 1996) suggesting that the vector is sufficiently hidden from immune surveillance. Several methods have been employed to enhance transfection of muscle fibres after intramuscular injection of DNA plasmids, by stabilising the vector in the extracellular compartment. Two

commonly used methods include the formulation of DNA with polyvinylpyrrolidone (PVP) (Alila et al. 1997), which has been used successfully to express human IGF-1 in rat muscle or the entrapment of plasmid in cationic liposomes (Liu, F. & Huang 2002; Uchida et al. 2002). Once in the target cell, DNA must then be transcribed. It has been reported that plasmid vectors containing the CMV promoter show a decline in expression after just 2 or 3weeks after injection (Wells et al. 1997). No such decline, however, was observed in nude mice 6-weeks post injection in this study, meaning that elimination of transfected muscle fibres by the immune system is a distinct possibility. This implies that a greater total amount of protein could be made available to motoneurons conferring putatively, greater neuroprotection, if the MGF plasmid was to be administered 2-3 weeks before injury, maximising exposure of motoneurons to increased MGF in this time-window prior to nerve avulsion. Removal of transfected muscle fibres, however, would reduce the capacity for transgene expression. This may be of particular relevance in the present study since the transgene encodes specifically rabbit MGF. Interestingly, it has been shown previously that induction of inflammation after repeated injection of phytohaemagglutinin into the snout muscle, which induces an inflammatory reaction in this tissue, leads to retrograde cell response in facial motoneurons (Mariotti et al. 2001). This leads to altered response to injury (Mariotti et al. 2002b), possibly by alerting damage surveillance mechanisms in the CNS and in the long-term affect the propensity of motoneurons to survive lethal nerve trauma. If muscle inflammation occurred in the present study, detrimental to long-term survival potential of motoneurons following nerve avulsion, then it would be due to transgene expression rather than immune reaction to the vector since no significant further loss of motoneurons was observed at 3-months in rats injected with a control plasmid, which contained no gene insert.

Irrespective of the nature of target perturbation induced by intramuscular injection of plasmid, the effect is likely only to operate prior to loss of contact between nerve and muscle, caused by either nerve avulsion or removal of tranfected muscle fibres by the immune system. This has considerable implications for proposed mechanisms of signaling induced and/or maintained by target-derived neurotrophic factors with regards to sustaining intracellular mechanisms independent of their availability. As long as MGF is present in a

given place at a specific time then irrespective of its long-term availability, only a finite level of neuroprotection will be achieved. It is conceivable also, that vector transported to cell bodies and successfully expressed could render motoneurons vulnerable to interaction with resident immune cells of the CNS (e.g. microglia). Removal of transfected motoneurons in such a manner, *i.e.* without mechanical damage to the nerve, has not been reported previously. Prolonged exposure of motoneurons to inflammatory cytokines however, is known to render motoneurons more vulnerable to axotomy-induced loss of facial motoneurons (Raivich et al. 2002). T-lymphocytes, as part of the cell-specific repertoire mediating antigen removal are a known source of TNF-α (Burger & Dayer 2002), and may cause degradation of motoneurons over time. This process may, in part, be responsible for loss of motoneurons in MGF treated rats beyond 3 months post injury but it remains unclear whether this would be caused by expression of foreign gene products. One way to suppress inflammation due to transgene expression could involve co-treatment of animals with the anti-inflammatory glucocorticoid dexamethasone. This has been used successfully in combination with adenoviral gene transgene expression in the facial nucleus to prolong transgene expression (Hermens & Verhaagen 1998). Given that the expression vector used in the present study contained a myosin light-chain enhancer cassette, it seems most likely that target muscle is the major source of transgene expression. The amount of MGF providing optimal levels of gene expression would be dependent ultimately on protein half-life, which is discussed in more detail in chapter 5, irrespective of whether protein is intrinsically unstable or invokes immune reaction resulting in breakdown of the transfected cell and its contents. Once this is known, adjustments may be made to the protocol (*e.g.* vector concentration, time allotted for transgene expression) so as to maximise transgene expression in as short time as possible before onset of degenerative processes outlined above. Many other factors are known to be important for muscle gene transfer. These include: needle type; speed of injection; volume of injection fluid; tonicity of injection fluid; type of solute; type of muscle; physiological condition of the muscle (Levy et al. 1996); age and sex of the animals (Wells & Goldspink 1992). Tonicity of injection fluid and type of solute were shown to affect motoneuron survival in chapter 3. It is possible that this information may be used to further optimise muscle gene transfer so as eliminate the possibility that inadequate transfection and gene expression were not contributory factors to

the decline of motoneuron survival beyond 1 month post nerve avulsion in rats subjected to MGF gene transfer prior to injury.

Lack of motoneuron survival in the MGF plasmid-injected rats beyond 1 month survival may not be related to inadequacies associated with gene transfer and may, instead, be representative of the changes in intracellular signaling and/or gene expression, or lack of them/it. This may be specific to the nature of altered response to injury due to exposure of motoneurons to increased MGF. Long-term motoneuron survival (up to 12 weeks) by neurotrophic factors has been reported in other models of adult motoneuron death. It is difficult however, to compare these studies directly because of different experimental protocols and methods of gene transfer that change ultimately the long-term availability of protein. Continuous transgene expression of GDNF was assured throughout the period of 12 weeks in proximally transected mouse motoneurons treated with an intracranially injected lentivirus (Hottinger et al. 2000) or with adenvirus to avulsed nerve stumps (Watabe et al. 2000). In contrast, dosing of rats with BDNF for just the first 4 weeks post injury was also enough to confer motoneuron survival for a total of 12 weeks after spinal root avulsion (Novikov et al. 1997). There are clearly, issues concerning long-term signaling invoked by short-term exposure, which cannot be discussed effectively until further information is known about the spatial and temporal availability of MGF to motoneurons in the current study (see chapter 5). There may be significant insight however, into relative mechanisms of action of MGF *versus* L.IGF-1 further to that apparent at 1 month post-injury. The present results show that adult motoneurons, despite prolonged cellular survival induced by MGF gene transfer, eventually succumb to the effects of nerve avulsion suggesting the failure to locate further neurotrophic interaction either intrinsically or via other sources. In contrast, motoneuron survival in rats subjected to L.IGF gene transfer was not significantly less at 3 months compared to that found at 1 month, and analysis of intra-group variation showed some rats had similar numbers of motoneurons ipsilaterally to the group mean value at 1 month. This could mean that L.IGF-1, through its putative interaction with apoptotic mechanisms and/or effect on nerve sprouting, can sustain long term survival of a smaller subset of motoneurons more effectively than MGF can sustain the survival of the majority of the facial motoneuron population. It is reasonable to suggest at this stage, therefore, that

MGF does not have a direct effect on motoneurons survival, *i.e.* interfering directly with the effector mechanisms of neuron death and/or survival. It may, instead, prime protective stress mechanisms common to all facial motoneurons, prior to injury. In the continued absence of further signals that interact directly with the 'survive-or-die' pathway, stress signals become overridden. This view however, is in conflict with that described earlier where full-length MGF may be cleaved to yield mature IGF-1 so as to materialise with the additive effect of both IGF-1 splice variants. In the face of evidence however, showing that systemic IGF-1 can enter through the blood-brain barrier (Pan & Kastin 2000; Reinhardt & Bondy 1994) and affect central neurons (Trejo et al. 2001), there is a distinct possibility that the apparent longer term activity of L.IGF-1 plasmid in the present study may be mediated via release of IGF-1 systemically. Only mature IGF-1 protein has been found in blood. The potential of skeletal muscle to act as an endocrine organ has been discussed previously (MacColl et al. 1999).

4.3.3 Lack of neuroprotection in neonatal rats

The present results show a complete lack of neuroprotection afforded by prior MGF or L.IGF-1 gene transfer, 1 week after injury in neonatal rats. Because of the apparent sensitivity of immature motoneurons to nerve axotomy, and in particular nerve avulsion (Yuan et al. 2000), the effects of gene transfer at earlier time points were examined. Prior injection of vector significantly reduced numbers of surviving motoneurons at 3 days compared to avulsion alone suggesting that gene transfer had a deleterious effect. However, it cannot be concluded that this was due to the effects of MGF and L.IGF-1 gene transfer *per se* since a control plasmid group was not examined on this occasion. However, on account of observations made immediately after injection and before injury, it was clear that substantial bruising throughout the poorly developed snout muscle in these animals marked considerable damage in this tissue. Muscle damage due to injection, irrespective of what it comprises, exposes potentially serious methological inadequacy in minimising equivocal observations. Such an outcome however, could not have been predicted on account of other studies (Baumgartner & Shine 1997; Ribotta et al. 1997) at the time of experimentation.

Baumgartner and colleagues (1998) gave 10μl injections (analogous to this experiment) of adenovirus-encoded GDNF into the 1-day-old rat snout muscle, only 2 days prior to injury. Furthermore, Ribotta and colleagues (1997) gave just 4μl volume into the snout region only 24 hours prior to injury. In these experiments, such treatment resulted in significant neuroprotection 7 days after injury. But differences in vector, the period allowed for gene expression and the method used for counting neurons may have had considerable influence in the differing outcome of these studies. Adenoviral vectors have greater transfection efficiency than plasmid vectors (Glatzel et al. 2000) so there would be more transfected muscle fibres with greater numbers of vector units per cell. A natural degree of neurotropism of these vectors allows gene transfer to motoneurons, ensuring retrograde transport of gene product from muscle and, therefore, long-term exposure of cell bodies to gene product despite loss of nerve-target interaction. Finally, allowing just 2 days after injection of an adenoviral vector avoids the immune response reported commonly with this type of vector.

A suggested mechanism for the decreased motoneuron survival in neonatal animals in present experiment following plasmid injection may involve inflammation of the injected muscle due to puncture wounds from the syringe needle and/or mechanical damage from the injected liquid, degradation of vector by the immune system, disruption of motoneuron interaction with their target resulting in prolonged deprivation of immature motoneuron cell bodies to target derived neurotrophic factors. This might result in substantial death of motoneurons at 3 days, in addition to that observed due to avulsion alone. A more appropriate experiment would have involved the application of MGF protein to the lesion site at the time of surgery. Due to limited stock of E-domain protein however, no such experiment was attempted further than that applied to adult animals (chapter 5). Additionally, this was deemed an unnecessary experiment given the initial interpretation of lack of effect of MGF gene transfer in these animals. To investigate the suggested mechanism in these animals, neuroglial markers in the brain would help indicate CNS damage in the facial nucleus after injection of vector alone such as that reported by Mariotti et al. (1999) following intramuscular injection of a muscle-inflammatory protein. These may be compared to age-matched controls subject to nerve avulsion alone.

In conclusion, MGF gene transfer 7 days prior to facial nerve avulsion conferred significant protection of motoneurons at 1 month post injury but the majority of surviving motoneurons were lost by 3 months. Interestingly, MGF gene transfer was significantly more effective at promoting motoneuron survival than L.IGF-1, which nonetheless, was able to protect approximately half of the motoneurons. Gene transfer in neonatal animals was inconclusive but raised the question of alternative mechanisms operating after intramuscular injection of vector.

Chapter-5

Mechanisms of Neuroprotection

5.1 Introduction

The experiments described in this chapter are concerned with understanding the mechanism of neuroprotection afforded by MGF and prior muscle damage, paying particular attention to endogenous gene expression of IGF-1 mRNAs as well as exogenous MGF following intramuscular injection of MGF plasmid. In addition, by examining correlates of retrograde cellular responses to nerve avulsion, clues may arise as to central changes in the stereotyped motoneuron reaction specific to particular neuroprotective treatments. Such information may have wider significance to the endogenous mechanism of MGF activity, and to understanding further the retrograde influence of skeletal muscle activity on motoneurons.

5.1.1 Transport of vector following intramuscular injection

Exogenous supply of the MGF gene in peripheral targets results in increased motoneuron survival (chapter 4). However, the temporal and spatial mechanism of events regarding MGF mRNA expression and protein availability leading to neuroprotection remains uncertain. The experiments described in the first part of this study are aimed at helping to decipher these events.

Gene transfer of neurotrophic factors to intact motoneurons leads to long-term survival following nerve injury. Expression of the gene within motoneuron cell bodies ensures *de novo* protein synthesis and, therefore, availability of the neurotrophic factor within the cell for as long as the vector remains viable for gene expression. This has been achieved in previous studies by use of viral vectors injected either into to target-muscle (Baumgartner & Shine 1997; 1998a 1998b; Millecamps et al. 2002; Ribotta et al. 1997) or intracranial injection into the vicinity of the facial nucleus (Hottinger et al. 2000). Unlike adenoviruses, which are the most commonly used form of viral vector in motoneuron gene transfer experiments, plasmid DNA vectors have no known receptor for tropic interaction with motoneurons. It has been shown nonetheless that plasmid vectors are transported retrogradely to cell bodies following intraneural injection or by direct application to

axotomised proximal nerve stumps (Johnson, I.P. et al. 1998; Sahenk et al. 1993). It has not been established whether plasmids may be transported retrogradely following intramuscular injection. The use of gene transfer in aiming to expose motoneurons to increased levels of MGF and L.IGF-1 requires characterisation, therefore, by confirming mRNA and/or protein expression derived specifically from the vector subsequent to intramuscular injection. mRNA expression in motoneuron cell bodies and/or target-muscle is investigated in the present study using a qualitative RT-PCR approach. Analysis of muscle, brainstem and other tissues will shed light on the extent of transport of the vector from its injection site.

5.1.2 Endogenous availability of MGF to injured motoneurons

Most neurotrophic factors have been shown to promote the survival of motoneurons following direct application to axotomised proximal nerve stumps. In order to confer neuroprotection, therefore, these neurotrophic factors are required by motoneurons at the time of injury. This does not necessarily dictate however, an endogenous role of promoting the survival of intact adult motoneurons. Following facial nerve avulsion, there can be no further influence of target-derived factors on motoneurons that once supplied them since only the mature IGF-1 hormone is found in the circulation. It is established already that elevated MGF gene expression in skeletal muscle is a result of increased muscular activity and damage/repair (McKoy et al. 1999; Yang, S. et al. 1996). Furthermore, prior intramuscular injection of a plasmid encoding MGF is associated with increased survival of adult facial motoneurons subjected to nerve avulsion (chapter-4). It is unclear however, whether MGF has a role in promoting motoneuron survival after nerve-muscle interaction has been disrupted.

In the absence of an antibody directed specifically to MGF, expression of MGF endogenously or following intramuscular injection of its gene cannot give information relating the presence of MGF protein within cell bodies to increased motoneuron survival. To acquire this information, the present study uses an alternative strategy that aims to expose the proximal nerve stumps of avulsed motoneurons to the putative active component of MGF, the E-domain peptide (Siegfried et al. 1992; Yang, S. & Goldspink 2002), by

placing close to the lesion site gelfoam soaked in this substance at the time of injury. Comparing motoneuron survival to that found in animals subjected to prior intramuscular injection of this peptide (this study) or the MGF gene (chapter 4) will give insight firstly, on the temporal relationship between MGF availability and motoneuron survival, secondly, on the mechanism of MGF signal transduction, and finally, on the therapeutic potential of MGF/E-peptide for repairing injured motoneurons.

5.1.3 Muscle damage

5.1.3.1 Stereotyped response of muscle to injury

Muscle degeneration occurs in two distinct phases: firstly, a non-inflammatory necrosis occurs within the muscle fibres coinciding with the breakdown of structural components and organelles of the muscle cell; and the second phase is characterised by infiltration of the muscle fibre by circulating macrophages, which remove the necrotic debris (Carlson & Faulkner 1983). Morphological studies with the myotoxic agent bupivacaine have revealed that the first stage of intrinsic degeneration (events prior to infiltration) is complete by about 12 hours after injection (Hall-Craggs 1974; Yoshimura & Schotland 1987). Over the course of the next 12-hours macrophages begin to infiltrate and by 3 days phagocytose the debris (Ishiura et al. 1983). Concomitant with these changes are the proliferation of myoblasts and subsequent formation of multinucleate myotubes. From 4 days, and proceeding for the next 7 days myotubes begin to grow and differentiate into fibres (Hall-Craggs 1974; Sadeh 1988). During this period, elevated IGF-1 mRNA has been reported in adult rats commencing from 5 days post injection, lasting until at least 15 days (Marsh et al. 1997). A 7 day period post injection was chosen in this study at which to inflict nerve injury. Morphological assessment and MGF *versus* L.IGF-1 *in situ* mRNA analysis, was carried out at 4 and 7 days post injection of bupivacaine, physiological saline or distilled water.

5.1.3.2 Expression of IGF-1 mRNA in the neuromuscular system

To give a better understanding of the paracrine/autocrine activity of MGF, it is advantageous to characterise more fully the relative preferences of mRNA transcripts in the neuromuscular system with regard to the endogenous availability of MGF to motoneurons. Alternative splicing of the IGF-1 gene occurs determining an array of mRNAs that requires some further explanation for the benefit of the present study. The rat IGF-1 gene has two known promoters, promotor 1 and promotor 2. These lay up-stream to exon 1 and 2 sequences respectively. The appearance of only one of either exon 1 (class-1 IGF-1) or exon 2 (class-2 IGF-1) in mRNA, spliced to exon 3, designates specific activity from promotor-1 and promotor 2 respectively. Both classes have been found in hepatic and non-hepatic tissues. Based on observations of relative tissue-selectivity and the differential effects of GH stimulation in hypophysectomised rats (Lowe et al. 1987) and during development (Adamo et al. 1988), it has been suggested that, in addition to splicing at the 3' end (see chapter 4), alternate leader exons (1 or 2) add further molecular basis for endocrine *versus* local action (LeRoith & Roberts 1991). Within class-1 IGF-1 mRNAs, there is further variation resulting from alternative 5'UTRs, which result presumably, from use of different transcriptional start points. These alternative 5'UTRs within exon 1 are characterised by the insertion or deletion of a 186 base-pair pseudo-exon (Lowe et al. 1987; Shimatsu & Rotwein 1987a).

Increased IGF-1 mRNA expression has been detected previously in muscle (Glazner et al. 1993; Glazner & Ishii 1995) and centrally (Hammarberg et al. 1998) in response to denervation. Detection in these studies however, was limited only to sequences specific to exons 3 and 4 so was not able to relate expression of IGF-1 to the autocrine/paracrine-acting MGF. Full knowledge of the wider complement of potential IGF-1 mRNAs in response to nerve injury has not until now been investigated. Such information would supplement prospective differential levels of neuroprotection afforded by intramuscular injections of L.IGF-1 and MGF transgenes. The current study uses qualitative RT-PCR to investigate the relative expression levels IGF-1 mRNAs in denervated muscle as well as in the brainstem. The knowledge gained will help us understand the extent to which the IGF-1 gene may be expressed in relation to localised tissue repair in the neuromuscular system, as

well as to optimise further gene therapy strategies for protecting intact motoneurons from subsequent lethal nerve trauma.

5.1.4 Retrograde cellular responses to motoneuron injury

A peripheral nerve lesion leads to a characteristic retrograde reaction with substantial changes in morphology, metabolism and gene expression of the injured motoneurons and the surrounding neuroglial cells (Kreutzberg 1996; Raivich et al. 1999a; Schwaiger et al. 1998). Such alterations can be observed using specific cellular markers, whose expression may aid identification of neuroprotective mechanisms elicited by the various modifications to the neuromuscular system described in the current thesis. This section reviews these changes emphasising particular correlates of cellular activation that are useful to these studies of neuroprotection.

5.1.4.1 Morphological Alterations

Changes in nerve cells resulting from the interruption of their axons (axotomy), were first described for neurons in the facial nucleus by Nissl in 1894. These are as follows: Nissl body dispersion (chromatolysis); decrease in size of Nissl bodies; eccentric nuclei; swelling of cell bodies; folding of the nuclear membrane and nuclear cap formation. These are all useful indicators of reaction to injury that may be visualised under the light microscope. Other observations including, changes in the number of organelles, abundance of free or bound ribosomes and re-organisation of the rough endoplasmic reticulum (rER) are seen only at the electron microscopic level. The precise changes that occur in the cell body, how quickly and their duration, depend on factors, amongst others, such as age and type of injury (distance of the lesion from the cell body) (Fawcett & Keynes 1990; Lieberman 1971; Soreide 1981a; 1981b; Watson 1974). Since the same avulsion will be introduced to all animal age groups, the latter is not an important variable to this study. However, the effect of age on the neuronal response to injury may be significant to this study because differences in response to neurotrophic factor treatment between animals of different ages

may be signified by changes in the morphological repertoire of events subsequent to injury (Soreide 1981a).

There are also axonal changes in response to injury. Axotomy of motoneuron axons results in the process classically referred to as Wallerian degeneration. This leads to the removal and recycling of axonal and myelin-derived material, and prepares the environment through which regenerating axons grow. Dividing Schwann cells remain inside the basal lamina tube that surrounded the original nerve fibre. Collectively, these structures are known as the endoneurial tubes or bands of Bunger. Preservation of this continuity between proximal nerve stump and target is a major determinant of successful axonal regeneration (Jessell 1991).

5.1.4.2 Metabolic Changes

In an attempt to regenerate their axons after peripheral lesion, motoneurons switch metabolic status from one that is required to maintain neurotransmission (function), to one that promotes the re-growth of axons to their targets (recovery). Such cellular activation is possible by recapitulating part of the embryonic program associated with axonal outgrowth and the regulation of neuronal differentiation (Aubert et al. 1995; Schwaiger et al. 1998). Some of the cell-specific and selective molecular changes that occur after injury are presented in **Table 5.1**.

Table 5.1

Correlates of protein activity and/or expression in motoneurons in response to peripheral nerve lesion. Protein with reduced activity/expression is highlighted in red.

Function	*Protein*	*Response*	*References*
Energy metabolism	Glucose-6-phoshate dehydrogenase	↑	(Harkonen & Kauffman 1974);
	6-phosphogluconate dehydrogenase	↑	Tetzlaff & Kreutzberg

			1984)
RNA synthesis	Ornithine decarboxylase	↑	(Gilad & Gilad 1983)
	Transglutaminase	↑	(Tetzlaff & Kreutzberg 1985;
	c-jun	↑	Tetzlaff et al. 1988b) (Haas et al. 1993)
Transcription factors	CREB	↑	(Herdegen et al. 1992)
Neurotransmission	Choline acetyltransferase	↓	{Piehl et al. 1993)
	Acetylcholinesterase	↓	(Hoover & Hancock 1985)
Cytoskeletal	Actin	↑	(Tetzlaff et al. 1988a)
	T-α1 tubulin	↑	(Tetzlaff et al. 1991)
	Neurofilament	↓	(Tetzlaff et al. 1988a; Tetzlaff et al. 1991)
Growth	GAP-43	↑	(Linda et al. 1992)
	Akt/PKB	↑	(Johnson, H. et al. 1995)
Neuropeptides	CGRP	↑	(Streit et al. 1989)
	Galanin	↑	(Arvidsson et al. 1990)

From the table, it is clear that a multitude of markers may be used to indicate cellular status after injury. Those used in this study are described below.

Growth-associated protein-43 (GAP-43) is found in the growth cones of developing motoneurons and, via protein kinase-C (PKC) -mediated signaling, appears to be necessary

for the extension of axons to their target organs (Gispen et al. 1991). It is normally expressed at high levels during development, and is down-regulated after contact with muscle has been made (Caroni & Becker 1992; Fitzgerald et al. 1991; Reynolds et al. 1991). It becomes re-expressed in motoneurons during adulthood after nerve injury (Linda et al. 1992; Mehta et al. 1993; Piehl et al. 1993) and furthermore, may be up-regulated by direct injection of growth factors into peripheral targets (Kobayashi et al. 1997; Lutz et al. 1999; Piehl et al. 1998a). It is therefore a good marker of motoneuron regeneration in response to injury.

Calcitonin gene-related peptide (CGRP) is a neuropeptide, which is up-regulated in surviving motoneurons after nerve injury (Arvidsson et al. 1990; Piehl et al. 1998b; Streit et al. 1989). Its function is not clear but CGRP content in motoneurons is positively correlated with the degree of intramuscular nerve sprouting seen after Botulinum toxin-induced paralysis (Tarabal et al. 1996b) and after injection of a nerve-sprouting agent such as ciliary neurotrophic factor (CNTF) (Tarabal et al. 1996a).

Akt or protein kinase-B is phosphorylated by the activated products of phosphoinosityl-3 kinase (PI-3K) signaling (Coffer et al. 1998; Dudek et al. 1997), substrates for which derive (amongst others) from receptor tyrosine-kinase-mediated signaling (Zheng et al. 2000). Many neurotrophic factors mediate their actions via this type of receptor signaling *in vitro* (Cheng et al. 2000; Dolcet et al. 2001; Franke, Yang, et al. 1995). Moreover, signaling via Akt has been shown to prevent injury-induced motoneuron death (Dolcet et al. 1999; Namikawa et al. 2000). CGRP and Akt represent two different classes of protein with different functions, both of which will be used in the present study as markers of motoneuron survival.

5.1.4.3 Neuroglial Response to Motoneuron Injury

In addition to neurons, the local neuronal microenvironment consisting mainly of glial cells is also affected by remote axonal lesion. Astrocytes react with strong morphological changes to different kinds of lesion (Kiefer et al. 1993). They change from a protoplasmic to a

fibrous cell shape and show hypertrophy, extending long processes far into the neuropil (Schwaiger et al. 1998). Such a reaction may be visualised easily with an astrocyte-specific marker such as glial fibriliary acidic protein (GFAP), whose expression levels increase after injury-induced activation (Neiss et al. 1992). In the rat facial nucleus, astrocytes persist wrapping neuronal cell bodies for up to a year after injury (Kreutzberg 1993) making them a suitable marker for extent of injury in this study.

Microglial cells, as the intrinsic immune cells of the brain, exhibit morphological alterations as an unspecific repertoire to defend the CNS from injury. There is a rapid migration and proliferation of microglia in the immediate vicinity of injured motoneurons (Graeber & Kreutzberg 1988; Graeber et al. 1988). Their cell body size increases after injury and becomes stellar-shaped (Kreutzberg & Raivich 2000). In this sense, microglial cells become activated. These stimulated cells move rapidly into close vicinity with neurons, and this is associated with the period during which neurons are stripped of their synaptic input. Microglia are useful indicators of inflammatory responses, the level of which can determine whether motoneurons survive or degenerate (Kalla et al. 2001).

Together, the neuroglial response may be indicative of availability of neurotrophic support centrally.

5.1.4.4 Motoneuron cell death

Motoneuron cell death following nerve axotomy has been characterised extensively in neonatal animals and is believed to occur by an apoptotic-like process (de Bilbao & Dubois-Dauphin 1996; Dubois-Dauphin et al. 1994; Li, L. et al. 1998), similar in morphology to the programmed cell death (PCD) described initially to characterise developmental neuron death (La Velle & La Velle 1958). It is a multi-step signaling pathway allowing the removal of individual cells in the absence of provoking immune and/or inflammatory reaction within the tissue of origin (Wyllie 1987). There are numerous markers that could be used potentially to signify this process. These are associated usually with its latter events to avoid

the possibility that prior to a specific point at which neurons reach competence and/or commitment to die, promotion of cellular survival may still be achieved through reversal or diversion of the apoptotic mechanism (Francois et al. 2001; Pena et al. 2001). Caspases are the effector molecules in the apoptotic pathway. These proteases have specific intracellular targets such as proteins of the nuclear lamina and cytoskeleton. Cleavage of these substrates leads to the demise of a cell. Activation of caspases appears to be a common feature of most cell-death programs (Lodish et al. 2000) and caspase-3 is activated following axotomy of neonatal faical motoneurons (Vanderluit et al. 2000). The active cleaved fragment of caspase-3 is used in the current thesis to indicate apoptotic-like cell death. It is reported that motoneuron death following nerve avulsion may occur, in part, through a necrotic-like process. Both apoptotic- and necrotic-like cell death result in the fragmentation of nuclear DNA (Charriaut-Marlangue & Ben Ari 1995), which may be detected synonymously by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (Gavrieli et al. 1992). This was used to detect DNA fragmentation and motoneuron death in the present thesis.

5.1.5 Specific aims

Establish the location(s) of vector-derived MGF mRNA expression *in vivo* following intramuscular injection of the plasmid into the snout muscle of adult rats.

To investigate whether MGF E-domain peptide can protect facial motoneurons when made available to avulsed proximal nerve stumps in adult rats.

To determine the nature of IGF-1 mRNA expression in denervated snout muscle and brainstem in adult rats.

To indicate the mechanism of neuroprotection afforded by prior muscle damage emphasising the role of muscle inflammation and endogenous expression of IGF-1 isoforms.

Characterise the retrograde response to facial nerve avulsion in order to help clarify possible mechanisms of neuroprotection/cell death.

5.2 Results

The materials and methods for these experiments can be found in chapter-2.

5.2.1 *In vivo* expression of plasmid MGF mRNA

To determine whether MGF mRNA is expressed from the plasmid pcDNA3.0 rbMGF *in vivo,* subsequent to intramuscular injection, RTPCR analysis was carried out using EX3-F and RV3.1-R primers, which amplify product specifically from this plasmid (**Table 2.2**). PCR products were separated and visualised under UV trans-illumination (**Fig. 5-1**).

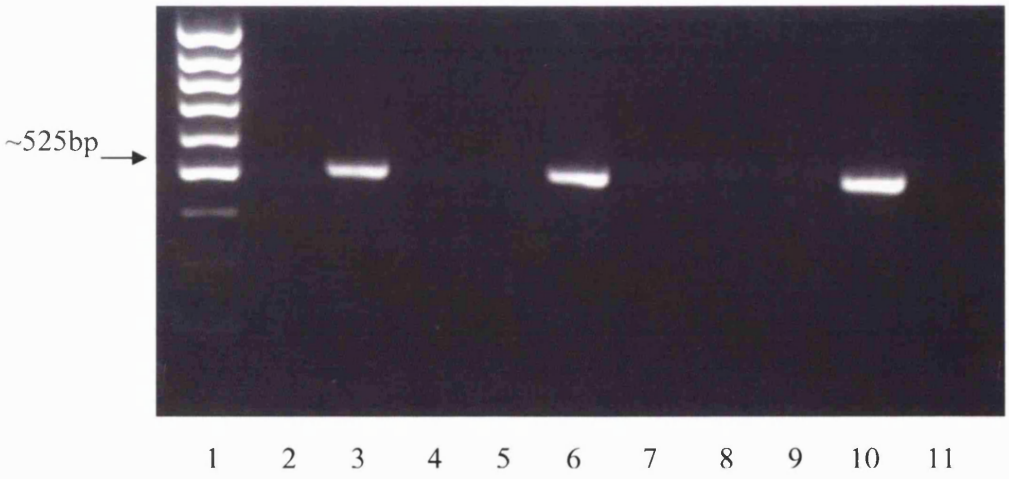


Figure 5-1 RTPCR amplification to detect plasmid-derived mRNA expression 7 days after intramuscular injection. Lane 1: 100bp marker; lane 2: water control; lane 3: 1μg pcDNA3.0 rbMGF (positive control); lane 4: liver; lane 5: blood; lane 6: snout muscle (injected side); lane 7: snout muscle (non-injected side); lane 8: total brainstem; lane 9: lumbar spinal cord; lane 10: tibialis anterior (injected side); lane 11: tibialis anterior (non-injected side).

A product of approximately 525bp was observed in the lanes corresponding to the injected-side of the snout and tibialis anterior muscles (lanes 6 and 10 respectively), 7 days after injection. These were similar in size to the product corresponding to the positive control

(lane 3). There were no amplification products in the non-injected muscle tissue or in the CNS (brainstem and spinal cord), liver or blood. PCR products were purified and sequenced. Sequence analysis revealed that the first 425bp of each of the amplification products was analogous to the published rabbit IGF-1Eb sequence (accession number X06108). The 70bp at the 3' end corresponded to the DNA sequence of pcDNA3.0. Further RTPCR analysis showed that this amplification product could be detected up to 3 weeks after injection into tibialis anterior (data not shown).

5.2.2 Effect of MGF E-domain protein on the survival of avulsed facial motoneurons

Rats were injected with either synthesized MGF E-domain peptide or recombinant human (rh)IGF-1 into the right snout muscle. After 7 days, the right facial nerve was avulsed. In another group of rats (MGFtoi, n=4), gelfoam soaked in MGF E-domain peptide was positioned at the site of axotomy at the time of surgery. All rats were allowed to survive for a period of 1 month prior to stereological and immunocytochemical analyses.

Table 5.2

Effect of MGF E domain on the survival of avulsed facial motoneurons in adult rats (300-350g) 1 month after injury. Mean totals of motoneurons per facial nucleus (± SEM).

	Experimental group					
	Normal°	*Avulsion only°*	*Avulsion + saline*	*Avulsion + L.IGF**	*Avulsion + MGF**	*Avulsion + MGFtoi*
Left (Contralateral)	2957 ± 172 (n=6)	3278 ± 150 (n=6)	3253 ± 135 (n=6)	3577 ± 174 (n=6)	3459 ± 290 (n=6)	3672 ± 78 (n=6)
Right (Ipsilateral)	3242 ± 325	806 ± 80	1380 ± 318	804 ± 78	792 ± 73	1835 ± 683

* injected into the snout muscle 7 days prior to nerve avulsion. *toi* = applied at time of injury. ° indicates that data is taken from **Table 3.5**

Estimates of motoneurons per facial nucleus for this experiment may be observed in **Table 5.2** and are represented graphically in **Fig. 5-2**. With 792 ± 73 and 804 ± 78 motoneurons in the ipsilateral facial nucleus respectively, injection of either MGF E-peptide or rhIGF-1 peptides 7 days prior to injury had no significant neuroprotective effect on the number of surviving facial motoneurons 1 month after nerve avulsion (806 ± 32 motoneurons). In contrast, injection of saline resulted in significantly more motoneurons ipsilaterally than rats treated to avulsion alone. When MGF E-peptide was applied to the injured nerve at the time of surgery however (gelfoam), an increase in the number of surviving motoneurons in the ipsilateral nucleus was observed. The mean number of motoneurons after injury in this group was significantly greater than that observed for animals to which either MGF E-peptide or rhIGF-1 had been injected intramuscularly 7 days prior to surgery (p=0.05).

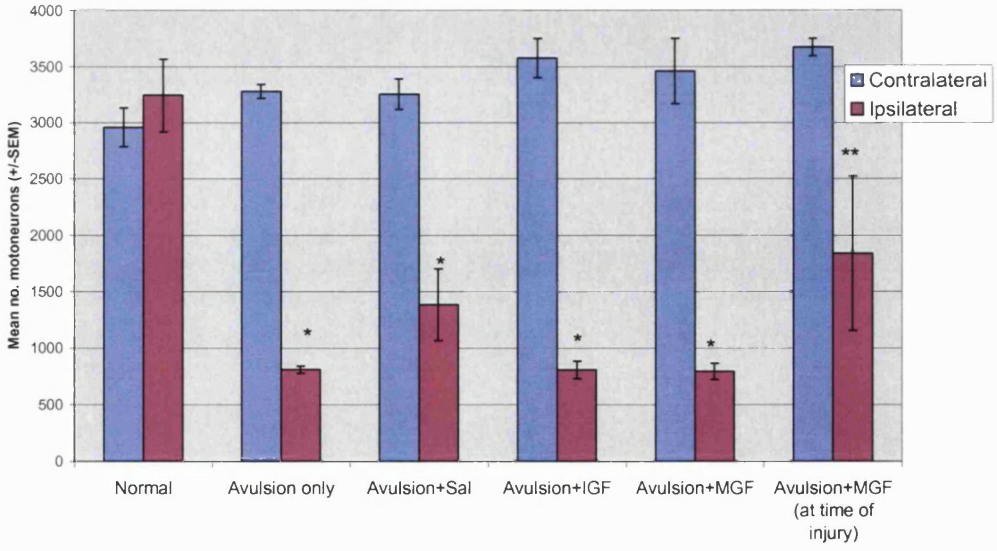


Figure 5-2 Effect of MGF E-peptide delivered either 7 days prior to or at the time of injury, on the survival of avulsed facial motoneurons 1 month after injury. Means are given plus and minus the standard error of the mean. * indicates significant motoneuron loss ipsilaterally compared to the mean of the combined left and right values in normal rats (Kruskal-Wallis and Mann-Whitney U-test, P=0.05). ** indicates significant increase in motoneuron survival ipsilaterally compared to the ipsilateral facial nucleus of rats injected with MGF peptide 7 days prior to avulsion (Mann-Whitney U-test).

To investigate further the nature of the increase in survival of motoneurons in animals treated with MGF E-peptide, values for individual rats were analsyed for variance around the calculated mean for the group (**Fig. 5-3**).

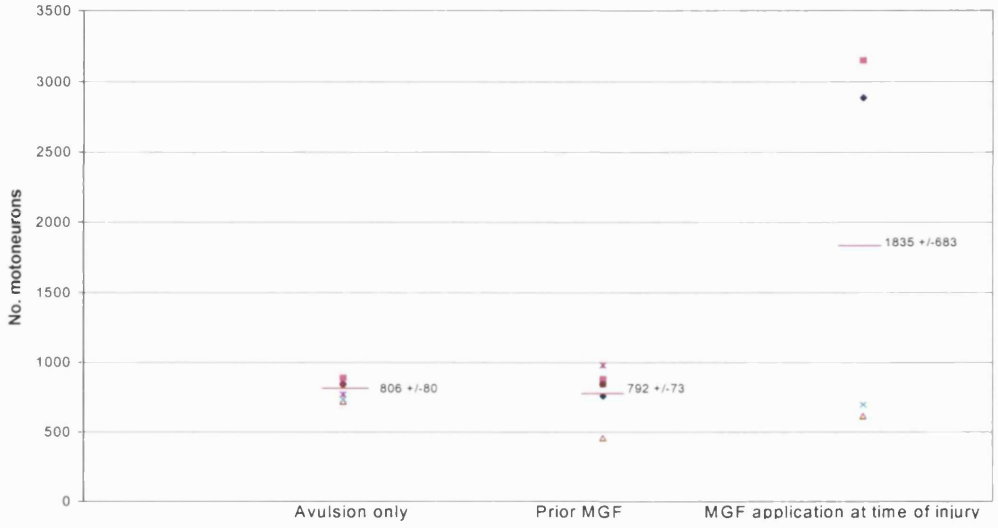


Figure 5-3 Plot to show the scatter of individual cell counts around the group mean. Red line with adjacent numerical value = mean no. motoneurons (+/- SEM)

The values for individual rats in the MGFtoi group are more variable than those rats that make up the group subjected to intramuscular injection of MGF E-peptide 7 days prior to injury (pre-MGF group). Two of the rats in the MGFtoi group have similar numbers of motoneurons in the ipsilateral facial nucleus to the mean values in both avulsion-only and pre-MGF groups (806 ± 80 and 792 ± 73 motoneurons respectively). However, the remaining 2 rats in this group have values that are similar to the mean number of motoneurons in the ipsilataral nucleus of MGF plasmid-treated rats 1 month after injury (2883 and 3147 *versus* 2807 motoneurons) where significant neuroprotection was observed in adult rats (**Fig. 4-5** and **Table 4.2**). Indeed, these two rats showed mean neuroprotection of 82% compared to the contralateral facial nucleus and 97% compared to normal animals.

5.2.3 Effect of muscle damage

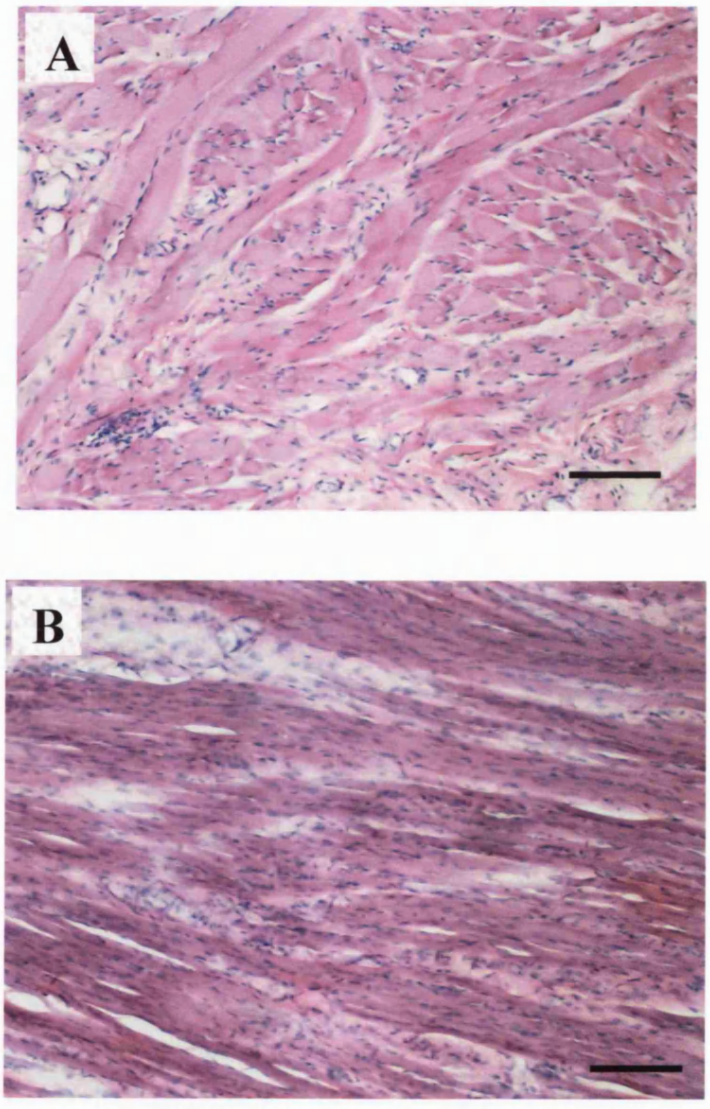
The peripheral and central effect of muscle damage was introduced to adult rats by the injection (2 per group) into the snout muscle with either distilled water or bupivacaine or physiological saline was characterised. A forth group remained untreated, which served as a control. One animal of each group was allowed to survive for 4 days prior to termination, while the remaining rat of each group survived for 7 days. After perfusion-fixation, snout muscles and brainstems were removed from all animals, sectioned and analysed for signs of muscle damage, retrograde cellular reaction and expression of MGF and L.IGF-1 mRNA expression *in situ* (muscle only).

5.2.4 Response to direct muscle injury

Haematoxylin and eosin histochemical staining revealed no change in motoneuron cell morphology either 4 or 7 days after muscle damage, irrespective of treatment. There appeared also not to be any qualitative change in number or shape of non-neuron-like cells in or in the vicinity of the facial nucleus. GFAP immuno-staining was unchanged regardless of what was injected into the snout muscle. Using the OX-42 monoclonal antibody (specific marker of reactive microglia), no specific signal could be traced due to high background (non-specific) staining by the secondary antibody. In the muscle, injection of distilled water resulted in massive infiltration of lymphocyte-like cells compared to control tissues. Injection of bupivacaine resulted in considerable lymphocytic-like infiltration at 4 days compared to controls although this was not as extreme as that observed following injection of distilled water. Specific degradation of muscle fibres was seen following injection of bupivacaine. Some lymphocytic-like infiltration was seen following injection with physiological saline at 4 days compared to controls. All of the observations in muscle observed at 4 days were qualitatively less at 7 days post injection.

5.2.4.1 Morphology

The micrographs in **Figure 5-4** show haematoxylin- and eiosin-stained cryosections through snout muscles of rats taken from each of the four groups described above at 4 days after treatment.



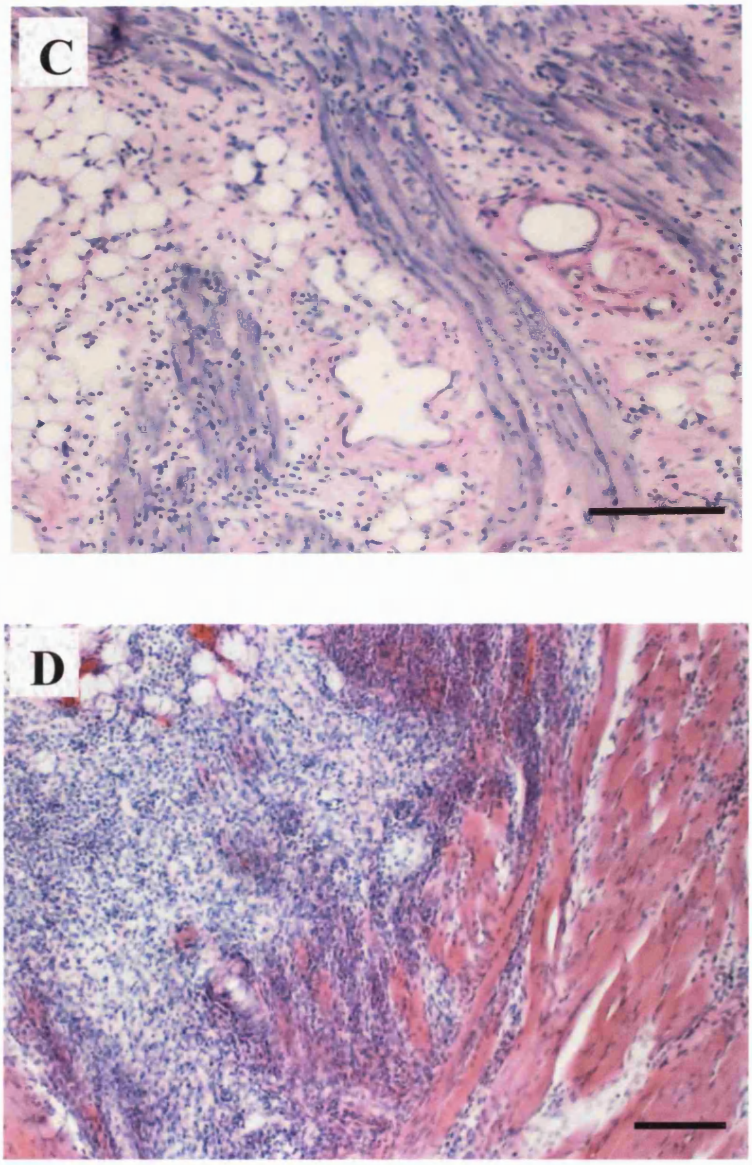
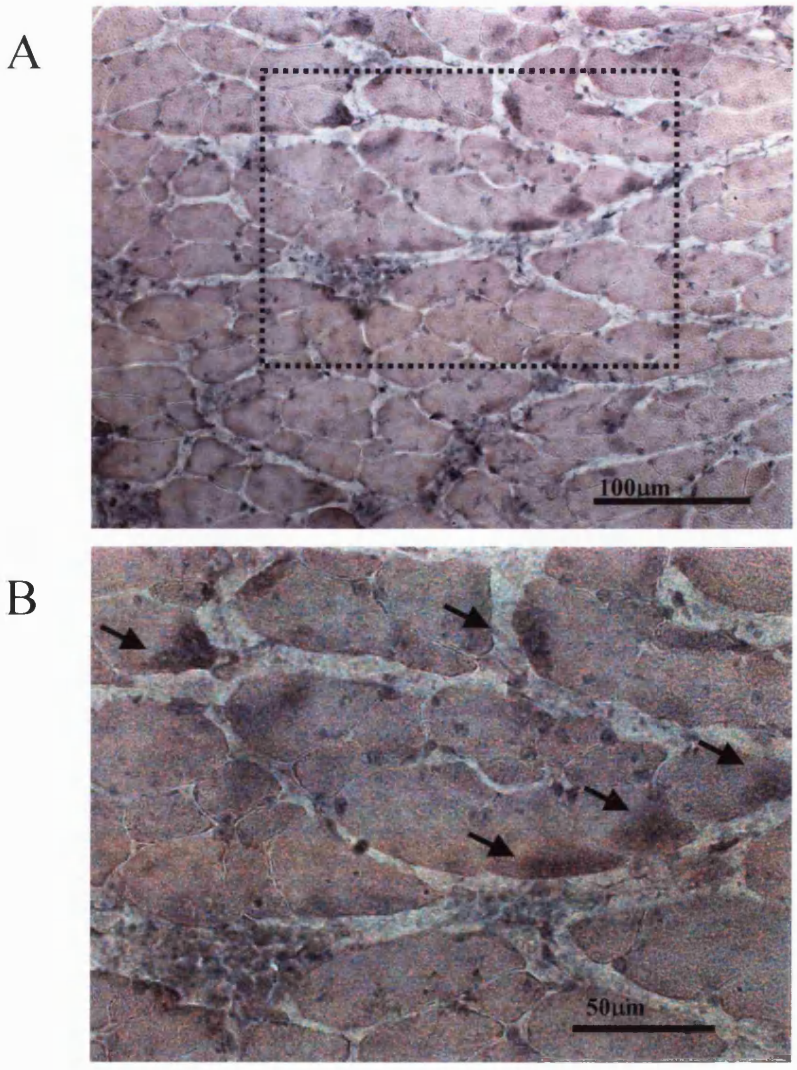


Figure 5-4 Muscle histology 4 days after intramuscular injection (A) Normal snout muscle. (B) 4 days after saline injection a slight increase in lymphocytic-like cells is seen. (C) 4 days after bupivacaine injection, muscle fibre damage and moderate lymphocytic-like infiltration is seen. (D) 4 days after distilled water injection, pockets of marked lymphocytic-like infiltration are seen. Cryosections stained with haematoxylin and eosin. Scale bar = 100μm

5.2.4.2 L.IGF-1 and MGF mRNA expression

DIG-labeled riboprobes specific for MGF and L.IGF-1 mRNA transcripts (for probe sequences see **Table 2.2** in chapter 2) were hybridised to perfused-fixed sections of damaged and non-damaged snout muscles from each rat as well as normal (undamaged) rats. In both sides, hybridisation with either MGF or L.IGF-1 anti-sense probes resulted in specific staining of selected muscle fibres in a compartmentalised pattern irrespective of prior treatment and time point. Such examples can be observed in **Fig. 5-5 A, B and C**. In sections treated with sense probe no such staining was observed (**D**).



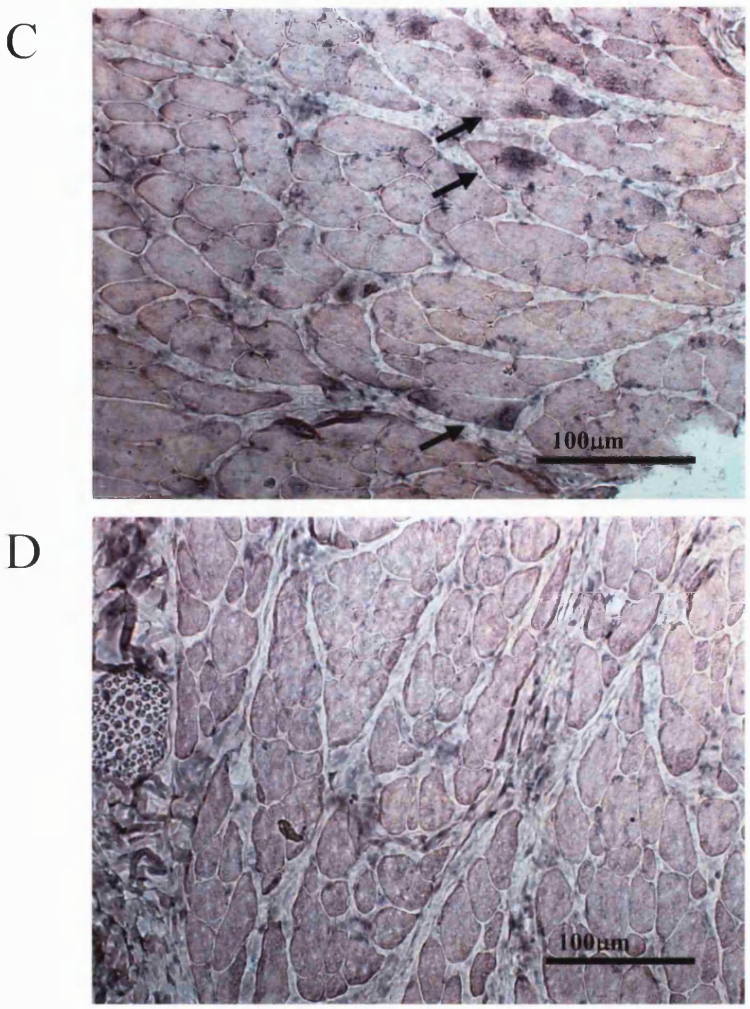


Figure 5-5 Sections through the snout muscle taken a 4 days after treatment hybridised with DIG-labeled riboprobes specific for either MGF (panels A-B) or L.IGF-1 (panel C). Alkaline phosphatase reaction product was detected in compartmentalised patterns within muscle fibres close to the cell membrane (indicated by arrows). No change in pattern or qualitative abundance of staining was observed which was specific to variable of treatment (including normal rats) or time (4 and 7 days). In sections hybridised with sense probe (panel D), such staining was not observed. Non-specific staining appeared to be localised to regions within the extracellular matrix. The rectangle outlined with dotted lines in panel A defines the field for the micrograph of higher magnification in panel B.

5.2.5 MGF and L.IGF-1 mRNA expression following denervation of the snout muscle

To provide information on the endogenous IGF-1 mRNA species is responsible for increasing the survival of adult motoneurons, the facial nerve was transected in 4 adult rats. Two animals survived for 1 day while the remaining two animals survived for 3 days. Total RNA was extracted separately from left and right (or contralateral and ipsilateral) sides of both brainstem and snout muscle. cDNA was synthesised using EX6-R for reverse transcriptase priming and analysed by semi-quantitative PCR amplification using optimised primer pairs (**Table 2.2**). These were designed specifically to detect all cDNAs corresponding to various mRNA species differing by the presence or absence of exon 5 (MGF), in combination with alternate splicing of either of exons 1 or 2 to the 5' end. PCR products were analysed by agarose gel electrophoresis, sequenced and compared to the published sequences in the NCBI database (accession numbers XO6107 and M15480) to confirm appropriate amplification.

5.2.5.1 MGF mRNA

To detect changes in mRNA of IGF Eb (MGF) specific primer pairs were designed to differentiate between those copies derived from promoter 1 (exon 1, including differential 5'UTRs) and those from promoter 2 (exon 2). These are illustrated schematically in **Fig. 5-6.**

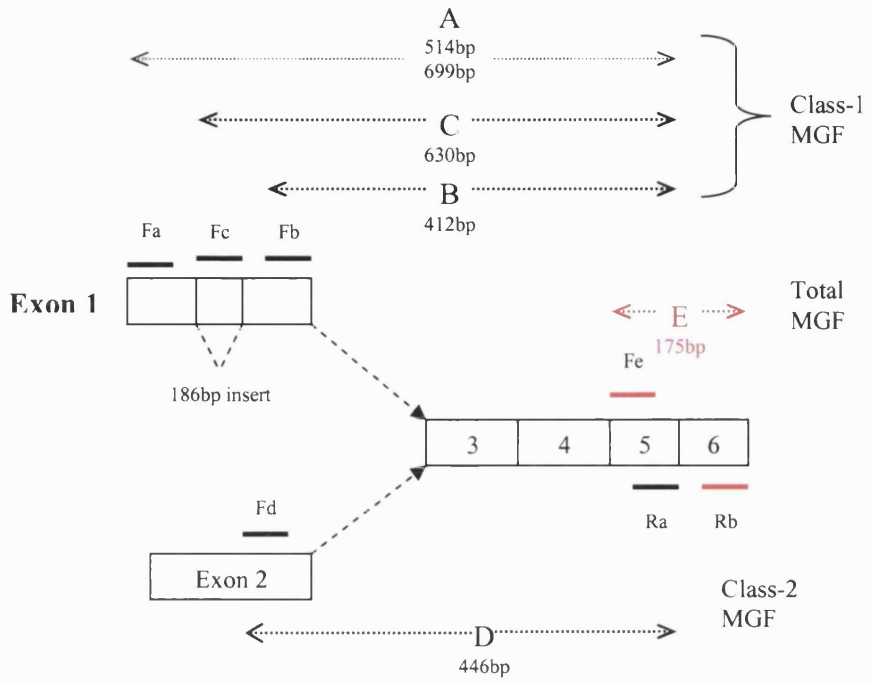


Figure 5-6 Schematic representation of the PCR strategy used to identify possible splicing in MGF mRNA, containing the 52bp insert of exon-5. Exon-1 contains an insert of 186bp giving rise to a possible extra mRNA species. Reaction A (primers Fa and Ra) results in 2 amplification products (with the presence and absence of the 186bp insert), while reaction B (primers Fb and Ra) is specific for the combination of both of these products. Reaction C (primers Fc and Ra) identifies only the mRNA containing exon-1 with the presence of 186bp insert. Reaction D (primers Fd and Ra) is specific for MGF containing exon-2. Reaction D (primers Fd and Rb) is designed to detect all copies of IGF Eb mRNA irrespective of the presence of either of exons-1 or -2. (Primer nomenclature: Ra=5UTR-F; Fb=UEX1-F; Fc=P186-F; Fd=EX2-F; Fe=EX5-F; Ra=EX5-R; Rb=EX-6-R. See **Table 2.2** for nucleotide sequences)

Results of gel electrophoresis of PCR products in each of reactions A-E are shown in **Fig. 5-7**. All PCR products were purified and sequenced to ensure that they matched to the published sequence in the NCBI database (accession number X06107) (Shimatsu & Rotwein 1987a).

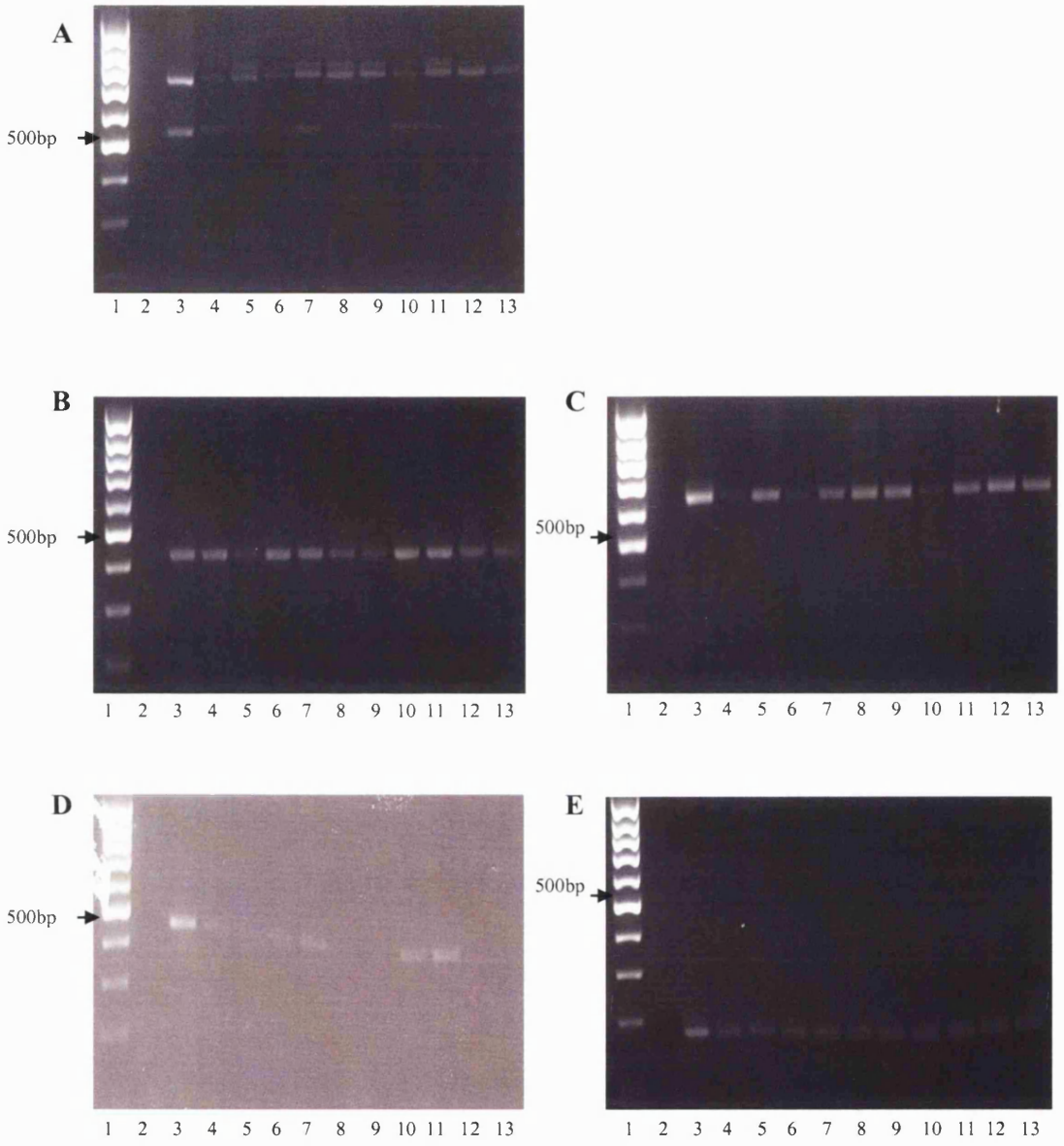


Figure 5-7 PCR amplification of MGF cDNA. Panels A-E correspond to PCR reactions (A-E) described schematically in Figure 5-6. Lane 1: 100bp marker; lane 2: negative control (water in replace of cDNA); lane 3: liver; lane 4: normal facial muscle (FM); lane 5: normal brainstem (BS); lane 6: 1d survival FM, contralateral side (Con); lane 7: 1d survival FM, ipsilateral side (Ips); lane 8: 1d BS Con; lane 9: 1d BS Ips; lane 10: 3d FM Con; lane 11: 3d FM Ips; lane 12: 3d BS Con; lane 13: 3d BS Ips.

cDNA relating to class-1 (186 insert) IGF-Eb (MGF) mRNA was qualitatively greater in denervated snout muscle compared to the contralateral side both 1 and 3 days after axotomy (panels A and C, lanes 6-7 and 10-11). This was confirmed by separate PCR reactions differing only in the forward primer: reaction A (panel A) detected the 186bp variants separately by hybridising a primer up-stream to this insert in the 5'UTR, whereas reaction C (panel C) detected only the 186-insert 5'UTR insert. However, total class-1 variants (with respect to MGF only), detected by using a forward primer for the coding sequence of the signal peptide (common to both 5'UTR variants), showed that there was no overall qualitative increase of this class of MGF mRNA in denervated snout muscle compared to the non-operated side (panel B). There was also no overall increase in MGF mRNA (panel E, lanes 6-7 and 10-11), irrespective of splicing at the 5' end, which was supported by the detection of staining in both operated and non-operated sides using a DIG-labeled riboprobe relating to all MGF variants for *in situ* hybridisation histochemistry. An example of this staining can be observed in **Fig. 5-8**. There were no other mRNA species affected by muscle denervation in either tissue or time-point. Interestingly, total class-1 mRNA was greater in normal snout muscle compared to normal brainstem (panel B, lanes 4-5), whereas, when only the longer 5'UTR variant is examined (panel C and larger band in panel A, lanes 4-5), this appears to be the predominant species in brainstem compared to snout muscle. Class-1 (186-deletion) and Class-2 MGF variants were undetectable in normal brainstem (panels A and D respectively).

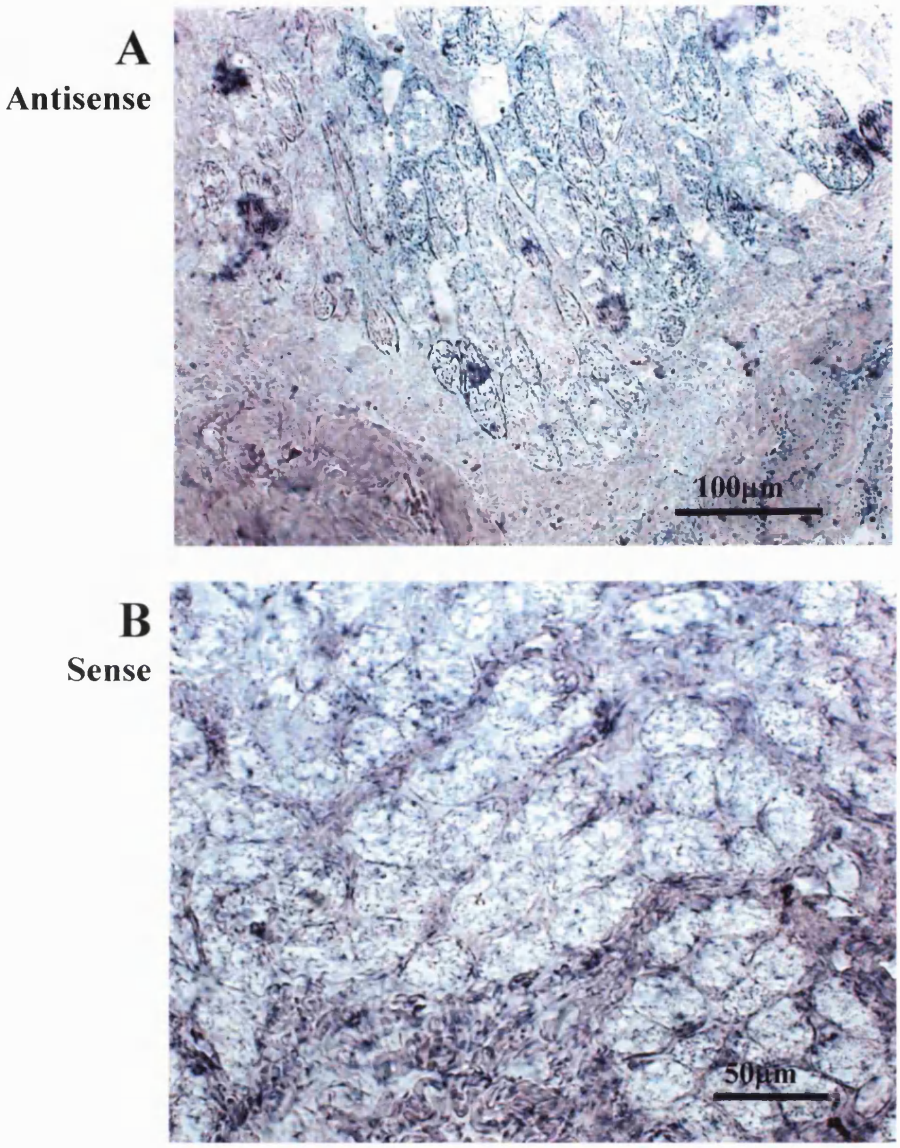


Figure 5-8 *In situ* hybridisation of an anti-sense (**A**) and sense (**B**) DIG-labeled cRNA probe specific to all MGF mRNA variants to sections through the snout muscle 3 days after facial nerve axotomy. The use of fresh frozen tissue has resulted in diminished tissue preservation and this resulted in high background staining. However, specific staining was localised to the perimeter of muscle fibres in discrete areas of the tissue section (A), irrespective of injury. Heavy background staining, which was observed in both antisense and sense, is present outside muscle fibres. By omitting the alkaline phosphatase anti-DIG Fab fragments from the protocol, background staining was no longer present.

5.2.5.2 L.IGF-1 mRNA

To detect changes in mRNA of IGF Ea (L.IGF-1) the same three forward primers used in the last section were used to differentiate between the mRNA subtypes relating to interchange between exons 1 (plus or minus 186bp insert) and 2. These were individually paired with a reverse primer designed to hybridise across the junction between exons 6 and 4, thus specific only for cDNA excluding the 52bp insert relating to exon 5. These reactions are illustrated schematically in **Fig. 5-9**.

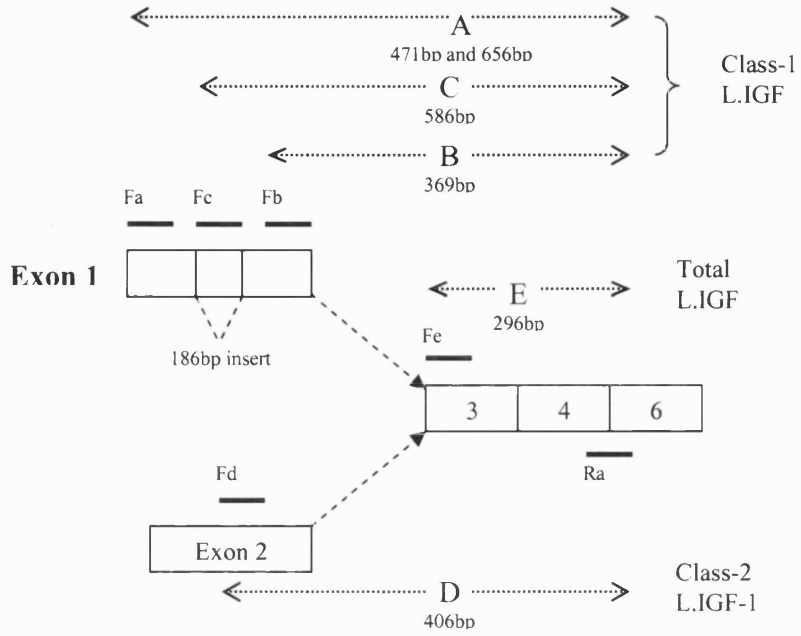


Figure 5-9 Schematic representation of the PCR strategy used to identify possible splicing in L.IGF (absence of exon 5). Exon 1 contains an insert of 186bp giving rise to an extra possible mRNA species. Reaction A (primers Fa and Ra) identifies two amplification products relating to exon 1 with and without the presence of the 186bp insert. Reaction B (primers Fb and Ra) is specific for the combination of these cDNA species while reaction C (primers Fc and Ra) is specific for only mRNA containing exon 1 with the presence of 186bp insert. Reaction D (primers Fd and Ra) is specific for L.IGF containing exon 2. Reaction E (primers Fe and Ra) was designed to detect all copies of L.IGF mRNA irrespective of the presence of either of exons 1 or 2. (Primer nomenclature: Ra=5UTR-F; Fb=UEX1-F; Fc=P186-F; Fd=EX2-F; Fe=RtEX3-F; Ra=L64-R. See **Table 2.2** for nucleotide sequences)

Results of gel electrophoresis of PCR products in each of reactions A-E are shown in **Fig. 5-10**. All PCR products were purified and sequenced to ensure that they matched to the published sequence in the NCBI database (accession number M15480) (Roberts et al. 1987).

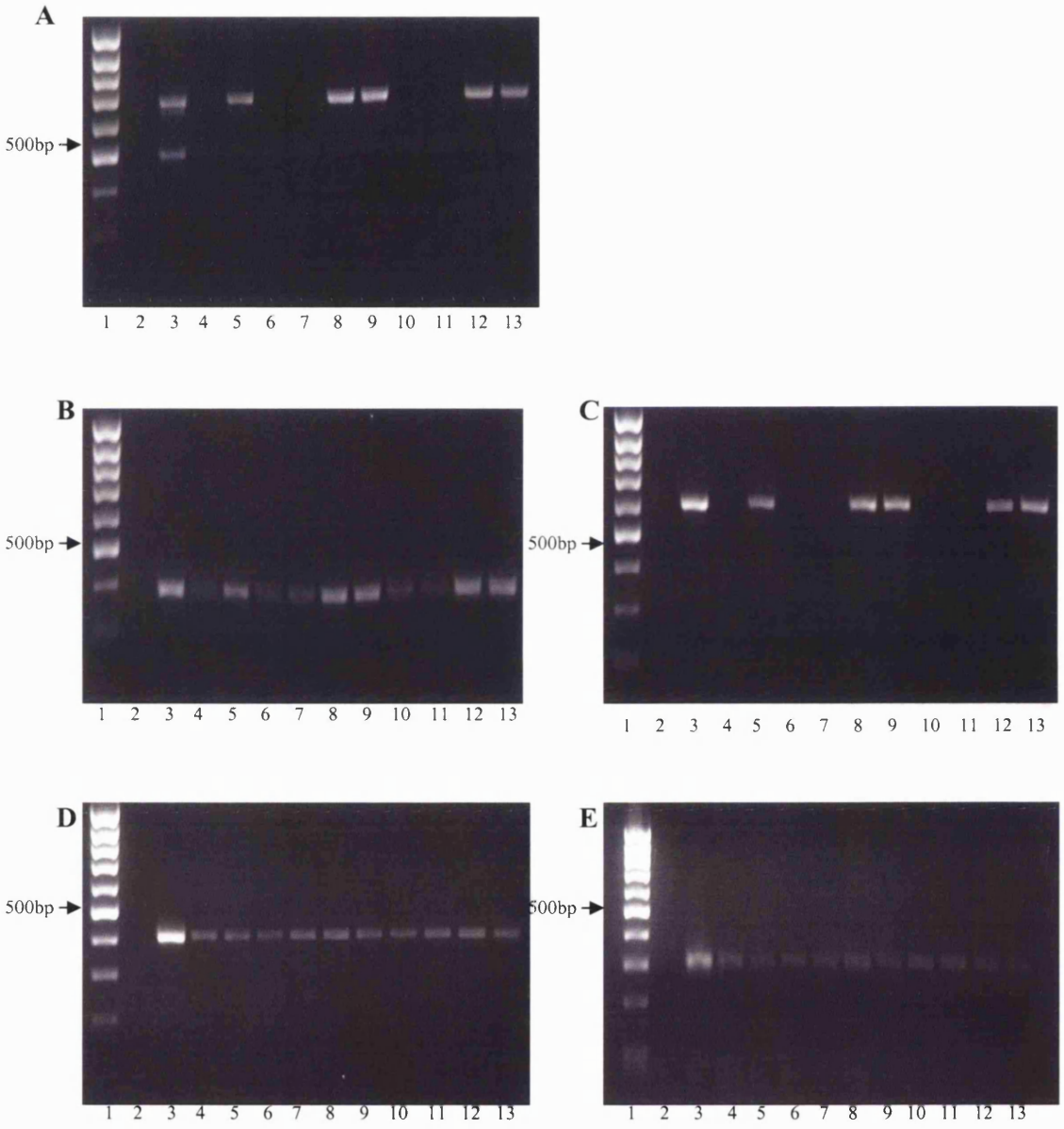


Figure 5-10 PCR amplification of L.IGF cDNA, which excludes exon 5. Panels A-E correspond to PCR reactions (A-E) described schematically in Figure 5-9. Lane 1: marker; lane 2: negative control; lane 3: liver; lane 4: normal facial muscle (FM); lane 5: normal brainstem (BS); lane 6: 1d survival FM, contralateral side (Con); lane 7: 1d survival FM, ipsilateral side (Ips); lane 8: 1d BS Con; lane 9: 1d BS Ips; lane 10: 3d FM Con; lane 11: 3d FM Ips; lane 12: 3d BS Con; lane 13: 3d BS Ips.

No qualitative change in any of the L.IGF-1 mRNAs was detected as a result of muscle denervation in either tissue or day post-surgery. In a fashion similar to analysis of MGF variants, primer Fa, which hybridises to a sequence upstream to the 186bp insert in exon -1, was paired with Ra to produce two amplification products corresponding to class-1 L.IGF-1 with either the presence or absence of the 186bp insert (**panel A**). 186-deletion class-1 L.IGF 1 (smaller product) was amplified to only trace level, to equal extent in all samples compared to that of liver (lane 3). The 186bp-insert variant (larger band) was amplified only in brainstem (lanes 5, 8-9, 12-13) with no difference between contralateral and ipsilateral sides after injury. These findings are confirmed using Fc primer against Ra, which detects only 186bp-insert variant (**panel C,** lanes 5, 8-9, 12-13). It is further reflected in the combined amplification of class-1 L.IGF-1 variants using primer Fb (specific for signal peptide coding sequence, common to both class-1 5'UTR variants) paired with Ra (**panel D**). Class-2 L.IGF-1 was detected in all samples (**panel E**). There are signs that the level of amplification of this variant is qualitatively decreased slightly on the contralateral side of the brainstem both 1 and 3 days after injury compared to the ipsilateral side (lanes 8 *versus* 9 and 12 *versus* 13) and to normal brainstem (lane 5). The relative difference in band intensity however, between operated and non-operated samples, is not as visually apparent as that observed between operated and non-operated snout muscles described for class-1 (186-insert) L.IGF-1 variant **panel C** of **Fig. 5-7**. Primers Fe and Rb were used to observe the tissue selective change in the amplification of total L.IGF-1 mRNAs after nerve injury irrespective of the presence of either of exons 1 and 2 (**panel E**). It can be seen that amplification products of approximately 296bp were obtained in all samples. No qualitative difference is observed in intensity of bands between samples. This is supported by *in situ* hybridisation of a DIG-labeled riboprobe specific for all transcripts with exon 5 deletion (**Fig. 5-11**).

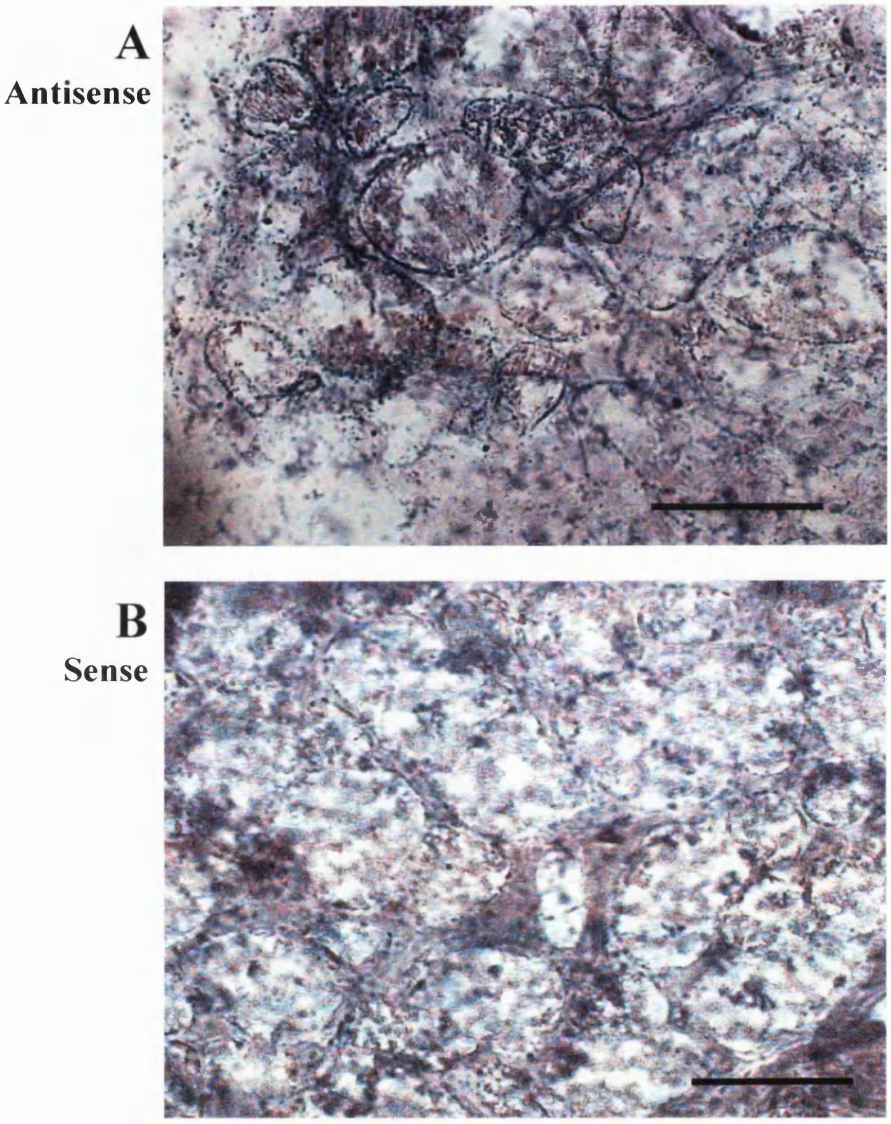


Figure 5-11 In situ hybridisation of an anti-sense (A) and sense (B) DIG-labeled cRNA probe specific all L.IGF-1 mRNA variants to sections through the snout muscle 3 days after facial nerve axotomy. The use of fresh frozen tissue has resulted in diminished tissue preservation and this resulted in high background staining. However, specific staining was localised to the perimeter of muscle fibres in discrete areas of the tissue section, irrespective of injury (A). Heavy background staining, which was observed in both anti-sense and sense, is present outside muscle fibres. Crystals of alkaline phosphate reaction product resulted from the need to incubate sections for prolonged periods despite frequent replacement of detection buffer. Scale bar = 50μm

5.2.6 Investigation into the retrograde reaction to injury

Several markers were chosen to investigate differences in retrograde reaction to peripheral injury as a result of the various treatments that have been tested in the present thesis. It was not possible to test all markers for all experiments. **Table 5.3** summarises the complement of markers used for individual studies. An antibody for the microglial-specific antigen OX-42 was also tested but failed to show specific immuno-detection. Immuno-absorption of CGRP and cleaved caspase-3 antibodies with an appropriate binding peptide blocked specific immune-staining (not shown). Such a peptide was not found for GFAP. Omitting either primary or secondary antibodies resulted in lack of specific immuno-detection.

Table 5.3

Range of specific cellular markers utilized to characterize the retrograde response to injury different treatment groups.

	Marker					
	CGRP	*GFAP*	*GAP-43*	*Akt-2*	*TUNEL*	*Caspase-3*
Neonate 1-, 3- and 7- days	✔	✔	✔	✔	✔	✔
Adult: MGF plasmid	✔	✔	✔		✔	✔
(1-month) *Adult:* MGF plasmid						
(3-month) *Adult*: prior muscle damage	✔	✔	✔			
Adult: MGF E-domain study	✔	✔	✔			
Ageing: AL	✔	✔	✔		✔	✔
Ageing: DR	✔	✔	✔		✔	✔

5.2.6.1 Correlates of retrograde response to facial nerve avulsion only

Morphology

At all ages, irrespective of diet or prior muscle damage, there was evidence of a retrograde response to injury in the ipsilateral facial nucleus. Most of the surviving motoneurons remaining in the ipsilateral facial nucleus contained crenated, eccentric nuclei with perinuclear caps of Nissl substance and vacuolated nucleoli.

Motoneuron survival

CGRP immuno-positive neurons were not counted but in the ipsilateral facial nucleus, there were qualitatively fewer CGRP immuno-positive motoneurons where this coincided with motoneuron loss. However, the remaining motoneurons in neonatal and adult rats showed qualitatively greater intensity of cytosolic staining compared to those found in the contralateral nucleus (for an example of CGRP immuno-positive staining, see **Fig. 5-16**). In ageing animals, irrespective of diet, qualitatively little or no increase in CGRP immuno-staining intensity was seen in the surviving motoneurons ipsilaterally at 1 month.

In situ hybridisation of a DIG-labeled RNA probe specific for Akt was located to motoneurons of the facial nucleus of neonatal rats. The number of stained neurons and level of staining intensity was assessed qualitatively. 1 day (**Fig. 5-12**) and 3 days (not shown) after nerve avulsion, a qualitative increase in number of intensely stained motoneurons was observed in the ipsilateral facial nucleus compared to the contralateral side. 3 days after injury, the number of labeled motoneurons in the ipsilateral nucleus was observed to be fewer than that at 1 day, which coincided with significant motoneuron loss at this time point. This probe was not tested on adult or ageing animals.

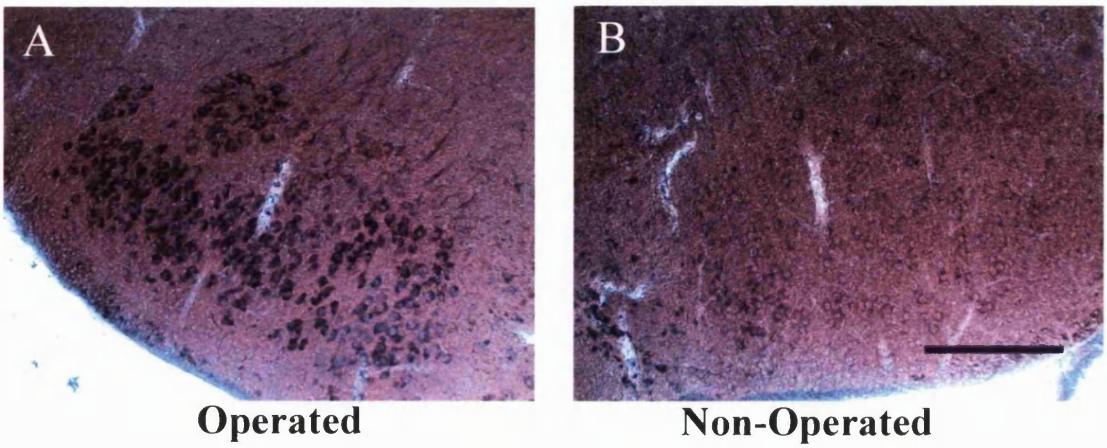


Figure 5-12 Brainstem sections through the facial nucleus of an neonatal rat at 1 day following facial nerve avulsion. Sections were hybridised with a DIG-labeled riboprobe specific for Akt. mRNA expression is increased on the operated side (A) compared to the non-operated side (B). Scale bar = 100μm

Motoneuron regeneration

In situ hybridization of a DIG-labeled RNA probe specific for GAP-43 was located to motoneurons of the facial nucleus in neonatal rats with high intensity compared to the contralateral side. The number of stained neurons and level of staining intensity was assessed qualitatively. In a manner similar to *in situ* hybridisation of Akt probe, an increase in the number of intensely stained motoneurons coinciding with motoneuron survival was observed in the ipsilateral facial nucleus 1 day (**Fig. 5-13**) and 3 days (not shown) after injury. Again, fewer numbers of labeled neurons were observed after 3 days. Staining was also seen ipsilaterally in adult (not shown) and ageing animals (A and B below) at 1 month post avulsion but was not found at all in the contralateral facial nucleus.

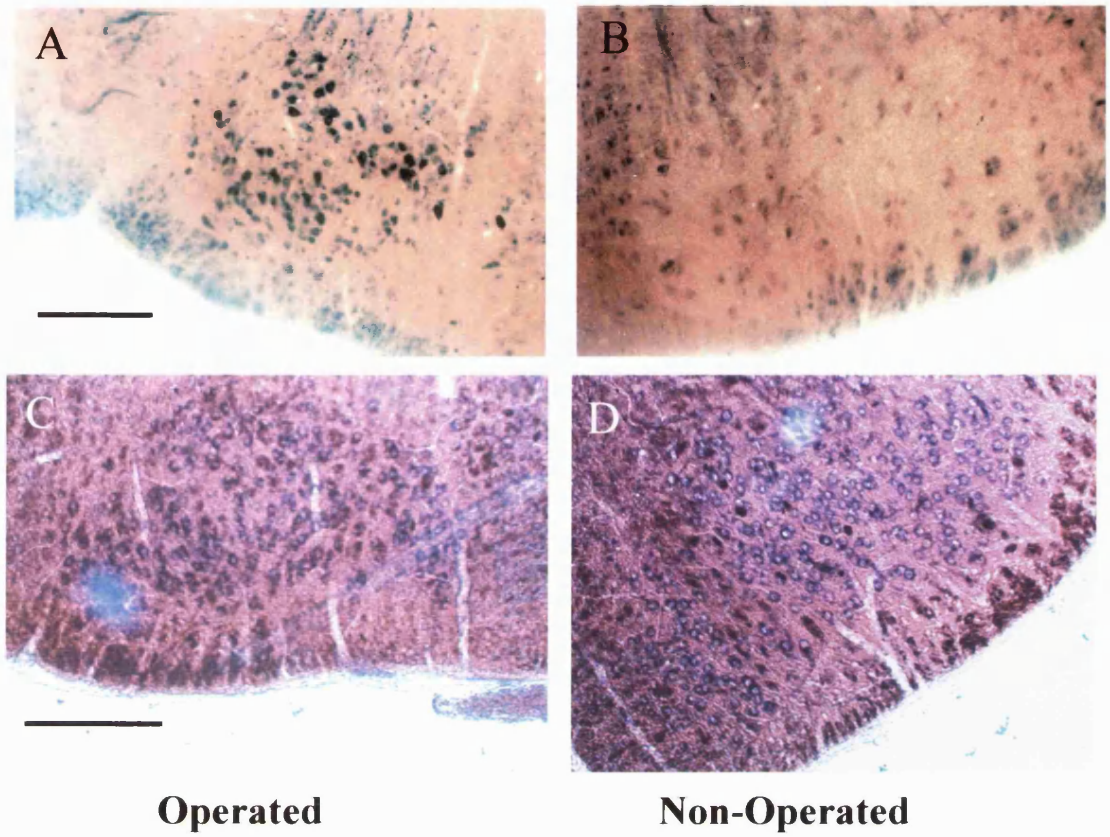


Figure 5-13 Brainstem sections through the facial nucleus of an ageing rat (A-B) at 1-month and a neonatal rat (C-D) at 1-day following facial nerve avulsion. Sections were hybridised with a DIG-labeled riboprobe specific for GAP-43. mRNA expression is increased on the operated side (A and C) compared to the non-operated side (B and D). Scale bar = 100μm

Neuroglial response

Astrocyte-specific immuno-staining for GFAP was qualitatively increased in and surrounding the region of the ipsilateral facial nucleus compared to the contralateral side in animals of all ages (**Fig. 5-14**). The astroglial reaction to injury was more pronounced in neonatal animals ipsilaterally 1 week after injury compared to adult animals, persisting 1 month after injury. In ageing animals there was generally a high level of GFAP-positive staining throughout the brainstem compared to animals of other age groups.

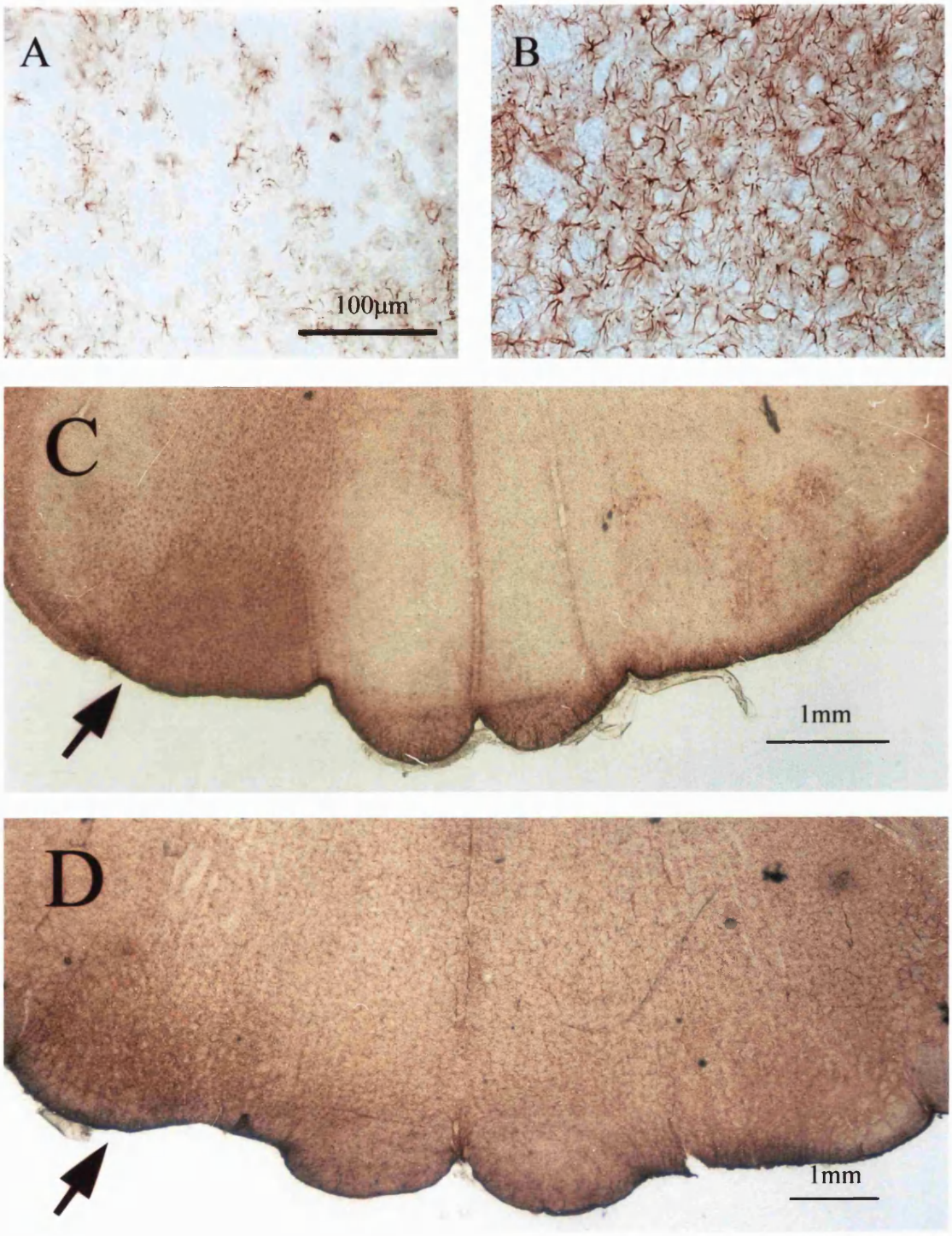

A
100µm
B
C
1mm
D
1mm

Figure 5-14 Sections through the brainstem at the level of the facial nucleus immune-stained for GFAP. High power magnification (A-B) shows immuno-positive cells of typically astroglial-like morphology at 1 month in adult rats. Staining is more intense with immuno-positive cells of greater abundance in the operated side in the neonatal rat, 7 days after injury. Low power magnification shows that the contrast in astroglial reaction to avulsion (operated [indicated with an arrow] *versus* non-operated side) is greater in the neonatal rat (C) compared to adult (not shown) and ageing rats (D)

Motoneuron death

TUNEL and cleaved caspase-3 staining was observed rarely in neonatal animals 7 days after avulsion, and not at all in adult or ageing animals 1 month after avulsion. On average, staining for both markers was found to be most abundant 3 days after injury. It can be observed in **Fig. 5-15 A** that cleaved caspase-3 immuno-reactivity is densely localised throughout selected cells of typical neuron-like morphology, extending into multi-polar, dendrite-like structures. Such staining was found only in the ipsilateral facial nucleus. TUNEL-positive nuclei of approximately 5μm were also observed ipsilaterally (**Fig. 5-15 B**). Such staining was located amongst neuron-like cells (as defined by haematoxylin counterstaining), only in the ipsilateral facial nucleus.

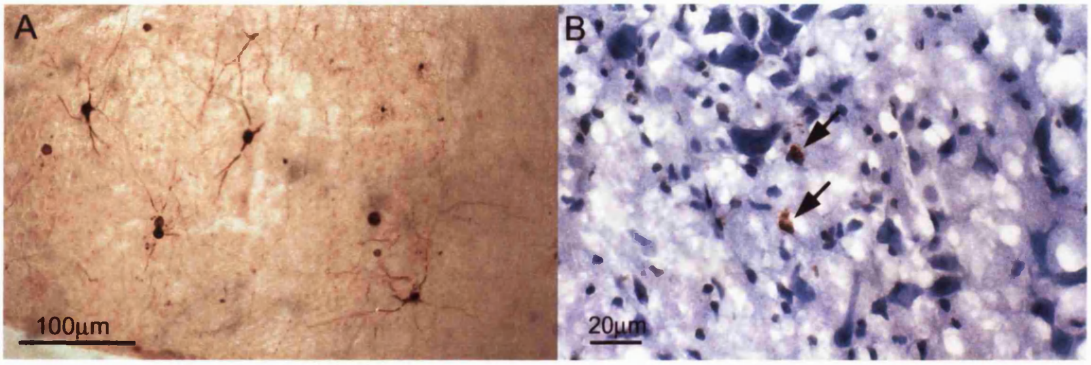


Figure 5-15 Evidence of apoptosis in 7 day-old rats, 3 days after injury. (A) Cells showing activated caspase-3-like immuno-staining are seen in the ipsilateral facial nucleus, whereas none were seen on the contralateral side (not shown). Immuno-positive cells have a typical neuron-like morphology. (B) TUNEL-stained nuclei in the ipsilateral facial nucleus.

Other indicators of apoptotic activity *in situ*, which were tested include; immuno-staining for Bad, and Bcl-2, and immuno-blotting for uncleaved caspase-3. Bad and Bcl-2 immuno-positive staining was not identified in any of the examined. Since TUNEL and cleaved caspase-3 were reliable as markers of cell death, no other marker in this category was included in determining the effect of plasmid injection.

5.2.6.2 Alterations in retrograde response to nerve avulsion correlating with treatments

Only observations that differed in their characteristics from those described for normal animals subjected to avulsion alone are noted. Except for CGRP, all other markers, where tested, were unaffected by diet (chapter 3), MGF plasmid (irrespective of survival period) (chapter-4), MGF E-domain peptide (chapter 5) and prior muscle damage (chapter 3). There tended to be a greater number of CGRP immuno-positive neurons ipsilaterally, showing increased staining intensity where this coincided with increased motoneuron numbers (as defined by stereology). Thus a qualitatively greater number of intensely stained CGRP immuno-positive motoneurons were easily observed ipsilaterally in rats subjected to all of the following treatments: prior MGF plasmid injection observed at 1 month in adult rats; MGF E-domain peptide (applied at the time of injury to avulsed nerve), where this coincided with increased motoneuron numbers; prior injection with distilled water. Exemplifying these observations, **Fig. 5-16** shows CGRP immuno-positive motoneurons in operated and non-operated facial nuclei of rats subjected to either nerve avulsion alone (A-B), avulsion with prior injection of distilled water (C-D) or MGF plasmid (E-F). In panels D and F, there is greater abundance of intensely stained neurons compared to panels C and E respectively.

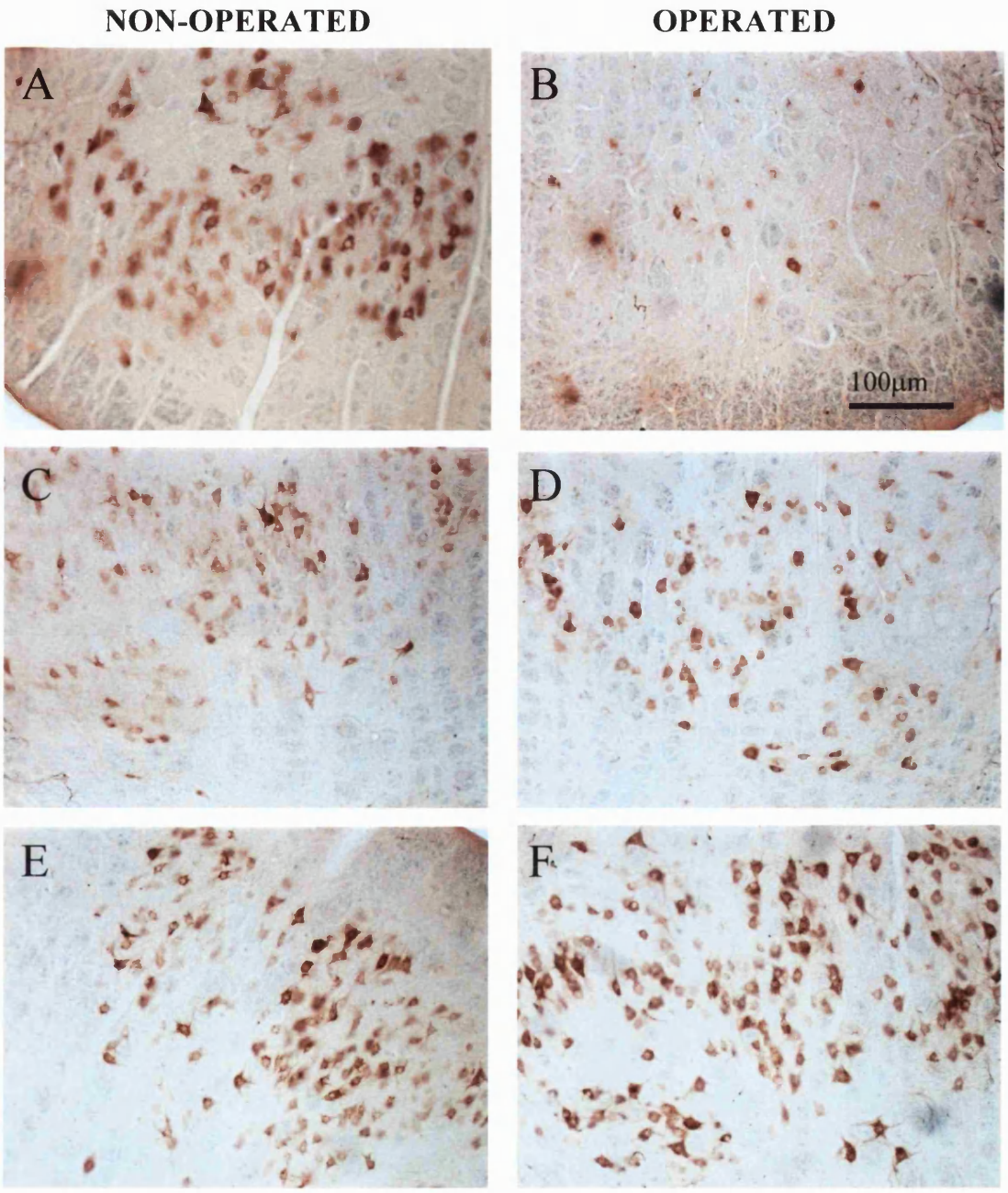


Figure 5-16 Sections through the brainstem at the level of the facial nucleus immuno-stained for CGRP. Sections were taken from adult rats subjected to avulsion only (A-B), prior muscle damage induced by injection of distilled water (C-D) or prior MGF gene transfer (E-F), seen at 1 month post injury. Motoneurons surviving facial nerve avulsion in rats subjected to prior muscle injury and MGF gene transfer (D and F) are qualitatively more intensely immuno-stained than motoneurons in the non-operated nucleus (C and E).

5.3 Discussion

The aim of the present study was to reveal experimentally the mechanisms by which neuroprotection is afforded by MGF and prior muscle damage. The main findings are firstly, that intramuscular injection of MGF plasmid results in gene expression only in muscle tissue; secondly, MGF E-peptide needs to be present at the time of injury in order to confer neuroprotection; thirdly, denervation results in perturbation of MGF mRNA expression in target muscle; and finally, the level neuroprotection seen following prior muscle damage corresponds to the observed level of peripheral inflammation.

The following discussion is presented in three sections corresponding firstly, to the mechanism by which exogenous MGF/E-peptide becomes available to motoneurons; secondly, to endogenous changes in the periphery following denervation of and direct damage to muscle, emphasising the role played by IGF-1 isoforms as defined by mRNA expression as well as muscle inflammation; and finally, to indications of other mechanisms which may mediate neuroprotection centrally.

5.3.1 Mechanism of protection by exogenous MGF/E-peptide

In the absence of an antibody specific for rabbit MGF produced from the pcDNA3.0 plasmid vector, it was not possible to confirm protein expression in this study. Interestingly, RT-PCR and PCR analysis confirmed presence of mRNA and plasmid DNA respectively, 7 days after injection, *i.e.* concomitant with the time of injury (chapter 4). This was located in the snout and tibialis anterior muscles into which the plasmid vector was injected but absent in liver, blood, non-injected muscles, brainstem and spinal cord. This means that after injection, the vector was retained and was stable in muscle cells at the time of injury and was not transported either into the blood or retrogradely to the CNS. mRNA continued to be expressed at least 21 days after injection, which is consistent with other studies (Alila et al. 1997; Flint et al. 1999) and indicates strongly that protein was synthesised in this tissue. On one occasion, DNA and mRNA were detected in the brainstem but this was a result that could not be repeated and was thus excluded from the data. It is conceivable however; that a

plasmid vector could have entered peripheral nerves through intramuscular injection. It was shown previously that a plasmid encoding the LacZ gene was transported and expressed in cell bodies after application to cut nerve stumps (Johnson, I.P. et al. 1998; Sahenk et al. 1993). In the present study, three separate injections were made into the facial muscle. It is possible, therefore, that on occasion intramuscular nerves may be ruptured upon penetration of the syringe needle into muscle tissue thus allowing low-level uptake of plasmid into selected nerve fibres. Complications of nerve function and viability following deep intramuscular injection of substances have been reported previously (Ling & Loong 1976; Streib & Sun 1981). In one study, injection of a range of substances was found to cause directly widespread axonal and myelin degeneration of the sciatic nerve in Wistar rats (Gentili et al. 1980).

It is not certain from these present results whether MGF protein was transported retrogradely from the snout muscle along axons to motoneuron cell bodies. Much of the work concerning signal transduction of neurotrophic factors has been done on the neurotrophins in sympathetic and sensory neurons *in vitro* (Ginty & Segal 2002; Neet & Campenot 2001; Reynolds et al. 2000) due to their amenability for culturing in compartmentalised culturing chambers (Campenot 1982). NGF for example, binds to its receptor, becomes internalised into an endosome vesicle and transported to cell bodies where they activate signaling for cellular growth or survival (Reynolds et al. 2000; Sandow et al. 2000). Receptors have also been found on the cell surface of cell bodies suggesting that these neurons are responsive to neurotrophins derived via secretion from adjacent cells (Whitmarsh & Davis 2001). Another study has shown that transport of NGF is not necessary for cellular activation (MacInnis & Campenot 2002). The experimental evidence for signal transduction and/or retrograde transport of neurotrophic factors along intact motoneurons is less clear. Neurotrophin receptor mRNAs are expressed in intact motoneurons during development (Seeburger & Springer 1993; McKay et al. 1996) during adulthood (Kobayashi et al. 1996) and through to ageing but no clear evidence suggests that their protein is expressed at the motor termini (Sheard et al. 2002). IGF-1R mRNA is found in injured motoneurons but its protein was absent (Gehrmann et al. 1994), suggesting that it may have been transported anterogradely for its expression in the periphery. IGF-1 immuno-reactivity has been reported in astroglia

centrally (Gehrmann et al. 1994; Hammarberg et al. 1998). Substantially less however, is known about IGF-1-mediated signal activation with respect to motoneuron injury/survival compared with that described for the neurotrophins.

When gelfoam soaked with MGF E-domain peptide was applied to the lesion site, substantial neuroprotection in half of the animals tested was observed. This suggests that motoneuron survival was promoted by a mechanism possibly involving retrograde transport of this protein followed by receptor activation, potentially in the CNS, similar to transport of I^{125} –labeled neurotrophic factors to motoneuron cell bodies following application to axotomised proximal nerve stumps as has been reported previously (Koliatsos et al. 1993; Yan et al. 1995). It is not known however, whether E-domain peptide or MGF is transported in intact motoneurons or whether transport is actually required. Intramuscular injection of MGF E-domain protein or L.IGF-1 7 days prior to injury had no effect on motoneuron survival 1 month after nerve avulsion. Lack of retrograde transport of the E-domain peptide in intact motoneurons may account for this observation. This is in contrast to MGF, putatively expressed in target muscle following gene transfer but able to afford increased motoneuron survival significantly. It is not known if, and under what circumstances, the alternative ligand-binding site for the E-peptide (Yang, S. & Goldspink 2002) is also present in intact motoneurons. It is tempting to suggest that the E-domain peptide can be transported only in the form of the uncleaved MGF protein, recognising the type-1 IGF receptor at the nerve termini of uninjured motoneurons. Following activation and/or internalisation of the MGF-IGF-1 receptor complex, it may be transported retrogradely thus allowing exposure of cell bodies to E-domain peptide, produced subsequent to proteolytic cleavage of MGF intracellularly. It is reasonable to speculate, therefore, that E-peptide is important only under conditions of injury. **Fig. 5-17** illustrates schematically a possible mechanism of how MGF becomes available and active during its passage from muscle to cell body.

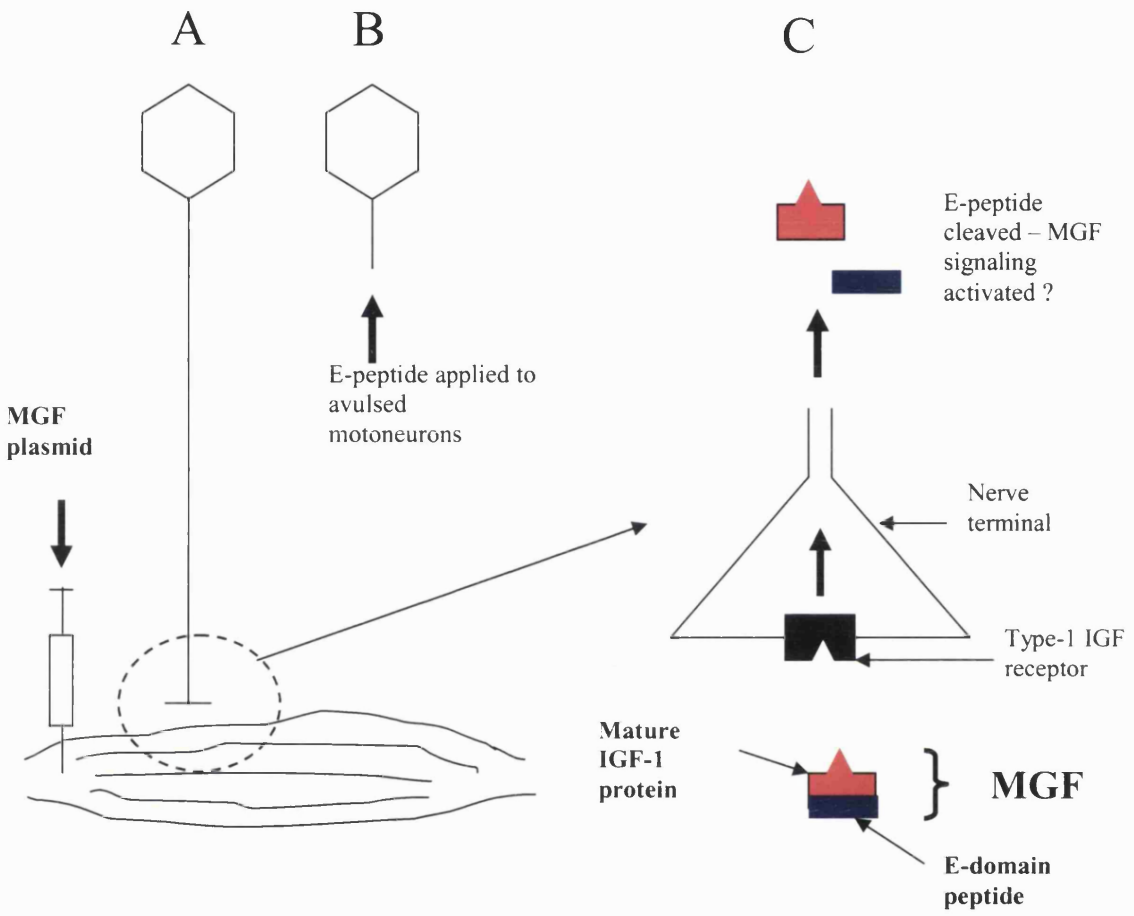


Figure 5-17 Schematic representation of MGF availability to intact motoneurons following intramuscular injection of MGF plasmid (A) and application of E-domain peptide to avulsed motoneurons (B). (C) A suggested mechanism for how MGF activity is mediated. MGF derived from the vector binds to the type-1 IGF receptor, becomes internalized and transported retrogradely towards the motoneuron cell body. Under conditions of injury, the E-peptide becomes cleaved and available to bind to separate ligand-binding site. This situation is mimicked possibly following application of the peptide to avulsed motoneurons.

There are certain parallels, therefore, between the activity and availability of MGF/E-peptide in avulsed motoneurons and availability of CNTF to motoneurons after injury.

CNTF is believed generally to be a lesion factor (Kuno 1993), available to motoneurons only upon rupture of the nerve where it is synthesised from myelinating Schwann cells (Sendtner et al. 1997). Likewise, cleaved E-domain peptide may have no access to motoneurons under physiological conditions. In contrast to CNTF however, it might still gain access to normal motoneurons through the potential binding of uncleaved MGF to the IGF-1 receptor. The receptor component to which CNTF binds, is found commonly in astrocytes (Monville et al. 2001; Reddington et al. 1995). It would be of great interest to characterise the spatial and temporal expression of the E-domain peptide receptor/ligand-binding site following injury of the facial nerve.

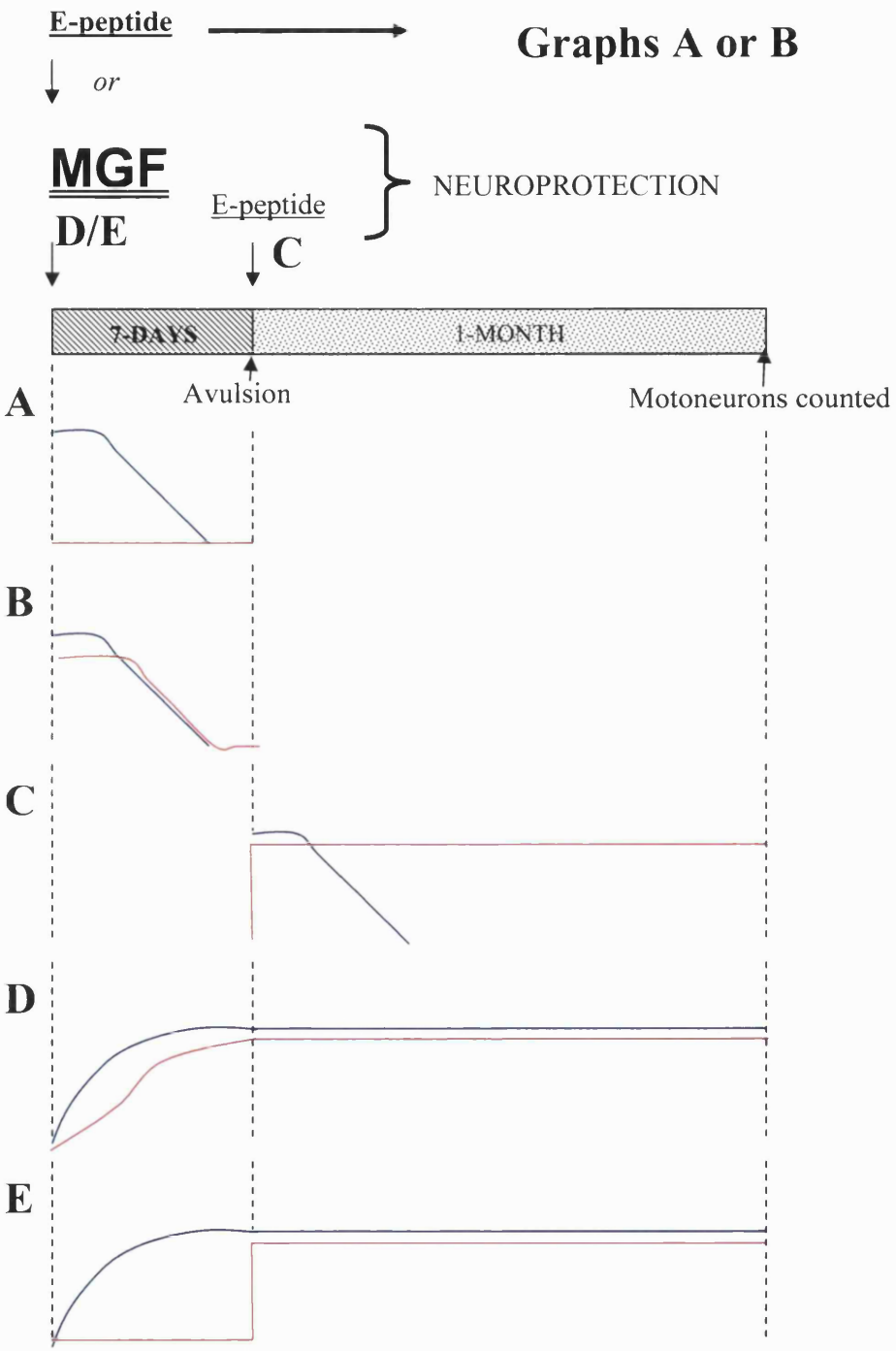
Following application of E-domain peptide to avulsed motoneuron stumps by use of gelfoam; all cell bodies would be potentially exposed directly to the protein. Conversely, vector-derived MGF would have access only to those motoneurons projecting to the snout muscle. Neuroprotection however, was not confined to those neurons. Protein expressed from the vector may be transported axonally with or without prior receptor activation, producing a by-stander effect on adjacent motoneurons and/or neuroglia within the facial nucleus. The mechanisms by which this phenomenon is mediated are not clear. For instance, application of horseradish peroxidase has been used previously to identify motor pools specific to muscle groups. This is in contrast to Fluoro-Gold tracer in the present study, which was found throughout the facial nucleus following intramuscular injection. Gap-junctions have been found in abundance in facial motoneurons of the rat (Yamamoto et al. 1991) and may provide a means for intercellular trafficking, yet motoneuron electronic coupling, known to be mediated by these inter-membrane structures (Kiehn & Tresch 2002), is known to decrease considerably postnatally in correlation with functional maturity of motoneurons and the muscular system (Walton & Navarrete 1991). It is perhaps more likely that Fluro-Gold leaks non-specifically through many types of membrane channel and subsequently taken-up by adjacent motoneurons. Nonetheless, such communication, apparent in the present study, is potentially a crucial parameter for motoneuron survival endogenously, or induced by exogenous application of neurotrophic factors.

It should be noted that when between-group analyses were applied, there was discrepancy between the Mann-Whitney U-test and ANOVA. Whereas the Mann Whitney test showed that there had been significant neuroprotection when E-peptide was applied at the time of injury compared to rats injected with the same peptide 7 days prior to injury, ANOVA revealed no significant variability amongst the data set. This may be indicative of the relative strengths of using a parametric test *versus* a non-parametric test that was discussed earlier in chapter 3. However, it is appreciated that in the current work, complete lack of motoneuron rescue in half of the animals treated with MGF E-peptide at the time of surgery, indicates strongly a degree of methological inconsistency. The result will therefore only be interpreted with respect to the half of the group showing neuroprotection, making the assumption that should it have been the case that all of the animals in this group had shown such neuroprotection, then no discrepancy between significance would have been apparent between the two statistical tests. It is possible that the protein-soaked gelfoam did not remain at the site in which it was placed for the duration of the experiment. Given that the gelfoam was not fixed into position, in accordance with other studies (Chai, et al. 1999; Schmalbruch & Rosenthal 1995; Watabe et al. 2000), it seems quite feasible that displacement of just a few millimeters could result in prolonged deprivation from MGF E-peptide. Furthermore, it cannot be guaranteed that the peptide was able to enter avulsed proximal nerve stumps distally via stylomastoid foramen. Plugging the stylomastoid foramen with gelfoam soaked in E-peptide and its subsequent suture to adjacent tissue may overcome this problem but was not applied in the present study. Ideally, future studies would use a vector encoding MGF with a fused marker protein that can be distinguished from host tissue with specific antibodies. Such a plasmid vector, analogous to that used in the present study but containing additionally a polyhistidine tag, was injected into an adult rat so as to investigate whether protein product was transported retrogradely. Despite the identification of vector-derived mRNA in the injected muscle at 7 days from this vector, preliminary immunoblots using an antibody directed against a designated protein sequence unique to the fusion protein produced unreliable data and was rejected. Use of this vector in cell cultures by others in this laboratory also showed that this vector was not functional (Yang, S. et al. unpublished). An alternative strategy to confirm plasmid expression is based on the activity of the expected products. The IGF-1 splice variants share in common the sequence for mature IGF-1 protein.

Although it has yet to be proven beyond reasonable doubt that E-domains are cleaved from the IGF pro-hormones *in vivo*, putative sites for such processing are encoded in both isotypes of IGF-1 (Nakayama 1997; Siegfried et al. 1992). If both vectors used in the current study are functional, intramuscular injection could result in increased GAP-43 in motoneuron cell bodies and/or nerve terminals as well as intramuscular nerve sprouting (Caroni & Grandes 1990; Flint et al. 1999). Such observation would help confirm, at least, that protein expression has occurred from the vector, which has resulted in signal transduction from L.IGF-1 over-expression but not necessarily either retrograde transport or synthesis of functional MGF/E-domain protein.

Another possibility, which may account for the lack of neuroprotection afforded by intramuscular injection of the MGF E-domain peptide 7 days prior to injury, is associated with protein half-life. Different half-lives of insulin-like growth factor 1 mRNAs that differ in length of 3' untranslated sequence have been reported previously (Hepler et al. 1990) but did not specify to which isoforms particular half-lives were matched. It seems reasonable that MGF E-peptide, whose half-life *in vitro* has not been determined experimentally, is broken down within a few hours or days after injection, either due to intrinsic (chemical) instability of the protein or degradation by the immune system. The latter is likely, on account of this synthesised peptide being based on the amino acid sequence of human MGF E-domain peptide. Furthermore, lack of glycosylation *in vitro* (Bach et al. 1990) and possible alteration of IGFBP binding properties (Goldspink 1999; Goldspink & Yang 2001) are consistent with a short protein half-life, promoting an active role mostly in the vicinity of its site of synthesis and adjacent cellular environment. A short protein half-life may have allowed the signal generated by temporary exposure of motoneurons to elevated MGF to be reversed to physiological levels prior to injury (**Fig. 5-18 A/B**) thus nullifying any signal for increased motoneuron survival that may be apparent only after nerve injury. Although it can be concluded undoubtedly that E-domain peptide must be present at the time of injury in order for it to promote motoneuron survival (**Fig. 5-18 C**), this does not rule out the possibility of signal activation in, or priming of normal motoneurons with respect to cellular defence mechanisms. This possibility is discussed in more detail later on. MGF gene transfer also fails to shed light on this matter despite presumably making available to normal

motoneurons elevated levels of MGF prior to injury (**Fig. 5-18 D/E**), ensuring any associated signal in normal motoneurons would be primed shortly before and during nerve injury. Some of the possibilities regarding the relationship between MGF/E-peptide availability and signal activation are illustrated in **Fig. 5-18**.



KEY: RED line = MGF signal activation, BLUE line = MGF/E-peptide content

Figure 5-18 Inferences on temporal regulation of MGF signal activity relative to protein availability following application of E-domain peptide either 7 days prior to avulsion (schematic graphs A and B) or at the time of injury (C), and prior MGF gene transfer (D and E). It is assumed that E-domain peptide/MGF has a finite half-life. It is known that if E-peptide is present at the time of injury then motoneurons are protected (C) but it is uncertain whether the associated signal is activated in normal motoneurons or if it is active only under conditions of injury. After a bolus application of E-peptide, therefore 7 days prior to avulsion, the signal may either be activated but reversed due to protein degradation before the lesion is given (B) or there is lack of any signal due to inactivity of E-peptide on normal motoneurons (A). Similarly, gene transfer leads to an accumulation of MGF (D and E, although this may not necessarily plateau as the graphs indicates), ensuring that MGF is available at the time of injury. But it cannot be deduced that the associated signal is activated before injury.

Ideally, with prior knowledge of a down-stream marker, associated unequivocally with MGF-induced signal activation, activity of MGF in normal motoneurons would be confirmed. In the absence of such a marker, localised application of function-blocking MGF-directed antibody shortly before surgery in rats subjected to prior MGF gene transfer would shed light on this matter. With MGF in a non-functional state, the response of these animals to nerve avulsion would be compared to that of animals subjected to MGF gene transfer but omitting the neutralising antibody. Unaltered promotion of motoneuron survival would suggest that MGF's intervention is not limited to the period after nerve injury.

In the absence of retrograde transport of plasmid, and irrespective of whether MGF acts on normal or injured motoneurons, a limitation with regard to MGF half-life would impose a situation where motoneuron survival is sustained by intracellular mechanisms independent of MGF availability. This has been shown previously where significant motoneuron survival was seen 6 weeks following a bolus dose of BDNF after C7 root avulsion (Chai et al. 1999). Dosing of rats with BDNF for just the first 4 weeks post injury was also enough to confer motoneuron survival for a total of 12 weeks after spinal root avulsion (Novikov et al. 1997). This suggests that to prevent motoneuron death following injury, the timing of neurotrophic factor administration is more critical than how much of or for how long the factor is available. Relating this to MGF, avulsed motoneurons could be exposed to MGF for a range of time periods subsequent to injury, identifying a crucial time-window during which

exposure to MGF is able to sustain motoneuron survival at 1 month. It is possible that MGF might be required only for a short period during this time-window. It is interesting that BDNF can activate cellular survival mechanisms even when applied up to 2 weeks subsequent to nerve injury (Kishino et al. 1997).

Acceptance of human MGF in rats as having potent neuroprotective properties, demonstrated in some of the animals of the current study, would require further investigation in order to compare them directly to the activities other potent neurotrophic factors such as GDNF. Firstly, the present experiment requires repetition with greater numbers of animals, making the appropriate adjustments to the surgical technique concerning fixation of gelfoam outlined earlier. In addition, MGF E-peptide should be one of many treatment groups, each testing a different neurotrophic factor on the same model of nerve avulsion. Where this has been done previously, GDNF was the only neurotrophic factor to promote significant motoneuron survival following C7 root avulsion in adult mice (Li, L. et al. 1995), rescuing approximately two-thirds of motoneurons. Other neurotrophic factors tested were BDNF and IGF-1 (mature protein), each showing slight but not significant neuroprotection at 6 weeks post injury. It would be advantageous, therefore, to examine the level of motoneuron survival afforded by MGF but counting motoneurons after 6 weeks. In addition, to gain wider recognition as a general neurotrophic factor, its action should be investigated in the neonatal rat since this has served as the standard model on which most neurotrophic factors have become characterised and subsequently compared (Oppenheim 1996). Activity in neonatal animals would be additive also to the current work because it would reveal more about the relative trophic dependencies of immature *versus* adult motoneurons.

5.3.2 Peripheral mechanisms

5.3.2.1 mRNA expression in response to muscle denervation

Extensive RT-PCR analysis confirmed that for each of the IGF splice variants L.IGF-1 (Ea) and MGF (Eb) (Lowe et al. 1988; Roberts et al. 1987), which differ by the absence or presence of a 52bp insert (exon 5) respectively, there are at least three further variants associated with transcriptional activity from the two known promoters in the rat IGF-1 gene. Two transcripts derive from promotor 1 with alternative 5'UTRs, differing by the presence or absence of an insert of 186bp (class-1 [+ or – 186] IGF-1 mRNA), and one from exon 2. In combination with the splicing associated with L.IGF-1 and MGF variants, at least six mRNA species may be derived theoretically, notwithstanding further variation determined by multiple polyA signals in the 3'UTR (Hoyt et al. 1992). All six 5' variants were detected in the present study, which were analogous to the published nucleotide sequences (Shimatsu & Rotwein 1987a; Roberts et al. 1987), showing a qualitative, selective abundance of one particular mRNA species in response to injury, and a general degree of selectivity between snout muscle and brainstem tissues.

A significant finding from this study revealed that relative levels of type-1 (+186) MGF mRNA are qualitatively higher in the denervated snout muscle 1 and 3 days after injury, compared to the non-injured side. This finding was reinforced by separate PCR reactions, with different primer sets. There appears to be reasonable evidence to show that this is the preferred 5'UTR species for generating type-1 MGF mRNAs, in denervated snout muscle. There is no overall increase however, in class-1 mRNAs relating to either of the L.IGF-1 or MGF isoforms, only an alteration in preference of exon 1 (+186) MGF transcript. This is possibly due to a lack of requirement for transcriptional activation. In the presence or absence of appropriate signals however, splicing activity may be down-regulated so as to increase relative abundance of this longer transcript. No physiological function has been confirmed to the existence of variant 5'UTRs within exon 1 but it has been speculated that such variant leader peptides might constitute a signaling mechanism determining either a

secretory or an endogenous pathway (LeRoith & Roberts 1991; Lund et al. 1991). It has been shown that the 5'UTR can affect mRNA translation due to its length, secondary structure and/or presence of AUG start codons up-stream of the reading frame (Kozak 1991a; 1991b). Other studies, in contrast, have showed that IGF-1 mRNA in general, *i.e.* not discriminating between class 1 or 2 and L.IGF-1/MGF variants, is increased, covering the time period used in this study, in denervated or Botulinum toxin-paralysed muscle (Caroni & Schneider 1994), following bupivacaine injection (Marsh et al. 1997), muscle subjected to nerve crush (Pu et al. 1999a) or induced to undergo longitudinal growth (Yang, S. et al. 1996). This is inconsistent with present findings, whereby change in total L.IGF-1 or MGF transcripts was not observed in the snout muscle. This may be a reaction that is specific to muscle fibres in the snout muscle or that the increase in total L.IGF-1 and MGF mRNAs is too subtle to be detected by this method. Since no quantitative analysis was applied, the contribution of the observed relative increase in expression level of a single transcript may be of little consequence to total mRNAs.

IGF-1 mRNA and protein was shown to be increased in previous studies, in the spinal cord (Hammarberg et al. 1998) and the facial nucleus (Gehrmann et al. 1994) *from* 3 days after nerve axotomy. This *is* consistent with present findings, in that change in total L.IGF-1 and MGF transcripts, irrespective of 5' variation, was not observed in the brainstem *prior* to 3 days post denervation. A qualitative increase of IGF-1 mRNA may have been detected had the animals in the present study been allowed to survive for a longer time-period. These survival periods were chosen in accordance with the hypothesis that MGF provides a means for short-term cellular repair, in contrast to L.IGF-1 transcripts that may be more important for long-term regeneration of muscle tissue (Goldspink 1999). It seems possible that either transcriptional activation for increased MGF production is not required by injured motoneuron cell bodies and snout muscle prior to 3 days post axotomy/denervation, or that MGF protein is increased by a post-transcriptional mechanism. The latter mechanism may indeed serve as a more rapid response to injury. Although translation efficiency rates have been shown to be inversely proportional to transcript lengths (Yang, H. et al. 1995) and may provide a molecular basis for translational control of IGF-1 biosynthesis (Hepler et al. 1990), the actual differences in transcript length, combined with the relatively weak

transcriptional elements that promotor 1 is known to contain (Wang et al. 1997; Werner, Stannard, et al. 1990), do not necessarily support rapid *de novo* transcription and synthesis of MGF in response to injury, compared to that of other isotypes (McKoy et al. 1999; Yang, S. et al. 1996). The present study shows that both L.IGF-1 and MGF mRNAs may be produced from promotor 1. In the absence of any direct link between promotor 1 usage and propensity of MGF mRNA production in favour of L.IGF-1 production, it seems reasonable to postulate that immediate increase of MGF may, therefore, be supplied in the short-term by decreased splicing of mRNAs. This would increase the relative abundance of MGF compared to L.IGF-1 mRNAs. It is likely that transcriptional activation is a rapid consequence of muscle damage (Yang, S. et al. 1996) but this may be secondary to changes in splicing activity. Analysis of tissue after a period of greater than 3 days may have shown increased total MGF transcripts compared to those for L.IGF-1. In the absence of *de novo* transcription however, changes in relative levels of these mRNAs may not be detected by the present method. Real-time qualitative RT-PCR is an alternative method that would measure actual mRNA abundance. The present study however, was designed only for detecting qualitative differences utilising a practical approach for increasing the chances of quenching amplification within the exponential phase of the PCR.

Irrespective of the search for specific responses to injury, the current results show a degree of tissue selectivity between class-1 and class-2 IGF-1 variants with respect to L.IGF-1 and MGF mRNAs. Class-2 MGF was untraceable, and presumably, absent in brainstem. Of class-1 MGF copies, the 5'UTR variant containing the 186bp insert predominated in brainstem, whereas the deletion variant showed tendency towards snout muscle. In total however, class-1 mRNAs predominated in snout muscle. Of class-1 L.IGF-1 variants, 5'UTR variants containing the 186bp insert predominate, again, in brainstem and were absent entirely from snout muscle. There was only slight tissue-selective expression of class-2 L.IGF-1 copies. There was no tissue-selective expression of total L.IGF-1 or MGF mRNA between normal tissues. The relative levels of exon-1 and exon 2 expression have been studied previously in normal tissue (LeRoith & Roberts 1991). Exon 2-containing mRNAs are expressed primarily in the liver (LeRoith & Roberts 1991) and seem to be involved with steady state IGF-1 expression, mostly to the effects of GH (Lowe et al. 1987). As a result,

these transcripts, whose activity is controlled mainly by promotor 2, primarily mediate the endocrine actions of IGF-1. In contrast, exon 1-containing mRNAs have been assigned to a more locally responsive role to short-term stimuli and to stimuli of greater variety, such as oestrogen, gonadotrophins and insulin (Mittanck et al. 1997; Murphy, L.J. et al. 1987). This is reflected by multiple transcriptional start sites (Hall et al. 1992) amongst weak promoter elements (Adamo et al. 1991) and the fact that exon 1-containing mRNA transcripts have been found in all tissues studied (LeRoith & Roberts 1991). Their transcripts, whose activity is controlled by promotor 1, are considered to encode the paracrine/autocrine form of IGF-1. It is generally recognised that the endocrine *versus* autocrine/paracrine paradigm is more pronounced with respect to tissue selective expression of exon 1 and exon 2 variants than that shown by the Ea (L.IGF-1) and Eb (MGF) variants (LeRoith & Roberts 1991). In summary, this is the first time that tissue-selective expression of differential IGF-1 mRNAs has been studied with respect to nerve axotomy and the first evidence of selection between alternative 5'UTRs of class-1 IGF-1 mRNAs in any tissue to date.

5.3.2.2 Direct muscle damage

It has been shown so far that muscle denervation causes perturbations in levels of MGF in target-muscle. There can be no further influence on motoneurons however, if there is no contact between nerve and muscle. By damaging target-muscle directly, the present study aimed to expose intact motoneurons to altered levels of MGF and other factors made available to motoneurons following muscle damage, which may affect the capacity of motoneurons to survive subsequent lethal nerve trauma. The following is a discussion of the possible mechanisms underlying increased motoneuron survival in rats subjected to prior muscle damage, cell count data for which was discussed in chapter 3.

In situ detection of IGF-1 mRNA was shown in both non-damaged muscle and damaged muscle fibres irrespective of treatment so it cannot be deduced unequivocally from the present data that L.IGF-1 and/or MGF transcripts were up-regulated specifically in response to muscle damage. As a result, there is no immediate explanation involving IGF-1 for the observed differences in neuroprotection. The reaction of skeletal muscle to localised

injection of bupivacaine has been documented extensively however (Carlson & Faulkner 1983; Hall-Craggs 1974), so it is surprising that intramuscular injection of bupivacaine, known to cause increased IGF-1 production in skeletal muscle (Marsh et al. 1997), and in particular, temporally regulated increase in MGF mRNA (Hill *et al.* in press), did not result in significant neuroprotection in the present study, especially in comparison to muscle injected with distilled water.

L.IGF-1 and MGF mRNA was found in muscle fibres. Irrespective of muscle damage, such staining was observed only in discrete areas of each tissue section and may correspond to a pattern consistent with accumulation at neuromuscular junctions. In the absence of co-staining with either α-Bungarotoxin or silver cholinesterase activity staining, such localisation cannot be confirmed but was similar to the pattern of staining in other published work (Newey et al. 2001) where mRNA was detected by *in situ* hybridisation. Such accumulation of mRNA would support the notion that transcription in large cells such as myofibres takes place in close vicinity to the site of protein requirement in a fashion similar perhaps to that occurring in motoneurons (Bassell et al. 1998; Mohr 1999). In this situation presumably, intact nerve fibres are attempting to regenerate. Increased IGF-1 in muscle is known to cause nerve sprouting (Caroni & Grandes 1990) and GAP-43 would have been a useful marker to confirm this due to its locality in regenerating growth cones. The pattern of *in situ* hybridisation staining of these sections (**Fig. 5-5**) is in contrast to that found after muscle denervation, where L.IGF-1 and MGF transcripts were located around the whole perimeter of muscle fibres, across the majority of the tissue section area (**Fig. 5-8 and 5-11**). This may be more closely consistent with non-specific secretion of protein in the absence of intact nerve fibres.

On balance, it seems that no true conclusion may be derived from this data regarding mRNA expression *in situ*. It remains uncertain, therefore, whether a genuine increase in mRNA is present at 7 days post injury, which is concomitant with the time chosen to avulse the facial nerve. mRNA analysis in snout muscle from 1 to 3 days following denervation or 4 to 7 days following direct muscle injury showed no evidence of increased expression of IGF-1 isoforms at the mRNA level. While it is possible that a post-transcriptional mechanism may

account for the lack of alteration in total L.IGF-1 and MGF mRNAs up to 3 days post denervation as described earlier, the considerable evidence for increased mRNA expression after this time-point (Caroni & Schneider 1994; Marsh, et al. 1997; Pu et al. 1999a; Yang, S. et al. 1996) suggests a degree of failure to detect accurately such changes by *in situ* hybidisation after direct muscle damage, in the present study. Cross-sectional profiles of muscle fibres, such as that observed for subjective analysis in the present study, are likely to expose just a few neuromuscular junctions, which limits severely the possibility of making accurate quantitative or qualitative measurements to compare relative levels of staining to undamaged tissue, and to tissue from animals subjected to alternative treatments. Due to the irregular morphology of musculature in the snout muscle however, it was not possible to obtain such profiles. Successful attempts to use the ribonuclease protection assay (RPA) were made but time constraints did not allow full optimisation of probes in liver tissue. Northern blotting was an attractive alternative but was rejected on the basis of too small tissue size, characteristic of the snout muscle, which would yield enough RNA only for a few blots.

In keeping with the considerable amount of information correlating muscle damage with increased levels of IGF-1 isoforms, there may be other mechanisms by which motoneurons gain increased access to such growth factors as a result of the respective treatments. The generation of an extracellular osmotic potential, an expected outcome of distilled water injection, would render membranes vulnerable to rupture irrespective of cell type. Therefore, intramuscular nerve fibres would be additionally subjected to such damage. This would allow free passage of IGF-1 isotypes and other contents of ruptured muscle fibres into the cytoplasm and may, therefore, by-pass some receptor-mediated mechanisms by which entry of neuroprotective substances released following bupivacaine injection may be constrained. Injection of physiological saline may cause only negligible muscle damage

Thus increased access of IGF-1 isoforms to damaged nerves may increase respective signaling in motoneuron cell bodies as shown schematically in **Fig. 5-19**.

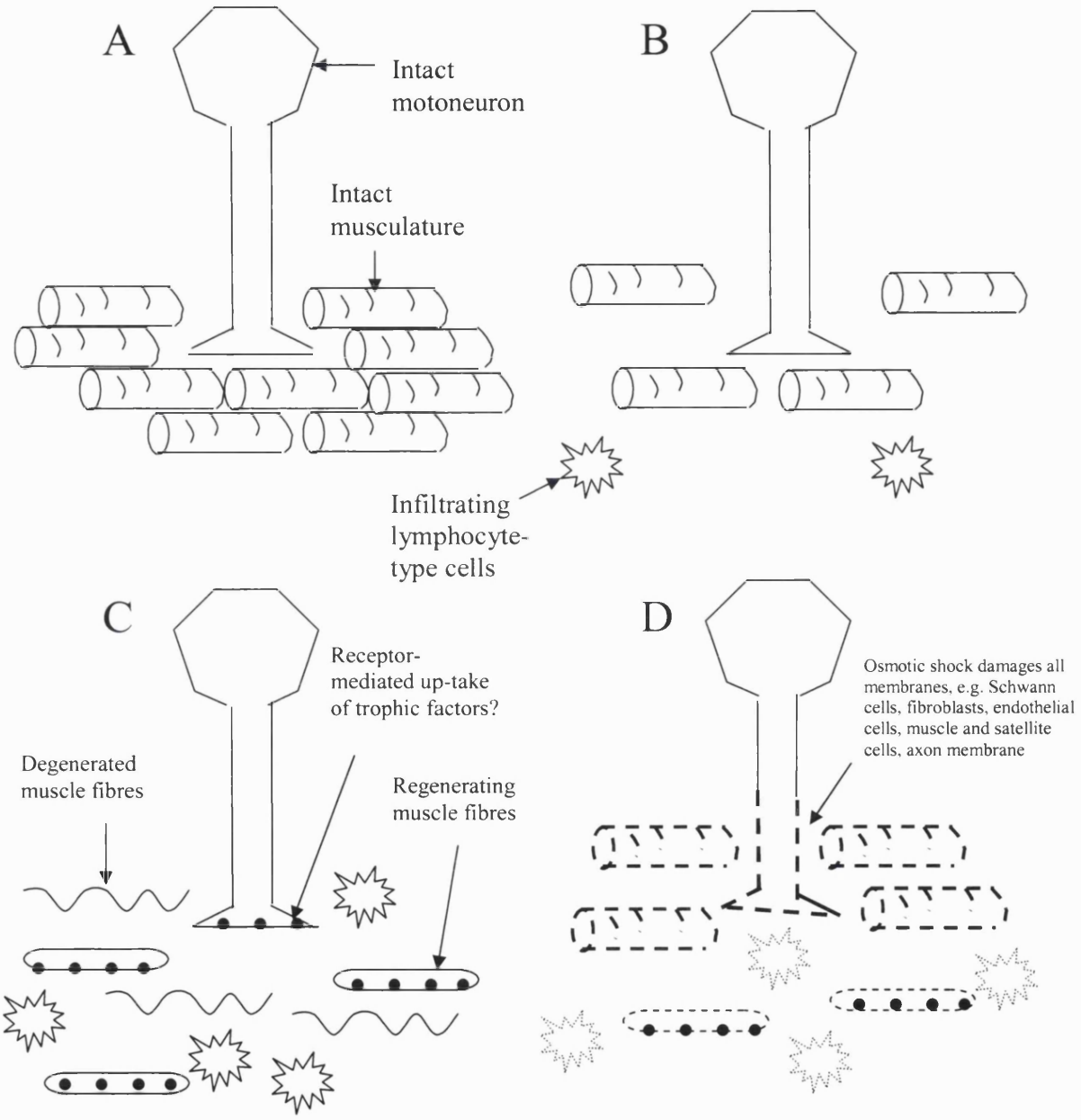


Figure 5-19 Possible mechanism of access for IGF-1 isoforms into motoneurons following injury of target-muscle. (A) Intact motoneurons are maintained, in part, by neurotrophic support derived from their target musculature. (B) Following injection of physiological saline, some mechanical displacement and slight damage of a few muscle fibres is seen with only limited influx of inflammatory cells. (C) After injection of the myotoxic agent bupivacaine, extensive degradation of muscle fibres and infiltration of lymphocyte-type cells is

seen followed by muscle regeneration. Motoneurons become exposed to neurotrophic factors and cytokines released from the target tissue but gain only limited access. (D) Injection of distilled water results in osmotic shock of cellular membranes causing rupture irrespective of cell type. Neurotrophic factors and cytokines released from ruptures muscle fibres and other cells, regenerating fibres and infiltrating cells have increased access to intramuscular nerves enabling neuroprotective mechanisms within motoneurons to be induced.

The present results showed that GAP-43 mRNA was not increased ipsilaterally after any of the injected solutions indicating that there was not enough damage to axons to induce this type of response associated commonly with elevated IGF-1 in the periphery (Flint et al. 1999; Piehl et al. 1998a). Therefore, other than observing relative infiltration of lymphocyte-type cells through morphological evaluation (see next section), there is no further evidence from the present study to prove that the mechanisms described above were apparent. Specific markers indicating the lack of synapses (e.g. lack of co-localisation at neuromuscular junctions of labeled α-Bungarotoxin and green fluorescent protein-expression in motoneuron axons previously manipulated to over express this protein) but presence of viable (non-ruptured) nerves (*e.g.* lack of leakage of green fluorescent protein from motoneuron axons) would be helpful in distinguishing between the illustrated processes. Additionally, salt solutions of equal volume but with graded osmotic potential may result in corresponding levels of neuroprotection. Nerve avulsion 4 days post-muscle damage would have also given more information about possible mechanisms since muscle damage at this time point was greater than that observed at 7 days post-injection. Considerable membrane repair may have already taken place during this period.

5.3.3 Other mechanisms

5.3.3.1 Response to peripheral inflammation

Unlike *in situ* hybridisation detection of IGF-1 mRNAs, the level of tissue damage apparent on observation of snout muscle sections stained with haematoxylin and eosin after 4 and 7 days parallels more closely the level of motoneuron rescue from avulsion-induced death. It

has been shown previously that induction of inflammation after repeated injection of phytohaemagglutinin into the snout muscle leads to retrograde cell response in facial motoneurons (Mariotti et al. 2001) and this alters the subsequent vulnerability of these motoneurons to axotomy (Mariotti et al. 2002b). Unlike the cited studies, which used the microglial marker (OX-42), immuno-staining for NADPH-diaphorase and *in situ* detection of Bcl-2, no retrograde cell reaction was detected in response to any of the treatments given in the present study. The markers used in this study were specific to microglia (OX-42), which failed to yield specific immuno-staining, GFAP for astroglial response and GAP-43 mRNA for signs of axonal regeneration (Bisby & Tetzlaff 1992), also known to be up-regulated specifically after increased IGF-1 in target tissue (Caroni & Grandes 1990). Morphologic assessment was also made from haematoxylin- and eosin-stained sections revealing no overt signs of retrograde cellular response to muscle injury. The present result demonstrates however, that muscle damage failed to elicit an astroglial response, which has not been examined previously after muscle damage, and also did not result in increased GAP-43 mRNA production. This is in agreement with another study, where GAP-43, also examined 4 days after treatment, was shown only to be increased after partial denervation, in an experiment where ineffective treatments included Botulinum toxin and bupivacaine (Bisby et al. 1996). Since some muscle treatments, which increase IGF-1 levels, do not increase GAP-43 in motoneurons it must be concluded that the IGF-1/GAP-43 link is not invariable. Although GAP-43 was not examined, motoneurons innervating partially denervated rat hindlimb muscles in another study remained susceptible to axotomy-induced cell death (Harding et al. 1998b). This indicates that mechanisms additional to increased IGF-1 levels contribute to neuroprotection.

Another mechanism by which muscle damage might increase motoneuron survival is by pre-conditioning central immune and inflammatory surveillance prior to nerve injury. Whether central surveillance of nerve damage may be determined, in part, by the level of peripheral inflammation remains uncertain. Interestingly, it has been reported previously that inflammation provoked in peripheral axons of sensory neurons by injection of bacterial toxin enhanced the regenerative capacity of central axons when subjected dorsal spinal root crush (Lu & Richardson 1991). Influx of macrophages and other inflammatory cells to the

vicinity of the dorsal root ganglia cells was responsible for the influence on regeneration (Richardson & Lu 1994).

Rupture of intramuscular motor nerve membranes, as a predicted outcome of mechanical and osmotic damage, would certainly expose motoneurons to a range of pro- and anti-inflammatory cytokines produced by circulating inflammatory cells, Schwann cells, fibroblasts and muscle cells. Resident mononucleated cells as well has neutrophils and macrphages from the general circulation are present in acutely injured muscle (Best & Hunter 2000; Tidball 1995). TNFα, interleukin-1 (IL-1), IL-6, IL-8, IL-10 are all known to be increased in plasma after strenuous exercise (Pedersen 2000). IL-6 in particular, has been shown to rescue motoneurons subjected to nerve axotomy in neonatal rats (Ikeda et al. 1996a) and whose mRNA is specifically increased in the injured facial nucleus (Kiefer et al. 1993), suggesting a role for IL-6 centrally (Streit et al. 1998). IL-6 shares the same receptor as CNTF and LIF, both of whom are also increased in response to muscle injury (Kurek et al. 1996a; 1996b) and which have substantial neurotrophic qualities in motoneurons (Hughes, R.A. et al. 1993; Sendtner et al. 1990). If the present results are due to the pre-conditioning of motoneurons with factors from damaged muscle, it would be interesting to determine if injection of a damaged muscle extract alone into the snout muscle of rats can replicate the results obtained by injection of substances such as water or bupivacaine.

IL-6 amongst numerous other cytokine mRNAs are also expressed in microglia reacting to facial nerve axotomy in adult rats, indicating a protective role for such proteins in motoneurons (Klein et al. 1997; Kloss et al. 1997; Raivich et al. 1999b; Schwaiger et al. 1998). Microglia are generally recognised as the first line of defence against a variety of pathologies (Gehrmann et al. 1995; Kreutzberg 1995) and thus considered to be as the most sensitive reacting cell-type. On reflection, there is good evidence to show the transduction of peripheral inflammatory signals to the CNS, upon which the brain's resident macrophages and immune cells may react as a non-specific defence mechanism.

It is unfortunate that information on microglial activity was not obtained in this study, despite the use of analogous antibody in other studies (Angelov et al. 1998; Graeber et al.

1998; Mariotti et al. 2001; 2002b). The conditions under which the OX-42 antibody was applied in the present study may have been problematic with regards to detecting successfully the type 3 complement receptor, which is the antigen specific to cells of the macrophage/microglial lineage (Robinson et al. 1986). Irrespective of staining procedures (see below), it is noteworthy that this cell surface protein is expressed usually under conditions of activation, i.e. microglia expressing phagocytotic phenotype. It is quite possible that the perturbations to target muscle described in the current study were not sufficient to induce such activity. Another marker of microglial activation/response to injury may have been more appropriate. For example, bromodeoxyuracil incorporation would have indicated cell proliferation in the facial nucleus – a response of microglia that has been described previously (Graeber et al. 1988) but which may not induce complement receptor expression at the cell surface.

Upon review of other studies in which this particular antibody has been used to detect microglia in the facial nucleus, the immunohistochemical staining protocol used appears to vary substantially with respect to tissue processing, fixation, section thickness, blocking conditions, antibody concentration and buffer-type/conditions. These are summarised in **Table 5.4**.

Table 5.4

Comparison of immunohistochemical staining protocols (up to the stage of application of primary antibody*) between the present study and those of previous studies.

	Study			
Conditions	*Present study*	*Angelov et al.*	*Graeber et al.*	*Mariotti et al.***
Section type/thickness	Vibratome/70μm	Vibratome/50 μm	Fresh frozen/20μm	Frozen (after perfusion)/40μm
Fixation	Perfused fixed in 4% PFA	Perfused fixed in 4% PFA	4% PFA, then graded acetones	Perfused fixed in 4% PFA
Permeabilisation	Triton-X 100	Triton-X 100	Not	No separate step

		and 0.5M NH_2Cl	specified	
Blocking conditions	5% BSA	5% BSA	Not specified	5% BSA in 0.3% Triton-X 100
Antibody conc./incubation	1/500 4% overnight	1/5,000 Room temperature	1/1,000 4°C overnight	1/500 4°C overnight
Dilution Buffer	TBS + Triton-X 100	TBS + 0.8% BSA	PBS + 0.1% BSA	PBS

* indicates that secondary antibody application and chromophore development stages are almost identical between all studies. ** indicates the staining procedure on which immobilsed sections in the present study were subjected, except sections were fresh frozen and cut at 10μm.

Not withstanding other conditions, it seems that the thickness of vibratome sections was probably not a significant factor in preventing immuno-staining in the present study since it is likely that at least some cells would have been stained towards the cut surfaces of each section. However, the other study to use such sections (Angelov et al. 1998) applied the antibody at a concentration one order of magnitude higher than the current study and this was left to incubate at room temperature. Such concentration is likely to have had a considerable effect of increasing binding specificity to this cell-surface antigen. For immobilised sections, the use of fresh frozen sections and subsequent on-slide fixation may have caused greater masking of antigen at the cell surface thus inhibiting antibody binding. Given that other antibodies were detected at the same time in the same tissues using the same detection reagents, it is unlikely that there was a problem with OX-42 antibody detection. This forces the conclusion that there may have been a problem with the primary antibody itself, such as denaturation. In retrospect, this could have been assessed using Western or immunoblot procedures.

Ineffective on Vibratome and fresh cryosections in the present thesis, OX-42 immuno-positive or lectin-stained sections may have yielded information on whether microglia can detect, and react to relatively mild perturbation of peripheral targets. Data revealing microglial response coordinating quantitatively with muscle inflammation would be highly

suggestive of a pre-conditioning mechanism invoked by muscle damage. Immuno-detection of NADPH-diaphorase, also known as nitric oxide synthase (NOS), would also give important data on metabolic status of motoneurons subsequent to muscle damage. NOS is responsible for production of nitric oxide, whose excessive accumulation in motoneurons is associated with increased oxidative stress and motoneurons destined to die (Novikov et al. 1995; Rossiter et al. 1996; Ruan et al. 1995). NOS was shown to be altered in response to target damage (Mariotti et al. 2001). The disruption of extracellular matrix such as that occurring putatively by displacement of fibres following injection of large volumes of fluid might be conducive to inflammatory/immune response in peripheral target tissue. A marker specific for extracellular proteins (*e.g.* fibroblast specific marker) could have been used to assess the regeneration of this tissue compartment in the absence of specific markers for nerve or muscle cell rupture. A generalised assay to measure cellular breakdown products of muscle cells, *e.g.* creatine kinase, or immune markers in blood would serve, perhaps, as the most practical tool for detecting relative levels of total tissue damage incurred by each of the treatments.

5.3.3.2 Central markers of retrograde reaction

Markers of retrograde cell response to nerve avulsion were used in all experiments where cell count data was obtained. It is not possible to make deductions from these findings about the mechanism of neuroprotection offered by MGF. Of all the cellular markers used in this study, CGRP was a key marker of motoneuron survival, usually identifying most motoneurons that remain in the facial nucleus after nerve avulsion. It is expected, therefore, that the pattern of staining in circumstances of neuroprotection, irrespective of whether rats have been treated with MGF plasmid, MGF E-domain protein, DR or muscle damage, does not differ in that the number of stained cells and staining intensity increased ipsilaterally 1 month after injury. Since the expression of CGRP remains non-specific to any single treatment all that can be concluded is CGRP expression correlates with nerve injury. It cannot be certain however, that CGRP expression is a prerequisite for neuroprotection. Numerous other studies have made similar observations (Blake-Bruzzini et al. 1997; Haas et al. 1990; Piehl et al. 1993; Simon et al. 2000). Increase in CGRP immuno-staining was seen

only in response to avulsion in adult animals but not seen in neonatal and ageing animals (irrespective of diet). This is inconsistent with that observed after ventral root avulsion, which showed that CGRP mRNA expression had returned to normal following an initial up-regulation induced by ventral root avulsion (Piehl et al. 1998b). The same study also showed a decrease in CGRP mRNA in neonatal rats operated on at the age of 3 to 7 days at the time of surgery. This is in contrast to the increase in immuno-staining seen in the present study in which operated neonates were 7 days old. It seems likely that this represents the period at which motoneurons begin to change to their mature phenotype. It has been suggested that CGRP is a mediator of glial cell activation after neuronal injury (Reddington et al. 1995) but it seems that it could be one of many such mediators because glial cell activation was not confined to instants of increased motoneuron survival with which increased CGRP expression was associated. It may help explain more elaborately however, a mechanism by which neonatal motoneurons become mature and, therefore, adopt their more robust nature with respect to motoneuron survival following axonal injury. Thus we see general increase in the level of activated astrocytes with age (see later).

Akt mRNA was used in neonatal animals only as a marker of neuronal survival that is associated with IGF-1 signaling (Zheng et al. 2000). An alternative use of these probes could have been as a marker for IGF-1-induced activity in response to either to MGF/L.IGF-1 gene transfer or muscle damage in adult animals. Experimental duplication would have been required in order to investigate this. Interestingly, up-regulation ipsilaterally of Akt and GAP-43 mRNA was observed in response to nerve avulsion in neonatal animals despite the fate of the majority of motoneurons at this age to die within 7 days post injury. Staining was seem also in adult and ageing animals (GAP-43 only) at 1 month post injury, which is consistent with other studies (Johnson, H. et al. 1995; Piehl et al. 1998b). Such mRNA up-regulation may form part of a repertoire of events in response to general cell stress as a last effort to avoid motoneuron death by attempting to extend new nerve sprouts to locate a source of neurotrophic support. Avulsed motoneurons have been shown to extend new nerve sprouts from cell bodies in response to exogenous BDNF (Novikov et al. 1997). It is not known whether these new nerve sprouts can lead to increased availability of neurotrophic factors from spurious sources to avulsed motoneurons. Although GAP-43 mRNA was not

increased in response to muscle damage alone, it cannot be dismissed that GAP-43 was not up-regulated in response to either MGF or L.IGF-1 plasmids alone. GAP-43 mRNA was shown previously to be up-regulated in hypoglossal motoneurons following IGF-1 gene transfer alone into the tongue of rats (Flint et al. 1999). Interestingly, prolonged expression of GAP-43 is associated with greater susceptibility to injury-induced neuronal death (Gagliardini et al. 2000) (Harding, Greensmith, et al. 1998a; 1999). It is possible, therefore, that the continued expression of GAP-43 at 1-month post avulsion is inhibitory with respect to motoneuron survival in adult and ageing animals.

Avulsion-induced facial motoneuron death in neonatal rats examined 3 days post-lesion was associated with cytoplasmic staining due to the cleavage of caspase-3 and the appearance of TUNEL-stained nuclei. While many of the caspase-3-immunostained cells had a typical neuronal appearance, the cellular identity of the TUNEL-stained nuclei remains unknown in the absence of additional neuronal or neuroglial markers. It would appear that neuronal death is in part, induced by the activation of the apoptotic pathway, as has been reported previously for neonatal motoneurons (de Bilbao et al. 2000; Li, L. et al. 1998; Vanderluit et al. 2000) . Such markers were not identified in the adult or ageing rats of the present study. This cannot be taken as evidence that apoptotic death is absent in adult and ageing rats, however, as the combination of short time-course of apoptosis, the long (1 month) survival time chosen, and the use of a sampling approach (rather than examining every section through the facial nucleus), is likely to have reduced severely the probability of detecting apoptotic motoneurons. There is uncertainty regarding the nature of motoneuron death in adult animals following nerve injury. Avulsion-induced death in adult animals has been described as apoptosis with necrotic-like features (Chai et al. 1999). TUNEL-stained nuclei however, would have failed to discriminate between the alternative modes of cell death because both sets of events terminate ultimately with DNA fragmentation, such was the case in a previous study that used TUNEL staining and morphologic observations (Martin et al. 1999). The real implications of not dying by apoptosis are associated with therapeutic intervention since motoneurons dying by necrosis would be unaffected by application of neurotrophic substances alleged to interact with the apoptotic pathway. Much work has suggested that neurotrophic factors may inhibit neurons *in vitro* from entering the active cell

death program (Comella et al. 1994). It remains unclear however; with necrotic-type death may be prevented by augmentation of the same cellular pathways operating in apoptotic-type neuronal death.

It is possible that the increased background immuno-staining, which were observed in ageing DR animals, reflects the astroglial hyperplasia that has previously been reported to occur with DR (Major et al. 1997). Astrocytes are known to be source of many neurotrophic factors (Dreyfus et al. 1999) and their hypertrophy might therefore reflect a shift in the balance of peripheral *versus* central neurotrophic support of motoneurons. This may be a significant concept with regard to predicting the likelihood of motoneuron survival subsequent to nerve injury. Their presence in sufficient numbers under physiological conditions, therefore, may create an environment permissive to motoneuron survival. A quantitative study of astroglial activation based on GFAP mRNA expression could help indicate a difference in central support when DR-fed rats are compared directly to *ad libitum*-fed rats of the same age.

Other markers that could have been useful indicators of neuroprotection include choline acetyletransferase (ChAT) and the low-affinity neurotrophin receptor p75. ChAT, for example, is used for synthesis of neurotransmitter acetylcholine (ACh). Down-regulation of ChAT is a retrograde effect of nerve axotomy indicating a switch in metabolism from neurotransmitter synthesis to cellular recovery. Local application of neurotrophic factors is usually associated with maintenance of ChAT levels in injured motoneurons (Alderson et al. 1996; Yan et al. 1995). It is unclear whether such a marker is a good indicator of neuroprotective mechanisms because motoneurons that fail to make the metabolic shift from a mode of function to one of recovery (i.e. inactivation of survival promoting signaling) may be more susceptible in the long-term to the effects of nerve axotomy (Schwaiger et al. 1998). In addition, neuroprotection of mature motoneurons recovering from nerve avulsion remain, perhaps, unlikely to re-establish ChAT levels to physiological levels and consequently, may not serve as a reliable indicator of motoneuron survival. p75 is another marker whose true value with respect to expression in motoneurons is not fully understood (Davies 1997). Whereas gene knock-out studies have indicated that p75 expression may increase the risk of

motoneurons dying following injury (Ferri et al. 1998), its expression has also been correlated with axon regeneration (Piehl et al. 1998b). Expression of p75 during motoneuron development is associated with programmed cell death and its re-expression in cell bodies of adult motoneurons after axotomy may represent the re-activation of the developmental program of gene transcription and has been postulated as a possible mechanism for selective motoneuron degeneration in murine and human MND (Lowry et al. 2001).

5.3.4 Conclusion

Intramuscular injection of a plasmid encoding MGF resulted in expression of mRNA derived from the vector at 7 days, coinciding with the time of nerve avulsion (chapter 4). The vector was neither transported retrogradely to motoneuron cell bodies nor to any other tissue tested. Application of gelfoam soaked in MGF E-domain peptide to avulsed motoneurons resulted in significant increase of motoneuron survival at 1 month and indicated that this putative active fragment of MGF needed to be present at the time of injury in order to confer neuroprotection.

Muscle denervation was effective at altering levels of MGF mRNA in muscle compared to the non-injured side. Interestingly however, there appears to be numerous mRNA species by which both MGF and L.IGF-1 may be derived whose tissue-selective expression may be important for regulating their differential activity and localised protein expression. Intramuscular injection of distilled water, bupivacaine and physiological saline caused considerable mechanical damage to muscle tissue and this seems to provide a means by which adult motoneurons become resistant to the effects of facial nerve avulsion. The greater the level of peripheral inflammation caused, the more substantial the level of neuroprotection. It remains uncertain whether MGF lies central to the protective effect on motoneurons of prior muscle damage. The retrograde reaction to perturbations of the peripheral target may be important for recruiting central neuroprotective mechanisms.

Chapter-6

General discussion

Intervention before the onset of neuropathology might be regarded as an ultimate goal for many fields of research into neuronal degeneration. Knowledge of those factors, which normally promote neuron survival, is crucial to such research. The general aim of the work described in this thesis is to provide information on the effect of prior perturbations of target muscle on the survival of axotomised facial motoneurons. This study has shown for the first time, in the same system using the same experimental model, that the target-dependence of motoneurons alters from the neonate to the adult to the ageing rat. It also shows that motoneuronal survival is affected by a locally acting isoform of IGF-1 (MGF), by dietary restriction and by prior muscle damage that is known to result in MGF expression. A common factor, which unites all these different experimental situations, is that they are all associated with altered levels of IGF-1. This general discussion will explore mechanisms regulating motoneuronal survival, which may be common to all the experimental studies described in this thesis.

Until recently, much of what we know about motoneuron survival in adult animals was based on the considerable amount of data that has accumulated on studies of the neonatal animal. Introduction of a suitable model of motoneuron loss in adult animals has meant that factors affecting motoneuron survival may be tested directly. The current thesis has shown that IGF-1 isoforms, DR and muscle damage affect motoneuron survival in adult rats and more importantly; and contrary to common belief, motoneurons remain sensitive to perturbations of target-derived neurotrophic support. It is reasonable to suggest that the locally acting splice variant of IGF-1 (MGF) may have a central role in determining motoneuron survival in adult rats. Its prominence seems not to be related to its potency as a potentially novel neurotrophic factor, although its activity compared to the most effective neurotrophic factor GDNF is notable. Rather, its endogenous role in skeletal muscle as an important regulator of muscle mass (Goldspink 1999; McKoy et al. 199; Yang, S. et al. 1996) may help to illuminate the relationship between nerve and muscle compared to other neurotrophic factors. As a result, the potential to alter the status of motoneuron viability simply by varying the activity of the peripheral target may be a significant advantage over applying neurotrophic factors exogenously. Therefore it seems appropriate to make some

informed judgments about the mechanism of action of MGF with regard to promoting motoneuron survival.

Common to all of the experiments (excluding the peptide study) carried out here has been the apparent influence on motoneuron survival of altering the physiological environment of nerve and muscle prior to avulsion. It has been shown that DR reduces motoneuron number (chapter 3). It is likely that prolonged muscle atrophy induced by stringent caloric restriction causes the loss of motoneurons that have a high target-dependence. DR may also alter intrinsic metabolism of the remaining motoneurons making them more resistant to cell death. DR is known however, to perturb age-related alterations in muscle IGF-1 (Sonntag et al. 1999) and this may affect target-muscle mass. It is of particular interest to the current thesis, therefore, to highlight this relationship as just one possible explanation for these findings. Neuroprotection was also afforded by exposing adult motoneurons to elevated MGF either by inducing muscle damage and/or repair in target tissue (chapter 3 and 5), or more directly by muscle gene transfer of MGF (chapter 4), prior to nerve injury. It remains uncertain whether the signaling mechanisms, amenable to alteration by MGF, may be modified under physiological conditions. However, there seems to be little doubt the MGF E-domain peptide must be present at the time of injury (chapter 5) in order for it to confer neuroprotection. These observations are consistent with MGF being transported retrogradely from the periphery to motoneuron cell bodies *via* interaction with the type-1 IGF receptor that is found in motoneurons (Gehrmann et al. 1994).

Application of E-domain peptide to avulsed motoneurons and prior muscle gene transfer of MGF also resulted in significant neuroprotection, whereas injection of the peptide into snout muscle had no effect on motoneurons. It is possible that the E-domain peptide is the active component of MGF, perhaps becoming cleaved only once internalised within motoneurons. Peptide injected into muscle, therefore, may only be myogenically active. On account of this, it would have been interesting to observe the effect of MGF peptide on muscle mass after nerve avulsion (denervation) compared to the non-injured side. Exogenous or endogenous modification of muscle-derived MGF availability to motoneurons must occur with motoneurons intact and undamaged. If MGF were such an important mediator of

motoneuron survival, however, it would be interesting to see if there are circumstances (age, injury) where MGF may be derived from alternative sources (*e.g.* CNS cells).

Short-term exposure to MGF or acute muscle damage resulted in considerable neuroprotection. Such acute sensitivity to these perturbations however, contrasts with the slow loss of motoneurons to nerve avulsion or DR, where target-derived neurotrophic support is decreased or removed. There appears to be no doubt that MGF expression is related to muscle activity and/or damage. MGF-induced neuroprotection may be mediated by altering responses to injury. Biological action of MGF should not be confused with a lesion factor, which is a term used to refer to the action of molecules such as CNTF on motoneurons, that become available to motoneurons only under conditions of nerve lesion (Sendtner et al. 1997). It is easier to reconcile MGF as a stress-signaling molecule readily available to motoneurons under conditions of increased peripheral activity that alerts them, and maybe other cell types including muscle fibres, to injury thus holding cellular activity in a state that promotes or facilitates motoneuron survival. It is not clear whether such stress priming may be effective for long-term neuroprotection. MGF was effective at protecting motoneurons for approximately 1 month although this might be affected by the method of gene transfer. It would be helpful to gain knowledge on the period for which motoneurons were protected by muscle damage, as this would give some indication of whether MGF may be considered an agent of muscle damage by sharing similar temporal regulation of motoneuron survival as that shown following intramuscular injection of the MGF gene. Prolonged priming due to continued peripheral perturbations may in fact be detrimental to the neural system in a manner similar to prolonged axonal sprouting by over-expression of GAP-43 rendering adult motoneurons more susceptible to nerve axotomy (Harding, Greensmith, et al. 1998a; 1999). MGF could be compared to a heat shock protein (HSP) but with the added activity of IGF-1, which may also be yielded from MGF. Protein chaperoning, which is a common action of HSPs, could be responsible for its well-documented neuroprotective effects (Kelly & Yenari 2002). It is interesting, therefore, that within the promotor 1 region of the rat IGF-1 gene, from which it was shown that MGF mRNA becomes altered in response to muscle denervation (chapter 5), also lie heat-shock elements (HSEs) (personal observations). HSEs are the putative DNA binding recognition

sites for heat-shock factors (HSFs), which have been found in unstressed spinal cord neurons (Stacchiotti et al. 1999). HSEs are found in promotor regions of HSP genes, whose expression is driven by the binding of HSFs to HSEs. In times of localised tissue stress it seems reasonable to suggest that HSFs also stimulate MGF synthesis as part of a repertoire of events leading to increased cellular survival. Such regulation of the IGF-1 gene has not yet been demonstrated but it is interesting that HSP70 and HSF-1 is induced in hepatocytes, the principle source of systemic IGF-1, as a result of calorie restriction (Heydari et al. 1996).

The current work showed that in the short-term, MGF elevation might be a result of a change in splicing (i.e. post-transcriptional), which would be further suited, with regard to rapid response, to a mode of action associated with cellular stress. From an evolutionary viewpoint, it seems reasonable that MGF resulted from an error in splicing giving rise to the E-domain peptide but with the initial transcriptional purpose of *de novo* synthesis of IGF-1. Following from this, it is tempting to speculate that the putative ligand-binding site (Yang, S. & Goldspink 2002) did not evolve for the sole purpose of selective affinity for the MGF E-domain peptide. Rather, the receptor could be a non-specific ligand-binding site receptive to many different stress-related proteins. It is of particular interest to establish the identity of the MGF receptor in order to work out the true mechanism of action of MGF. In attempting to parallel over-expression of the MGF gene with the effects of direct muscle damage, it is appropriate to highlight the importance of the non-specific retrograde reaction to peripheral inflammation as this may have general relevance to protection of motoneurons and other neural systems. A component of the retrograde reaction is the microglial response (Schwaiger et al. 1998). Peripheral inflammation has been shown to induce such changes in the CNS (Mariotti et al. 2001), some aspects of which are deemed to be protective (Raivich et al. 1999a). By causing sub-lethal peripheral damage, the protective signal may be primed so as to heighten motoneuron surveillance of injury thus affecting subsequent vulnerability to axonal damage (Mariotti et al. 2002b). It is not known whether MGF signaling derived from the target muscle may also affect central responses to injury, or if it acts independently of this mechanism. Pre-conditioning might also explain other phenomena, namely, enhanced motoneuron loss in neonatal animals as a result of intramuscular injection of vector, and the mechanisms of DR, in similar terms of a non-specific response to peripheral perturbations. It

is possible that injection of neonatal snout muscle with vector caused a significant retrograde reaction, although there is no data from the present thesis to support this. Increased motoneuron loss compared to non-injected neonates may be explained by greater sensitivity of immature motoneurons to perturbation of target tissue, which is an observation that has been well documented. It is thus tempting also to speculate that long-term muscle atrophy caused by DR in the present study induces prolonged retrograde effects centrally, recruiting over time neuroglia with protective properties, enhancing the capacity of remaining motoneurons at 24-months to withstand nerve avulsion.

All the phenomena that have been described in the present thesis are highly suggestive of the maintained sensitivity of adult motoneurons to alterations of their interaction with target-tissue. While such target-derived support persists as a significant factor promoting survival, motoneurons remain vulnerable to age-related changes in muscle activity. Mimicking a switch from peripheral to central neurotrophic support, in a manner not detrimental to motoneuron viability could be a crucial new strategy to overcome age- and disease-related motoneuron loss associated with a decline in target-derived neurotrophic support. In the absence of adequate information on the action of MGF, this might be a more general and, thus, effective approach to pre-conditioning motoneurons prior to lethal trauma in prognostic situations. Intriguingly however, it has been shown that age-related loss of skeletal muscle function is correlated with the inability of this muscle to express MGF mRNA in response to muscle ablation injury (Owino et al. 2001). The alternative approach, of course, is aimed at reversing the slow-acting degenerative changes synonymous with ageing using a strategy equally prolonged in nature to incur the desired maintenance of target-derived neurotrophic support. Maintaining activity of skeletal muscle throughout the lifetime of an organism could be the most effective and least invasive method of achieving this. Crucially, acute and chronic exercise has been shown to attenuate age-associated resistance to IGF-1 (Willis et al. 1997), restoring its activity in skeletal muscle of old mice (Willis et al. 1998). It is interesting too that GDNF is increased in skeletal muscle following bupivacaine injection (Sakuma et al. 2001) and chronic exercise, which may be responsible for activity-dependent re-modeling of neuromuscular junctions and may aid recovery from injury and disease (Wehrwein et al. 2002). On balance however, switching to central

neurotrophic support may be the preferable option since it is inevitable that muscle activity will decline at some stage, and that this may correlate with increasing resistance to nerve damage as shown by the effect of DR.

Experiments with DR have proven to be useful with regards to furthering understanding of age-related motoneuronal loss. The consistency with which the response to DR was observed is interesting because there appears to be the basis for a model for disease-related motoneuron degeneration. Existing mouse models for motoneuron degeneration are based either on mutations of a known gene such as SOD-1 or loci such as *wobbler*, *mnd* and *pmn* mice. All these share the common feature of motoneuron degeneration and have been used as models for human MNDs. There are inconsistencies however, regarding their phenotype of motor deficit with which they are related. Additionally, the issue of applicability to all types of MND is highly contentious since the most commonly used of these models, the SOD-1 mutant, has been modeled specifically to mimick subpopulations of human familial MND, which only make up about ~10% all cases. It has become increasingly accepted that MND may represent simply a collection of similar pathologies, dictated perhaps, by programmed pathways of selective motoneuron degeneration, differing only in genetic irregularity, rendering them more vulnerable to the toxic effects of ageing. As a result, much of what we know about the aetiology of human MND is based on these animal models. Although age-of-onset and disease duration are considered to be consistent between most sufferers, notable exceptions exist such as that early-onset MND is less aggressive in nature, and that this is associated with prolonged disease duration. Such observations are highly suggestive of extrinsic factors that control the vulnerability of motoneurons to succumb to toxic effects, related to specified genetic pre-disposition, to which motoneurons in all MND subjects are putatively exposed. Medical advances have prolonged human life so as to expose new lifespan-determining genes that otherwise would have been eliminated, arguably, by processes of natural selection. Extrinsic factors associated with target-derived neurotrophic support can be investigated in the DR model of motoneuron loss, shown for the first time in this study (Aperghis et al. 2003), free from genetic complications associated with other models that show pre-determined, intrinsic (genetic) vulnerability to motoneuron loss. Although, it is appreciated that certain genetic susceptibilities amongst a population of

animals subjected to DR are likely to become exposed. Nonetheless, work of the present thesis has shown unequivocally that target muscle continues to play an essential role with regards to neurotrophic support, a relationship, which could be exploited with regards to preventing neurodegeneration.

It has been shown that motoneurons in pre-symptomatic mice carrying the human G93A mutation in the SOD-1 gene are more susceptible to axotomy-induced motoneuron loss (Mariotti et al. 2002a) indicating that the toxic effects of this mutation act from an early age. Thus, studies investigating the effects of target-derived neurotrophic factors in the models mentioned previously, are susceptible to misinterpretation or, at least, serve as less powerful models to investigate factors that determine the survival and maintenance of normal motoneurons. The DR model, therefore, has application across the spectrum of age- and disease-related motoneuron pathologies. Additionally, the current work stresses the requirement to treat all motoneurons as equally vulnerable to sporadic alterations of their environment, associated particularly, with nerve-muscle interaction.

It is worthy of discussion that all the treatments for MND that are based on exposing motoneurons to elevated levels of target-derived neurotrophic factors have failed to prevent motoneuron degeneration in human clinical trials (Glaser 1997). Present intervention is aimed at preventing further degeneration after onset of disease. IGF-1 has been the main candidate for such therapy, aiming to stimulate nerve sprouting and re-establish nerve-muscle contact. It is questionable, however, whether injured motoneurons are able to respond in the same way as normal motoneurons to exogenous neurotrophic factors. It was shown that the MGF E-domain peptide needs to be present during injury in order to confer motoneuron survival and could be offered as a more effective therapy as long as local availability to motoneurons is achieved. Likewise, it will be extremely useful for short-term survival of motoneurons following nerve injury, especially where lesion is sustained near root junctions of the spinal cord. The ultimate goal however, is to prevent motoneuron degeneration prior to disease onset or at least, before nerve-muscle interaction has been lost, while the potential to manipulate motoneurons with muscle-based perturbations persists. Such a target is currently unrealistic with respect to sufferers of sporadic MND and other

related conditions because prior knowledge of disease occurrence would be a prerequisite for treatment based on the findings of the present study. Not until the genetic dispositions of all MND are deciphered, could such therapies be considered specifically to prevent or delay disease onset. That is not to say that a lifestyle avoiding over-eating and maintaining moderate physical activity should be recommended only for those with prognosis to develop degenerative motor neuropathies. Motoneuron degeneration during ageing may be unavoidable, particularly if survival is extended way beyond the lifespan of the species. Diet, MGF and certain aspects of muscle damage, thus, may be a useful basis on which to develop preventative measures to maintain motoneuron viability and in some cases perhaps to extend lifespan by maintaining appreciable motor function into old age. Future studies would aim to prevent disease pathology in mouse MND disease models, such as that over-expressing the mutant human SOD-1 gene, using the neuroprotective treatments established in the present thesis.

While MND remains universally fatal and virtually untreatable, it is hoped that the new information acquired from the present investigations may serve, at least, to shed more light on the relationship between nerve and muscle with respect to sustained motoneuron survival. Clearly the discovery that a growth/repair factor, produced in response to mechanical signals, has also neuroprotective properties, poses many questions. Several of these questions need to be answered before the mechanism of MGF is understood and subsequently considered for therapeutic application.

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