THE EFFECT OF PROLONGED AXONAL GROWTH ON THE VULNERABILITY OF DEVELOPING AND ADULT MOTONEURONS TO CELL DEATH

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By

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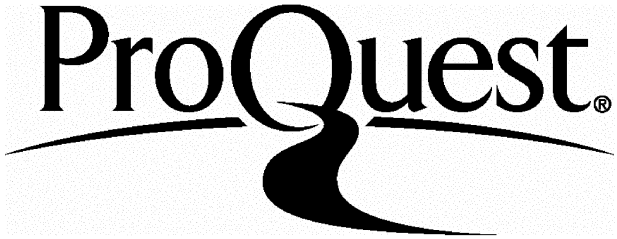

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ABSTRACT

Motoneurons are known to die following neonatal nerve injury. Prematurely enhancing transmitter release from developing nerve terminals induces motoneurons to become more resistant to subsequent nerve injury, whereas reducing transmitter release increases their susceptibility. In this Thesis I examine the relationship between the vulnerability of motoneurons to cell death and motoneuron phenotype, in developing and adult animals.

In developing rats, increasing transmitter release from nerve terminals at birth resulted in the premature transition of motoneurons from a growth into a transmitting state. In contrast, decreasing transmitter release delayed this transition by maintaining motoneurons in a growth state, which increased their vulnerability to cell death. These results indicate that the target-induced up-regulation in transmitter release from developing nerve terminals, determines motoneurone phenotype and vulnerability to cell death.

Adult motoneurons survive peripheral nerve injury and are able to withstand a period of growth associated with regeneration. However, even adult motoneurons may be more vulnerable to cell death when forced to grow for a prolonged period. The results of this Thesis show that extensive and prolonged growth caused by nerve injury in transgenic mice that over-express the growth-associated protein GAP-43, results in the progressive loss of motoneurons, possibly due to an increased vulnerability to excitotoxic cell death.

Finally, in this Thesis I also examined the possibility that the extensive sprouting of motoneurons that initially survive in Amyotrophic Lateral Sclerosis may contribute to the progressive motoneuron degeneration that is a feature of this disease. The results show that inducing axonal growth during the presymptomatic phase of the disease in a transgenic mouse model of ALS increases the rate of disease progression.

These results indicate that continued growth of motor axons renders both developing and adult motoneurons more vulnerable to cell death and implicates the excessive sprouting of motoneurons as a contributing factor in ALS.

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CHAPTER 1

GENERAL INTRODUCTION

During early embryonic development, motoneurons and muscle fibres develop independently. However, following neuromuscular contact, motoneurons become critically dependent upon a functional interaction with their target muscle, which is maintained up until the end of the first postnatal week. At present, the nature of the retrograde signal that promotes the development and survival of developing motoneurons is unclear. Therefore, this Thesis examines whether functional events that occur at the neuromuscular junction are important for regulating the development and survival of motoneurons. Furthermore, this Thesis also addresses whether gaining a better insight into the factors that regulate the survival of developing motoneurons may also improve our understanding of those conditions in which adult motoneurons degenerate, for example in amyotrophic lateral sclerosis (ALS).

The purpose of this Chapter is to highlight the sequential series of developmental events that result in the formation of a mature neuromuscular system and in particular the mechanisms that direct these dynamic changes. Furthermore, the factors involved in the survival of developing motoneurons are described. The second section of this Chapter describes the adaptive changes that the motoneuron undergoes in response to axonal injury and following partial denervation of muscle territory. Finally, the last section describes a number of possible mechanisms, which may play a role in the death of developing motoneurons following disruption of neuromuscular interaction. In addition, the current hypotheses on mechanisms of adult motoneuron degeneration in ALS are reviewed.

1.1. Early motoneuron and muscle development

During early development of the vertebrate central nervous system, motoneuron development occurs independently from interaction with the target muscle. The establishment of connectivity between motoneurons and their target muscle can be described in sequential steps. Motoneurons differentiate and become organized into specific motor pools located within individual motor columns. Motor axons exit the spinal cord through the ventral roots, and select major pathways into the limb or towards axial muscles. Next, axons innervate and arborize within specific muscle targets in a stereotyped manner. The first section of this introduction will describe in more detail the initial stages of motoneuron development and establishment of neuromuscular contact.

1.1.1. Specification of motoneurons

The central nervous system is first recognisable in vertebrate embryos as a flat sheet of cells called the neural plate, which subsequently folds to form the neural tube. Within the ventral regions of the neural tube, motoneurons arise from a common source of mitotically active progenitors in the ventricular layer (reviewed by Tanebe and Jessell, 1996; Bronner-Fraser and Fraser, 1997). Commitment to motoneuron identity is imposed by an extrinsic signal, Sonic Hedgehog (Shh), which is produced by the subajacent notochord and floorplate (Marti et al, 1995; Roelink et al, 1995; Chiang et al, 1996). The concentration gradient established by secreted Shh directs the differentiation of progenitor cells into specific neuronal types (Ericson et al, 1997). Progenitor cells respond to Shh signalling by translating the specific

concentration of the signals into a patterned expression of transcription factors. Recent studies have provided evidence that a group of DNA-binding homeodomain proteins expressed by the ventral progenitor cells interpret the graded Shh signals (Briscoe et al, 1999, 2000). The combined action of three homeodomain proteins restricts the generation of motoneurons to a single progenitor domain and direct the expression of down-stream factors such as the homeodomain protein MNR2 (chick) or HB9 (mouse), which functions as a dedicated determinant of motoneuron identity (Tanabe et al, 1998; Arber et al, 1999; Thaler et al, 1999; Jessell, 2000). Functional significance for the MNR2 homeodomain transcription factor was demonstrated by the finding that ectopic expression of MNR2 is sufficient to activate a program of somatic motoneuron differentiation, and the enzyme choline acetyltransferase (ChAT) for transmitter synthesis and by the extension of axons that exit from the ventral spinal cord (Tanabe et al, 1998).

1.1.2. Control of motoneuron subtypes

Vertebrate limb muscles are innervated by motoneuron pools that are localized in highly stereotyped positions, spanning two to four segments of the spinal cord (Landmesser, 1978a; Hollyday, 1980). During embryonic development of higher vertebrates all spinal motoneurons derived from a single ventral progenitor domain acquire distinct subtype identities based on the position of their cell body in the spinal cord. One level of organization is the alignment of motoneurons with common target muscles into longitudinally orientated columns. For example, median motor column (MMC) neurons innervate trunk muscles, whereas motoneurons in the lateral motor column (LMC), project to limb muscles (Landmesser, 1980). At a second

organizational level, neurons within the motor columns are segregated into medial and lateral divisions and project axons along different trajectories (Landmesser. 1978a). At a third level discrete pools of motoneurons exist within each division of the LMC and innervate specific muscles in the limb (Landmesser, 1978b). The identity of the signals that generate motoneuron diversification along the rostrocaudal axis is presently unknown. However, the paraxial mesoderm is implicated in generating positionally restricted signals that determine motoneuron subtypes (Ensini, et al, 1998).

A number of findings have raised the possibility that the expression of LIM homeodomain transcription factors specifies the identity of motoneurons and selects distinct axonal pathways prior to the onset of axon outgrowth (Eisen, 1991; Tsuchida, et al, 1994; Appel et al, 1995). So far, by analysing the combined expression of LIM genes, not only can the major classes of motoneuron be distinguished but also the medial or lateral identity of limb motoneurons (Tsuchida et al, 1994; Sharma et al, 1998; Sockanathan and Jessell, 1998). Furthermore, the onset ETS gene expression at the time of limb innervation has been shown to specify individual motor pools within the LMC (Lin et al, 1998).

1.1.3. Axonal pathfinding

Prior to the period of axonal growth, motoneurons have acquired distinct identities that determine specific axonal pathways and synaptic targets, which is reflected by the regulated expression of appropriate growth cone receptors. In addition, motor axon pathfinding occurs in a series of steps that is dependent on the differential

action of guidance cues. Firstly, the floorplate of the neural tube secretes diffusible chemorepellants (netrin-1 and semaphorins), which deflects motor axons into the periphery at specific exit points (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995). Secondly, motor axons emerge from the spinal cord at embryonic day 11.5 in the rat (Filogamo and Gabella, 1967) and form the periodic arrangement of motor nerves through repulsive and attractive activities of molecules derived from the adjacent somites (Keynes and Stern, 1984; Oakley and Tosney, 1993; Fredette et al, 1996).

The ability of specific guidance cues to generate topographic organization from the spinal cord to the muscle masses has been the subject of many investigations. A classical series of experiments have used surgical perturbations to force motor axons into foreign territory to test the pathfinding ability of motor axons. When motoneurons were displaced along the anterior-posterior axis by rotating portions of the spinal cord, motor axons were able to alter their pathways and project to their correct muscles (Hollyday and Hamburger, 1977; Lance-Jones and Landmesser, 1980, 1981). Similarly, rotation of the limb bud about the dorsal-ventral axis also resulted in the innervation of the correct muscles, even though motor axons now entered the limb from abnormal positions (Ferguson, 1983). These findings point to involvement of limb derived diffusible factors in the guidance of motor axons to their eventual targets. Indeed, the chemoattractive activity of limb muscle appears to be mediated by hepatocyte growth factor (Ebens et al, 1996), although many other unknown molecules are likely to be involved. In contrast, following larger surgical displacements, such as rotations of the entire lumbosacral spinal cord, most motoneurons appeared unable to respond to these cues and thus innervated a number

of incorrect muscles (Landmesser and O'Donovan, 1984b). Interestingly, motor axons still followed anatomical pathways appropriate to these foreign muscles, which support the notion that there are defined "highways" in place, that are demarcated by inhibitory guidance cues (Ebens et al, 1996; Kitsukawa et al, 1997). If motor axons are displaced too far from these highways, they cannot find the correct pathway.

1.1.4. Early development of skeletal muscle

Most skeletal muscle in vertebrates develops from progenitor cells present in somites, which arise by segmentation of the paraxial mesoderm on either side of the neural tube. Over the past few years, numerous studies have investigated the role of secreted signalling molecules in determining the fate of somatic cells. Most notable are Wnts produced by the dorsal neural tube, and Sonic hedgehog from the notochord and floorplate of the neural tube, which are positive effectors of myogenesis (reviewed by Tajbakhsh and Buckingham, 2000). Markers of myogenic specification belong to the family of basic helix-loop-helix (bHLH) transcription factors. These include proteins such as MyoD, and Myf5, which are responsible for activating muscle specific genes (Ott et al, 1991; Pownall and Emerson, 1992). Furthermore, the expression of the myoD and myf5 genes can trigger muscle development in non-myogenic cells such as fibroblasts (Choi et al, 1990; Weintraub, 1993).

All muscle cells produced by somites have their origin in the dermatomyotome located in the dorsal part of the somite. The limb musculature develops from the lateral dermatomyotome, following the migration of undifferentiated myogenic

precursor cells into the limb buds (Chevallier et al, 1977; Williams and Ordahl, 1994). After these cells leave the proliferative cell cycle they are committed to becoming myoblasts (Tello, 1917, 1922) and fuse with each other to form multinucleated myotubes (Holtzer et al, 1957; Yaffe and Feldman, 1965). Just prior to fusion, the metabolic activity of myoblasts increases in preparation for contractile activity (Schudt et al, 1975; Holland and MacLennan, 1976). For fusion to occur, intracellular Ca^{2+} levels have to increase in myoblasts (Shainberg et al, 1969; Fambrough and Rash, 1971; Pryzbylski et al, 1989). During the formation of multinucleated myotubes, the synthesis of contractile proteins and production of acetylcholine receptors (AChR) increases (Allen and Pepe, 1965; Fambrough and Rash, 1971; Sytkowski et al, 1973; Dryden et al, 1974). Myotubes at this stage are also capable of producing active electrogenic responses and conduct them along the length of the fibre (Kano and Shimada, 1973; Purves and Vrbová, 1974). In terms of muscle fibre diversification, primary myotubes can be distinguished as fast or slow at early embryonic stages, which indicates that myoblast proliferation, myotube formation and limb muscle patterning occurs independently from motoneuron innervation (Fredette and Landmesser, 1991). However, the formation of secondary myotubes is heavily dependent on functional innervation (Condon et al, 1990; Wilson and Harris, 1993).

1.2. The structural and functional development of neuromuscular synapses

Motor axons innervate developing muscles as myoblasts are fusing to form myotubes. Once the growth cone of the axon contacts a newly formed myotube, synaptic transmission commences quickly. Initially, the efficacy of transmission is

very low, reflecting the absence of pre- and postsynaptic specializations. During the early period of synapse formation at the neuromuscular junction (NMJ), the presynaptic terminal lacks identifiable active zones and the postsynaptic membrane contains immature acetylcholine receptors, which are present along the entire length of the myotube. Over a protracted period of time, a fully functional synapse is established, in which both nerve and muscle are greatly transformed. The following section will describe the important changes that occur as a mature neuromuscular synapse is established, and will discuss the mechanisms that are involved in synaptic differentiation and functional maturation.

1.2.1. Presynaptic differentiation at the NMJ

The early events in synaptogenesis involve the transformation of a highly motile growth cone into a stable nerve terminal. Initially, the newly formed synapses are simple bulbous enlargements, which have close membrane appositions with an extracellular gap of <10 nm (Linden, et al, 1988; Buchanan, et al, 1989). These enlargements contain few synaptic vesicles and no recognizable active zones. Over the next few days, there is a gradual formation of the active zone, which involves the localization and assembly of transmitter related proteins, including voltage-dependent Ca^{2+} channels, vesicle docking proteins and clustering of synaptic vesicles (Nakajima et al, 1980; Takahashi et al, 1987; Buchanan et al, 1989; Lupa and Hall, 1989). These changes, accompanied by an increase in synaptic volume and area, underlie large increases in frequency of spontaneous exocytosis and the number of vesicles that release transmitter in response to stimulation (Dennis et al, 1981). It is currently unclear what signals promote presynaptic differentiation. However, a

number of *in vitro* studies have implicated membrane-bound cell adhesion molecules in the early events of synaptogenesis (Dai and Peng, 1995; Porter et al, 1995; Campagna et al, 1995, 1997; Chang et al, 1997). For example, latex beads coated with basic fibroblast growth factor (bFGF) stimulates the clustering of synaptic vesicles when applied to neurites (Dimario et al, 1989). Furthermore, basal lamina derived components induce presynaptic differentiation in the absence of the postsynaptic cell (Glicksman and Sanes, 1983; Son et al, 1999). Evidence that the basal lamina protein, laminin β1 is a crucial organizer of synaptogenesis *in vivo* came from the observation that the NMJs in mutant mice lacking this protein exhibited relatively unbranched nerve terminals with no active zones and impaired synaptic transmission (Noakes et al, 1995). Taken together, these findings indicate that direct interactions between surface-bound factors and growth cones are likely to be responsible for the early-induced events of synaptogenesis.

Even though physical contact with the target muscle can influence presynaptic development, increasing evidence suggests that later steps of differentiation require synaptic transmission and postsynaptic activation. For example, mutant mice that lack the acetylcholine (ACh) synthesizing enzyme, choline acetyltransferase (ChAT), displayed significant presynaptic abnormalities (Misgeld et al, 2002). In addition, preventing postsynaptic differentiation by disrupting essential proteins involved in ACh receptor clustering (see next section, 1.2.2.) results in limited presynaptic differentiation and continuous synaptic remodeling (Gautam et al, 1995; DeChiara et al, 1996). Thus, postsynaptic differentiation seems to be dispensable for initial presynaptic differentiation but is required for presynaptic stabilization so that synapses can mature (Nguyen et al, 2000). However, although the nature of this

activity-dependent signal is unknown, it is likely to have the ability to induce an elevation of intracellular Ca^{2+} in the presynaptic terminal (Dai and Peng, 1993).

1.2.2. Postsynaptic differentiation

Developmental programs ensure that motor axons are guided to the correct muscle and also prepare growing axons and target muscles for initial contact. Before axons reach their target muscle, myotubes assemble functional acetylcholine receptors (AChRs) and insert them along their entire surface, with the occasional area of high receptor density – so called "hot spots" (Fischbach and Cohen, 1973). However, motor axons do not preferentially innervate these hot spot areas (Anderson and Cohen, 1977; Frank and Fischbach, 1979). Instead, the pattern of innervation appears to depend on changing levels of adhesion molecules on both nerve and muscle that deliver axons to the right region of the muscle fibre (Rutishauser and Landmesser, 1991; Landmesser, 1996).

Following innervation, postsynaptic differentiation proceeds, resulting in the clustering of AChRs beneath the motor nerve terminal, with the density of extrajunctional AChRs declining to very low levels (Fambrough, 1979; Salpeter and Loring, 1985, Salpeter et al, 1988). The motoneuron-derived protein, agrin, has been strongly implicated in directing postsynaptic differentiation in vivo. Agrin is a large protein synthesized by the motoneuron, transported in motor axons, and released from nerve terminals, where it is incorporated into the basal lamina of the synaptic cleft (McMahan, 1990; Cohen and Godfrey, 1992; Reist et al, 1992). Agrin is necessary for organizing developing synapses, since post-synaptic differentiation is

profoundly impaired in agrin-deficient mutant mice (Gautam et al, 1996). Moreover, experimental introduction of agrin into denervated muscles results in the formation of a remarkably complete postsynaptic apparatus in precise apposition to the deposits of agrin (Cohen et al, 1997; Meier et al, 1997).

Agrin stimulates postsynaptic differentiation by activating MuSK, a transmembrane tyrosine kinase that is selectively expressed in skeletal muscle and concentrated at synaptic sites (Jennings et al, 1993; Valenzuela et al, 1995). MuSK knockout mice display neuromuscular defects similar to those of agrin-deficient mice, despite normal levels of AChR expression (DeChiara et al, 1996). Furthermore, agrin is unable to induce AChR clustering in muscle cells of MuSK mutant mice (Glass et al, 1996), but agrin sensitivity is restored following the introduction of wild-type MuSK into MuSK -/- myotubes (Zhou et al, 1999; Herbst and Burden, 2000). Together, these results demonstrate that MuSK is a crucial component for agrin induced AChR clustering. However, the intracellular mechanisms down-stream of MuSK are poorly understood, although rapsyn, a cytoplasmic protein, is a required intermediate on the pathway that couples MuSK activation to AChR clustering (Gautam et al, 1995).

The synaptic accumulation of AChRs is also a product of selective transcription of AChR subunit genes by myonuclei at synaptic sites. These synaptically localized myonuclei not only transcribe AChR subunit genes at a faster rate than nonsynaptic myonuclei, but also express the mature combination of AChR subunits that results in receptor channels with larger conductance and shorter mean opening times (Merlie and Sanes, 1985; Brehm and Henderson, 1988; Goldman and Staple, 1989; Sanes et al, 1991). Two molecules have been proposed as nerve-derived inducers of synapse-

specific transcription: calcitonin gene-related peptide (CGRP) and neuregulin-1 (Nrg-1). CGRP is present in dense core vesicles and released upon stimulation (Uchida et al, 1990; Changeux et al, 1992). Application of CGRP to cultured myotubes stimulates AChR synthesis (New and Mudge 1986; Fontaine et al, 1987). The splice variant of Nrg-1, which induces AChR synthesis in cultured myotubes, is termed ARIA (AChR inducing activity; Usdin and Fischbach, 1986). Like agrin, ARIA is expressed by motoneurons, secreted into the synaptic cleft and interacts with transmembrane receptors located on the postsynaptic membrane at the NMJ (Moscoso et al, 1995; Rimer et al, 1998). However, a direct role for ARIA in synapse formation *in vivo* has not yet been demonstrated.

During development of the NMJ, the density of extrasynaptic AChRs begins to decline, in conjunction with AChR clustering at the synapse (Merlie et al, 1984, Tsay and Schmidt 1989). This phenomenon appears to be regulated by electrical activity, since direct electrical stimulation of denervated muscle prevents the build up of extrasynaptic AChRs, whereas pharmacological blockade of synaptic transmission induces AChR synthesis in innervated muscle (Lomo and Westgaard, 1975; Goldman et al, 1988).

1.2.3. Early neuromuscular transmission

The study of early physiological events during synaptogenesis has been facilitated by the use of co-culture preparations, in which the timing of nerve-muscle contacts can be precisely determined. In Xenopus nerve–muscle cultures, axonal growth cones are capable of spontaneous and evoked quantal neurotransmitter release even before

making physical contact with a myotube (Hume et al, 1983; Young and Poo, 1983; Sun and Poo, 1987). Specifically, growth cones release quanta of ACh at very low frequencies prior to muscle contact (Young, 1986). However, within a few minutes of contact with the muscle membrane, the amplitude and frequency of spontaneous ACh release increases markedly (Chow and Poo, 1985; Xie and Poo, 1986; Evers et al, 1989). This increase in transmitter release is not due to secreted factors from the muscle, since the excised patch of muscle membrane by itself is able to induce spontaneous ACh release (Xie and Poo, 1986). The precise nature of the surface component responsible for inducing transmitter release is unknown, but a contact induced increase of cytosolic Ca^{2+} at the growth cone is likely to be involved. Indeed, it has been shown that there is an immediate increase in the level of free Ca^{2+} in the growth cone at the point of muscle contact (Dai and Peng, 1993). In turn, the rise in intracellular calcium may also initiate presynaptic differentiation at the NMJ, including the insertion of voltage-sensitive Ca^{2+} channels required for evoked transmitter release (Funte and Haydon, 1993).

Cell-cell communication during synaptogenesis may also occur via gap junctions, which would allow the direct exchange of intracellular messengers. This would allow the initial events of synaptogenesis to be directed by signals generated by the developing pre-and postsynaptic membranes at the NMJ. Although gap junctions are usually formed between cells of the same type, they have been observed forming transiently between motoneurons and muscle cells in Xenopus and chick cultures (Bonner, 1989; Allen and Warner, 1991).

Transmission in the newly formed neuromuscular synapse is very different from that seen in the adult. Initially, there is a wide variability in the amplitudes of spontaneous postsynaptic currents, with most endplate potentials (EPPs) recorded from embryonic muscles having a slow rise time and small amplitude (Dennis et al, 1981; Pilar et al, 1981). This is probably due to the low density of AChRs postsynaptically, and immature presynaptic terminals that contain few synaptic vesicles (Takahashi et al, 1987). As a consequence, many early synapses are subthreshold for muscle fibre excitation (Jones and Ridge, 1987). During embryonic and postnatal development, both the frequency of spontaneous MEPPs and the quantal content of the EPPs increase dramatically, until high safety margins are achieved (Diamond and Miledi, 1962; Kelly, 1978; Dennis et al, 1981). This is associated with an increase in the amount of ACh-synthesizing enzyme, ChAT (Burt, 1975; O'Brien and Vrbová, 1978; Phelps et al, 1984) and an up-regulation in the expression of synaptic vesicle genes (Campagna et al, 1997; Plunkett et al, 1998).

1.3. Target dependence of motoneurons

The initial stages in the development of motoneurons, such as their specification, proliferation and axonal growth, occur independently of the target muscle. However, once neuromuscular contact is established, motoneurons become dependent on their target muscle for their maturation and survival. This target dependence persists in most mammals during the early postnatal period. However, the nature of the mechanism by which the muscle promotes motoneuron maturation and survival is unresolved. The following section will describe experiments that demonstrated the target dependence of motoneurons during embryonic development. Furthermore, the

influence of neuromuscular activity on the timing and extent of naturally occurring motoneuron death will be discussed. Finally, the role of muscle-derived factors in motoneuron development will be assessed.

1.3.1. Motoneuron cell death during development

The regulation of neural development by muscle-derived signaling began with the demonstration by Storey (1909) that removal of limb bud targets in the chick embryo resulted in the loss of neurons that normally innervated the limbs. Thus, following the removal of the forelimb bud on embryonic day 2 (E2), Storey observed that muscle nerves failed to develop and that there was a marked reduction in the number of brachial motoneurons. At the time, these results were mistakenly interpreted as a failure of neuronal differentiation. After several variations of limb removal experiments in the 1930's and 1940's, it was finally concluded that the target muscle provides trophic signals that promote and maintain neuronal survival (Hamburger and Levi-Montalcini, 1949). Furthermore, it was discovered that a proportion of spinal motoneurons (~50%) undergo degeneration during normal development (Hamburger, 1958, 1975), and so the effect of limb bud removal was reinterpreted as an exaggeration of this normal period of programmed cell death (PCD). In support of the proposal that the target muscle regulates motoneuron survival, it was found that increasing the amount of target tissue by transplantation of a supernumery limb, rescued a substantial fraction of the neurons that would otherwise have died (Hollyday and Hamburger, 1976). PCD is now recognized as a prominent feature of embryonic development and affects most neuronal populations. The massive loss of spinal motoneurons during an advanced stage of differentiation probably reflects a

process that corrects the number of neurons necessary to match the target size. Other explanations, such as the elimination of aberrant connections are unlikely for motoneuron populations, since axonal projections are specified prior to outgrowth and few errors are made (Lance-Jones and Landmesser, 1980, 1981; Oppenheim, 1981).

1.3.2. Role of neuromuscular activity in embryonic motoneuron death

Motoneuron death that occurs during embryonic development coincides with the onset of muscle innervation and neurally-mediated embryonic movements (Hamburger, 1948, 1975; Oppenheim, 1987). This temporal coincidence between motoneuron death and postsynaptic activation indicates that neuromuscular activity is important for regulating motoneuron numbers during embryonic development. Indeed, numerous studies have shown that disrupting neuromuscular activity during the period of PCD has a significant effect on motoneuron survival. For example, postsynaptic block of neuromuscular activity using (+)-tubocurarine or α-bungarotoxin during the period of normal motoneuron death, rescues virtually all motoneurons from PCD (Pittman and Oppenheim, 1978, 1979). Furthermore, the survival of these rescued motoneurons can be maintained as long as activity remains blocked. However, after treatment is stopped and activity recovers, the rescued motoneurons undergo a delayed cell death. Consistent with these findings, naturally occurring motoneuron death is almost completely abolished in mutant mice lacking the ACh-synthesizing enzyme, ChAT, since neuromuscular transmission is specifically and completely blocked in these mice (Misgeld et al, 2002). Conversely, increasing muscle activity by direct electrical stimulation of the hindlimb accelerates

the rate of motoneuron death (Oppenheim and Nunéz, 1982). In addition, increasing neuromuscular activity with carbachol (an acetylcholine receptor agonist) or with eserine (an anti-acetylcholinesterase) increases motoneuron death (Oppenheim and Maderudt, 1981).

After the initial findings that activity blockade rescued motoneurons, further analysis revealed that there were increased numbers of axons and synapses in the limb muscles (hyperinnervation) of activity-blocked embryos before any significant loss of motoneurons (Oppenheim et al, 1989). On the basis of these findings Oppenheim (1989) proposed that this hyperinnervation rescued motoneurons by allowing an increased access to muscle-derived trophic factors via nerve terminals. Further support for this proposal has come from studies, which showed that an increase in the number of motoneurons was accompanied by an increase in intramuscular branching following the disruption of postsynaptic specializations (Banks et al, 2001; Terrado et al, 2001).

1.3.3. Neurotrophic factors

Neurotrophic factors are important molecules that regulate neural survival and development (reviewed by Oppenheim, 1996; Sendtner et al, 2000; Huang and Reichardt, 2001). Nerve growth factor (NGF), the first such factor to be characterized, promotes the survival of sensory and sympathetic neurons and is now recognized as the prototypical target-derived neurotrophic factor (reviewed by Levi-Montalcini, 1987). The discovery that NGF was necessary for the survival of specific populations of embryonic neurons, led to the proposal that neurons compete for

neurotrophic agents that are supplied by their targets in limited amounts. This proposal also known as the 'Neurotrophic Hypothesis,' provides a useful framework for understanding why only a proportion of developing neurons, in most neuronal populations, survive following target innervation.

NGF is regarded as a prototypical neurotrophic factor because it meets all the criteria needed to qualify as a specific neurotrophic factor involved in regulating the survival of discrete populations of neurons. Many other neurotrophic factors have subsequently been identified and proposed to play a similar role to NGF in other neuronal populations. However, unless these molecules meet the criteria that were originally established by work on NGF, they remain classed as putative neurotrophic factors (Levi-Montalcini, 1987; Barde, 1988; Oppenheim, 1989). The criteria required for neurotrophic factor classification includes: (1) neuronal cell death is prevented by its continuous supply (2) it is synthesized in limited amounts by the target tissue of the very neurons that need it for survival (3) it binds to specific receptors and is internalized by the nerve terminals of contacting neurons (4) it is transported in a retrograde direction to the cell body of the neuron.

As described above (see 1.3.1.), during embryonic development, motoneurons are dependent upon interaction with their target muscle for their survival. It is therefore possible that muscle-derived neurotrophic factors produced in limited amounts regulate the survival of embryonic motoneurons during the period of naturally occurring cell death (or PCD). In support, it has been shown that muscle extract and muscle-derived proteins promoted the survival of dissociated embryonic motoneurons in culture (Calof and Reichardt, 1984; Dohrmann et al, 1986, 1987;

Arakawa et al, 1990). Furthermore, muscle extracts administered systemically to chick embryos in ovo were able to rescue motoneurons from naturally occurring cell death (Oppenheim et al, 1988; McManaman et al, 1990; Houenou et al, 1991). Using this in vivo assay, a number of putative neurotrophic factors derived from a number of tissues, have been tested for survival promoting effects on motoneurons (Oppenheim et al 1992, 1993, 1995). Although many of these factors were unsuccessful, brain derived neurotrophic factor (BDNF), and neurotrophin-4 (NT-4) were able to rescue a large proportion of motoneurons from PCD (Henderson et al, 1993; Oppenheim, et al, 1993). Both these putative neurotrophic factors, which are classed as neurotrophins, show structural similarity to NGF and act through the low affinity p75 receptor and a family of receptor tyrosine kinases expressed by developing motoneurons (Klein et al, 1990, 1991; Cordon-Cardo et al, 1991). In addition, the most potent survival factor was the glial cell-derived neurotrophic factor (GDNF), which rescued a significant number of motoneurons from PCD and following embryonic axotomy (Oppenheim et al, 1995). This result indicates that non-target derived neurotrophic support may also play an important role in the survival of developing neurons.

Because developing motoneurons remain dependent upon target interaction during the early postnatal period (Schmalbruch, 1984; Lowrie et al, 1987), a popular in vivo model used to investigate the trophic requirements of developing motoneurons is neonatal axotomy. A number of neurotrophic factors have been shown to temporarily support spinal and cranial motoneurons when directly delivered to the cut nerve after neonatal axotomy. These include: GDNF, BDNF, NT-3, and the cytokine factor, CNTF (Sendtner et al, 1990, 1992; Henderson et al, 1994; Zurn et al, 1994; Yan et al,

1992, 1995). Neonatal motoneurons are also protected from axotomy-induced cell death if they are transduced with adenoviral vectors carrying neurotrophic factor genes (Gravel et al, 1997; Baumgartner and Shine 1997). However, neuroprotection from axotomy-induced cell death by directly applied neurotrophic factors in neonates is transient, lasting only 2-3 weeks (Zurn et al, 1994; Eriksson et al, 1994; Vejsada et al, 1995). Equally, adenoviral mediated expression only resulted in a transient neuroprotective effect (Gravel et al, 1997; Baumgartner and Shine 1997). Longer-term survival effects have been reported after repeated application of GDNF following neonatal axotomy, although only 40% of injured facial motoneurons were rescued (Matheson et al, 1997). Furthermore, transduction of the target muscle with GDNF resulted in the permanent survival of a substantial number of motoneurons following nerve-crush at birth (Baumgartner and Shine, 1998). This long-term effect was attributed in part to the diffusion of GDNF from the muscle to the regenerating axons and also an improved capacity for rescued motoneurons to regenerate, since following nerve-crush, the endoneurial sheath remains intact and axons retain access to the distal nerve.

Another approach to investigate the role of neurotrophic factors in motoneuron survival was the use of mice in which the genes encoding either the potential trophic factor or its receptor(s) were deleted. It was found that knocking out the genes for BDNF, NT-3 or NT-4 had negligible effects on motoneuron survival (Ernfors et al, 1994; Farinas et al, 1994; Jones et al, 1994; Liu et al 1995; Conover et al, 1995). However, mice lacking the gene for trkB, which is the receptor for BDNF and NT-4, showed a 35% reduction in lumbar motoneurons (Klein et al, 1993). Similarly, mice lacking the survival promoting factors CNTF or leukemia inhibitory factor (LIF), do

not display motoneuron loss (Masu et al, 1993), whereas null mutant mice for components of their respective receptors, CNTFRα (DeChiara et al, 1995), or LIFR (Li et al, 1995), resulted in the loss of 40-50% of spinal and cranial motoneurons. These results indicate that these receptors are utilized by multiple ligands in vivo, which have yet to be identified. Furthermore, although exogenous delivery of GDNF has been shown to rescue developing motoneurons from axotomy-induced cell death (Henderson et al, 1994; Zurn et al, 1994; Yan et al, 1995), deleting the gene for GDNF or its primary co-receptor (GFRα1), is reported to have no effect on the number of surviving motoneurons in these mutant mice (Moore et al, 1996; Sánchez et al, 1996; Cacalano et al, 1998). Therefore, these findings put into question the physiological role of GDNF in maintaining the survival of developing motoneurons.

Overall, the results described above suggest that developing motoneurons require multiple factors from diverse sources for optimal survival. It has been hypothesized that subpopulations of motoneurons based on their function or connectivity require different neurotrophic factors for their survival (Oppenheim, 1996). Furthermore, motoneurons may also require distinct factors at different stages of development. At present the evidence supporting this hypothesis is limited. Indeed, even a mutant mouse lacking BDNF, NT-3 and NT-4 only has a 20% deficit in facial and spinal motoneurons (Liu and Jaenisch, 2000). It therefore appears that the neurotrophic hypothesis formed on the basis that target-derived NGF regulates the survival of sensory and sympathetic neurons, does not explain the target-dependence of developing motoneurons. Consequently, is has been argued that non-target-derived neurotrophic factors are important survival molecules for motoneurons, which act in concert with muscle-derived neurotrophic factors (Oppenheim, 1996). Nevertheless,

just how these other sources of trophic factors act together with target-derived factors is poorly understood. An alternative view to the neurotrophic hypothesis is the possibility that as a result of an interaction between the developing motoneuron and its target muscle, the phenotype of the motoneuron changes and as a consequence matures into a target independent cell (Lowrie and Vrbová, 1992; Greensmith and Vrbová. 1996). This theory forms the basis of experiments in Chapter 1, where it is described in more detail.

1.4. Postnatal development of the motor unit

Even after neuromuscular synapses have been established, the emergence of the motor unit in its adult form is not yet complete. Thus, individual muscle fibres in newborn rodents are innervated at a single endplate by several motor axons, whereas in adult mammals each muscle fibre is innervated by a single motor axon. To achieve this adult form of connectivity, the superfluous axons at each endplate retract in a process called synapse elimination. The follow section will describe the changes to motor unit connectivity that occur during the early postnatal period and the activity-dependent mechanisms that are involved in the process of synapse elimination.

1.4.1. Polyneuronal innervation

The term "motor unit" was coined by Sherrington (1904), and is defined by the motoneuron cell body, its axon, and all the muscle fibres it innervates. In adult mammalian muscles, each muscle fibre is innervated at a single endplate by one motor axon and therefore only activated by one motoneuron. However, during late

embryonic development, following the initial contact of one motor axon with the muscle fibre, newly arriving axons are preferentially guided to the region of nerve-muscle contact. This process continues for several days and results in many motor axons converging at a single endplate. This polyneuronal (or multiple) innervation was first described by Redfern (1970) in the diaphragm muscle of newborn rats using electrophysiological recordings and subsequently observed in the hindlimb muscles of the rat (Brown et al, 1976; O'Brien et al, 1978).

1.4.2. Synapse elimination at the mammalian NMJ

One hallmark of the developmental refinement in synaptic circuitry is the elimination of superfluous axonal branches and their synapses. This important developmental event is called synapse elimination, and occurs throughout the nervous system (Crepel et al, 1976; Hubel et al, 1977; Lichtman and Purves, 1980). To date, this process has been analyzed in greatest detail at the skeletal NMJ, owing to its accessibility and relative simplicity compared to synaptic connection in the brain. Although neuromuscular synapses are established well before birth, the transition from multiple to single innervation typically does not occur until the postnatal period (Bennett and Pettigrew, 1974; Brown et al, 1976; O'Brien et al, 1978). For example, in the soleus muscle of the rat, muscle fibres that have single innervation begin to appear 8-10 days after birth and by about 16 days of age, all muscle fibres have lost their multiple innervation (O'Brien et al, 1978, 1982). However, there is some degree of variation in the time of onset and rate of synapse elimination between muscles, which is largely linked to the rostro-caudal sequence of motoneuron development and muscle activity (Bixby and Van Essen, 1979). Thus, in the rat, synapse

elimination is complete by days 10-11 in the intercostal muscles (Dennis et al, 1981), 14-18 days in the diaphragm (Redfern, 1970), 16-20 days in the soleus (Benoit and Changeux, 1975; Brown et al, 1976; O'Brien et al, 1978), and by 21 days in the lumbrical muscles (Betz et al, 1979).

The process of synapse elimination is now thought to be due to the retraction of superfluous axonal branches and not linked to motoneuron death, since the period of PCD is complete at birth (Lance-Jones, 1982), and the size but not the number of motor units decrease during the period of synapse elimination in most muscles (Brown et al, 1976). Furthermore, extensive morphological studies have shown that each axonal branch undergoes atrophy, detachment and withdrawal from the endplate region, leading to the gradual separation of the muscle territories that each axon occupies (Korneliussen and Jansen, 1976; Ostberg and Vrbová, 1977; Riley, 1977; Bixby, 1981; Balice-Gordon et al, 1993).

1.4.3. Role of activity in synapse elimination

It was first noticed by Redfern (1970) that synapse elimination coincides with a general increase in the locomotor activity of the animal. Thus, in order to investigate whether muscle activity plays a role in synapse elimination various experiments altered the overall activity levels in the muscle and/or motoneurons during the period of synapse elimination. Reducing activity by tenotomising the muscles (Benoit and Changeux, 1975) or by blocking neuromuscular transmission using botulinum toxin (Brown et al, 1981a) or α-bungarotoxin (Sohal and Holt, 1980; Duxson, 1982), delays the loss of superfluous nerve terminals. Furthermore, selectively reducing

mechanical activity, whilst maintaining electrical activity, by interfering with the excitation-contraction coupling using dantrolene sodium, also reduces the rate of elimination of polyneuronal innervation (Greensmith et al, 1998).

In contrast, increasing neuromuscular activity by chronic stimulation of the nerve (O'Brien et al, 1978; Thompson, 1983) or using the inhibitor of acetylcholinesterase, di-isopropyl fluoro phosphate (Duxson and Vrbová, 1985), both accelerate the process of synapse elimination. Furthermore, denervation of synergistic muscles resulting in compensatory hyperactivity, also increases the rate of elimination in overactive muscles (Zelena et al, 1979).

More recently, a number of studies have focused on whether the pattern of motoneuron activity rather than the total amount of activity in important in modulating synapse elimination. One significant change that occurs during the early postnatal period is the timing of postsynaptic activation by different motor axons innervating a muscle fibre. At birth, spontaneous motor activity is low and motoneurons fire synchronously (Navarrete and Vrbová, 1983; Personius and Balice-Gordon, 2001). However, after the first postnatal week motor unit activity becomes asynchronous (Buffelli et al, 2002), which is mediated at least in part by the uncoupling of gap-junction communication between motoneurons (Walton and Navarrete, 1991; Chang et al, 1999). It is possible that this switch to asynchronous activity, allows muscle fibers to discriminate among axon terminals on the basis of their relative activity, selectively strengthening more active inputs, and weakening less active ones. This idea has received support from a variety of experiments that examined the effect of altering the normal pattern of neuromuscular activity on

synapse elimination. For example, replacing the normal activity patterns with synchronous stimulation of the regenerating nerve suppresses the elimination of multiple innervation at newly formed synapses in adult muscle (Busetto et al, 2000). Alternatively, the effect of asynchronous postsynaptic activation was tested by focally blocking acetylcholine receptors in small regions of singly innervated adult NMJs and therefore desynchronizing neuromuscular activity (Balice-Gordon and Lichtman, 1994). When a small region of the NMJ was saturated with the irreversible blocking agent α-bungarotoxin, the blocked acetylcholine receptors gradually disappeared and overlying nerve terminals were eliminated. In contrast, when all the acetylcholine receptors within the NMJ were uniformly blocked, no loss of nerve terminals or acetylcholine receptors was observed. This showed that the focal loss of nerve terminals was not due to receptor inactivation per se but rather to differences in activity sensed by postsynaptic cells. Thus, the size and strength of the active region determined the likelihood of the inactive region being eliminated.

1.4.4. Mechanisms of synapse elimination

There are currently at least three general models that could explain the activity dependent nature of synapse elimination. The first model was developed on the basis that muscle activity appears to a more important event in regulating its own innervation, compared to a possible competitive interaction between motor axons contacting the endplate. This was demonstrated by a number of experiments that manipulated muscle activity without interfering with motoneuron activity. For example, blocking acetylcholine receptors during the period of rapid synapse elimination, using curare or α-bungarotoxin, temporarily arrests the loss of

neuromuscular contacts (Srihari and Vrbová, 1978; Duxson, 1982). Moreover, increasing muscle activity by inhibiting the enzyme acetylcholinesterase, results in a greater loss of neuromuscular contacts than normally seen (O'Brien et al, 1982; Duxson and Vrbová, 1985). It is proposed that muscle activity regulates its synaptic inputs by activating proteolytic enzymes that destabilizes the weaker nerve terminals, whilst allowing the more stable terminals to remain (O'Brien et al, 1978, 1980, 1984). More specifically, the proteolytic breakdown of cytoskeletal proteins is dependent upon the influx of calcium ions (Ca^{2+}) into the nerve terminal, which activates the calcium activated neural protease (CANP). Evidence for the role of Ca^{2+} and CANP in synapse elimination has been provided by a number of *in vitro* and *in vivo* studies. For example, exposure of soleus muscles from 9 day old rats *in vitro* to acetylcholine resulted in a reduction in neuromuscular contacts (O'Brien et al, 1980; Zhu and Vrbová, 1992). This effect of acetylcholine was reduced when the muscles were pre-incubated with a calcium chelator before being exposed to acetylcholine, or when L-type Ca^{2+} channels were blocked by nifedipine (Zhu and Vrbová, 1992). In addition, treating the nerve terminals of the soleus muscle in vitro with the broad spectrum protease inhibitor, leupeptin, in the presence of acetylcholine and Ca^{2+}, preserved the normal complement of nerve terminals per endplate (O'Brien et al, 1980, 1982). Furthermore, leupeptin locally applied to the soleus muscle *in vivo*, also delayed synapse elimination (Connold et al, 1986).

This model of synapse elimination was further developed to include a link between muscle activity and CANP activation (Vrbová et al, 1988). In this model, as a consequence of neuromuscular activity, muscle fibres release K^+ in to the synaptic cleft (Hohfeld et al, 1981), which is likely to cause depolarization of the nerve

terminal, resulting in an influx of Ca^{2+} via voltage-gated calcium channels. This results in an increase in Ca^{2+} in nerve terminals, which activate CANP and leads to the breakdown of cytoskeletal proteins and the subsequent withdrawal of smaller more vulnerable nerve terminals.

A second model, proposes that after postsynaptic receptor activation, a local protective signal is generated for which axons compete. This signal or "synaptotrophic agent" (Snider and Lichtman, 1996) is released in an activity-dependent manner, so that the axon better able to generate muscle fibre activation would be selectively rewarded. In fact, several muscle-derived factors are capable of retarding synapse elimination when applied to postnatal muscles (Caroni and Becker, 1992; Kwon et al, 1995; Kwon and Gurney, 1996). For example, in transgenic mice that overexpress GDNF, synapse elimination is delayed for several weeks (Nguyen et al, 1998). However, interpreting the results of these experiments is complicated by the fact that many of these factors have potent effects on axonal sprouting, and so may increase signs of multiple innervation, without having any direct effect on synaptic competition.

Lastly, a third model also involves retrograde influences, but postulates that postsynaptic activation initiates two kinds of signal within the postsynaptic cell, one protective and one destabilizing (Nguyen and Lichtman, 1996; Lichtman and Colman, 2000). This model incorporates more recent work on the activity-dependence of synapse elimination, which suggests that the asynchronous activation of postsynaptic receptors may be the primary factor in establishing a competitive interaction between nerve terminals (Balice-Gordon and Lichtman, 1994; Busetto et

al, 2000). Thus, after acetylcholine receptor activation, a local protective signal is generated that prevents active synapses from destabilizing themselves. The second signal is an intersynaptic punishment signal that acts to destabilize neighbouring synapses that are not active at the same time. However, the nature of these signals has yet to be identified.

1.5. Development of motor activity

During the first few weeks of postnatal life, motor activity in rodents undergoes a dramatic change reflected by the emergence of weight bearing capacity of limbs, coordinated locomotor activity, postural orientation and righting reflexes. During this time, spinal motoneurons acquire mature electrophysiological characteristics, receive afferent innervation from supraspinal sources such as the corticospinal tract and display developed dendritic architecture. The following section will briefly describe the development of motor activity in terms of behavioural observations and the correlating electrophysiological properties of motoneurons.

1.5.1. Behavioural studies

During embryonic development of vertebrates, spontaneous movements can be observed. In the chick embryo, the first spontaneous movements appear at embryonic day 3.5 (E3.5) and consist of movements of the head followed by those of the trunk (Hamburger and Balaban, 1963). At about E6 the limbs begin to move in a jerky and uncoordinated fashion. A general characteristic of embryonic movements is their periodicity, phases of high motor activity alternate with inactive phases (Hamburger

and Balaban, 1963). A marked increase in movement occurs at around E9, reaching a plateau of sustained activity at E13-E17 (Hamburger et al, 1965). In the rat embryo stereotyped movements progress from lateral head flexion, seen at E15.5 (Windle and Baxter, 1936) to generalized mass movements at E16 onwards (Narayanan, et al, 1971).

At birth, the rat pup displays very low levels of locomotor activity and the limbs are flaccidly extended and abducted away from the body. Between postnatal days 4-9 (P4-P9), spontaneous locomotor activity consists of "pivoting", whereby the more active forelimbs act as paddles to propel the rat forward. Pivoting is gradually replaced by crawling between P7-P14 (Altman and Sudarshan, 1975). The adult pattern of locomotion, characterized by an adduction of the hindlimbs during the stance phase and smooth movements of the trunk and the limbs, is present from P16 (Westerga and Gramsbergen, 1990). It is important to note that during postnatal development the acquisition of postural control is the limiting factor for the development of locomotion, since it is necessary to counteract gravity and to stabilize body segments during movement (Massion, 1994). Experimentally, locomotion can be evoked during the early postnatal period, by lifting the animal in the air, or placing it in a water pool, which reduces postural constraints and demonstrates that neural networks underlying locomotion are functional at birth (Cazalets et al, 1990; Walton et al, 1992; Fady et al, 1998).

1.5.2. Electrophysiological properties of motoneurons

Electrophysiological evidence for the first embryonic movements in the chick was provided by extracellular recordings in situ from the lumbosacral spinal cord. It was found that neuronal activity in the spinal cord is concomitant with observed limb movements and this activity was generated by intrinsic circuits within the spinal cord (Ripley and Provine, 1972). This neuronal activity is characterized by a high degree of synchronous discharging (Provine, 1971). More specific changes were revealed by electromyographic (EMG) recordings. At 7 days of incubation (E7) in the chick embryo, both flexor and extensor muscles of the leg are activated synchronously and that coordinated reciprocal activation of antagonists emerges gradually after E9-10 (Bekoff, 1976; Landmesser and O'Donovan, 1984a; O'Donovan and Landmesser, 1987). Spontaneous movements with similar characteristics to those observed in the chick embryo are seen in mammalian fetuses (Narayanan et al, 1971).

The most pronounced changes in locomotor activity are seen during the first 3 weeks of postnatal development, as postural systems exert a controlling influence over the spinal cord, and allow adult patterns of locomotion (Altman and Sudarshan, 1975; Westergna and Gramsbergen, 1990). Simultaneous EMG recordings from hindlimb flexor and extensor muscles in freely moving rat pups were used to characterize the changes in activation patterns of motoneurons to these muscles during this period (Bursian and Sviderskaya, 1971; Navarrete and Vrbova, 1983; Brocard et al, 1999).

During the first postnatal week, the activity of motor units in soleus muscle (extensor) is very different to that seen in the adult, since at rest no activity was

recorded and during spontaneous locomotion the soleus is only intermittently active. At this stage the aggregate EMG activity of the soleus is not significantly different from the EDL muscle (flexor). By P14, postural EMG activity in soleus is established, with the motor units firing tonically at rest and the aggregate activity being almost 3 times greater than the fast flexor muscles. Fast motoneurons innervating the EDL muscle continue to discharge in a phasic manner but significantly increase their firing rate during the first 2-3 weeks of postnatal development. Thus, by P21, the qualitative EMG pattern of adult locomotion, characterized by reciprocal tonic activity in soleus and phasic activity in EDL, is fully established (Navarrete and Vrbova, 1983). Furthermore, it is now well established that the electrical properties of motoneurons also determines the contractile properties of the muscles fibres they innervate (Vrbová, 1963; Salmons and Vrbová, 1969; Lomo et al, 1974; reviewed by Gundersen, 1998; Buonanno and Fields, 1999). Thus, using a cross-reinnervation method in which the motor nerve supplying the slow soleus muscle was connected to the fast flexor digitorum longus muscle and vice versa, it was shown that the fast muscles supplied by a slow nerve became slow contracting and the slow muscles supplied by a fast nerve became fast contracting (Vrbová, 1963; Salmons and Vrbová, 1969). In addition to the changes in contractile characteristics, a number of biochemical changes also occurred. For example, following the connection of a slow motor nerve to a fast muscle, there is an increase in oxidative enzyme activity with a simultaneous decrease in the enzyme activities of glycogenolysis, glycolysis and lactate metabolism (Romanul and Van der Meullen, 1967).

The development of adult patterns of motor activity can be explained by marked changes in the intrinsic electrophysiological properties of motoneurons in combination with the source and distribution of synaptic inputs that activates them. First, motoneurons develop the capacity to fire repetitively during the early postnatal period. For example, in vivo spinal cord recordings have shown that before birth, a prolonged depolarizing current, produces only one action potential, whereas the same stimulus triggers repetitive firing in motoneurons a few days after birth (Xie and Ziskind-Conhaim, 1995; Gao et al, 1998). It is possible that the inability of embryonic motoneurons to fire repetitively is due to long-lasting subthreshold depolarization thereby prolonging sodium channel activation (Gao and Ziskind-Conhaim, 1998; Rothe et al, 1999). The development of repetitive firing requires an up-regulation in the expression of repolarizing conductances via voltage- and Ca^{2+}-activated K^+ channels, which shortens the action potential duration by modulating the time for hyperpolarization. Secondly, there is evidence of continued reorganization of synaptic inputs to motoneurons during the first few postnatal weeks (Conradi and Ronnevi, 1975; Gilbert and Stelzner, 1979; Ronnevi, 1979). In particular, descending brainstem pathways undergo developmental changes in distribution and transmitter synthesis of their terminals in the spinal cord (Loizou, 1972; Commissiong, 1983; Aramant et al, 1986). Furthermore, the earliest corticospinal projections are established after birth, reaching the lumbosacral spinal cord between P9-P14 (Hicks and D'Amato, 1975; Donatelle, 1977; Schreyer and Jones, 1982). Thus, the functional specialization of the motoneuron may be regulated by afferent synaptic activity associated with maturation of descending and interneuronal inputs to motoneurons.

1.6. Plasticity of the motor unit

During the early postnatal period, developing motoneurons are critically dependent upon a functional interaction with their target muscle. Disrupting this interaction during the first few days of postnatal development, compromises the maturation and survival of developing motoneurons (see Chapter 2). However, in the adult animal, motoneurons survive disruption of functional connections following nerve injury and are able to regenerate their peripheral axons in order to resume neuromuscular interaction. These injured neurons undergo a number of structural, metabolic and functional changes, which can be viewed as an anabolic response appropriate for repair of cellular damage and axon regeneration. Furthermore, a second case of motor unit plasticity occurs following partial denervation of a muscle. The response of the remaining motoneurons is to send out axonal branches (sprouts) in order to reinnervate the denervated muscle territory, thereby expanding its peripheral field. The following section will describe in greater detail the changes that occur to the motoneuron following direct nerve injury and after partial denervation.

1.6.1. Cell body responses to axonal injury

Following axonal injury, the cell body of surviving motoneurons undergo a number of characteristic morphological changes within a few hours of injury, which is referred to as the "retrograde response" or "axon reaction" (reviewed by Lieberman, 1971). The main morphological event is the apparent disappearance of the cytoplasmic basophilic material (Nissl substance), due to the dissolution of the ribosomes and ordered arrays of rough endoplasmic reticulum from the cell body.

This was first described by Nissl in 1892 and termed "chromatolysis" by Marinesco (1896). Dissolution of the Nissl substance is the morphological correlate of enhanced protein synthesis (Cragg, 1970), which is associated with increased nucleic acid production (Watson, 1974). Furthermore, chromatolysis is more severe when axon lesions are close to the cell body (Marinesco, 1896). This indicates that chromatolysis is a metabolic response, which is quantitatively proportional to the amount of axonal growth required to re-establish connections with the target muscle. Interestingly, if no functional regeneration occurs, the rate of protein synthesis declines, whereas if functional neuromuscular interaction is restored, a second burst of nucleic acid synthesis and metabolism takes place (Watson, 1970). Other morphological changes indicative of increased metabolism include an increase in nucleolar volume (Lavelle and Lavelle, 1958; Watson, 1965, 1968) and cell swelling (Brattgard et al, 1957; Lavelle and Lavelle, 1958; Watson, 1969), which is associated with increased RNA synthesis and protein content respectively.

The onset of regeneration is associated with an alteration in gene expression as the motoneuron enters a "growth phenotype" from a mature "transmitting phenotype" (reviewed by Fawcett and Keynes, 1990; Vrbová et al, 1995; Fu and Gordon, 1997). In general, the proteins associated with regeneration are the same as those produced by motoneurons during embryonic development. Thus, when the peripheral axon is cut, motoneurons reduce their expression of proteins associated with the synthesis of the neurotransmitter acetylcholine (e.g. choline acetyltransferase: Borke et al, 1993; Rende et al, 1995; Jacobsson et al, 1998). In addition, proteins associated with transmitter release such as the synaptobrevin isoform, VAMP-1 are down-regulated (Jacobsson et al, 1998), and those that are involved with acetylcholine degradation

(e.g. acetylcholinesterase; Engel and Kreutzberg, 1986). Concurrent with the down-regulation of transmitter related proteins there is an up-regulation of proteins involved with axonal growth. An example is the re-synthesis of the growth associated protein, GAP-43, which is also produced in high levels in neurons in the developing nervous system (Jacobson et al, 1986).

1.6.2. Growth associated proteins

There is now a large body of evidence consistent with the possibility that GAP-43 may be an intrinsic competence factor for nerve growth and plasticity (reviewed by Benowitz and Routtenberg, 1997; Skene, 1989). GAP-43 is a phosphoprotein that is transported by fast axoplasmic flow to the axonal growth cone or presynaptic terminal, where it is phosphorylated by protein kinase C (Skene and Willard, 1981; Aloyo et al, 1983; Akers and Routtenberg, 1985). GAP-43 is enriched at the interface between growth cone receptors and the cytoskeleton, where once phosphorylated influences cytoskeletal dynamics, including actin polymerization (Meiri and Gordon-Weeks, 1990; He et al, 1997). Its expression in neurons correlates closely with axonal elongation, synaptic remodeling and nerve sprouting both during development and in the adult following nerve injury (Skene et al, 1986; Neve et al, 1988; Verhaagen et al, 1988; Woolf et al, 1990; Tetzlaff et al, 1991). During postnatal development, down-regulation of GAP-43 expression to undetectable levels coincides with the termination of synapse elimination following the generation of superfluous nerve terminals on individual muscle fibres (Caroni and Becker, 1992). Thus, in the neuromuscular system, GAP-43 down-regulation corresponds to the specific termination of a nerve growth state in motoneurons.

More compelling evidence for the role of GAP-43 in axonal growth came from the generation of transgenic mice, which overexpress GAP-43 in the adult. These mice express high levels of the GAP-43 transgene for up to 3 months of age and as a consequence display extensive spontaneous motor nerve sprouting at the adult neuromuscular junction during the same period (Aigner et al, 1995). In addition, injury-induced nerve sprouting and terminal arborization was greatly potentiated in these mice. Furthermore, *in vitro* studies have shown that blocking GAP-43 function using antisense GAP-43 nucleotides or intracellular antibodies inhibited neurite extension in cultured DRG neurons, neuroblastoma and PC-12 cells (Shea et al, 1991; Aigner and Caroni, 1993). However, GAP-43 gene inactivation studies in mice did not reveal defects that would directly support the hypothesis that GAP-43 plays an essential role in promoting axon elongation or nerve sprouting (Strittmatter et al, 1995; Stretawan and Kruger, 1998; Zhu and Julien, 1999). Thus, although GAP-43 -/- mice exhibited high postnatal mortality, defects in the formation of axonal projections were restricted to growth cone guidance at specific choice points that generate specific topographic maps. For example, retinal axons were seen to take a random course upon reaching the optic chiasm resulting in grossly abnormal connections (Zhu and Julien, 1999). These results suggest that GAP-43 is involved in translating signals that are important for growth cone guidance.

1.6.3. Cytoskeletal proteins

An alteration in the pattern of gene expression following axonal injury also results in the synthesis of cytoskeletal proteins that are normally expressed by developing neurons. The fibrillar proteins that make up the axon are the actin containing

filaments, microtubules, which are tubulin polymers and neurofilaments that are essential for establishing the caliber of large myelinated axons (Shwartz, 1979; Hoffman and Lasek. 1980; Hoffman et al, 1987; Lee et al, 1993). Following nerve injury, the synthesis of neurofilament protein and expression of neurofilament mRNA decreases during axonal regeneration (Hoffman and Lasek, 1980; Oblinger and Lasek, 1988; Tetzlaff et al, 1988). This is associated with a decrease in the diameter of the regenerating nerve fibre (Hoffman et al, 1984, 1987). In order to lengthen the axon shaft, the neuron begins to synthesize and transport larger amounts of actin and microtubules (Tetzlaff et al, 1988; Jacob and McQuarrie, 1991). Specifically, axotomized motoneurons re-express mRNA for the microtubule, Tα1 tubulin, which is normally only present in developing neurons, where it is down-regulated upon target contact (Miller et al, 1987, 1989). Also, mRNA for β-actin is increased in regenerating neurons (Tetzlaff et al, 1991; Lund and McQuarrie, 1996).

Overall, these qualitative and quantitative changes in gene expression can be considered to be a process of de-differentiation from a mature transmitting state to an immature growth state akin to the pattern of gene expression in developing neurons (Hoffman and Cleveland, 1988).

1.6.4. Axonal regeneration and target reinnervation

The success of peripheral nerve regeneration depends critically on the growth environment in the distal stump following removal of axonal and myelin debris. When axons are physically disconnected from their cell body, they degenerate, due to their dependence on the cell body for the materials required for normal metabolic

turnover (Zelená, 1972; Droz et al, 1973; Lasek and Hoffman, 1976). The process of degeneration in the distal portion of the axotomized nerve was first described by Waller (1850) and termed Wallerian degeneration. Wallerian degeneration is characterized by axonal and myelin breakdown induced by the activation of axonal proteases and calcium influx (Schlaepfer and Freeman, 1980). Primarily it is the Schwann cells that degrade or phagocytose myelin and axonal debris within the first 3 days of injury (Fernandez-Valle at al, 1995; Liu et al, 1995; Perry et al, 1995). Thereafter, macrophages are recruited to injury site and combine with Schwann cells to remove the debris by phagocytosis in the distal nerve stump (Beuche and Friede, 1986; Stoll et al, 1989; Reichert et al, 1994). The importance of macrophages in axonal degradation was seen in the C57BL/Ola mutant mouse, where an impaired macrophage invasion, resulted in delayed Wallerian degeneration (Lunn et al, 1989). Furthermore, active removal of myelin by macrophages is essential for axonal regeneration because a number of myelin-derived proteins are inhibitory to neurite growth (reviewed by Bandtlow and Schwab, 2000).

After the phagocytic removal of axon and myelin debris, proliferating Schwann cells extend cytoplasmic process, which form the Schwann cell column or band of Bünger (Bünger, 1891). Schwann cell columns serve as effective pathways for the growth of the regenerating axons. The Schwann cells play a dual role in this process. First, they express a variety of adhesion molecules on their surface plasma membrane, which act as substrates for extending neurites. These include the immunoglobulin superfamily, e.g. neural cell adhesion molecule (N-CAM) and L1, and the cadherin superfamily, e.g. N-cadherin and E-cadherin (Daniloff et al, 1986; Martini and Schachner, 1988; Shibuya et al, 1995). Second, denervated Schwann cells produce a

number of neurotrophic factors including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), cilliary neurotrophic factor (CNTF), and glial derived neurotrophic factor (GDNF) (reviewed by Ide, 1996; Fu and Gordon, 1997). However, there is little evidence that neurotrophic factors synthesized and released by Schwann cells directly promote the growth of regenerating axons.

Other components of the damaged nerve, fibroblasts and basal lamina, are also involved in promoting axon regeneration. The role of the basal lamina in nerve regeneration has been thoroughly examined using acellular nerve grafts from which Schwann cells have been removed by freeze-thaw treatment, leaving empty basal lamina tubes. Regenerating axons will only grow through the acellular graft when accompanied by migrating Schwann cells from the proximal nerve stump (Gulati, 1988; Hall, 1986a; Ide et al, 1983). If the migration of live Schwann cells into the acellular grafts is prevented by cytotoxic agents, axons fail to regenerate (Hall, 1986b). Even so, molecules in the basal lamina, such as laminin and fibronectin have been shown to be potent promoters of neurite growth in culture (Rogers et al, 1983; Bozyczko and Horwitz, 1986). Indeed, regeneration across acellular grafts is severely impaired when treated with laminin blocking antibodies (Sandrock and Matthew, 1987; Toyota et al, 1990).

Following axotomy or nerve-crush, axons close to the proximal nerve stump die back to the first node of Ranvier, from which the first regenerating axonal sprouts are seen as early as 5 hours after injury (Friede and Bischhausen, 1980; McQuarrie, 1985; Tomatsuri, 1993). The first nerve fibres emerging from the proximal nerve stump undergo extensive branching, with a single axon reported to have given rise to 50-

100 branches (Ranson, 1912). However, these branches often form spirals that later disappear (Peroncito, 1907). Sprouts from the nodes of Ranvier extend through their basal lamina tubes in the proximal nerve stump, traverse the narrow gap of connective tissue between the proximal and distal stumps, and finally enter the distal stump. From the parent axon within the supportive environment of the distal stump, an average of 5 daughter axonal sprouts per parent axon start to regenerate, which comprise a "regenerating unit" (Morris, 1972; Bray and Aguayo, 1974; Jenq and Coggeshall, 1985). Regenerating axons grow down the endoneurial tubes towards their targets, through the space between the basal lamina and the Schwann cell membrane, which supply the necessary molecules for growth cone extension (Nathaniel and Pease, 1963; Haftek and Thomas, 1968; Scherer and Easter, 1984). Subsequent axonal enlargement and maturation is dependent upon contact with the target, which also regulates the withdrawal of superfluous daughter axons (Aitken et al, 1947; Gordon and Stein, 1982).

The success of peripheral nerve regeneration and the extent of recovery of muscle function are also dependent upon the type of nerve injury. For example, nerve crush injuries, in which the endoneurial tubes and Schwann cell basal lamina remain intact, are associated with accurate regeneration and full recovery of function (Haftek and Thomas, 1968; Sunderland, 1978). Under these conditions, the normal numbers and diameters of myelinated fibres are often fully reconstituted (Gutmann and Sanders, 1943), with the normal mosaic distribution of muscle fibre types in the reinnervated muscle often recovered (Warszawski et al, 1975). In contrast, following complete nerve transection, when the endoneurial tubes are disrupted, the number of regenerating fibres that are successful in re-establishing functional contacts is far

more variable. This is because the growth environment in the gap between the proximal and distal nerve stumps is often less than optimal. Furthermore, if the endoneurial tubes are disrupted, regenerating axons may be guided to inappropriate targets (Brushart and Mesulam, 1980; Thomas et al, 1987).

When the regenerating motor axon reaches the skeletal muscle, intramuscular branching occurs, and these axonal branches are guided by terminal Schwann cell processes to their original motor endplate region (Gutmann and Young, 1944; Son and Thompson, 1995a). In addition, experimental analysis of reinnervation revealed that synaptic basal lamina also contains cues that guide selective reinnervation of synaptic sites and differentiation of growth cones into motor nerve terminals at those sites (Sanes et al, 1978, Glicksmann and Sanes 1983; Kuffler, 1986). Furthermore, once neuromuscular contact is made, spontaneous and evoked release of acetylcholine increases rapidly, mediated by retrograde signals from the muscle (Xie and Poo, 1986; Hall and Sanes, 1992). Similarly, reinnervation of a mature denervated muscle recapitulates the developmental process of multiple innervation of muscle fibres by several different axons, followed by the elimination of most of these superfluous inputs (Werle and Herrera, 1988, 1992; Rich and Lichtman, 1989).

1.6.5. Sprouting and nerve terminal remodeling

The peripheral territory of a motor unit is not rigidly determined since as well as having the flexibility to reduce its size during development (synapse elimination) a motoneuron has the ability to expand its peripheral field when a skeletal muscle becomes partially denervated (Brown and Ironton, 1978; Fisher et al, 1985; Lowrie

et al, 1985). Motor unit plasticity in the adult is revealed following partial denervation, where the remaining axons sprout immediately to occupy some or all of the muscle fibres that lose their nerve supply, thereby preventing a substantial loss of muscle force (Weiss and Edds, 1946; Hoffman, 1950; Edds and Small, 1951; Edds, 1953). Two types of sprouting have been characterized: nodal sprouting, where intact axons grow collateral sprouts from the nodes of Ranvier (Edds, 1949, 1950), and terminal sprouting where axons extend from intact nerve terminals to adjacent denervated endplates (Hoffman, 1950). Another form of sprouting can also be seen, which arises close to the nerve terminal and is called preterminal sprouting. However, since it is difficult to distinguish between nodal and preterminal sprouting, both types of sprouting are commonly referred to as collateral sprouting (Herrera and Grinnell, 1981).

Although partial denervation is an effective stimulus for sprouting, resulting in extensive collateral and terminal sprouting (reviewed by Brown et al, 1981b; Wernig and Herrera, 1986), there is a limit to the extent that each motoneuron can expand to innervate the denervated muscle territory. Thus, the enlargement of motor units is restricted to a limit of five to eight-fold, so that sprouting compensates for up to 85% of the loss of motor units (Thompson and Jansen, 1977; Brown and Ironton, 1978; Rafuse et al, 1992). Therefore, when less than 20% of motor units are present in denervated muscle, sprouting cannot fully compensate for muscle fibre denervation and muscle weakness becomes evident (Luff et al, 1988; Rafuse and Gordon, 1996). Following muscle paralysis less robust sprouting occurs. For example, blocking neuromuscular activity using botulinum toxin causes profuse terminal but not collateral sprouting (Duchen and Strich, 1968; Duchen, 1970; Ironton et al, 1978;

Brown et al, 1981a). Similarly, blocking postsynaptic activation using α-bungarotoxin primarily results in terminal sprouting (Holland and Brown, 1980). Furthermore, axonal sprouting induced by partial denervation or muscle paralysis can be prevented by direct electrical stimulation of the muscle (Brown and Holland, 1979; Gardiner et al, 1984; Tam et al, 2001). Therefore, axonal sprouting appears to be induced by inactive muscle fibres in an attempt to restore neuromuscular activity.

More recently it has become clear that Schwann cells at the denervated or paralyzed endplate region play an important role in triggering and guiding axonal sprouts from innervated endplates. A number of immunohistochemical studies have demonstrated that following partial denervation, the terminal Schwann cells once associated with the retracted nerve terminal, dedifferentiate and extend long filamentous processes that bridge between the denervated and innervated endplates (Reynolds and Wolf, 1993; Son and Thompson, 1995a, b; Son et al, 1996). Furthermore, it appears that when a Schwann cell process (SCP) contacts an innervated endplate, it induces the nerve terminal at this endplate to form an axonal sprout that grows along the process back to the denervated endplate where it forms a new synapse (Son and Thompson, 1995a). In support of the view that SCPs have the ability to induce sprouting, it was found that SCPs emanating from an axonless nerve transplanted onto the surface of a normal muscle, induced sprouting from the nerve terminals in the endplate zone on the host muscle (Son and Thompson, 1995b). Moreover, paralysis of the rat soleus muscle using botulinum toxin induced the growth of nerve sprouts, and all these sprouts were associated with SCPs (Son and Thompson, 1995b).

What causes Schwann cells to dedifferentiate and extend processes to induce axonal sprouting is unresolved. However, the answer may lie in fact that Schwann cells are sensitive to neuromuscular activity. Terminal Schwann cells express acetylcholine receptors that normally respond to acetylcholine release from nerve terminals by influx of calcium (Jahromi et al, 1992; Resit and Smith, 1992; Reynolds and Wolf, 1993). In addition, synaptic blockade results in a rapid up-regulation of glial fibrillary acidic protein (GFAP) in terminal Schwann cells (Georgiou et al, 1994). Thus, it is possible that in the absence of acetylcholine, a combination of a decrease in calcium influx and up-regulation of GFAP in terminal Schwann cells maybe the trigger for extending processes from denervated endplates. In addition to their role in providing physical guidance for extending axonal sprouts, Schwann cells may also produce sprout-inducing substances. For example, in light of the capacity of neurotrophic factors to induce sprouting in normally innervated muscles (Gurney et al, 1992; Kwon and Gurney, 1994; Siegel et al, 2000) it is possible that the expression of these factors is altered upon denervation, which in turn induces motoneuron sprouting.

Finally, the source of the stimulus for sprouting may be the inactive muscle fibres themselves (Brown et al, 1978; Slack and Pocket, 1981; Pockett and Slack, 1982). Putative sprouting factors released from inactive muscle fibres include, insulin like growth factors (Caroni and Grandes, 1990), neural cell adhesion molecules (Gurney et al, 1986; Sanes et al, 1986) and neurocrescin (Nishimune et al, 1997).

1.7. Mechanisms of cell death after neonatal nerve injury

During embryonic and early postnatal development, motoneurons are dependent upon target contact for their survival (see sections 1.3.and 2.1). Thus, following injury to the peripheral sciatic nerve in neonatal rats, a large proportion of motoneurons die (Romanes, 1946). The follow section will describe the characteristic features of degenerating neonatal motoneurons after nerve injury, and the possible mechanisms that may be involved in motoneuron death.

1.7.1. Apoptotic motoneuron cell death

The death of motoneurons during embryonic programmed cell death occurs by a process of apoptosis (see 1.3.1. also reviewed by Oppenheim et al, 1991). Apoptosis can be characterized on the basis of morphological and biochemical characteristics. Morphologically, apoptosis is characterized by cell shrinkage, compaction of cytoplasmic organelles, condensation and fragmentation of the nucleus (Kerr et al, 1972; Wyllie et al, 1980). Biochemically, apoptosis can be detected following the activation of preprogrammed intracellular suicide machinery. The central components in this pathway are caspases, which are evolutionary conserved cysteine proteases (Marks and Berg, 1999). Caspases are synthesized within cells as inactive pro-enzymes (procaspases) that in response to an apoptotic signal are cleaved to produce the catalytically active protease (Nicholson and Thornberry, 1997). A subgroup, the effector caspases (caspase-2, -3, -6, -7) are responsible for executing the final breakdown of the cell by cleaving cytoskeletal components and regulatory

enzymes, and activating nucleases that ultimately degrade DNA (Janicke et al, 1998; Thornberry and Lazebik, 1998).

Apoptosis results from interplay between positive and negative regulators of cell survival (Ellis et al, 1991). Recently, it has become clear that proteins of the Bcl-2 family play a central role in regulating caspase activation (reviewed by Pettman and Henderson, 1998). The Bcl-2 gene family members encode proteins with either anti-apoptotic or pro-apoptotic roles. One key member of this family is Bax, which is thought to be necessary to induce the apoptotic death of many neurons during development (Deckwerth et al, 1996; reviewed by Merry and Korsmeyer, 1997). In contrast, Bcl-2 and Bcl-x_L are anti-apoptotic proteins that can protect neurons from cell death during development and following injury (Martinou et al, 1994; Parasandian et al, 1998). The relative levels of pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family have been proposed to be the primary determinant in the decision to die (Oltvai, 1993). The mechanistic basis of this proposal is based on the fact that Bcl-2 and Bcl-x_L inactivate Bax by heterodimerization.

Following neonatal axotomy of sciatic motoneurons and facial motoneurons the largest proportion of cell death occurs in the first 2-3 days after injury (de Bilbao and Dubois-Dauphin, 1996a; Rossiter et al, 1996; Lawson and Lowrie, 1998). These axotomized motoneurons die by apoptosis as demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and in situ end labeling (de Bilbao and Dubois-Dauphin, 1996a; Rossiter et al, 1996; Olivera et al, 1997; Lawson and Lowrie, 1998). Evidence for the involvement of caspases in motoneuron apoptosis has been provided by studies that showed an increase in

caspase-3 mRNA and protein expression in axotomized motoneurons of neonatal animals (Vanderluit et al, 2000; Kalmar et al, 2002). In support of the proposal that caspase activation is involved in the death of axotomized neonatal motoneurons, caspase gene deletion has been shown to delay the death of axotomized motoneurons (de Bilbao et al, 2000; Vanderluit et al, 2000). Furthermore, additional experiments have shown that general or specific caspase inhibition reduced the number apoptotic motoneurons following axotomy or avulsion of neonatal motoneurons (de Bilbao and Dubois-Dauphin, 1996b; Chan et al, 2000). In addition, over-expression of Bcl-2 in transgenic mice protects facial motoneurons from axotomy-induced cell death (Dubois-Dauphin et al, 1994) and can block the DNA fragmentation generated by neonatal nerve injury (de Bilbao and Dubois-Dauphin, 1996a).

As previously discussed (see 1.3.3.), a variety of neurotrophic factors have been shown to transiently support the survival of motoneurons following neonatal nerve injury. Recently, significant progress in the understanding of signaling pathways, which mediate the survival promoting effects of neurotrophic factors has been made (reviewed by Kaplan and Miller, 2000; Sendtner et al, 2000). Binding of neurotrophic factors to their trk receptors, elicits a variety of signaling responses depending on the receptor and the cell type. Initiation of this signaling depends on the cytosolic adapter protein Shc, as they can couple trk-signaling to the small GTP-binding protein Ras, which is responsible for the majority of neurotrophin-dependent survival (reviewed by Kaplan and Miller, 1997; 2000). Ras does not directly promote survival rather it functions by activating multiple signaling pathways. The two pathways most implicated in neuronal survival are the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein (MAP) kinase pathways.

Activation of P13K leads to the subsequent activation of the serine-threonine kinase Akt, which mediates much of the survival promoting effects of P13K, by preventing the pro-apoptotic protein BAD from interacting with Bcl-x_L (Zha et al, 1996; Datta et al, 1997). Bcl-x_L can now exert it survival-promoting activity by heterodimerizing with Bax, thereby increasing the relative levels of anti-apoptotic proteins in the cell (Yang et al, 1995). The role of the MAP kinase pathway is neuronal survival is unclear, however the result of the signaling cascade appears to increase the activity or expression of anti-apoptotic proteins (Aloyz et al, 1998; Liu et al, 1999). It is necessary to note that most of the understanding of the signaling pathways downstream of neurotrophin receptor binding comes from in vitro experiments on the PC-12 cell line and sensory and sympathetic neurons, which are dependent on NGF for their survival. Thus, it is unclear whether these same pathways are involved in the survival promoting effects of neurotrophic factors applied to motoneurons after nerve injury in neonatal rats.

1.7.2. Excitotoxic cell death

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (Cotman et al, 1988). Glutamate acts postsynaptically at several receptor types that are named for their prototypic pharmacological agonists (Dingledine et al, 1999). One major subtype of the ionotrophic glutamate receptor is the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, a ligand-gated cation channel with crucial roles in synaptic physiology and some disease processes. The other major subtype is the N-methyl-D-aspartate (NMDA) receptor, which is ligand-gated and contains channels that are highly permaeable to calcium ions (Ca^{2+}). Prolonged activation of glutamate receptors results in increased intracellular

calcium ions (Ca^{2+}) and activation of oxidant-generating pathways (Choi, 1994; Dugan and Choi, 1994). Thus, excess activation of glutamate receptors can cause cell death via alterations in cytosolic free Ca^{2+} homeostasis ('excitotoxicity'). Excitotoxicity has been implicated in several neurodegenerative disorders including ischaemia, epilepsy, and Huntington's chorea (Choi, 1988).

It has been shown for motoneurons that NMDA receptors mediate the slow component of excitatory postsynaptic currents (Konnerth et al, 1990) and that the application of NMDA elicits a locomotor like pattern of activity in neonatal rat spinal cord preparations (Kudo and Yamada, 1987). Furthermore, NMDA receptor activation is required for activity-dependent development of motoneurons (Kalb and Hockfield, 1990). Because of the expression of NMDA receptors by neonatal motoneurons, excitotoxicity has been implicated in the death of injured neonatal motoneurons. Evidence to suggest that injured developing motoneurons may be exposed to excessive excitation came from a study that examined the activity pattern of motoneurons that survived neonatal nerve-crush (Navarrete and Vrbová, 1984). It was found that motoneurons that survive target separation during the early postnatal period were more active, which may be related to a permanent reduction in their cell body size (Lowrie et al, 1987). Furthermore, these motoneurons also exhibited a permanent enhancement of spinal reflexes involving the regenerated flexor motoneurons (Navarrete et al, 1990). It has been proposed that during the early postnatal period neuromuscular interaction induces the motoneuron to acquire characteristics that enables it to survive the increasing levels of excitatory input in the developing spinal cord (Lowrie and Vrbová, 1992). This hypothesis is supported by a number of findings, which demonstrated that disrupting neuromuscular

interaction during the early postnatal period rendered developing motoneurons more vulnerable to the toxic effects of glutamate (Greensmith et al, 1994a, 1995a). This evidence is described in more detail in Chapter 3 (see 3.1.3.). In addition, developing motoneurons undergo significant changes in their morphology and excitability during the early postnatal period, which may be regulated by neuromuscular interaction. For example, the large number of synapses on motoneurons is reduced by approximately half during the first several weeks of postnatal life in the cat (Conradi and Ronnevi, 1975). The withdrawal of filopodial growth associated processes from the motoneuron somatodendritic surface takes place during the first few days after birth (Dekkers and Navarrete, 1998). Significantly, the excitability of motoneurons decreases during the early postnatal period, such that NMDA-induced currents decrease (Hori and Kanda, 1996). This probably reflects changes in NMDA receptor subunits so that immature channels are replaced by their more mature counterparts (Monyer et al, 1994; Stegenga and Kalb, 2001). Thus, the decline in the excitability of developing motoneurons may be necessary to enable them to withstand the ever increasing amounts of excitatory inputs in the spinal cord (Provine, 1971; Hicks and D'Amato, 1975; Donatelle, 1977; Schreyer and Jones, 1982; Navarrete and Vrbová, 1983; Westerga and Gramsbergen, 1990).

1.8. Pathological cell death of motoneurons

The motor neuron diseases, an etiologically heterogeneous group of disorders, are characterized by weakness (muscle atrophy) and/or spastic paralysis, reflecting the selective involvement of lower motoneurons and/or upper motoneurons, respectively (see Wong et al, 1998). These include: spinal bulbar muscular atrophy (SBMA; or

Kennedy's disease), and spinal muscular atrophy (SMA), which selectively affects lower motoneurons, whereas hereditary spastic paraplegia (HSP), involves predominantly upper motoneurons. Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease and involves both lower and upper motoneurons. Several genetic mutations associated with ALS have been identified. Most significantly, was the discovery that 15-20% of patients with autosomal-dominant familial ALS (FALS) have missense point mutations in the gene encoding the cytosolic enzyme Cu/Zn superoxide dismutase (SOD1; Deng et al, 1993; Rosen et al, 1993). This led to the generation of several transgenic mouse models that express the mutant form of the human SOD1 gene, which develop the clinical and pathological hallmarks similar to that seen in human ALS patients (Gurney et al, 1994; Ripps et al, 1995; Wong et al, 1995; Bruijn et al, 1997). A detailed description of these mutant SOD1 mice and the toxicity associated with the aberrant function of mutant SOD1 can be found in Chapter 4 (section 4.1.1. and 4.1.2). In addition, a number of other mechanisms have been suggested to play a role in the pathogenesis of ALS and are discussed in the following section.

1.8.1. Apoptotic death

Recent evidence suggests that neuronal death in human ALS occurs through a process of apoptosis and that this cell death pathway appears to have a major contribution to the pathogenesis of ALS. This is supported by morphological descriptions of motoneuron degeneration in the spinal cord of sporadic ALS (SALS) cases, which showed that motoneurons had shrunk considerably, with only an extremely condensed nucleus and contracted cytoplasm remaining (Martin et al,

1999). Several studies have also provided evidence of DNA fragmentation in selectively vulnerable CNS regions of SALS cases, as determined by in situ end labeling of DNA strand breaks (Migheli et al, 1994a; Yoshiyama et al, 1994; Ekegren et al, 1999).

Another body of evidence supporting a role for apoptosis in motoneuron death comes from studies showing an increased expression of certain apoptosis related molecules in ALS. For example, the apoptosis related Par-4 protein was significantly increased in the lumbar spinal cords of ALS patients compared to controls (Pedersen et al, 2000). In addition, the levels of pro-apoptotic proteins Bax and Bak were increased in the mitochondrial-enriched membrane compartment of the motor cortex and spinal cord of ALS patients (Martin, 1999). In contrast, the levels of the anti-apoptotic protein Bcl-2 was decreased in the same region in these ALS patients (Martin, 1999).

There is also compelling evidence that motoneuron death involves apoptosis in FALS, caused by mutations in the SOD1 gene. A number of studies have examined the levels of Bcl-2 family members in the mutant SOD1 transgenic mouse models of FALS. In asymptomatic G93A SOD1 mice, expression of anti-apoptotic Bcl-2 and Bcl-x_L and proapoptotic Bad and Bax was similar to normal mice (Vukosavic et al, 1999). However, in symptomatic G93A SOD1 mice, there was a reduced expression of Bcl-2 and Bcl-xl, whereas expression of Bad and Bax was increased. Similar results were also found using the G86R SOD1 mice, which showed decreased Bcl-xl expression in conjunction with increased Bax immunoreactivity in the lumbar spinal cord compared to wild-type controls (Gonzalez de Aguilar et al, 2000). Further support for the role of Bcl-2 came from a study in which Bcl-2 was overexpressed in

G93A SOD1 mice. The overexpression of Bcl-2 delays the onset of motor symptoms by retarding the loss myelinated nerve fibres and the death of spinal cord motoneurons, resulting in prolonged survival (Kostic et al, 1997).

Members of the caspase family are central components in the apoptotic death pathway and play an important role in the pathogenesis of central nervous system disorders (Hara et al, 1997; Fink et al, 1999; Springer et al, 1999). A recent report provided evidence for caspase activation in human ALS (Martin, 1999). More detailed studies investigating the role of caspases in the pathogenesis of FALS have used mutant SOD1 mouse models of FALS. The activation of caspase-1 and capsase-3 in motoneurons and astrocytes has been reported using three models of ALS, the G93A, G37R, and G85R SOD1 mice (Pasinelli et al, 1998, 2000; Li et al, 2000). Furthermore, expression of mutant caspase-1 that dominantly inhibits caspase-1 activity (Friedlander et al, 1997), or chronic infusion of a broad caspase inhibitor (zVAD-fmk; Li et al, 2000), prolonged the survival of G93A SOD1 mice. Interestingly, two recent studies have demonstrated that caspases in these mice are activated in sequence (Pasinelli et al, 2000; Vukosavic, et al, 2000). Caspase-1 is activated months before activation of caspase-3 in G37A SOD1 mice, and before any detectable loss of motoneurons (Pasinelli et al, 2000). Caspase-3 activation matched the timing of motoneuron loss and was found within neurons showing shrunken cell bodies and nuclei (Pasinelli et al, 2000; Vukosavic et al, 2000), which suggests that caspase-3 mediates the terminal stages apoptosis. Furthermore, caspase-3 is especially prominent within astrocytes of G85R and G93A SOD1 mice (Li et al, 2000; Pasinelli et al, 2000), which raises the possibility that astrocytes themselves are direct target for SOD1-mediated toxicity. Moreover, early caspase-1 activation in

astrocytes may cause inflammation and astrocytosis resulting in impaired astrocytic function that may ultimately contribute to motoneuron damage.

1.8.2. Excitotoxicity

Glutamate-mediated excitotoxicity has been suggested to play a role in motoneuron degeneration in ALS (reviewed by Ludolph et al, 2000; Shaw and Eggett, 2000; Heath and Shaw, 2002). As elevated levels of glutamate may be neurotoxic, it is vital that glutamate is cleared quickly from the synaptic and extracellular environment. For spinal motoneurons, rapid recovery of glutamate from the synaptic cleft is accomplished by the glutamate transporter (excitatory amino acid transporter; EAATs) present in astrocytes. Loss of the EAAT2 (also known as GLT-1; glial glutamate transporter) transporter results in increased extracellular glutamate, and excitotoxic degeneration of motoneurons (Rothstein et al, 1996; Maragakis and Rothstein, 2001), and this has been suggested to be a possible contributor to increased levels of glutamate in ALS. Indeed, up to 60-70% of sporadic ALS patients have a 30-95% reduction in the astroglial transporter EAAT2 in the motor cortex and spinal cord (Rothstein et al, 1995). In addition, elevated levels of extracellular glutamate have been found in the cerebrospinal fluid of some ALS patients, and it is possible that this is due to the loss of the EAAT2 protein (Rothstein et al, 1990; Shaw et al, 1995). The decreased level of EAAT2 is unlikely to be attributed to cell death, since no significant astroglial loss occurs (Rothstein et al, 1995; Maragakis and Rothstein, 2001). Furthermore, the absence of EAAT2 protein in ALS material was not accompanied by a corresponding reduction in EAAT2 mRNA and there is no evidence to suggest that mutations within the coding region of EAAT2 commonly

underlie sporadic or familial ALS (Bristol and Rothstein, 1996; Aoki et al, 1998; Jackson et al, 1999). Thus, the absence of EAAT2 may be explained by aberrant mRNA species resulting from RNA processing errors. Indeed, abnormal mRNA transcripts have been reported in a subset of sporadic ALS cases including intron retention and exon skipping splice variants (Lin et al, 1998). However, a recent study showed aberrant EAAT2 transcripts to be equally present in ALS patients and in control cases (Meyer et al, 1998). In view of this evidence it appears that the loss of EAAT2 is most likely a secondary effect of the disease process and other reasons for increased glutamatergic activity must be found.

It is possible that activation of glutamate receptors could prove toxic without any underlying abnormality in the expression or modulation of glutamate or its receptors (reviewed by Shaw and Eggett, 2000). For example, it is possible that the selective vulnerability of motoneurons to glutamate-induced excitotoxicity is due to an unusual Ca^{2+}-permeability mediated by AMPA receptors (Lu et al, 1995; Carriedo et al, 1996; Terro et al, 1998; Van den Bosch et al, 2000). The AMPA glutamate receptor consists of four subunits, GluR1-GluR4. The GluR2 subunit is of particular functional significance because it determines the Ca^{2+}-permeability of the receptor. If the GluR2 subunit is absent the AMPA receptor complex allows Ca^{2+} to enter the cell (Jonas and Burnashev, 1995). Therefore, motoneurons that do not express GluR2 may be more vulnerable to glutamate-mediated excitotoxicity. However, studies investigating the expression of the GluR2 subunit in motoneurons have produced conflicting results. In situ hybridization has shown that both upper and lower motoneurons in humans have a low expression of GluR2 mRNA (Williams et al, 1997). In addition, low levels of expression of the GluR2 protein have been

demonstrated in human motoneurons (Shaw et al, 1999). Furthermore, the expression of mutant SOD1 in embryonic mouse motoneurons *in vitro* resulted in a greatly increased vulnerability to glutamate toxicity mediated by calcium permeable AMPA/kainate receptors (Roy et al, 1998; Kruman et al, 1999). On the other hand, a number of studies have reported the strong presence of GluR2 in human and rat spinal motoneurons (Jackowec et al, 1995; Tomiyama et al, 1996; Temkin et al, 1997). Furthermore, GluR2 protein was present on motoneurons in the spinal cord of SOD1 transgenic mice (Morrison et al, 1998b).

A further feature of motoneurons that may render them unusually susceptible to excitotoxicity is their relatively low expression of calcium binding proteins. Human motoneurons that are vulnerable in ALS do not express the calcium binding proteins parvalbumin and calbindin D28K. However, motoneurons of the oculomotor nucleus and Onuf's nucleus, which express these calcium-binding proteins, are relatively spared in ALS (Ince et al, 1993; Alexianu et al, 1994; Elliott and Snider, 1995). Thus, calcium-binding protein may play an important role in protecting neurons from calcium-mediated toxicity following activation of glutamate receptors. In support, a recent study demonstrated that expressing the calcium-buffering protein parvalbumin in motoneurons could protect these cells *in vitro* from excitotoxicity induced by AMPA receptor activation (Van den Bosch et al, 2002).

1.8.3. Aberrant protein aggregation

Intraneuronal inclusions in the proximal axon and cell body of degenerating motoneurons are pathological hallmarks of ALS and are also prominent in mutant

SOD1 mice prior to the period of motoneuron degeneration. These protein aggregates mainly take the form of ubiquitinated protein accumulations, called skein-like inclusions, and neurofilament rich spheroids (Leigh et al, 1988; Manetto et al, 1988). However, it is not known whether these aggregates are central to disease pathogenesis or harmless by products. The following section will describe in turn the role of ubiquitin and neurofilaments in protein aggregation toxicity.

Ubiquitin is a small protein of 76 amino acids and is crucial in the degradation of many cytosolic and nuclear proteins (reviewed by Hochstrasser, 1996). Ubiquitin functions by binding to other proteins through an ATP-dependent process, which form ubiquitinated complexes that can be degraded by a cascade of proteolytic enzymes (reviewed by Alves-Rodrigues, et al 1998). Significantly, the intraneuronal inclusions in ALS contain ubiquitinated protein conjugates (Leigh et al, 1988; Lowe et al, 1988; Leigh et al. 1991), indicating a dysfunction in the process of ubiquitin-dependent proteolysis. These pathological hallmarks have been shown to form in the cytoplasm of motoneurons well before the onset of the symptomatic phase of the disease (Schiffer et al, 1991; Tu et al, 1996; Stieber et al, 2000). However, at present it is not understood how these cytoplasmic inclusions develop or whether they are the cause or consequence of neuronal dysfunction (Al-Chalabi et al, 2000; Julien et al, 2001). In general, ubiquitin proteins do not acculumulate in healthy cells and are rapidly degraded by the ubiquitin ATP-dependent pathway. It has been suggested that oxidative stress and the formation of reactive oxygen species may generate aggregates by inducing abnormal protein modifications that result in ubiquitinated aggregates of high molecular weight (Montine et al, 1996; Figueiredo-Pereira 1998). In addition, oxidative stress elevates the expression of genes associated with the

ubiquitin ATP-dependent pathway (Jungmann et al, 1993; Uney et al. 1993). Therefore, factors causing cell stress may lead to a malfunction or overload of the ubiquitin ATP-dependent pathways that normally respond to cell stress. An inability of the cell to degrade damaged proteins, which have been ubiquitinated may result in protein accumulation and ultimately cell death due the sequestration of essential cellular components.

Neurofilament proteins constitute a major component of the cytoskeleton of neurons and have significant roles in maintenance of cell shape, axonal caliber and axonal transport. There are three neurofilament subunits of differing molecular weights: neurofilament light (NF-L; 61 KDa), medium (NF-M; 90 KDa) and heavy (NF-H; 110 KDa). Neurofilaments are synthesized in the cell body and transported down the axon by slow axonal transport, where they are progressively phosphorylated.

Perturbations in the normal metabolism of neurofilaments are implicated in the pathogenesis of ALS, since accumulations of phosphorylated neurofilaments in the cell body and proximal axons of motoneurons is a characteristic feature of ALS pathology (Manetto et al, 1988; Munoz et al, 1988; Sobue et al, 1990; Hirano, 1991). The first experimental evidence that neurofilaments may be involved in the pathogenesis of ALS came from transgenic mice, which overexpress NF-L or NF-H. Both these mice developed perikaryal neurofilament accumulations reminiscent of those found in human ALS (Cote et al, 1993; Xu et al, 1993). However, in both cases there was no significant loss of motoneurons, but mice expressing mutations in the NF-L subunit was sufficient to induce massive neurofilament accumulations and

spinal motoneuron death (Lee et al, 1994). Thus, disorganized neurofilament expression can provoke ALS-like pathology and motoneuron degeneration.

To examine the contribution of neurofilaments to familial ALS caused by mutations in the SOD1 gene, mice expressing mutant SOD1 were crossed with transgenic mice with altered neurofilament protein content. Surprisingly, it was found that mice overexpressing G37A mSOD1 and human NF-H had an extended life span of approximately 6 months, which represents a 65% increase in longevity (Couillard-Despres et al, 1998). In addition, increasing the synthesis of NF-L subunit also powerfully slows the progression of mSOD1 induced ALS (Kong and Xu, 2000). This beneficial effect of neurofilament overexpression appears to be dependent on the degree of perikaryal neurofilament accumulation (Eyer et al, 1998; Williamson et al, 1998). It has been postulated that neurofilaments may act as calcium chelators, since they have multiple calcium binding sites and therefore can potentially protect motoneurons against rises in intracellular calcium resulting from oxidative stress and mitochondrial dysfunction (Lefebvre and Mushynski, 1987, 1988). Alternatively, on the basis that mutant SOD1 mice have impaired slow axonal transport (Collard et al, 1995; Zhang et al, 1997; Williamson and Cleveland, 1999), it has been suggested that accumulation of neurofilaments in the perikarya of motoneurons may alleviate 'axonal strangulation' caused by neurofilament accumulation in the axon (Williamson and Cleveland, 1999). Further support for the possibility that inhibition of slow axonal transport is sufficient to cause motoneurons degeneration came from a recent study, which showed that disrupting slow axonal transport resulted in neurofilament accumulations and late-onset motoneuron degeneration (LaMonte et al, 2002).

Overall, this evidence suggests that a number of factors involving neurofilament expression and/or axonal transport represent possible risk factors that underlie the selective vulnerability of motoneurons in ALS. However, at present there is no direct evidence to suggest that protein aggregation is the primary cause of ALS.

CHAPTER 2

THE EFFECT OF MANIPULATING TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION ON THE EXPRESSION OF CHAT AND GAP-43 IN DEVELOPING RAT MOTONEURONS

2.1. INTRODUCTION

During the early postnatal period developing motoneurons are dependent upon functional interaction with their target muscle for their survival and maturation. Recent evidence indicates that a critical event in motoneuron development is the transformation from a growing into a transmitting cell, a transition that is dependent on interaction with the target muscle. It has been postulated that a failure to undergo this transformation during a critical period of development compromises the motoneurons ability to survive within the mature central nervous system (CNS). Therefore, in order to determine whether local events at the neuromuscular junction (NMJ) influence the development of motoneurons during the early postnatal period, this study investigates whether manipulating transmitter release from motor nerve terminals in neonatal rats alters the normal pattern of gene expression in motoneurons of those proteins associated with growth and transmission.

2.1.1. Postnatal development of motoneurons

The dependence of motoneurons on their target continues into the early postnatal period. For example, during the first few days of postnatal development, disconnecting rat motoneurons from their muscle fibres following axotomy or nerve-crush results in the death of a large proportion of motoneurons (Romanes, 1946; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987). The degree of motoneuron cell death is dependent upon the age of the animal at the time of target separation and the duration for which neuromuscular interaction is disrupted. Thus, sciatic nerve-crush in newborn rats results in the loss of as many as 60-70% of

motoneurons innervating hindlimb muscles (Lowrie et al, 1987), with this number decreasing with age, such that by five days after birth all motoneurons are capable of surviving this insult (Lowrie et al, 1982). Increasing the time of target deprivation by axotomizing the nerve (Sendtner et al, 1992) or applying a more proximal crush (Lieberman, 1974), results in a greater loss of motoneurons, as the time needed to re-establish interaction with the target muscle is increased. Therefore, during the first five postnatal days, motoneurons in the rat undergo fundamental changes in development that enable them to survive target separation and these changes are dependent upon neuromuscular interaction.

Recent evidence suggests that the transition of a motoneuron from a target-dependent to a target-independent state not only requires neuromuscular contact, but also a functional interaction. For example, preventing neuromuscular activity during the early postnatal period by paralyzing muscle with α-bungarotoxin (α-BTX), maintains developing motoneurons in an immature state, such that nerve injury at 5 days of age results in the death of motoneurons that would normally survive (Burls et al, 1991). Furthermore, temporary paralysis of rat soleus muscles shortly after birth results in a significant loss of soleus motoneurons (Greensmith and Vrbová, 1992). The degree of motoneuron death is also dependent upon the length of neuromuscular disruption, so that more motoneurons die after a prolonged paralysis (Greensmith and Vrbová, 1992). In view of the rostro-caudal development of the spinal cord, it has also been shown that following neonatal muscle paralysis, motoneurons located in a more caudal position are more affected by temporary loss of interaction with their muscle fibers, resulting in a larger proportion of motoneuron death (Greensmith et al, 1997). Overall these results indicate that it is not the axonal injury per se that

causes motoneurons to die, but rather the interruption of neuromuscular interaction during a critical period of development that prevents the motoneuron from differentiating into a mature and target-independent state. It is therefore clear that interaction with the muscle is a crucial factor in the development and survival of motoneurons.

The mechanism by which target interaction promotes the development and survival of motoneurons remains unclear. Numerous studies have focused on the possibility that target-derived neurotrophic factors are essential for the maintenance and survival of developing motoneurons (Sendtner et al., 1992; Yan et al., 1992; Henderson et al., 1993). However, there is also evidence to indicate that functional events occurring at the NMJ also play a significant role in motoneuron development (Greensmith and Vrbová, 1996). During the early postnatal period there is a marked elevation in motor activity, which is accompanied by a steep increase in the expression of proteins associated with neuromuscular transmission, such as choline acetyltransferase (ChAT) and acetylcholinesterase (Burt, 1975; O'Brien and Vrbová, 1978; Phelps et al., 1984). Concomitant with this up-regulation in the cholinergic phenotype of the motoneuron, there is a decrease in the levels of proteins associated with axonal growth such as growth-associated protein-43 (GAP-43) and α-tubulin (Caroni and Becker, 1992). Thus, there is a clear switch in the pattern of expression of transmitter and growth related proteins during a period of development that coincides with the time that motoneurons become independent of target contact for their survival. Moreover, this switch in the phenotype of developing motoneurons is dependent not only on target contact, but also on neuromuscular activity, since it fails to take place when neuromuscular interaction is prevented (Caroni and Becker,

1992). It is therefore possible that the role of the target during the early postnatal period is to induce this switch in motoneuron phenotype. Indeed, it is well known that one of the first events that take place when developing nerve terminals first make contact with their muscle fibres is an immediate up-regulation in transmitter release (Chow and Poo, 1985; Xie and Poo, 1986). Therefore, it has been postulated that the transformation of a motoneuron from growing into a transmitting cell is essential for its maturation and survival. If motoneurons fail to undergo this change in phenotype within a critical period of development and remain immature, they also fail to acquire properties that are necessary to survive the increasing demands placed upon them within the developing CNS (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996).

2.1.2. Target interaction and transmitter release

In order to test the hypothesis that interaction with the target during the early postnatal period induces a change in the phenotype of the motoneuron that is essential for motoneuron development and survival, a series of experiments have been carried out where transmitter release from motor nerve terminals has been manipulated and the effects on motoneuron survival examined. The neural potassium channel blocker 4-aminopyridine (4-AP) is a pharmacological agent known to cause an increase in transmitter release at the adult and neonatal NMJ, by enhancing the influx of calcium ions (Ca^{2+}) into the nerve terminal (Lundh, 1978; Thesleff, 1980; Okamoto et al, 1992). Prematurely enhancing transmitter release at the nerve terminals of neonatal rats, by applying 4-AP, induces a proportion of motoneurons to become resistant to target separation, that were otherwise destined to die

(Greensmith et al, 1996). The converse study examined the effect of reducing transmitter release from motor nerve terminals at birth. This was achieved by using a magnesium sulphate ($MgSO_4$) treatment, which is known to antagonise the influx of Ca^{2+} and therefore depress transmitter release (Katz and Miledi 1969; Cooke et al, 1973). It was shown that application of $MgSO_4$ alone caused a large proportion of motoneurons to die. Furthermore, reducing transmitter release rendered a larger number of motoneurons unable to withstand target separation at an age that normally does not result in cell death (Greensmith et al, 2000). It therefore appears that manipulating the levels of transmitter release at the nerve terminals, influences the rate of motoneuron development, which determines whether the motoneuron can survive target separation.

2.1.3. Hypothesis to be tested

Although previous work has shown that manipulating transmitter release from developing nerve terminals affects motoneuron survival, there is presently no evidence to show that motoneuron phenotype is altered. Therefore, the aim of this study is to determine whether pharmacologically manipulating transmitter release from developing motor nerve terminals, alters the normal developmental pattern of proteins associated with growth and transmission. As a marker of the functional state of the motoneuron, the activity of ChAT is measured in the endplate region of both soleus and extensor digitorum (EDL) muscles and in the spinal cord at a number of time-points during normal development and following the manipulation of transmitter release. As a marker of the growth state of the motoneuron, the levels of GAP-43 protein in the sciatic nerve was assessed during normal development and

following the manipulation of transmitter release. The results suggest that by regulating transmitter release, the target determines the developmental pattern of gene expression within motoneurons, and in this way influences the vulnerability of motoneurons to injury-induced cell death.

2.2. METHODS

2.2.1. Surgical procedures

Neonatal Sprague-Dawley rat pups of both sexes were used in this study (Biological Services, University College London, UK). All experiments were carried out under Licence from the Home Office in accordance with The Animals (Scientific Procedures) Act 1986, and Institute of Neurology guidelines. Throughout this study, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Newborn rats were anaesthetised with halothane, and under sterile conditions, a strip of silicon rubber (Dow Corning, 3140 RTV) containing either 4-AP or $MgSO_4$ was inserted into the lateral compartment of the right hindlimb, between that soleus and flexor hallucius longus muscles. This implantation procedure occurred within 3-6 hrs of birth. Control animals were treated with implants that contained sodium chloride (NaCl). After recovery from anaesthesia, the pups were returned to their mother.

2.2.2. Preparation of implants

The implants were prepared as follows: sterile NaCl was finely ground together with either 4-AP or $MgSO_4$. The mixture was added to a silicon rubber solution and mixed thoroughly. This final mixture was allowed to dry and subsequently stored at 4°C. Small strips weighing 0.5 mg were cut from the silicon rubber for implantation into the newborn rats. The final amount of 4-AP and $MgSO_4$ in each silicon strip was approximately 2.4 μg and 2.5 mg respectively. The use of implants such as these has

been previously shown to cause no physical damage to muscle fibres (Connold et al., 1997).

2.2.3. ChAT immunocytochemistry

The effect of treatment with 4-AP or $MgSO_4$ on the levels of ChAT protein in sciatic motoneurons was assessed at various ages during the first 3 postnatal weeks using immunocytochemistry. The animals were terminally anaesthetized (4% choral hydrate; 1ml/100g body weight, i.p.) and perfused transcardially with a fixative solution containing paraformaldehyde (4% in phosphate buffer saline (PBS), pH 7.3). The spinal cords were removed, post-fixed for 3 hours in the same fixative and cryoprotected in sucrose (30% in PBS) overnight at 4°C. The lumbar region of the spinal cord was identified (L3 to L6) and serial 20 μm transverse sections were cut on a cryostat. The spinal cord sections were then stained for ChAT immunoreactivity. The sections were blocked in 3% horse serum for 2 hrs and incubated overnight at 4°C with an anti-ChAT polyclonal antibody (Chemicon; dilution 1:200). Negative control sections, in which the primary antibody was omitted, were also prepared. After thorough washing in PBS with 0.1% Trition-X-100 (PBS-T), the sections were incubated for 2 hrs at room temperature with biotinylated secondary antibodies (dilution 1:100; Vector) and then incubated for 1 hr in Avidin-Biotin complex (ABC; Vector). They were processed using horseradish peroxidase (HRP) as the chromagen and 3.3'-diaminobenzidine (DAB) as the substrate.

2.2.4. ChAT assay

The effect of treatment of motor nerve terminals with 4-AP or $MgSO_4$ on the enzymatic activity of ChAT was measured at various ages during the first three postnatal weeks. ChAT activity was assessed using a sensitive radiochemical procedure in which [1-^{14}C] acetyl-CoA and choline were used as substrates and the radioactivity of the reaction product, [1-^{14}C] acetylcholine was measured (see Fonnum, 1975). Tissue collected from the control and treated animals included the soleus and EDL muscles, as well as the treated side of the ventral spinal cord (L4-L6 segment). Each tissue was collected from at least 3 control and 3 treated animals at each age, pooled, and stored in liquid nitrogen. Homogenates (5% w/v) of the control and treated tissue were prepared in 10 mM ethylenediamine tetra-acetate, pH 7.4, containing 0.5% Triton X-100 to ensure total release of enzyme activity. Ten microlitres of the homogenates were assayed radiochemically. The incubation medium contained (final conc.): 0.4 mM [1-^{14}C] acetyl-CoA (50.0 mCi/mM; Amersham), 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4) and 0.1 mM eserine. The reaction was initiated by mixing each homogenate with twenty-five microlitres of the incubation medium, and transferring this solution to a 37°C water bath for 1 hour. The reaction was terminated by adding 4.5 ml of 10 mM sodium phosphate buffer (pH 7.4, 4°C). All samples were assayed in duplicate including the appropriate blanks. The solution was then emptied into a scintillation vial and 2 ml of acetonitrile containing 0.5% tetraphenylboron (Kalignost) and 10ml of toluene scintillation mixture (0.5% 2,5-diphenyloxazole and 0.02% 1,4-bis[Phenyl-2-oxazolyl]-benzene;2,2-p-Phenylene-bis[phenyloxazole]) was added. The vial was shaken gently for 1 minute to extract the ACh into the toluene phase, leaving the

acetyl-CoA in the aqueous phase. The two layers were allowed to separate for 10 minutes before measurement in a Beckman liquid scintillation spectrometer. Enzymatic activity was calculated as disintegrations per minute (DPM) of C^{14}. The assay was repeated to ensure that similar and reproducible pattern of ChAT activity was obtained for each experimental condition.

2.2.4. Gel electrophoresis and immunoblotting

The effect of treatment with either 4-AP or $MgSO_4$ on the developmental expression of total GAP-43 was assessed by Western blot analysis. For each time point, the sciatic nerves on the treated side from at least 3 treated and 3 control animals were pooled. One per cent (w/v) homogenates were prepared in 2% (w/v) SDS, 2 mM EDTA, 2 mM EGTA, 5 mM Tris pH 6.8 and 0.01% PMSF. Debris was removed by low speed centrifugation (3000 RPM for 3 minutes) and the supernatant stored at -80 °C. The protein concentration of the supernatant was determined using a Coomassie-based assay (Bio-Rad), with the absorbance measurements made at 750 nm. BSA was used as the standard (Sigma). Samples containing 100 μg of protein were diluted 1:1 in SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% (wt/vol) SDS, β-mercaptoethanol and 1.0% bromophenol blue), heated at 95°C for 3 minutes and electrophoresed using a 12% acrylamide SDS gel (Laemmli, 1970). Proteins were then electroblotted onto nitrocellulose membrane using a buffer consisting of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. For immunodetection, non-specific binding sites were firstly blocked by immersing the membrane in a blocking reagent (5% (wt/vol) nonfat milk, 1% (v/v) rabbit serum in phosphate buffered saline/0.1% Triton (PBS-T) overnight at 4°C. For GAP-43 detection, a monoclonal

antibody (9-1, E12; Boehringer) was added at a dilution of 1:2000 in PBS-T and the membrane incubated for 2 hours. The blots were washed three times in PBS-T and incubated for 1 hour in the presence of a secondary antibody conjugated to horseradish peroxidase (Dako, 1:1000 dilution), and visualised using chemiluminescent detection reagents (ECL, Amersham). The blots were exposed to x-ray film until the appropriate band density was visualised. Each blot was repeated at least twice to ensure that a reproducible pattern for the levels of GAP-43 was obtained, and semi-quantitative analysis carried out using an Image Analysis system (KS300, Imaging Associates, UK).

2.2.5. Motoneuron survival

The effect of treating hindlimb muscles with $MgSO_4$ at birth on the survival of motoneurons was assessed in rats of 10 and 21 days of age. In 10 day old rats, the survival of motoneurons within the sciatic motor pool was assessed by counting the number of Nissl-stained (gallocyanin) motoneurons. This method has previously been used to count motoneurons within the sciatic pool (White et al, 2000). In 21 day old rats, in addition to counting the number of Nissl-stained motoneurons, the survival of motoneurons innervating the soleus muscle was established by retrograde transport with horseradish peroxidase (HRP). In rats at this age, injections of HRP can be made reliably into both the untreated and as well as $MgSO_4$ treated soleus muscles.

Assessing the number of Nissl-stained motoneurons in the sciatic motor pool

The animals were terminally anaesthetized (4% choral hydrate; 1ml/100g of body weight, i.p.) and transcardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The spinal cords were removed, and the lumbar enlargement post-fixed in the same fixative for 4 h, and cryoprotected in sucrose (30% in 0.1 M PBS) for 12 h at 4°C. The entire lumbar spinal cord (L1-L6) was fixed vertically onto a cryostat chuck and coronal, serial sections (20 μm) were mounted onto gelatinized slides and stained for Nissl (gallocyanin). The number of Nissl-stained motoneurons in every 3rd section within the sciatic motor pool in both the treated and untreated ventral horns was counted.

Criteria for counting sciatic motoneurons

The sciatic motor nerve in the rat is made up of a number of individual nerve branches (peroneal, tibial, sural, and the medial and lateral gastronemius nerves), which innervate the muscles of the lower limb. Comprehensive mapping of the localization of motor nuclei supplying the hindlimb muscles of the rat has been performed using retrograde transport of HRP (Nicolopoulos-Stournaras and Iles, 1983; Swett et al, 1986). These authors found that motor nuclei of the sciatic nerve are present in the dorsolateral portion of the ventral horn, forming a longitudinal column starting at L3 and terminating at the L5-L6 boundary. In addition, the total number of sciatic motoneurons was found to be approximately 2000 (Swett et al, 1986).

Using these detailed descriptions as guide for the location of the sciatic motor pool, motoneuron survival was assessed by counting the number of Nissl-stained sciatic motoneurons in both ventral horns. Only the large polygonal neurons with a clear nucleolus and a distinctly labeled cytoplasm were incorporated into the counts. These criteria are likely to result in an underestimation of the total sciatic population, since small gamma motoneurons innervating intrafusal muscle fibres are likely to be omitted. However, these criteria would also prevent interneurons from being incorporated, especially when the sciatic motor pool extends more medially within the ventral horn. The criteria for defining the start of the sciatic motor pool in the L3 spinal segment was the appearance of a small number of Nissl-stained motoneurons in the dorsolateral portion of the ventral horn. These motoneurons correspond to the most rostral portion of the peroneal motor column. Motoneurons in the caudal region of the L2 spinal cord are mostly located in the ventrolateral region of the spinal cord and acts as a marker for the area just prior to the start of the sciatic motor pool.

As the sciatic pool extends caudally, the motor nucleus of the tibial nerve starts in a more medial position at the L3-L4 boundary relative to the peroneal nucleus. Both the peroneal and tibial nuclei maintain this position in the dorsolateral portion of the ventral horn and become maximally developed in L4 and L5 respectively. The lateral gastronemius (LG) nucleus appears in mid-L4 and extends to mid-L5, and occupies an area more ventral to that of the tibial nerve. The medial gastronemius nucleus is located in a more lateral position relative to the LG and extends from mid-L5 to rostral L6. Finally, the sural nucleus is confined to L5 and is widely scattered in the same region as the tibial nucleus.

When assessing sciatic motoneuron number using this method, a potential source of error may come from incorporating hamstring motoneurons into the counts, since they are also present in the L4 and L5 segments. Fortunately, the majority of hamstring motoneurons are more ventrally located relative to the sciatic motor pool, however there is some degree of overlap. In order to reduce this source of error, where the sciatic motor pool is not clearly defined, only those motoneurons in the dorsolateral position within the ventral horn are counted. This method is likely to omit hamstring motoneurons and also some gastronemius motoneurons, which may result in an underestimation of the total number of sciatic motoneurons.

Finally, the criteria for defining the end of the sciatic motor pool, is the significant reduction of motoneurons present in the ventral horn and the appearance of a small tightly defined motor pool in an extreme dorsolateral position. These motoneurons are present in the L6 segment and form the plantar nerves, which innervate the intrinsic muscles of the foot and were not incorporated into the counts.

Overall, using this method does not result in an accurate assessment of total sciatic motoneuron number, rather only a estimate of the proportion of sciatic motoneurons surviving in the treated/operated ventral horn compared to the contralateral ventral horn.

Retrograde labeling of motoneurons

In 21 day old rats, in addition to counting the number of Nissl-stained motoneurons, the survival of motoneurons innervating the soleus muscle was also established by

retrograde labeling with horseradish peroxidase (HRP). In rats at this age, injections of HRP can be made reliably into both the untreated as well as $MgSO_4$ treated soleus muscles. Under halothane anaesthesia and sterile conditions, HRP (Type VI; Sigma) was injected into the exposed soleus muscles of both hindlimbs using a Hamilton microsyringe (2 μl of 15% HRP solution per 100g body weight). Twenty-four hours later the animals were terminally anaesthetized (4% choral hydrate; 1ml/100g body weight, i.p.) and perfused transcardially with a fixative solution containing glutaraldehyde (2.5% in Millonigs phosphate buffer (MPB), pH 7.3). The spinal cords were removed, post-fixed for 3 hours in the same fixative and cryoprotected in sucrose (30% in MPB) overnight at 4°C. The lumbar region of the spinal cord was identified and the spinal cord cut at L3 and L6. A fine micropin was inserted into the left dorsal horn along the entire length of this segment in order to identify the control side. Serial 30 μM transverse sections were cut on a freezing microtome and collected in a staining tray immersed in MPB. The free-floating sections were processed for HRP histochemistry using a modified Hanker-Yates method (see Appendix 1; Hanker et al, 1977). The sections were then mounted onto gelatinized slides and lightly counterstained with a Nissl-stain (gallocyanin). The HRP-labeled motoneurons in each ventral horn were counted under a light microscope. For both Nissl and HRP-labeled motoneurons, in order to prevent counting the same cell twice in consecutive sections, only those motoneurons in which the nucleolus was clearly visible under higher magnification (x 40) were included in the counts. As an index of motoneuron survival, the number of HRP and Nissl-stained motoneurons on the operated side of each ventral horn was expressed as a percentage of the number on the control side.

2.2.6. Statistical analysis

The results from these experiments were analyzed using the Mann-Whitney U test for comparison of independent samples. Two-tailed tests were used in all instances, and significance was set at $P<0.05$.

2.3. RESULTS

2.3.1. Developmental pattern of ChAT immunoreactivity in motoneurons

Motoneurons within the lumbar spinal cord at the level of the sciatic pool (L4-L6) were processed for ChAT immunoreactivity at various postnatal ages. Figure 1 shows examples of spinal cord sections immunostained for ChAT at different developmental ages. As can be seen in Figure 1, motoneurons located within the sciatic motor pool at postnatal day 1 (P1) displayed a low level of ChAT immunoreactivity within their somata and dendrites. By P5, the level of ChAT-positive staining was qualitatively higher in sciatic motoneurons. The intensity of ChAT staining in motoneurons continued to increase after P5, and by P14 motoneurons stained intensely for ChAT. There was no detectable increase in ChAT immunoreactivity from P14 to P21, which indicates that adult levels of ChAT in sciatic motoneurons are reached by the second postnatal week.

2.3.2. ChAT activity in muscle and spinal cord during normal development

The activity of ChAT was assayed in normal soleus and EDL muscles and the ventral spinal cord of rats aged between 1 and 21 days. The results are summarized in Figure 2. ChAT activity in the soleus muscle (Fig. 2A) remained low until postnatal day 7 (P7) but increased significantly between P7 and P12, when ChAT activity reached the maximum level observed during the 3 week period studied. Thus, within the first 2 weeks of postnatal development there is a 2-fold increase in ChAT activity in the soleus muscle. This finding is consistent with previous results (Wong et al, 1999).

Figure 1. Photomicrographs of the normal development of ChAT immunoreactivity in motoneurons

The photomicrographs show representative transverse sections (20 μm) of the ventral horn of the lumbar spinal cord processed for ChAT immunoreactivity. The expression of ChAT was assessed between 1 and 21 days of development. The developmental pattern of ChAT immunoreactivity at P1, P5, P10, P14, and P21, is shown in **A-E,** respectively. In newborn rats (P1), motoneurons located within the sciatic motor pool of the lumbar spinal cord showed low levels of ChAT immunoreactivity within their somata and dendrites. At P5 (**B**), the level of ChAT immunoreactivity in motoneurons was qualitatively higher than seen at P1 (**A**). By P21 (**E**), motoneurons were intensely ChAT positive. Scale bar = 40 μm.

Figure 1

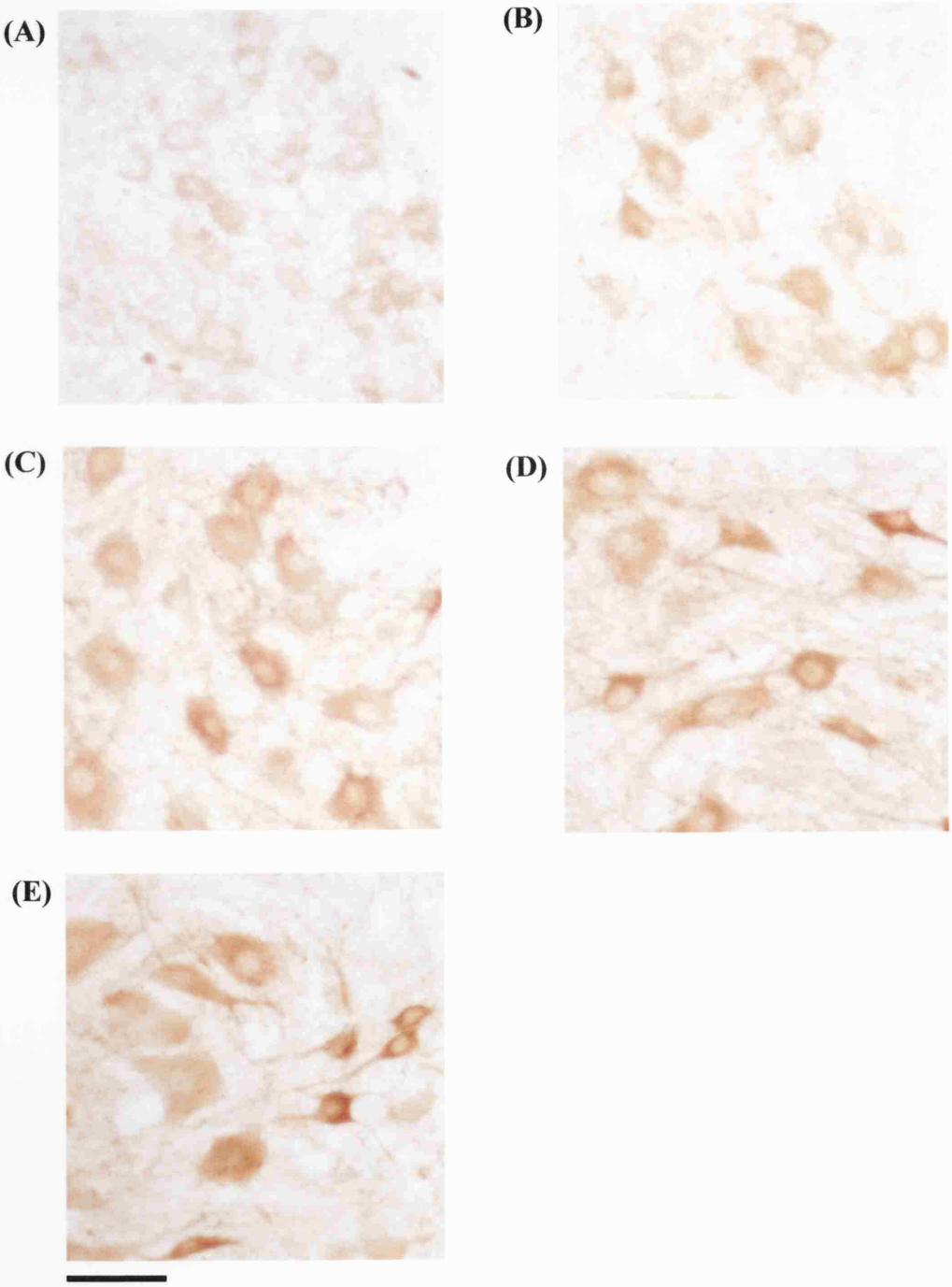
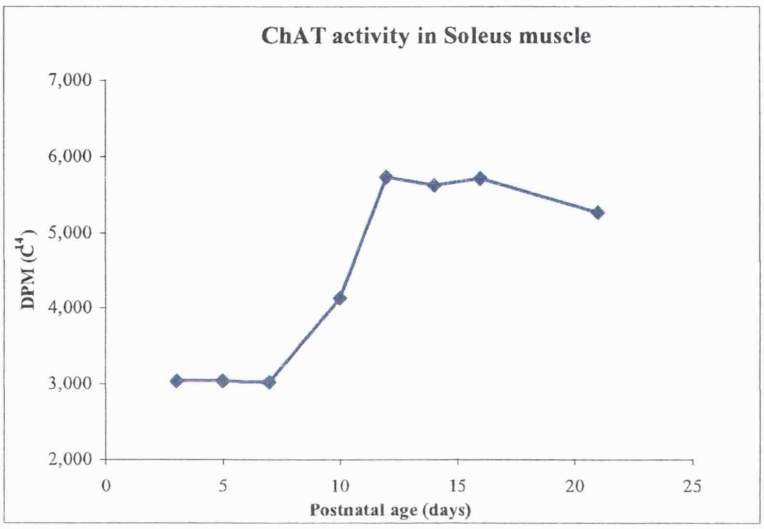


Figure 2. ChAT activity in muscle and spinal cord during normal development

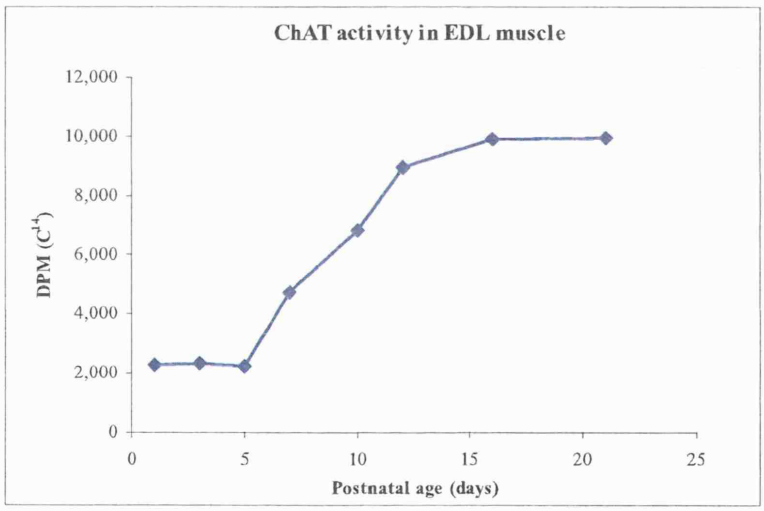
The graphs show the activity of ChAT [disintegrations per minute (DPM) of C^{14}] in (A) soleus muscles, (B) extensor digitorum longus (EDL) muscles and (C) ventral spinal cords (L4-L6) of normal rats at various ages during the first 3 weeks of postnatal development. For each tissue, samples from at least 3 animals were removed, pooled at each of the ages, and the samples assayed using a radiochemical assay. Each assay was run in duplicate, and the average DPM of C^{14} taken for each age.

Figure 2

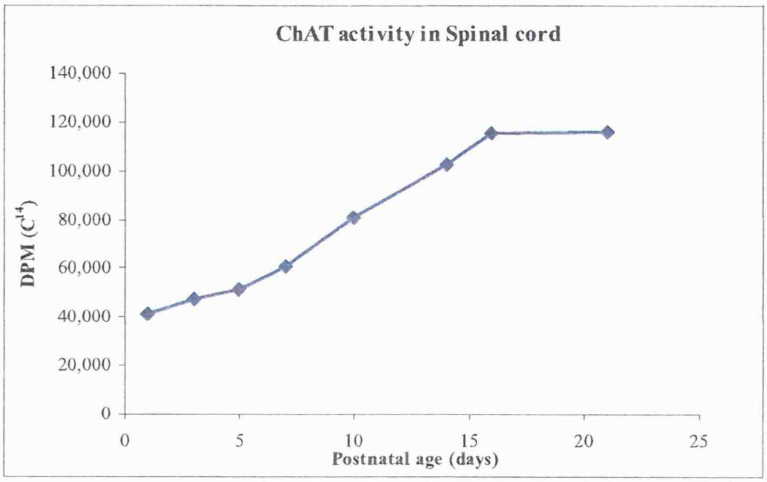
(A)


ChAT activity in Soleus muscle
DPM (C^{14})
7,000
6,000
5,000
4,000
3,000
2,000
0
5
10
15
20
25
Postnatal age (days)

(B)


ChAT activity in EDL muscle
DPM (C^{14})
12,000
10,000
8,000
6,000
4,000
2,000
0
0
5
10
15
20
25
Postnatal age (days)

(C)


ChAT activity in Spinal cord
DPM (C^{14})
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
0
5
10
15
20
25
Postnatal age (days)

The activity of ChAT in the EDL muscle (Fig. 2B) followed a similar time course to that observed in soleus, although overall levels of ChAT activity were greater. In addition, although the initial increase in ChAT activity occurred earlier in EDL, at P5, the maximum level of ChAT was not reached until P16. Thereafter, ChAT activity remained high. Thus, in EDL there is a 5-fold increase in ChAT activity within the first 2 weeks of postnatal development. ChAT activity in the ventral horn (Fig. 2C) was significantly greater than that observed in either soleus or EDL, and activity increased steadily during the first 3 weeks of postnatal development.

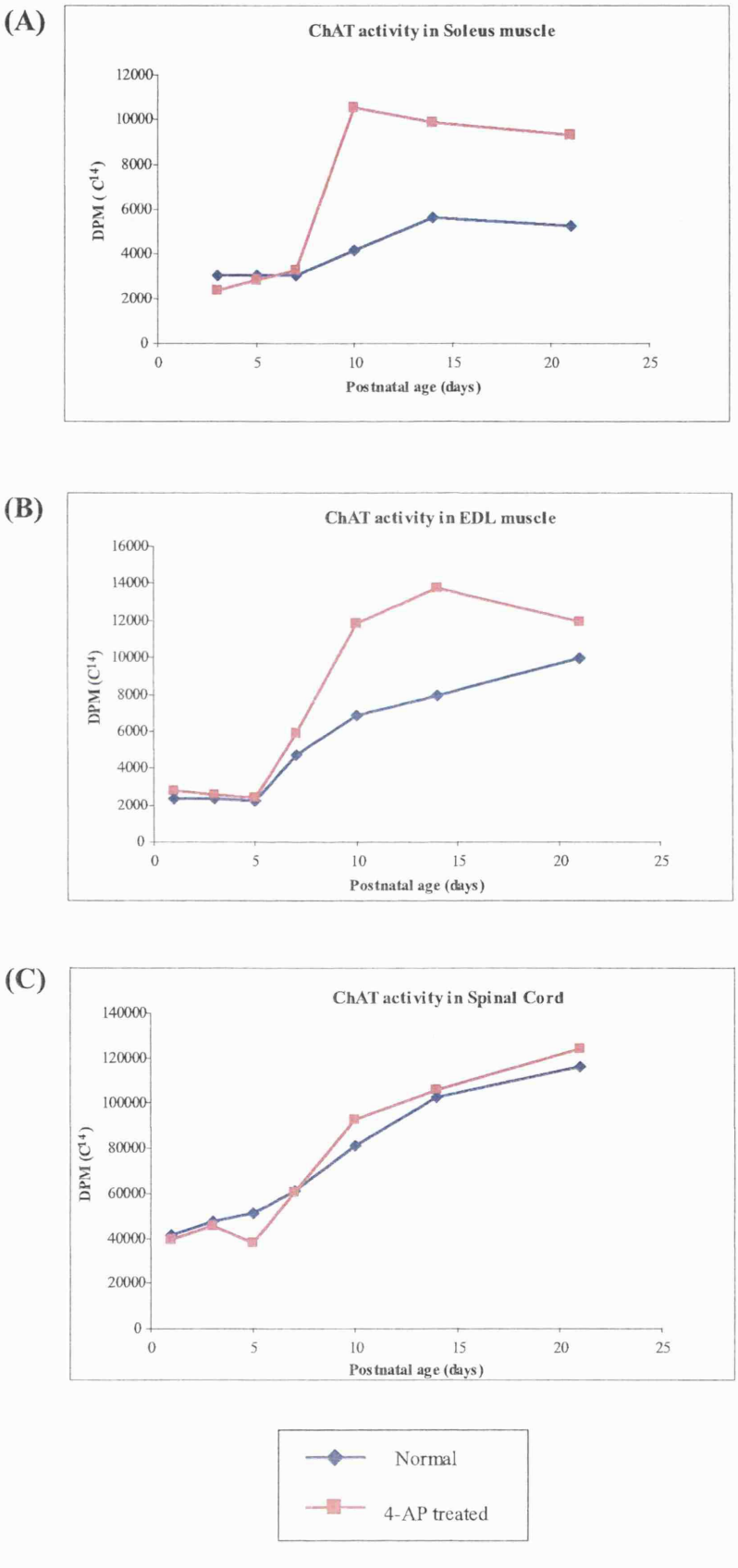
2.3.3. The effect of treatment with 4-aminopyridine on the activity of ChAT in muscle and spinal cord

The activity of ChAT was assayed in 4-aminopyridine (4-AP) treated soleus and EDL muscles and the ventral spinal cord of rats aged between 1 and 21 days. The results are summarized in Figure 3. Treatment of the hindlimb muscles with 4-AP during the first days of life had a marked effect on ChAT activity in both the soleus and EDL muscles (Fig. 3, A and B). For the first few days following treatment with 4-AP, ChAT activity in both soleus and EDL is at a similar level to that observed in untreated muscles. However, after P7 in 4-AP treated soleus and P5 in EDL muscles, ChAT activity increased dramatically to levels far in excess of normal. This effect was more pronounced in the soleus muscle than the EDL. Thus, between P7 and P10 there was a rapid 5-fold increase in ChAT activity in the 4-AP treated soleus compared to only a marginal increase in normal muscles during the same period. Even 3 weeks after the initial treatment of soleus muscles with 4-AP, the level of ChAT activity was over 2-fold greater in 4-AP treated soleus compared to normal. In

Figure 3. The effect of treatment with 4-AP on ChAT activity in muscle and spinal cord

The graphs show the activity of ChAT [disintegrations per minute (DPM) of C^{14}] in muscle and spinal cord collected from 4-aminopyridine (4-AP; K^+ channel blocker) treated rats, as compared to normal untreated rats. The treated animals received a silicone strip containing 4-AP, which was implanted into one hindlimb at birth (P0). The soleus and extensor digitorum longus (EDL) muscles from at least 3 animals were removed from the treated hindlimb and each muscle type pooled at each of the ages. For the spinal cord, only the ventral horn on the treated side (L4-L6) from at least three animals was removed and then processed in the same way as the muscles. Each assay was run in duplicate, and the average DPM of C^{14} taken for each age.

Figure 3


(A)
ChAT activity in Soleus muscle
DPM (C^{14})
Postnatal age (days)
(B)
ChAT activity in EDL muscle
DPM (C^{14})
Postnatal age (days)
(C)
ChAT activity in Spinal Cord
DPM (C^{14})
Postnatal age (days)
Normal
4-AP treated

addition, the age at which the maximum level of ChAT activity was reached, was earlier in 4-AP treated soleus muscles compared to normal, so that activity peaked at P10 in 4-AP treated muscles compared to P14 in normal muscles.

As can be seen in Figure 3B, in 4-AP treated EDL muscles, ChAT activity increased dramatically between P5 and P14, reaching levels far in excess of normal. Thus, by P10, there was a 6-fold increase in ChAT activity in 4-AP treated EDL muscles compared to a 3-fold increase in normal muscles. This increase in ChAT activity in 4-AP treated EDL muscles was sustained until P14, after which ChAT activity began to decline towards normal by P21.

In contrast, inserting a silicon implant containing 4-AP into the hindlimb, had no effect on overall ChAT activity in the ventral spinal cord on the treated side compared to ChAT activity in normal ventral spinal cord (Fig. 3C). This result indicates that 4-AP released from the implant primarily affected those muscles situated in close proximity to the implant. Thus, many of the hindlimb muscles, which are innervated by motoneurons located in the lumbar spinal cord (L4-L6), were unaffected by the treatment, which may have masked the increases in ChAT activity seen in the soleus and EDL muscles.

2.3.4. The effect of treatment with magnesium sulphate on the activity of ChAT in muscle and spinal cord

The activity of ChAT was assayed in magnesium sulphate ($MgSO_4$) treated soleus and EDL muscles and the ventral spinal cord of rats aged between 1 and 21 days.

The results are summarized in Figure 4. In contrast to the effect of 4-AP on ChAT activity, treatment of the hindlimb muscles with $MgSO_4$ at birth resulted in a reduction in ChAT activity in both soleus and EDL muscles during the first 3 weeks of postnatal development (Fig. 4, A and B). This effect was far more pronounced for soleus muscle than EDL muscle. Thus, at the earliest time point measured (P3), ChAT activity in $MgSO_4$ treated soleus muscle was substantially lower than normal. Furthermore, this reduced level of ChAT activity in $MgSO_4$ treated soleus muscles was present at all ages during the first 3 postnatal weeks, such that a similar reduction in ChAT activity was present at P21 as that seen at P3.

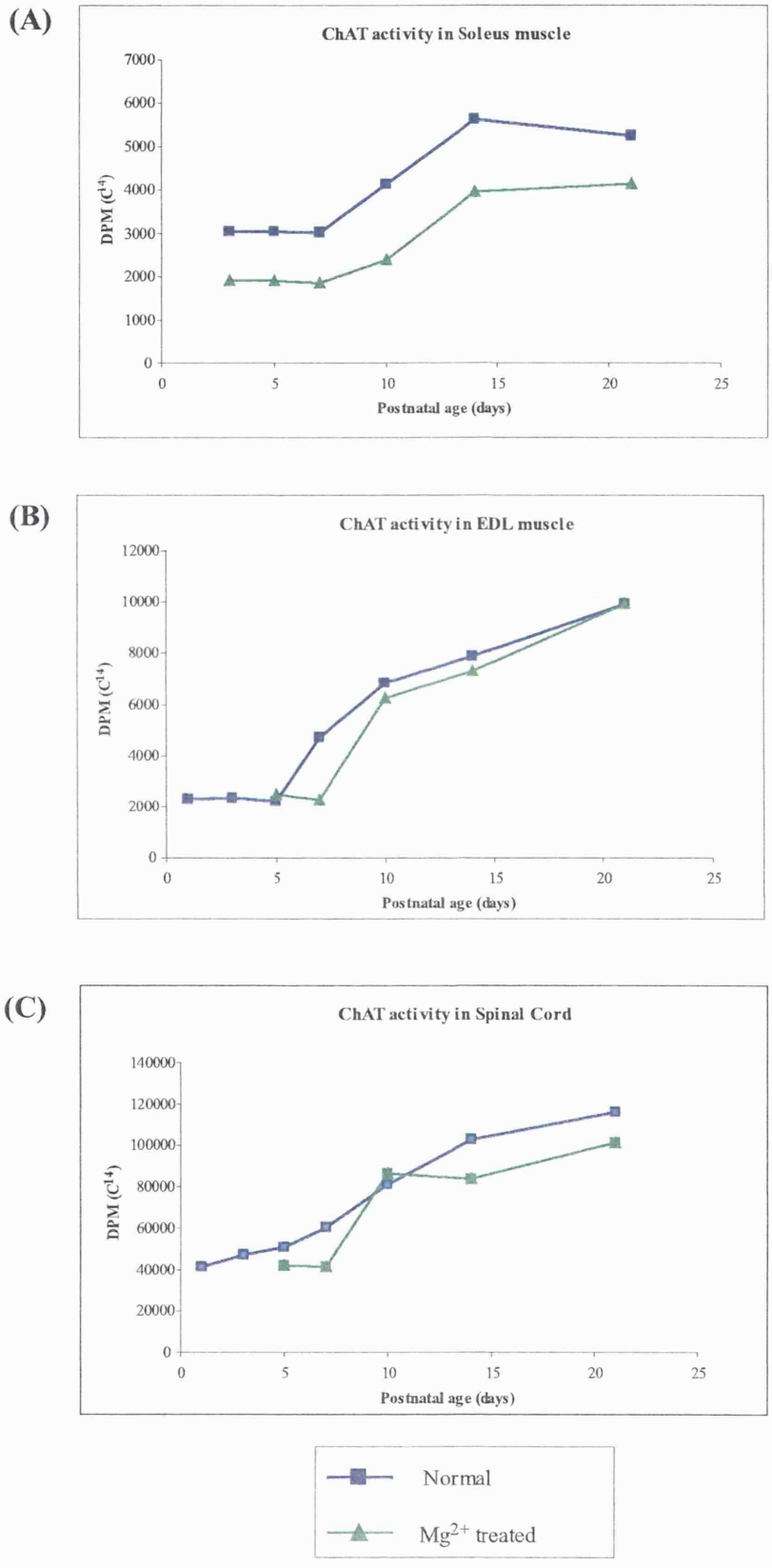
In comparison to the soleus muscle, the effect of $MgSO_4$ treatment on ChAT activity in EDL muscle was less significant (Fig.4B). Thus, at P5, there was no difference in ChAT activity between $MgSO_4$ treated and normal EDL muscles. In normal EDL muscles, there is a substantial increase in ChAT activity between P5 and P7. However, following $MgSO_4$ treatment, this increase is suppressed and no rise in ChAT activity is seen in EDL muscles. This delay in the increase in ChAT activity is only transient, since after P7, ChAT activity in $MgSO_4$ treated EDL muscles increases dramatically to reach near normal levels by P10.

Finally, the effect of treating hindlimb muscles on ChAT activity in ventral horn neurons (L4-L6) was assessed at various time ages during the first 3 postnatal weeks. It can be seen in Figure 4C, that ChAT activity in the ventral horn on the treated side was slightly lower than normal between P5 and P7, but increased dramatically to normal levels by P10. However, between P10 and P21, ChAT activity in the treated

Figure 4. The effect of treatment with $MgSO_4$ on ChAT activity in muscle and spinal cord

The graphs show the activity of ChAT [disintegrations per minute (DPM) of C^{14}] in muscle and spinal cord collected from magnesium sulphate ($MgSO_4$; Ca^{2+} channel blocker) treated rats, as compared to normal untreated rats. The treated animals received a silicone strip containing $MgSO_4$, which was implanted into one hindlimb at birth (P0). The soleus and extensor digitorum longus (EDL) muscles from at least 3 animals were removed from the treated hindlimb and each muscle type pooled at each of the ages. For the spinal cord, only the right side of ventral horn (L4-L6) from at least three animals was removed and then processed in the same way as the muscles. Each assay was run in duplicate, and the average DPM of C^{14} taken for each age.

Figure 4


(A)
ChAT activity in Soleus muscle
DPM (C^{14})
Postnatal age (days)
(B)
ChAT activity in EDL muscle
DPM (C^{14})
Postnatal age (days)
(C)
ChAT activity in Spinal Cord
DPM (C^{14})
Postnatal age (days)
Normal
Mg^{2+} treated

ventral horn failed to increase to the same extent as normal, and ChAT activity remained lower than normal during this developmental period.

2.3.5. Developmental changes in GAP-43 levels in the sciatic nerve during normal development

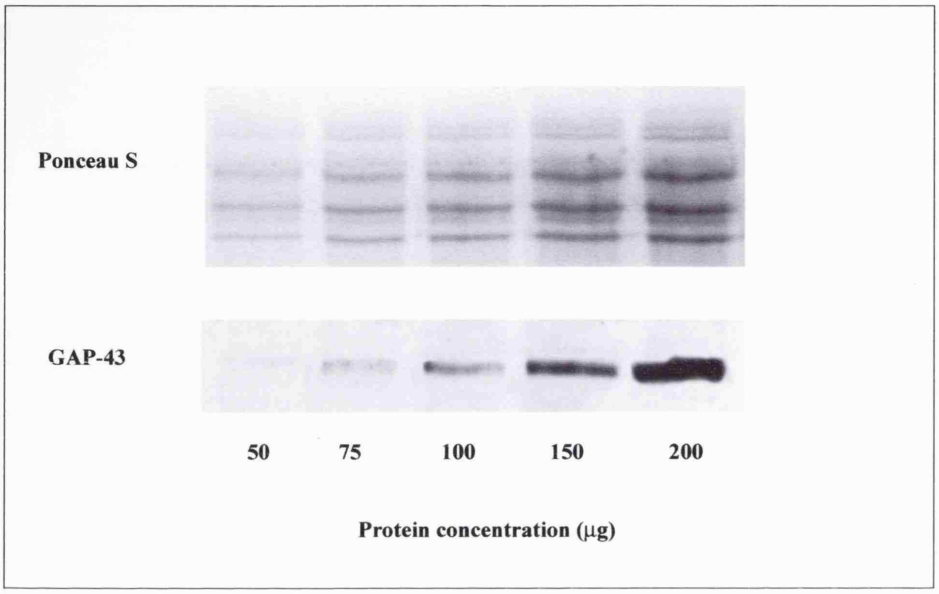
GAP-43 levels in sciatic nerves from normal animals were assessed at various ages during the first 3 weeks of postnatal development, using Western blot analysis. First, preliminary experiments were carried out to establish the relationship between band pixel density and protein concentration in immunoblots, using an anti-GAP-43 monoclonal antibody (9-1, E12; Boehringer). A range of protein concentrations (50-200 μg) of normal sciatic nerve were run on SDS/PAGE and transferred to nitrocellulose. To ensure the correct running of the gel and protein transfer, the nitrocellulose membrane was stained with Ponceau S in order to visualize all protein bands (Fig. 5A). Next, the membrane was rinsed in PBS-T to remove the ponceau S stain and probed with an antibody to GAP-43. The GAP-43 antibody was found to react specifically with a unique band that migrates at 48 KDa (Fig. 5A), which is similar to the molecular mass of GAP-43. In addition, this monoclonal antibody has previously been shown to provide a reliable and specific indicator of total GAP-43 abundance (Skene and Virag, 1989). The GAP-43 band density was quantified using an image analysis system, which digitized the GAP-43 bands, and measured the pixel density. The dose-response curve in Figure 5B shows a linear relationship between the pixel density of GAP-43 bands and the amount of total protein, within the range of 50-200 μg.

Figure 5. Linear range of responses of the Western blot for GAP-43

(A) Normal rat sciatic nerve samples containing 50, 75, 100, 150, 200 μg of total protein, were separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Transferred proteins were visualized using Ponceau S and then specifically probed with an anti-GAP-43 monoclonal antibody. (B) Densitometric quantification of the band pixel density corresponding to GAP-43 showed linear responses between 50 and 200 μg of total protein loaded.

Figure 5

(A)


Ponceau S
GAP-43
50
75
100
150
200
Protein concentration (µg)

(B)

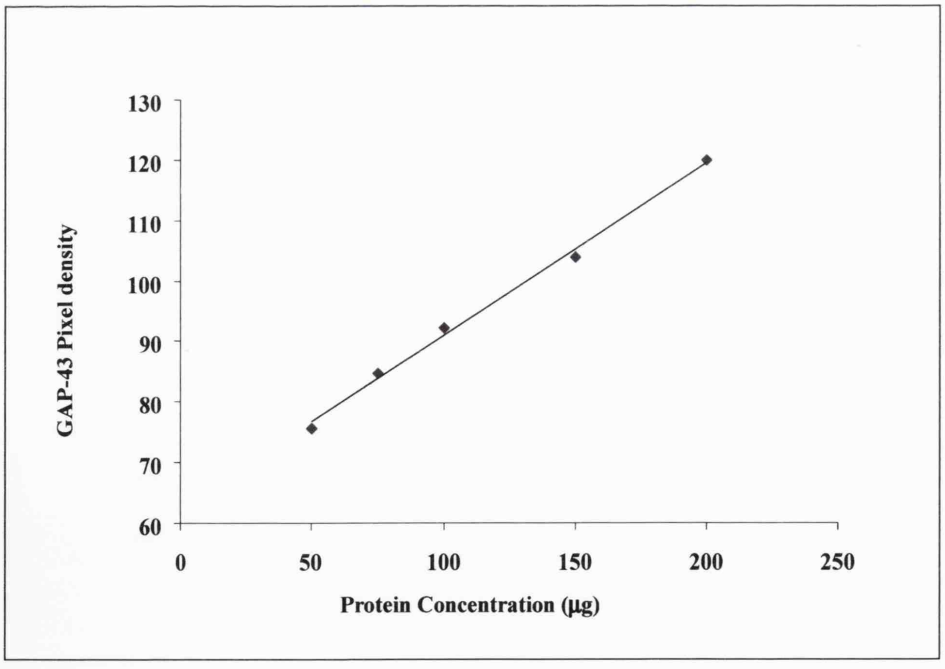
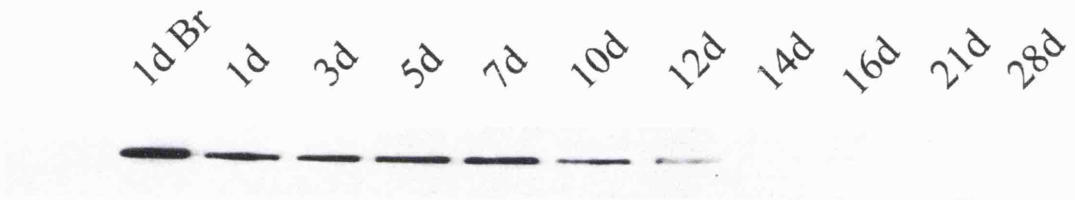

GAP-43 Pixel density
130
120
110
100
90
80
70
60
0
50
100
150
200
250
Protein Concentration (µg)

Figure 6. Western blot analysis of GAP-43 levels in normal sciatic nerve

The Western blot show levels of GAP-43 in sciatic nerve from normal rats aged between 1 and 28 days. Sciatic nerves from at least 3 animals were pooled at each age, homogenized, and the proteins separated on 12% SDS-polyacrylamide gel (100 μg per lane). Proteins were then transferred onto nitrocellulose membrane and probed with a GAP-43 monoclonal antibody. Western blots were run in duplicate to confirm the result. Neonatal rat brain (1d Br; 100 μg) was used as a positive control since it is known to contain high levels of GAP-43 (Skene et al, 1986).

Figure 6



The levels of GAP-43 in sciatic nerves of rats aged between 1 and 28 days of age was determined by Western blot analysis and an example of such a blot is shown in Figure 6. Neonatal rat brain was used as a positive control, since it contains high levels of GAP-43 (Skene et al, 1986). It can be seen that GAP-43 is present at a high level in sciatic nerves at P1, and remains at a relatively high level until P7. Between P7 and P12 there is a marked decline in GAP-43 levels. After P12, GAP-43 protein was undetectable.

2.3.6. The effect of treatment with 4-AP and $MgSO_4$ on GAP-43 levels in sciatic nerve

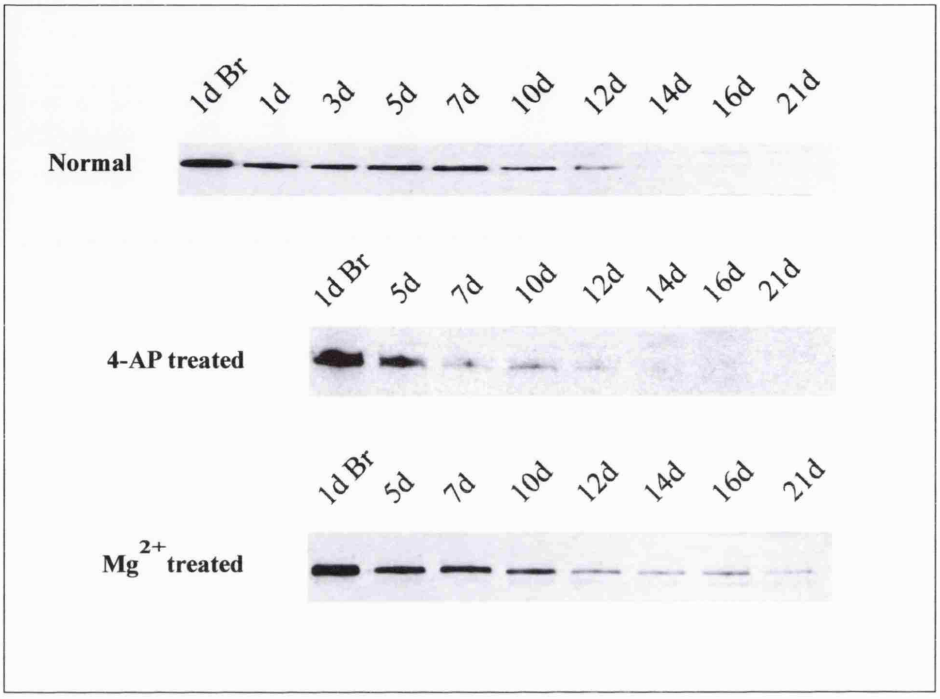
The effect of treatment with either 4-AP or $MgSO_4$ on GAP-43 levels in the sciatic nerve was examined next. As can be seen from the Western blot in Figure 7A, treatment with 4-AP had a marked effect on the developmental levels of GAP-43 in the sciatic nerve. As previously discussed, GAP-43 levels in normal sciatic nerve are relatively high between P1-P7, and decline to low levels by P12. In contrast, following 4-AP treatment the decline of GAP-43 levels occurs at a much earlier developmental age, such that GAP-43 levels declined rapidly after P5 and was barely detectable at P7. The bar graph in Figure 7B shows the densiometric quantification of the GAP-43 bands, which are represented as a percentage of the GAP-43 levels in neonatal brain, which is taken as 100%. It can be seen that following 4-AP treatment the level of GAP-43 in sciatic nerve declines from 80% at P5 compared to that of the control, to 30% by P7. In normal sciatic nerve GAP-43 levels are maintained at over 75% during the first 10 postnatal days, before declining to 40% at P12. Thus, in 4-

Figure 7. Western blot analysis of GAP-43 levels in 4-AP and $MgSO_4$ treated sciatic nerve

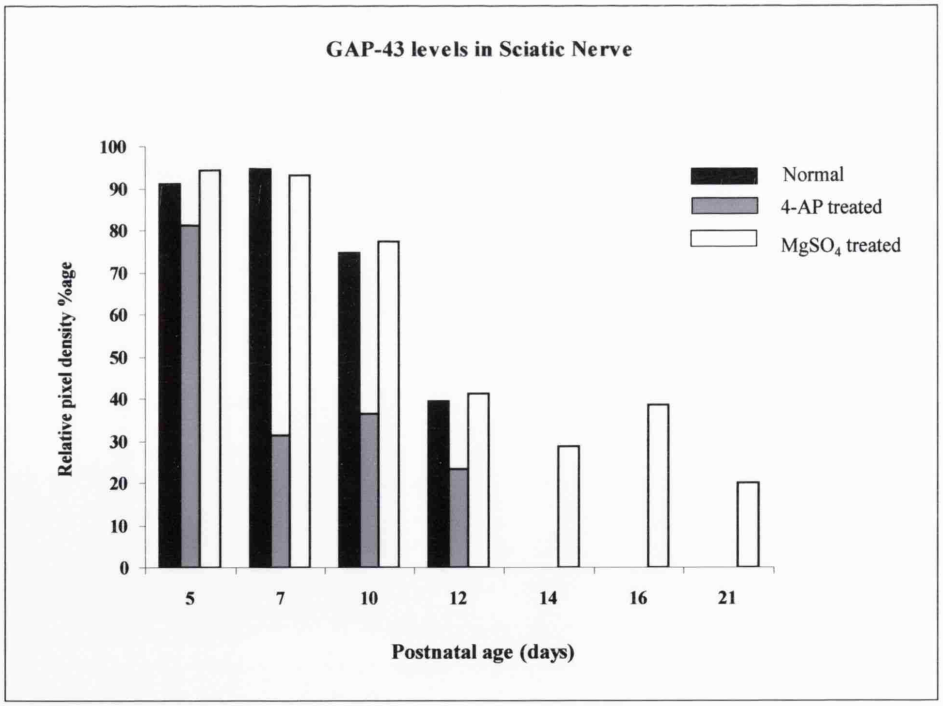
(A) The Western blots show levels of GAP-43 in sciatic nerve from normal rats and rats whose hindlimb muscles had been treated with 4-AP or $MgSO_4$. Sciatic nerves on the treated side from at least 3 treated and 3 control animals were pooled at each age, homogenized, and separated on 12% SDS-polyacrylamide gel (100 μg per lane). Proteins were then transferred onto nitrocellulose membrane and probed with a GAP-43 monoclonal antibody. Western blots were run in duplicate to confirm the result. Neonatal rat brain (1d Br; 100μg) was used as a positive control since it is known to contain high levels of GAP-43 (Skene et al, 1986). (B) Densitometric quantification of the Western blots for GAP-43 in sciatic nerve of normal (black bars), 4-AP treated (shaded bars), and $MgSO_4$ treated (open bars) animals. The values for GAP-43 at each age are plotted as percentages relative to the GAP-43 levels of neonatal brain, which are defined as 100%.

Figure 7

(A)


1d Br
1d
3d
5d
7d
10d
12d
14d
16d
21d
Normal
1d Br
5d
7d
10d
12d
14d
16d
21d
4-AP treated
1d Br
5d
7d
10d
12d
14d
16d
21d
Mg2+ treated

(B)


GAP-43 levels in Sciatic Nerve
Relative pixel density %age
100
90
80
70
60
50
40
30
20
10
0
5
7
10
12
14
16
21
Postnatal age (days)
Normal
4-AP treated
MgSO4 treated

AP treated animals there is a premature reduction in GAP-43 levels in the sciatic nerve.

In contrast, as can be seen in Figure 7, treatment of the hindlimb muscles with $MgSO_4$ prolonged the duration for which GAP-43 was present in sciatic nerve. Thus, between P14 and P21, GAP-43 levels in $MgSO_4$ treated sciatic nerve remained detectable, whereas GAP-43 protein in normal sciatic nerve was undetectable after P12. This result indicates that $MgSO_4$ treatment prolonged the presence of GAP-43 in a proportion of sciatic motoneurons.

2.3.7. Motoneuron survival following treatment with $MgSO_4$

A previous study has shown that treatment of motor nerve terminals with $MgSO_4$ results in the death of developing motoneurons (Greensmith et al, 2000). However, in these experiments, motoneuron survival was only assessed 10 weeks after treatment. The results of the present study show that $MgSO_4$ treatment results in a decrease in ChAT activity in soleus and EDL muscles for up to 3 weeks after treatment. In order to establish whether the decrease in ChAT activity in $MgSO_4$ treated muscles is due to a real reduction in enzymatic activity of ChAT or to a loss of motoneurons, the effect of $MgSO_4$ treatment on motoneuron survival was examined during the period of development under study. Therefore, following treatment of the hindlimb muscles with $MgSO_4$ at birth, motoneuron survival was assessed in rats of 10 and 21 days of age. In 10 day old rats, motoneuron survival was established by counting the number of Nissl-stained motoneurons in the sciatic motor pool in the ventral horn on both the treated and contralateral control sides under a light microscope. Motoneuron survival

was also assessed in 21 day old rats, by counting Nissl-stained motoneurons as well as retrograde labeled motoneurons innervating the soleus muscle. Retrograde labeling of motoneurons is reliable and reproducible in animals of this age, even after treatment of muscle with implants as used in this study. The number of HRP and Nissl-stained motoneurons in both the treated and contralateral ventral horns was counted.

An example of a spinal cord section from a 21 day old rat, treated with $MgSO_4$ at birth, and processed for HRP histochemistry, is shown in Figure 8. The results of motoneuron survival as assessed by counting Nissl-stained motoneurons are summarized in Table 1, which shows that treatment with $MgSO_4$ at birth does not result in motoneuron death in animals of 10 days of age. Thus, the number of Nissl-stained motoneurons in the ventral horn of the treated side was 99% (± 1.3 S.E.M n=4) of that in the contralateral control side (NS, $P>0.05$). However, in animals of 21 days of age treated with $MgSO_4$ at birth, there were significantly fewer motoneurons in the treated ventral horn compared to the contralateral side. Thus, in animals of 21 days of age only 85% (± 1.7 S.E.M n=4) of Nissl-stained motoneurons survived following $MgSO_4$ treatment ($P<0.05$). Moreover, as can be seen in Table 2, there was a greater loss of retrograde labeled motoneurons that innervated the soleus muscle. Thus, only 56% (± 4.7 S.E.M n=3) of HRP-labeled motoneurons in the soleus motor pool survived following $MgSO_4$ treatment ($P<0.05$). In control rats, which were treated with implants containing NaCl, there was no loss of motoneurons and 98% (± 3.7 S.E.M n=4) of HRP-labeled motoneurons survived (NS, $P>0.05$).

Figure 8. Photomicrograph of an adult spinal cord section from a $MgSO_4$ treated rat

(A) The photomicrograph shows a transverse section of a spinal cord from a 21 day old rat in which the right hindlimb was treated with $MgSO_4$ at birth. The control side was identified by the insertion of a fine micropin into the control dorsal horn prior to processing. (B) and (C) show high power images of the ventral horns on the control-side and treated sides of the spinal cord. It can be seen that there are more HRP-labeled motoneurons in the control ventral horn compared to the ventral horn on the treated side. The presence of Nissl-stained (gallocyanin) motoneurons can also be seen within the sciatic pool. Scale bars for (A) = 250μm, (B) and (C) = 50μm.

Figure 8

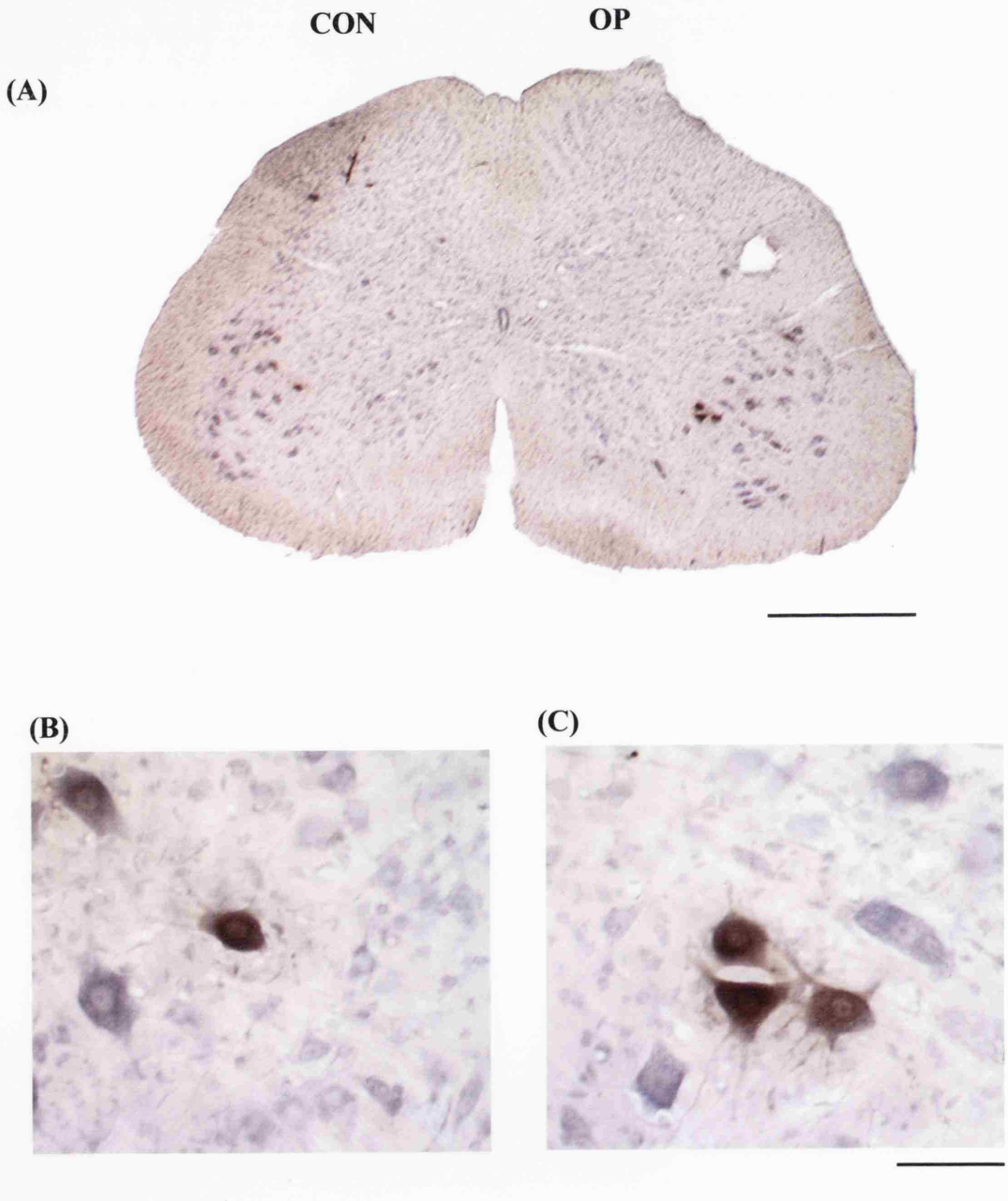


Table 1. The effect of $MgSO_4$ application on the survival of sciatic motoneurons

The Table summarizes the results of the mean number of Nissl-stained motoneurons in the sciatic motor pool 10 and 21 days after treatment with either NaCl or $MgSO_4$. Following treatment with $MgSO_4$, the number of sciatic motoneurons in the treated ventral horn on the operated side did not differ significantly from the number in the control ventral horn at 10 days of age (*$P>0.05$). However, by P21 the number of sciatic motoneurons has decreased significantly in the treated side compared to the contralateral control side (**$P<0.05$).

Table 1

Treatment	Age (days)	Number of Nissl-stained motoneurons		
		Control ventral horn	Operated ventral horn	% operated/control
NaCl only mean ± S.E.M (n=4)	10	587± 6.6	583± 18.5	99 ± 3.3
$MgSO_4$ only mean ± S.E.M (n=4)	10	588 ± 12.7	583 ± 11.1	99 ± 1.3*
$MgSO_4$ only mean ± S.E.M (n=4)	21	580 ± 6.0	491 ± 12.7	85 ± 1.7**

Table 2. The effect of $MgSO_4$ application on the survival of soleus motoneurons

The Table summarizes the results of the mean number of retrogradely labeled soleus motoneurons after treatment with either NaCl or $MgSO_4$. Following treatment with $MgSO_4$, the number of HRP-labeled soleus motoneurons had decreased significantly in the treated ventral horn on the operated side compared to the contralateral control side by 21 days of age (*$P<0.05$). Treatment with NaCl did not result in a loss of HRP-labeled soleus motoneurons.

Table 2

Treatment	Age	Number of HRP labeled motoneurons		
		Control ventral horn	Operated ventral horn	% operated/control
NaCl only mean ± S.E.M (n=4)	Adult	52 ± 4.3	51 ± 3.0	98 ± 3.7
$MgSO_4$ only mean ± S.E.M (n=3)	21days	66 ± 7.2	36 ± 1.5	56 ± 4.7*

Thus, following $MgSO_4$ treatment at birth, motoneuron death does not occur until at least 10 days after treatment, although significant death takes place thereafter. Therefore, the reduction in ChAT activity observed in $MgSO_4$ treated muscles in the present study during the first 2 postnatal weeks is likely to reflect a true decrease in ChAT activity, and not a loss of motoneurons.

2.4. DISCUSSION

The results of this study demonstrate that during normal development, ChAT activity and the amount of ChAT protein in spinal motoneurons increases dramatically during the first few weeks of postnatal development. Concomitant with the increase in ChAT levels, the levels of GAP-43 in sciatic nerve decrease during the same period. Furthermore, treating motor nerve terminals of neonatal rats with agents that have been shown to alter transmitter release from presynaptic terminals changes the normal developmental pattern of expression of ChAT and GAP-43 in developing motoneurons. These results indicate that motoneuron development during early postnatal life may be regulated by local events that occur at the NMJ. It is therefore possible that one important function of neuromuscular interaction is to up-regulate transmitter release from motor nerve terminals, which in turn promotes motoneuron development and survival.

2.4.1. Normal developmental changes of ChAT and GAP-43 in motoneurons

Motoneurons communicate with muscle cells by secreting the neurotransmitter acetylcholine (ACh, Dale et al, 1936). The synthesis of ACh is critically dependent upon the enzyme ChAT, which determines the production of ACh depending on functional demands (Tucek et al, 1982) and is recognized as a definitive marker for cholinergic neurons (Fonnum, 1973). At birth, the level of ChAT activity in the rat spinal cord is relatively low compared to that seen in the adult (Burt, 1975). This is most likely due to the structural and functional immaturity of spinal motoneurons at this time. For example, the newly formed presynaptic terminals at the NMJ are

simple bulbous enlargements that contain few synaptic vesicles and poorly developed active zones (Takahashi et al, 1987; Buchanan et al, 1989; Lupa and Hall, 1989). Consequently, the quantal content of transmitter release is relatively low (Lowrie et al, 1985), with both spontaneous MEPPs and nerve evoked EPPs being small in amplitude (Dennis et al, 1981; Pilar et al, 1981). Furthermore, at birth, the rat pup displays very little evidence of locomotor activity (Altman and Sudarshan, 1975). However, during the first 3 postnatal weeks, the structural development of pre-and postsynaptic terminals at the NMJ, results in a dramatic increase in both the frequency of spontaneous MEPPs and the quantal content of EPPs (Diamond and Miledi, 1962; Kelly, 1978; Lowrie et al, 1985). In addition, locomotor activity increases and adult patterns of motoneuron firing are established (Navarrete and Vrbová, 1983; Westergna and Gramsbergen, 1990). The results of the present study show that coincident with this marked increase in motor activity, the intensity of ChAT-positive staining increases dramatically in sciatic motoneurons during the first 2 postnatal weeks. Consistent with this immunocytochemical data, this study also demonstrates an exponential increase in ChAT activity in the lumbar spinal cord. Taken together, these results confirm previous observations that describe a similar increase in ChAT levels in the spinal cord during the early postnatal period (Burt, 1975; Phelps, et al, 1984).

Interestingly, the present results also show a significant increase in ChAT immunoreactivity in sciatic motoneurons between P1 and P5, which correlates with the period in which the vulnerability of motoneurons to injury-induced cell death declines (Lowrie et al, 1982, 1987). In addition, during the first 10 postnatal days most ChAT-positive motoneurons in the sciatic motor pool displayed relatively

homogenous levels of staining intensity. However, by P14 the smaller motoneurons were commonly more intensely stained, whereas the largest motoneurons often exhibited weaker and more variable intensity of staining. This variability in ChAT staining intensity could be associated with differences in amounts of transmitter required by different sizes of motoneurons. There is evidence that smaller motoneurons that generally stain more intensely have a lower firing threshold, and are relatively more excitable than larger motoneurons (Henneman et al, 1965a, b). Further evidence supporting the idea that the level of ChAT activity is directly related to motoneuron activity has been shown in experiments on spinal cord cell cultures. It was found that increasing cellular activity using depolarizing agents significantly increased ChAT activity (Ishida and Deguchi, 1983).

This study also examined the developmental changes of ChAT activity in specific motor pools located within the lumbar spinal cord that innervate soleus and EDL muscles. In accordance with the exponential increase in ChAT activity in the lumbar spinal cord, ChAT activity in the endplate region of both the soleus and EDL muscles also increases dramatically during the first 2 postnatal weeks. It has previously been shown that the quantum content of transmitter release in soleus muscles increases between 2-5 fold between P5 and P18 (Lowrie, et al, 1985). Therefore, this increase in quantum content is reflected in the capacity of soleus motoneurons to synthesize the components needed for transmitter production and thereby an increase in ChAT activity.

It is well known that during the first few weeks of postnatal development the electrophysiological properties of lumbar motoneurons undergo significant changes

in order to establish adult patterns of locomotor activity (Navarrete and Vrbová, 1983; Walton and Navarrete; 1991; Walton et al, 1992; Gao and Ziskind-Conhaim, 1995, 1998). Recently, several lines of evidence indicate that a differential maturation of motoneurons belonging to different motor pools occurs during the early postnatal period. First, the development of dendritic fields of motoneurons innervating the extensor muscles lags behind that of the antagonistic motoneurons (Curfs et al, 1993). Second, during the first 5 postnatal days, changes in excitability of motoneurons innervating extensor muscles compared to flexor muscle in the rat hindlimb are less pronounced. Third, the vulnerability of soleus motoneurons has been shown to be higher that of flexor motoneurons during the few days after birth (Sanusi et al, 1998). Fourth, the disappearance of polyneuronnal innervation of muscle fibres occurs earlier in EDL muscles than in soleus muscles (Brown et al, 1976; Balice-Gordon and Thompson, 1988). Consistent with these findings, this study shows that the increase in ChAT activity seen during the first 2 postnatal weeks occurs at an earlier age in the flexor muscle (EDL), than the extensor muscle (soleus).

This study also examined the developmental expression of a growth-associated protein in sciatic nerve. The phosphoprotein GAP-43 was chosen as a marker of neuronal growth because of its strong association with promoting axonal elongation in developing neurons and synaptic terminal formation (Skene and Willard, 1981; Skene et al, 1986; Benowitz and Routtenberg, 1997). However, unlike many other growth associated proteins, GAP-43 levels in developing neurons do not decline immediately after target contact (Hesselmans et al, 1989; Reynolds et al, 1991; Caroni and Becker, 1992). In addition, GAP-43 is maintained in areas of the CNS

that are associated with plasticity (Neve et al, 1988; Benowitz et al, 1990). This suggests that the function of GAP-43 is not confined purely to axonal growth. Thus, in the neuromuscular system, GAP-43 is not only expressed when axons are initially growing towards their target muscles, but are maintained long after target contact, and well into the early postnatal period. However, soon after the first postnatal week, GAP-43 mRNA levels decline quite rapidly (Caroni and Becker, 1992). The period of development during which this decline in GAP-43 occurs, coincides with the period during which polyneuronal innervation is lost (Brown et al, 1976). For example, in rat hindlimb muscles, between postnatal days 8-14, there is a rapid decrease in the number of muscle fibres innervated by more than one axon terminal (O'Brien et al, 1978; Jansen and Fladby, 1990). During the same developmental period there is also a decrease in the expression of GAP-43 mRNA (Caroni and Becker, 1992). Consistent with these findings, the present study found that GAP-43 protein levels in sciatic nerve decreased rapidly at the onset of the normal period of synapse elimination period.

Overall, the present findings suggest that during the early postnatal period, developing motoneurons undergo a coordinated shift in the expression of transmitter and growth related proteins, such that a dramatic up-regulation in motoneuron ChAT levels appears to switch off the expression of GAP-43.

2.4.2. The effect of 4-AP and $MgSO_4$ on ChAT activity in developing motoneurons

The next series of experiments in the present study examined whether motoneuron development is regulated by events that occur in the periphery as a result of functional interaction. It is postulated that the target muscle induces an up-regulation in transmitter release from developing motor nerve terminals, which subsequently alters the phenotype of the motoneuron (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996). Therefore, in order to modify local events at the motor nerve terminals, various pharmacological agents, which have been shown to alter transmitter release were applied to the NMJs of muscles in the rat hindlimb at birth. The effect of these treatments on motoneuron development was assessed by determining ChAT activity levels in treated muscles.

The results show that application of 4-AP to nerve terminals of neonatal muscles significantly increases the activity of ChAT present in nerve terminals contacting both the soleus and EDL muscles. This elevation in ChAT activity was sustained for many days following the application of 4-AP, such that ChAT activity did not begin to return to normal levels for 15-20 days. The effect of 4-AP on ChAT activity in soleus and EDL muscles was not reflected by changes in ChAT activity within the spinal cord. This result indicates that treatment of the hindlimb muscles with 4-AP only affected those muscles that were in close proximity to the implant. Thus, the proportion of motoneurons affected by the treatment is likely to be relatively small compared to the total number of cholinergic neurons with the ventral spinal cord, and as a result, the high baseline levels of ChAT activity in the spinal cord may mask any

increases in a subpopulation of motoneurons. Furthermore, since the overall levels of ChAT in the spinal cord were not increased following 4-AP treatment is unlikely that 4-AP acts systemically to increase ChAT activity. However, within muscles treated with 4-AP there is likely to be a significant increase in the release of transmitter. It is well established that 4-AP potentiates transmitter release at the NMJs of neonatal vertebrates (Okamoto et al, 1992). 4-AP

is a neural K^+ channel blocker, which has been shown to enhance the influx of Ca^{2+} into nerve terminals, resulting in a sustained increase in quantal transmitter release (Thesleff, 1980; Muller, 1986). Inducing increased levels of transmitter release by applying 4-AP is likely to increase the demand for ACh synthesis and result in the up-regulation of ChAT to cope with the rise in functional demand. In addition, it is likely that as a consequence of up-regulating ChAT expression, other proteins required for neurotransmission are also up-regulated by the motoneuron. For example, the vesicular acetylcholine transporter and ChAT have been found to share a common gene locus and regulatory elements for gene transcription. Thus, their coordinated expression forms part of the synthesis/sequestration pathway required for cholinergic transmission (reviewed by Eiden, 1998). Furthermore, it has been shown that transfecting neuroblastoma cells with a contruct containing cDNA for rat ChAT, resulted not only in an increase in the synthesis of ACh but also the expression of the synaptic-vesicle associated protein, synapsin I (Bignami, 1997).

In contrast to the effect of 4-AP treatment, following $MgSO_4$ treatment, ChAT activity was reduced during the first 2 postnatal weeks in both the soleus and EDL muscles. This is likely to reflect a decrease in functional demand imposed on the nerve terminals innervating these muscles. Mg^{2+} is known to antagonize the influx of Ca^{2+} into nerve terminals and therefore acts to depress transmitter release from treated nerve terminals (Katz and Miledi, 1969; Cooke et al, 1973). Furthermore, since Ca^{2+} is implicated in initiating a cascade of intracellular events, which lead to maturation of transmitter release components (Henderson et al, 1984; Ghosh and Greenberg, 1995), reducing Ca^{2+} influx into the nerve terminals may delay normal presynaptic maturation, which normally occurs during the first few postnatal days

(Linden et al, 1988). Therefore, altering the ability of nerve terminals to release ACh, may in turn prevent the normal up-regulation of ChAT during the first 2 postnatal weeks, since the amount of ACh released during this period is likely to be reduced.

It has previously been shown that altering the local environment at the NMJ, by blocking neuromuscular activity at birth, results in a significant loss of motoneurons (Greensmith and Vrbová, 1992). It is therefore possible that the lower levels of ChAT activity in soleus and EDL muscles seen during the early postnatal period is due to loss of motoneurons. However, this was found not to be the case, since the present results show that following $MgSO_4$ treatment no motoneuron loss was evident at P10, but by P21 significant motoneuron loss had occurred. Thus, it is clear that during the first 10 days of postnatal development the reduction in ChAT activity observed in nerve terminals contacting $MgSO_4$ treated muscles, is not due motoneuron death.

In contrast to the effect of 4-AP, following $MgSO_4$ treatment, ChAT activity in the spinal cord was significantly reduced at P7, and then recovered to normal levels by P10. Furthermore, substantially lower levels of ChAT were evident in the treated spinal cord compared to normal between P10 and P21. This later decline in ChAT activity corresponds closely to period of motoneuron death following $MgSO_4$ treatment and is therefore likely to reflect the loss of motoneurons within the spinal cord. However, it was previously argued, in the case of 4-AP, that the pharmacological effects were localized to muscles close to the implant, and therefore the overall levels of ChAT activity within the spinal cord may mask the increases observed in muscles affected by the treatment. It is therefore possible that the

concentration of $MgSO_4$ used in this study was able to affect muscles further from the implant compared to 4-AP. In support of this possibility, it was found that following $MgSO_4$ treatment 15% of sciatic motoneurons had died by P21 (see Table 1), which indicates that muscles other than the soleus and EDL were affected. Furthermore, the fact that ChAT levels in the spinal cord at P7 were lower than normal also indicates that ChAT activity is reduced in many muscles innervated by motoneurons within the lumbar spinal cord (L4-L6).

A further observation that requires explanation is the immediacy in which 4-AP and $MgSO_4$ affected the levels of ChAT activity. For example, the effect of $MgSO_4$ application on the activity of ChAT in the soleus and EDL muscle occurred at a much earlier age (P2) than the effect of 4-AP (P7). This is probably due to the different mechanisms by which these pharmacological agents act. Application of $MgSO_4$ results in the immediate blockade of Ca^{2+} channels on the presynaptic terminal (Katz and Miledi, 1969), which in turn is likely to result in the immediate reduction in ChAT activity and synthesis. In contrast, the application of 4-AP to the NMJ would not necessarily lead to an immediate up-regulation in ChAT production. Instead there may be a period during which there is sufficient ChAT present in the

nerve terminals to cope with an increased demand for ACh synthesis. However, after some time, the motoneuron would be required to synthesize additional ChAT in order to sustain the increase in transmitter release. This may explain the latent effect of 4-AP compared to $MgSO_4$ on ChAT activity in soleus and EDL muscles.

2.4.3. The effect of 4-AP and $MgSO_4$ on GAP-43 levels in sciatic nerve

This study also examined the effect of manipulating transmitter release at neonatal motor nerve terminals on the developmental expression of GAP-43 protein in sciatic nerve. The present study found that treatment with 4-AP increases the rate at which GAP-43 levels in the sciatic nerve decline. $MgSO_4$ treatment also had a significant effect on GAP-43 levels in the sciatic nerve, so that levels were maintains for a longer period that normal. At present the widely held view is that synaptic refinement of neuromuscular contacts is an activity-dependent process, since blocking neuromuscular activity, either pre or postsynaptically, results in a delay in the elimination of polyneuronal innervation of muscle fibres (Thompson et al, 1979; Duxson, 1982; Callaway and Van Essen, 1989; Greensmith and Vrbová, 1991). Conversely, increasing neuromuscular activity results in an acceleration of the process of synapse elimination during the early postnatal period (O'Brien et al, 1978; Thompson, 1983). Furthermore, the expression of GAP-43 in motoneurons appears to be linked to synapse elimination at the NMJ, since GAP-43 expression declines rapidly with the onset of elimination of polyneuronal innervation. In addition, preventing synapse elimination by blocking neuromuscular activity, also maintained the expression of GAP-43 in motoneurons (Caroni and Becker, 1992). Therefore, the present results suggest that manipulating neuromuscular activity using 4-AP and

$MgSO_4$, interferes with the normal course of synapse elimination during the early postnatal period, and thereby alters the normal time-course of GAP-43 down-regulation in developing motoneurons.

2.4.4. The effect of manipulating transmitter release on the maturation and survival of developing motoneurons

It is well established that functional contact with the muscle plays an important role in the development and survival of motoneurons (Romanes, 1946; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987). However, the mechanism by which the target promotes changes in the cell body of the motoneuron is unclear. Recent studies have focused on the possibility that functional events that occur at the motor nerve terminals may regulate the development of motoneurons. Specifically, it has been postulated that an important role of the target muscle is to up-regulate the release of transmitter from developing presynaptic terminals (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996). Indeed, it is well known that one of the first events that take place when developing nerve terminals first make contact with their muscle fibres is an immediate up-regulation in transmitter release (Chow and Poo, 1985; Xie and Poo, 1986). Furthermore, during the early postnatal period there is a marked elevation in motor activity, which is accompanied by an increase in proteins associate with neurotransmission (Burt, 1975; O'Brien and Vrbová, 1978; Phelps et al, 1984). On the basis of these findings, previous experiments have examined whether manipulating transmitter release at developing nerve terminals has a significant effect on the survival of motoneurons. It has been shown that prematurely enhancing ACh release by treatment of the NMJ with 4-AP, renders motoneurons

resistant to subsequent nerve injury (Greensmith et al, 1996). In this study it was demonstrated that nerve injury at 3 days of age results in the death of fewer motoneurons if preceded by 4-AP treatment. In contrast, reducing transmitter release by treatment with $MgSO_4$ prolongs the period in which motoneurons are vulnerable to nerve injury. Moreover, $MgSO_4$ treatment alone, results in significant motoneuron death, which is confirmed by results of the present study (Greensmith et al, 2000). It was suggested that the effect of manipulating transmitter release on the vulnerability of motoneurons to injury-induced cell death is due to a shift in normal pattern of expression of growth and transmitter related proteins. This transition of developing motoneurons from growing into transmitting cells has been postulated to be a critical event in the maturation of developing motoneurons. Failure to undergo this transformation within a critical period of development renders developing motoneurons vulnerable to the increased level of excitatory input in the maturing CNS (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996). The present results support this proposal, since 4-AP treatment of motor nerve terminals at birth resulted in a premature transition of developing motoneurons from a growth into a transmitting state. Conversely, $MgSO_4$ treatment delayed this transition and resulted in the death of motoneurons.

2.4.5. Conclusions

Taken together, the results of this study show that prematurely increasing transmitter release from motor nerve terminals of developing rats, by treatment with 4-AP, up-regulates the activity of ChAT in developing motoneurons, with a premature reduction in levels of the growth-associated protein GAP-43. Conversely, decreasing

transmitter release by treatment with $MgSO_4$ reduces the levels of ChAT activity in developing motoneurons during the first 3 postnatal weeks of development and maintains GAP-43 expression for longer than normal. Thus, manipulating transmitter release at motor nerve terminals alters the rate at which motoneurons up-regulate their cholinergic phenotype and as a consequence affects their transformation from growing into transmitting cells. It is likely that this change in phenotype is important for the maturation of developing motoneurons, which may determine their vulnerability to nerve injury.

CHAPTER 3

THE EFFECT OF PROLONGED AXONAL GROWTH ON THE VULNERABILITY OF ADULT MOTONEURONS TO CELL DEATH

3.1. INTRODUCTION

There is substantial evidence indicating that maintaining developing motoneurons in a growth state by nerve injury or muscle paralysis, causes them to die (see Greensmith and Vrbová, 1996). In contrast, similar insults to adult motoneurons does not result in their death, which demonstrates that adult motoneurons are able to survive in a growth state for a certain period of time. Nevertheless, recent studies have shown that inducing prolonged and extensive axonal growth of adult motoneurons also compromises their survival (Harding et al, 1999; White et al, 2000). However, the mechanism by which these growing adult motoneurons die is unknown. Previous studies have implicated glutamate-mediated excitotoxicity as a mechanism that underlies the death of injured developing motoneurons (Mentis et al, 1993, Greensmith et al, 1994a, b). Whether adult motoneurons, which are forced to maintain a growth state for a prolonged period, are also vulnerable to glutamate-mediated excitotoxicity is examined in this Chapter. In addition, this study also investigates whether extensively growing adult motoneurons develop pathological features normally associated with amyotrophic lateral sclerosis (ALS).

3.1.1. The vulnerability of developing motoneurons to conditions that extends their normal period of growth

It is well established that developing motoneurons die if their peripheral axons are injured during the period of development when these cells are still in a growth state and their transmitting state has yet to be fully developed (Romanes, 1946; Schmalbruch et al, 1984; Lowrie et al, 1987). By disconnecting these developing

motoneurons from their target, their growth state is maintained. Thus, the developing motoneuron is prevented from undergoing the transition from a growth state into a fully transmitting state, which has been postulated to be a critical event in determining its continued survival (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996). Furthermore, the results presented in Chapter 2 indicate that this transition in motoneuron phenotype is regulated by an increase in transmitter released from developing nerve terminals, which is likely to occur as a consequence of a functional interaction with their target muscle fibres. Specifically, the results in Chapter 2 suggest that disrupting neuromuscular interaction by depressing transmitter release from motor nerve terminals using $MgSO_4$, maintains motoneurons in a growth state, which causes those motoneurons to die. Further evidence supporting the proposal that maintaining developing motoneurons in a growth state, also maintains their vulnerability to cell death, came from a number of experiments that used a variety of manipulations to keep motoneurons growing. For example, blocking neuromuscular transmission either presynaptically with botulinum toxin or postsynaptically with α-bungarotoxin, delays the normal time-course of GAP-43 down-regulation and prolongs the period in which muscle fibres were polyneuronally innervated (Greensmith and Vrbová, 1991; Caroni and Becker, 1992). Furthermore, following α-bungarotoxin treatment at birth, motoneurons that innervated paralyzed muscles die when their axons are injured in 5 day old animals (Burls et al, 1991), which does not occur in normal animals of the same age. Moreover, it was subsequently shown that simply paralyzing muscles with α-bungarotoxin during the first week of postnatal development significantly reduced the number of surviving motoneurons (Greensmith and Vrbová, 1992).

Surgical methods have also been used to extend the growth phase of developing motoneurons. Partial denervation of the extensor digitorum longus and tibialis anterior muscles in 3 day old rats prevents the normal phase of synapse elimination of intact motoneurons and maintains those motoneurons in a state in which they are vulnerable to injury-induced cell death (Harding et al, 1998). Finally, repeated nerve injury, first carried out in rats of 5 days of age, followed by the same injury 6 days later, also results in motoneuron death (Lowrie and Vrbová, 2001). Taken together, these experiments demonstrate that developing motoneurons that are forced to maintain their growth state remain vulnerable to cell death, which is likely to be a reflection of their delayed maturation.

3.1.2. Prolonged axonal growth and death of adult motoneurons

In contrast to developing motoneurons, adult motoneurons do not die following injury to their peripheral nerve. This demonstrates that mature motoneurons are able to withstand at least a certain period of growth associated with regeneration. Even so, a number of studies have recently examined whether adult motoneurons are also vulnerable to cell death if their axons are induced to grow for a prolonged period. It has been shown that even adult motoneurons die if their axons are subjected to repeated stimuli for growth. For example, following a conditioning lesion, adult motoneurons die after a second nerve injury (Arvidsson and Aldskogius, 1982). However, in these experiments adult motoneurons have been repeatedly injured and the injury itself may have directly caused motoneurons to die. In order to address this issue, a recent experiment prolonged the growth of adult motor axons without direct nerve injury (White et al, 2000). In this study, motor axons in adult rats were

induced to grow by paralyzing the muscle with botulinum toxin followed 3 weeks later by partial denervation. It was found that the combination of these two stimuli for axonal growth resulted in the death of approximately 30% of motoneurons. However, neither of these procedures alone resulted in loss of motoneurons.

Further support for the hypothesis that extending the period of axonal growth of adult motoneurons results in an increased vulnerability to cell death, comes from experiments using a transgenic mouse that over-expresses the growth associated protein, GAP-43 (Harding et al, 1999). Motor axons in this transgenic mouse undergo a period of profuse spontaneous sprouting, which is only reduced by 3 months of age. Nerve injury carried out during this period of sprouting results in a dramatic loss of motoneurons. However, no motoneurons die if the nerve is injured at 3 months of age, when the axons are no longer sprouting. These results taken together indicate that continued growth of adult motor axons may render adult motoneurons more vulnerable to cell death.

3.1.3. Mechanisms implicated in the death of growing motoneurons

It has been proposed that developing motoneurons, which are maintained in a growth state by disrupting functional neuromuscular interaction, are vulnerable to the increase in levels of excitatory input known to occur in the developing spinal cord (see 1.7.2.). There is now substantial evidence implicating glutamate-mediated excitotoxicity as a mechanism that contributes to the death of target-deprived neonatal motoneurons. Firstly, it has been shown that nerve injury during the early postnatal period renders motoneurons more susceptible to the excitotoxic effects of

the glutamate agonist, N-methyl-D-aspartate (NMDA), which was exogenously applied (Greensmith et al, 1994a). However, this susceptibility of injured motoneurons to NMDA is temporary, since motoneurons that are allowed to reinnervate their target and resume neuromuscular interaction become resistant to NMDA (Greensmith et al, 1994a). In addition, maintaining developing motoneurons in a growth state by paralyzing the muscle using α-bungarotoxin, also results in an increased vulnerability of motoneurons to increased levels of excitatory input (Greensmith et al, 1995a). Conversely, treatment with dizocilpine maleate (MK-801), a NMDA receptor antagonist, can prevent the death of motoneurons that normally occurs after nerve injury at birth (Mentis et al, 1993; Greensmith et al, 1994b). Similarly, treating injured neonatal motoneurons with the non-competitive NMDA antagonist, magnesium sulphate, prevents the death of a proportion of motoneurons (Greensmith et al, 1995b).

Several studies have also provided evidence that target-deprived neonatal motoneurons undergo apoptotic cell death, which predominantly occurs in the first 2-3 days after injury (de Bilbao and Dubois-Dauphin, 1996a; Rossiter et al, 1996; Lawson and Lowrie, 1998). Furthermore, the specific molecules responsible for the apoptotic death of injured neonatal motoneurons have recently been identified. For example, the cysteine protease, caspase-3, which is thought to be a central component in the apoptotic cascade, is activated in injured neonatal motoneurons (Vanderluit et al, 2000; Kalmar et al, 2002). Moreover, caspase-3 gene deletion delayed the death of axotomized neonatal motoneurons (Vanderluit et al, 2000).

Overall these findings indicate that developing motoneurons induced to maintain their growth state, die by apoptosis and that glutamate mediated-excitotoxicity may be significantly involved in this process of cell death. Therefore, it is also possible that similar mechanisms may also be involved in the death of adult motoneurons, which are forced to grow for a prolonged period.

Additional factors that may contribute to the vulnerability of adult motoneurons firstly relate to the size of motoneurons compared to other groups of cells. Motoneurons, especially those present in the lumbar spinal cord, are large cells with an elaborate dendritic architecture, and a long axonal process, each of which can innervate many hundred-muscle fibres. As a consequence of these anatomical features it is likely that motoneurons have high-energy demands, which necessitates a high level of mitochondrial activity. Mitochondria are central to the maintenance of cell function and generate the bulk of cellular ATP required for cellular homeostasis (reviewed by Duchen, 2000). However, mitochondria are also the main sites for the generation of reactive oxygen species (ROS), which is closely linked to mitochondrial respiration. ROS, such as superoxide anions ($O2^{-}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^{-}$) can induce lethal cellular damage by lipid peroxidation, damage to cell membranes and to DNA (Coyle and Puttfarcken, 1993). Cellular damage caused by the excessive production of ROS is commonly referred to as oxidative stress, which has been implicated as a mechanism involved in the pathogenesis of motoneuron degeneration in amyotrophic lateral sclerosis (ALS) (Ferrante et al, 1997; Bogdanov et al, 1998). It is also possible that oxidative stress may play a role in the death of excessively growing motoneurons, since prolonged axonal growth may increase levels of mitochondrial

activity associated with increased metabolic demands, resulting in higher levels of ROS production and cellular damage.

Another important function of mitochondria is to regulate Ca^{2+} levels in the cytosol, since it has long been known that elevated levels of cytosolic free Ca^{2+} results in the activation of harmful biochemical processes within the cell (Choi, 1988; Duchen, 1999). The cause of excessive Ca^{2+} influx into the cell is now thought to be due to the over-stimulation of NMDA and selective AMPA/kainate receptors. This process is known as glutamate-mediated excitotoxicity (Choi, 1988; Choi, 1994) and has been implicated in the death of motoneurons in ALS, due to the loss of astroglial glutamate transporters, which act to regulate extracellular glutamate concentrations (see Chapter 1, section 1.8.2.). It is also possible that activation of glutamate receptors in growing adult motoneurons could prove toxic without any abnormalities to glutamate modulation. For example, NMDA receptors as well as sub-populations of AMPA/kainate expressed by adult motoneurons are highly permeable to Ca^{2+} when activated by glutamate (see Chapter 1, section 1.8.2.). The resulting influx of Ca^{2+} in conjunction with oxidative stress caused by excessive metabolic demands may result in mitochondrial dysfunction and a breakdown in cellular homeostasis. Furthermore, mitochondrial injury may lead to the release of pro-apoptotic factors, which initiate the cascade to apoptotic cell death (reviewed by Crompton 2000).

A further feature of motoneurons that may render them susceptible to the damaging effects of Ca^{2+} influx is their relatively low expression of calcium binding proteins, calbindin-D28K and/or parvalbumin (Celio, 1990). Motoneurons relatively deficient in these calcium-binding proteins (spinal motoneurons) degenerate early in ALS,

whereas those that express high levels of these proteins (Onuf's nucleus and extraocular motoneurons) are relatively spared (Ince et al, 1993; Alexianu, 1994; Elliott and Snider, 1995; Nimchinsky et al, 2000). Thus, the inability of growing spinal motoneurons to buffer calcium through the expression of calbindin-D28K and/or parvalbumin, may result in a heavier burden on mitochondria to regulate cytosolic levels of Ca^{2+}, which in turn may result in cell death.

Finally, another factor that may also contribute to death of growing adult motoneurons is the formation of protein aggregates or inclusions. Such inclusions are common pathological abnormalities found in motoneurons of patients with amyotrophic lateral sclerosis (ALS; for further detail see 1.8.3.), in which sprouting of motor axons is known to occur (Wohlfart, 1957; Parhad et al, 1992). In particular, the formation of ubiquitin skein-like inclusions may develop in growing adult motoneurons, since ubiquitin mRNA is up-regulated in axotomized motoneurons during regeneration (Savedia and Kiernan, 1994). Furthermore, the formation of ubiquitin inclusions is thought to be a consequence of cell stress, leading to a malfunction or overload of the ubiquitin dependent pathways (Jungmann et al, 1993; Uney et al, 1993; Montine et al, 1996; Figueiredo-Pereira 1998). Therefore, it is possible that the stress placed upon extensively growing adult motoneurons coupled with the normal up-regulation of ubiquitin following nerve injury, may result in an overload in ubiquitin protein and as a consequence cause the formation of ubiquitin inclusions.

3.1.4. A model of prolonged axonal growth

In order to explore the mechanisms involved in the death of adult motoneurons induced to undergo prolonged growth, this study examined transgenic mice that constitutively express GAP-43 in adult neurons, which results in motor axon sprouting being maintained well into adult life (Aigner et al, 1995). GAP-43 expression is strongly implicated as an intrinsic competence factor for nerve growth and plasticity (for evidence see 1.6.2.). The expression of GAP-43 in adult neurons was achieved by inserting chick GAP-43 cDNA linked to the mouse Thy-1 expression cassette into the mouse genome (Aigner et al, 1995). This system provided a relatively late activation of the GAP-43 transgene (P4-P10), which was expressed at high levels until P60. In both GAP-43 transgenic and wild-type mice, endogenous GAP-43 was down-regulated at P8-10. In contrast, adult GAP-43 mice expressed significant levels of GAP-43 between P8-60, which resulted in the prominent sprouting of motor nerves at the NMJ. Ultraterminal sprouting was detected at P20 and was maximal at around P30. By P90 no sprouting at the NMJ was detected. Significantly, although expression of GAP-43 from P8 onwards resulted in motor axon sprouting, this is did not affect the period of synapse elimination nor any obvious alterations in neuromuscular transmission. Furthermore, the increased expression of GAP-43 did not result in motoneuron death. However, a recent study has shown that injury to the sciatic nerve of adult GAP-43 mice during the period of maximal sprouting results in significant motoneuron death (Harding et al, 1999). Thus, it appears that maintaining adult motoneurons in a growth state confers a vulnerability to a second stimulus for growth. In this Chapter, the possible

mechanism(s) involved in the death of adult motoneurons in GAP-43 mice is examined.

3.1.5. Hypotheses to be tested

It is well documented that developing motoneurons are vulnerable to nerve-injury induced cell death (see review by Greensmith and Vrbova, 1996). The period for which immature motoneurons are vulnerable to cell death closely correlates with the period in which they are still undergoing axonal growth. The present Chapter investigates whether adult motoneurons, which are forced to grow for a prolonged period are also vulnerable to cell death. A model of continued axonal growth in the adult animal is provided by a transgenic mouse, which overexpresses GAP-43. Expression of the GAP-43 transgene results in prominent sprouting of motor axons at the NMJ up until 3 months of age. It has been established that sciatic nerve injury carried during this period of sprouting results in the death of a significant proportion of adult soleus motoneurons (Harding et al, 1999). The present study examines: i) the time-course of motoneuron death in these animals; ii) the mechanisms involved in this degeneration, including the possibility that motoneuron death occurs by apoptosis, the role of excitotoxicity, and whether these adult motoneurons whose axons are sprouting exhibit similar pathological features to that seen in motoneurons of patients with ALS, namely ubiquitin inclusions.

3.2. METHODS

3.2.1. Animals and genotyping by PCR

Adult transgenic mice expressing chick GAP-43 (wt3 strain) and adult wild-type littermates were used throughout this study. Stocks of transgenic animals were maintained as heterozygotes by crossing transgenic with non-transgenic mice. They were genotyped by PCR amplification of the transgene from genomic DNA. Mice were anaesthetized with halothane and ~1 cm of tail was removed with a new scalpel blade. The tail snips were digested with proteinase K (500μg/ml; Boehringer) in 300 μl TNES buffer (50 mM Tris, pH 8, 100 mM EDTA, 100 mM NaCl, 1% lauryl sulfate) overnight at 55°C. Digests were then centrifuged at 13,000 rpm for 3 min, and 10 μl of a 1:50 dilution of the supernatant was used for the PCR. Each reaction tube contained 10 μl of 10 x PCR buffer, 25 ng each primer (sequences 5' GCC TCG AGG ATC ATG CTG TGC TGT 3' and 5' GCC TCG AGG ATC CCC GGG AGT CAT 3'), 2 μl each dNTP (10 μM), 6 μl $MgCl_2$ (25 μM), 2 units *Taq* polymerase (Promega), and 10 μl tail digest in a total volume of 100 μl. These were then temperature cycled as follows: 5min at 94°C, 1 min at 64°C, 1 min at 72°C, x 1; 1 min at 94°C, 1 min at 64°C, 1 min at 72°C, x 3; 20 s at 94°C, 1 min at 64°C, 1 min at 72°C, x 36; 1 min at 94°C, 1 min at 64°C, 10 min at 72°C, x 1. The PCR products were assessed by agarose gel electrophoresis.

3.2.2. Surgery

All experimental animals used in this study were cared for in accordance with the British Government Home Office and our institutional guidelines. Throughout this study, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Under sterile conditions, 1 month old transgenic mice and wild-type littermates were anaesthetized with halothane and the sciatic nerve was exposed in one hindlimb. Using a fine pair of Watchmaker forceps the sciatic nerve was crushed at the mid-thigh level. Following the crush, the nerve was examined to ensure that the endoneurial sheath was intact but translucent.

In some animals, 7 days after sciatic nerve injury the mice received a sterotactic injection of either NMDA (2 µl, 50mM) or sterile saline (2 µl, 0.9% NaCl), into the lumbar region of the spinal cord, as previously described (Greensmith et al, 1994a). Under sterile conditions and hypnorm/midazolam (7-10 ml/kg i.p) anaesthesia, a partial laminectomy was performed to expose the lumbar spinal cord (L4-L5 level) on the injured side. Using a Hamilton syringe and a micromanipulator, a stereotactic injection was made into the ventral horn of the spinal cord on the same side as the nerve injury. Following the injury the wound was rinsed with sterile saline and a piece of gelatin sponge (Spongistan) placed over the area of the laminectomy in order to aid wound repair.

In a separate experimental group, immediately following nerve injury, the mice were treated with riluzole (16mg/kg i.p) or sterile saline, daily for up to 10 weeks.

3.2.3. Motoneuron survival

The effect of nerve injury on motoneuron survival and vulnerability to excitotoxic insults was examined in adult transgenic and wild-type mice. In addition, the effect of inhibiting endogenous glutamate release by treatment with riluzole on the survival of injured motoneurons was also assessed. The animals were anaesthetized with chloral hydrate (4%; 1ml/100g) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffer saline (PBS). The spinal cords were removed, the lumbar region post-fixed for 4 h in the same fixative, and cryoprotected in sucrose (30% in 0.1 M PBS). The entire lumbar spinal cord (L1-L6) was serially sectioned (20 μm) and stained for Nissl (gallocyanin). The number of Nissl-stained motoneurons in every 3rd section within the sciatic motor pool in both the operated and contralateral ventral horns was counted (see Figure 9). The criteria for the identification of Nissl-stained sciatic motoneurons were similar to that described in Chapter 2 (see section 2.2.5.). However, in this case, the work by McHanwell and Biscoe (1981) on the localization of motoneurons supplying the hindlimb muscles of the mouse was used as a guide. These authors found that the position of the motonuclei forming the sciatic motor pool was very similar to that of the rat except for the longitudinal location, which in most cases was in a more caudal position in the spinal cord.

3.2.4. Motoneuron size distribution

Following nerve injury and treatment with riluzole, the mean size and size distribution of surviving motoneurons was assessed. Motoneuron soma area was determined with the aid of an image analysis system (KS200; Imaging Associates U.K). The sciatic motor pool in the operated and control ventral horn in each section was visualized and captured onto computer. The motoneuron soma area was measured by tracing the perimeters of each Nissl-stained motoneuron soma that contained a clear nucleolus and distinctly stained cytoplasm (see Figure 11). All motoneurons in both the operated and contralateral ventral horn in every 6^{th} section were measured.

3.2.5. Immunocytochemistry

Spinal cord sections were processed for either activated caspase-3 or ubiquitin immunoreactivity. The activated form of caspase-3 is used as a marker of apoptosis and the presence of caspase-3 in the spinal cord of GAP-43 and wild-type mice was assessed at 24 and 48 hrs after sciatic nerve-crush. Ubiquitin immunocytochemistry was performed on spinal cord sections from GAP-43 and wild-type mice 1, 3, 5 and 10 weeks after sciatic nerve-crush. For each marker, spinal cord sections from at least 3 animals for each time-point following nerve injury were processed for immunoreactivity.

Spinal cord sections (20 μm) were first blocked in 3% goat serum for 2 hrs and incubated overnight at 4°C with either the primary antibody (PharMingen; dilution

1:1500), specific for the activated form of the caspase-3 enzyme only or ubiquitin (DAKO; dilution 1:400). Negative control sections, in which the primary antibody was omitted, were also prepared. After thorough washing in phosphate buffered saline with 0.1% Trition-X-100 (PBS-T), the sections were incubated for 2 hrs at room temperature with biotinylated secondary antibodies (dilution 1:100; Vector) and then incubated for 1 hr in Avidin-Biotin complex (ABC; Vector). They were processed using horseradish peroxidase (HRP) as the chromagen and 3.3'-diaminobenzidine (DAB) as the substrate.

3.2.6. Statistics

The results were analyzed using the Mann-Whitney U test for comparison of independent samples. Two-tailed tests were used in all instances, and significance was set at $P<0.05$.

3.3. RESULTS

3.3.1. The effect of nerve injury on caspase-3 activation in adult wild-type and GAP-43 mice

It has previously been shown that following injury to the sciatic nerve in newborn rats, apoptotic motoneuron death is greatest 24-48 hours after injury (Lawson and Lowrie, 1998; Kalmar et al, 2002). Therefore, this study examined whether injury to growing adult motoneurons in GAP-43 mice also results in the apoptotic cell death of motoneurons. As a marker of apoptosis, the presence of the active form of caspase-3 was assessed in spinal cord sections of GAP-43 and wild-type mice, 24 and 48 hrs after sciatic nerve injury. No caspase-3 immunoreactive motoneurons were detected in the spinal cords of both wild-type and GAP-43 mice, at either time-point after nerve injury.

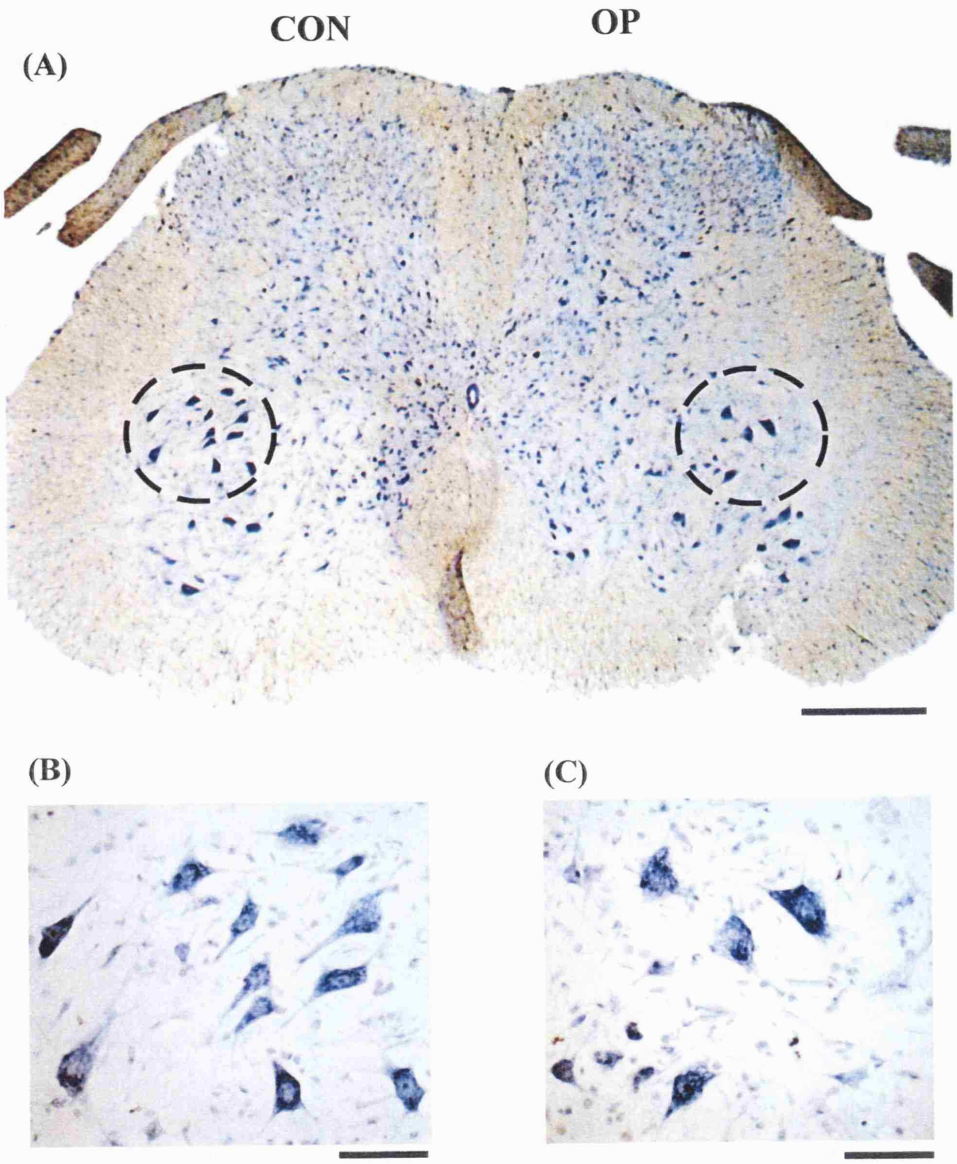
3.3.2. The effect of nerve injury on motoneuron survival in adult wild-type and GAP-43 mice

Following sciatic nerve injury in 4 week old GAP-43 transgenic mice, the effect on motoneuron survival within the sciatic motor pool was assessed at various intervals later. An example of a spinal cord section from a GAP-43 mouse stained for Nissl, 10 weeks after nerve injury is shown in Figure 9. The Figure shows the location and appearance of motoneurons in the operated and control ventral horns within the lumbar spinal cord. As an index of motoneuron survival the number of Nissl-stained sciatic motoneurons in the operated ventral horn was expressed as a percentage of the

Figure 9. Photomicrograph of an adult spinal cord section from a transgenic mouse that overexpresses the growth associated protein GAP-43

(A) A cross-section of a lumbar spinal cord from an adult GAP-43 mouse, 10 weeks after unilateral sciatic nerve-crush. The broken lines demarcate the approximate location of the sciatic motor pool in the ventral horn. (B) and (C) show the ventral horns at higher magnification. It can be seen that there are fewer Nissl-stained motoneurons in the ventral horn on the operated side (OP) compared to the contralateral control side (CON). Scale bars, A = 250μm, B and C = 50μm

Figure 9



number in the contralateral control ventral horn. In wild-type mice, following nerve injury at 4 weeks, motoneuron survival was assessed 10 weeks later. As shown in Figure 10, adult nerve injury did not result in motoneuron death in wild-type mice and 10 weeks after injury 99% (± 2.2 S.E.M., n=4) of sciatic motoneurons survive. In GAP-43 mice, motoneuron survival was examined 1, 3, 5, 10, and 17 weeks after nerve injury. The results of the time-course of motoneuron death are summarized in Figure 10. It can be seen that following nerve injury in adult GAP-43 mice, there is no significant loss of motoneurons for several weeks after injury so that even 5 weeks later 90% (± 4.5 S.E.M., n=5) of motoneurons survive. However, between 5 and 10 weeks post injury, there is a significant decrease in motoneuron survival, and 10 weeks after injury only 66% (± 2.7 S.E.M., n=5) of sciatic motoneurons remain. There is significantly less than present in wild-type mice 10 weeks after injury ($P<0.05$) and also significantly less than in GAP-43 mice 5 weeks after nerve injury ($P<0.05$). However, no further loss of motoneurons occurs after 10 weeks, so that 17 weeks after injury 68% (± 3.3 S.E.M., n=3) of sciatic motoneurons survive.

3.3.3. The effect of nerve injury on motoneuron size and size distribution in adult wild-type and GAP-43 mice

In order to examine whether motoneurons of different sizes are particularly vulnerable to nerve injury in adult transgenic mice, the mean soma area of the surviving sciatic motoneurons was established at various intervals after injury. All Nissl-stained sciatic motoneurons in both the operated and contralateral control ventral horns were measured in every 6th spinal cord section of both wild-type and GAP-43 mice. An example of the measurement of motoneuron soma area using

Figure 10. Motoneuron survival following sciatic nerve-crush in adult GAP-43 mice

The sciatic nerve was crushed in one hindlimb of GAP-43 mice at 1 month of age. One, 3, 5, 10 and 17 weeks later the effect of nerve injury on motoneuron survival was assessed by counting the number of Nissl-stained motoneurons within the sciatic pool, in both the operated and contralateral control ventral horns. The bar diagram shows the mean number of motoneurons in the operated ventral horn expressed as a percentage of that in the contralateral control ventral horn. Note the progressive nature of motoneuron death, with the majority of motoneuron loss occurring between 5-10 weeks after nerve injury. Error bars are S.E.M.

Figure 10

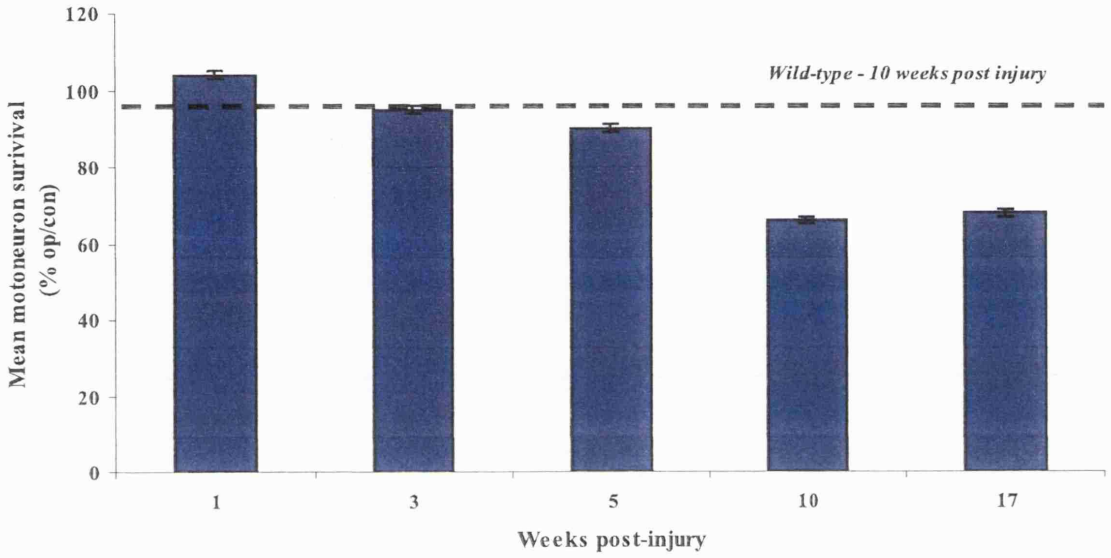

120
100
80
60
40
20
0
Mean motoneuron surivival (% op/con)
Wild-type - 10 weeks post injury
1
3
5
10
17
Weeks post-injury

image analysis is shown in Figure 11. The results of the mean areas of motoneurons are summarized in Table 3. It can be seen that following nerve injury in wild-type mice, there is no significant difference between the mean areas of operated and control motoneurons, 10 weeks after injury. The Table also shows that there is no significant difference in mean area of operated and control motoneurons in GAP-43 mice at 3 or 5 weeks after injury. However, between 5 and 10 weeks after injury in GAP-43 mice, there is a significant decrease in the mean area of motoneurons within the operated ventral horn. Thus, 10 weeks after injury in GAP-43 mice the mean area of operated motoneurons is 425 μm^2 ($\pm$ 2.7 S.E.M., n=4) compared to 511 μm^2 ($\pm$ 18.6 S.E.M., n=4) for control motoneurons ($P<0.001$). In addition, between 3 and 5 weeks after injury in GAP-43 mice there is a slight increase in the mean area of both operated and control motoneurons. This is probably related to the overall growth of these mice between 7 and 9 weeks of age.

To further investigate the possibility of selective motoneuron degeneration, the distribution of motoneuron sizes in wild-type and GAP-43 mice following nerve injury was examined. Figure 12 shows the size distribution of sciatic motoneurons in wild-type mice, 10 weeks after injury. It can be seen that there is a similar distribution of motoneuron areas in the operated and control ventral horns. The results in Table 4 confirm this observation and shows that there are no significant differences between the numbers of motoneurons in the operated and control ventral horns for all size ranges. Figure 13 shows the size distribution of sciatic motoneurons in the control and operated ventral horns of GAP-43 mice at 3, 5 and 10 weeks after nerve injury. In GAP-43 mice, there is no difference in the distribution of motoneuron soma areas in operated and control ventral horns 3 and 5 weeks after

Figure 11. Example of Nissl-stained motoneurons in the sciatic pool of a GAP-43 transgenic mouse

The photomicrograph show examples of Nissl-stained motoneurons located within the sciatic motor pool of an adult GAP-43 transgenic mouse. The motoneuron soma area was measured with the aid of an image analysis system. In order to measure soma area, the perimeters of each Nissl-stained motoneuron soma were traced as shown in the photomicrograph. Only those motoneurons, which display a clear nucleolus and distinctly stained cytoplasm, were assessed for soma size. Scale bar = 50 μm.

Figure 11

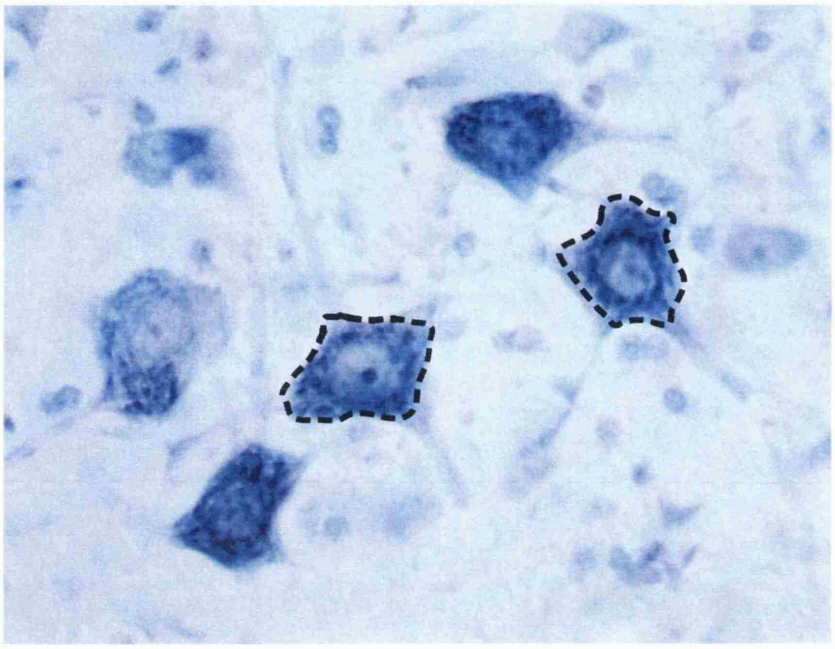


Table 3. The effect of nerve injury on mean motoneuron area in adult GAP-43 and wild-type mice

At one month of age the sciatic nerve was crushed in one hindlimb of GAP-43 and wild-type control mice. For GAP-43 mice, motoneuron size was determined, 3, 5 and 10 weeks after nerve-injury. In wild-type mice, motoneuron size was determined 10 weeks after injury. Motoneuron soma area was measured by tracing the perimeter of each Nissl-stained motoneuron that contained a distinctly stained nucleolus and cytoplasm. All motoneurons in both operated and contralateral ventral horns in every 6th section were measured. The Table shows that in GAP-43 mice there is no significant difference in mean motoneuron area of operated and control motoneurons 3 or 5 weeks after injury. However, at 10 weeks post-injury, there is a significant decrease in mean motoneuron area of motoneurons within the operated ventral horn (*$P<0.001$). In wild-type mice, nerve injury does not result in a significant change in mean motoneuron area in the operated ventral horn compared to the control ventral horn.

Table 3

		Mean motoneuron area (μm^2)	
	Time after injury	Control ventral horn mean +/- S.E.M	Operated ventral horn mean +/- S.E.M
GAP-43 mice	3 weeks (n=3)	475 ± 9.5	471 ± 9.2
	5 weeks (n=4)	538 ± 9.2	533 ± 9.1
	10 weeks (n=4)	511 ± 18.6	425 ± 2.7*
Wild-type mice	10 weeks (n=3)	484 ± 18.8	505 ± 26.3

Figure 12. The distribution of motoneurons soma size following nerve injury in adult wild-type mice

At one month of age the sciatic nerve was crushed in one hindlimb of wild-type mice. Motoneuron soma area was measured by tracing the perimeter of each Nissl-stained motoneuron that contained a distinctly stained nucleolus and cytoplasm. All motoneurons in both operated and contralateral ventral horns in every 6^{th} section were measured 10 weeks after nerve injury. Motoneuron size was measured in at least 3 animals. The data was plotted on a frequency histogram (Error bars = S.E.M.). The histogram shows that there is no significant change in motoneuron size distribution in the operated ventral horn compared to the control ventral horn, 10 weeks after nerve injury.

Figure 12

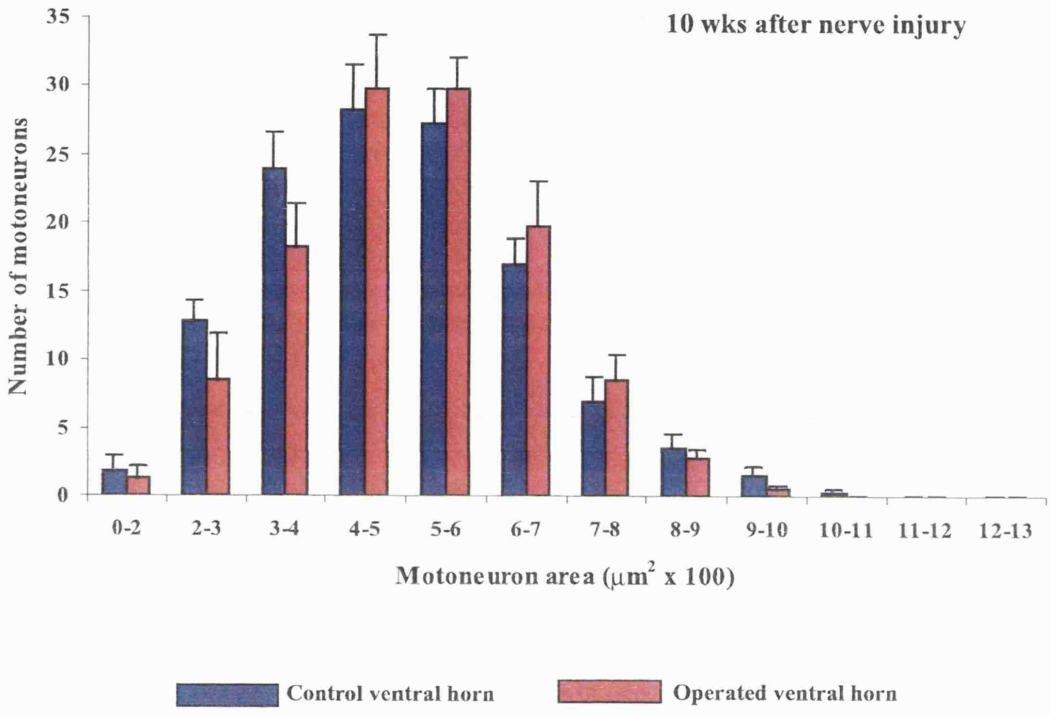

10 wks after nerve injury
Number of motoneurons
35
30
25
20
15
10
5
0
0-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
Motoneuron area (μm² x 100)
Control ventral horn
Operated ventral horn

Table 4. Motoneuron size distribution following nerve injury in adult GAP-43 and wild-type mice

The Table summarizes the results from sizing Nissl-stained motoneurons following nerve injury in adult GAP-43 and wild-type mice. The size of all motoneurons in both the operated (OP) and control (CON) ventral horns was measured in GAP-43 mice, 3, 5 and 10 weeks after nerve injury. For wild-type mice, motoneurons were sized 10 weeks after nerve injury. The mean number of motoneurons in each size range is given for the operated and control ventral horns in each group (n= number of animals; ± = S.E.M.). The Table shows that 10 weeks after nerve injury there is a significant reduction in the number of motoneurons in the larger size ranges (*$P<0.002$).

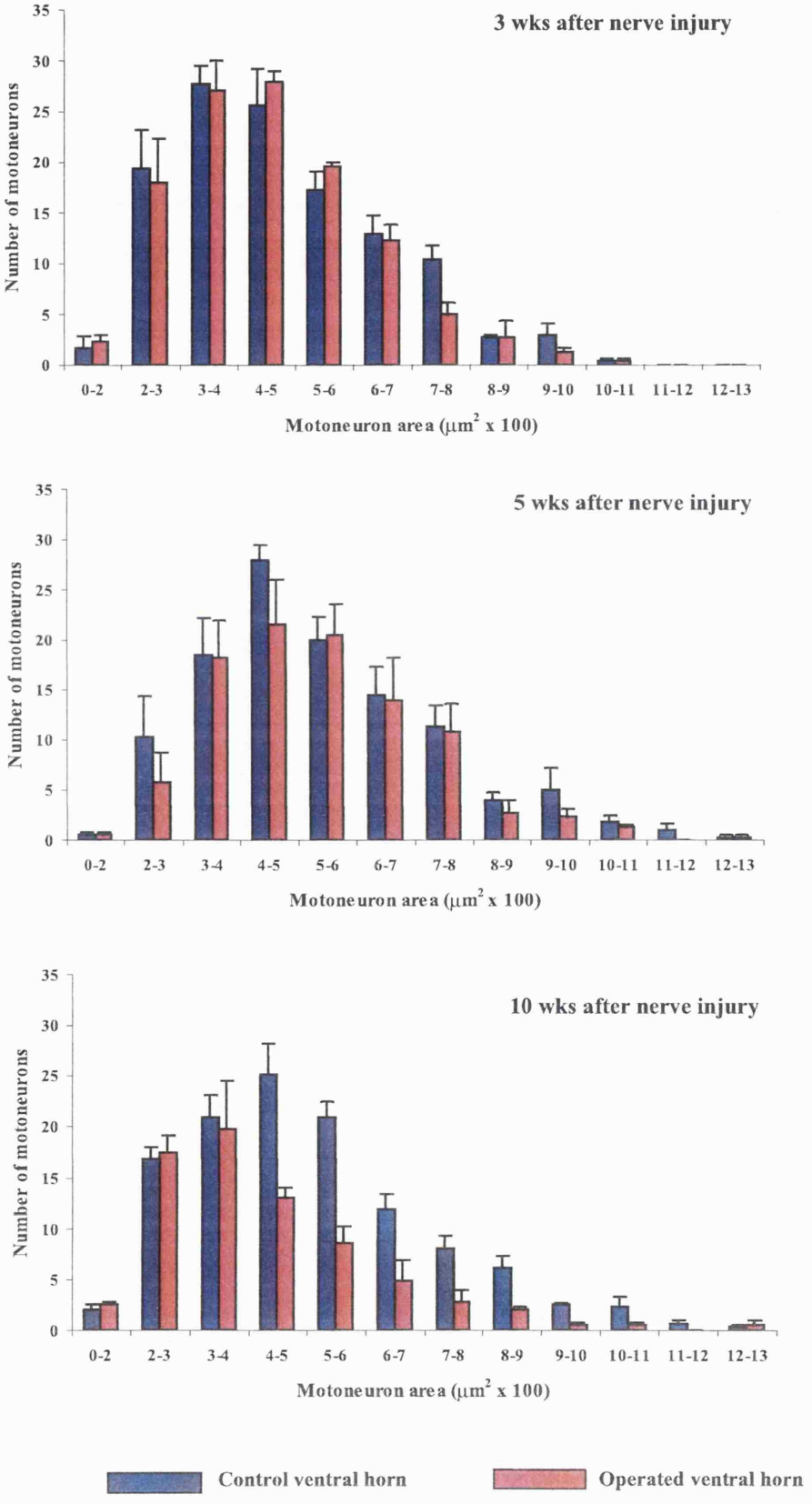
Table 4

Motoneuron area (μm^2)	Motoneuron size distribution (mean ± S.E.M)							
	Time after injury in GAP-43 mice						Time after injury in wild-type mice	
	3 weeks (n=3)		5 weeks (n=4)		10 weeks (n=4)		10 weeks (n=3)	
	CON	OP	CON	OP	CON	OP	CON	OP
0-400	49 ± 6.7	47 ± 7.4	29 ± 7.6	25 ± 6.5	40 ± 1.7	40 ± 4.1	39 ± 4.0	28 ± 6.2
401-700	56 ± 5.1	60 ± 2.6	63 ± 3.5	56 ± 4.9	58 ± 5.1	26 ± 2.6*	73 ± 1.7	81 ± 6.6
701-1300	16 ± 2.2	9 ± 2.0	23 ± 2.9	17 ± 4.4	20 ± 2.6	6 ± 1.0*	12 ± 3.1	12 ± 2.7

Figure 13. The distribution of motoneuron soma size following nerve injury in adult GAP-43 mice

At one month of age the sciatic nerve was crushed in one hindlimb of GAP-43 mice. Motoneuron soma area was measured by tracing the perimeter of each Nissl-stained motoneuron that contained a distinctly stained nucleolus and cytoplasm. All motoneurons in both operated and contralateral ventral horns in every 6^{th} section were measured at 3, 5 and 10 weeks after injury. Motoneuron size was measured in at least 3 animals for each time-point after nerve injury. The data was plotted on a series of frequency histograms (Error bars = S.E.M.). The histograms show that there is no significant change in motoneuron size distribution in the operated ventral horn compared to the control ventral horn, 3 and 5 weeks after nerve injury. However, by 10 weeks after injury, there is a clear reduction in the number of motoneurons in the larger size ranges.

Figure 13


3 wks after nerve injury
Number of motoneurons
0
5
10
15
20
25
30
35
0-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
Motoneuron area (μm² x 100)
5 wks after nerve injury
Number of motoneurons
Motoneuron area (μm² x 100)
10 wks after nerve injury
Number of motoneurons
Motoneuron area (μm² x 100)
Control ventral horn
Operated ventral horn

nerve injury. However, by 10 weeks post-injury, there is a clear reduction in the number of larger motoneurons. Thus, 10 weeks after injury there are significantly fewer motoneurons in the operated ventral horn compared to the control ventral horn within the size ranges: 401-700 μm^2 and 701-1300 μm^2 (see Table 4; $P<0.002$). Therefore, the decrease in mean motoneuron size, 10 weeks after injury, most likely reflects the selective loss of larger motoneurons rather than an overall decrease in the average size of motoneurons.

3.3.4. The effect of exogenous application of NMDA on the survival of injured motoneurons in adult wild-type and GAP-43 mice

Next the vulnerability of adult motoneurons to excitotoxicity was examined in wild-type and GAP-43 mice. Seven days after adult nerve injury, wild-type and GAP-43 mice received an injection of NMDA or sterile saline into the ventral horn on the operated side. The effects of this treatment on motoneuron survival were initially examined 10 weeks later. The results are summarized in Figure 14. It can be seen that the dose of NMDA used in this study was not toxic to injured motoneurons in wild-type mice, since 99% (± 2.7 S.E.M., n=3) of motoneurons survive. In addition, uninjured motoneurons in GAP-43 mice were also able to tolerate the dose of NMDA used. However, after nerve injury, motoneurons in GAP-43 mice show an increased vulnerability to the excitotoxic effects of glutamate, since significantly fewer motoneurons survive following application of the glutamate agonist NMDA, than after nerve injury alone ($P<0.03$). Thus, in GAP-43 mice 10 weeks after nerve injury, 65% (± 3.9 S.E.M., n=4) of motoneurons survive, whereas nerve injury

Figure 14. The effect of NMDA application following nerve injury on motoneuron survival in adult GAP-43 and wild-type mice, assessed 10 weeks later

The sciatic nerve was crushed **(NC)** in one hindlimb of GAP-43 mice **(tg)** at 1 month of age. Seven days after nerve injury, GAP-43 mice and wild-type mice **(wt)** received an injection of NMDA or saline directly into the ventral horn on the operated side. Ten weeks later the effect of NMDA treatment on motoneuron survival was assessed by counting the number of Nissl-stained motoneurons within the sciatic pool, in both the operated and contralateral control ventral horns. The bar diagram shows the mean number of motoneurons in the operated ventral horn expressed as a percentage of that in the contralateral control ventral horn. It can be seen that significantly fewer motoneurons survive following NMDA application compared to nerve injury alone (*$P<0.03$). Also, note that dose of NMDA used was tolerated by motoneurons in GAP-43 mice and wild-type mice, since no motoneuron loss was detected. Error bars = S.E.M.

Figure 14

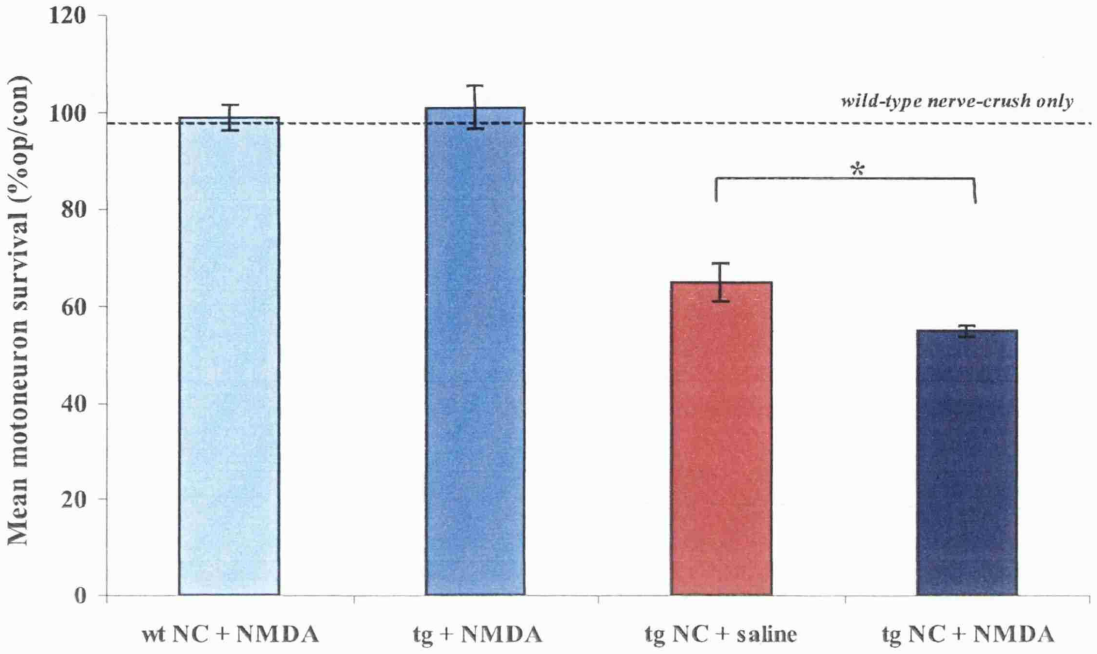
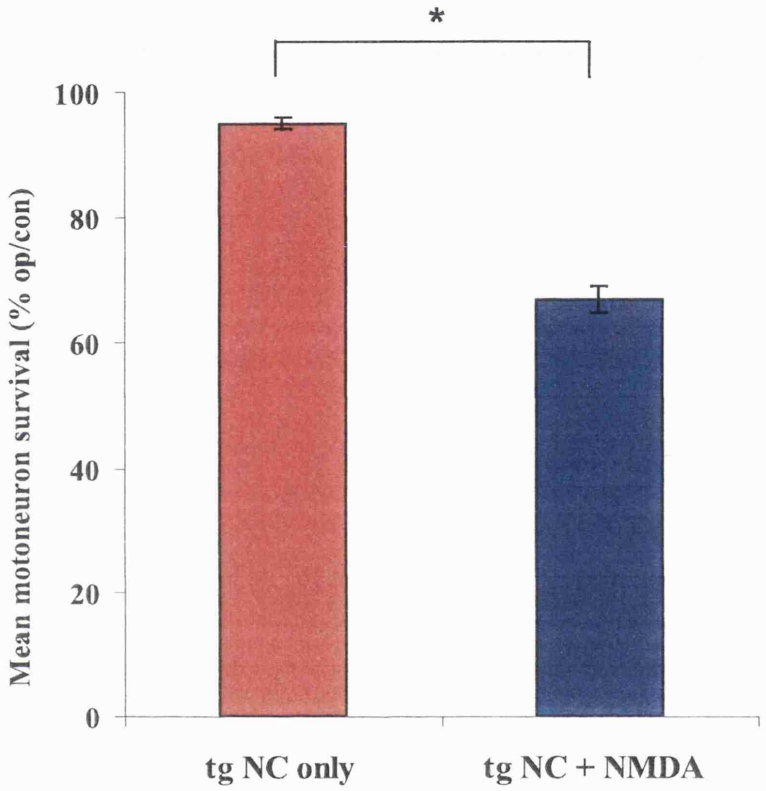

120
100
80
60
40
20
0
Mean motoneuron survival (%op/con)
wild-type nerve-crush only
*
wt NC + NMDA
tg + NMDA
tg NC + saline
tg NC + NMDA

Figure 15. The effect of NMDA application following nerve injury on motoneuron survival in adult GAP-43 mice, assessed 3 weeks later

The sciatic nerve was crushed **(NC)** in one hindlimb of GAP-43 mice **(tg)** at 1 month of age. Seven days after nerve injury, NMDA was injected directly into the ventral horn on the operated side. Three weeks later the effect of NMDA treatment on motoneuron survival was assessed by counting the number of Nissl-stained motoneurons within the sciatic pool, in both the operated and contralateral control ventral horns. The bar diagram shows the mean number of motoneurons in the operated ventral horn expressed as a percentage of that in the contralateral control ventral horn. It can be seen that in GAP-43 mice, 3 weeks after injury, significantly fewer motoneurons survive following NMDA application compared to nerve injury alone (*$P<0.05$). Error bars = S.E.M.

Figure 15


*
100
80
60
40
20
0
Mean motoneuron survival (% op/con)
tg NC only
tg NC + NMDA

followed by NMDA treatment resulted in the survival of only 55% (± 1.1 S.E.M., n=4) of motoneurons.

Since nerve injury in GAP-43 mice increases the vulnerability of injured motoneurons to NMDA mediated excitotoxicity, the next investigation examined whether treatment with NMDA accelerates the rate of death of injured motoneurons in GAP-43 mice. Therefore, motoneuron survival was assessed 3 weeks after nerve injury and treatment with NMDA. The results are summarized in Figure 15, which shows that 3 weeks after nerve injury alone in GAP-43 mice, almost all motoneurons survive (95%, ± 0.9 S.E.M., n=3). However, 3 weeks after nerve injury and NMDA treatment in GAP-43 mice, only 67% (± 2.1 S.E.M., n=4) of motoneurons survive. These results suggest that exogenous application of NMDA to injured motoneurons in GAP-43 mice accelerates the death of motoneurons that were destined to die following nerve injury alone.

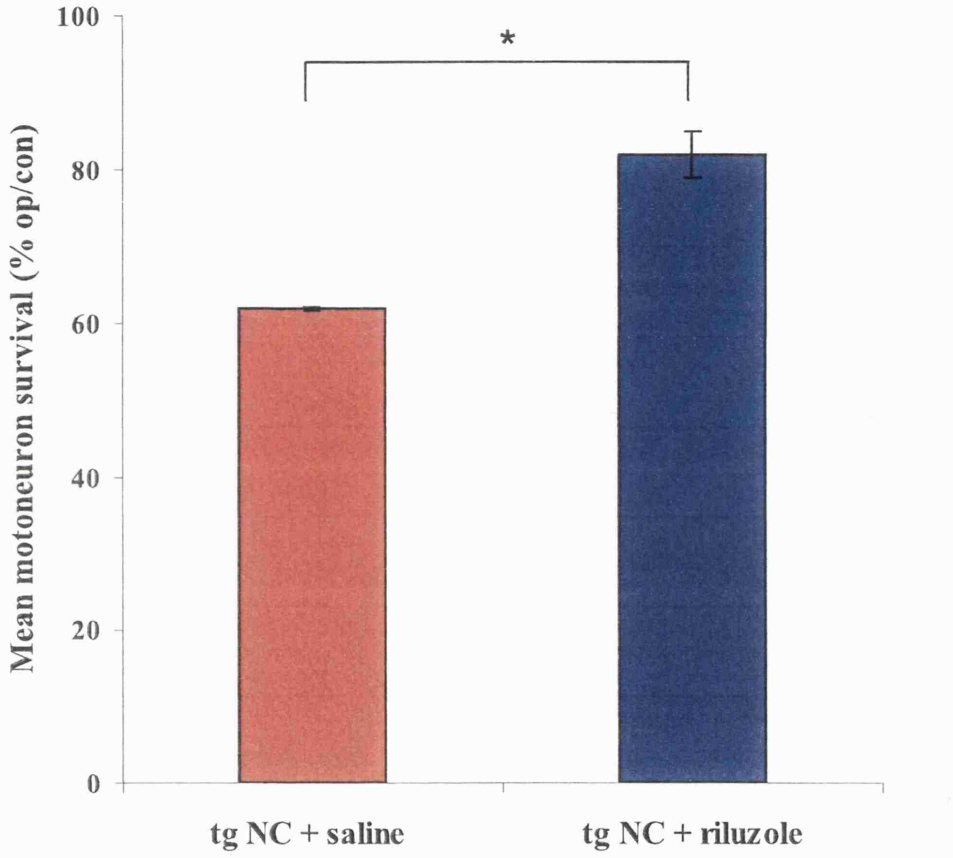
3.3.5. The effect of treatment with riluzole on motoneuron survival and size distribution following nerve injury in adult wild-type and GAP-43 mice

Since nerve injury in adult GAP-43 mice increases the vulnerability of motoneurons to glutamate excitotoxicity, in the next set of experiments the effect of treatment with the glutamate release inhibitor riluzole on motoneuron survival was examined. The results are summarized in Figure 16, which shows that following treatment with riluzole daily for 10 weeks significantly more motoneurons survive following nerve injury in adult GAP-43 mice. Thus, in GAP-43 mice treated with saline for 10 weeks after nerve injury only 62% (± 0.3 S.E.M., n=3) of sciatic motoneurons survive,

Figure 16. The effect of riluzole treatment following nerve injury on motoneuron survival in adult GAP-43 mice

The sciatic nerve was crushed **(NC)** in one hindlimb of GAP-43 mice **(tg)** at 1 month of age. Immediately after nerve injury, the mice were treated with a daily dose of riluzole (16 mg/kg, i.p.) for up to 10 weeks. The effect of riluzole treatment on motoneuron survival was assessed by counting the number of Nissl-stained motoneurons within the sciatic pool, in both the operated and contralateral control ventral horns. The bar diagram shows the mean number of motoneurons in the operated ventral horn expressed as a percentage of that in the contralateral control ventral horn. It can be seen that following nerve injury in GAP-43 mice, significantly more motoneurons survive following riluzole treatment compared to nerve injury alone (*$P<0.05$). Error bars = S.E.M.

Figure 16


100
80
60
40
20
0
Mean motoneuron survival (% op/con)
*
tg NC + saline
tg NC + riluzole

whereas following riluzole treatment 82% (± 3.0 S.E.M., n=5) of motoneurons survive. This increase in motoneuron survival following riluzole treatment is significant ($P<0.05$).

The results presented above indicate that following nerve injury in GAP-43 mice, the larger motoneurons are particularly vulnerable to injury-induced cell death. Therefore, the next assessment sought to determine whether treatment with riluzole rescued these larger motoneurons. The mean motoneuron size and size distribution in GAP-43 mice was therefore established 10 weeks after nerve injury and treatment with either saline or riluzole. The results show that following riluzole treatment the mean area of injured motoneurons is 482 μm^2 (± 22.4 S.E.M., n=4) compared to 423 μm^2 (± 7.3 S.E.M., n=2) following saline treatment. Thus, following treatment with riluzole the mean motoneuron area is similar to that of uninjured motoneurons, which was found to be 487 μm^2 (± 13.0 S.E.M., n=4). In addition, in order to examine whether riluzole treatment saved selective populations of injured motoneurons, size distribution analysis was performed. Figure 17 summarizes the effect of riluzole or saline treatment on the size distribution of sciatic motoneurons in the operated and control ventral horns of GAP-43 mice, 10 weeks after nerve injury. The size distribution graphs show that the loss of motoneurons in the larger range of sizes, which occurs after nerve injury (Fig. 17B), is prevented following riluzole treatment (Fig. 17A). However, there still appears to be a substantial loss of motoneurons in the operated ventral horn in the mid-range (400-500 μm^2) following riluzole treatment, compared to the control ventral horn (Fig. 17B). However, in the riluzole treated group no significant differences were found between the mean motoneuron sizes in the operated ventral horn compared to the control ventral horn for all 3 grouped size

Figure 17. The effect of riluzole on motoneuron size distribution following nerve injury in adult GAP-43 mice

At one month of age the sciatic nerve was crushed in one hindlimb of GAP-43 mice. These mice were then treated with a daily dose of riluzole (16 mg/kg, i.p.) or sterile saline for up to 10 weeks. Motoneuron soma area was measured by tracing the perimeter of each Nissl-stained motoneuron that contained a distinctly stained nucleolus and cytoplasm. All motoneurons in both operated and contralateral ventral horns in every 6^{th} section were measured 10 weeks after injury. The data was plotted as frequency histograms (Error bars = S.E.M.). (A) The histogram shows the effect of daily riluzole treatment on motoneuron size distribution 10 weeks after nerve injury. (B) The histogram shows the effect of nerve injury and saline treatment on motoneuron size distribution. It can be seen that the number of larger motoneurons surviving nerve injury following riluzole treatment is greater than in the saline treated group.

Figure 17

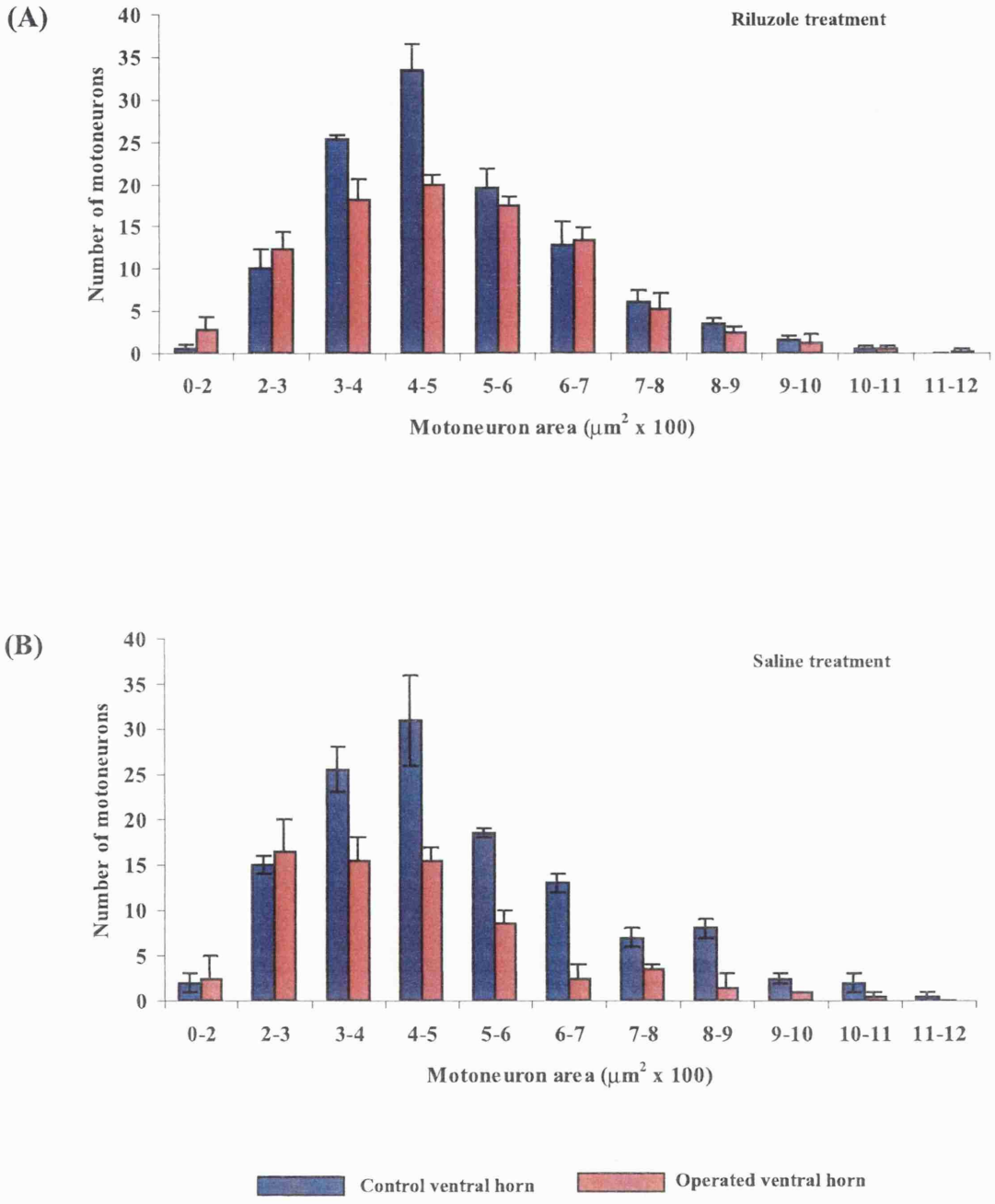

(A)
Riluzole treatment
Number of motoneurons
40
35
30
25
20
15
10
5
0
0-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
Motoneuron area (μm² x 100)
(B)
Saline treatment
Number of motoneurons
40
35
30
25
20
15
10
5
0
0-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
Motoneuron area (μm² x 100)
Control ventral horn
Operated ventral horn

Table 5. The effect of riluzole on motoneuron size distribution following nerve injury in adult GAP-43 mice

The Table summarizes the results from sizing Nissl-stained motoneurons following daily treatment with riluzole or saline, in adult GAP-43 mice. The size of all motoneurons in both the operated (OP) and control (CON) ventral horns was measured in GAP-43 mice, 10 weeks after nerve injury (NC; n = number of animals; ± = S.E.M.). The Table shows that in the saline treated group, 10 weeks after nerve injury there is a significant reduction in the number of motoneurons in the larger size ranges (*$P<0.05$). However, in the riluzole treated group there are no significant differences between the sizes of motoneurons in the operated and control ventral horns for all size ranges.

Table 5

Motoneuron size distribution (mean ± S.E.M)				
Motoneuron area (μm^2)	NC + Saline (n=2)		NC + Riluzole (n=4)	
	CON	OP	CON	OP
0-400	42 ± 4.5	35 ± 1.5	36 ± 2.8	33 ± 5.0
401-700	63 ± 3.5	27 ± 1.5*	62 ± 6.5	51 ± 3.0
701-1200	20 ± 3.0	7 ± 1.5*	12 ± 1.6	10 ± 3.1

ranges (0-400, 401-700, and 701-1300 μm^2; see Table 5). In contrast, in the saline treated group, nerve injury in GAP-43 mice results in a significant loss of motoneurons in both mid range (401-700 μm^2) and larger range (701-1300 μm^2) of motoneuron sizes (see Table 5). Therefore, these results indicate that riluzole treatment has prevented the death of a proportion of large motoneurons that would otherwise die as a consequence of nerve injury in GAP-43 mice.

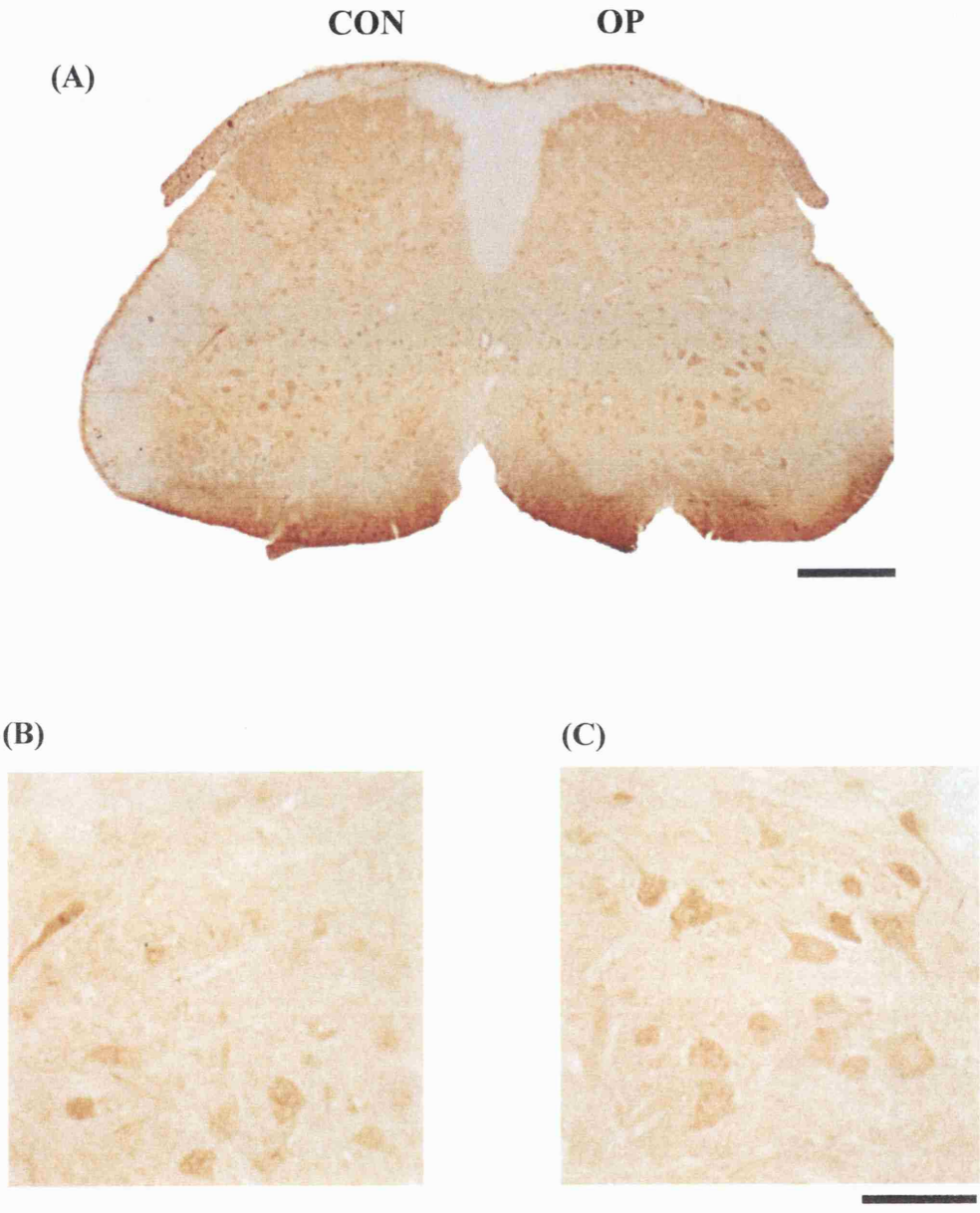
3.3.6. Ubiquitin immunoreactivity in motoneurons following nerve injury in adult wild-type and GAP-43 mice

In order to investigate another mechanism that may contribute to motoneuron loss following nerve injury in GAP-43 mice, the possible development of cytoplasmic inclusions was assessed in motoneurons of GAP-43 mice following injury. Therefore, the presence of ubiquitin immunoreactivity was examined in spinal cords of wild-type and GAP-43 mice, 1, 3, 5, and 10 weeks after nerve injury. Figure 18 shows an example of ubiquitin immunoreactivity in the spinal cord of a wild-type mouse, 1 week after nerve injury. It can be seen that there is a greater proportion of ubiquitin-positive motoneurons in the operated compared to the control ventral horn. This pattern of ubiquitin staining was seen in a majority of spinal cord sections processed and is therefore unlikely be due to a smaller number of motoneurons in the ventral horn on the unoperated side. The presence of a larger number of ubiquitin positive injured motoneurons compared to uninjured motoneurons in the control ventral horn was also seen in GAP-43 mice, 1 week after injury. However, at no time after nerve injury (1, 3, 5 and 10 weeks) were ubiquitin positive inclusions (skein-like inclusions) found in injured sciatic motoneurons of GAP-43 and wild-type mice.

Figure 18. The effect of nerve injury in adult wild-type mice on the expression of ubiquitin in sciatic motoneurons

(A) The photomicrograph shows a cross-section (20 μm) of the lumbar region of a 5-week old wild-type mouse, 1 week after a unilateral sciatic nerve-crush. The spinal cord section was stained for ubiquitin immunoreactivity using a polyclonal antibody. It can be seen that there is an increase in the number of ubiquitin-positive motoneurons in the operated (OP) ventral horn compared to the control (CON) ventral horn. (B) and (C) show a higher magnification of the sciatic motor pools in the operated and control ventral horns. The pattern of ubiquitin immunoreactivity in this section is representative of that seen in a majority of spinal cord sections processed and is likely to reflect an increase in the expression of ubiquitin in injured motoneurons, rather than fewer motoneurons being present in the control ventral horn. Scale bars, A = 250 μm, B and C = 50 μm.

Figure 18



3.4. DISCUSSION

The results presented in this Chapter, show that sciatic nerve injury carried out during the period of axonal sprouting in adult GAP-43 mice results in a progressive loss of sciatic motoneurons, the majority of which occurs between 5 and 10 weeks after nerve injury. In addition, larger motoneurons appear to be selectively vulnerable to nerve injury. Furthermore, glutamate-mediated excitotoxicity may play a role in this motoneuron degeneration. These findings therefore suggest that large motoneurons forced to grow for a prolonged period are more vulnerable to excitotoxic cell death than normal.

These results confirm those of a previous study, which have shown that nerve injury in adult GAP-43 mice results in a dramatic loss of soleus motoneurons (Harding et al, 1999). The findings of the present study are also consistent with other experiments, which have demonstrated that repeated stimuli for axonal growth result in motoneuron death even in adult rats (Arvidsson and Aldskogius, 1982; White et al, 2000). Furthermore, Purkinje cells which overexpress GAP-43 in vivo (Buffo et al, 1997) and in vitro (Wehrle et al, 2001) have also been shown to be vulnerable to injury. Conversely, absence of GAP-43 dramatically increases the resistance of sensory neurons to apoptotic stimuli in vitro (Gagliardini et al, 2000).

3.4.1. Progressive loss of injured motoneurons in GAP-43 mice

In these experiments, motoneuron death occurs progressively between 5-10 weeks after nerve injury in adult GAP-43 mice. This protracted period of injury-induced

motoneuron death suggests that it is the extensive growth of motor axons rather than an increased vulnerability to the nerve injury per se that is the primary factor involved with motoneuron loss. Following axonal injury, the motoneuron cell body undergoes a marked increase in mRNA synthesis and altered gene expression resulting in a transition from a mature "transmitting" phenotype to a "regenerating" phenotype (reviewed by Fu and Gordon, 1997). Specifically, motoneurons reduce their expression of enzymes associated with neurotransmission (e.g. choline acetyltransferase; ChAT; Borke et al, 1993; Kou et al, 1995; Rende et al, 1995; Jacobsson et al, 1998) and up-regulate proteins associated with axonal elongation (e.g. GAP-43 Skene and Willard, 1981; Skene, 1989). Thus, mature adult motoneurons that are regenerating display a similar phenotype to developing motoneurons. Indeed, developing motoneurons are known be vulnerable to cell death when growing (Lowrie et al, 1987) and the period for which they remain vulnerable can be extended by forcing them to continue to grow (Greensmith et al, 1992; Harding et al, 1998; Greensmith et al, 2000). A nerve crush injury in adult GAP-43 mice leads to a marked potentiation of the regenerative capacity of injured motoneurons resulting in accelerated axonal growth (Aigner et al, 1995). Therefore, motoneurons in GAP-43 mice, which are spontaneously sprouting, appear to be primed to a further stimulus for growth. This accelerated axonal growth is likely to be similar to that seen following a conditioning lesion, which also significantly increases the rate of axonal regeneration (McQuarrie & Grafstein, 1973) due to the elevated expression of cytoskeletal proteins (Tetzlaff et al, 1996). However, the metabolic demands placed upon motoneurons, which undergo accelerated growth may be a factor that contributes to the loss of injured motoneurons in GAP-43 mice. The progressive loss of motoneurons that occurs 5-10 weeks after nerve injury

possibly reflects the inability of motoneurons to withstand this amount of growth for a prolonged period. Moreover, as motoneurons die following nerve injury in GAP-43 mice, surviving motoneurons sprout to innervate the denervated muscle territory, thereby enlarging their peripheral fields (Brown and Holland, 1979) and undergo increased levels of growth. Furthermore, in normal rats, extensive sprouting occurs for many weeks after muscle reinnervation (Ostberg et al, 1986) and this is likely to be increased in GAP-43 mice. Indeed, it has been shown that the extent of terminal arborisation after muscle reinnervation is increased in GAP-43 mice (Aigner et al, 1995). Thus, muscle reinnervation by collateral sprouting is a slow process, which in turn, may place motoneurons under higher levels of metabolic stress for many weeks after muscle reinnervation.

However, it is important to note that the spontaneous sprouting caused by the expression of the transgene in uninjured GAP-43 mice does not, in the long term, result in the loss of motoneurons (Aigner et al, 1995). The expression of the GAP-43 transgene results in prominent axonal sprouting at the NMJ for 2-3 months following the down-regulation of endogenous GAP-43 (Aigner et al, 1995). However, it has previously been shown that collateral sprouting of motoneurons in mice can occur without significant intervention of metabolic changes in the cell body (Bisby et al, 1996). In contrast, the metabolic changes that occur in the cell body of injured motoneurons in response to the requirements of axonal regeneration and functional reinnervation are likely to result in higher metabolic demands, which may contribute to the death of injured motoneurons in GAP-43 mice.

3.4.2. Selective vulnerability of larger motoneurons after injury in GAP-43 mice

This present study also shows that motoneurons with a larger soma area appear to be selectively vulnerable to nerve injury in GAP-43 mice, since the mean motoneuron area decreased significantly in the operated compared to the control ventral horn during the period of motoneuron death (5-10 weeks after injury). In addition, size distribution analysis of surviving motoneurons shows a significant reduction in the number of motoneurons within the larger soma size ranges. However, there are a number of alternative interpretations, which need to be addressed. Firstly, it is possible that following nerve injury motoneuron atrophy occurs stochastically across all soma sizes, resulting in a complete shift of the distribution of motoneuron sizes to the left. If then a proportion of those motoneurons, which have undergone atrophy, are lost, the resulting motoneuron size distribution may show a spurious loss of only larger motoneurons. However, atrophy followed by an unselective loss of motoneurons across all size ranges, is likely to result in a greater number of motoneurons within the smaller soma size ranges and this was not found to be the case (see Figure 13). A second possibility could be that only larger motoneurons undergo atrophy after nerve injury, followed by a selective loss of smaller motoneurons. This would also result in a decrease in average motoneuron size and the apparent loss of larger motoneurons after the period of motoneuron death. However, again this interpretation seems unlikely since size distribution analysis does not show any significant reduction in the size of larger motoneurons at 5 weeks after injury, which is just prior to the period of motoneuron death. Therefore, the decrease in mean motoneuron size 10 weeks after injury most likely reflects the

selective loss of larger motoneurons rather than a specific atrophy of larger motoneurons.

A number of factors might account for this selective loss of larger motoneurons. It has been argued above, that one of the contributing factors to motoneuron death after injury may be the metabolic burden placed upon motoneurons, which undergo an extensive and prolonged period of growth. It is possible that larger motoneurons, which are known to have larger peripheral axons and innervate larger peripheral muscle territories (Henneman and Olson, 1965a; Henneman, 1985), are placed under a higher degree of stress during regeneration following nerve injury compared to

smaller motoneurons. Nerve injury induces a marked increase in mRNA synthesis to produce the cytoskeletal proteins necessary for the regenerating axons to re-establish contact with their original muscle fibres (reviewed by Ide, 1996). Even after muscle reinnervation, the expression of neurofilaments is also up-regulated to increase the final diameter of the axon, which is determined by the size of the muscle target they reinnervate (Hoffman and Cleveland, 1988). Thus, larger motoneurons will require a greater regenerative response to nerve injury than smaller motoneurons, which in turn may increase the metabolic burden placed on larger motoneurons, resulting in their selective vulnerability.

3.4.3. Injured motoneurons in GAP-43 mice show an increased susceptibility to excitotoxic cell death

Developing motoneurons which are forced to maintain their growth state as a consequence of nerve injury or muscle paralysis have been shown to be more susceptible to the excitotoxic effects of the glutamate agonist, NMDA (Greensmith et al, 1994a; Greensmith et al, 1995). Moreover, treatment with dizocilpine maleate (MK-801), an NMDA antagonist, can prevent this death of motoneurons induced by injury or paralysis (Mentis et al, 1993; Greensmith et al, 1994b). Therefore, this study also examined whether adult motoneurons, which are forced into an extensive and prolonged phase of growth are also more vulnerable to excitotoxicity than normal. The results presented in this Chapter suggest that injured motoneurons in GAP-43 mice have an increased vulnerability to the excitotoxic effects of glutamate, since more motoneurons die following the application of NMDA, and the rate of motoneurons loss is also increased. The dose of NMDA used in this study is non-

toxic, since application of NMDA to uninjured motoneurons in wild-type, as well as GAP-43 mice, did not result in loss of motoneurons. Moreover, nerve injury in wild-type mice did not increase the vulnerability of injured motoneurons to excitotoxicity.

Several factors may account for this increased vulnerability of injured growing motoneurons in GAP-43 mice to excitotoxicity. Glutamate is the main excitatory neurotransmitter in the spinal cord (Davidoff et al, 1967; Cotman et al, 1988) and spinal motoneurons express high levels of glutamate activated NMDA receptors (Tolle et al, 1993) and Ca^{2+} permeable AMPA receptors (Williams et al, 1997). Therefore, application of the glutamate agonist NMDA permits high level influx of Ca^{2+} into the cell, which has been shown to be toxic to neurons (Choi, 1988; Choi et al, 1988; Meldrum and Garthwaite, 1990). The retrograde reaction of motoneurons to axonal injury is to undergo a series of structural and metabolic events, which alters gene expression from that required for neurotransmission to growth and cell repair (Fu and Gordon, 1997). This shift in phenotype results in a transient swelling of the cell body, a decrease in size of dendritic trees, and a reduction in the number of synaptic boutons contacting the injured motoneuron (Blinzinger and Kreutzberg, 1968 Sumner and Sutherland, 1973; Brannstrom et al, 1992a; Brannstrom and Kellerth, 1998). These structural changes to the motoneuron dendritic surface area and synaptic input can be seen as a rescue effort to reduce the metabolic burden on the cell, which is undergoing increased levels of protein production involved with axonal elongation. Furthermore, there is also a significant reduction of monosynaptic excitatory potentials in lumbar motoneurons (Eccles et al, 1958; Kuno and Llinas, 1970a), which may be a result of the removal of excitatory input after axotomy. However, while synaptic transmission may be depressed, axotomized motoneurons

are hyperexcitable (Campbell, 1944; Kuno and Llinas, 1970b). Therefore, treating injured motoneurons with NMDA during this period of hyperexcitability, and increasing oxidative stress, due to the higher levels of Ca^{2+} influx, may increase the motoneurons vulnerability to cell death.

However, this does not explain why injured motoneurons in GAP-43 mice are more susceptible to NMDA application compared to normal wild-type mice. As previously discussed, the metabolic stress placed upon the regenerating motoneurons in GAP-43 mice is likely to be greater than normal, due to the accelerated rate of axonal growth. Therefore, any additional demands placed upon the cell such as that generated by NMDA application, will have a more deleterious effect on those motoneurons, which are already under higher levels of stress. Similar mechanisms may also be involved in the increased vulnerability of injured developing motoneurons to glutamate excitotoxicity previously reported (Mentis et al, 1993; Greensmith et al, 1994a, 1994b; Greensmith et al, 1995).

Application of exogenous NMDA, as used in these experiments, does not reflect the level of physiological excitation motoneurons may receive following nerve injury, since there is evidence to suggest that the level of synaptic activation actually decreases following adult nerve injury. For example, it has been shown that the number of synaptic profiles on axotomized motoneurons declines in the first few days following nerve injury (Brannstrom et al, 1992a). Another feature of axotomized neurons is a reduction in the dendritic surface area (Blinzinger and Kreutzberg, 1968; Sumner and Sutherland, 1973). Even so, it is clear that injured motoneurons in GAP-43 mice die by a mechanism that is likely to involve glutamate

excitotoxicity. Therefore, how might excitotoxicity play a role in the demise of injured motoneurons in GAP-43 mice? Significantly, it has been shown that these changes induced by injury to adult motoneurons may be reversed following restoration of functional neuromuscular contact. For example, in axotomized spinal motoneurons, reinnervation is followed by the restitution of dendritic surface area (Brannstrom et al, 1992b). Furthermore, the most proximal dendrites are capable of attracting new boutons to such an extent that their total exceeds that found in uninjured motoneurons (Brannstrom and Kellerth, 1998). This partial recovery of synaptic input and dendritic surface area after reinnervation is likely to result in an increase in excitatory input 1-2 weeks after nerve injury. This may explain the progressive nature of motoneuron death after injury in GAP-43 mice, since extensive motoneuron growth is likely to continue in conjunction with the re-established levels of excitatory input, thereby placing the motoneuron under higher levels of metabolic stress.

When assessing the possible role of excitotoxicity in motoneuron death in GAP-43 mice, it is also important to consider the effect of adult nerve injury on glutamate receptor expression. Although adult motoneurons normally survive peripheral nerve injury and therefore do not necessarily show a change in their vulnerability to glutamate excitotoxicity, previous reports have indicated that there are changes in glutamate receptor subunit expression. Excitatory transmission in motoneurons is mediated by both the AMPA/kainate and NMDA receptors. AMPA receptors consist of different combinations of GluR1, GluR2, GluR3, and GluR4 subunits. A previous study has shown that sciatic nerve injury induces a large decrease in GluR2/3-immunoreactivity compared with GluR-4 in motoneuron somata. (Alvarez et al,

2000). This change in AMPA receptor expression may result in higher levels of Ca^{2+} influx, since AMPA receptors lacking the GluR2 subunit increases the channels permeability to Ca^{2+} (Hollmann et al, 1991) These differences in the composition of AMPA channels after nerve injury may explain the more prolonged excitatory postsynaptic potentials observed in axotomized motoneurons (Kuno and Linas, 1976b; Mendell et al, 1976), and therefore has the potential to initiate excitotoxic effects (Pellegrini-Giampietro et al. 1997; Shaw et al, 1999). In contrast, it has been shown with the use of in situ hybridisation and immunohistochemistry that a reduction in the expression of the NMDA subunit NR1 was paralleled by a decrease in the NR1 subunit protein after sciatic nerve transection (Piehl et al, 1995). The fact that NMDA receptors consist of at least one NR1 subunit (Hollmann and Heinemann, 1994), it was concluded that NMDA receptors are down-regulated in axotomized motoneurons. However, there is a time-dependent functional change in the NMDA receptor, resulting in a reduction of Mg^{2+} block of NMDA responses following axonal injury to motoneurons (Furukawa, et al, 2000). This suggests that injured motoneurons acquire immature characteristics of plasticity with respect to the sensitivity of NMDA receptors to extracellular Mg^{2+} and a long-term increase in the susceptibility to Ca^{2+}-mediated excitotoxicity.

Overall, axonal injury to motoneurons induces a complex series of structural and metabolic changes that may result in reducing glutamatergic input, thereby attenuating synaptic transmission and reducing excitotoxic influences. However, from the evidence presented above, these changes are unlikely to be completely protective against excitotoxic events, since injured motoneurons undergo restoration of synaptic inputs and dendritic surface area upon muscle reinnervation.

Furthermore, the increased expression of calcium permeable AMPA receptors and possible hyperexcitability produced by a decrease in Mg^{2+} block of NMDA responses after injury may confer a significant vulnerability to excitotoxicity.

3.4.4. Protective effects of riluzole on injured motoneurons in GAP-43 mice

The present results show that treatment with riluzole rescues a proportion of motoneurons that normally die following sciatic nerve injury in GAP-43 mice. Riluzole is a compound that reduces glutamatergic excitation and has neuroprotective effects on injured neurons in a number of experimental models. For example, riluzole can reduce the damage caused by ischemia in the brain and spinal cord (Pratt et al, 1992; Lang-Lazdunski et al, 1999), protect motoneurons in vitro from glutamate induced toxicity (Rothstein and Kuncl, 1995; Estevez et al, 1995) and in vivo following ventral root avulsion (Nogradi and Vrbová, 2001). Moreover, riluzole can apparently improve the survival of ALS patients, especially those with bulbar onset (Bensimon et al, 1994; Meininger et al, 1997). Thus, the results of the present study support the idea that glutamate excitotoxicity may be a contributing factor to the death of motoneurons, which have been forced to undergo extensive and prolonged growth. So how might riluzole exert its neuroprotective effects? Riluzole acts to reduce excitation through a complex mechanism involving the blockade of voltage-activated Na^{+} channels, interfering with ionic influx through NMDA channels and inhibition of presynaptic glutamate release (Chéramy et al, 1992; Debono, et al, 1993; Hebert et al, 1994). Therefore, riluzole is capable of interfering both pre- and postsynaptically with glutamatergic neurotransmission, thereby reducing the levels of Ca^{2+} influx into the damaged motoneuron. There is direct

evidence that activation of glutamate receptors and the influx of Ca^{2+} result in the formation of reactive oxygen species (ROS), which have been implicated in initiating both neural apoptosis and necrosis (Mattson et al, 2000). High levels of Ca^{2+} passing through NMDA receptors and voltage-gated Ca^{2+} channels lead to impaired function of the mitochondrial electron transport system, and subsequent ROS formation. These ROS then attack critical cellular components (Azbill et al, 1997), resulting in cell death.

The results of this study suggest that inducing extensive and prolonged axonal growth may exert a high metabolic burden upon the regenerating motoneuron. This could result in high levels of oxidative stress, and coupled with the influx of Ca^{2+} via NMDA and AMPA receptor activation, may cause mitochondrial dysfunction, which ultimately results in the death of injured motoneurons. Riluzole may exert its neuroprotective effects by reducing glutamatergic excitation, thereby reducing oxidative stress placed upon the growing motoneuron. This proposal is supported by the finding that sciatic nerve injury results in high oxidative stress due to the abnormal accumulation of metabolically active mitochondria in the perikaryon of injured motoneurons (Martin et al, 1999). Furthermore, riluzole treatment was found to improve mitochondrial function, and enhance glutamate and glucose uptake following neuronal injury (Mu et al, 2000).

3.4.5. Ubiquitin expression in injured motoneurons

Ubiquitin is known to play a crucial role in the degradation of many cytosolic, nuclear and rough endoplasmic proteins (Hochstrasser, 1996). Following injury to

adult motoneurons, the cell body undergoes chromatolysis, which results in considerable swelling of the cell body, and reflects an increase in protein synthesis and cellular metabolism required to restore the motoneurons peripheral axon (Watson, 1974). This increase in the rate of protein synthesis may place more demands on the ubiquitin-proteosome system. As a consequence the expression of ubiquitin is therefore likely to increase. Indeed, the results of this study show that 1 week after sciatic nerve-crush the levels and number of ubiquitin positive motoneurons appears to be greater in the operated ventral horn compared to the control ventral in wild-type mice. This result is consistent with other findings, which have shown an increase in ubiquitin mRNA expression in motoneurons of the hypoglossal nuclei, 1 week after nerve injury (Savedia and Kiernan, 1994). Moreover, these authors also found that ligating the transected nerve, thereby preventing axonal regeneration, significantly reduced the normal increase in ubiquitin expression. These results suggest that ubiquitin-mediated proteolysis is a metabolic process involved in axonal regeneration. However, it appears that the ubiquitin-proteosome system is able to withstand the continued demands for growth associated protein synthesis in GAP-43 mice following nerve-injury, since at no time were ubiquitin immunoreactive skein-like cytoplasmic inclusions observed in the cell bodies of injured motoneurons.

3.4.6 Conclusions

In summary, the results presented in this Chapter show that sciatic nerve injury carried out during the period of axonal growth in GAP-43 mice results in the progressive death of motoneurons. Furthermore, size distribution analysis indicates

that motoneurons in the larger size range are selectively affected. It also appears that excitotoxicity contributes to the death of motoneurons, since application of exogenous NMDA to injured motoneurons results in a greater loss of motoneurons and an accelerated rate of death in GAP-43 mice. Consistent with these findings are results, which show that treating these injured motoneurons with the glutamate release inhibitor, riluzole, rescues a proportion of motoneurons that were destined to die. On the basis of these findings, it is possible that motoneurons which are forced to grow extensively and for a prolonged period are subjected to high metabolic stress due to the demands of the regenerative process coupled with excitatory input, which may ultimately result in mitochondrial dysfunction and cell death.

These findings may have important implications for our understanding of the progressive nature of ALS, since the extensive motor axon sprouting known to occur during the disease process may exacerbate the oxidative stress that these diseased motoneurons are required to tolerate. This may contribute to the vulnerability of surviving motoneurons to cell death, resulting in a rapid decline of motor function and reducing the life-span of the patient.

CHAPTER 4

THE EFFECT OF INDUCING AXONAL GROWTH ON DISEASE PROGRESSION IN A MOUSE MODEL OF MOTOR NEURON DISEASE

4.1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of neurons in the motor system. During the relentless course of the disease, extensive growth and enlargement of the motoneurons peripheral territory occurs to compensate for the initial death of a proportion of motoneurons, thereby re-establishing innervation of denervated muscle fibres. Recent findings demonstrate that even the survival of healthy adult motoneurons is compromised by the prolonged growth of motor axons. Therefore, the present study examines whether the continued growth of motor axons is a key factor responsible for the rapid degeneration of motoneurons in ALS.

4.1.1. ALS and superoxide dismutase

ALS is the most common variant of motor neuron disease affecting adults, having a prevalence of ~5 per 100,000 individuals (Smith and Appel, 1995). The primary hallmark of ALS is the selective death of motoneurons in the spinal cord, brain stem, and motor cortex. The loss of motoneurons leads to progressive atrophy of skeletal muscles, paralysis and ultimately death, within 2 to 5 years of clinical onset (Cleveland and Rothstein, 2001). The majority of ALS cases are sporadic (SALS; 90%) with unknown aetiology, but in the remaining cases, the disease is inherited in an autosomal dominant manner and is known as familial ALS (FALS; reviewed by Gaudette et al, 2000). The majority of both familial and sporadic ALS cases are clinically indistinguishable and share common pathological features such as abnormal protein accumulations in degenerating motoneurons. An important insight

into the pathogenesis of FALS came from the discovery of mutations in the gene encoding for Cu/Zn superoxide dismutase 1 (SOD1) in a subset of patients with familial ALS (Deng et al, 1993; Rosen et al, 1993). SOD1 is a ubiquitous cytosolic enzyme involved in the conversion of superoxide anion to hydrogen peroxide (Fridovich, 1995), and is thought to protect against cellular damage by scavenging toxic superoxide radicals (Bowling et al, 1993). To date, over 90 mutations have been discovered spanning all exons of the SOD1 gene, most of these resulting in the substitution of one amino-acid (Orrell, 2000).

4.1.2. Mutant SOD1 toxicity

Several transgenic mice have been generated that express the FALS-linked mutant human SOD1 gene (i.e. G93A mice and G85R mice) and develop the clinical and pathological hallmarks similar to that seen in ALS patients (Gurney et al, 1994; Ripps et al, 1995; Wong et al, 1995; Bruijn et al, 1997). Despite the production and extensive study of these transgenic mouse models of FALS, the mechanism by which mutant SOD1 causes the selective degeneration of motoneurons still remains unclear. Initially, mutant SOD1 toxicity was believed to be a result of compromised enzymatic activity leading to accumulation of harmful superoxide anions (Borchelt et al, 1994; Tsuda et al, 1994; Fujii et al, 1995). However, the picture is more complex, since this idea is not supported by measurement of enzymatic activity and by transgenic mouse studies. For example, the G1H line of mice that expresses mutant human SOD1 (Gly93→Ala; G93A) at approximately 5 times the endogenous level, developed progressive motor neuron disease despite markedly elevated SOD1 activity levels (Gurney et al, 1994). This finding was extended by the generation of 3

other mice that expressed different SOD1 mutations, which resulted in a large degree of variation with respect to enzymatic activity, polypeptide half-life and resistance to proteolysis, and these variables did not correlate with age of onset or disease progression (Ripps et al, 1995; Wong et al, 1995; Bruijn et al, 1997). In addition, a mouse completely deficient in SOD1 activity, by deletion of the entire coding sequence, develops normally and shows no sign of motor neuron disease (Reaume et al, 1996). These studies suggest that mutations in SOD1 cause motor neuron disease through a gain of toxic properties and not a loss of function. Several hypotheses have been proposed concerning the toxic gain of function of mutant SOD1. One proposal is that misfolding of SOD1 induced by mutations would allow the access of abnormal substrates such as peroxynitrate, resulting in the nitration of tyrosine residues (Beckman et al, 1993). Another hypothesis suggests that SOD1 mutants have enhanced ability to use hydrogen peroxide as a substrate to generate toxic hydroxyl radicals (Wiedau-Pazos et al, 1996). These aberrant activities of mutant SOD1 would increase oxidative stress in neurons, leading to cellular dysfunction and death. In agreement, there is evidence of elevated hydroxyl radical activity in mutant SOD1 mice (Bogdanov et al, 1998) and of oxidative damage in neuronal tissue of sporadic and familial ALS (Ferrante et al, 1997). However, the pathogenic pathway of mutant SOD1 toxicity has not been causally linked to neuronal damage in ALS, and the selective degeneration of motoneurons has not been resolved.

A number of other mechanisms that may contribute to motoneuron degeneration in ALS have been proposed. These include, apoptosis, excitotoxicity, and protein aggregation, which are described in detail in Chapter 1 (see 1.8). However, it is currently unclear, which of these mechanisms plays a primary role in the

pathogenesis of ALS. Furthermore, current knowledge of the pathogenic mechanisms of ALS is largely based on studies with mutant SOD1, which is responsible for only 2% of all ALS cases. Therefore, to gain a better understanding of the factors involved in causing motoneuron loss in ALS, a detailed analysis of the progression of the disease process in terms of motoneuron pathology, motor deficits and motoneuron loss may help to delineate the sequence of events leading to motoneuron degeneration.

4.1.3. Characterization of G93A mutant SOD1 mice

Several different strains of mutant SOD1 transgenic mice have been created (Gurney et al, 1994; Ripps et al, 1995; Wong et al, 1995; Bruijn et al, 1997). These differ in the SOD1 mutation analyzed, the species in which the mutant SOD1 is placed and the level of expression of the mutant protein; all of which affects the timing and severity of motor neuron disease.

The experiments described in this Chapter were carried out using SOD1-G93A transgenic mice (TgN (SOD1-G93A) Gur1). These G93A mice are healthy until ~90 days of age, at which time they develop motor symptoms such as hyper-reflexia, crossed spread of spinal reflexes and shaking in the limbs when suspended (Chiu et al, 1995). Over the next month the mice ~~grow~~ become weaker, which develops into progressively worsening paresis involving primarily the hindlimbs with atrophy of the skeletal musculature. At the end-stage of the disease (~130 days) the mice are severely paralyzed as a result of loss of motoneurons from the ventral spinal cord (Tu et al, 1996). Importantly, transgenic mice that express the wild-type form of human

SOD1 at comparable protein and activity levels to that seen in the transgenic mice expressing the mutant form, never develop motor neuron disease (Dal Canto and Gurney, 1995; Tu et al, 1996).

Extensive pathology develops in the spinal cord well before the display of overt symptoms of motor neuron disease. As early as 30 days of age, subtle vacuolar changes are seen in motor axons where they enter ventral roots, and insoluble protein complexes are present in the spinal cords of G93A transgenic mice, discerned by biochemical assay (Chiu et al, 1995; Johnston et al, 2000). Fragmentation of the Golgi apparatus is also observed in asymptomatic mice, 2 months before the first clinical signs of the disease (Mourelatos et al, 1996). By 60 days of age, vacuolar changes have extended to the motoneuron cell body, which are derived from the swelling of the mitochondria intermembrane space and rough endoplasmic reticulum (Wong et al, 1995; Dal Canto and Gurney, 1994). By 90 days of age, when the first clinical symptoms can be seen, G93A mice have developed cytoskeletal spinal cord pathology, which is strikingly similar to that seen in patients with ALS. For example, neurofilament (NF) rich spheroids were identified in the spinal cord and most prominently in motoneuron processes (Tu et al, 1996). Ubiquitinated cytoplasmic inclusion bodies some of which contain aggregated SOD1 were also seen and increased in intensity by 111 days (Tu et al, 1996; Hall et al, 1998). In human ALS, cytoplasmic inclusions immunoreactive for ubiquitin and SOD1 are commonly found in the perikarya of motoneurons (Sasaki et al, 1992; Migheli et al, 1994b; Ince et al, 1996; Shibata et al, 1996). Therefore, the initial accumulation of NF-rich spheroids and ubiquitin inclusions correlates very closely with disease onset.

To determine the onset and progression of motor deficits in transgenic mouse models of FALS, a number of motor-related behavioural tasks have been used (Barneoud et al, 1997; Kong and Xu, 1998). For example, an objective assay to measure muscle strength consisted simply of measuring the time that a G93A mouse was able to hang onto a wire at various times during the course of the disease (Kong and Xu, 1998). Furthermore, this assay was used to correlate motor deficits with the development of motoneuron pathology and motoneuron loss. It was found that during the stage of rapidly declining muscle strength very few motoneurons had been lost. The majority of motoneuron death occurred in the next stage defined by a slow and protracted period of declining muscle strength leading to paralysis. Furthermore, the onset of muscle weakness correlated with massive mitochondrial vacuolation in motoneurons. Therefore, the onset of motor deficits is not due to motoneuron degeneration, which suggests that mitochondrial damage may be the cause of motoneuron dysfunction at the early stages of the disease. Indeed, it has been shown that mutant SOD1 is present in the vacuolated mitochondria of G93A mice (Jaarsma et al, 2000; Mattiazzi et al, 2002) and therefore may be the cause of defective mitochondria seen in mutant SOD1 mice (Carri et al, 1997; Browne et al, 1998; Kruman et al, 1999).

However, conflicting evidence has shown that motoneuron loss occurred during the presymptomatic stage of the disease in G93A transgenic mice (Feeney et al, 2001). This discrepancy may be due to variation in transgene copy number in these G93A mice, which is supported by the difference seen in the age of disease onset in these 2 studies. In addition, the first sign of motor deficits seen in the Kong and Xu (1998) study may precede the first clinical symptoms (fine hindlimb tremor) normally used to define the onset of the disease. Indeed, this was found to be the case for a number

of behavioural tests, which detected motor deficits in G93A mice well before the first clinical symptoms of the disease (Barneoud et al, 1997).

How the pattern of motoneuron loss in mouse models of FALS compares to that in human FALS patients is difficult to assess due to the paucity of studies conducted during the early stages of the disease. However, it has been shown that patients with substantial chronic denervation can maintain normal muscle twitch strength by compensatory mechanisms until about 70-80% of motor units are lost (McComas et al, 1971). Therefore, it has been suggested that there is a long preclinical phase of motoneuron loss in ALS that is hidden by compensation before the onset of symptoms (Swash and Ingram, 1988). Alternatively, normal numbers of motoneurons are maintained until the disease process results in a sudden, rapid and widespread death of motoneurons with the onset of symptoms. Recent clinical studies have shown that a majority of presymptomatic SOD1 carriers (17 out of 19) maintained a full complement of motor units up until the symptomatic phase of the disease (Aggarwal and Nicholson, 2001, 2002). This suggests that the first sign of motor deficit in ALS patients is due to a rapid loss of motoneurons just prior to the symptomatic phase and not the result of a gradual loss of motoneurons.

4.1.4. Motoneuron sprouting in ALS

Denervation of skeletal muscle, a major manifestation of lower motoneuron involvement in ALS, is accompanied by compensatory reinnervation, a process that initially enables muscle strength to be preserved in the face of continuing motoneuron degeneration (Wohlfart, 1957; Stalberg, 1982; Schmied et al, 1999).

Furthermore, a 2-4 fold increase in GAP-43 mRNA expression was found in spinal motoneurons of ALS patients (Parhad et al, 1992). This indicates that the mechanism which leads to motoneuron degeneration in ALS does not compromise the motoneurons capacity for expressing growth associated proteins and sending out collateral sprouts to denervated muscle fibres. Significantly, a recent study has shown that local reinnervation by collateral and ultraterminal sprouting occurs in hindlimb muscles of G93A transgenic mice, at least one month prior to the onset of symptoms of the disease (Frey et al, 2000). This suggests that as a result of some underlying motoneuron dysfunction generated by mutant SOD1, synaptic weakening and functional loss occurs prior to motoneuron death. Furthermore, during the symptomatic phase of the disease, and concurrent with motoneuron death, more extensive axonal sprouting is likely to occur, resulting in enlarged motor units. However, over the course of the disease this sprouting becomes ineffective in compensating for the rapid death of motoneurons (Dengler et al, 1990). Therefore, it is possible that the additional demands placed upon the diseased motoneuron to engage in a growth phase and then support an enlarged peripheral field, may ultimately result in the rapid death of motoneurons and accelerate the rate of disease progression. Indeed, there are several lines of evidence to show that even healthy adult motoneurons are more vulnerable to cell death when induced to grow for a prolonged period (see Chapter 3; Arvidsson and Aldskogius, 1982; Harding et al, 1999; White et al, 2000).

4.1.5. Hypothesis to be tested

In ALS, the progression of the disease is extremely rapid. In humans, the disease results in fatality within 2-5 years of clinical onset. An equivalent disease time-

course is also seen in transgenic mice that express the FALS-linked mutant SOD1 gene (G93A). The purpose of the present study is to investigate whether compensatory sprouting by surviving motoneurons in ALS, contributes to the rapid progression of the disease. To examine this possibility, motoneurons in G93A mice were induced to grow a number of weeks before the onset of motoneuron death. At 90 days of age, when the first clinical symptoms of the disease can be seen, the effect of inducing growth on the rate of progression of the disease was assessed using a number of physiological parameters and by counting the number of surviving motoneurons. In addition, the normal progression of the disease in G93A mice was assessed using the same methods in order to establish baseline data for which to compare the effect of inducing axonal growth. Furthermore, the baseline data is also used to clarify whether the onset of motor deficits in G93A mice is due to motoneuron loss or motoneuron dysfunction.

4.2. METHODS

4.2.1. Animals and genotyping by PCR

Transgenic mice with a G93A mutation of the human SOD1 gene (TgN[SOD1-G93A]1Gur) were purchased from the Jackson Laboratory (Bar Harbor, ME), and maintained by breeding male heterozygous carriers to female B6SJLF1 hybrids. The G93A line of transgenic mice was originally made and characterized by Gurney et al, (1994) and now contain an estimated copy number of 14-17.

The SOD1 G93A transgenic mice were identified using polymerase chain reaction (PCR) amplification of the transgene from genomic DNA. Mouse DNA was prepared for PCR as previously described (see Chapter 3, 3.2.1). For PCR, 10 µl of tail digest was added to the reaction mixture, which contained 10 x PCR buffer, 25 ng each primer (sequences 5' CAT CAG CCC TAA TCC ATC TGA 3' and 5' CGC GAC TAA CAA TCA AAG TGA 3'), 2 µl each dNTP (10 µM), 6 µl $MgCl_2$ (25 µM), 2 units *Taq* polymerase (Promega), and in a total volume of 100 µl. These were then temperature cycled as follows: 3 min at 95°C x 1, 30 sec at 95°C, 30 sec at 60°C, 45 sec at 72°C x 35, and 2 min at 72°C x 1. The PCR products were assessed by agarose gel electrophoresis. Throughout this study only male G93A transgenic mice and male wild-type littermates were used. All experimental procedures were carried out under Licence from the Home Office in accordance with The Animals (Scientific Procedures) Act 1986, and Institute of Neurology guidelines.

4.2.2. Surgery

All surgical procedures were carried out under sterile conditions, using halothane anaesthesia. At 42 days of age both G93A transgenic mice and wild-type littermates were divided into 3 groups: i) unoperated, ii) nerve-crush, iii) partial denervation. In the nerve-crush group, the sciatic nerve was exposed in one hindlimb, and crushed at the mid-thigh level using a fine pair of Watchmaker forceps. Following the crush, the nerve was examined to ensure that the endoneurial sheath was intact but translucent. In the partial denervation group, the L4 and L5 spinal nerves were exposed by making an incision just above the iliac crest on one side of the animal, and parting the pre-vertebral muscles. For partial denervation of the extensor digitorum (EDL) muscle in one hindlimb, the L4 spinal nerve was transected and a small portion of the nerve removed in order to prevent reinnervation.

4.2.3. Isometric tension measurements

All animals in the nerve-crush and partial denervation groups were prepared for EDL muscle tension recordings at 90 days of age. In the unoperated group, tension recording were carried out for both tibialis anterior (TA) and EDL muscles at 90 and 130 days of age.

Animals were anaesthetized with choral hydrate (4%, 1ml/100g body weight, i.p.) and the TA and EDL muscles in both hindlimbs were prepared for isometric tension recordings. The distal tendons of EDL and TA were dissected free of surrounding tissue. Both hindlimbs were securely fixed to a table with stainless steel pins, and the

distal tendons attached to isometric force transducers (Dynamometer UFI Devices) using a silk thread. The sciatic nerve was exposed, sectioned and all its branches cut apart from the deep peroneal nerve, which innervates the EDL and TA muscles. The motor nerve was placed over bipolar silver stimulating electrodes. Isometric contractions were elicited by stimulating the distal part of the cut end of the motor nerve using a pulse width of 0.02 ms pulse duration at supramaximal intensity or by direct stimulation of the muscle using 2 ms pulses. The muscle length was adjusted to obtain maximum twitch tension. Tetanic contractions were elicited by stimulation of the muscle at 40, 80 and 100 Hz for 500 ms. As an indicator of muscle denervation, in some cases measurements of maximal tetanic tension elicited by indirect stimulation were compare to the tension elicited by direct stimulation of the muscle.

4.2.4. Assessment of motor unit number and size

To estimate the number of motor units in EDL muscles in both hindlimbs, the motor nerve was stimulated by single pulses every 4 seconds. The stimulus strength was gradually increased to obtain stepwise increments in twitch tension as individual motor axons were recruited. This was continued until a plateau was reached, when all motor axons were presumed to have been recruited. The number of stepwise increments were counted and taken as an estimate of the number of motor axons in the nerve. Assessing motor unit number by indirectly stimulating through the motor nerve (sciatic) only allows a rough estimate of the true number. This is because it is assumed that each increment in tension is due to the excitation of an additional motor unit. There are two reasons why this assumption may not be true. First, is that several

motor axons might have similar thresholds, firing or not firing together, and hence behave as a single large unit, resulting an underestimation of total motor unit number. A second reason why this assumption may not hold is the possible introduction of experimenter bias. Thus, when a very small increment in tension occurs the experimenter must decide whether it represents an actual recruitment of a motor unit or just a subtle change in the force produced by the same motor unit. Obviously the sensitivity of the recording equipment will all so be a factor in determining the overall motor unit number.

To circumvent these problems, a more accurate method for estimating motor unit number is termed ventral root splitting, whereby individual motor units are functionally isolated. They are judged to be a single unit if the twitch tension showed all-or-none behaviour on stimulating the ventral root filament at the threshold voltage and showed no increase in when the voltage stimulus is increased by 50% (see Lewis et al, 1982). The total number of motor units innervating a muscle is then calculated by dividing the mean twitch tension of the sample motor units by the maximal twitch tension of the muscle. However, ventral root splitting was not used in this study, due the difficulty in keeping unhealthy mice alive for the time needed using this more invasive technique.

An estimate of motor unit size was calculated by dividing the maximal tetanic tension elicited, by the number of motor units of an individual muscle.

4.2.5. Muscle contractile properties

In those unoperated animals in which disease progression was assessed at 90 and 130 day of age, 2 other physiological parameters were also measured to determine any changes in the contractile properties of muscle in G93A mice during the course of the disease. Time to peak (TTP) was calculated by measuring the time taken (ms) for a muscle to elicit peak twitch tension. Time to half relaxation (½ RT) was calculated as the time taken (ms) for the peak twitch tension to decrease to half its original value.

4.2.6. Fatigue resistance

At the end of tension recordings, the EDL muscles were tested for their resistance to fatigue during a period of repetitive stimulation. The muscle was stimulated indirectly via the motor nerve at a frequency of 40 Hz for 250 ms every second for 3 minutes. The calculated fatigue index **(FI)** represents the decline in muscle force following a 3 minute period of stimulation.

$$\mathbf{FI} = \frac{\text{Initial tetanic tension} - \text{Tetanic tension after stimulation (3 min)}}{\text{Initial tetanic tension}}$$

4.2.7. Muscle histology

Following all tension experiments, the soleus, EDL and TA muscles were removed from both hindlimbs, weighed and frozen in isopentane cooled in liquid nitrogen.

The oxidative metabolism of muscle fibres in G93A and wild-type mice was assessed in EDL muscles of G93A and wild-type mice at 90 and 130 days of age. Transverse sections (10 μm) were cut from the middle third of EDL muscles of G93A and wild-type mice at 90 and 130 days of age. The muscle sections were processed for succinate dehydrogenase (SDH) activity (see Nachlas et al, 1957).

4.2.8. Motoneuron survival

The number of sciatic motoneurons in G93A and wild-type mice was assessed at 90 and 130 days of age. In addition, the effect of experimentally inducing axonal growth on motoneuron survival was examined in G93A and wild-type mice at 90 days of age. The animals were anaesthetized with chloral hydrate (4%; 1ml/100g) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffer saline (PBS). The spinal cords were removed, the lumbar region post-fixed for 4 h in the same fixative, and cryoprotected in sucrose (30% in 0.1 M PBS). The entire lumbar spinal cord (L1-L6) was serially sectioned (20 μm) and stained for Nissl (gallocyanin). The number of Nissl-stained motoneurons in every 3rd section within the sciatic motor pool in both ventral horns was counted as previously described in Chapter 2 and 3 (see sections 2.2.5. and 3.2.3.). When assessing the number of Nissl-stained motoneurons only the large polygonal neurons with a clear nucleolus and a

distinctly stained cytoplasm were incorporated into the counts. As an index of motoneuron survival in the experimental groups, the number of Nissl-stained motoneurons on the operated side of each spinal cord was expressed as a percentage of the number on the contralateral control-side.

4.2.9. Statistical analysis

The results from these experiments were analyzed using the Mann-Whitney U-test for comparison of independent samples and significance was set at $P<0.05$.

4.3. RESULTS

4.3.1. Physiological assessment of motor deficits and motoneuron survival in G93A and wild-type mice at 90 and 130 days of age

Previous studies have clearly described the course of disease progression of G93A mice in terms of clinical symptoms, neuropathology and motor deficits measured by behavioural tasks. The aim of the present study is to investigate whether inducing motoneurons to grow prior to the period of motoneuron death, accelerates the progression of the disease. Therefore, it was necessary to first establish the normal rate of disease progression in G93A mice. This was achieved using a number of physiological assessments, which were carried out for both the TA and EDL muscles at the approximate symptomatic onset of the disease (90 days) and the end stage of the disease (130 days). In addition, the survival of motoneurons within the sciatic motor pool was also assessed at the same time points in G93A mice, in order to examine whether a decline in motor function closely correlates with a loss of motoneurons.

4.3.1.1. Isometric tension recordings

Twitch and tetanic tension recordings were carried out on TA and EDL muscles of G93A transgenic and wild-type mice at 90 and 130 days of age. Figure 19 shows examples of records of twitch and tetanic contractions from EDL muscles of G93A and wild-type mice. The results of mean muscle force output for EDL and TA muscles are shown in Table 6. These results are also illustrated in Figure 20, where

Figure 19. Twitch and tetanic contractions from EDL muscles of G93A and wild-type mice

Examples of oscilloscope traces of single twitch and tetanic contractions from EDL muscles elicited by stimulation of their motor nerves. (A) EDL muscle of a 90 day old wild-type mouse. (B) and (C) EDL muscles of G93A transgenic mice at 90 and 130 days of age respectively. The tetanic contractions were elicited at 40, 80 and 100 Hz for 500 ms.

Figure 19

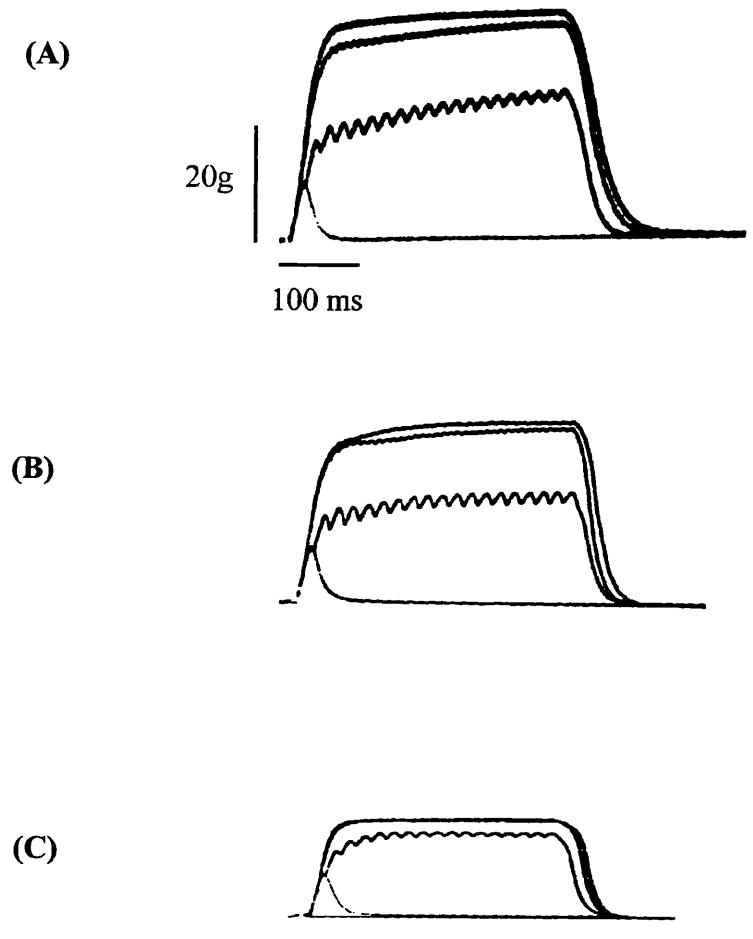


Table 6. Twitch and tetanic forces elicited by EDL/TA muscles of G93A and wild-type mice at 90 and 130 days of age

Muscle forces were measured *in vivo* by attaching the distal tendon of the muscle to a force transducer. The motor nerve was stimulated with a single square pulse of 0.02 ms duration to elicit a twitch tension. Stimulation of the muscle with increasing frequencies (40, 80 and 100 Hz) was used to produce maximum tetanic tensions. The Table shows the mean twitch and tetanic tensions elicited by EDL and TA muscles of G93A **(tg)** and wild-type **(wt)** mice at 90 and 130 days of age (mean ± S.E.M; n=number of muscles). The force produced by muscles of G93A mice is also expressed as a percentage of the age-matched wild-type control mice **(% tg/wt)**. Significance (Mann-Whitney U test), G93A compared to wild-type *P<0.004; ** P<0.001.

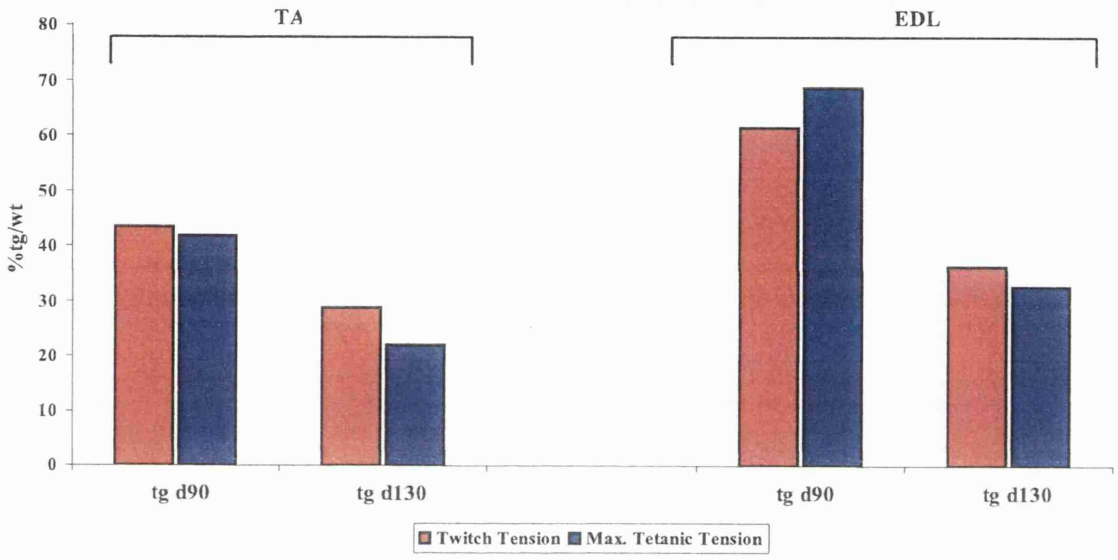
Table 6

Muscle	Age	Twitch tension (g) wt	Twitch tension (g) tg	Twitch tension (g) % tg/wt	Tetanic Tension (g) wt	Tetanic Tension (g) tg	Tetanic Tension (g) %tg/wt
EDL	90	11.4	7.0*	61.4	36.0	24.7**	68.6
	mean ± S.E.M.	± 1.3	± 0.4		± 2.4	± 1.5	
		(n=8)	(n=15)		(n=8)	(n=15)	
	130	11.3	4.1**	36.3	41.7	13.7**	32.9
	mean ± S.E.M.	± 0.8	± 0.4		± 2.0	± 1.0	
		(n=6)	(n=12)		(n=6)	(n=11)	
TA	90	38.9	16.9**	43.4	136.0	56.7**	41.7
	mean ± S.E.M.	± 3.3	± 1.3		± 9.4	± 5.0	
		(n=8)	(n=13)		(n=8)	(n=13)	
	130	44.3	12.7**	28.7	157.2	34.5**	21.9
	mean ± S.E.M.	± 1.2	± 2.3		± 5.5	± 6.0	
		(n=6)	(n=12)		(n=6)	(n=12)	

Figure 20. Twitch and tetanic tensions elicited by EDL and TA muscles of G93A and wild-type mice

The bar chart summarises the results of both twitch and tetanic forces produced by EDL and TA muscles following stimulation of their motor nerve. The mean muscle force produced by EDL/TA muscles of G93A **(tg)** mice is expressed as a percentage of those muscles in age matched wild-type **(wt)** control mice **(% tg/wt)**. Note it can be seen that the reduction in muscle force produced by muscles from G93A mice compared to wild-type mice is much greater for TA than EDL.

Figure 20


TA
EDL
80
70
60
50
40
30
20
10
0
%tg/wt
tg d90
tg d130
tg d90
tg d130
Twitch Tension
Max. Tetanic Tension

the values for muscle force of G93A mice are expressed as a percentage of the age-matched wild-type controls. No error bars are given in Figure 20, since mean values for G93A and wild-type mice are compared. Table 6 shows that in G93A mice at 90 days of age, both the twitch and tetanic forces of EDL and TA muscle were significantly weaker than in age-matched wild-type mice. Thus, EDL muscles in G93A mice at 90 days of age produced a twitch force that was only 61.4% of that produced by EDL muscles of wild-type mice. Similarly, the tetanic force produced by G93A EDL muscles was 68.6% of that produced by EDL muscles of wild-type mice. Interestingly, the decrease in muscle force at 90 days of age in G93A mice was considerably greater for TA muscles compared to EDL muscles. Thus, the twitch and tetanic force produced by TA muscles of G93A mice at 90 days of age was only 43.4% and 41.7% respectively, compared to that produced by TA muscles of wild-type mice. By 130 days, which marks the beginning of the paralysis stage of the disease, the force produced by muscles in G93A mice was considerably weaker than in wild-type mice. Thus, by 130 days the tetanic force produced by EDL and TA muscles in G93A mice was approximately 70% and 80% (respectively) less than in muscles from wild-type mice of the same age.

4.3.1.2. Muscle weights

Following isometric tension recordings, the soleus (SOL), EDL and TA muscles were removed and weighed. The results are summarized in Table 7 and Figure 21, where the muscle weights for G93A mice are expressed as a percentage of the age-matched wild-type control mice. It can be seen that the muscle weights for both soleus and EDL in G93A mice were not significantly different from wild-type mice

Table 7. Muscle weights of soleus, EDL and TA muscles in G93A and wild-type mice at 90 and 130 days of age

Muscle weights of soleus (SOL), EDL and TA were measured. The Table shows the mean muscle weights (mg) of SOL, EDL and TA muscles in G93A **(tg)** and wild-type **(wt)** mice at 90 and 130 days of age (mean ± S.E.M; n=number of muscles). The muscle weights in G93A mice are also expressed as a percentage of the age-matched wild-type control mice **(% tg/wt)**. Significance, (Mann-Whitney U test), G93A compared to wild-type * $P<0.002$; ** $P<0.001$.

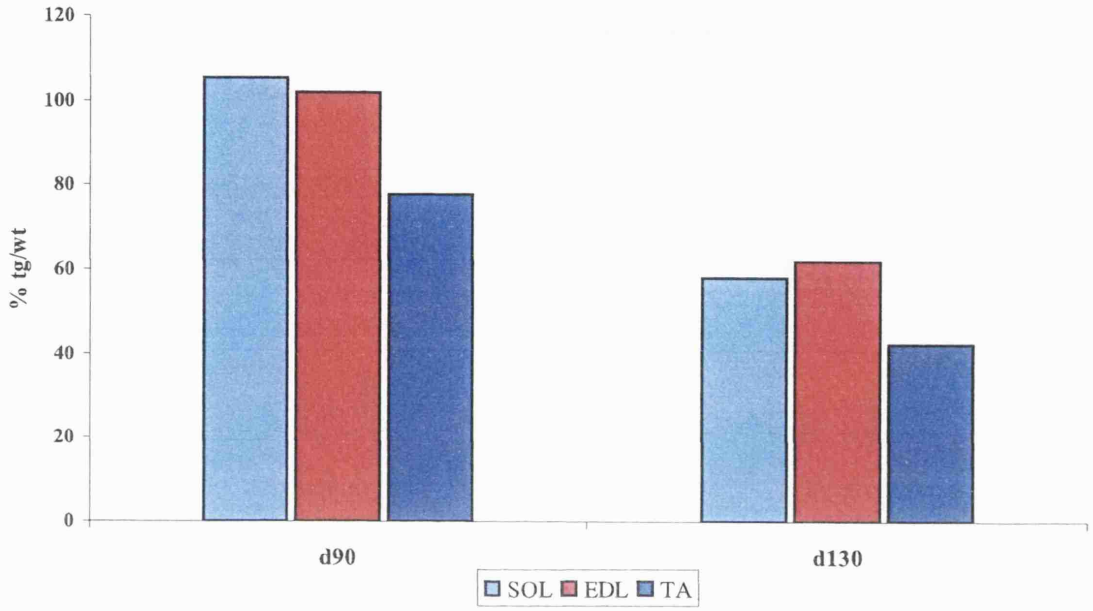
Table 7

		Muscle weight (mg)		
Muscle	**Age (days)**	**wt**	**tg**	**% tg/wt**
SOL	**90**	**7.6**	**8.0**	**105.3**
	mean ± S.E.M.	± 0.7	± 0.5	
		(n=6)	(n=17)	
	130	**8.6**	**5.0***	**58.1**
	mean ± S.E.M.	± 0.3	± 0.6	
		(n=6)	(n=8)	
EDL	**90**	**10.6**	**10.8**	**101.9**
	mean ± S.E.M.	± 0.4	± 1.9	
		(n=7)	(n=17)	
	130	**12.6**	**7.8***	**61.9**
	mean ± S.E.M.	± 0.4	± 0.5	
		(n=6)	(n=10)	
TA	**90**	**56.0**	**43.6****	**77.9**
	mean ± S.E.M.	± 1.4	± 1.8	
		(n=7)	(n=17)	
	130	**68.1**	**28.9***	**42.4**
	mean ± S.E.M.	± 2.3	± 2.5	
		(n=6)	(n=10)	

Figure 21. Muscle weights of soleus, EDL and TA muscles in G93A and wild-type mice at 90 and 130 days of age

The bar chart summarizes the results of muscle weights of SOL, EDL and TA from G93A **(tg)** and wild-type mice **(wt)**. The weights of the muscles from G93A mice are expressed as a percentage of that from wild-type mice **(% tg/wt)**. Note the smaller relative weight of TA muscles in G93A mice compared to wild-type control mice at 90 days of age. The muscle weights of both the SOL and EDL are similar at 90 days of age in G93A and wild-type mice. However, by 130 days of age, for all muscles examined, the muscle weights are considerably smaller in G93A mice compared to wild-type mice.

Figure 21



at 90 days of age. However, even at 90 days of age, the TA muscle in G93A mice weighed 77.9% of the TA muscle in wild-type, which is significantly less ($P<0.002$). Between 90 and 130 days of age, for all muscles examined, there was a sharp decrease in muscle weight as can be seen in Table 7 and Figure 21. Thus, the soleus muscle weight in G93A mice decreased from 105.3% at 90 days of age to 58.1% at 130 days of age compared to the age matched wild-type muscle weight. A similar decrease in muscle weight occurred for the EDL muscle in G93A mice, which decreased from 101.9% at 90 days of age to 61.9% compared to the EDL muscle weight of age-matched wild-type mice. Finally, the TA muscle in G93A mice at 130 days of age weighed approximately 60% less than the TA muscle in age-matched wild-type mice.

4.3.1.3. Motor unit number

The number of motor units in EDL muscles in wild-type and G93A mice was established by counting the number of stepwise increments of force produced in response to increasing the intensity of stimulation of the motor nerve. Examples of such recording taken from EDL muscles of wild-type mice at 130 days of age and G93A mice at 90 and 130 days of age are shown in Figure 22. Table 8 summarizes the results. It can be seen in Table 8 that the number of EDL motor units in wild-type mice at 130 days of age was 26.6 (± 0.4 S.E.M., n=14). This number of EDL motor units was also taken to be the same for 90 day old wild-type mice. In G93A mice at 90 days of age, the number of EDL motor units was approximately 15% less than in wild-type mice, so that the number of EDL motor units was 22.7 (± 1.2 S.E.M., n=17). Between 90 and 130 days of age, the number of EDL motor units in G93A

Figure 22. Examples of recordings of incremental recruitment of motor units in EDL muscles of G93A and wild-type mice

Representative recordings of the stepwise increments in twitch force elicited in response to gradually increasing the intensity of stimulation applied to the motor nerve to the EDL muscle. The number of increments of force gives an estimate of the number of motor units in the EDL muscle. (A) shows a recording of the number of increments in twitch tension in EDL muscle of a wild-type mouse at 130 days of age. (B) and (C) shows recordings from EDL muscles of G93A mice at 90 and 130 days respectively.

Figure 22

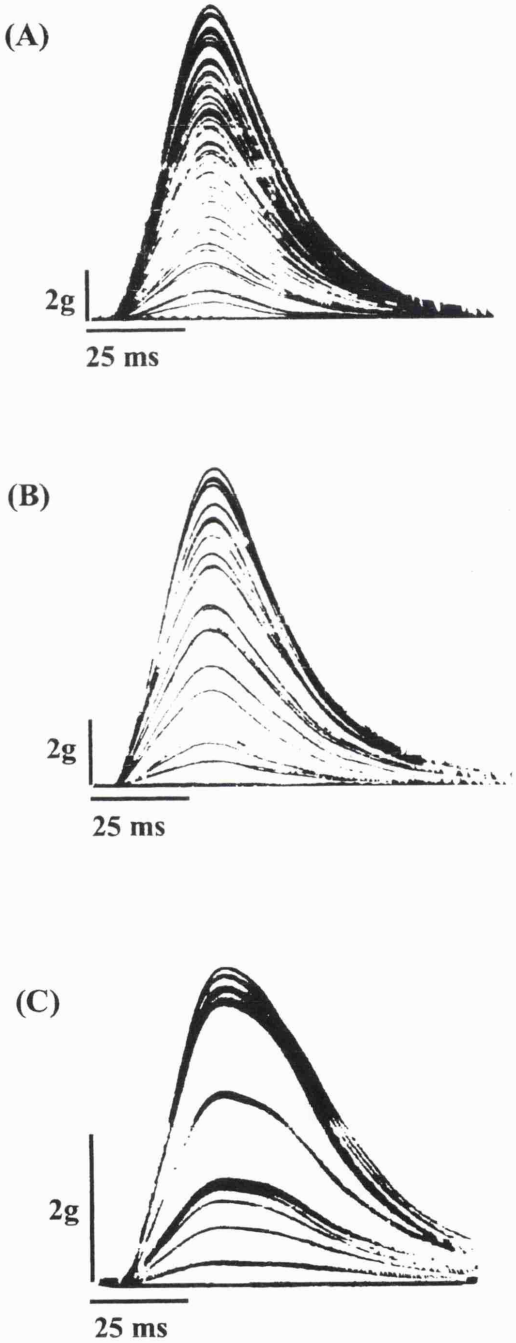


Table 8. The number of motor units in EDL muscles of adult wild-type mice and G93A mice at 90 and 130 days of age

Motor unit number is estimated by counting the number of stepwise increments in twitch force produced in response to stimulating the motor nerve with stimuli of increasing intensity. The Table shows the mean number of motor units in EDL muscles in G93A mice **(tg)** at 90 and 130 days of age and adult wild-type mice **(wt)**. The mean number of motor units in EDL muscles of G93A mice is also expressed as a percentage of the mean number in wild-type mice **(% tg/wt)**. Significance (Mann-Whitney U test), G93A compared to wild-type * $P<0.05$ ** $P<0.001$.

Table 8

		No. of motor units		
Muscle	**Age (days)**	**wt**	**tg**	**% tg/wt**
EDL	**90**	**26.6**	**22.7***	**85.3**
	mean ± S.E.M.	**± 0.4**	**± 1.2**	
		(n=14)	(n=17)	
	130	**26.6**	**10.7****	**40.2**
	mean ± S.E.M.	**± 0.4**	**± 1.0**	
		(n=14)	(n=11)	

mice decreased by approximately 60% compared to wild-type mice, so that only 10.7 (± 1.0 S.E.M., n=11) EDL motor units remained. At both 90 and 130 days of age in G93A mice the number of EDL motor units was significantly smaller than in the EDL muscles of adult wild-type mice ($P<0.05$ and $P<0.001$, respectively).

4.3.1.4. Muscle fatigue and muscle contractile properties

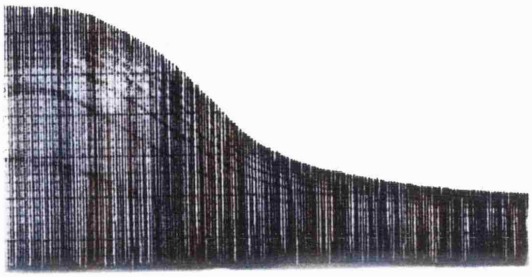
The fatigue resistance of EDL muscles in wild-type and G93A mice was examined in mice of 90 and 130 days of age. Figure 23 shows representative recordings of fatigue traces of EDL muscles in wild-type and G93A mice, obtained by repetitive stimulation of the motor nerve to the EDL muscle. The fatigue index is calculated from these traces as the reduction of tension following 3 minutes of stimulation. A high fatigue index, that is a value close to 1, indicates that the muscle is relatively less fatigue resistant compared to a muscle with a lower fatigue index, which is relatively more fatigue resistant. The results are summarized in Table 9. It can be seen that at 90 days of age, the EDL muscle was more fatigue resistant in G93A mice compared to wild-type mice of the same age. Thus, the mean fatigue index for EDL muscles in G93A mice was 0.70 (± 0.03 S.E.M., n=13) compared to 0.79 (± 0.03 S.E.M., n=7) in wild-type. This represents a difference of 11% in the fatigue resistant of EDL muscles in G93A mice compared to wild-type mice. However, this difference was not found to be statistically significant ($P>0.05$). Between 90 and 130 days of age, the fatigue resistance of EDL muscles in G93A mice increases dramatically compared to wild-type mice. Thus, the fatigue index for EDL muscles in G93A mice was 0.42 (± 0.04 S.E.M., n=11) compared to 0.76 (± 0.03 S.E.M., n=6) in wild-type mice. This represents a 45% difference in fatigue resistant for EDL

Figure 23. Fatigue resistance of EDL muscles in adult wild-type mice and G93A mice at 90 and 130 days of age

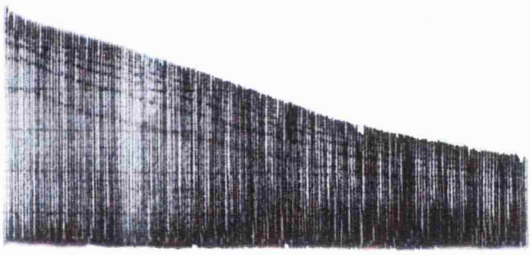
Representative isometric tension recordings of EDL muscles following repetitive stimulation via the motor nerve for 3 minutes (40 Hz for 250 ms every second). (A) shows a recording of tension elicited by an EDL muscle from a wild-type mouse at 130 days of age. (B) and (C) shows recordings from EDL muscles of G93A mice at 90 and 130 days respectively. Note the rapid decline in muscle force of the EDL muscle in adult wild-type mice. However in G93A mice of 130 days of age the initial force is maintained throughout the period of repetitive stimulation.

Figure 23

(A)



(B)



(C)

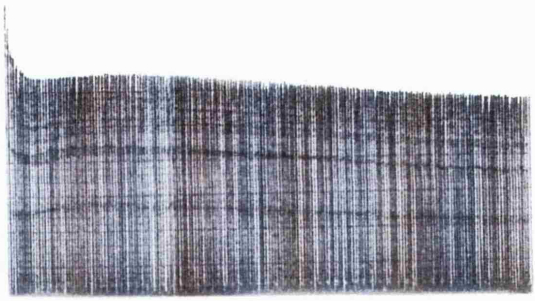


Table 9. Fatigue index of EDL muscles in G93A and wild-type mice at 90 and 130 days of age

The fatigue index is calculated as the reduction in force elicited by the muscle following stimulation for 250 ms every second for 3 minutes. The Table shows the mean fatigue index of EDL muscles in wild-type **(wt)** and G93A **(tg)** mice at 90 and 130 days. The fatigue index of EDL muscles in G93A mice is expressed as a percentage of the fatigue index in wild-type mice **(% tg/wt)**. Significance (Mann-Whitney U test), G93A compared to wild-type * $P<0.001$.

Table 9

Muscle	Age (days)	Fatigue index		
		wt	tg	% tg/wt
EDL	**90**	**0.79**	**0.70**	**88.6**
	mean ± S.E.M.	**± 0.03**	**± 0.03**	
		(n=7)	(n=13)	
	130	**0.76**	**0.42***	**55.3**
	mean ± S.E.M.	**± 0.03**	**± 0.04**	
		(n=6)	(n=11)	

muscles in G93A mice compared to wild-type mice, which was found to be highly significant ($P<0.001$).

The contractile properties of both EDL and TA muscles were also assessed in G93A and wild-type mice at 90 and 130 days of age. The results are summarized in Table 10, which shows that there was no significant difference in the time course of contraction (time to peak) for either EDL or TA muscles at 90 and 130 days in G93A mice compared to age-matched wild-type mice. In addition, there was no significant difference in the time course of relaxation (½ time to relaxation) for EDL and TA muscles in G93A mice at 90 days of age compared to wild-type mice. However, the TA muscle in 90 day old G93A mice, shows a trend towards a slower rate of relaxation, since the ½ time to relaxation was 54.6 ms (± 3.6 S.E.M, n=15) in G93A mice compared to 45.4 (± 2.2. S.E.M, n=8) in wild-type mice. By 130 days of age, both EDL and TA muscles in G93A mice had a significantly slower rate of relaxation compared to wild-type mice ($P<0.04$ and $P<0.002$, respectively).

4.3.1.5. Succinate dehydrogenase activity

To establish whether the changes in fatigue resistance observed in G93A mice were accompanied by a change in the oxidative metabolism of muscle fibres, EDL muscles were frozen and processed for succinate dehydrogenase (SDH) histochemistry. Figure 24 show examples of frozen transverse sections of EDL muscles from a wild-type mouse at 130 days of age (Fig. 24A) and G93A mice at 90 and 130 days of age (Fig. 24, B and C). It is clear that the EDL muscle of wild-type mice consists of mixture of muscles fibres with varying oxidative capacity, resulting

Table 10. Contractile properties of EDL and TA muscles in G93A and wild-type mice at 90 and 130 days of age

The Table shows the time to peak and half time to relaxation of EDL and TA muscles in G93A and wild-type mice at 90 and 130 days of age. The times were calculated from recordings of singe twitch contractions following indirect stimulation of the muscle via the motor nerve. The times calculated for EDL and TA muscles in G93A **(tg)** mice are also expressed as a percentage of the age-matched wild-type **(wt)** control mice **(% tg/wt)**. Significance (Mann-Whitney U test), G93A compared to wild-type *$P<0.04$; ** $P<0.002$.

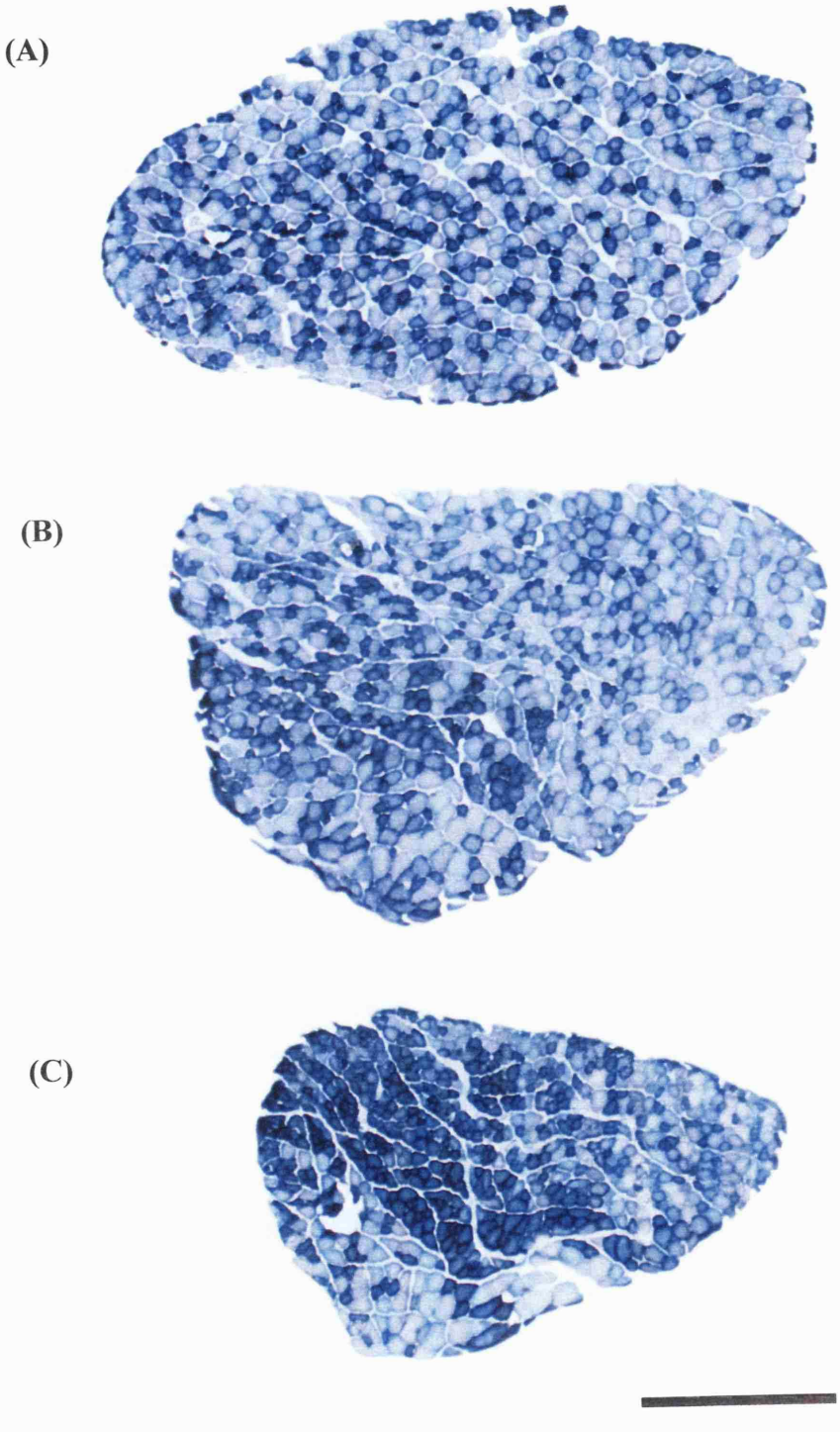
Table 10

		Time to peak (ms)		1/2 time to relaxation (ms)	
Muscle	**Age (days)**	**wt**	**tg**	**wt**	**tg**
EDL	**90**	**26.6**	**25.3**	**45.7**	**45.3**
	mean ± S.E.M.	**± 1.4**	**± 0.5**	**± 2.5**	**± 1.5**
		(n=8)	(n=17)	(n=8)	(n=15)
	130	**26.3**	**29.7**	**46.3**	**59.8***
	mean ± S.E.M.	**± 0.6**	**±1.4**	**±1.7**	**± 3.8**
		(n=6)	(n=12)	(n=6)	(n=12)
TA	**90**	**27.3**	**25.6**	**45.4**	**54.6**
	mean ± S.E.M.	**± 1.1**	**± 1.2**	**± 2.2**	**± 3.6**
		(n=8)	(n=15)	(n=8)	(n=15)
	130	**25.1**	**28.3**	**41.7**	**67.8****
	mean ± S.E.M.	**± 1.4**	**± 1.4**	**± 2.2**	**± 4.5**
		(n=6)	(n=12)	(n=6)	(n=12)

Figure 24. Succinate dehydrogenase activity in EDL muscles of an adult wild-type mouse and G93A mice at 90 and 130 days of age

Photomicrographs of transverse sections of EDL muscles stained for succinate dehydrogenase (SDH) activity. (A) shows a section from an adult wild-type mouse. (B) and (C) shows sections from G93A mice at 90 and 130 days respectively. Scale bar = 1 mm.

Figure 24



in a mosaic pattern of dark and light staining. The majority of muscle fibres are only lightly stained for SDH, reflecting a low oxidative metabolism and are classed as fast twitch fibres (Stein and Padykula, 1962). The darkly stained muscle fibres have a high oxidative metabolism and are likely to be slow twitch fibres. In EDL muscles from G93A mice at 90 days of age, there are already signs of fibre type reorganization at 90 days age, with darkly stained fibres grouping, indicating that denervation and reinnervation may be occurring in a localized area. However, by 130 days of age, the EDL muscles in G93A mice are clearly smaller in cross-sectional area, contain a greater proportion of muscle fibres that staining darkly for SDH and there is a more pronounced fibre type grouping.

4.3.1.6. Motoneuron survival in G93A mice

To determine whether motoneuron loss had occurred at the start of the symptomatic phase of the disease, the number of sciatic motoneurons was assessed in the spinal cords of G93A mice at 90 days of age and compared to the number in adult wild-type mice at 130 days of age. In addition, the extent of motoneuron loss in G93A mice was assessed at the end-stage of the disease (130 days of age). Figure 25 shows examples of spinal cord sections from an adult wild-type mouse (Fig. 25A) and G93A mice at 90 and 130 days of age (Fig. 25, B and C). The Figure shows the location and appearance of motoneurons within the sciatic motor pool in the lumbar region of the spinal cord. It can be seen that there are fewer Nissl-stained motoneurons in the spinal cords of 90 and 130 day old G93A mice compared to adult wild-type mice. The number of Nissl-stained motoneurons in each ventral horn was counted in adult wild-type mice and G93A mice at 90 and 130 days of age. Table 11

Figure 25. Photomicrographs of spinal-cord sections from an adult wild-type mouse and G93A mice at 90 and 130 days of age

The photomicrographs show cross-sections of the lumbar region of mouse spinal cords, which have been stained for Nissl. The broken lines demarcate the approximate location of the sciatic motor pool in the ventral horns. (A) a cross-section of a spinal cord from an adult wild-type mouse. (B) and (C) show spinal cord sections from G93A mice at 90 and 130 days of age respectively. Scale bar = 250 μm.

Figure 25

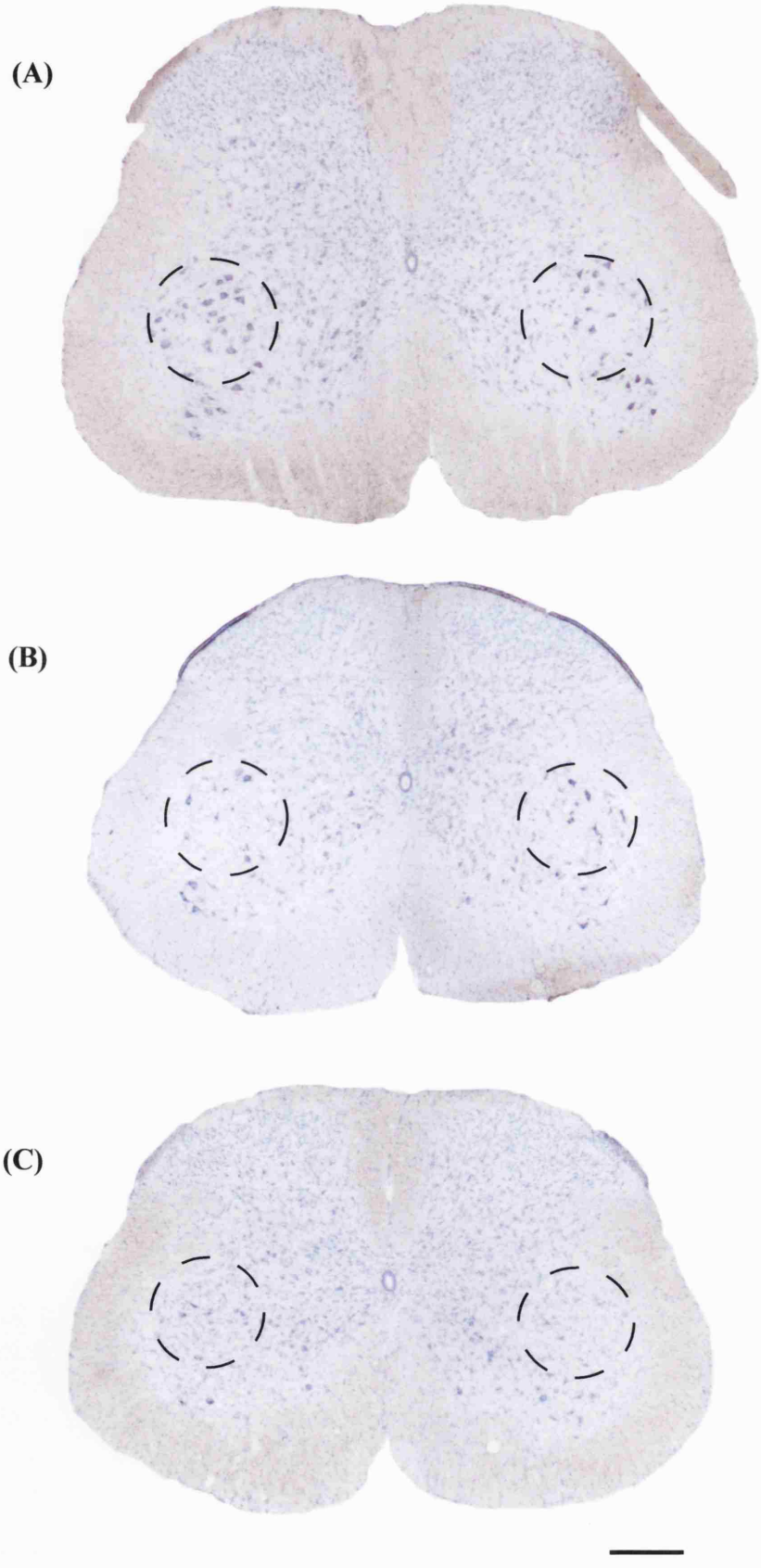


Table 11. The number of sciatic motoneurons in adult wild-type mice and G93A mice at 90 and 130 days of age

Motoneuron survival was assessed by counting the number of Nissl-stained motoneurons in every 3rd section within the sciatic motor pool in both ventral horns. The Table shows the mean number of motoneurons present in adult wild-type mice and G93A mice at 90 and 130 days of age. The number of motoneurons in G93A **(tg)** mice is expressed as a percentage of the number of motoneurons in adult wild-type **(wt)** control mice **(% tg/wt)**. Significance (Mann-Whitney U test), G93A compared to wild-type * $P<0.03$.

Table 11

	No. of sciatic motoneurons		
Age (days)	**wt**	**tg**	**% tg/wt**
90	**433**	**264**	**61.0***
mean ± S.E.M.	**± 7.8**	**± 10.5**	
	(n=4)	(n=4)	
130	**433**	**126**	**29.1***
mean ± S.E.M.	**± 7.8**	**± 9.5**	
	(n=4)	(n=4)	

summarizes the results and shows that in G93A mice at 90 days of age only 264 (± 10.5 S.E.M., n=4) motoneurons are present in the sciatic motor pool compared to 433 (± 7.8 S.E.M., n=4) in adult wild-type mice. This represents a significant loss of motoneurons in G93A mice by 90 days of age since only 61.0% of motoneurons survive ($P<0.03$). By 130 days of age, only 126 (± 9.5 S.E.M., n=4) motoneurons in the sciatic motor pool survive in G93A mice, which represents only 29.1% of the number of motoneurons present in adult wild-type mice. Thus, a dramatic loss of motoneurons occurs between 90 and 130 days of age in G93A mice, such that over 50% of motoneurons in the sciatic motor pool die within that period of time (i.e. 264 ± 11.0 S.E.M., n=4, to 126 ± 10.0 S.E.M., n=4).

4.3.2. The effect of inducing axonal growth on disease progression in G93A mice

The previous results of this Chapter have shown that at the symptomatic onset of the disease (90 days of age) in G93A mice there is a significant decline in motor function and also significant motoneuron degeneration in the lumbar spinal cord. In the next section of this Chapter, the effect of inducing axonal growth on disease progression in G93A mice was investigated. In order to induce growth, 2 methods were used: i) nerve injury or ii) partial denervation. The effect of each of these stimuli for growth will be reported in turn.

4.3.2.1. The effect of nerve injury on disease progression in G93A mice

In order to induce axonal growth, the sciatic nerve was crushed in one hindlimb of G93A mice during the presymptomatic phase of the disease (42 days of age) and the effect of this injury on motor function and motoneuron survival was examined.

4.3.2.1.1. Isometric tension recordings and muscle weights following nerve injury

Following sciatic nerve injury in G93A and wild-type mice at 42 days of age, isometric tension recordings were carried out when the animals were 90 days of age. In both groups of animals, twitch and maximal tetanic tensions of the operated and contralateral EDL muscles were established. In addition, following the completion of in vivo tension recordings, EDL muscles from both hindlimbs of G93A and wild-type mice were removed and weighed. The mean force output and weights for EDL

muscles are shown in Table 12. These results are summarized in Figure 26, where the values of the operated muscles are expressed as a percentage of the unoperated contralateral control muscles. Table 12 shows that following nerve injury in wild-type mice both the twitch and tetanic forces elicited by the operated EDL muscles were weaker compared to the contralateral control EDL muscles (62.8% ± 4.2 S.E.M., n=3; 86.2% ± 6.1 S.E.M., n=5, respectively). In contrast, in G93A mice following nerve injury, both twitch and tetanic forces produced by operated EDL muscles were greater compared to contralateral control EDL muscles (107.7% ± 16.9 S.E.M., n=3; 134.2% ± 23.2 S.E.M., n=3, respectively). However, the differences in mean force output between the operated and control EDL muscles in G93A and wild-type control mice following nerve injury was not significantly different due to a high degree of variability in the G93A mice group (NS $P>0.05$). This is probably due to variation in the age of disease onset. No significant differences were seen between operated and contralateral control EDL muscle weights in G93A and wild-type mice following nerve injury (NS $P>0.05$).

4.3.2.1.2. Motor unit number and size following nerve injury

The muscle force results indicate that following nerve injury there is a slower decline in muscle function in G93A mice. To examine the possibility that this was due to a greater number of functional motoneurons in these mice, the number of motor units in EDL muscles was determined by counting the number of stepwise increments of force produced in response to increasing the intensity of stimulation via the motor nerve. In addition, motor unit size was estimated by calculating the force developed by individual motor units in EDL muscles. The mean motor unit number and size in

Table 12. The effect of nerve injury on muscle force and weight in G93A and wild-type mice at 90 days of age

Following sciatic nerve injury, EDL muscle forces were measured *in vivo* by attaching the distal tendon of the muscle to a force transducer. The motor nerve was stimulated with a single square pulse of 0.02 ms duration to elicit twitch tension. Stimulation of the muscle with increasing frequencies (40, 80 and 100 Hz) was used to produce maximum tetanic tensions. The Table shows the mean twitch and tetanic forces elicited by EDL muscles of G93A **(transgenic)** and wild-type mice at 90 days of age (mean ± S.E.M; n=number of animals). In addition, mean EDL muscle weights from G93A and wild-type mice are also shown. The values of the operated EDL muscle are also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**.

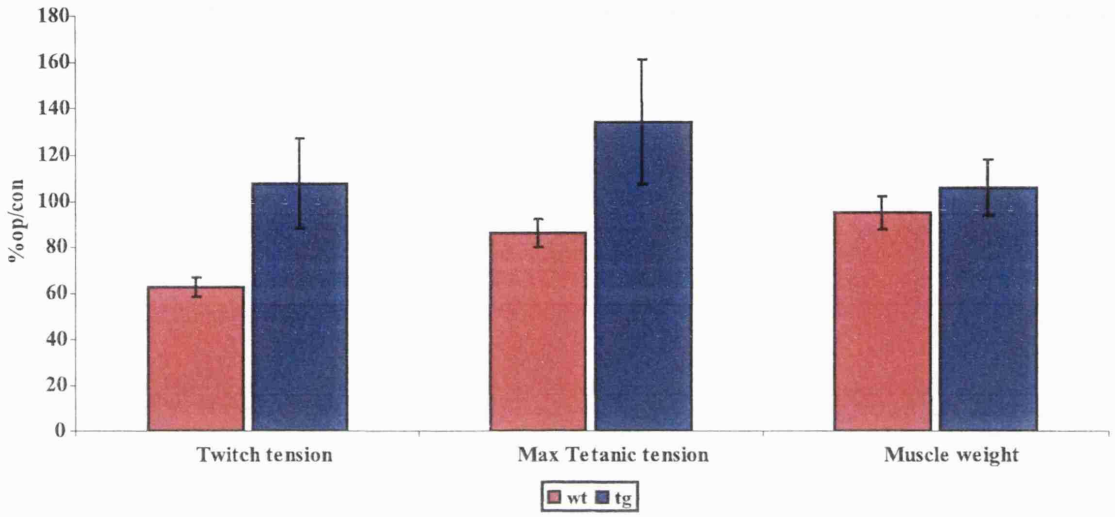
Table 12

Mice	Twitch tension (g)			Tetanic tension (g)			Muscle weight (mg)		
	CON	OP	% OP/CON	CON	OP	% OP/CON	CON	OP	% OP/CON
Wild-type mean ± S.E.M. (n=5)	**11.3** ± 2.2	**6.9** ± 1.1	**62.8** ± 4.2	**37.9** ± 3.4	**32.0** ± 1.8	**86.2** ± 6.1	**11.0** ± 0.2	**10.3** ± 0.9	**95.0** ± 7.1
Transgenic mean ± S.E.M. (n=3)	**6.5** ± 0.5	**6.9** ± 1.2	**107.7** ± 19.6	**18.6** ± 1.5	**24.2** ± 3.2	**134.2** ± 27.0	**8.6** ± 1.0	**8.9** ± 0.2	**106.0** ± 12.2

Figure 26. The effect of nerve injury on muscle force and weight in G93A and wild-type mice at 90 days of age

The bar chart summarizes the results of muscle force and muscle weight following nerve injury in wild-type and G93A mice. Both twitch and tetanic forces were elicited by stimulation of the EDL muscle via the motor nerve. The values for the operated EDL muscle are expressed as a percentage of the contralateral control EDL muscle **(% OP/CON)** in both G93A **(tg)** and wild-type mice **(wt)** at 90 days of age. The error bars show the standard error of the mean.

Figure 26


180
160
140
120
100
80
60
40
20
0
%op/con
Twitch tension
Max Tetanic tension
Muscle weight
wt
tg

EDL muscles is shown in Table 13. These results are also summarized in Figure 27, where the values of the operated muscle are expressed as a percentage of the unoperated contralateral control muscle. Table 13 shows that following nerve injury the number of motor units in operated EDL muscles was not significantly different to the contralateral control muscle in both G93A and wild-type mice at 90 days of age (NS $P>0.05$). The results in Table 13 also show the mean motor unit size in wild-type and G93A mice following nerve injury. It can be seen that there was an increase in motor unit size in EDL muscles of G93A mice following nerve injury. Thus, the force elicited by individual motor units of the reinnervated EDL muscle after nerve injury in G93A mice was 143.7% (± 25.5 S.E.M., n=3) compared to the contralateral control EDL muscle. In contrast, the force of individual motor units in the operated EDL muscle compared to the contralateral control in wild-type mice was 92.0% (± 7.4 S.E.M., n=5). However, there is no significant difference between the relative motor unit sizes following nerve injury between G93A and wild-type control mice (NS $P>0.05$). Even so, the trend towards a slower decline in motor function following nerve injury in G93A mice is more likely to be due to a larger motor unit size in the reinnervated EDL muscle compared to the contralateral control muscle, rather than a greater number of functional motor units.

4.3.2.1.3. Muscle fatigue following nerve injury

The effect of nerve injury on the fatigue resistance of EDL muscles in 90 day old wild-type and G93A mice was examined. Figure 28 shows examples of fatigue traces of control and operated EDL muscles from wild-type and G93A mice at 90 days of age. From traces such as these a fatigue index for each EDL muscle was

Table 13. The effect of nerve injury on motor unit number and size in G93A and wild-type mice at 90 days of age

Following nerve injury at 42 days of age in wild-type and G93A mice, the number of EDL motor units was established from records of stepwise increments in twitch force produced in response to stimulating the motor nerve with increasing intensity. Motor unit size was calculated by dividing the maximal tetanic tension elicited by the number of motor units of an individual muscle. The Table shows the mean number of motor units and motor unit size of EDL muscles in G93A **(transgenic)** and wild-type mice at 90 days of age (mean ± S.E.M; n=number of animals). The values of the operated EDL muscle are also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**.

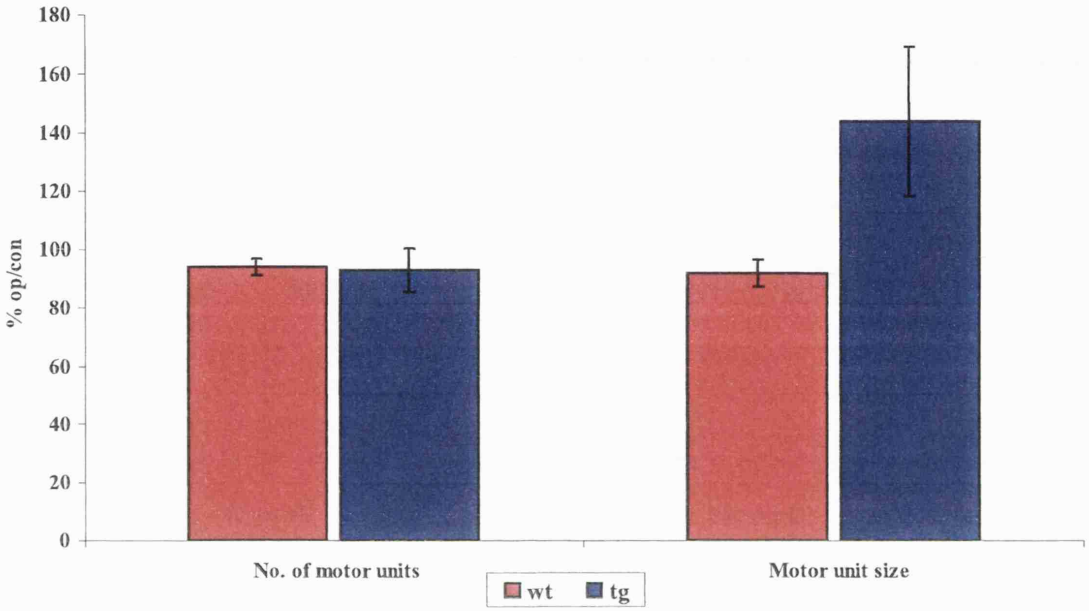
Table 13

Mice	No. of motor units			Motor unit size		
	CON	OP	% OP/CON	CON	OP	% OP/CON
Wild-type	**27**	**26**	**94.2**	**1.4**	**1.2**	**92.0**
mean ± S.E.M.	± 0.4	± 0.8	± 2.8	± 0.13	± 0.04	± 7.4
(n=5)						
Transgenic	**22**	**20**	**93.0**	**0.9**	**1.2**	**143.7**
mean ± S.E.M.	± 2.3	± 1.7	± 4.5	± 0.2	± 0.1	± 25.5
(n=3)						

Figure 27. The effect of nerve injury on motor unit number and size in G93A and wild-type mice at 90 days of age

The bar chart summarizes the results of motor unit number and size in EDL muscles following nerve injury in wild-type and G93A mice at 90 days of age. The number of motor units and size of individual motor units in the operated EDL muscle is expressed as a percentage of that in the contralateral control EDL muscle **(% OP/CON)** in both G93A **(tg)** and wild-type mice **(wt)** at 90 days of age. The error bars show the standard error of the mean.

Figure 27


180
160
140
120
100
80
60
40
20
0
% op/con
No. of motor units
Motor unit size
wt
tg

calculated. A high fatigue index, that is a value close to 1, indicates that the muscle is relatively fatiguable compared to a muscle with a low fatigue index, which is relatively fatigue resistant. The results are summarized in Table 14. It can be seen that following nerve injury, operated EDL muscles in G93A mice were more fatigue resistant compared to the contralateral control muscles. Thus, in G93A mice the fatigue index for the operated EDL muscles was 0.46 (± 0.20 S.E.M., n=3) compared to 0.69 (± 0.06 S.E.M., n=3) in the contralateral muscle at 90 days of age. In contrast, the fatigue resistance of operated EDL muscles in wild-type mice was similar to the contralateral control muscles (0.70 ± 0.07 S.E.M., n=5; 0.78 ± 0.04 S.E.M., n=5, respectively). However, nerve injury did not significantly increase the fatigue resistance of EDL muscles in G93A mice compared to wild-type mice (NS $P>0.05$).

4.3.2.1.4. Succinate dehydrogenase activity following nerve injury

To establish whether the increase in fatigue resistance of EDL muscles following nerve injury in G93A mice was accompanied by a change in the oxidative metabolism of muscle fibres, EDL muscles were frozen and processed for succinate dehydrogenase (SDH) histochemistry. Figure 29 shows examples of EDL muscle sections stained for SDH activity from wild-type (Fig 29A) and G93A mice (Fig. 29B), following nerve injury. The mosaic distribution of muscle fibres with varying oxidative capacity can be clearly seen in both contralateral control EDL muscles of G93A and wild-type mice. In the operated EDL muscles the proportion of darkly stained muscle fibres from G93A mice was greater compared to wild-type mice. This indicates that following nerve injury there is a more extensive conversion of fast (low

Figure 28. The effect of nerve injury on fatigue resistance of EDL muscles in G93A and wild-type mice at 90 days of age

Following nerve injury, EDL muscles of wild-type and G93A mice were prepared for a fatigue test. The fatigue resistance of EDL muscles was assessed by indirectly stimulating the muscle via the motor nerve for 3 minutes (40 Hz for 250 ms every second). (A) shows records of tension elicited by the control **(CON)** and operated **(OP)** EDL muscles of wild-type mice at 90 days of age. (B) shows records of tension elicited by control and operated EDL muscles of G93A mice at 90 days of age. Note the resistance to fatigue of the reinnervated EDL muscle in G93A mice compared to wild-type mice.

Figure 28

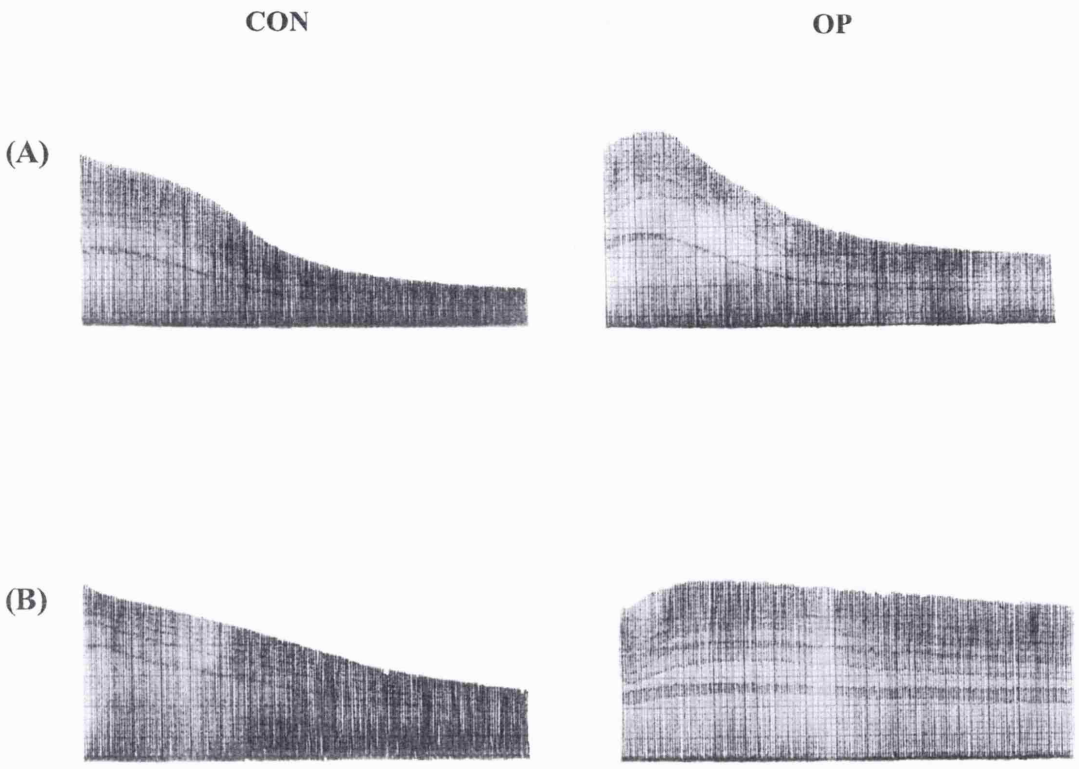


Table 14. The effect of nerve injury on muscle fatigue resistance in G93A and wild-type mice at 90 days of age

The fatigue index is calculated as the reduction in force elicited by the muscle following stimulation for 250 ms every second for 3 minutes. A high fatigue index represents a fatiguable muscle, whereas a low index represents of a fatigue resistant muscle. The Table shows the mean fatigue index of EDL muscles in 90 day old G93A **(transgenic)** and wild-type mice following nerve injury. The values of the operated EDL muscle are also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**.

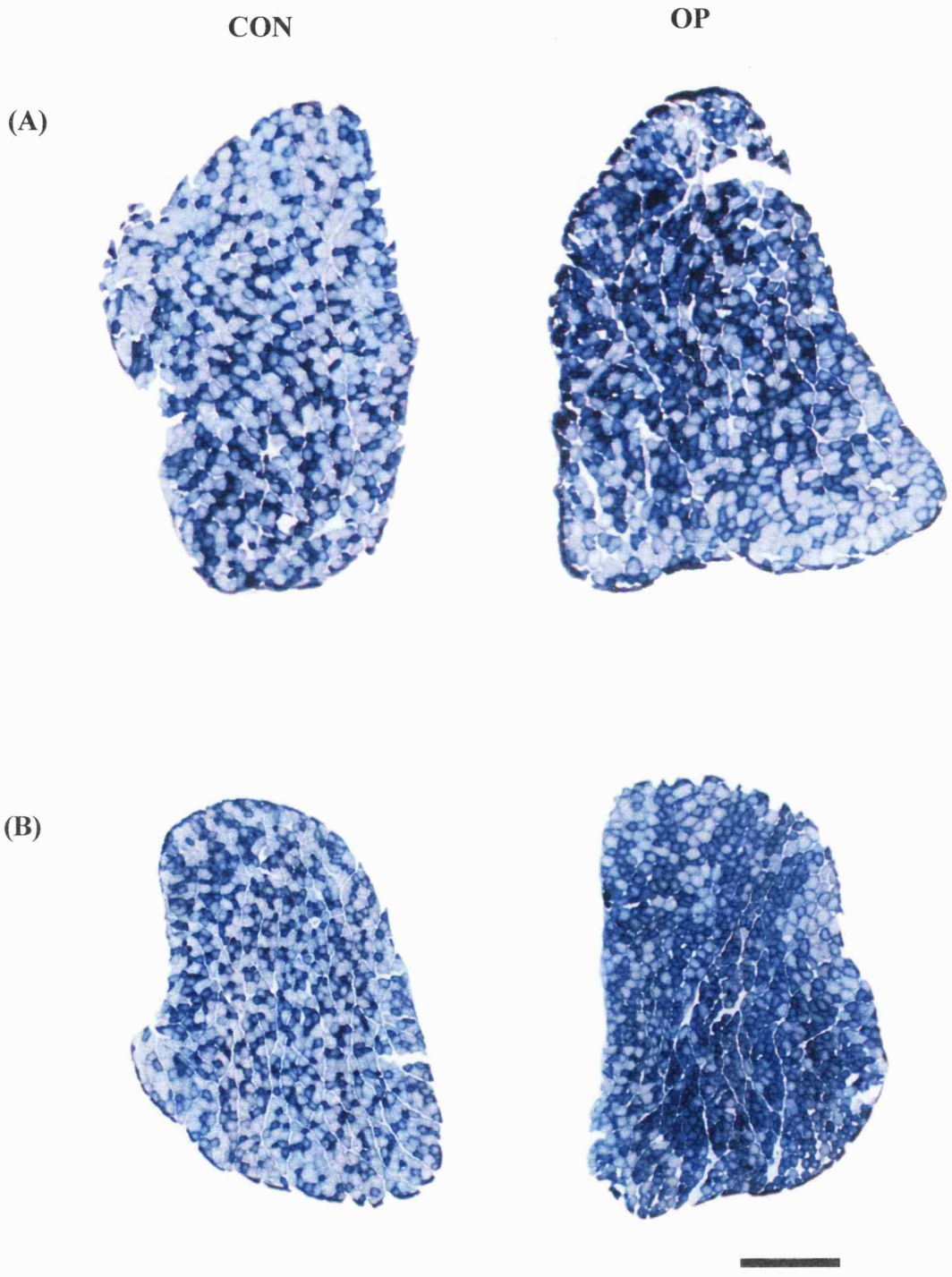
Table 14

Mice	Fatigue index		
	CON	OP	% OP/CON
Wild-type	**0.78**	**0.70**	**89.7**
mean ± S.E.M.	± 0.04	± 0.07	± 11.2
(n=5)			
Trangenic	**0.69**	**0.46**	**62.5**
mean ± S.E.M.	± 0.06	± 0.20	± 25.6
(n=3)			

Figure 29. The effect of nerve injury on succinate dehydrogenase activity in EDL muscles of G93A and wild-type mice at 90 days of age

Representative photomicrographs of transverse sections of EDL muscles stained for succinate dehydrogenase (SDH) activity. (A) shows both control **(CON)** and operated **(OP)** EDL muscle sections from a wild-type mouse. (B) shows sections from the control and operated EDL muscle of a G93A mouse. Scale bar = 1 mm.

Figure 29



oxidative capacity) to slow fibre types (high oxidative capacity) in EDL muscles of G93A mice compared to wild-type mice.

4.3.2.1.4. Motoneuron survival following nerve injury

To determine whether inducing axonal growth by nerve injury alters the rate of motoneuron degeneration in G93A mice, motoneuron survival was assessed in the spinal cords of G93A and wild-type mice at 90 days of age. As an index of motoneuron survival the number of Nissl-stained sciatic motoneurons in the operated ventral horn was expressed as a percentage of the number in the contralateral control ventral horn. The results are summarised in Table 15, which shows that after nerve injury in G93A mice only 66.7% (± 1.8 S.E.M., n=4) of sciatic motoneurons survived in the operated ventral horn compared to the contralateral control ventral horn. Following nerve injury in wild-type mice, there was no significant difference in the number of motoneurons in the operated ventral horn compared to the contralateral control ventral, since 96.5% (± 3.9 S.E.M., n=4) of motoneurons survived. The percentage reduction in motoneuron survival in G93A mice compared to wild-type mice following nerve injury was significant ($P<0.03$). Thus, nerve injury in G93A mice accelerated the death of motoneurons.

4.3.2.2. The effect of partial denervation on disease progression in G93A mice

The final section of this Chapter investigates the effect of inducing axonal growth on disease progression in G93A mice by partial denervation. In order to more closely model the type of axonal growth that occurs in ALS during the course of the disease,

Table 15. The effect of nerve injury on motoneuron survival in G93A and wild-type mice at 90 days of age

The effect of nerve injury on motoneuron survival was assessed at 90 days of age by counting the number of Nissl stained motoneurons in every 3rd section within the sciatic motor pool in both ventral horns of G93A and wild-type mice. The Table shows the mean number of sciatic motoneurons present in operated **(OP)** and control **(CON)** ventral horns of both G93A and wild-type mice. The number of motoneurons in the operated ventral horn is also expressed as a percentage of that in the control ventral horn **(%OP/CON)**. Significance (Mann-Whitney U test), G93A **(transgenic)** compared to wild-type * $P<0.03$.

Table 15

Mice	No. of sciatic motoneurons		
	CON	OP	% OP/CON
Wild-type	**221**	**213**	**96.5**
mean ± S.E.M.	± 10.7	± 7.4	± 3.9
(n=4)			
Transgenic	**147**	**99**	**66.7***
mean ± S.E.M.	± .5.5	± 5.5	± 1.8
(n=4)			

the EDL muscle in G93A mice was partially denervated during the presymptomatic phase the disease (42 days of age) by transecting the L4 spinal nerve. The response of the remaining intact axons is to undergo collateral sprouting and enlargement of their motor unit size (Brown et al, 1981b; Rafuse et al, 1992). Similarly, compensatory axonal sprouting is thought to occur in ALS following the initial loss of motoneurons (Wohlfart, 1957; Parhad et al, 1992). Therefore, the effect of partial denervation on motor function and reinnervation was examined in G93A mice at the start of the symptomatic phase of the disease (i.e. 90 days of age).

4.3.2.2.1. Isometric tension recordings and muscle weights following partial denervation

Following partial denervation of EDL muscles in G93A and wild-type mice at 42 days of age, isometric tension recordings were carried out when the animals were 90 days of age. In both groups of animals, twitch and maximal tetanic tensions of the operated and contralateral EDL muscles were established. In addition, following the completion of in vivo tension recordings, EDL muscles from both hindlimbs of G93A and wild-type mice were removed and weighed. The mean force output and weights for EDL muscles are shown in Table 16. These results are summarized in Figure 30, where the values of the operated muscles are expressed as a percentage of the unoperated contralateral control muscles. The Table shows that following partial denervation the mean twitch and tetanic forces elicited by operated EDL muscles in G93A mice were weaker than the contralateral control muscles (57.5% ± 7.6 S.E.M., n=4; 56.6% ± 10.6 S.E.M., n=4, respectively). In contrast, in wild-type mice, partial denervation of EDL muscles did not result in a similar decline in muscle force. Thus,

Table 16. The effect of partial denervation on EDL muscle force and weight in G93A and wild-type mice at 90 days of age

Following partial denervation, EDL muscle forces were measured *in vivo* by attaching the distal tendon of the muscle to a force transducer. The motor nerve was stimulated with a single square pulse of 0.02 ms duration to elicit a twitch tension. Stimulation of the muscle with increasing frequencies (40, 80 and 100 Hz) was used to produce maximum tetanic tensions. The Table shows the mean twitch and tetanic forces elicited by EDL muscles of G93A **(transgenic)** and wild-type mice at 90 of age (mean ± S.E.M; n=number of animals). In addition, mean EDL muscle weights from G93A and wild-type mice are also shown. The values of the operated EDL muscle are also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**. Significance (Mann-Whitney U test), G93A compared to wild-type * $P<0.02$, ** $P<0.01$.

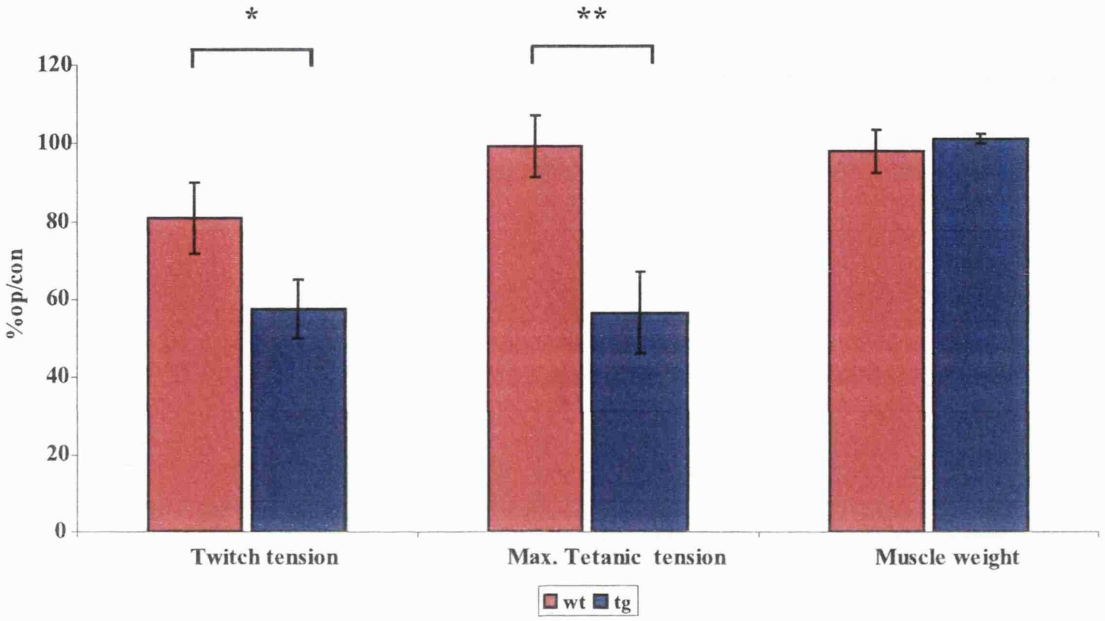
Table 16

Mice	Twitch tension (g)			Tetanic tension (g)			Muscle weight (mg)		
	CON	OP	% OP/CON	CON	OP	% OP/CON	CON	OP	% OP/CON
Wild-type	**10.0**	**7.7**	**80.8**	**29.6**	**28.9**	**99.2**	**10.3**	**10.0**	**98.1**
mean ± S.E.M.	± 1.9	± 0.9	± 9.2	± 4.3	± 4.2	± 7.9	± 0.9	± 0.7	± 5.5
(n=4)									
Transgenic	**8.1**	**4.8**	**57.5***	**28.0**	**16.6**	**56.6****	**9.0**	**9.1**	**101.3**
mean ± S.E.M.	± 0.8	± 1.1	± 7.6	± 4.8	± 5.1	± 10.6	± 1.0	± 0.2	± 1.2
(n=4)									

Figure 30. The effect of partial denervation on EDL muscle force and weight in G93A and wild-type mice at 90 days of age

The bar chart summarizes the effect of partial denervation on force and weight of EDL muscles. Both twitch and tetanic forces were elicited by stimulation of EDL via the motor nerve. The values of the operated EDL muscle are expressed as a percentage of the contralateral control EDL muscle **(% OP/CON)** in both G93A **(tg)** and age-matched wild-type control mice **(wt)** at 90 days of age. The error bars show the standard error of the mean. Significance (Mann-Whitney U test), G93A compared to wild-type * $P<0.02$, ** $P<0.01$

Figure 30


*
**
120
100
80
60
40
20
0
%op/con
Twitch tension
Max. Tetanic tension
Muscle weight
wt
tg

the twitch force elicited by the operated EDL muscles in wild-type mice was 80.8% (± 9.2 S.E.M., n=4) of the contralateral control. In addition, there was no loss of tetanic force following partial denervation in wild-type mice, since the operated EDL muscles produced 99.2% (± 7.9 S.E.M., n=4) of the tetanic force elicited by the contralateral control muscles. Thus, the decline in twitch and tetanic forces of partially denervated EDL muscles compared to contralateral control muscles in G93A mice were significantly greater than in wild-type mice ($P<0.02$ and $P<0.01$, respectively). However, the decline in muscle force following partial denervation in G93A mice was not reflected by a similar decline in muscle weight (NS $P>0.05$).

4.3.2.2.2. Functional reinnervation following partial denervation

To determine the extent of functional reinnervation of muscle fibres following partial denervation of EDL muscles in G93A and wild-type mice at 90 days of age, tetanic tensions were measured with alternate direct and indirect stimulation of the muscle and a functional innervation ratio (FIR) was calculated as tetanic tension evoked by indirect stimulation divided by tetanic tension evoked by direct stimulation.. The results are summarized in Table 17, which shows that following partial denervation in G93A mice the FIR for operated EDL muscles is lower than in the contralateral control muscles. Thus, indirect stimulation of the operated EDL muscle in G93A mice only elicited 73% (± 1.0 S.E.M., n=4) of the force produced by direct stimulation, whereas indirect stimulation of the control EDL muscle elicited 103% (± 0.05 S.E.M., n=4) of the force produced by direct stimulation. This difference in the FIR between operated and control EDL muscles in G93A mice was significant ($P<0.05$). In contrast, in wild-type mice, no significant difference in the FIR of

Table 17. Functional reinnervation of EDL muscles following partial denervation in G93A and wild-type mice at 90 days of age

The functional reinnervation ratio (FIR) was calculated by measuring the maximal tetanic tension evoked by indirect stimulation divided by the tension evoked by direct stimulation. Assessment of functional reinnervation was carried out at 90 days of age in G93A **(transgenic)** and wild-type mice, 7 weeks after partial denervation of EDL muscles The Table shows the mean FIR of EDL muscles following partial denervation (mean ± S.E.M; n=number of animals). The FIR of the operated EDL muscle is also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**. Significance (Mann-Whitney U test), G93A operated muscles compared to control muscles * $P<0.05$.

Table 17

Mice	FIR		
	CON	OP	% OP/CON
Wild-type	**0.99**	**0.91**	**92.8**
mean ± S.E.M.	± 0.03	± 0.06	± 6.7
(n=4)			
Transgenic	**1.03**	**0.73***	**70.7**
mean ± S.E.M.	± 0.05	± 0.10	± 8.8
(n=4)			

operated and control EDL muscle was found, since the extent of functional reinnervation in the operated EDL muscle was near complete. Thus, the FIR in operated EDL muscles in wild-type mice was 0.91 (± 0.06 S.E.M., n=4) compared to 0.99 (± 0.03 S.E.M., n=4) in contralateral control muscles. However, following partial denervation in G93A mice, the FIR of operated EDL muscles compared to the control muscles (70.7% ± 8.8 S.E.M., n=4) was not significantly different to that found in wild-type mice (92.8% ± 6.7 S.E.M., n=4).

4.3.2.2.3. Muscle fatigue following partial denervation

The effect of partial denervation on the fatigue resistance of EDL muscles in 90 day old wild-type and G93A mice was examined. As a measure of fatigue resistance, a fatigue index for each EDL muscle was calculated by subtracting the force produced at the end of 3 minutes of stimulation from the force produced at the beginning of the fatigue test. This value was then divided by the initial force produced by the muscle. A high fatigue index, that is a value close to 1, indicates that the muscle is relatively fatiguable compared to a muscle with a low fatigue index, which is relatively fatigue resistant. The results are summarized in Table 18. It can be seen that following partial denervation in G93A mice, the fatigue resistance of the operated EDL muscles was similar to the contralateral control muscles (0.79 ± 0.03 S.E.M., n=3; 0.74 ± 0.03 S.E.M., n=3, respectively). Following partial denervation in wild-type mice, the fatigue resistance of operated EDL muscles was also not significantly different to the contralateral control muscles (0.83 ± 0.03 S.E.M., n=3; 0.82 ± 0.05 S.E.M., n=3, respectively). Thus, partial denervation in G93A mice did not alter the fatigue resistance of operated EDL muscles at 90 days of age.

Table 18. The effect of partial denervation on muscle fatigue resistance in G93A and wild-type mice at 90 days of age

The fatigue index is calculated as the reduction in force elicited by the muscle following stimulation for 250 ms every second for 3 minutes. A high fatigue index represents a fatiguable muscle, whereas a low index represents of a fatigue resistant muscle. The Table shows the mean fatigue index of EDL muscles in 90 day old G93A **(transgenic)** and wild-type mice following partial denervation. The values of the operated EDL muscle are also expressed as a percentage of the control unoperated EDL muscle **(% OP/CON)**.

Table 18

Mice	Fatigue index		
	CON	OP	% OP/CON
Wild-type	**0.82**	**0.83**	**101.2**
mean ± S.E.M.	± 0.05	± 0.03	± 7..9
(n=3)			
Transgenic	**0.74**	**0.79**	**108.7**
mean ± S.E.M.	± 0.05	± 0.03	± 25.6
(n=3)			

4.3.2.2.4. Succinate dehydrogenase activity following partial denervation

To examine whether the poor functional reinnervation that occurs in EDL muscles following partial denervation in G93A mice is reflected by a change in the oxidative metabolism of muscle fibres, EDL muscles were frozen and processed for succinate dehydrogenase (SDH) histochemistry. Figure 31 show examples of EDL muscle sections stained for SDH activity from G93A mice, following partial denervation. The mosaic distribution of muscle fibres with varying oxidative capacity can be clearly seen in the contralateral control EDL muscle of a G93A mouse. However, in the operated EDL muscle the even distribution of dark and lightly stained muscle fibres was disrupted by partial denervation. Furthermore, in the central portion of the EDL muscle, the muscle fibres are almost devoid of staining, which suggests that a substantial area of the EDL muscle lacks innervation.

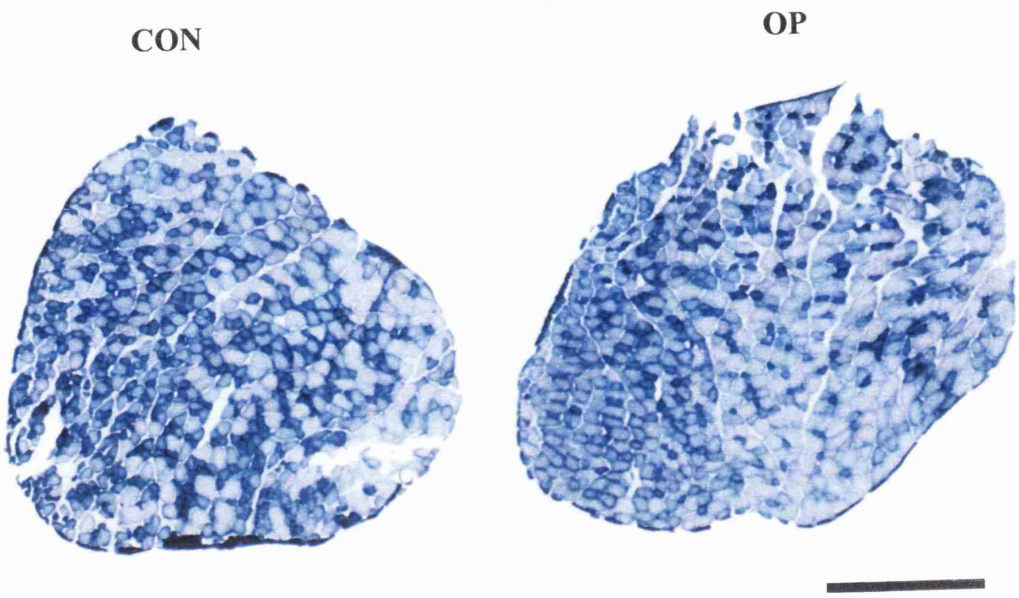
4.3.2.2.5. Motor unit number and motoneuron survival following partial denervation

To examine the possibility that partial denervation in G93A increases the decline in muscle force due to a greater loss functional motoneurons, the number of motor units in EDL muscles was assessed. Unfortunately, it was difficult to obtain recordings of motor units following partial denervation, as there was simultaneous firing of motoneurons at a set voltage. This may be due to the extensive sprouting and synaptic remodelling that occurs for many weeks following partial denervation.

Figure 31. The effect of partial denervation on succinate dehydrogenase activity in EDL muscles of G93A mice at 90 days of age

Representative photomicrographs of transverse sections of control **(CON)** and operated **(OP)** EDL muscles from a G93A mouse stained for succinate dehydrogenase (SDH) activity. Scale bar = 1 mm

Figure 31



An alternative method to assess motoneuron survival is to count the number of Nissl-stained motoneurons in each ventral horn of the spinal cord at the level of the sciatic motor pool. However, in this case, this method of assessing motoneuron survival is not appropriate, since partial denervation carried out by transection of the L4 nerve may affect motoneuron survival, as observed as a consequence of nerve injury presented earlier in this Chapter. Therefore, any additional motoneuron death that may be caused by inducing axonal growth of motoneurons in the intact L5 spinal nerve cannot be separated from the effect of L4 transection.

4.3.3. Summary of results

The present study examined the progression of disease in a transgenic mouse model of FALS (TgN (SOD1-G93A) Gur1) using physiological and morphological methods. This study also investigated whether inducing motoneurons to grow prior to the period of motoneuron death in G93A mice, accelerated the progression of the disease.

(A) Progression of disease in G93A mice

At the approximate symptomatic onset of the disease in G93A mice (90 days of age) compared to age-matched wild-type mice there was: **(1)** a decline in the forces produced by both EDL and TA muscles, **(2)** a significant decrease in TA muscle weight but not soleus and EDL muscles, **(3)** a 15% decline in the number of EDL motor units, **(4)** a small increase in the fatigue resistance of EDL muscles, **(5)** no significant differences in the contractile properties of EDL muscles, **(6)** evidence of fibre type grouping, and **(7)** a 39% reduction in the number of sciatic motoneurons.

By the end-stage of the disease in G93A mice (130 days of age) compared to age-matched wild-type mice there was: **(1)** a 70-80% reduction in the force produced by EDL and TA muscles, **(2)** a considerable loss of muscle weight for TA, EDL and soleus muscles, **(3)** a 60% reduction in the number of EDL motor units, **(4)** a significant increase in the fatigue resistance of EDL muscles, **(5)** a slower rate of relaxation for both EDL and TA muscles, **(6)** a substantial increase in the SDH activity of EDL muscle fibres, and **(7)** a 71% reduction in the number of sciatic motoneurons.

(B) The effect of nerve injury on disease progression

The effect of sciatic nerve-crush in G93A mice carried out during the presymptomatic phase of the disease (42 days of age) was characterized at 90 days using both physiological and morphological methods. The results show that nerve injury in G93A mice does not have the same deleterious affects as observed in wild-type mice following nerve injury. Thus, nerve injury in G93A mice: **(1)** tends to slow the decline in muscle force in the EDL muscle, **(2)** does not exacerbate the loss of EDL motor units, but increases the average motor unit size, **(3)** increases the fatigue resistance of the EDL muscles, which also exhibit higher levels of SDH activity. However, nerve injury in G93A mice, **(4)** accelerates the overall loss of sciatic motoneurons, since only 67% of motoneurons survived in the operated ventral horn compared to the contralateral ventral horn.

(C) The effect of partial denervation on disease progression

The effect of partial denervation in G93A mice carried out during the presymptomatic phase of the disease (42 days of age) was also characterized at 90 days. The results show that partial denervation in G93A mice results in poorer muscle function: **(1)** increases the decline in EDL muscle force, **(2)** reduces the extent of functional innervation in EDL muscles, but **(4)** does not alter the fatigue resistance of EDL muscles.

4.4. DISCUSSION

Since the development of mouse models of FALS, numerous studies have investigated the neuropathological features of the disease, the time-course of behavioural motor deficits, and the underlying causes of motoneuron degeneration. However, there are relatively few studies that relate to the physiological changes in motor function during the course of the disease, and which examine the progressive nature of motoneuron degeneration. Therefore, the aim of the present study was to characterize the progression of motoneuron disease in G93A transgenic mice in terms of muscle physiology and motoneuron degeneration. Secondly, the influence of axonal growth and sprouting as a possible contributory factor in the death of motoneurons was also examined.

4.4.1. Time-course of motoneuron disease in G93A mice

The results of this Chapter show that at the approximate onset of the symptomatic phase of the disease in G93A mice (90 days of age) significant motor deficits are present in hindlimb muscles. Thus, at P90, the forces produced by both TA and EDL muscles in G93A mice were significantly weaker than in age-matched wild-type mice. Interestingly, the decrease in muscle force was considerably greater for TA muscles than EDL muscles. For example, the maximal tetanic tension elicited by TA muscles in G93A mice declined to 42% and in EDL muscles to 69%, compared to age-matched wild-type mice. This difference in muscle force between TA and EDL muscles was also reflected by a significant decline in the weight of TA muscles, whereas the weight of the EDL muscles was unchanged. Furthermore, at P90,

assessment of muscle contractile characteristics revealed that the ½ time to relaxation was significantly slower in TA muscles but unaffected in EDL muscles. Although the contractile characteristics of EDL muscles were unaffected at this stage of the disease, there was a trend towards an increase in the fatigue resistance of EDL muscles in G39A mice. There was also evidence of fibre type grouping in EDL muscles, which indicates that re-specification of muscle fibre properties was occurring due to a process of denervation followed by reinnervation. However, changes in muscle fibre properties were assessed in terms of the levels of SDH activity, which only indicates the oxidative capacity, but not the structural composition of the muscle fibre. Therefore, to gain a clearer understanding of the changes in muscle fibre type in SOD1 mice, further experiments should assess a range of aerobic and anaerobic enzyme activities as well as structural components such as the different myosin isoforms, which largely determine the contractile characteristics of muscle fibres.

Overall, these various physiological and morphological assessments reveal that at the onset of the symptomatic phase of the disease in G93A mice, the disease process has reached a level where muscle function is significantly impaired and a number of muscle characteristics are altered.

The present study also examined whether the decrease in muscle force was concomitant with a decrease in motoneuron survival. It was found that only 15% of EDL motor units were lost in G93A mice at P90, compared to wild-type mice. Thus, this reduction in motor unit number does not closely correlate with the decline in muscle function observed, since EDL tetanic force decreased by 31%. This suggests

that collateral reinnervation of muscle fibres by surviving motoneurons was unable to compensate for the small loss of motoneurons. This is somewhat surprising, since extensive muscle denervation (>50%) in humans and rodents is normally compensated for by the enlargement of peripheral fields by intact motoneurons, which maintain normal muscle strength (Weiss and Edds, 1946; McComas et al, 1971; White et al, 2000). In addition, the present experiments also found that the number of motoneurons in the sciatic pool of G93A mice decreased by 39% compared to age-matched wild-type littermates. This is consistent with other studies,

which have reported a similar loss of motoneurons in the lumbar region of the spinal cord in a number of different mouse models of ALS (Chiu et al, 1995; Bruijn et al, 1997; Morrison et al, 1998). Overall, the assessment of motoneuron survival at P90 in G93A mice indicates that there is a selective vulnerability of sciatic motoneurons at this stage of the disease process, since the number of EDL motor units, which are located within the sciatic motor pool, only decreased by 15%, whereas the number of sciatic motoneurons decreased by 39%. Furthermore, a previous study has shown that the number of gastrocnemius motoneurons only decreased by 25% in G93A mice at P90 (Azzouz et al, 1997). Moreover, the present results have also shown that muscle function is far more impaired in TA muscles compared to EDL muscles in G93A mice at P90. This suggests that there is a more extensive decline in the number of functional motoneurons innervating the TA muscle compared to the EDL muscle, which may, in part, account for the significant decline in the overall numbers of sciatic motoneurons. It is possible that the underlying disease process is accelerated in motoneurons with larger peripheral fields, since larger motor units may have higher metabolic demands and higher levels of stress, which may exacerbate the underlying toxicity. Thus, it is possible that the average motor unit size in TA muscles is larger than in EDL muscles, which may account for the more severely impaired function of TA muscles compared to EDL muscles in G93A mice. Further experiments are required to examine this possibility.

Between P90 and P130, the disease progressed rapidly in G93A mice. Thus, by P130 (end-stage of the disease) there was a severe loss of maximum tetanic force in both TA and EDL muscles (71% and 78% respectively), and a considerable decrease in the number of functional motor units in EDL muscles (60%). At this stage in the

disease process the decline in the number of sciatic motoneurons (70%) more closely correlated with the loss of EDL motor units, indicating that that even those motoneurons which were relatively unaffected by the disease process at P90, also undergo degeneration by the end-stage of the disease. In addition, at P130, the EDL muscles were highly fatigue resistant, which was reflected by an increase in the oxidative capacity of a large proportion of muscle fibres (high SDH activity) and a significant increase in the time to contract and relax of both EDL and TA muscles.

It has long been thought that there may be a long preclinical phase of motoneuron loss in ALS during which the disease is not symptomatic because of compensatory mechanisms such as sprouting and reinnervation by surviving motoneurons (Swash and Ingram, 1988). However, the results of the present Chapter suggest that collateral or terminal nerve sprouting by less affected motoneurons is not able to compensate for an initial loss of motoneurons, since the force produced by EDL muscles had declined significantly by P90 in G93A, whereas the decline in motor units in EDL muscles was relatively small. Although, it appears that surviving motor units are unable to compensate for the initial loss of motoneurons, diseased motoneurons in G93A mice do retain their capacity to send out collateral axons in response to denervated muscle territory (Chiu et al, 1995; Frey et al, 2000). Therefore, it appears that the process of axonal sprouting and reinnervation in G93A mice may be functionally limited. This possibility is supported by evidence that many ALS patients with modestly enlarged motor unit potentials also had smaller than normal twitch forces (Schmied et al, 1999).

Interestingly, evidence of axonal sprouting and denervated endplates has been shown in hindlimb muscles of G93A mice during the presymptomatic phase of the disease and prior to motoneuron loss (Chiu et al, 1995; Frey et al, 2000). Indeed, indirect evidence of the denervation/reinnervation process is also shown by the present study, since the even distribution pattern of muscle fibre types normally seen in EDL muscle is disrupted and evidence of grouping of slow fibres can be clearly seen (Fig. 24B). Therefore, synaptic destabilization and progressive denervation before the onset of motoneuron loss may also explain the decline in muscle function. Consistent with this proposal, reports of early neuromuscular junction defects have been shown in a dog model of ALS (Pinter et al, 1995, 1997) and in ALS patients (Maselli et al, 1993).

Previous findings have shown that the earliest evidence of pathology in G93A mice is neuronal vacuolation, which is evident as early as 37 days in motor axons, and in cell bodies of motoneurons at 45 days (Chiu et al, 1995; Mourelatos et al, 1996). These vacuoles are thought to be derived from dilation and fragmentation of mitochondria (Dal Canto and Gurney, 1995; Wong et al, 1995). Similar signs of abnormal mitochondria have also been found in motoneurons of ALS patients (Hirano et al, 1984; Nakano and Hirano, 1987). This evidence suggests that mitochondria are at the centre of the degenerative process and that mitochondria are one of the earliest targets of mutant SOD1 toxicity (Carri et al, 1997; Kong and Xu, 1998; Kruman, 1999). Thus, mitochondrial dysfunction may contribute significantly to early functional deficits observed in G93A mice, which occur prior to substantial motoneuron death, by impairing energy metabolism in vulnerable motoneurons.

Overall, the results of the present study have shown that at the start of the symptomatic phase of the disease (P90) in G93A mice, there was a loss of muscle force for both TA and EDL muscles, a small loss of EDL motor units, an increase in EDL fatigue resistance/oxidative metabolism, and a substantial loss of sciatic motoneurons. These results indicate that following an initial loss of motoneurons in G93A mice, the compensatory reinnervation process by surviving motoneurons fails to maintain muscle function. It is possible that early pathological events, such as mitochondrial dysfunction, contribute to poor functional recovery after denervation.

By the end stage of the disease (P130) in G93A mice, physiological assessments showed a considerable decline in muscle function, such that muscle force, and motor unit number declined rapidly between P90 and P130. In addition, the EDL muscle became highly fatigue resistant, and contained many muscle fibres with high oxidative metabolism, which was reflected by slower contractile properties. Finally, massive motoneuron degeneration occurred in the sciatic motor pool, such that only 29% of motoneurons survived by P130.

4.4.2. Inducing axonal growth on disease progression in G93A mice

Both immature and adult motoneurons have been shown to be vulnerable to cell death when their axons are forced to grow for a prolonged period (Romanes, 1946; Lowrie et al, 1982; Harding et al, 1999; White et al, 2000). It is possible that continued sprouting and growth of motor axons that occurs in patients with ALS, contributes to the vulnerability of diseased motoneurons, which results in a rapid progression of the disease. Therefore, the present study examined the effect of

experimentally inducing axonal growth on disease progression in a mouse model of ALS (G93A). Axonal growth was induced by either sciatic nerve injury or partial denervation during the presymatomatic phase of the disease (42 days) in G93A mice.

4.4.2.1. The effect of sciatic nerve injury on disease progression in G93A mice

The results of the present Chapter have shown that following nerve injury in G93A mice there was a surprising trend towards an improved function in the EDL muscle. Thus, at P90 both the twitch and tetanic force of the operated EDL muscle was greater than the EDL muscle on the contralateral side (107% and 134%, respectively). However, due the small numbers of SOD1 mice analyzed, and the high degree of variability within this group, no firm conclusions can be made concerning the apparent beneficial effects of nerve injury on EDL muscle force in SOD1 mice.

In contrast, the same injury in wild-type mice resulted in a decline in EDL muscle function, such that the twitch and tetanic forces were weaker for the operated EDL muscle compared to the contralateral control muscle (63% and 86%, respectively). In addition, the present study also examined whether nerve injury would accelerate the loss of motoneurons in G93A mice. The results showed that nerve injury had no effect on the number of motor units in EDL muscles, in both wild-type as well as G93A mice. However, nerve injury in G93A mice resulted in an accelerated loss of sciatic motoneurons, since only 67% of motoneurons in the operated ventral horn survived compared to the contralateral ventral horn, whereas no loss of sciatic motoneurons occurred in wild-type mice, since 97% of motoneurons survived. Overall, these results show that inducing motoneurons to grow in G93A mice by

nerve injury accelerates the rate in which sciatic motoneurons degenerate. However, there also appears to be a selective vulnerability of motoneurons within the sciatic motor pool, since no additional loss of EDL motor units was found at P90 in G93A mice. Furthermore, the surviving EDL motor units

tended to generate more force than those on the contralateral side, which indicates that those injured motoneurons innervating EDL muscles were in a relatively healthier state compared to uninjured motoneurons. Moreover, the fatigue resistant and oxidative capacity of reinnervated EDL muscle fibres also increased following nerve injury, which indicates that the activity of injured motoneurons changed from a predominantly phasic pattern to a tonic pattern.

So what factors may account for the improved EDL muscle function following injury? One possibility is linked to the metabolic state of the motoneuron during the early stages of the regenerative process. Following nerve injury, the motoneuron cell body decreases in size (Vanden Noven et al, 1993), dendrites retract (Standler and Bernstein, 1982) and the number of synapses contacting the motoneuron declines (Blinzinger and Kreutzberg, 1968; Brannstrom and Kellerth, 1998). Furthermore, a decrease in axonal diameter also occurs immediately after injury (Gillespie and Stein, 1983; Gordon et al, 1991). These morphological changes probably reflect an overall atrophic state of the motoneuron during the period that the motoneuron is disconnected from the muscle, which may result in lower metabolic activity and cell stress. This idea is supported by evidence that shows that motoneurons with large axonal diameters are more profoundly affected than motoneurons with small axonal diameters in G93A mice (Klivenyi et al, 1999). In addition, large motoneurons innervating the gastronemius muscle have been shown to be selectively lost at the end stage of the disease in G93A mice (Moharjeri et al, 1998). It is therefore possible that the lower metabolic activity of injured motoneurons in G93A mice, results in a delay in the pathological process and thereby an improvement in EDL muscle force following reinnervation. However, the beneficial effect of nerve injury is only likely

to be a transient phenomenon, since re-establishment of functional connections with the muscle, will also result in the return of high metabolic demands required by intramuscular sprouting, an enlarged peripheral territory, renewed synaptic inputs and neuromuscular transmission, which may ultimately exacerbate the disease process in motoneurons.

A previous report has shown that axotomizing and ligating the sciatic nerve to prevent muscle reinnervation in G93A mice, reduced the extent of axon degeneration at the end-stage of the disease, leading to an increase in the number of surviving axons in the axotomized nerve compared to the control nerve (Kong and Xu, 1999). In contrast, the present study shows that nerve injury in G93A mice increases the extent of motoneuron degeneration as early as the start of the symptomatic phase of the disease. This difference is likely to be due to the fact that in the present study regenerating sciatic motoneurons were not prevented from reinnervating the target hindlimb muscles. By allowing functional reinnervation, this is likely to impose a greater metabolic demand than if the nerve was ligated, which may increase the vulnerability of diseased motoneurons to cell death.

Another possible explanation for the improvement in the force generated by the reinnervated EDL muscle in G93A mice is that the stability of the neuromuscular contacts is greater following reinnervation, since denervated muscle is very receptive to establishing neuromuscular synapses by regenerating motor axons (Prof. G. Vrbová, personal communication). In contrast, during normal disease progression in animal models of FALS there is progressive synaptic weakening and denervation at neuromuscular synapses prior to motoneuron loss (Dengler et al, 1990; Chiu et al,

1995; Pinter et al, 1995, 1997; Frey et al, 2000). Thus, following reinnervation of the EDL muscle, there may be an improvement in the efficacy of neuromuscular transmission, and a reduction in the extent of denervation. Indeed, the present results show that following reinnervation of EDL muscles in G93A mice there is an increase in motor unit size. However, the enlargement of motor units following reinnervation may, in the longer-term, actually accelerate the rate of motoneuron degeneration due to an increase in the metabolic requirements of motoneurons with larger peripheral territories. This explanation may account for the increase in sciatic motoneuron degeneration following nerve injury. However, why EDL motoneurons are less vulnerable to nerve injury compared to other motoneurons in the sciatic motor pool remains unclear.

4.4.2.2. The effect of partial denervation on disease progression in G93A mice

The present study also examined the effect of inducing axonal growth by partial denervation on disease progression in G93A mice. This method of inducing axonal sprouting is a more suitable model for assessing the effects of axonal growth of disease progression, since motoneurons are not directly injured. Furthermore, the resulting partial denervation of EDL muscle induces compensatory sprouting by intact motoneurons, which more closely reflects the type of growth that occurs following muscle denervation caused by the progressive loss of motoneurons in ALS.

The results of this Chapter have shown that following partial denervation in G93A mice, there was a decline in the function of EDL muscles. Thus, at P90, the twitch

and tetanic forces decreased by 42% and 43% respectively in the reinnervated EDL muscles compared to the contralateral control muscles. In contrast, partial denervation of EDL muscles in wild-type did not result in a significant decline in muscle force, which is consistent with findings of other studies (Weiss and Edds, 1946; Rafuse et al, 1992; White et al, 2000). In addition, the degree of reinnervation was assessed in both G93A and wild-type mice by measuring the relative force produced by indirect stimulation of the reinnervated EDL muscle compared to direct stimulation. It was found that following partial denervation in G93A mice the force elicited by indirect stimulation of reinnervated EDL muscle was only 68% of that produced by direct stimulation, whereas in the unoperated EDL muscle the value was 96%. Thus, the functional innervation ratio in operated EDL muscles compared to control muscles was 71%, which indicates that there was incomplete reinnervation in G93A following partial denervation. In wild-type mice, there was near complete reinnervation, since the functional innervation ratio of the operated EDL muscle was comparable with the control EDL muscle. Furthermore, there was also histochemical evidence that substantial areas of the operated EDL muscle in G93A lacked innervation, since many muscle fibres were almost devoid of SDH staining.

One possible explanation for the decline in muscle function following partial denervation in G93A mice is that due to the formation of larger motor units following collateral reinnervation by intact motoneurons, the metabolic demands placed upon these motoneurons are much greater than those with smaller territories. This in turn may cause an increase in the levels of oxidative stress and accelerate the degenerative process in vulnerable motoneurons. Oxidative stress is thought to be a factor involved in cellular dysfunction in ALS (Ferrante et al, 1997; Liu et al, 1998),

with mitochondria being the predominant source of oxidative free radicals. Because production of free radicals is directly correlated with metabolic activity (Beal, 1996), the increased metabolic demands of a motoneuron within an enlarged peripheral territory, may result in a higher production of free radicals. Thus, the substrate for the aberrant activities of mutant SOD1, which has been hypothesized to further generate free radicals and oxidative stress (Beckman et al, 1993; Wiedau-Pazos et al, 1996), may be increased in motoneurons following partial denervation. As a result, these motoneurons may be unable to support the expanded peripheral territory, and therefore withdraw synaptic terminals in an attempt to reduce metabolic stress, leaving many muscle fibres denervated and resulting in a decline in muscle force. Alternatively, motoneurons in G93A mice that are forced to expand their peripheral field, may degenerate at a faster rate, due to the damage caused by higher metabolic demands. Unfortunately, this study was unable to ascertain whether this was the case due to difficulties in recruiting individual EDL motor units at this stage following partial denervation, since there was simultaneous firing of motoneurons at a set voltage.

In support of the proposal that enlarged motor units compromise motoneuron function in G93A mice, a recent study has shown that even healthy motoneurons with chronically enlarged motor units fail to cope with the increased metabolic demands and progressively withdraw nerve terminals (Tam et al, 2002). Furthermore, clinical observations have shown patients who have suffered from poliomyelitis, a viral illness which causes motoneuron death, can develop further weakness, muscle atrophy, and fatiguability (post-polio syndrome) many years after the initial loss of motoneurons (Halstead and Rossi, 1987). It has also been

postulated that these symptoms are a result of further motoneuron loss caused by the metabolic stress placed upon motoneurons that support enlarged muscle territories (Dalakas, 1995). In support, symptomatic post-polio syndrome is experienced primarily by patients who suffered extensive motoneuron loss at the time of their original illness, which suggests that the development of enlarged motor units is necessary to cause further motoneurons loss (Cashman et al, 1987; Maselli et al, 1992).

4.4.3. Conclusions

Overall, the results of this study provide a physiological and morphological analysis of disease progression in a transgenic mouse model of FALS (mSOD1, G93A). Furthermore, this study also investigated whether axonal sprouting known to occur as a compensatory mechanism following the death of motoneurons in ALS, increases the vulnerability of motoneurons and thereby contributes to the rapid progression of the disease. The results show that inducing axonal growth by nerve injury prior to motoneuron death in G93A mice increases the extent of sciatic motoneuron death by the start of the symptomatic phase of the disease (P90). However, there appears to be a differential vulnerability of sciatic motoneurons, since the number of EDL motor units was unaffected and there was also a trend towards improved EDL muscle function. In contrast, inducing axonal growth by partial denervation in G93A mice, which more closely models compensatory sprouting in ALS, resulted in a substantial decline in muscle force and functional reinnervation. Taken together, these results suggest that growth associated with compensatory sprouting may accelerate disease progression in ALS. It can be argued that it is not growth per se that increases the vulnerability of motoneurons to cell death, but the enlarged motor unit size that

occurs as a result of axonal sprouting. Thus, the enlarged peripheral territories of motoneurons in ALS may accelerate disease progression by placing motoneurons under higher levels of metabolic stress, which may exacerbate the underlying disease process.

CHAPTER 5

GENERAL DISCUSSION

5.1. Aims of the Thesis

This Thesis examined the hypothesis that prolonged axonal growth increases the vulnerability of developing and adult motoneurons to cell death. It is well established that developing motoneurons are critically dependent upon functional interaction with their target muscles for their survival. This target dependence declines during the early postnatal period, which correlates with transformation of motoneurons from growing into fully transmitting cells. It has been postulated that a muscle-induced increase in transmitter release from developing motor nerve terminals is an essential step in promoting the transformation of motoneurons into mature transmitting cells. Therefore, in the first Chapter of this Thesis, I examined whether manipulating transmitter release at the NMJ altered the developmental expression of proteins associated with growth and transmission in developing motoneurons, in order to establish the role of target interaction in ensuring motoneuron development.

Evidence suggests that maintaining developing motoneurons in a growth state by disrupting neuromuscular contacts, renders them vulnerable to glutamate-mediated excitotoxicity. Since adult motoneurons are also vulnerable to cell death when forced to grow for a prolonged period, it is possible that the same mechanisms implicated in the death of developing motoneurons may also underlie the death of extensively growing adult motoneurons. Therefore, in the second Chapter of this Thesis, I also investigated a number of possible mechanisms that may contribute to the death of adult motoneurons forced to grow as a consequence of nerve injury in transgenic mice induced to overexpress the growth-associated protein, GAP-43.

Since adult motoneurons are known to undergo periods of extensive axonal growth in conditions such as ALS, it is possible that this growth of axons actually contributes to the progressive neurodegeneration observed in this disorder. Therefore, in the final Chapter, I examined whether experimentally inducing motoneurons to undergo axonal sprouting, accelerates the progression of motor neuron disease in mutant SOD1 mice.

5.2. The role of neuromuscular interaction in the development of motoneurons

Numerous studies have clearly demonstrated that during the early postnatal period, functional contact with the muscle is critical for the continued survival of developing motoneurons (Romanes, 1946; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987; Greensmith and Vrbová, 1992). For example, following sciatic nerve injury in neonatal rats, a large proportion of motoneurons die (Lowrie et al, 1987). However, this vulnerability to nerve injury declines rapidly during the first postnatal week, so that by 5 days after birth, most sciatic motoneurons are able to survive the same injury (Lowrie et al, 1982). Thus, sciatic motoneurons undergo a dramatic change during the first 5 days of postnatal development, which is dependent on contact with their target muscle. Furthermore, it appears the transition of a motoneuron from a target-dependent into a target-independent state not only requires physical contact with the target, but also a functional interaction between motoneuron and muscle. For example, blocking neuromuscular activity at birth, by paralyzing the muscle with α-bungarotoxin (α-BTX), results in the death of motoneurons (Greensmith and Vrbová, 1992).

However, the mechanism by which neuromuscular interaction promotes the development and survival of the motoneuron is unclear. One approach to resolving this question is to explore the possibility that functional events that occur at the NMJ, may play a critical role in changing the motoneuron from a target-dependent into a mature target-independent state. One of the most significant changes in function occurs when the growth cone encounters its target muscle cell. This interaction results in a marked retrograde effect demonstrated by an immediate increase in spontaneous acetylcholine (ACh) release (Chow and Poo, 1985; Xie and Poo, 1986; Evers et al, 1989). With further synaptic differentiation there is an increase in the frequency of spontaneous minature endplate potentials (MEPPs), and the quantal content of EPPs (Diamond and Miledi, 1962; Kelly, 1978; Dennis et al, 1981; Lowrie et al, 1985). These changes at the motor nerve terminal are reflected in the cell body, where an increase in the ACh-synthesizing enzyme ChAT is observed (Phelps et al, 1984). Furthermore, following the increase in transmitter release, superfluous synaptic contacts are lost through the retraction of nerve branches (Bennett and Pettigrew, 1974; Brown et al, 1976; O'Brien et al, 1978). This period of synapse elimination is accompanied by a down-regulation of growth associated proteins such as GAP-43, α-tubulin and the microtubule MAP1B (Reynolds et al, 1991; Caroni and Becker, 1992; Ma et al, 1997). This down-regulation of growth associated proteins is dependent on neuromuscular activity, since their levels fail to decline when neurotransmission is blocked (Caroni and Becker, 1992) and retraction of nerve terminals does not occur (Sohal and Holt, 1980; Brown et al, 1981; Duxson, 1982; Caroni and Becker, 1992). Thus, the functional emphasis of the motoneuron is greatly altered by events that occur at the nerve terminals, and these events are dependent upon neuromuscular interaction. This change in the phenotype of the

motoneuron from a growing into a fully transmitting cell closely corresponds with the period during which motoneurons lose their dependence on target interaction. Indeed, the results presented in Chapter 2, confirm this, by demonstrating that ChAT protein levels in the cell bodies of sciatic motoneurons increase significantly during the first 5 postnatal days, a period in which developing motoneurons lose their vulnerability to injury-induced cell death. In addition, levels of ChAT activity at the developing motor nerve terminals also increase dramatically at the end of the first postnatal week. Furthermore, there appears to be a direct relationship between an increase in ChAT activity in nerve terminals and axonal growth, since GAP-43 levels in motor axons decline soon after the increase in ChAT activity. Overall these findings suggest that an increase in transmitter release from developing nerve terminals is a critical event in promoting motoneuron maturation, which is likely to be induced by active neuromuscular interaction. Furthermore, it has been postulated that this target-induced change in gene expression within motoneurons is essential for their survival (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996).

Recent evidence supporting the possibility that muscle-induced transmitter release promotes motoneuron survival has come from experiments which examined the effect of manipulating transmitter release at the NMJ on the survival of motoneurons. It was shown that decreasing transmitter release from developing nerve terminals *in vivo* by treatment with $MgSO_4$ (a Ca^{2+} channel blocker) resulted in motoneuron death (Greensmith et al, 2000). Conversely, prematurely enhancing ACh release by treatment of the NMJ with 4-AP (a neural K^+ channel blocker), protected motoneurons from axotomy-induced cell death (Greensmith et al, 1996). Moreover, results presented in Chapter 2, extend these observations and demonstrate that 4-AP

treatment of motor nerve terminals at birth results in a premature transition of developing motoneurons from a growth into a transmitting state. In contrast, $MgSO_4$ treatment at birth delays this transition and subsequently results in the death of motoneurons. Thus, manipulating transmitter release at motor nerve terminals alters the rate at which motoneurons up-regulate their cholinergic phenotype and as a consequence affects their transformation from growing into transmitting cells. It is likely that this change in phenotype is important for the maturation of developing motoneurons, which may determine their vulnerability to nerve injury.

5.3. What is the nature of the retrograde signal that induces transmitter release?

In recent years, much attention has focused on elucidating the factors involved in the regulation of neuronal development and survival. It is now well established that target-derived neurotrophins play an essential role in maintaining the survival of several neuronal populations (Cohen et al, 1954; Korsching and Thoenen, 1983; reviewed by Levi-Montalcini and Angeletti, 1968; Thoenen, 1991). For developing motoneurons, a variety of neurotrophins including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), have all been found to promote the survival of motoneurons both in culture (Henderson et al, 1993, 1994) and following neonatal axotomy (Sendtner et al, 1992; Yan et al, 1992). However, neuroprotection from axotomy-induced cell death by directly applied neurotophic factors is only transient (Zurn et al, 1994; Eriksson et al, 1994; Vesjsada et al, 1995). Interestingly, one effect that is common to all these factors that maintain motoneuron survival is the enhanced expression of the cholinergic phenotype of the motoneuron.

For example, neurotrophins can stimulate the expression of ChAT in the motoneuron cell body in vitro (Martinou et al, 1992; Wong et al, 1993), and can prevent the loss of ChAT expression that is normally triggered by axotomy (Yan et al, 1995; Friedman et al, 1995; Wang et al, 1997). It is possible that the survival promoting effect of neurotrophins may be attributable to the enhanced expression of ChAT, which may result from an ability to increase transmitter release from growth cones of developing and axotomized motoneurons. This possibility is supported by studies that identified neurotrophins as potent neuromodulators at developing neuromuscular synapses (reviewed by Fitzsimonds and Poo, 1998; Schinder and Poo, 2000). For example, acute treatment of nerve-muscle cultures with BDNF or NT-3 rapidly increased both spontaneous and evoked transmitter release (Lohof et al, 1993). Furthermore, the production of NT-4 in rat skeletal muscle was found to depend on muscle activity (Funakoshi et al, 1995), and also enhance evoked synaptic transmission and developing neuromuscular synapses (Wang and Poo, 1997). These results indicate that neurotrophins may be crucial molecules in an activity-dependent feedback mechanism that potentiates transmitter release from developing nerve terminals, which in turn induces further motoneuron maturation.

Another possibility is that a physical interaction between developing nerve terminal and muscle may be partly responsible for the induction and maturation of transmitter release. Indeed, cell adhesion molecules or extracellular matrix proteins (ECM) have been implicated in mediating contact-dependent presynaptic changes during synaptogenesis, since the muscle membrane by itself is capable of inducing spontaneous ACh release from the growth cone (Xie and Poo, 1986). An indication of the nature of these membrane-bound components that regulate transmitter release

has been provided by studies investigating the mechanism of stretch-induced enhancement of transmitter release ~~Transmitter release~~ from frog motor nerve terminals is known to be powerfully regulated by muscle length (Fatt and Katz, 1952). It has been reported that this phenomenon can be suppressed by interfering with the binding of integrins to their natural ligands (Chen and Grinnell, 1995, 1997). Integrins are heterodimeric glycoproteins, which function predominantly as receptors for components of the ECM (Hynes, 1992). Peptides containing the arginine-glycine-aspartic acid (RGD) sequence are known to disrupt the binding of integrins to their ligands present on the ECM (Ruoslahti and Pierschbacher, 1987) and therefore interrupt the bidirectional mechanical signalling implicated in enhancing transmitter release (Chen and Grinnell, 1997). A recent finding indicates that integrin-ECM interactions at the developing NMJ may also be involved in enhancing transmitter release, since blocking integrin-ECM binding at the NMJ delayed the normal developmental increase in ChAT and significantly, resulted in the death of a large proportion of motoneurons (Wong et al, 1999). Thus, integrins may act as mechanotransducers at the NMJ, and may possibly play a role in both regulating transmitter release and motoneuron development.

5.4. Prolonged axonal growth results in the death of adult motoneurons

Results presented in Chapter 2 suggest that disrupting neuromuscular interaction at birth by pharmacologically depressing transmitter release maintains developing motoneurons in a growth state, which increases the vulnerability of these motoneurons to cell death. This interpretation is supported by a number of studies, which also found motoneuron death when neuromuscular interaction was prevented

at birth using a variety of surgical and pharmacological manipulations (Lowrie et al, 1987; Greensmith et al, 1992; Harding et al, 1998; Greensmith et al, 2000). Moreover, stabilizing neuromuscular contacts following neonatal nerve injury or muscle paralysis, by treatment with leupeptin (a neural protease inhibitor) rescued developing motoneurons from cell death (Harding et al, 1996, 1999).

In contrast to developing motoneurons, adult motoneurons survive injury to their peripheral axons, and resume functional interaction with the muscle following a period of regeneration, which involves the transient re-expression of growth associated proteins (Hoffman and Lasek, 1980; Tetzlaff et al, 1988; 1991). Nevertheless, it has been shown that even adult motoneurons die if their axons are subjected to repeated stimuli for growth (Arvidsson and Aldskogius, 1982; White et al, 2000). Thus, it is possible that extending the period in which adult motoneurons are in a growth state renders them vulnerable to cell death. Evidence supporting this proposal came from experiments on transgenic mice that overexpress the growth associated protein GAP-43. Motor axons in these transgenic mice undergo a period of profuse axonal sprouting during early adult life (Aigner et al, 1995). It has been shown that nerve injury carried out in these transgenic mice, during the period of profuse sprouting, resulted in the death of motoneurons innervating the soleus muscle (Harding et al, 1999). Consistent with these findings, the results in Chapter 3 have shown that nerve injury carried out in GAP-43 transgenic mice during the time of maximal sprouting, resulted in the death of a significant proportion of motoneurons in the sciatic motor pool. Interestingly, it was also found that these motoneurons died progressively, with the majority of motoneuron loss occurring 5-10 weeks after nerve injury. This result indicates that it is not nerve injury per se that

causes motoneuron to die, but the extensive growth that occurs as a result of nerve injury. Furthermore, this study also revealed that larger motoneurons in the sciatic motor pool appear to be more vulnerable to cell death. Given the fact that larger motoneurons have larger peripheral axons and larger motor unit territories (Henneman and Olson, 1965), it is likely that the regenerative effort required by larger motoneurons to re-establish their original motor unit size is greater than that required by smaller motoneurons. Taken together, it is possible that adult motoneurons, which are induced to grow extensively and for a prolonged period, die as a consequence of the metabolic stress generated by the requirements of re-establishing functional connections with their target muscle.

5.5. Are extensively growing adult motoneurons vulnerable to excitotoxic cell death?

There is substantial evidence to suggest that glutamate-mediated excitotoxicity plays a role in the death of developing motoneurons that are maintained in a growth state as a consequence of muscle inactivity or nerve injury (Mentis et al, 1993; Greensmith et al, 1994a, 1994b; Greensmith et al, 1995b). Therefore, the experiments presented in Chapter 4 examined whether excitotoxicity also plays a role in the death of extensively growing adult motoneurons. The results show that nerve injury in GAP-43 transgenic mice renders regenerating adult motoneurons vulnerable to the excitotoxic effects of the glutamate agonist, NMDA. Furthermore, treating injured motoneurons in GAP-43 mice with riluzole, a compound that reduces glutamatergic transmission (Chéramy et al, 1992; Debono, et al, 1993; Hebert et al, 1994), rescues a proportion of motoneurons that would otherwise die. These results

taken together support the hypothesis that adult motoneurons, which undergo prolonged and extensive growth, are more vulnerable to excitotoxicity than normal.

It has previously been proposed that as a result of disrupting neuromuscular interaction at birth, developing motoneurons are maintained in a growth state, which prevents them from acquiring mature characteristics that normally enables them to cope with increasing levels of excitatory input (Lowrie and Vrbová, 1992; Greensmith and Vrbová, 1996). NMDA receptors present on neonatal motoneurons have been implicated in mediating excitotoxic events, since their prolonged activation by glutamate results in an increase in free intracellular calcium, thereby activating Ca^{2+}-mediated cell death pathways (Kudo and Yamada, 1987; Konnerth et al, 1990; Choi, 1994; Dugan and Choi, 1994). Furthermore, during the early postnatal period, NMDA-induced currents in rat motoneurons have been shown to decrease (Hori and Kanda, 1996), which may be a reflection of immature NMDA receptors being replaced by more mature counterparts (Kalb et al, 1992; Monyer et al, 1994; Hughes et al, 1997; Stegenga and Kalb, 2001). Thus, it is possible that this change in NMDA receptor expression fails to occur in developing motoneurons when neuromuscular interaction is disrupted, resulting in an increased vulnerability to excitotoxicity.

This proposal, however, does not readily explain the observation that growing adult motoneurons die when forced to grow for a prolonged period, since adult motoneurons down-regulate the expression of NMDA receptors following nerve injury (Piehl et al, 1995). Furthermore, morphological evidence has demonstrated that there is reduction in the number of synaptic contacts onto injured motoneurons

and a decrease in the size of their dendritic tree (Blinzinger and Kreutzberg, 1968 Sumner and Sutherland, 1973; Brannstrom et al, 1992a; Brannstrom and Kellerth, 1998). Together these changes to injured adult motoneurons can be seen as a mechanism to reduce the metabolic burden on the cell.

In order to explain why extensive and prolonged growth of adult motoneurons in GAP-43 mice results in a vulnerability to excitotoxicity, is important to consider the changes that occur many days after nerve injury, since the results presented in Chapter 3 also show that motoneuron death does not occur until 5-10 weeks after injury. One important event that may result in increased levels of cell stress following nerve injury is functional reinnervation. A previous report has shown that following a nerve-crush injury in rats, functional recovery was seen 9-10 days later (Lowrie et al, 1990). The same injury in GAP-43 mice is likely result in earlier muscle reinnervation, since it has been shown that overexpressing GAP-43 causes a faster rate of axonal regeneration (Aigner et al, 1995). During the period of muscle reinnervation, extensive terminal arborization occurs for many days in order to restore motor unit territories (Ostberg et al, 1986; Aigner, 1995). Furthermore, soon after reinnervation, injured motoneurons recover their dendritic surface area and synaptic inputs (Brannstrom et al, 1992b; Brannstrom and Kellerth, 1998). It is possible that excessive levels of growth, associated with an increased rate of regeneration and intramuscular sprouting, coupled with the restoration of excitatory input, may together place these injured motoneurons under high levels of metabolic stress, which may result in their progressive death.

5.6. Is the progressive nature of ALS a consequence of the continued growth of motor axons?

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective degeneration of motoneurons that manifests as a progressive decline in muscle function leading to paralysis and premature death (reviewed by Julien, 2001). Although the majority of ALS cases are sporadic and due to unknown causes, about 5-10% are familial (FALS), of which ~20% are associated with mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1; Deng et al, 1993a; Rosen et al, 1993). This important discovery led to the generation of transgenic mouse models that express mutant forms of the human SOD1 gene (e.g. the G93A mutation). These mutant SOD1 transgenic mice, all develop the clinical and pathological hallmarks of human ALS, and their production has initiated an intense period of research into elucidating the mechanisms involved in the pathogenesis of ALS. Although numerous possible mechanisms have been put forward, the exact cause of ALS is still poorly understood.

In this Thesis I have explored an alternative approach to understanding motoneuron degeneration in ALS, by focusing on the cause of the progressive nature of this disease. The results presented in Chapter 3 have shown that extensively growing motoneurons are more vulnerable to cell death. These findings may have important implications for our understanding of the progressive nature of motoneuron death in ALS, since extensive axonal sprouting by surviving motoneurons occurs in ALS in order to reinnervate the denervated territory vacated by the degenerated motoneurons (Wohlfart, 1957; Stalberg, 1982; Schmied et al, 1999). Furthermore, recent evidence

has shown that axonal sprouting occurs at least one month prior to motoneuron loss in hindlimb muscles of G93A mice, which is associated with synaptic weakening and denervation (Frey et al, 2000). Thus, compensatory sprouting by motoneurons in ALS may be responsible for the rapid progression of the disease.

To examine this hypothesis, in Chapter 4 axonal growth was induced prior to motoneuron death in transgenic mouse model of FALS (G93A). The effect of experimentally inducing axonal growth on the rate of disease progression in G93A was investigated using both physiological and morphological methods. The results presented in Chapter 4, indicate that axonal growth associated with axonal regeneration and collateral reinnervation increases the vulnerability of motoneurons in G93A mice to cell death, resulting in an increase in the rate of disease progression.

How axonal growth causes the rapid progression of the disease in G93A mice is not clear. However, one possible explanation is that following a period of compensatory sprouting, the peripheral territory innervated by the remaining motoneurons increases, which places these motoneuron under higher levels of metabolic stress. Increasing the metabolic demands on motoneurons, may cause an increase in oxidative stress, and accelerate the disease process in G93A mice. In support of this possibility, it is recognized that motoneurons in ALS may be particularly vulnerable to oxidative stress generated by the aberrant catalytic function of mutant SOD1, which leads to excessive production of damaging free radical species (Beckman et al, 1993; Wiedau-Pazos et al, 1996; Yim et al, 1996; Liu et al, 1998; Beal et al, 1998). Furthermore, there is also evidence to suggest that free radical damage is prominent in mitochondria, where a majority of cellular superoxide is produced (Higgins et al,

2002; Mattiazzi et al, 2002). Thus, collateral reinnervation that occurs in ALS in order to compensate for an initial loss of motoneurons may place surviving motoneurons under higher levels of metabolic stress, which in turn increases the production of free radicals (Beal et al, 1996), resulting in a breakdown of mitochondrial function and thereby precipitating the degenerative process.

5.7. Conclusions

The results of this Thesis show that functional events that occur at the motor nerve terminal during the early postnatal period can influence the development and survival of motoneurons. Although the precise mechanism by which neuromuscular interaction promotes the survival of developing motoneurons is unclear, the present results suggest that a muscle-induced up-regulation in transmitter release from motor nerve terminals is a critical event that regulates the maturation and survival of developing motoneurons. Furthermore, previous evidence suggests that disrupting neuromuscular interaction at birth, maintains developing motoneurons in a growth state, which cause those motoneurons to die (Lowrie et al, 1987; Greensmith et al, 1992; Harding et al, 1998; Greensmith et al, 2000). The results presented in this Thesis also show that inducing adult motoneurons to grow extensively for a prolonged period results in progressive motoneuron death, which is possibly mediated by an increased vulnerability to excitotoxicity. Thus, prolonging axonal growth in both developing and adult motoneurons increases the vulnerability of these motoneurons to cell death.

Importantly, the present results also indicate that compensatory sprouting by motoneurons in ALS may precipitate the neurodegenerative process, resulting in an increase in the rate of disease progression. Therefore, strategies that aim to reduce excessive axonal growth and sprouting in neurodegenerative conditions such as ALS may represent a novel approach that aims if not to cure this disease, to at least delay disease progression.

APPENDIX 1

Modified Hanker-Yates method:

1. Wash sections briefly (60 sec) in distilled water
2. Incubate sections in cobalt/Nickel solutions for 15 minutes
3. Wash sections in distilled water and rinse in 2 changes of Millonig's phosphate buffer (MPB) for 10 minutes
4. React sections in Hanker-Yates solution for 10-25 minutes, stopping before the background staining darkens
5. Rinse in 2 changes of MPB for 5 minutes each and place in distilled water

Solutions:

1. **MPB**

$NaH_2PO_42H_2O$	19.08g
1M NaOH	96.3 ml

Make up to 1 litre with distilled water. Adjust to pH 7.3 and store at 4°C.

2. **Cobalt/Nickel solution**

1% cobalt chloride	300 ml
1% ammonium nickel sulphate	200 ml

Mix together before use

3. Cacodylate buffer

0.1M sodium cacodylate	500 ml
0.2M HCl	

Adjust to pH 5.1. Make up to 2 litres with distilled H_2O. Store at 4°C.

4. Hanker-Yates solution

Hanker-Yates combines reagent (Sigma)	150 mg
Cacodylate buffer (pH 5.1)	100 ml
30% H_2O_2	1 drop

Make up just before use and discard after 1 hour.

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