Degenerative and regenerative changes after loss of 
neuromuscular interaction during early postnatal life

A thesis submitted to the University of London for the degree 
of Doctor of Philosophy in the Faculty of Science

by

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January 1997
Acknowledgements

I would like to thank my supervisor Professor Gerta Vrbová for her support and enthusiasm throughout the years. I am grateful to have had the opportunity to work with her. I would also like to thank my co-supervisor Dr Patrick Anderson for his support and advice.

There are many people who have helped me both intellectually and practically throughout the PhD, and I would particularly like to thank Dr Linda Greensmith for her invaluable help and advice, and Dr Angela Connold and Jim Dick for their constant help and support. I am grateful to Mark Turmaine and Suhel Miah for their help, and for making electron microscopy enjoyable. Thanks to Claire White, Dr Junedah Sanusi, and Dr Jo Deckers for interesting discussions, help and all the other things that fellow PhD students share in times of stress. Finally, thanks to Fiona Wardle for her careful proof reading and useful criticism.
Abstract

Young motoneurones are critically dependent upon functional interaction with their target muscle for their normal development, and if deprived of target contact a large proportion die. This thesis is concerned with changes induced by loss of nerve-muscle interaction during early postnatal life, and examines the possibility that motoneurones which are not allowed to mature during this critical period are more vulnerable.

It was found that stabilising neuromuscular contacts on reinnervation after nerve crush at birth or prolonged neonatal muscle paralysis, by inhibiting the calcium-activated neutral protease with leupeptin, rescued motoneurones that would otherwise die. In the case of nerve crush this lead to a significant improvement in muscle recovery. The time-course of motoneurone death following a prolonged period of neonatal muscle paralysis was studied, and motoneurones were observed to die by 3 weeks of age. The structural changes in the nerve to soleus following neonatal nerve injury or muscle paralysis were examined, and it was found that although motor and sensory myelinated axons were lost in the nerve after nerve injury, only motor myelinated axons were lost after muscle paralysis.

Finally, the thesis explored the possibility that motoneurones supplying a partially denervated muscle may remain in a growing mode for longer, and could be susceptible to nerve crush injury at a time when they would normally survive this insult. It was found that the remaining motoneurones supplying the EDL and TA muscles, after these muscles had been partially denervated at 3 days of age, died when
the sciatic nerve was crushed at 9 days of age.

The results of this study illustrate the importance of nerve-muscle interaction in the developing neuromuscular system, and show that this is a time when even a slight perturbation can have permanent effects.
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Chapter 1. General Introduction

Preface

Young motoneurones are critically dependent upon functional interaction with their
target muscle for their normal development, and if deprived of target contact a large
proportion die. In this thesis, the neuromuscular system was studied after interfering
with nerve-muscle interaction by: a) sciatic nerve crush at birth where there is a loss
of motoneurones and consequently a severe impairment of muscle function; b) after
muscle paralysis by blocking the postsynaptic acetylcholine receptor with α-
bungarotoxin, which also results in motoneurone loss although there is only a
functional loss of neuromuscular interaction without injury to the motor nerve; and
c) after neonatal partial denervation soon after birth where the muscle loses a
proportion of its innervation, but is still innervated by motoneurones not directly
affected by the original insult.

Chapter 2 studies the effect of stabilising neuromuscular contacts by inhibiting
the calcium-activated neutral protease on reinnervation after nerve crush at birth or
during recovery from neonatal muscle paralysis. It then explores the time-course of
motoneurone death following neonatal paralysis. The structural changes of the nerve
to soleus following nerve crush at birth or neonatal muscle paralysis were compared.
Chapter 2 therefore compares the effects of nerve crush and muscle paralysis.

Chapter 3 explores the possibility that the motoneurones supplying a partially
denervated muscle may remain in a growing mode for longer than usual, and could
be susceptible to nerve injury at a time when they would normally survive this insult.

This thesis is therefore concerned with the regenerative and degenerative changes after loss of neuromuscular interaction during early postnatal life, and examines the possibility that motoneurones and muscle fibres which are not allowed to mature during this critical period are more vulnerable.

The development of the mammalian neuromuscular system and its response to injury are described in this chapter.

1) Development of the neuromuscular system

a) Motoneurone development

Motoneurones, in the motor columns of the ventral horn, originate from neuroblasts in the ventricular zone of the neural tube (Langman and Hadden, 1970; for review see Bennett, 1983), and their initial development is independent of their target (Hamburger, 1958; Hughes and Tschumi, 1958). It is thought that the eventual phenotype of motoneurone precursors is influenced by epigenetic factors, and is not wholly determined by the muscle fibres they originally innervate (Leber et al, 1990). Even before their axons reach their target muscles motoneurones express choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) (Phelps et al, 1984), but for the increase in and maintenance of the levels of enzyme expression required for acetylcholine (ACh) synthesis, target interaction is necessary (Giacobini-Robechi et
al, 1975; Betz et al, 1980; reviewed by Vaca, 1988). The up-regulation of ChAT seen in development (Burt, 1975; O'Brien and Vrbová, 1978; Pilar et al, 1981; Phelps et al, 1984) is to some extent dependent on activity of the motoneurone, as decreasing activity by blocking action potentials with tetrodotoxin prevents the developmental increase in ChAT activity (Brenneman et al, 1983), while chronic K⁺ or NMDA receptor induced membrane depolarisation increases ChAT activity (Ishida and Deguchi, 1983; Brenneman et al, 1990).

Several proteins which are not normally expressed in adult or mature neurones, are predominantly expressed during neuronal growth and development. These proteins, associated with periods of axonal and dendritic elongation and periods of synaptic rearrangement, are often re-expressed following injury in later life. For example, the gene for GAP-43 is expressed during development, and in most neurones its expression is down regulated as they mature. GAP-43 is found in growth cones (Skene et al, 1986), and is involved in axonal elongation and synaptic rearrangement. It is thought to alter the response of neurones to extracellular signals (see Skene, 1989). The expression of GAP-43 is increased in retinal ganglion cells following injury to the optic nerve in toads (Skene and Willard, 1981), and GAP-43 expression is upregulated in motoneurones following axotomy and paralysis (Piehl et al, 1995). Tubulin synthesis is high during axonal outgrowth, and one isoform is expressed at higher levels during development and regeneration of axons (Lewis et al, 1985; Miller et al, 1987). Although tubulin is a major component of the axonal cytoskeleton, it is transported to the site of growth slowly, and as such may limit the rate of elongation
of axons (Hoffman and Lasek, 1975). A further example of a developmentally expressed group of cytoskeletal proteins are the microtubule associated proteins (MAPs). These proteins, which regulate the stability of microtubules in axons and dendrites, are involved in determining neuronal shape and regulating the balance between rigidity and plasticity (see Matus, 1988, 1990).

The excitability and firing pattern of motoneurones changes with development, and from the onset of electrical excitability Na⁺ channels are present in the motoneurone soma and axon, and their numbers increase with age (Ziskind-Conhaim, 1988; McCobb et al, 1990). Ca²⁺ currents in early embryonic chick motoneurones are mainly of the low-threshold T-type, and later the embryonic motoneurones have mainly high-threshold calcium currents (L and N-type) (McCobb et al, 1989). Neonatal motoneurones have both high threshold Ca²⁺ conductances, necessary for the after-depolarisation phase of the action potential (Walton and Fulton, 1986), and low-threshold Ca²⁺ conductances (Harada and Takahashi, 1983; Walton and Fulton, 1986; Berger and Takahashi, 1990). The spontaneous oscillations in membrane potential seen soon after birth are due to the presence of the low-threshold Ca²⁺ conductance, and this conductance may also account for the burst firing of neonatal motoneurones seen in vivo and in vitro (Navarrete and Vrbová, 1983; Navarrete and Walton, 1989). The vast array of K⁺ channels seen in adult motoneurones allows a fine control in excitability and firing patterns (Schwindt and Crill, 1984; Rudy, 1988; Kiehn, 1991), and in motoneurones of the neonatal rat both the $I_K$ (voltage-dependent K⁺ current responsible for repolarisation of the action potential) and $I_A$ (transient) types of K⁺
currents are present, along with calcium dependent K^+ currents (Takahashi, 1990). The somatodendritic membrane in developing embryonic motoneurones is sensitive to the excitatory amino acid neurotransmitter glutamate (O'Brien and Fischbach, 1986a,b,c,d), but the information regarding the time course of membrane chemosensitivity in development to other neurotransmitters is scarce.

The properties of developing motoneurones change with age, as they differentiate into the functional sub-classes which will later be distinguished by their electrophysiological properties and recruitment order (Naka, 1964; Kellerth et al, 1971; Fulton and Walton, 1986). The synaptic inputs to motoneurones are reorganised during the first few weeks of postnatal life in mammals (Conradi and Ronnevi, 1975; Ronnevi, 1979; Gilbert and Stelzner, 1979; Stelzner, 1982), and it is possible that this maturation of the afferent synaptic input may contribute to the changes of motoneurone firing patterns during early development and co-ordinate functional specialisation (Navarrete and Vrbová, 1983).

It is well established that there is a close relationship between motoneurones and their target muscles (Shorey, 1909; Hamburger, 1958). It is thought that the survival of embryonic motoneurones is in some way regulated by interaction with their target, although the nature of this target dependency is not clear. The suggestion that motoneurone survival may be dependent upon the release of a trophic factor from the muscle (Oppenheim, 1991) is discussed later.

In addition, it has been proposed that as a result of continued interaction with
the target the phenotype of the developing motoneurone changes, rendering it less
target dependent. This change may be a consequence of alterations of the growing
terminal. When a growth cone contacts a target muscle fibre it is changed from
a growing into a secreting structure, neurite elongation stops, and transmitter release
from the growth cone is rapidly increased (Frank and Fischbach, 1979; Xie and Poo,
1986). This is shown by a dramatic increase in the frequency of miniature endplate
potentials, as well as the appearance of quantal release of ACh. With continued
contact with the muscle the quantal content of the endplate potential, and the activity
of ChAT in the motor nerve endings rapidly increases (Diamond and Miledi, 1962;
Kelly, 1978; O’Brien and Vrbová, 1978; Lowrie et al, 1985). At the same time, many
nerve branches and synaptic contacts are eliminated, thus reducing the size of the
peripheral field of the motoneurone. These changes in the motoneurone’s terminal
expression are reflected in the motoneurone cell body by an increase in the proteins
associated with the secretion of ACh, and down-regulation of growth-associated
proteins such as GAP-43 and tubulin. If neuromuscular transmission is blocked then
the growth-associated proteins are not down-regulated (Caroni and Becker, 1992) and
nerve terminal retraction does not occur (Duxson, 1982; Greensmith and Vrbová,
1991). Therefore, the motoneurone cell body is greatly influenced by changes at its
terminals, and as the terminals change from growing to transmitting structures the cell
body responds appropriately. This transition of the motoneurone from a growing cell
to a transmitting one may affect some features that are important for its survival, and
these associated changes are likely to be essential for the motoneurone’s development
into a target-independent structure able to function in the mature CNS. For example, it could be necessary for the motoneurone to redistribute or down-regulate its glutamate receptors, in order to withstand the increased afferent excitatory input which occurs in the developing spinal cord (Lowrie and Vrbová, 1992). The expression of one of the glutamate receptors, the NMDA receptor, has been shown to change during development (Kalb et al, 1992; Piehl et al, 1995), as do the types of NMDA-receptor subunits which are expressed, so that immature channels are replaced by their more mature counterparts (Monyer et al, 1994). The target’s role in this developmental change in the motoneurone, is suggested by results that show that if neuromuscular contact is denied during the first week of life motoneurones are susceptible to the excitotoxic effects of exogenously applied NMDA (Greensmith et al, 1994a). Furthermore, motoneurones that are destined to die can be rescued if their NMDA receptors are blocked (Mentis et al, 1993; Greensmith et al, 1994b). Target deprivation may also affect other membrane characteristics of the motoneurone. Motoneurones destined to die following axotomy are also rescued if their Na\(^{+}\) channels are blocked (Casanovas et al, 1996), indicating a complex change in the motoneurone phenotype when deprived of target contact.

Interaction with the target is therefore essential for the motoneurone to mature and cope with the normal levels of excitation that exist within the mature CNS.
b) Naturally occurring cell death

The naturally occurring cell death of neurones is a feature of embryonic development in vertebrates, and is thought to regulate neuronal numbers and to control the interaction between different populations of neurones and their targets (See Oppenheim, 1991). In the chick embryo there is a period of target dependent cell death in which 50-60% of motoneurones in the spinal cord die (Hamburger, 1975). These cells die by apoptosis (see Oppenheim, 1991), and this death occurs within a precise period during the development of the embryo, usually when the axons arrive at their target (Hamburger, 1975; Landmesser and Pilar, 1974) as shown by retrograde labelling experiments (Chu-Wang and Oppenheim, 1978). The period of cell death ceases before birth in most vertebrates (Lance-Jones, 1982; Hardman and Brown, 1985; Bennett et al, 1983).

Following removal of limb buds in early chick and amphibian embryos there is a severe depletion of spinal motoneurones and sensory ganglia (Shorey, 1909), and when limb buds are transplanted to non-limb regions there is an increase in the number of sensory ganglia innervating the transplant (Detwiler, 1924). Furthermore, when limb buds are removed from chick embryos there is a decrease in the number of motoneurones which is proportional to the amount of target removed (Hamburger, 1939). Thus, there appears to be a quantitative relationship between the number of neurones and the amount of target tissue present in the periphery.

Paralysis of chick embryos with curare or α-bungarotoxin (α-BTX) during the period of naturally occurring cell death reduces the degree of motoneurone cell death
(Pittman and Oppenheim, 1978, 1979; Oppenheim and Maderdrut, 1981), but if the cause of paralysis is removed before the end of the normal period of cell death, then cell death quickly ensues (Oppenheim, 1981). Increasing activity during this period by electrical stimulation of the chick hindlimb (Oppenheim and Nunez, 1982) or by using carbachol (a nicotinic receptor agonist) or eserine (an anti-acetylcholinesterase) (Oppenheim and Maderudt, 1981) accelerates motoneurone death.

c) Muscle development

There is a population of mesenchymal cells in the developing embryo which leave the proliferative cell cycle to become myoblasts (Tello, 1917, 1922). Those mesenchymal cells which become committed myogenic cells are able to synthesize muscle-specific proteins such as myosin and actin (Konigsberg, 1963; Holtzer and Sanger, 1972). Myoblasts line up and fuse with each other to form long multi-nucleated myotubes (Moscona, 1957; Yaffe and Feldman, 1965). This fusion is dependent upon the entrance of Ca^{2+} ions into the cell and the subsequent increase in intracellular calcium levels (Shainberg et al, 1969; Fambrough and Rash, 1971; Patterson and Prives, 1973; Pryzbylski et al, 1989). These cross-striated cells differentiate further, with nuclear migration to the periphery of the cell and an increase in protein synthesis, to eventually form muscle fibres (for review see Wakelam, 1985).

As the muscle cells develop into their unique multinucleated state, so too does their membrane properties and ion channel complement. Acetylcholine receptor molecules are synthesized and incorporated into the myoblast and myotube membrane
(Fambrough and Rash, 1971; Sytkowski et al, 1973; Dryden et al, 1974), and although the membrane potential of myotubes is low and increases with time (Fischbach et al, 1971; Dryden et al, 1974), these immature cells are able to produce electrogenic responses (Kano and Shimada, 1973; Purves and Vrbová, 1974). The time course of the emergence of these responses is consistent with the appearance of Na\(^+\) channels in the membrane (Kidokoro, 1975). Immature muscle cells have L and T type Ca\(^{2+}\) channels (Kano et al, 1989; Gonoi and Hasegawa, 1988) and the T type channels, which are not present in adult muscle fibres, are sensitive to very small changes in membrane potential, close to the resting potential, and their presence may be essential in development to allow Ca\(^{2+}\) entry. K\(^+\) channels are present in developing muscle cells, and there is a shift from the predominance of low-conducting channels in the myoblast, to high-conducting K\(^+\) channels in the myotube, and this shift is not dependent on myoblast fusion. ATP-dependent K\(^+\) channels are not present in the myotube and their development is critically dependent on myoblast fusion (Zemková et al, 1989). This dependence could be related to the increase in the proportion of contractile proteins which occurs after fusion (Allen and Pepe, 1965; Przybilski and Blumberg, 1966), and the ATP-dependent channels may have a protective influence if the muscle fibre is threatened with energy depletion.

Adult mammalian muscles are composed of different types of muscle fibres, which can be distinguished by their isozymic forms of contractile proteins such as myosin heavy chains (MHC) and troponin (for review see Pette and Staron, 1990). By studying the type of MHC isoforms in developing muscle fibres it is possible to
observe the heterogeneity of developing muscles. In vitro experiments showed that myotubes can be isolated in different states, depending on their expression of fast and slow MHC isoforms, and this led to the proposal that the fate of muscle fibres is predetermined (Miller and Stockdale, 1987; Miller, 1991). However, there are many epigenetic factors which could generate phenotypic diversity in developing myotubes, such as stretch (Vandenburgh et al, 1990). If the postural function of the rat soleus muscle is impeded then the number of slow MHC muscle fibres do not increase as they normally would soon after birth (Lowrie et al, 1987). Furthermore, in fast muscles which are reinnervated by their own nerve after perinatal nerve injury, it is seen that fast fibres are converted into slow fibres as they are reinnervated by axons originally innervating slow motor units (Lowrie et al, 1988). This work illustrates the importance of environmental and neuronal factors in the development of muscle phenotype.

d) Axonal growth

Once in position, nerve cells extend neurites which make connections within their local environment. Neurite growth requires a supply of structural molecules from the cell body, and guidance towards the target. The growth cone, first identified by Ramón y Cajal (Ramón y Cajal, 1929), is a swelling at the tip of the neurite from which exploratory filopodia and lamellipodia arise, and it is essential for determining the direction of neurite growth. Filopodia are motile structures containing filamentous actin, and they “explore” the local environment for directional cues (Bray and Bunge,
Neurite elongation occurs at the tip of the growth cone (Bray, 1973) and is achieved by the intra-axonal transport of macromolecular materials produced in the cell body and assembled into new membrane at the leading edge of the growth cone (Bray, 1973; Bunge, 1973; Bray and Bunge, 1973; Lasek and Katz, 1987). Addition to the axolemma does not occur along the established part of the axons (Letourneau, 1982; Landis, 1983) until the target is contacted. Adhesion by the filopodia and contraction by actin and myosin filaments results in a forward pull of the tip of the neurite (Bray, 1973; Bunge, 1977; Letourneau, 1981), an event which involves Ca^{2+} (Anglister et al, 1982).

Motor axons leave the ventral horn of the spinal cord to innervate muscles in the periphery (Tello, 1917). In the chick embryo this is seen to occur at the time of limb bud development (Hollyday and Hamburger, 1977), and in the rat foetus axons leave the ventral horn on embryonic day 11.5 (Filgamo and Gabella, 1967). With further development the axonal projection pattern gradually achieves a recognisably adult state, as demonstrated by retrograde labelling techniques using horseradish peroxidase (HRP) (Landmesser, 1978) and DiI (Snider and Palavali, 1990). The direction of axonal outgrowth is specific, and when axons were misrouted in the chick embryo by removal or rotation of a part of the spinal cord, the axons still usually selected their correct target (Lance-Jones and Landmesser, 1980a,b; Lance-Jones and Landmesser, 1981a,b). There are nevertheless limits to the path-finding ability of axons, and transplanted duplicate limbs are aberrantly innervated by nerves which would normally innervate the distal portions of the original limb (Whitelaw and
The mechanisms directing axonal outgrowth are not clearly understood. The growing nerves could respond to variety of environmental cues including mechanical guidance cues (Bray, 1982), changes in adhesiveness (Nardi, 1983), the extracellular matrix (Schinstine and Cornbrookes, 1990), electrical (Jaffe, 1979, 1981) and tropic gradients (Gundersen and Barrett, 1980). There are “guidepost” cells in invertebrates which play a role in growth cone attraction and guidance (Taghert et al, 1982; O’Connor et al, 1990), and it is possible that in vertebrates Schwann cells could play a similar role (Keynes, 1987). Schwann cells may migrate into developing muscles and determine the intramuscular nerve growth (Noakes and Bennett, 1987), but they are not essential for the guidance of growing axons (Grim et al, 1992). Materials that repel growth cone elongation are thought to be important for the achievement of accurate path finding (Patterson, 1988; Fan et al, 1993; Igarashi et al, 1993; McKerracher et al, 1994; Goshima et al, 1995). It is possible that in peripheral nerves pioneer axons may represent the guideline structure for other axonal growth cones. After the removal of motoneurones early in the development of chick embryos the remaining sensory axons could not find their way to the target, suggesting that motor axons lead the way in mixed nerves of these embryos (Landmesser and Honig, 1986; Scott, 1988). Furthermore, motor growth cones are larger and more elaborate than the growth cones of sensory axons (Landmesser and Honig, 1986). Growth cones use proteases to facilitate extension through the tissue matrix (Seeds et al, 1992).

Once the growth cone has arrived at and recognised its target, growth is
arrested and the growth cone is transformed into an axon terminal by axon-target interactions.

e) Axons and Schwann cells

From a very early stage in development, axons and Schwann cells depend on interaction with each other for their full differentiation (Bunge and Wood, 1987). Axonal growth is enhanced by Schwann cell secretions such as nerve growth factor (NGF), laminin, heparan sulphate proteoglycan and other growth promoting substances (Baron Van Evercooren et al, 1982; Varon et al, 1984; Unsicker et al, 1985), and normal axonal function is dependent on the formation of axonal sheaths by Schwann cells (Speidel, 1964; Webster and Favilla, 1984; Ard et al, 1985; Bunge et al, 1986). Conversely, Schwann cell proliferation is enhanced by the provision or mediation of mitogenic factors by axons (Baron Van Evercooren et al, 1987; Bunge and Wood, 1987), and the type of Schwann cell ensheathment is determined by the axon (Weinberg and Spencer, 1975; Aguayo et al, 1976; Bray et al, 1981). Furthermore, axons contribute to the full expression of the Schwann cell phenotype by influencing Schwann cell production of basal lamina and other extracellular matrix components (Mehta et al, 1985; Bunge et al, 1986).

With the growth of the length and girth of peripheral axons, there is a change in the neurofilaments from an immature to a mature form resulting in the coexpression and up-regulation of all three neurofilament proteins, including the heavy protein which is absent from neurofilaments earlier in development (Schlaepfer and Bruce,
In the rat this occurs 5 to 24 days after birth (Schlaepfer and Bruce, 1990). The ensheathment of axons by Schwann cells progresses from the situation where each Schwann cell initially surrounds a relatively large bundle of thin axons, with the surrounding of ever smaller bundles as the Schwann cells proliferate (Gamble, 1976; Webster and Favilla, 1984), to the peak of Schwann cell proliferation soon after birth at which point the Schwann cells associated with myelinated fibres stop dividing and those associated with unmyelinated fibres continue to divide (Brown and Asbury, 1981; Griffin, 1990). Once a Schwann cell has become committed to an axon, it synthesizes and secretes the components of basal lamina (Armati-Gulsan, 1980). The basal laminae first assemble around “Schwann cell families” when each Schwann cell surrounds large groups of axons, and later in development they form individual tubes around single axons (Webster and Favilla, 1984). Individual axons become myelinated and small groups of axons remain unmyelinated.

Whether or not a Schwann cell will form a myelin sheath depends on the type of axon with which it interacts (Bray et al, 1981), and it is large diameter axons, usually above 1 μm in diameter, which become myelinated (Matthews, 1968). The myelin sheath is created by the apposed layers of the Schwann cell plasmolemma (the mesaxon) which wrap around the axon (Robertson, 1962; Webster and Favilla, 1984; Bunge et al, 1986), and myelination proceeds proximodistally with the terminal internodes being myelinated last. Axonal diameter, myelin thickness and conduction velocities all increase during maturation and growth (Vejsada et al, 1985; Fraher et al, 1990). Unmyelinated axons are ensheathed by a Schwann cell column that encases
several axons in deep furrows indented in its surface (Landon and Hall, 1976; Webster and Favilla, 1984), and mature Schwann cells with unmyelinated axons still preserve some of the characteristics common to all immature glial cells, e.g. expression of L1 and N-CAM (Mirsky et al, 1986) and expression of the low-affinity neurotrophic receptor (P75) (Yan and Johnson, 1987; Verge et al, 1989). The fascicles of mammalian nerves are surrounded by continuous multilayered sleeves, the perineurium, consisting of concentric layers of flattened perineurial cells (Thomas, 1963) linked by tight junctions, with each perineurial layer covered by basal lamina on both sides (Low, 1976). Large nerves are enclosed by 10 to 15 perineurial layers, and this decreases as the nerve branches approach their termination.

f) Development of the neuromuscular junction

Acetylcholine (ACh) released from the motor nerve ending is the means by which the motor nerve communicates with the muscle, and the acetylcholine receptor (AChR) provides the functional connection between nerve and muscle. Study of the development of the AChR is extensive (for review see Schuetze and Role, 1987), and much is known about the receptor's structure, assembly, and insertion into the surface membrane (Fambrough, 1979; Anderson, 1986; Merlie and Smith, 1986; Salpeter and Loring, 1986). AChR's are present in the membrane of myoblasts (one of the characteristics distinguishing myogenic cells from mesenchyme) and after fusion of myoblasts into myotubes the receptors form clusters, the formation of which can be
artificially induced by focal stimulation of the muscle fibre membrane (Jones and Vrbová, 1974; Peng et al, 1981). These clusters are stabilised in the endplate region of the muscle surface (where neuromuscular contacts are established) at the onset of activity, and extrajunctional AChRs disappear at this time. This is dependent on nerve induced muscular activity, so that when embryonic or neonatal muscles are paralysed the disappearance of extrajunctional AChRs does not occur (Gordon et al, 1974; Burden, 1977a, b).

Neuromuscular contact at the endplate is thought to provide a signal or “trace” which induces and allows the endplate to develop and mature further. This “trace” could be in the form of a synapse organising factor in the basal lamina (Nitkin et al, 1987), and such a molecule, “agrin” does cause cultured AChR aggregation (Fallon and Gelfman, 1989; Nastuk and Fallon, 1993) and is present in the motoneurone soma (Magill-Solc and McMahan, 1988).

AChR channels in immature endplates have longer opening times than in mature endplates, and the reduction in opening time as the junction develops is regulated by the nerve and in particular the muscular activity induced (reviewed by Changeux, 1991). Denervation soon after birth inhibits the normal developmental appearance of fast channels at the neuromuscular junction.

The enzyme acetylcholinesterase (AChE), whilst being present in muscle cells very early on in their embryonic development, accumulates in the vicinity of axon growth cone invasion on the sarcolemma of the myotome (Tennyson et al, 1973), and becomes more prominently localised as the motor endplate matures (Kupfer and
Koelle, 1951; Lentz, 1969; Tóth and Karcsiú, 1979; Brzin et al, 1981). Extrajunctional patches of AChE on the sarcolemma disappear concomitantly and are not detectable in innervated postnatal or adult muscles. Simultaneously with this biochemical organisation, the subsynaptic sarcolemma and muscle fibre undergo morphological differentiation (Kelly and Zacks, 1969; Bennett and Pettigrew, 1974; Brzin et al, 1981). AChE accumulation at the neuromuscular junction is influenced by innervation and activity, and the enzyme does not accumulate if the neonatal muscle is denervated (Zelená and Szentágothay, 1957) or paralysed with curare (Gordon et al, 1974). The previous site of nerve-muscle contact in muscle denervated at birth does however display a small level of AChE accumulation (Sketelji et al, 1991), some AChR accumulation (Slater, 1982), and a degree of growth of synaptic folds (Brenner et al, 1983).

Although the neuromuscular junction undergoes profound developmental changes to facilitate neuromuscular transmission, muscular contraction can be elicited by motor nerve stimulation at a time when the axon profiles are not closely associated with the AChR clusters, and when synaptic specialisation has not yet occurred (Landmesser and Morris, 1975; Dahm and Landmesser, 1991). Before muscle contact growth cones release very little ACh, but once a myogenic cell is contacted there is an increase in the amplitude and frequency of spontaneous ACh release (Xie and Poo, 1986). Transmitter release is probably induced by target contact, and is characterised by the appearance and organisation of synaptic vesicles into clusters at the active zones of the plasma membrane where neurotransmitter release preferentially occurs.
(Kullberg et al, 1987; Ceccarelli and Hurlbut, 1980; Buchanan et al, 1989). The depolarisations induced in the muscle by the initial release of ACh resemble mature end plate potentials (Xie and Poo, 1986) but they are bigger and can trigger muscle contractions (Jaramillo et al, 1988). This muscular response is likely to be essential for the further differentiation of the muscle fibres, as it represents the first form of neuromuscular interaction. Soon after this time the neurone can respond to a depolarizing event with a pulse of synchronous ACh secretion (Evers et al, 1989).

A number of synaptic vesicle-associated phosphoproteins, called the synapsins, have been implicated in the formation and maturation of developing synapses, and the regulation of transmitter release from mature terminals (for review see Greengard et al, 1993). In particular, synapsin I has been observed to induce ultrastructural changes in the synapse, thus accelerating its maturity (Valtorta et al, 1995), and this illustrates the kind of molecular mechanisms that could play a role in synaptic maturation.

g) Development of the motor unit

Embryonically, a full complement of motor axons innervate relatively few myogenic cells, and these developing muscle cells are multiply innervated. These early muscle fibres are mainly large diameter primary myotubes spanning the length of the muscle (for review see Kelly, 1983). As the muscle develops further smaller secondary myotubes appear within the same basal lamina as the primary myotubes, and these fuse with other myoblasts to increase in length and eventually form their own separate attachments with the muscle tendons (Ross et al, 1987). The primary and secondary
myotubes communicate via gap junctions (Kelly, 1983), and both myotubes often initially share a proportion of terminals which are later transferred wholly to the secondary fibre as development progresses (Duxson et al, 1986). The question of what makes a given terminal compatible with a particular secondary myotube, and therefore what presupposes the selection of which terminals will be shared and later transferred, could be in part answered by the characteristics of secondary myotubes. Because of their higher input resistance, immature AChRs, and lack of AChE, the secondary myotubes give large depolarisations for even small amounts of transmitter release, and they also have much longer endplate potentials than primary myotubes and more mature cells (Sheard et al, 1991). It is likely that more mature and therefore more active nerve terminals will retract if they contacted a secondary myotube, and these myotubes would favour connection with a less mature inactive terminal, which indeed seems to be the case (Sheard et al, 1991). Even during this period of multiple innervation there is some degree of segregation of fast and slow muscle fibres in individual motor units (Jones and Ridge, 1987; Jones et al, 1987a,b; Fladby and Jansen, 1990), and this preliminary layout is later modified and refined as activity and the environment change muscle fibre phenotype.

In the adult, each muscle fibre is supplied by a single axon, and the innervation ratio represents the number of muscle fibres supplied by each motoneurone. In late embryonic and early postnatal life, each muscle fibre is polyneuronally innervated (Redfern, 1970), and the motoneurones thus have a higher innervation ratio than in the adult (Bagust et al, 1973). In the adult more active motoneurones, and therefore more
active motor units, have a smaller innervation ratio than less active motor units. This phenomenon is consistent with the observation that blocking the action potentials of developing motor units, and thus decreasing their activity, increased the motor unit territory (Callaway et al., 1987). In the second and third week of life the level of neuromuscular activity in the rat increases, and the polyneuronal innervation is eliminated to produce adult motor unit size (Navarrete and Vrbova, 1983, 1993). If neuromuscular activity is increased further by chronic electrical stimulation of the nerve the rate of loss of polyneuronal innervation is accelerated (O'Brien and Vrbova, 1978). This is also true if muscle activity is increased pharmacologically, without activating the motor nerve, by blocking ACh hydrolysis (Duxson and Vrbova, 1985).

If activity is reduced, by tenotomy (Benoit and Changeux, 1975; Riley, 1978), spinal cord transection (Miyata and Yoshioka, 1980), or spinal cord isolation (Zelená et al., 1979; Caldwell and Ridge, 1983) the rate of loss of polyneuronal innervation is reduced. This also occurs if muscle activity is reduced by blocking neural or neuromuscular transmission with tetrodotoxin (Thompson et al, 1979), α-BTX (Duxson, 1982), and botulinum toxin (Brown et al, 1981b) in rats, and curare and α-BTX in chicks (Srihari and Vrbova, 1978). It appears therefore, that the activity of the muscle influences its own innervation.

During muscle activity K⁺ is released from the muscle fibre and accumulates in the synaptic cleft (for review, see Lowrie et al, 1989; Navarrete and Vrbova, 1993), leading to the depolarisation of the endplate region. This would open Ca²⁺ channels and may allow the entry of enough Ca²⁺ from the extracellular compartment to
activate the calcium-activated neutral protease (CANP), which is present in the nerve terminal (Tashiro and Ishizaki, 1982; Ishizaki et al, 1983). CANP has a high affinity for cytoskeletal elements, e.g. axonal neurofilament proteins (Lasek and Hoffman, 1976; Lasek and Black, 1977; Pant et al, 1979; Schlaepfer, 1979; Pant and Gainer, 1980; Croall and DeMartino, 1991) and may be involved in the turnover and breakdown of these proteins (Schlaepfer, 1979; Schlaepfer and Freeman, 1980). CANP is involved in the elimination of nerve terminals (O'Brien et al, 1982, 1984; for review, see Navarrete and Vrbová, 1993). This model could explain the mechanism by which synaptic input is regulated by the target muscle, and could account for the preferential loss of smaller terminals observed during synapse elimination (O'Brien et al, 1984; Duxson and Vrbová, 1985). Ca^{2+} transients may be greater in smaller terminals because of their bigger surface-to-volume ratio, and the terminals may also contain less cytoskeletal protein. Small terminals would therefore be particularly susceptible to an increase in the depolarisation of the post-synaptic membrane. The selection of nerve terminal elimination may also be determined by the maturity of the endplate. Primary myotubes are bigger and more mature than secondary myotubes, and they can sustain a higher degree of polyneuronal innervation (Sheard et al, 1991).
2) Response of the neuromuscular system to injury

a) Motoneurone survival

During early postnatal development motoneurones remain dependent upon contact with their target for their survival. The extent of motoneurone death induced by nerve injury during this period of development depends on several factors, including the age of the animal at the time of nerve injury and the duration for which neuromuscular interaction is prevented. Sciatic nerve crush injury at birth results in the loss of about 90% of sciatic motoneurones (Lowrie et al, 1987; Greensmith et al, 1994b) and injury at 3 days of age still results in the death of the majority of the motoneurones (Greensmith et al, 1996a). The next few days of life sees the rapid decline in the motoneurone's dependence on target contact for its survival, and if the motoneurones remain in contact with their target for at least 5 days after birth then they are able to survive nerve injury (Lowrie et al, 1982; Greensmith et al, 1994a). The duration for which motoneurones are deprived of target contact is another crucial factor in determining motoneurone survival. There is a much greater chance for motoneurone survival following a brief period of target deprivation, than after a prolonged period of target separation. After axotomy of the nerve at birth, when there is little reinnervation of the target muscle, almost no motoneurones survive (Schmalbruch, 1984). After nerve crush at birth reinnervation of the target muscle is permitted, and the chances of motoneurone survival are greatly improved (Lowrie et al, 1987; Greensmith et al, 1994b). If a nerve is injured further away from the muscle, more
motoneurones die than when the nerve is injured close to the muscle (Lieberman, 1974; Greensmith et al, 1994b). When the site of injury is further from the muscle the period before target contact is re-established is longer than if the injury is closer to the muscle. Further evidence for the importance of the duration of target separation on motoneurone survival is provided from experiments where innervation was delayed by inflicting a second injury at the same site (Lowrie, 1990). In this case, motoneurone death was greater than when the nerve was injured only once. The distance of the injury site from the cell body did not change in this experiment, but the period of separation of the motoneurone from the muscle was prolonged. Therefore, it seems that the extent of motoneurone death after nerve injury is indeed influenced by the duration for which the interaction between the motoneurone and the target is prevented, and not just injury to the axon itself.

b) Wallerian degeneration

Axons are dependent on the transport of macromolecules from their place of synthesis in the cell body, as they cannot synthesize proteins and lipids themselves (Zelena, 1972; Droz et al, 1973; Lasek and Hoffman, 1976). If an axon is disconnected from its cell body it undergoes Wallerian degeneration (Waller, 1850). Wallerian degeneration results in the removal of axonal and myelin-derived material, and prepares the environment to allow the growth of regenerating axon. The axon and myelin degenerate and leave behind Schwann cells, which divide inside the basal lamina tube that surrounded the original nerve fibre. The empty Schwann cell columns
surrounded by basal lamina are called endoneurial tubes or bands of Büngner. The Wallerian degeneration of unmyelinated nerve fibres occurs in a similar process. There is debate as to the direction of axon degeneration after nerve injury (for review, see Sunderland, 1978), but there is evidence to suggest that this occurs in a proximodistal direction starting at the point of injury, as the distal stump will continue to function after disconnection for a time dependent on the length of the distal stump (Miledi and Slater, 1968, 1970; Ljubinska, 1975). The axon degenerates because of nutrient depletion (Ljubinska, 1964, 1975) and the death of the mitochondria (Webster, 1962). Mitochondrial death, and so failure of oxidative phosphorylation, results in the loss of the membrane potential and thus the accumulation of Ca\(^{2+}\) ions in the axon as electrical and chemical gradients are disrupted. The calcium causes microtubular depolymerisation and the activation of CANP, and if CANP is inhibited axonal degeneration is prevented (Schlaepfer and Freeman, 1980).

Much of the axoplasm synthesized in the cell body is supplied to the nerve by slow axonal transport, including the cytoskeletal elements actin, tubulin, and neurofilament subunits (Hoffman and Lasek, 1975). Particulate matter such as membranous organelles for membrane turnover, transmitter synthesis and release, and axonal metabolism, are transported by fast axonal transport (McEwen and Grafstein, 1968; Cuenod et al, 1972; Droz, 1975; Grafstein, 1977). Only fast transport continues after nerve injury, and so in this case neuromuscular transmission occurs until the transmitter is depleted (Feldberg, 1943; Dahlstrom, 1967; Lubinska, 1964; Lasek, 1970), and a longer distal stump maintains transmission for a longer period (Miledi
and Slater, 1970). If an isolated distal stump is stimulated, its transmitter is depleted faster and transmission fails more rapidly (Gerard, 1932; Card, 1977).

Before nerve regeneration can occur the distal stump must be broken down. The clearance of myelin and its breakdown products is facilitated by the invasion of macrophages into the degenerating nerve stump (Ramon y Cajal, 1928; Holmes and Young, 1942; Weinberg and Spencer, 1978). The relative roles of Schwann cells and macrophages in removing degenerating debris have been partially resolved in experiments where a piece of mouse nerve was placed in a chamber the pore size was sufficiently small to not allow the entry of macrophages (Beuche and Friede, 1984; Scheidt and Friede, 1987). In this case the Schwann cells did not proliferate, and although the myelin was discarded it was not phagocytosed. However, when macrophages were allowed access to the nerve, they actively phagocytosed myelin. The nature of the injury dictates the ease of macrophage entry, and after crush injury the Schwann cell columns are left intact and the speed of degeneration is slower than after nerve section (Lunn et al, 1990). The Schwann cell continuity could also provide metabolic support to the isolated axon, and in experiments in vitro where the cell cultures were artificially metabolically supported, severed peripheral axon stumps could survive, grow, and fuse to the growing axon (Boeke, 1950). Wallerian degeneration eventually leads to the breakdown of neurotubules and neurofilaments, the phagocytosis of the remnants of the axon, and the division of Schwann cells (reviewed by Gutmann, 1958; Alt, 1976; Sunderland, 1978; Salzer and Bunge, 1980; Hall, 1989; Salonen et al, 1988). Interleukin 1 is released as the nerve degenerates,
and this induces the synthesis of NGF by Schwann cells and other non-neuronal cells is the distal stump (Lindholm et al, 1987; Brown et al, 1991; Matsuoka et al, 1991). The Schwann cell proliferation that occurs during the early phase of Wallerian degeneration could be triggered by macrophages which secrete various cytokines, including interleukin 1 (Rotshenker et al, 1992), known to promote nerve regeneration (Guenard et al, 1991).

The importance of macrophage invasion is seen in the mutant C57BL/6/Ola mouse, in which a greatly reduced infiltration of injured nerves by circulating macrophages results in delayed Wallerian degeneration (Lunn et al, 1989), although degeneration does eventually occur (Brown et al, 1992). There is a lack of Schwann cell proliferation around the intact axolemma in the distal stump of injured nerves in Ola mice (Thomson et al, 1991), and during normal Wallerian degeneration the stimulus for Schwann cell division could be the substances released by the degenerating nerves (Salzer et al, 1980; Clemence et al, 1989).

c) Peripheral nerve regeneration

i) Changes in the cell body

At the onset of regeneration following axotomy, the surviving cell bodies express different genes and their protein products, these generally being the same as those associated with axonal growth during development. Growth-associated proteins, tubulin, and actin all have their synthesis enhanced. The induction of GAP-43 is very rapid after axotomy and decreases following reinnervation (Skene et al, 1986;
Verhaagen et al, 1988; Karns et al, 1987). GAP-43, an axonally transported phosphoprotein which resides inside the membrane of developing and regenerating axons particularly near the growth cone (Meiri et al, 1988), is a substrate for protein kinase C and could play an important role in nerve fibre growth. The tubulin genes T alpha 1 (Miller et al, 1987) and class II beta, are highly expressed during axonal development, down-regulated in the adult, and are re-induced during regeneration (Miller et al, 1989; Hoffman and Cleveland, 1988). Conversely, neurofilament protein which is expressed during development once axons have reached their target and are expanding radially, is decreased during regeneration (Hoffman et al, 1987; Oblinger and Lasek, 1988). Thus, there is a decrease in the diameter of the proximal part of regenerating axons.

ii) Nerve growth

Within a few hours of axotomy the cut tip of the axon swells, inflated by smooth endoplasmic reticulum, mitochondria and eventually microtubules, and the first regenerating axonal sprouts are produced, usually at the node of Ranvier (Hopkins and Slack, 1981; McQuarry, 1985). Regenerating sprouts are found as early as 5 hours after the injury (Tomatsuri et al, 1993). These sprouts grow down the endoneurial tubes towards their targets, with their growth cones in contact with Schwann cell basal lamina on one side and Schwann cell membrane on the other (Nathaniel and Pease, 1963; Haftek and Thomas, 1968; Scherer and Easter, 1984). Axon-Schwann cell attachment is mediated by a number of adhesion molecules, including the
immunoglobulin superfamily, e.g. N-CAM and L1, and the cadherin superfamily, e.g. N-cadherin and E-cadherin, and axon-basal lamina contact is mainly mediated by laminin (for review see Bixby and Harris, 1991; Letourneau et al, 1994). These adhesion molecules are down-regulated in mature myelinated nerves, but are still expressed in mature unmyelinated fibres (Martini, 1994). There is the opportunity for both myelinated and unmyelinated axons to branch distal to the site of transection (Bray and Aguayo, 1974; Jenq et al, 1987), although not all these branches survive. The regenerating axons extend as far as the target organs (Ann et al, 1994).

Nerve regeneration depends on protein synthesis in the Schwann cells of the peripheral nerve (reviewed by Aguayo, 1985). In the PNS the regenerating axons pass through an environment consisting of Schwann cells and their basal laminae, fibroblasts, collagen, and early during regeneration they encounter axonal debris, degenerating myelin, and phagocytic cells. If Schwann cells are not present, then the growth of the regenerating sprouts is either stopped or reduced. This is demonstrated in experiments where a frozen peripheral nerve, in which the Schwann cells are dead but their basal laminae intact, is implanted into a host animal. Axonal regeneration only occurs with the concurrent migration of host Schwann cells into the graft (Anderson et al, 1983; Hall, 1986a; Gulati, 1988), and if the migration of the host’s Schwann cells into the nerve graft is prevented by cytotoxic agents, axons fail to regenerate (Hall, 1986b).

The Schwann cell basal lamina, containing laminin and fibronectin which promote neurite growth in culture (Rogers et al, 1983; Bozyczo and Horwitz, 1986),
is thought to play a role in axon regeneration (Ide et al, 1983). Indeed, an antibody to a laminin-heparan sulphate proteoglycan complex inhibits the growth of neurites on sections of peripheral nerves (Sandrock and Matthew, 1987). However, Schwann cells developed in culture free medium where little or no basal lamina is present, still promote regeneration (Ard et al, 1987). The regeneration promoting properties of Schwann cells can be blocked by using antibodies to the cell surface adhesion molecules, L1/Ng-CAM, N-cadherin, and integrins all together (Bixby et al, 1988; Seilheimer and Schachner, 1988), although none block the promotion when used alone. Schwann cells can attract regenerating axons from a distance (Ramon y Cajal, 1928; Politis et al, 1982), and this is inhibited by the addition of antibodies to laminin in Schwann cell conditioned media (Chiu et al, 1986; Lander et al, 1985). As well as forming a scaffolding for regenerating axons by expressing adhesion molecules on the surface plasma membrane, Schwann cells also produce various trophic factors for regenerating axons (Bunge and Bunge, 1983).

The remyelination of regenerated axons starts after about 8 days and proceeds in much the same way as during development. If an axon was originally myelinated then it will be remyelinated, regardless of whether or not its Schwann cell was originally myelinating (Weinberg and Spencer, 1975; Aguayo et al, 1976).

The success of functional recovery following regeneration depends on the accuracy of regeneration. After nerve crush injury the endoneurial tubes and Schwann cell basal lamina are left intact (Haftek and Thomas, 1968), and consequently nerve fibres regenerate more accurately following crush rather than cut injuries, as they are
able to remain in their parent tubes and are guided directly back to their targets (Sunderland, 1978; Totosy de Zepetnek et al, 1992).

**iii) Target reinnervation**

Once the regenerating axons reach their target muscle they follow the Schwann cell guidelines within the muscle before terminating, usually at the original motor endplate, and new neuromuscular contacts are established between themselves and the denervated muscle fibres (Gutmann and Young, 1944). The innervation of mammalian muscles is topographically organised (Brown and Booth, 1983; Bennet and Lavidis, 1984; Hardman and Brown, 1985; Laskowski and Sanes, 1987), and the maintenance of this selective organisation is challenged following nerve injury and reinnervation. The pattern of muscle reinnervation is more selective after nerve injury in neonatal rats, than after nerve injury in adult rats (Bernstein and Guth, 1961; Aldskogius and Thomander, 1986; Hardman and Brown, 1987; Laskowski and Sanes, 1988), suggesting that positional cues exist in immature but not more mature muscle, or that positional cues can be more effectively utilised by immature regenerating axons rather than axons from mature motoneurones (reviewed by Grinnell and Herrera, 1981; Purves and Lichtman, 1985).

Only axons that release ACh can establish functional contact with skeletal muscle (Gutmann and Young, 1944), and nerves releasing other transmitters can not do so. Once the nerve contacts the muscle, spontaneous and evoked release of ACh increases rapidly (Xie and Poo, 1986). This increase could be mediated by retrograde
signals from the muscle which trigger a rise in intracellular levels of Ca\(^{2+}\) in the growth cone (Hall and Sanes, 1992). On reinnervation, like during development, many of the endplates are polyneuronally reinnervated, and with time the excess terminals are eliminated (McArdle, 1975; Jansen and Van Essen, 1975; Letinsky et al, 1976; Rotshenker and McMahan, 1976; Gorio et al, 1983; Rich and Lichtman, 1989). These newly formed neuromuscular junctions have similar features to immature synapses, and some time is required before they attain their adult characteristics. Following the re-establishment of the nerve-muscle contacts, the changes that occurred in the motoneurone and muscle during the period of separation and regeneration are reversed. However, the regenerated axons do not always return to the muscle fibres they originally supplied, and as such the distribution of motor unit territory is altered on reinnervation (Kugelberg et al, 1970; Peyronnard and Charron, 1980; Totosy de Zepetnek et al, 1992; Bodine-Fowler et al, 1993). Large motoneurones reinnervate more muscle fibres than small ones (Totosy de Zepetnek et al, 1992), and the re-establishment of motor unit size is determined by activity dependent interactions with the target muscle fibres.
Aim of study

This thesis is concerned with the regenerative and degenerative changes after loss of neuromuscular interaction during early postnatal life. The first question asked is whether or not stabilising the new neuromuscular contacts formed by reinnervating axons after neonatal nerve crush leads to increased motoneurone survival. This is compared with the effect of stabilising neuromuscular contacts after a period of time when functional neuromuscular interaction was interrupted by neonatal muscle paralysis. Next, the time course of motoneurone death following prolonged neonatal muscle paralysis is studied, and compared with that seen after nerve crush. The structural changes in the nerve to soleus after nerve crush at birth are examined, and compared with those changes observed after neonatal muscle paralysis. This study therefore provides a direct comparison between nerve crush at birth and neonatal muscle paralysis, two methods used to interrupt neuromuscular interactions during the early postnatal period.

Finally, the possibility that motoneurones supplying a partially denervated muscle may remain in a growing mode for longer, and could be susceptible to a nerve crush injury at a time when they would normally survive this insult, was investigated.
Chapter 2. Motoneurone survival and the structure of the nerve to soleus after temporary loss of functional neuromuscular interaction during early postnatal development in rats.

2.1. Introduction

Young mammalian motoneurones are critically dependent upon functional interaction with their target muscle for their normal development and if deprived of target contact a large proportion die (Romanes, 1946; Zelená and Hník, 1963; Lowrie et al, 1982; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987; Crews and Wigston, 1990). After injury to the sciatic nerve in newborn rats there is a loss of motoneurones and consequently a severe impairment of muscle function (Bueker and Meyers, 1951; Zelená and Hník, 1963; McArdle and Sansone, 1977). Although some motoneurones survive neonatal nerve injury and their axons find their way back to the muscle, the recovery of the reinnervated muscles is very poor, partly because of the loss of motoneurones but also because the injured axons are unable to expand their peripheral field (Lowrie et al, 1982; Lowrie et al, 1987; Dick et al, 1995). Following nerve injury during early postnatal development the muscle is separated from its nerve at a time when it is differentiating and is therefore deprived of those influences that normally bring about its maturation (Buller et al, 1960; Vrbová, 1963; Close, 1964; Brown, 1973). Thus, motoneurones and the muscle fibres they innervate depend upon each other; motoneurones that are deprived of their target during a critical period of their
development will die, and muscle fibres that are not functionally innervated fail to
differentiate (Lowrie et al, 1982; Lowrie et al, 1987; Greensmith and Vrbová, 1992;
Lowrie and Vrbová, 1992).

The extent of motoneurone death induced by neonatal nerve injury depends on
several factors. These include the age of the animal at which the injury is inflicted
(Romanes, 1946; Zelená and Hník, 1963; Lowrie et al, 1982; Schmalbruch, 1984;
Lowrie et al, 1987), the site and type of injury and the duration of disruption of nerve-
muscle contacts (Kashihara et al, 1987; Lowrie, 1990; Lowrie et al, 1990). The
importance of the type of injury is probably associated with the opportunity that the
injured motoneurone has to re-establish contact with the target muscle. Fewer
motoneurones die if they are permitted to reinnervate their target muscle than when
reinnervation is prevented (Kashihara et al, 1987). This is consistent with findings that
show that limb amputation or nerve section result in massive motoneurone death but
that some motoneurones survive following a nerve crush injury inflicted close to the
muscle (Crews and Wigston, 1990). Another critical factor for the survival of
motoneurones, even when reinnervation is permitted, is the duration for which the
motoneurone is separated from its target. More motoneurones die when reinnervation
is delayed either by repeated nerve injury (Lowrie, 1990) or by injuring the axons
further away from the muscle (Lowrie et al, 1990; Greensmith et al, 1994b). When the
injury site is far from the muscle, the regenerating axons have further to grow before
they can reinnervate the target muscle and so there is a longer delay before
neuromuscular interaction is resumed (Lowrie et al, 1990). Nerve injury effectively
delays the maturation of the motoneurone, and when the duration of loss of target interaction is prolonged then the motoneurone is maintained in its growing state and is not allowed to mature into its transmitting mode. For example, the acquisition of the motoneurones transmitter phenotype is delayed following target disruption. It has been shown that after target disruption levels of growth-associated proteins remain high (Caroni and Becker, 1992) and proteins related to transmission remain low (Hebb and Silver, 1963; Watson, 1970; Burt, 1975). The transition from a growing into a transmitting cell is an important step in the maturation of the motoneurone, which may play a crucial role in determining the response of the cell to injury.

These considerations already indicate that it is not the nerve injury itself that causes motoneurone death, but the loss of nerve-muscle interaction. Blocking the postsynaptic acetylcholine receptor with α-bungarotoxin (α-BTX) also results in loss of neuromuscular interaction although there is no direct injury to the nerve. Recently it was shown that when the soleus muscle was paralysed with α-BTX at a critical stage of postnatal development a large number of motoneurones to soleus died (Greensmith and Vrbová, 1992), and the degree of motoneurone death was dependent upon the length of time that neuromuscular interaction was disrupted, so that more motoneurones died after prolonged paralysis (Greensmith and Vrbová, 1992).

Most of the available evidence indicates that developing motoneurones have a better chance to survive target deprivation if they rapidly resume interaction with their target. This can occur only if their axons reinnervate the muscle and their terminals are able to establish and maintain contact with the muscle fibres.
Nevertheless, some injured motoneurones die even after their axons have reached the muscle (Kashihara et al, 1987; Lowrie et al, 1994), possibly because their terminals are unable to find or maintain contact with the muscle fibres. In this study we examined the possibility that these motoneurones could be rescued if the formation of new neuromuscular contacts made by the reinnervating axons could be maintained and supported.

It has previously been shown that the withdrawal of synaptic terminals during both normal development and sprouting of nerve terminals after partial denervation is brought about by a process that is Ca\(^{2+}\) dependant, and involves the participation of a calcium-activated neutral protease (CANP) (O’Brien et al, 1984; Connold et al, 1986; Vrbová and Fisher, 1989; Connold and Vrbová, 1994). CANP is present in axons and nerve terminals where it is bound to the cytoskeleton (Tashiro and Ishizaki, 1982; Ishizaki et al, 1983). It has a high affinity for cytoskeletal elements, e.g. axonal neurofilament proteins (Lasek and Hoffman, 1976; Lasek and Black, 1977; Pant et al, 1979; Schlaepfer, 1979; Pant and Gainer, 1980; Croall and Demartino, 1991), and may be involved in the turnover and degradation of these proteins (Schlaepfer, 1979; Schlaepfer and Freeman, 1980).

Muscle activity has been shown to play an important role in the rate of synapse elimination. For example, during development the rate of synapse elimination is accelerated by increased muscle activity (O’Brien et al, 1978), and is slowed down by decreased activity (Benoit and Changeaux, 1975; Zelená et al, 1979; Caldwell and Ridge, 1983; Callaway et al, 1987). Increased muscle activity results in an increased
Ca\(^{2+}\) concentration in the terminal, which could activate CANP and thus induce an increase in the degradation of the axonal cytoskeleton and an acceleration of axonal retraction (O'Brien et al, 1978; Connold et al, 1986). Indeed, the rate of axonal retraction is decreased if Ca\(^{2+}\) levels are manipulated so as to prevent them reaching high enough concentration in nerve terminals to activate CANP (Connold et al, 1986).

Leupeptin is a potent CANP inhibitor (Suzuki et al, 1981) which can penetrate into cells and enter axon terminals (Roots, 1983). Leupeptin also increases the complexity of nerve terminals at the neuromuscular junction (Swanson and Vrbová, 1987). More nerve-muscle contacts are maintained during a period of synapse elimination when CANP is inhibited by leupeptin (Connold et al, 1986; Connold and Vrbová, 1994). When locally applied to soleus muscles of rats that had been partially denervated at 4-6 days it induced an expansion of motor unit territory that was over and above that due to partial denervation alone (Vrbová and Fisher, 1989). It was suggested that this expansion was due to leupeptin inhibiting CANP and thus preventing the normally occurring elimination of some nerve terminals, allowing them to maintain contact with the muscle fibres and to complete their development to maturity (Vrbová and Fisher, 1989). This is consistent with earlier findings where leupeptin was found to prevent the elimination of polyneuronal innervation in vitro (O'Brien et al, 1984; Vrbová et al, 1988) and in vivo (Connold et al, 1986).

Leupeptin has been observed to decrease neurofilament protein breakdown in goldfish optic tectum (Roots, 1983). By inhibiting CANP, leupeptin blocks the normal degradation of the axonal cytoskeleton, and as such it is possible that it is responsible
for the decrease in synapse elimination described above.

It has been shown that when the soleus muscle of newborn rats is paralysed with α-BTX, there is a few days later a sharp reduction in the number of neuromuscular contacts, followed by an increase (Greensmith and Vrbová, 1991). In muscles treated with α-BTX axons are likely to sprout (Holland and Brown, 1980), and the cell body thereby is likely to be kept in a growing mode. Due to the neonatal paralysis there was a permanent loss of motoneurones observed in the adult (Greensmith and Vrbová, 1992). In addition it has been recently shown in partially denervated muscles treated with α-BTX that many newly formed sprouts contacted the denervated muscle fibres, but these contacts were lost upon recovery of neuromuscular activity (Connold and Vrbová, 1994). Furthermore, the loss of these contacts was prevented when they were treated with leupeptin (Connold and Vrbová, 1994). In this study we examined the effects of stabilising those neuromuscular contacts which would otherwise be lost as the soleus muscle regains its activity after a period of neonatal paralysis.

The time-course of motoneurone death after nerve crush in neonatal rats has been examined (Lowrie et al, 1994), and it was found that most of the cell death occurred within 6 days after the initial injury. These motoneurones were mainly in the more caudal part of the motor column, and cells from the more cranial part died later, i.e., between 6 and 12 days after the injury. This indicates that those cells in the upper, more mature part of the spinal cord survived longer after nerve crush. It is these motoneurones, which perhaps reinnervate the muscle but then subsequently die, that
could possibly benefit from stabilisation of neuromuscular contacts upon reinnervation. The time-course of motoneurone death after neonatal muscle paralysis is much slower than after sciatic nerve crush. Following paralysis for only 24-48 hours at birth no loss of motoneurones was observed in 3 week old animals, and only by 10 weeks were there significantly fewer motoneurones on the α-BTX treated side. When the neonatal soleus muscle was paralysed for longer, the degree of motoneurone death at 10 weeks of age was greater than that observed after a short lasting paralysis (Greensmith and Vrbová, 1992). However, whether or not the time-course of motoneurone death was different after prolonged paralysis as compared to a brief paralysis, i.e. occurs at a different rate, had not previously been established, and is investigated in this study.

There are several differences between disrupting neuromuscular interactions by nerve injury or by muscle paralysis. For example, after sciatic nerve crush injury at birth all the efferent and afferent fibres supplying the muscle are disrupted, and there is a loss of motoneurones and consequently a severe impairment of muscle function. Blocking the postsynaptic acetylcholine receptor with α-BTX for a few days after birth also results in motoneurone loss, but in this case the insult is primarily motor and the afferent fibres are not directly affected. In this study the structure of the nerve to soleus in adult rats was examined after neuromuscular interaction was prevented neonatally by either nerve injury or by induction of muscle paralysis. The number and size of myelinated and unmyelinated axons has been thoroughly studied with electron microscopy, and tranverse sections through nerves are a routine method
to demonstrate axon-Schwann cell relations and how the structure of the nerve changes after injury. It was of interest to see if the structure of the nerve to soleus was different after a mixed afferent and efferent fibre disruption, than to a purely efferent insult.

This study provides a direct comparison between nerve crush at birth and neonatal muscle paralysis. The questions asked in this chapter were: a) Could motoneurones be rescued after the loss of target contact during the neonatal period by stabilising neuromuscular contacts at critical time-points when it is thought that the loss of synaptic contacts is related to the long-term motoneurone death? If so, then what are the similarities and differences between the functional response of the neuromuscular system after nerve crush at birth as compared to prolonged neonatal muscle paralysis in this situation. b) What is the time-course of motoneurone cell death after prolonged neonatal muscle paralysis, and how does this compare to that seen after nerve crush at birth? Finally, c) What are the structural changes in the nerve to soleus after loss of target contact by nerve crush at birth, and how do these compare to those changes observed after prolonged neonatal muscle paralysis?
2.2. Materials and methods

1) Surgery

Experiments were carried out on Sprague Dawley rats (Biological Services, UCL, UK)

a) Sciatic nerve crush

New-born rats were anaesthetized with halothane and under sterile conditions the sciatic nerve was exposed in the right hindlimb on the day of birth. The nerve was crushed just proximal to the division of the sciatic nerve into the tibial and common peroneal nerves, with a pair of fine watchmaker's forceps. Using a dissecting microscope (Zeiss) the nerve was examined after the crush to ensure that the epineurial sheaths were intact. In some rats, used to examine the structure of neonatal peripheral nerves, a fine suture (Ethicon, 0.1) was placed at the crush site to mark it. The incision was then closed and sutured with fine thread (Ethicon, 0.7), and after recovery from the anaesthetic the animals were returned to their mothers.

b) Muscle paralysis

i) Preparation of the implant.

Sterile sodium chloride (NaCl) was finely ground, mixed with a known amount of α-BTX, and added to an appropriate amount of liquid silicone rubber (Dow Corning, 3140 RTV, non-toxic) in a fume cupboard. The solution was thoroughly mixed to form a homogenous paste. The paste was spread into a thin film and left to dry for at
least 24 hours at room temperature, and then stored at 4°C. Small strips were cut from the dried plastic. For a new-born rat a strip would typically weigh about 0.3mg (containing about 7μg of α-BTX and 120μg of NaCl) and for a 3 day old rat 0.6mg strips were used (14μg of α-BTX and 240μg of NaCl). The silicone strips were similar to those used previously in this laboratory (Duxson, 1982; Greensmith and Vrbová, 1991, 1992). Implants containing only NaCl were prepared for control experiments.

ii) Implantation of α-BTX containing silicone rubber strips

Under halothane anaesthesia and sterile conditions a small longitudinal incision was made in the skin and fascia to reveal the underlying muscles in the right hindleg of rats within 3-6 hours of birth. A small silicone strip containing α-BTX was implanted between the soleus and flexor hallucis longus muscles, taking care to avoid the point of nerve entry to soleus. These implants usually remained in position so that there was no need to secure them. The skin was closed and sutured with fine thread (0.4 Ethicon) and the animal was left to recover from the anaesthetic before being returned to its mother. A second α-BTX containing silicone strip was implanted alongside the soleus muscle of the same leg 3 days later in order to prolong the duration of paralysis, at which time the original implant was checked for position and removed. The implants have previously been shown to cause no damage to the muscle (Greensmith and Vrbová, 1991). Control animals received silicone implants containing only NaCl in the same manner at these time points.

The degree of paralysis produced by this procedure has been previously
assessed in this laboratory, and was checked by calculating the ratio of indirect:direct tetanic tension for soleus muscles taken from animals treated with α-BTX at birth (Greensmith and Vrbová, 1991). This ratio gives an estimate of the proportion of the muscle which is functionally innervated. The soleus muscle was completely paralysed for 24 hours after implantation of each strip, and the muscle gradually recovered with neuromuscular transmission being restored 8-9 days postnatally following the application of α-BTX to the surface of the soleus muscle at 2 time-points: at birth and at 3 days of age (Greensmith and Vrbová, 1992).

c) Application of the protease inhibitor

i) Preparation of the implant

Leupeptin (Sigma) was added to an appropriate amount of liquid silicone rubber. The solution was thoroughly mixed to form a homogenous paste, spread into a thin film and left to dry for at least 24 hours at room temperature. Leupeptin is a small peptide (N-acetyl-L-leu-L-leu-arg-al) which is known to inhibit the activity of CANP (Connold et al, 1986; Connold and Vrbová, 1994) and to some extent that of thrombin (Liu et al, 1994). Leupeptin is routinely applied to the surface of muscles in this way, and the time-course of its release has been characterised (Connold et al, 1986). As with the α-BTX containing silicone strips, small strips were cut from the dried plastic which was stored at 4°C.
ii) Implantation of leupeptin containing silicone rubber strips

After either nerve crush injury at birth, or neonatal paralysis of the soleus muscle, a leupeptin-containing silicone strip was applied to the surface of the soleus muscle.

Six to 7 days after the sciatic nerve crush, when the regenerating axons reached the muscle, the rats were re-anaesthetized with halothane and a silicone strip weighing 1mg and containing approximately 70μg of leupeptin was inserted between the soleus and flexor hallucis longus muscles. The procedure was repeated three days later, when the animals were 9 to 10 days old. During this operation the location of the first implant was checked and it was then removed. The control group of animals received a 1mg silicone strip containing NaCl at the same ages.

In rats previously treated with α-BTX at birth and 3 days of age, a leupeptin containing silicone strip weighing 1mg was applied to the surface of the soleus muscle once the rats were 6 days old. The second α-BTX containing strip was checked for position and removed at this time.

d) Ventral root section

In a group of rats where the nerve to soleus was analysed using electron microscopy, ventral root section was carried out in some animals 1 week prior to removal of the nerve from the animal. This was done to assess the motor and sensory component of axons in the nerve to soleus, as by the time that the animals were perfused 1 week later the motor axons would have died.

Adult rats were anaesthetised with halothane and the lumbar region of the
vertebral column was exposed. Laminectomy of the lumbar region of the vertebral column was carried out by cutting the vertebral arches and exposing the spinal cord. A small incision was made in the dura-mater to expose the dorsal and ventral roots of the spinal cord, and all the ventral roots at the lumbar level of the spinal cord were cut. A small piece of Spongostan was placed over the exposed spinal cord to protect the cord and promote healing, and the muscle layers and skin were closed with sutures. Antibiotic powder (Cicatrin) was liberally applied to the wound, a painkiller (Buprenorphine) was injected, and the rat was left to recover from anaesthesia before being returned to its cage.

2) Tension recording experiments

Two to 4 month old rats were prepared for isometric tension recordings of muscle contraction (Lowrie et al, 1982). The animals were anaesthetized with 4.5% chloral hydrate solution (1ml/100g body weight, i.p.) and the soleus muscles on both the operated and contralateral sides exposed. The distal tendons were attached to tension transducers (PYE UNICAM) and the leg was rigidly secured to the table. Isometric contractions were elicited by square-wave pulses of 0.02ms duration and supramaximal intensity applied to the nerve to soleus, via silver-wire electrodes. The length of the muscle was adjusted for optimal twitch tension.

The number of motor units in the operated and in some cases the contralateral, unoperated control muscle, was determined by grading the stimulus intensity to the nerve and recording step-wise increments in twitch tension due to successive
recruitment of motor units (Fisher et al, 1989; Connold and Vrbová, 1991). Twitch tensions were used to determine motor unit number to avoid the fatigue which may develop during repeated tetanic contractions.

Tetanic contractions of the muscle were then recorded to determine maximal muscle force. Contractions were elicited by trains of stimuli lasting 700-900ms at a frequency of 10, 20, 40 and 80Hz. The effect of the treatment was assessed by expressing the tension developed by the operated muscle as a percentage of that developed by its contralateral control. This was done to account for the difference in size observed between rats. The muscles and nerves were kept moist throughout the experiments with saline and all experiments were carried out at room temperature (23°C).

The mean motor unit tetanic force was calculated by dividing the number of motor units found in each muscle into the maximum tetanic tension for that muscle. This was expressed as a percentage of the mean of motor unit force calculated from the contralateral muscle, using the same method.

Variations in body weight will influence muscle weight and force. The variation in body weights of female rats between the ages of 2 to 4 months is significantly less than that of males, and therefore motor unit force was assessed in this single sex group only.
3) Retrograde labelling of motoneurones

a) Horseradish peroxidase (HRP)

HRP is a chemical substance used to localise neuronal pools. Once injected into a muscle, it is taken up by motor nerve endings and is retrogradely transported along the axon to the neuronal cell body where it can be visualised by the use of histochemical methods. In this study the method developed by Harker et al, 1977 was used. To identify the motor pool of neurones which supplies this muscle HRP was injected into the soleus muscle of each hindlimb. The pool of motoneurones in the lumbar spinal cord which supply the soleus muscle has previously been characterised by this method (Lowrie et al, 1987; Greensmith and Vrbová, 1992). It is possible that after injection the HRP could spread to the surrounding muscles and distort cell counts (Burke et al, 1977; McHanwell and Biscoe, 1981). The muscle was rinsed following injection to minimise spread. Cells were counted within the documented position of the soleus motoneurone pool to minimise this error (Lowrie et al, 1987).

b) Injection of HRP

When the animals were either 3 weeks old or at least 10 weeks old they were anaesthetized with halothane, and under sterile conditions an incision was made in the skin and fascia of the hindlimb to reveal the underlying muscles. The soleus muscle was identified and injected with a 10% solution of HRP (Sigma Type Vf) in sterile saline (0.9%) using a fine needle and Hamilton syringe. The amount of HRP injected was calculated according to the weight of the animal. In a 100g rat the soleus muscle
weighs approximately 50mg, and 2μl of HRP was injected per 50mg of muscle. After completing the injection the muscle was rinsed with sterile saline to remove any HRP that had leaked from the point of injection. The incision was closed and sutured with fine silk thread (0.7 Ethicon), and the animal was left to recover from the anaesthetic before being returned to its cage.

c) Removal of spinal cord

24 hours after HRP injection the animals were terminally anaesthetized with chloral hydrate (4.5%, 1ml/100g body weight, i.p.) and prepared for transcardiac perfusion. The heart was exposed and an incision was made in the right atrium to allow the escape of perfusate. The apex of the heart was cut, a blunt needle was secured in the left ventricle with artery forceps, and the animal was perfused with 0.9% saline until the liver looked pale. The animal was then perfused with 2.5% glutaraldehyde in Millonigs phosphate buffer (pH 7.3). One hundred mls of fixative was used for every 100g body weight.

The spinal cord was removed and post-fixed in 2.5% glutaraldehyde for a period of 2-4 hours at 4°C. The cord was then transferred into a 30% sucrose solution prepared with Millonigs phosphate buffer (pH 7.3) and left overnight at 4°C, to cryoprotect the tissue and remove the fixative.

d) HRP histochemistry

The lumbar region of the spinal cord between the L2 and L6 ventral roots was excised
and a fine micropin was pushed through the left dorsal horn to mark the control side of the cord. The cord was mounted on a freezing microtome and serial sections were cut at 50µm and collected into individual wells of a tray immersed in Millonig's phosphate buffer. The sections were processed for HRP histochemistry using a modified Hanker-Yates method (Hanker et al., 1977): see Appendix I for full protocol. The sections were mounted onto gelatinised slides and left to dry overnight at 37°C. 

Gallocyanin was used to counterstain the RNA and Nissl substance of the sections (Culling, 1963): see Appendix I. The sections were then dehydrated, cleared, and coverslips were applied using Permount mounting medium. The slides were left at 37°C to dry.

e) Microscopy

The sections were examined under a light microscope, and the retrogradely labelled soleus motoneurone pool was located under a low power objective (X2.5). The soleus pool is located in the medial part of the ventral horn of the spinal cord (Nicolopoulos-Stournaras and Iles, 1983; Lowrie et al., 1987; Greensmith and Vrbová, 1992). The HRP labelled cells were carefully examined under high magnification (X40) and those cells which were in the soleus motor pool and had a clearly visible nucleolus were counted. In view of the thickness of the sections it is unlikely that the nucleolus would appear in consecutive sections of the same labelled motoneurone. This was done on both sides of the cord and the total number of labelled neurones on the operated side was expressed as a percentage of that on the control side.
f) *Fluorescent labelling*

In some rats the motoneurones supplying the soleus muscle were retrogradely labelled with the fluorescent dyes fast blue (FB) and diamidino yellow (DiY) (Illing) following prolonged neonatal muscle paralysis with α-BTX. A group of 5 day old rats were anaesthetised with halothane and under sterile precautions a solution of 2% FB and 2% DiY in distilled water was injected into the soleus muscles on both sides using a fine Hamilton syringe. The muscles were rinsed and the skin closed with sutures. The rats were returned to their mother upon recovery. The rats were terminally anaesthetised and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer at 3 time-points: 7 days, 14 days, and 21 days of age. The spinal cords were removed and 20μm thick serial sections were cut onto gelatinised slides using a cryostat. The slides were left to dry and coverslips were applied using Citifluor mounting medium. Fluorescent motoneurones were counted on both sides of the spinal cord in every fourth section using a fluorescent microscope.

4) **Electron microscopy**

a) *Fixation and tissue processing*

Rats were terminally anaesthetised and transcardially perfused with a mixture of 2% paraformaldehyde and 2% glutaraldehyde fixative (for full method of transcardial perfusion see section 3c). The nerve to soleus was removed from both sides and placed in fixative overnight. After washing in 0.1M phosphate buffer the nerves were fixed with a secondary fixative, 1% OsO₄ in 0.1M phosphate buffer, and then stained
with 2% uranyl acetate. The nerves were dehydrated with increasing concentrations of alcohol, and care was taken to ensure that the dehydration time was constant. After being transferred into propylene oxide, the specimens were embedded in Araldite. (For full protocol see Appendix II).

**b) Preparation of sections and structural analysis**

Semi-thin sections (1μm) were cut with a glass knife on a microtome (Reichert Ultracut) and dried onto glass slides. The sections were stained with toluidine blue, and the location of the nerve within the block was established with a light microscope. The block was shaved down and adjacent ultra-thin sections (70-90nm) were cut with a diamond knife on an ultramicrotome and collected on mesh grids and single slot grids coated with a thin formvar plastic film. The sections were counterstained with lead citrate.

The specimens were viewed in a transmission electron microscope (Joel EM1010) and montages of the transverse section of the nerve were constructed by taking adjacent photographs. The montages were typically made at a magnification of X3000, in order to clearly visualise unmyelinated axons and accurately measure the areas of myelinated fibres. Myelinated and unmyelinated axons were counted, and the areas of myelinated axons were calculated by tracing around the axons with a digitising tablet connected to a computer. The areas of myelinated axons were collated on a computer, and frequency histograms of the distribution of myelinated axonal area were produced by grouping the areas into bin widths.
2.3. Results

A. Motoneurone death caused by temporary loss of functional neuromuscular interactions during early postnatal development in rats can be reduced by stabilising neuromuscular contacts.

The effect of stabilising neuromuscular contacts after either neonatal nerve injury or neonatal muscle paralysis was studied here. The first part of the this section includes experiments where the sciatic nerve was crushed at birth, and this was followed by treatment of the neuromuscular junctions with the protease inhibitor leupeptin. The second part includes experiments where the soleus muscle was paralysed in neonatal rats, and was then treated with leupeptin.

1) The effect of leupeptin on motoneurone survival and recovery of muscle function after neonatal nerve injury in rats.

Twenty Sprague Dawley rats had their sciatic nerves crushed in one hindlimb at birth. Eight of these animals were subsequently treated on the injured side with an implant containing leupeptin at the time of reinnervation 6-9 days later (Naidu et al, 1996). The remaining 12 rats received treatment with NaCl-containing silicone strips.
a) Changes in muscle tension.

The difference in recovery of muscle tension following sciatic nerve crush at birth, and sciatic nerve crush at birth with subsequent treatment of the muscle with leupeptin, was assessed. Muscle tension is a good indication of the functional recovery of the neuromuscular system.

When the animals were at least 8 weeks old they were anaesthetised and tension recording experiments were carried out. In both groups of animals twitch and maximum tetanic tensions of the operated and contralateral soleus muscles elicited by stimulation of their motor nerves were established. Figure 1 shows examples of records of twitch and tetanic contractions from the operated and control soleus muscles taken from rats from the same litter. The tension records were obtained from (A) reinnervated NaCl-treated muscle along with (B) the contralateral unoperated control muscle, and (C) reinnervated leupeptin-treated muscle along with (D) the contralateral unoperated control muscle. Note the different scale bars for records (A) and (C). Figure 1 indicates that the force output of the leupeptin-treated muscle was greater than that of the NaCl-treated muscle. The mean values for both groups, along with the mean body weight of each group are summarised in Table 1. The absolute values of twitch and tetanic tension are influenced by the weight of the animals, and these were slightly different in the NaCl- and leupeptin-treated groups. In order to avoid the differences in body weight from interfering with the results, the values for each of the parameters measured were calculated as a percentage of the contralateral control muscle in each animal, and are shown in Table 1 (%op/con). This Table shows
Figure 1. Twitch and tetanic contractions from adult soleus muscles of NaCl-treated and leupeptin-treated animals after nerve injury at birth.

Each trace shows single twitch and tetanic contractions from the soleus muscles of two littermates elicited by stimulation of their motor nerves: (A) reinnervated NaCl-treated muscle with (B) the contralateral unoperated control muscle, and (C) reinnervated leupeptin-treated muscle with (D) the contralateral unoperated control muscle. Note the different scale bars for (A) and (C). The tetanic contractions were elicited at 20, 40 and 80Hz. The leupeptin-treated muscle in this example developed a maximum tetanic tension of 40g after nerve injury at birth, compared with that of 6.9g in the NaCl-treated muscle.
Figure 1

**NaCl treated**

A

2g

B

Con.
50g

---

**Leupeptin treated**

C

10g

D

50g

400ms
Table 1. Summary of the changes in twitch tension and maximal tetanic tension after sciatic nerve crush at birth with NaCl-treated and leupeptin-treated animals.

Muscle tension was recorded in vivo. This Table summarises the mean twitch and maximal tetanic tension recorded from contralateral unoperated muscles (con) and soleus muscles after a sciatic nerve crush at birth (op) with NaCl- and leupeptin-treated animals. The results are also expressed as \%op/con. The Table also shows the mean body weight for each group.

* Denotes a value for \%op/con of the leupeptin-treated group which is significantly different to that from the NaCl-treated group.
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<td>± SEM (n=12)</td>
<td>± 20</td>
<td>± 2.1</td>
<td>± 0.9</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>424</td>
<td>34.9</td>
<td>8.6</td>
</tr>
<tr>
<td>± SEM (n=8)</td>
<td>± 48</td>
<td>± 3.7</td>
<td>± 1.2</td>
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</table>
that following nerve injury at birth leupeptin-treated muscles were able to generate twice the force of the muscles treated with NaCl. The mean twitch tension in leupeptin-treated muscles was 26.6% (± 4.4 S.E.M., n=8) of the contralateral control muscles, whereas the mean twitch tension developed by muscles treated with NaCl was only 13.7% (± 3.5 S.E.M., n=12) of control (Mann-Whitney U-test, P<0.01). The mean maximal tetanic tension produced by the leupeptin treated muscles was 23.8% (± 4.7 S.E.M., n=8) of the contralateral control muscles, whereas the mean maximal tetanic tension in those muscle treated with NaCl was only 9.5% (±1.6 S.E.M., n=12) of control (Mann-Whitney U-test, P<0.01). The block diagrams in Figure 2 summarise the changes in (a) twitch tension and (b) maximal tetanic tension after sciatic nerve crush at birth with NaCl-treated and leupeptin-treated animals.

b) Changes in muscle weight.

The soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were removed from both sides of the rat and weighed. The mean values for both groups are summarised in Table 2. The absolute values of muscle weight are influenced by the weight of the animals, and so the values measured for the treated side were calculated as a percentage of the contralateral control muscle in each animal (%op/con). Table 2 shows that following nerve injury at birth those soleus muscles treated with leupeptin weighed twice as much as the muscles treated with NaCl (Mann-Whitney U-test, P<0.01). However, the weights of the EDL and TA muscles in leupeptin-treated animals were similar to the weight of those in the animals treated with NaCl.
Figure 2. Changes in twitch tension and maximal tetanic tension after sciatic nerve crush at birth followed by NaCl or leupeptin treatment.

The block diagrams show the mean values of (a) twitch tension and (b) maximal tetanic tension developed by soleus muscles after neonatal nerve injury and subsequent treatment with NaCl or leupeptin. The tensions recordings were made when the animals were 3-6 months old, and the tension developed by the treated muscle was expressed as a percentage of that developed by the contralateral control muscle (%op/con). The error bars denote the standard errors of the mean (S.E.M.). Both twitch tension and maximal tetanic tension is higher in animals where the soleus muscle was treated with leupeptin, than in those animals which were treated with NaCl.
Figure 2

**a**

Twitch tension

<table>
<thead>
<tr>
<th>% op/con</th>
<th>NaCl treated</th>
<th>Leupeptin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**b**

Maximal tetanic tension

<table>
<thead>
<tr>
<th>% op/con</th>
<th>NaCl treated</th>
<th>Leupeptin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Summary of weights of the soleus, EDL and TA muscles after sciatic nerve crush at birth followed by NaCl or leupeptin treatment of the soleus muscle.

The soleus, EDL and TA muscles of 3-6 month old rats were removed on both sides after the completion of each tension recording experiment and weighed. Following nerve injury at birth those soleus muscles treated with leupeptin weighed twice as much as the muscles treated with NaCl (Mann-Whitney U-test, P<0.01). The weights of those EDL and TA muscles from animals treated with leupeptin were not significantly different to those treated with NaCl after nerve injury at birth. This was to be expected as the leupeptin was applied only to the surface of the soleus muscle. The results are also expressed as %op/con.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>Soleus</th>
<th></th>
<th></th>
<th>EDL</th>
<th></th>
<th></th>
<th>TA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td>0.1538</td>
<td>0.0451</td>
<td>28.6</td>
<td>0.1483</td>
<td>0.0297</td>
<td>20.3</td>
<td>0.5084</td>
<td>0.0768</td>
<td>14.9</td>
</tr>
<tr>
<td>± SEM (n=12)</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>±3.9</td>
<td>± 0.008</td>
<td>± 0.003</td>
<td>±1.9</td>
<td>± 0.040</td>
<td>± 0.007</td>
<td>± 0.8</td>
</tr>
<tr>
<td>Leupeptin treated</td>
<td>0.2122</td>
<td>0.1076</td>
<td>53.3</td>
<td>0.2077</td>
<td>0.0447</td>
<td>21.3</td>
<td>0.7967</td>
<td>0.1325</td>
<td>16.5</td>
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<tr>
<td>± SEM (n=8)</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>±8.2</td>
<td>± 0.019</td>
<td>± 0.007</td>
<td>±2.4</td>
<td>± 0.073</td>
<td>± 0.02</td>
<td>± 1.7</td>
</tr>
</tbody>
</table>
This was to be expected as the leupeptin was applied only to the surface of the soleus muscle, and the weight of EDL and TA muscles therefore is unlikely to be affected by leupeptin. The block diagram in Figure 3 summarises the changes in muscle weight of the soleus, EDL, and TA muscles after sciatic nerve crush at birth followed by NaCl or leupeptin treatment.

c) Motor unit numbers.

The question whether the observed differences in muscle tensions between the 2 groups of rats was due to a difference in the number of motoneurones supplying the muscles or to the size of their motor units was addressed next. The number of motor units in soleus muscles of adult rats that had their nerves injured at birth and were treated with either leupeptin or NaCl was assessed. Motor unit numbers were estimated by counting the increments in twitch tension in response to stimulation of the soleus nerve by gradually increasing the stimulus intensity. Figure 4 shows examples of such recordings obtained from a soleus muscle denervated at birth and subsequently treated with either NaCl (A) or leupeptin (B). The results are summarised in Table 3. In the leupeptin-treated animals the number of remaining motor units in the soleus muscle was 7.6 (± 0.7 S.E.M., n=8), whereas the NaCl treated soleus had only 3.4 (± 0.4 S.E.M., n=10) motor units. This difference is significant (Mann-Whitney U-test, P<0.002) and is illustrated in Figure 5. The value of 3.4 motor units in the NaCl treated muscles is in good agreement with that reported in a previous study after nerve crush at birth (Dick et al, 1995). Thus it appears that
Figure 3. Weights of soleus, EDL and TA muscles after sciatic nerve crush at
birth followed by NaCl or leupeptin treatment of the soleus muscle.

The soleus, EDL and TA muscles from 3-6 month old rats were removed from both
sides of the animal after completion of each tension recording experiment and
weighed. The block diagram shows the mean values for the weight of the operated
muscle expressed as a percentage of the contralateral control muscle, of each of the
muscles after sciatic nerve crush injury at birth followed by NaCl or leupeptin
treatment of the soleus muscle. The error bars indicate the standard errors of the mean.
Figure 3

![Graph showing muscle weight (% of control) for Soleus, EDL, and TA muscles treated with NaCl and Leupeptin.](Image)

- **NaCl treated**
- **Leupeptin treated**
Figure 4. The number of motor units in the soleus muscle after sciatic nerve crush injury at birth followed by treatment with either NaCl or leupeptin.

This Figure shows examples of twitch tension elicited by stimulating the motor nerve to the soleus muscle with pulses of increasing stimulus intensity. The number of increments of force gives an estimate of the number of motor units in the soleus muscle. This Figure shows recordings from a soleus muscle after a sciatic nerve crush injury at birth followed by (A) NaCl treatment, or (B) leupeptin treatment. Note the different scale bars. It can be seen that in these examples that only 2 increments of force can be elicited in the NaCl-treated muscle, and 9 in the leupeptin-treated muscle.
Figure 4

A

B

NaCl treated

Leupeptin treated

1g

5g

100ms
Table 3. Summary of motor unit numbers in soleus muscles after sciatic nerve crush at birth followed by NaCl or leupeptin treatment.

Twitch tensions were elicited by stimulating the motor nerve to the soleus muscle with pulses of increasing stimulus intensity. The number of increments of force gives an estimate of the number of motor units in the soleus muscle. The Table shows the mean number of motor units found in operated soleus muscle after sciatic nerve crush injury at birth followed by NaCl or leupeptin treatment. There were significantly more increments of force in the soleus muscles treated with leupeptin (Mann-Whitney U-test, P<0.002). The results are also expressed as %op/con. The number of motor units on the control side was estimated to be 30, and there were 30 motor units on the control side of those rats which were analysed in this experiment.
<table>
<thead>
<tr>
<th></th>
<th>Mean motor unit number</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con</td>
<td>op</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>30</td>
<td>3.4</td>
<td>11.0</td>
</tr>
<tr>
<td>(n=10)</td>
<td>(estimate)</td>
<td>± 0.4</td>
<td>± 1.3</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>30</td>
<td>7.6</td>
<td>24.8</td>
</tr>
<tr>
<td>(n=8)</td>
<td>(estimate)</td>
<td>± 0.7</td>
<td>± 2.1</td>
</tr>
</tbody>
</table>
Figure 5. Motor unit number of the soleus muscle after sciatic nerve crush at birth followed by NaCl or leupeptin treatment.

The block diagram illustrates the increase in the %op/con of motor unit numbers seen in the soleus muscle of animals that received a nerve injury at birth followed by leupeptin treatment as compared to those treated with NaCl after the same injury. This represents an increase in the survival of α-motoneurones in those animals where the soleus muscle was treated with leupeptin. The error bars indicate the standard errors of the mean.
application of leupeptin to the soleus muscle at the time of reinnervation rescues some motoneurones to soleus that would have otherwise died.

d) Motor unit force.

The mean motor unit force was calculated by dividing the maximum tetanic tension by the number of motor units. The number of motor units in the control soleus muscles was taken to be 30. This estimate is based on previous results obtained in this laboratory (Connold and Vrbová, 1991) and agrees well with values reported by others for the rat soleus (Zelená and Hník, 1963; Close, 1964; Gutmann and Hanzlíková, 1966; Brown et al, 1976). The results are summarised in Table 4. The Table shows that the mean motor unit tension in the leupeptin-treated muscles was 123.7% (± 17 S.E.M., n=4) of the contralateral control muscles, whereas the motor unit tension in those muscles treated with NaCl was 89.9% (± 16 S.E.M., n=9) of control. The difference in mean motor unit force between the leupeptin- and NaCl-treated groups, however, is not significant (Mann-Whitney U-test). Thus, the increase in force output in the leupeptin-treated group is mainly due to an increased motoneurone survival.
Table 4. Summary of mean motor unit force in soleus muscles after sciatic nerve crush at birth followed by NaCl or leupeptin treatment.

Motor unit force was calculated by dividing the maximum tetanic tension of each soleus muscle by the number of motor units supplying that muscle. In contralateral untreated soleus muscles (con) the number of motor units was estimated to be 30. Variations in body weight influence muscle weight and force, and since individual motor unit force is calculated by dividing total muscle force by the number of motor units present in the muscle, only female rats were used to assess motor unit force. The variation in body weights of female rats between the ages of 2 to 4 months is significantly less than that of males, and therefore motor unit force was assessed in this single sex group only. The difference in the mean motor unit force produced by soleus muscles after neonatal nerve injury and subsequent treatment by NaCl or leupeptin is not significant, possibly because of the small number of animals, since the motor units in the leupeptin-treated soleus muscles do seem to be considerably stronger.
Table 4

<table>
<thead>
<tr>
<th></th>
<th>Mean motor unit tension</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>4.7</td>
<td>4.1</td>
<td>89.9</td>
</tr>
<tr>
<td>(n=9)</td>
<td>± 0.37</td>
<td>± 0.7</td>
<td>± 16.0</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>4.9</td>
<td>5.8</td>
<td>123.7</td>
</tr>
<tr>
<td>(n=4)</td>
<td>± 0.8</td>
<td>± 0.8</td>
<td>± 17.0</td>
</tr>
</tbody>
</table>
2) The effect on motoneurone survival and muscle function of neonatal muscle paralysis of the soleus muscle followed by leupeptin treatment.

Nineteen Sprague Dawley rats were anaesthetised at birth and had a silicone implant containing α-BTX placed alongside the soleus muscle in the right leg. The procedure was repeated when the pups were 3 days old, at which time the first implant was removed. In this way, the soleus muscle was paralysed during the early neonatal period. When the pups were 6 days old, at the time when the soleus muscle was beginning to regain its activity, the pups were reanaesthetised and silicone implants containing either leupeptin or NaCl were placed alongside the treated soleus muscles. Previous results indicate that the majority of the loss of synaptic contacts, which is observed in neonatally paralysed soleus muscles, occurs on recovery from the paralysis. Therefore leupeptin was applied to the soleus muscles when they recovered from the effects of the α-BTX induced paralysis, i.e., when the pups were 6 days old (Greensmith and Vrbová, 1991).

Once the rats were at least 8 weeks old the number of motoneurones in the soleus pool and the tension characteristics of the soleus muscle were evaluated.

a) The effect of neonatal muscle paralysis followed by treatment with leupeptin on the long term survival of motoneurones to the soleus muscle.

Once the animals were at least 8 weeks old, HRP was injected into the soleus muscle on both sides and the number of motoneurones in the ventral horn of the spinal cord
supplying the soleus muscle was assessed. The mean number of retrogradely labelled motoneurones in the soleus pool of the untreated side was 54.4 (±3.3 S.E.M., n=11). This result is consistent with results obtained by others (Lowrie et al, 1987; Burls et al, 1991; Greensmith and Vrbová, 1992) and agrees with estimates by others using different methods (Eisen et al, 1974; Andrew et al, 1975). After treatment of the soleus muscle with α-BTX at birth there were 38.4 (±2.8 S.E.M., n=5) retrogradely labelled motoneurones, and 58.2 (±3.1 S.E.M., n=5) labelled motoneurones on the untreated control side. Thus, the number of soleus motoneurones on the α-BTX treated side decreased to 66.9% (±6.2 S.E.M., n=5) of the control side. This reduction was significant (Mann Whitney U-test, p<0.04). The results are shown in Table 5.

In those animals where α-BTX paralysis of the soleus muscle was followed by treatment with leupeptin, the number of labelled motoneurones on the treated side was 61.5 (±4.6 S.E.M., n=4), and that on the contralateral untreated control side was 59 (±3.8 S.E.M., n=4). The results are presented in Table 5 and show that no significant motoneurone death occurred in motoneurones to paralysed soleus muscles that were subsequently treated with leupeptin. The difference in the survival of soleus motoneurones after neonatal muscle paralysis and subsequent treatment with either NaCl or leupeptin is significant (Mann-Whitney U-test, P<0.033), and is illustrated in Figure 6.
Table 5. Motoneurone survival in adult rats after neonatal paralysis of the soleus muscle followed by treatment with either NaCl or leupeptin.

The number of motoneurones labelled with HRP in the soleus pool on each side of the spinal cord after neonatal paralysis of the soleus muscle by application of α-BTX to the surface of that muscle at birth and at 3 days of age, and subsequent treatment with either NaCl or leupeptin was assessed in adult rats. The number of retrogradely labelled soleus motoneurones on the treated side of the spinal cord was expressed as a percentage of the number of labelled soleus motoneurones in the untreated control side. This Table shows that there are significantly less labelled cells on the treated side of the spinal cord after prolonged neonatal paralysis and subsequent treatment with NaCl (Mann-Whitney U-test, P<0.04). However, after treatment with leupeptin there were as many labelled cells on the treated side of the spinal cord as the control side. The Table shows the means and the standard errors of the means (± S.E.M.).
Table 5

<table>
<thead>
<tr>
<th></th>
<th>Control side</th>
<th>Operated side</th>
<th>% op/con</th>
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<tr>
<td>NaCl treated</td>
<td>58.2</td>
<td>38.4</td>
<td>66.9</td>
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<tr>
<td>(n=5)</td>
<td>± 3.1</td>
<td>± 2.8</td>
<td>± 6.2</td>
</tr>
<tr>
<td>Leupeptin treated</td>
<td>59</td>
<td>61.5</td>
<td>103.7</td>
</tr>
<tr>
<td>(n=4)</td>
<td>± 3.8</td>
<td>± 4.6</td>
<td>± 2.0</td>
</tr>
</tbody>
</table>
Figure 6. Long term motoneurone survival after neonatal paralysis of the soleus muscle followed by treatment with NaCl or leupeptin.

The block diagram illustrates the number of motoneurones remaining in the soleus pool of the spinal cord of adult rats after neonatal muscle paralysis followed by treatment with NaCl or leupeptin, expressed as a percentage of the control side. There are significantly more motoneurones after treatment with leupeptin than after treatment with NaCl (Mann Whitney U-test, P<0.033).
Figure 6

![Graph showing the comparison of motor neuron survival between NaCl treated and Leupeptin treated samples. The graph indicates a significant difference in survival, with the Leupeptin treated group showing a much higher percentage.]
b) The effect of neonatal muscle paralysis followed by treatment with leupeptin on muscle function.

i) Changes in muscle tension.

After a period of α-BTX induced paralysis of the soleus muscle in neonatal rats, the soleus muscle was subsequently treated with either leupeptin or NaCl, when the paralysis was wearing off and a high rate of synapse elimination is thought to occur (Greensmith and Vrbová, 1991). When the animals were at least eight weeks old they were anaesthetised and tension recording experiments were carried out. In animals where the soleus muscle was paralysed and subsequently treated with leupeptin or NaCl, twitch and maximum tetanic tensions of the treated and contralateral untreated soleus muscles elicited by stimulation of their motor nerves were established. Figure 7 shows examples of records of twitch and tetanic contractions from the treated and contralateral untreated control soleus muscles taken from rats from the same litter. The tension records were obtained from soleus muscles paralysed at birth and subsequently treated with NaCl (A) or leupeptin (C). (B) and (D) are tension records from contralateral control soleus muscles. Figure 7 indicates that the force output of soleus muscles that had been paralysed at birth and subsequently treated with leupeptin was the same as that of the NaCl-treated muscles. The mean values for both groups are summarised in Table 6. This Table shows that following muscle paralysis at birth leupeptin-treated muscles generated forces similar to those of muscles treated with NaCl. The mean twitch tension in leupeptin-treated muscles was 69.6% (±8.6
Figure 7. Twitch and tetanic contractions from adult soleus muscles after paralysis of the neonatal soleus muscle followed by treatment with NaCl or leupeptin.

Each trace shows single twitch and tetanic contractions from the soleus muscles of two littermates elicited by stimulation of their motor nerves: (A) adult soleus muscle after neonatal paralysis followed by treatment with NaCl with (B) the contralateral unoperated control muscle, and (C) adult soleus muscle after neonatal paralysis followed by treatment with leupeptin with (D) the contralateral unoperated control muscle. The tetanic contractions were elicited at 20, 40 and 80Hz. The records shown are digitized images processed by a computer.
Figure 7

**NaCl-treated**

**Op.**

<table>
<thead>
<tr>
<th>Force (g)</th>
<th>Time (ms)</th>
</tr>
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<tbody>
<tr>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>960</td>
<td>1280</td>
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<td>1600</td>
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**Con.**

<table>
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<th>Time (ms)</th>
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<tbody>
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<td>320</td>
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<tr>
<td>960</td>
<td>1280</td>
</tr>
<tr>
<td>1600</td>
<td></td>
</tr>
</tbody>
</table>

**Leupeptin-treated**

**Op.**

<table>
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<th>Time (ms)</th>
</tr>
</thead>
<tbody>
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<td>320</td>
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<td>960</td>
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**Con.**

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<tr>
<td>960</td>
<td>1280</td>
</tr>
<tr>
<td>1600</td>
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</table>
Table 6. Summary of the changes in twitch tension and maximal tetanic tension in adult soleus muscle after neonatal muscle paralysis subsequently followed by either NaCl or leupeptin treatment.

Muscle tension was measured in vivo by attaching the distal tendon of the soleus muscle to a transducer. The nerve to soleus was stimulated and the contraction recorded on an oscilloscope. The tension transducers were calibrated with weights at the settings of sensitivity used in the experiment, and the amount of force developed was calculated by comparing the deflection recorded on the oscilloscope screen. This table summarises the mean twitch and maximal tetanic tension recorded from contralateral unoperated adult soleus muscles (con) and adult soleus muscles after neonatal paralysis and subsequent treatment with NaCl or leupeptin (op). Mean body weight is shown for each group. The results are also expressed as %op/con. The NaCl- and leupeptin-treated groups are not significantly different.
Table 6

<table>
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<tr>
<th></th>
<th>Mean body weight (g)</th>
<th>Twitch tension</th>
<th>Maximal tetanic tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>± 54.6</td>
<td>± 4.8</td>
<td>± 11.8</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>± 3.7</td>
<td>± 4.3</td>
<td>± 8.6</td>
</tr>
</tbody>
</table>

| NaCl-treated        | 419     | 47.2   | 31.2     | 69.2    | 254.6  | 178.4    | 71.1    |
| Leupeptin-treated   | 318     | 38.2   | 25.4     | 69.6    | 211.8  | 138.0    | 65.3    |
S.E.M., n=5) of the contralateral control muscles, and the mean twitch tension in those muscles treated with NaCl was 69.2% (±11.8 S.E.M., n=5) of control. The mean maximal tetanic tension produced by the leupeptin treated muscles was 65.3% (±0.9 S.E.M., n=5) of the contralateral control muscles, and the mean maximal tetanic tension in those muscle treated with NaCl was 71.1% (±6.0 S.E.M., n=5) of control. There was no significant difference in muscle force production. The block diagrams in Figure 8 summarise results obtained for (a) twitch tensions and (b) maximal tetanic tensions produced by soleus muscles after neonatal muscle paralysis followed by treatment with NaCl and leupeptin.

**ii) Changes in muscle weight.**

The soleus, EDL and TA muscles were removed from both sides of the rat and weighed. Table 7 shows that after neonatal paralysis the soleus muscles in the adult were smaller than the untreated contralateral control muscles, and this reduction in weight was similar in rats where the paralysed soleus muscle was subsequently treated with leupeptin, and those treated with NaCl. In both groups of animals the treated soleus muscles were significantly smaller than the untreated control muscles (Mann-Whitney U-test, p<0.004). Treatment with leupeptin did not have any effect on the reduction of soleus muscle weight seen after neonatal muscle paralysis. The weights of the EDL and TA muscles on the treated side of the animal were normal in both groups of rats. This was to be expected as only the soleus muscle was paralysed at birth and subsequently treated with leupeptin. The histogram in Figure 9 summarises
Figure 8. Changes in twitch tension and maximal tetanic tension in adult soleus muscles after neonatal muscle paralysis followed by either NaCl or leupeptin treatment.

The block diagrams show the average values of %op/con for (a) twitch tension and (b) maximal tetanic tension in adult soleus muscles after neonatal muscle paralysis subsequently followed by treatment with either NaCl or leupeptin. The error bars denote the standard errors of the mean (S.E.M.). Twitch tension and maximal tetanic tension is similar in muscles treated with leupeptin, as in those treated with NaCl.
Figure 8

(a) Twitch tension

(b) Maximal tetanic tension
Table 7. Summary of the changes in weight of the soleus, EDL and TA muscles of adult rats after neonatal paralysis of the soleus muscle subsequently followed by either NaCl or leupeptin treatment.

The soleus, EDL and TA muscles were removed on both sides of adult rats after the completion of each tension recording experiment and weighed. The table shows that in both groups the treated soleus muscles were significantly smaller than the untreated control muscles (Mann-Whitney U-test, p<0.004). Treatment with leupeptin did not have any effect on the reduction of soleus muscle weight seen after neonatal muscle paralysis. The weights of the EDL and TA muscles on the treated side were normal in both groups of rats. The results are also expressed as %op/con.
<table>
<thead>
<tr>
<th></th>
<th>Soleus</th>
<th></th>
<th></th>
<th>EDL</th>
<th></th>
<th></th>
<th>TA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
<td>con (g)</td>
<td>op (g)</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td>0.2165</td>
<td>0.1740</td>
<td>80.2</td>
<td>0.1988</td>
<td>0.2015</td>
<td>101.2</td>
<td>0.7706</td>
<td>0.7786</td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>± 0.022</td>
<td>± 0.023</td>
<td>± 6.2</td>
<td>± 0.025</td>
<td>± 0.026</td>
<td>± 2.1</td>
<td>± 0.076</td>
<td>± 0.074</td>
</tr>
<tr>
<td>Leupeptin treated</td>
<td>0.1672</td>
<td>0.1338</td>
<td>80.3</td>
<td>0.1465</td>
<td>0.1539</td>
<td>105.1</td>
<td>0.5684</td>
<td>0.5893</td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>± 0.005</td>
<td>± 0.008</td>
<td>± 5.1</td>
<td>± 0.004</td>
<td>± 0.005</td>
<td>± 3.2</td>
<td>± 0.009</td>
<td>± 0.011</td>
</tr>
</tbody>
</table>
Figure 9. Muscle weight of the soleus, EDL and TA muscles in adult rats after neonatal paralysis of the soleus muscle subsequently followed by NaCl or leupeptin treatment.

The soleus, EDL and TA muscles were removed on both sides of the animal after completion of each tension recording experiment and weighed. The histogram shows the average values for %op/con of each of the NaCl-treated and leupeptin-treated soleus muscles after neonatal muscle paralysis. The error bars denote the standard errors of the mean.
Figure 9

![Muscle weight (% op/con)](image)

- **Soleus**
- **EDL**
- **TA**

- **NaCl treated**
- **Leupeptin treated**
the changes in muscle weight of the soleus, EDL, and TA muscles after paralysis of the soleus muscle and subsequent treatment of that muscle with leupeptin or NaCl.

iii) Motor unit numbers.

The numbers of motor units in soleus muscles of adult rats after neonatal muscle paralysis and subsequent treatment with either leupeptin or NaCl was assessed. Figure 10 shows examples of such recordings obtained from a soleus muscle paralysed at birth and subsequently treated with either NaCl (A) or leupeptin (B). The results are summarised in Table 8. In those animals in which the paralysed soleus muscle was subsequently treated with leupeptin the number of remaining motor units in the soleus muscle was 29.8 (±1.0 S.E.M., n=5), whereas the NaCl-treated soleus had only 21 (±0.7 S.E.M., n=5) motor units. This difference is significant (Mann-Whitney U-test, P<0.028) and is in accordance with the number of retrogradely labelled motoneurones found previously in this study (See results section A.2.a). The difference is illustrated in Figure 11.

Thus it appears that application of leupeptin to the soleus muscle when it resumes activity after α-BTX induced paralysis, rescues the motoneurones to soleus, a proportion of which would otherwise die. However, the leupeptin-treated muscles were unable to recover after the neonatal period of paralysis, and they remained permanently weak though they had a full complement of α-motoneurones.
Figure 10. The number of motor units in the adult soleus muscle after neonatal paralysis and subsequent treatment with leupeptin or NaCl.

This Figure shows examples of twitch tension elicited by stimulating the motor nerve to the soleus muscle with pulses of increasing stimulus intensity. The number of increments of force give an estimate of the number of motor units in operated adult soleus muscle after neonatal paralysis and subsequent treatment with (A) NaCl and (B) leupeptin. It can be seen that in these examples there are 21 units in the NaCl-treated muscle, and 32 units in the leupeptin-treated muscle after neonatal paralysis.
Figure 10

A

10g

NaCl treated

B

10g

Leupeptin treated

100ms
Table 8. Motor unit numbers in adult soleus muscle after neonatal paralysis and subsequent treatment with NaCl or leupeptin.

Twitch tensions were elicited by stimulating the motor nerve to the soleus muscle with pulses of increasing stimulus intensity. The number of increments of force give an estimate of the number of motor units in operated soleus muscle. This table shows the average number of motor units found in operated adult soleus muscle after neonatal muscle paralysis and subsequent treatment with NaCl or leupeptin. There were significantly more motor units in those muscles treated with leupeptin (Mann-Whitney U-test, P<0.028).
Table 8

<table>
<thead>
<tr>
<th></th>
<th>Mean motor unit number</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control side</td>
<td>Operated side</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td>30</td>
<td>21</td>
<td>70.0</td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>(estimate) ±0.7</td>
<td>±2.4</td>
<td></td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>30</td>
<td>29.8</td>
<td>99.3</td>
</tr>
<tr>
<td>± SEM (n=5)</td>
<td>(estimate) ±1.0</td>
<td>±3.4</td>
<td></td>
</tr>
</tbody>
</table>

Figure 11. Mean motor unit number in adult soleus muscle after neonatal paralysis subsequently followed by treatment with NaCl or leupeptin.

This block diagram illustrates the significantly greater number of motor units seen in adult soleus muscles after neonatal paralysis and subsequent treatment with leupeptin, as compared to soleus muscles treated with NaCl after the same insult. This represents an increase in the survival of α-motoneurones in the leupeptin-treated muscles. The dotted line indicates the normal number of soleus motor units. The error bars denote the standard errors of the mean.
Figure 11

Motor unit numbers

NaCl treated  Leupeptin treated

10 20 30
iv) Motor unit force.

The motor unit force was calculated for each animal by dividing the maximum tetanic tension by the number of motor units. The number of motor units in the control soleus muscles was taken to be 30. This estimate is based on previous results obtained in this laboratory (Connold and Vrbová, 1991) and agrees well with values reported by others for the rat soleus (Zelená and Hnik, 1963; Close, 1964; Gutmann and Hanzlíková, 1966; Brown et al, 1976). The results are summarised in Table 9. The Table shows that the mean motor unit tension in the leupeptin-treated muscles was 66.0% (±3.1 S.E.M., n=5) of the contralateral control muscles, whereas the motor unit tension in those muscles treated with NaCl was 100.3% (±5.6 S.E.M., n=9) of control. The mean motor unit force of the leupeptin-treated group was significantly less than that of the NaCl-treated group (Mann-Whitney U-test, p<0.004).

v) Contractile properties.

The average time taken to reach peak tension (time-to-peak) and the average time taken to relax to half peak tension (half-relaxation) was calculated from the single twitch recordings of the paralysed soleus muscles subsequently treated with NaCl or leupeptin, and the contralateral control muscles, and the results are summarised in Table 10. There was no significant difference between the leupeptin-treated group and the NaCl-treated group.
Table 9. Mean motor unit force of adult soleus muscle after neonatal muscle paralysis and subsequent treatment with NaCl or leupeptin.

Motor unit force was calculated by dividing the maximum tetanic tension of a soleus muscle by the number of motor units supplying that muscle. In contralateral untreated soleus muscles (con) the number of motor units was estimated to be 30. The mean motor unit force was significantly less in the leupeptin-treated group than in the NaCl-treated group (Mann Whitney U-test, P<0.004).
<table>
<thead>
<tr>
<th></th>
<th>Mean motor unit tension</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (g)</td>
<td>op (g)</td>
<td>% op/con</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td>8.5</td>
<td>8.5</td>
<td>100.3</td>
</tr>
<tr>
<td>± SEM</td>
<td>± 0.7</td>
<td>± 0.6</td>
<td>± 5.6</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>7.1</td>
<td>4.7 ± 0.2</td>
<td>66.0</td>
</tr>
<tr>
<td>± SEM</td>
<td>± 0.1</td>
<td>± 3.1</td>
<td></td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Contractile properties of adult soleus muscle after neonatal paralysis and subsequent treatment with either NaCl or leupeptin.

The soleus muscle was paralysed during the neonatal period and then treated with either NaCl or leupeptin. The Table shows the average time-to-peak and half-relaxation times, calculated from traces of single twitch contractions in the adult rat. The value calculated for each operated muscle was expressed as a percentage of its contralateral control muscle.
<table>
<thead>
<tr>
<th></th>
<th>Mean time to peak</th>
<th>Mean half relaxation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con (ms)</td>
<td>op (ms)</td>
</tr>
<tr>
<td><strong>NaCl-treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>56.8</td>
<td>65.5</td>
</tr>
<tr>
<td>(n=5)</td>
<td>± 4.4</td>
<td>± 5.1</td>
</tr>
<tr>
<td><strong>Leupeptin-treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>63.9</td>
<td>74.6</td>
</tr>
<tr>
<td>(n=5)</td>
<td>± 2.8</td>
<td>± 6.2</td>
</tr>
</tbody>
</table>
B. Motoneurone survival after the prevention of neuromuscular interaction by prolonged neonatal muscle paralysis.

To compare prolonged neonatal muscle paralysis with nerve crush at birth as ways of blocking neuromuscular interaction, it is important to establish the difference in the time course of motoneurone death following these insults. The time course of motoneurone death following nerve crush has already been established (Lowrie et al, 1994).

It has previously been shown that many motoneurones to the soleus muscle die following prolonged neonatal muscle paralysis, and that this death is apparent by 8-10 weeks of age (Greensmith and Vrbová, 1992). However, the time course of motoneurone cell death following prolonged neonatal paralysis has not been previously established and is studied here. Previously, no motoneurone death was observed in 3 week old animals following a brief period of neonatal muscle paralysis, but by 10 weeks of age a significant proportion of the motoneurones had died (Burls et al, 1991; Greensmith and Vrbová, 1992). This indicates that the time course of motoneurone death following neonatal paralysis is very prolonged and the death occurs a long time after the original insult. Furthermore, more motoneurones died by 10 weeks when the period of neonatal muscle paralysis was prolonged (Greensmith and Vrbová, 1992). It was of interest whether the time-course of motoneurone death changed when the period of neonatal paralysis was prolonged, and so rats were examined at 1, 2 and 3 weeks following prolonged neonatal muscle paralysis.
A group of Sprague Dawley rats anaesthetised at birth, some of which had a silicon implant containing \( \alpha \)-BTX placed alongside the soleus muscle in the right leg and the remaining pups received implants containing only NaCl. The procedure was repeated when the pups were 3 days old, at which time the first implant was removed. In this way, the soleus muscle was paralysed during the early postnatal period.

The motoneurones supplying the soleus muscle were fluorescently retrogradely labelled with a mixture of fast blue (FB) and diamidino yellow (DiY) in six of these rats. The fluorescent dyes were injected at 5 days of age. The animals were perfused and the number of remaining soleus motoneurones counted at 3 time-points: 7 days, 14 days and 21 days of age. The results of this study are shown in Table 11. Although the numbers of motoneurones established from the 7 day old and 14 day old animal are quite high, probably due to the spread of the FB/DiY, these results indicate that the soleus motoneurones do not die until the animals are about 3 weeks old after neonatal muscle paralysis.

In order to establish more accurately the degree of motoneurone death in 3 week old animals following prolonged neonatal muscle paralysis, the motoneurones supplying the soleus muscle were retrogradely labelled with HRP. HRP was injected into the soleus muscle on both sides of 3 week old rats following prolonged neonatal muscle paralysis, and the number of motoneurones in the ventral horn of the spinal cord supplying the soleus muscle was assessed. The mean number of retrogradely labelled motoneurones in the soleus pool, calculated from the untreated sides of the rats was 55.5 (±3.1 S.E.M., \( n=10 \)).
Table 11. Motoneurone survival in rats up to 3 weeks old after paralysis of the neonatal soleus muscle by application of α-BTX to the surface of that muscle at birth and 3 days of age.

Neonatal rats had their soleus muscle paralysed by application of α-BTX containing silicone strips to the surface of that muscle at birth and 3 days of age. When the rats were 5 days old a solution containing 2% fast blue and 2% diamidino yellow was injected into the soleus muscle on both sides, and rats were transcardially perfused at 3 different time-points: 7 days, 14 days and 21 days of age. The number of fluorescently labelled motoneurones was counted, and although there was spread in the 7 day and 14 day old rats, there was no evidence of motoneurone loss following neonatal paralysis of the soleus muscle until the rats were 21 days old. The average values are shown as well as the raw data. These data illustrate the problem encountered with spread of fluorescent probes, or that HRP labelling is incomplete.
Table 11

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Operated</th>
<th>%op/con</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 day old</td>
<td>92.5</td>
<td>97</td>
<td>106.6</td>
</tr>
<tr>
<td>(Average and</td>
<td>(81, 104)</td>
<td>(98, 96)</td>
<td>(120.9, 92.3)</td>
</tr>
<tr>
<td>raw data)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 day old</td>
<td>71</td>
<td>91.5</td>
<td>127.2</td>
</tr>
<tr>
<td>(Average and</td>
<td>(60, 82)</td>
<td>(70, 113)</td>
<td>(116.6, 137.8)</td>
</tr>
<tr>
<td>raw data)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 day old</td>
<td>57</td>
<td>44.5</td>
<td>78</td>
</tr>
<tr>
<td>(Average and</td>
<td>(57, 57)</td>
<td>(35, 54)</td>
<td>(61.4, 94.7)</td>
</tr>
<tr>
<td>raw data)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
This result is consistent with results obtained by others (Lowrie et al., 1987; Burls et al., 1991; Greensmith and Vrbová, 1992) and agrees with estimates by others using different methods (Eisen et al., 1974; Andrew et al., 1975). The spread of HRP when injected into 3 week old soleus muscles is much less than the spread of FB/DiY when injected into 5 day old muscles. After treatment of the soleus muscle with α-BTX at birth there were 39.2 (±2.6 S.E.M., n=6) retrogradely labelled motoneurones remaining in the soleus pool in the ventral horn of the spinal cord, and 60.3 (±1.9 S.E.M., n=6) retrogradely labelled cells on the control side. Thus, the number of soleus motoneurones on the α-BTX treated side decreased to 64.9% (±3.8 S.E.M., n=6) of the control side. This reduction was significant (Mann Whitney U-test, p<0.02). In those animals treated with implants containing only NaCl there were 97.1% (±3.1 S.E.M., n=4) of cells on the treated side of the spinal cord compared to those on the control side. The results are shown in Table 12. Thus, the motoneurone death observed with the α-BTX containing implants was indeed due to the presence of α-BTX and not the implant itself.

In the present study, the number of motoneurones which survive following prolonged neonatal muscle paralysis is the same 3 weeks after the original insult as it is at 10 weeks. Thus, following prolonged neonatal paralysis, the time course of motoneurone cell death is faster than when the muscle is paralysed for a shorter period of time.

It has previously been observed that the motoneurones supplying soleus muscle of 10 week old rats which had been paralysed for a prolonged period neonatally, were
Table 12. Motoneurone survival in 3 week old rats after paralysis of the neonatal soleus muscle by application of α-BTX to the surface of that muscle at birth and 3 days of age.

Neonatal rats had their soleus muscle paralysed by application of α-BTX containing silicone strips to the surface of that muscle at birth and 3 days of age. Control rats had NaCl-containing silicone strips implanted at the same ages. When the rats were 3 weeks old HRP was injected into the soleus muscle on both sides, and the rats were transcardially perfused 24 hours later. The spinal cords were removed and processed for HRP histochemistry. The Table shows the number of remaining motoneurones on the α-BTX treated side of the spinal cord is significantly less than that on the control side. There was no reduction in the number of motoneurones after treatment with NaCl-containing silicone strips.
Table 12

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Operated</th>
<th>%op/con</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl treated</td>
<td>51.7</td>
<td>50.5</td>
<td>97.1</td>
</tr>
<tr>
<td>(n=4)</td>
<td>± 3.5</td>
<td>± 4.6</td>
<td>± 3.1</td>
</tr>
<tr>
<td>α-BTX treated</td>
<td>60.3</td>
<td>39.2</td>
<td>64.9</td>
</tr>
<tr>
<td>(n=6)</td>
<td>± 1.9</td>
<td>± 2.6</td>
<td>± 3.8</td>
</tr>
</tbody>
</table>
significantly smaller than the contralateral control motoneurones (Greensmith and Vrbová, 1992). It was of interest whether this difference in size of motoneurones on the treated and control sides would be apparent in 3 week old rats following prolonged paralysis.

The areas of the retrogradely labelled soleus motoneurones were calculated on the treated and control sides of the spinal cords from 3 week old rats which had undergone a prolonged neonatal period of muscle paralysis. The cells were visualised using a camera lucida and the motoneurone areas calculated by tracing around the cells with a mouse connected to a digitising tablet and computer. Figure 12 shows a frequency histogram constructed by combining the results found on the treated side, and superimposing this trace upon the results found on the control sides. The distributions of motoneurone area from both sides were not significantly different (Kolmogorov-Smirnov test), and the mean area of motoneurones from the treated side was not significantly different to that of the control. It appears that by 3 weeks following prolonged neonatal muscle paralysis the surviving motoneurones on the treated side are of a similar size to those on the control side, but as the animal grows up the motoneurones on the control untreated side grow to their adult size and those neurones on the treated side remain small.
Figure 12. Distribution of motoneurone areas in 3 week old rats after paralysis of the neonatal soleus muscle by application of α-BTX to the surface of that muscle at birth and 3 days of age.

Neonatal rats had their soleus muscle paralysed by application of α-BTX containing silicone strips to the surface of that muscle at birth and 3 days of age. When the rats were 3 weeks old HRP was injected into the soleus muscle on both sides, and the rats were transcardially perfused 24 hours later. The spinal cords were removed and processed for HRP histochemistry. The areas of the HRP labelled motoneurones were measured on the treated and control sides with a digitising tablet connected to a computer. The values obtained for individual rats were pooled together, and the distribution of the areas of labelled motoneurones on the treated and control sides was plotted as a frequency histogram. There was no significant difference between the labelled motoneurone areas on the treated and control sides.

The frequency histogram was produced by collating the motoneurone areas from 6 rats. There was a total of 362 motoneurones on the control untreated side, and 235 neurones on the α-BTX treated side.
Figure 12

Histogram comparing Bungarotoxin treated and Control samples.
C. Structural changes of the nerve to soleus induced by interference with nerve muscle interaction during early postnatal life.

Young motoneurones are critically dependent upon functional interaction with their target muscle for their normal development, and if deprived of target contact a large proportion die. After sciatic nerve crush injury at birth all the efferent and afferent fibres supplying the muscle are disrupted, and there is a loss of motoneurones and consequently a severe impairment of muscle function. Blocking the postsynaptic acetylcholine receptor with α-BTX for a few days after birth also results in motoneurone loss, but in this case the insult is primarily motor and the afferent fibres are not directly affected. Here the structure of the nerve to soleus in adult rats after neuromuscular interaction was prevented neonatally by these two methods was examined. It was interest whether or not the structure of the nerve to soleus was different after a mixed afferent and efferent fibre disruption, than to a primarily efferent insult.

In animals where neuromuscular interaction was interfered with during the neonatal period the structure of the nerve to soleus was examined in the adult rat. Neonatal Sprague Dawley rats were anaesthetised and received either a sciatic nerve crush at birth, or their soleus muscle was paralysed with 2 successive implants of α-BTX containing silicone strips. Some rats were left unoperated. When the rats were at least 10 weeks old they were terminally anaesthetised and transcardially perfused with fixative. The nerve to soleus was dissected from both sides and processed for
examination in the transmission electron microscope. Photographs were taken and a montage of the nerve to soleus was constructed. From this the number of myelinated and unmyelinated axons were counted, and the areas of the myelinated axons were calculated using a digitising tablet connected to a computer.

1) Normal adult nerve to soleus.

The normal nerve to soleus was examined from unoperated adult rats to establish the numbers of myelinated and unmyelinated axons, and to see how the myelinated axonal areas are distributed. The ultrastructural appearance of a normal soleus nerve is illustrated in Figure 13. The Figure shows a transverse section through a nerve to soleus. The number of myelinated axons in the nerve to soleus of adult rats was 158.7 (±13.1 S.E.M., n=6), and that of unmyelinated axons 231.1 (±17.1 S.E.M., n=6). This agrees with values reported by others (Zelená and Hník, 1963; Fugleholm et al, 1992). The results are shown in Table 13. The appearance and numbers of axons found in the control nerves from the contralateral unoperated sides of experimental animals were not significantly different from those in the nerve to soleus of unoperated animals.

The areas of myelinated axons from the normal nerves typically ranged from 1-30μm², and when plotted as a frequency histogram were seen to follow an approximate bimodal distribution. Figure 14 shows a frequency distribution for a normal nerve to soleus. The nerve to soleus is a mixed nerve, containing motor as well as sensory myelinated axons. Whether the myelinated axons seen in the soleus were motor or sensory was therefore studied next.
**Figure 13.** Electron micrograph of a normal adult nerve to soleus.

Electron micrograph showing a transverse section through the nerve to soleus from an unoperated adult rat. The nerve comprises of a single fascicle containing myelinated axons (eg, large arrows) and many bundles of unmyelinated axons (eg, small arrows).
Table 13. Number of myelinated and unmyelinated axons in the adult nerve to soleus after loss of neuromuscular interaction during the neonatal period.

Adult rats were transcardially perfused with fixative and the nerve to soleus was removed and processed for electron microscopy. This procedure was carried out with the following groups of rats: 1) Normal unoperated rats; 2) Rats which were anaesthetised and subject to ventral rhizotomy 1 week before fixation; 3) Rats which had received a sciatic nerve crush at birth; 4) Rats which had received a nerve crush at birth and additionally had their ventral roots cut 1 week before fixation; 5) Rats which had their soleus muscle paralysed by the application of $\alpha$-BTX containing silicone strips to the surface of the muscle at birth and at 3 days of age.

Montages of the cross sections of the nerves were prepared at a magnification of X3000, and the numbers of myelinated and unmyelinated axons were counted.
<table>
<thead>
<tr>
<th></th>
<th>Myelinated axons</th>
<th>Non-myelinated axons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal nerve to soleus</td>
<td>158.7 ± 13.1</td>
<td>231.3 ± 17.1</td>
</tr>
<tr>
<td>(Mean ± SEM, n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve to soleus 1 week</td>
<td>87 (90, 84)</td>
<td>268 (273, 263)</td>
</tr>
<tr>
<td>after ventral root (VR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>section (Average and raw data)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve to soleus after</td>
<td>55.5 ± 4.6</td>
<td>267.5 ± 33.9</td>
</tr>
<tr>
<td>nerve crush at birth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NCB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mean ± SEM, n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve to soleus after</td>
<td>17 (15, 19)</td>
<td>275 (316, 234)</td>
</tr>
<tr>
<td>NCB 1 week after VR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>section (Average and raw data)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve to soleus after</td>
<td>133 ± 5.0</td>
<td>154.5 ± 2.3</td>
</tr>
<tr>
<td>prolonged neonatal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>muscle paralysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mean ± SEM, n=4)</td>
<td></td>
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Figure 14. Frequency histogram of the areas of myelinated axons in a normal adult nerve to soleus.

Adult rats were transcardially perfused with fixative and the nerve to soleus was removed and processed for electron microscopy. A montage of the cross section of the nerve was prepared at a magnification of X3000 and the areas of myelinated axons were measured using a digitising tablet connected to a computer. The distribution of the range of myelinated axonal area was plotted as a frequency histogram.
In order to distinguish between motor and sensory axons, motor axons were selectively removed by cutting the L3, 4, 5, and 6 ventral roots of the spinal cord 1 week prior to removal of the nerve to soleus. The number of remaining sensory fibres was counted. The electron micrograph in Figure 15 shows a transverse section through an adult nerve to soleus 1 week after ventral root section. The degenerating axons were predominantly myelinated and appeared to include large and small diameter fibres. The average number of myelinated fibres remaining was 87 (n=2). This represents a loss of about 70 myelinated motor axons, and agrees quite well with the recognised number of motoneurones in the soleus pool (Zelená and Hník, 1963; Close, 1964; Gutmann and Hanzliková, 1966; Brown et al, 1976; Connold and Vrbová, 1991). The average number of unmyelinated axons remaining in the nerve to soleus after ventral root section was 268 (n=2). This number is not significantly different from the number of unmyelinated fibres found in a normal adult nerve to soleus. Table 13 shows the results.

The areas of the remaining myelinated sensory fibres were measured and were found to range between 1-30μm². In each bin width the number of sensory fibres was subtracted from the total number of myelinated fibres (in intact axons) to attain an estimate of the size distribution of the motor fibres. The distribution of the missing motor fibres was superimposed upon the distribution of fibres in the normal nerve to soleus, and this is shown in Figure 16. Figure 16 therefore approximately demonstrates the distribution of the motor component of myelinated axons in the nerve to soleus. It can be seen that there is a large range of areas of motor fibres and
Figure 15. Electron micrograph of an adult nerve to soleus 1 week after ipsilateral ventral root section.

Electron micrograph showing a transverse section through the nerve to soleus from an adult rat in which the ipsilateral L3,4,5, and 6 ventral roots had been transected 7 days prior to fixation. The degenerating axons are predominantly myelinated and appear to include large (large arrows) and small (crossed arrows) diameter fibres. Bundles of intact unmyelinated axons are also present (small arrows).
Figure 16. Frequency histogram illustrating the approximate motor component of an adult nerve to soleus.

Adult rats were anaesthetised and the ventral roots were exposed on 1 side of the spinal cord and cut. One week later the rats were transcardially perfused with fixative and the nerve to soleus was removed from the operated side and processed for electron microscopy. A montage of the cross section of the nerve was prepared at a magnification of X3000 and the areas of myelinated axons were measured using a mouse connected to a digitising tablet and a computer. The distribution of the range of axonal area of the remaining myelinated axons was subtracted from the frequency histogram created from a normal nerve to soleus. The remaining frequency histogram therefore approximately represented the range in axonal area of the motor myelinated fibres (those fibres which were missing after ventral root section), and this histogram was superimposed upon the frequency histogram of the normal nerve to soleus. The 2 rats used for this comparison were of similar sex, age and weight.
Figure 16

[Bar chart showing frequency distribution of myelinated axon area for normal soleus nerve and motor component of nerve to soleus.

Legend:
- Normal soleus nerve
- Motor component of nerve to soleus

Myelinated Axon Area

Frequency

0 5 10 15

10um² 20um² 30um²]
that motor fibres are found within both “groups” of the bimodal distribution observed in the control nerve to soleus.

2) Nerve to soleus in adult rats after nerve crush at birth.

An electron micrograph of a transverse section through a nerve to soleus of an adult animal that had an ipsilateral sciatic nerve crush injury at birth is shown in Figure 17. In adult rats which had received a sciatic nerve crush at birth there were 55.5 (±4.6 S.E.M., n=4) myelinated axons remaining in the nerve to soleus. Therefore, only 35% of myelinated axons remained after nerve crush at birth, when compared to a normal nerve to soleus, and this difference is statistically significant (Mann-Whitney U-test, p<0.005). There were 267.5 (±33.9 S.E.M., n=4) unmyelinated axons in the nerve to soleus of adult animals after nerve crush at birth, and this number is not significantly different from that in the normal nerve to soleus. The numbers of myelinated and unmyelinated fibres are shown in Table 13. There are about 60 motoneurones in the soleus pool, and so it is expected that there would be about 60 myelinated motor axons in the nerve to soleus. After nerve crush at birth the nerve to soleus loses about 100 myelinated axons, and so after this neonatal injury there is a loss of both motor and sensory myelinated fibres.

The structure of the adult nerve to soleus after nerve crush at birth is quite different to that of a normal soleus nerve from an adult rat. The nerve following neonatal injury is split up into smaller perineurial compartments, with the perineurium invaginating inwards, usually towards the vessel which runs along the nerve. The
Figure 17. Electron micrograph of an adult nerve to soleus from a rat which received an ipsilateral sciatic nerve crush at birth.

Electron micrograph showing a transverse section through the nerve to soleus from an adult animal in which the ipsilateral sciatic nerve had been crushed at birth. Perineurial cells (arrow heads) divide the nerve into several compartments, each containing a few myelinated axons (large arrows) together with bundles of unmyelinated axons (small arrows).
formation of perineurial compartments is a usual response of the injured peripheral nerve. In the example of a nerve to soleus after nerve crush at birth shown in Figure 17 there are 7 perineurial compartments.

The areas of the remaining myelinated axons in the nerve to soleus of adult rats after nerve crush at birth were much smaller than those in a normal nerve to soleus, and typically ranged from 0.5-12μm². It is quite usual for regenerated axons after peripheral nerve injury to be smaller than the original axons (Bueker and Meyers, 1951; Zelená and Hník, 1960, 1963; Jörgensen and Dyck, 1979). The size distribution of the myelinated axons which remain following nerve crush injury were compared to those in an unoperated nerve, and this is shown in Figure 18.

The motor component of the nerve to soleus after nerve crush at birth was estimated in adults. The ventral roots of adult rats which had received a sciatic nerve crush injury at birth were cut 1 week before perfusion. An example of such a nerve to soleus is shown in Figure 19. There was an average of 17 (n=2) myelinated and 275 (n=2) unmyelinated fibres left after ventral root section. Table 13 shows the results.

The areas of the remaining myelinated axons were smaller than those after nerve crush at birth without ventral root section, and typically ranged from 0.5-7μm². The areas were subtracted from the nerve crush axon area profile, and the size distribution of the motor component of the nerve to soleus was superimposed upon the histogram for the nerve to soleus after nerve crush at birth, creating a frequency histogram which is shown in Figure 20. Of those remaining axons after nerve crush at birth, it appears that the larger axons are sensory, and the motor axons which
Figure 18. Frequency histogram of the areas of myelinated axons in an adult soleus nerve from a rat which received a sciatic nerve crush at birth, and a normal adult soleus nerve.

Neonatal rats had their sciatic nerve crushed at birth, and when they were at least 8 weeks old they were transcardially perfused with fixative and the nerve to soleus was removed and processed for electron microscopy. A montage of the cross section of the nerve was prepared at a magnification of X3000 and the areas of myelinated axons were measured using a digitising tablet connected to a computer. The distribution of the range of myelinated axonal area was plotted as a frequency histogram. This image was superimposed upon the frequency histogram produced from a normal nerve to soleus, using the same magnification and bin width. Both rats were of similar sex, age and weight.
Figure 18

Control nerve to soleus

Nerve to soleus after nerve crush at birth

Frequency

Myelinated Axon Area

15

10

5

0

10 \mu m^2

20 \mu m^2

30 \mu m^2
Figure 19. Electron micrograph of an adult nerve to soleus from a rat which received an ipsilateral sciatic nerve crush at birth and additionally had its ventral roots cut 1 week before fixation.

Electron micrograph showing a transverse section through the nerve to soleus from an adult rat in which the ipsilateral sciatic nerve had been crushed at birth, and additionally in which the ipsilateral L 3, 4, 5, and 6 ventral roots had been transected 7 days prior to fixation. The degenerating axons are predominantly myelinated and appear to include large and small diameter fibres. Bundles of intact unmyelinated axons are also present (small arrows). Perineurial cells (arrow heads) divide the nerve into several compartments.
Neonatal rats had their sciatic nerve crushed at birth. Once the rats were at least 8 weeks old they were anaesthetised and the ventral roots were exposed on the operated side of the spinal cord and cut. One week later the rats were transcardially perfused with fixative and the nerve to soleus was removed and processed for electron microscopy. A montage of the cross section of the nerve was prepared at a magnification of X3000 and the areas of myelinated axons were measured using a digitising tablet connected to a computer. The distribution of the range of axonal area of the remaining myelinated axons was subtracted from the frequency histogram created from an adult nerve to soleus after nerve crush at birth. The remaining frequency histogram therefore approximately represented the range in axonal area of the motor myelinated fibres (those fibres which were missing after ventral root section), and this histogram was superimposed upon the frequency histogram of the adult nerve to soleus after nerve crush at birth. The 2 rats used for this comparison were of similar sex, age and weight.
Figure 20

Nerve to soleus after NCB
Motor component of nerve to soleus after NCB

Myelinated axon area

The adult soleus of new-born rats was paralysed during the neonatal period as described previously. When the animals were 3 weeks old they were re-examined. A cross-section from such an animal is shown in Figure 21.

The number of myelinated axons remaining in the adult nerve to soleus after neonatal paralysis was 155 ± 33 (S.E.M., n=4), and this is significantly less than the number of myelinated axons observed in normal soleus (Mann-Whitney U-test, p<0.05). This represents a 50% loss of myelinated axons, and correlates very closely with the number of motoneurones which are lost after neonatal muscle paralysis, as estimated by retrograde labelling with tracer injections (see results section A). Therefore, after neonatal paralysis there is a loss of a proportion of the small, unmyelinated sensory or sympathetic fibres in the nerve to soleus. This could be because the muscles are smaller, e.g. less blood vessels and connective tissue.
survive the injury are relatively small.

3) Nerve to soleus in adult rats after neonatal muscle paralysis.

The soleus muscle of new-born rats was paralysed during the neonatal period as described previously. When the rats were at least 10 weeks old they were transcardially perfused and the ipsilateral nerve to soleus was removed and processed for transmission electron microscopy. An electron micrograph of a transverse section through the nerve to soleus from such an animal is shown in Figure 21.

The number of myelinated axons remaining in the adult nerve to soleus after neonatal muscle paralysis was 133 (±5 S.E.M., n=4), and this is significantly less than the number of myelinated axons found in a normal nerve to soleus (Mann Whitney U-test, p<0.05). This represents a loss of about 24 myelinated axons, and correlates very closely with the number of motoneurones which are lost after neonatal muscle paralysis, as estimated by retrograde labelling with horseradish peroxidase (see results section A). It therefore appears that only myelinated motor fibres are lost after neonatal muscle paralysis. The number of unmyelinated axons remaining in the adult nerve to soleus after neonatal muscle paralysis was 154.5 (±2.3 S.E.M., n=4). This is significantly less than the number of unmyelinated fibres found in a normal nerve to soleus. The numbers of myelinated and unmyelinated axons are shown in Table 13. Therefore after neonatal paralysis there is a loss of a proportion of the small unmyelinated sensory or sympathetic fibres in the nerve to soleus. This could be because the muscles are smaller, e.g. less blood vessels and connective tissue.
Figure 21. Electron micrograph of an adult nerve to soleus after neonatal paralysis of the soleus muscle.

Electron micrograph showing a transverse section through the nerve to soleus from an adult animal in which the soleus muscle had been paralysed during the neonatal period by application of α-BTX at birth and 3 days of age. The nerve comprises a single fascicle containing myelinated axons (large arrows) and many bundles of unmyelinated axons (small arrows).
The areas of the remaining myelinated fibres after neonatal paralysis were calculated, and a frequency histogram was plotted. Figure 22 shows such a histogram superimposed upon a histogram of the size distribution of myelinated axons found in a normal nerve to soleus. The Figure shows that the areas of the remaining myelinated fibres after neonatal muscle paralysis ranged between 1-30μm², and were quite similar to the areas of the myelinated fibres in a normal nerve to soleus. The remaining myelinated fibres after neonatal paralysis followed an approximate bimodal distribution.

After sciatic nerve injury at birth the soleus nerve loses a large proportion of its motor and sensory myelinated fibres, but the number of unmyelinated fibres is not affected. However, after neonatal muscle paralysis, there is a loss of only motor myelinated fibres and the sensory myelinated fibres are unaffected, but there is a significant loss of unmyelinated fibres.

4) Neonatal peripheral nerve structure.

Neonatal rats were transcardially perfused at 4 days of age and the soleus nerve was collected and processed for electron microscopy. Another group of rats had their sciatic nerve crushed on 1 side at birth, and the crush site was marked with a fine suture. Four days later the rats were perfused and the tibial nerve was collected from both sides and processed for electron microscopy.
Figure 22. Frequency histogram showing the distribution of myelinated axon area of normal nerve to soleus, and a nerve to soleus after neonatal paralysis of the soleus muscle.

Neonatal rats had their soleus muscle paralysed by the application of α-BTX containing silicone strips to the surface of the muscle at birth and at 3 days of age. Once the rats were at least 8 weeks old they were anaesthetised and transcardially perfused with fixative, and the nerve to soleus was removed and processed for electron microscopy. A montage of the cross section of the nerve was prepared at a magnification of X3000 and the areas of myelinated axons were measured using a digitising tablet and a computer. The distribution of the range of myelinated axonal area was plotted as a frequency histogram. This image was superimposed upon the frequency histogram produced from a normal nerve to soleus, using the same magnification and bin width. Both rats were of similar sex, age and weight.
Figure 22

The electron microscopy in Figure 22 shows a transverse section through the nerve to soleus from a 3-day-old rat. The normal 70 mm² myelinated axons, and many bundles of unmyelinated nerves. The normal nerve has not yet fully myelinated, as there are many unmyelinated axons. After treatment with botulinum toxin (BTX), the myelinated axons decrease in size, and the unmyelinated axons increase in size.

The graph in Figure 22 shows the distribution of myelinated axon areas. The normal nerve shows a peak at around 15 mm², while the nerve after BTX shows a peak at around 5 mm². The results suggest that BTX treatment leads to a decrease in myelinated axon size and an increase in unmyelinated axon size.

The data in the graph are presented as histograms, with the x-axis showing the myelinated axon area in square micrometers (μm²) and the y-axis showing the frequency of occurrence.
a) Nerve to soleus from a 4 day old rat.

The electron micrograph in Figure 23 shows a transverse section through the nerve to soleus from a 4 day old rat. The nerve contains 83 myelinated axons, and many bundles of unmyelinated axons. The nerve is not yet fully myelinated, as there are many more myelinated fibres present in the adult nerve to soleus.

b) Regenerating axons 4 days after nerve crush at birth.

The electron micrographs in Figure 24 show part of a transverse section through the tibial nerve of 4 day old rats which had received a unilateral sciatic nerve crush at birth, A) from the contralateral control side, and B) from the ipsilateral side 1.5mm distal to the crush site. Many myelinated axons and bundles of unmyelinated axons are present in the tibial nerve from the contralateral control side. The tibial nerve on the injured side contains large numbers of regenerating unmyelinated axons all associated with Schwann cell cytoplasm. There were less Schwann cell nuclei present in the regenerating nerve, suggesting either death of Schwann cells following nerve injury or lack of division. Most of the Schwann cells in the unoperated nerve were associated with myelinated fibres.
Figure 23. Electron micrograph of the normal nerve to soleus from a 4 day old rat.

Electron micrograph showing a transverse section through the nerve to soleus from a 4 day old rat. The nerve comprises a single fascicle containing 83 myelinated axons (large arrows) and many bundles of unmyelinated axons (small arrows).
Figure 24. Electron micrograph of the tibial nerve 4 days after sciatic nerve crush at birth.

Electron micrographs showing part of the transverse section through the tibial nerve of a 4 day old rat. Figure 24A - Unoperated nerve contains many myelinated axons (large arrows) and bundles of unmyelinated axons (small arrows). Figure 24B - Tibial nerve 1.5mm distal to the site where the nerve was crushed at birth. Notice the nerve contains large numbers of regenerating unmyelinated axons (small arrows) all associated with Schwann cell cytoplasm. A macrophage (M) and several fibroblasts (F) are visible. The number of Schwann cell nuclei in the section is much lower than in the unoperated nerve where most Schwann cells are associated with myelinated fibres.
2.4. Discussion

The results of the first part of the present study show that after sciatic nerve injury at birth, some motoneurones to the soleus muscle are rescued by inhibiting the activity of the calcium activated neutral protease (CANP) with leupeptin at the time of reinnervation, and thereby stabilising the formation of new neuromuscular contacts. In addition to, and possibly as a consequence of, this increase in motoneurone survival, the leupeptin-treated soleus muscles lose less weight and suffer a smaller reduction of force output after neonatal injury than untreated muscles or muscles treated with NaCl. In view of previous findings on the ability of leupeptin to maintain neuromuscular contacts destined to be lost (Connold et al, 1986; Vrbová and Fisher, 1989; Connold and Vrbová, 1994), it is likely that treatment with leupeptin during the period of reinnervation results in preservation of a larger than usual number of neuromuscular contacts. It thus appears that the maintenance of neuromuscular contacts achieved by leupeptin treatment rescues some motoneurones destined to die after nerve injury and improves recovery of muscle function.

The next part of this study shows that motoneurones that would otherwise die after paralysis of the soleus muscle by α-BTX at birth, are rescued by subsequent application of leupeptin to the muscle. In this case the leupeptin was applied to the muscle at a time when muscle activity was returning, and when neuromuscular contacts are most likely to be lost (Connold and Vrbová, 1991). Despite the rescue of motoneurones, muscle function after the period of paralysis is not improved by
leupeptin treatment. Consequently, the average size of motor unit territory is less in leupeptin-treated muscles with an apparently full complement of motoneurones, than in those NaCl-treated muscles where the reduction of muscle function after paralysis is reflected by a reduction in motoneurone number.

After nerve crush at birth there is a proportion of motoneurones which it appears cannot be rescued and die very soon after the injury (Lowrie et al, 1994). Perhaps the same applies to the immature denervated muscle fibres after nerve crush. Indeed, many apoptotic muscle fibres have been observed after neonatal nerve injury (Kamińska, 1996). During a period of neonatal muscle paralysis the muscle is inactive, as it is after nerve crush, but in this case all the motoneurones can be rescued. However, it is possible that a proportion of muscle fibres have died as a result of the inactivity induced by α-BTX. Recent work by Greensmith et al has indeed shown that 20% of muscle fibres are lost following a transient period of paralysis, and this loss is increased to 45% when the period of paralysis is prolonged (Greensmith et al, 1996b). This loss of muscle fibres, and the ineffectiveness of leupeptin to increase the function of the soleus muscle after neonatal paralysis even though all the motoneurones to soleus are rescued, indicates that a proportion of muscle fibres have died.

Previous results show that during both normal development and regeneration, newly formed neuromuscular contacts can be rescued by treatment with leupeptin, an inhibitor of CANP and serine proteases (Connold et al, 1986; Connold and Vrbová, 1994; Liu et al, 1994). Leupeptin locally applied to soleus muscles of rats that had
been partially denervated at 4 to 6 days of age induced an expansion of motor unit territory which was over and above the increase in size that would be due to partial denervation alone (Vrbová and Fisher, 1989). At 4 to 6 days of age individual motor units are expanded and achieve their smaller adult size by 18 days. It was therefore suggested that the increase in motor unit size observed after partial denervation and treatment with leupeptin was due to leupeptin inhibiting CANP and thus preventing the normally occurring elimination of nerve terminals and consequent reduction of motor unit size (Vrbová and Fisher, 1989). Thus, by protecting these immature nerve terminals that would have otherwise been withdrawn, leupeptin enabled them to maintain contact with the muscle fibres and consequently complete their development to maturity (Vrbová and Fisher, 1989). This possibility is consistent with earlier findings in which leupeptin retarded the elimination of polyneuronal innervation both in vitro (O'Brien et al, 1984) and in vivo (Connold et al, 1986).

CANP is an enzyme found in the peripheral (Schlaepfer, 1979; Kamakura et al, 1983) and central nervous system (Schlaepfer and Freeman, 1980). Leupeptin is known to be a potent inhibitor of both CANP and serine proteases and can enter nerve terminals (Roots, 1983; Connold et al, 1986; Liu et al, 1994). The activity of CANP is Ca^{2+} dependent and it is known that the maintenance of neuromuscular contacts can be influenced by changes in Ca^{2+} concentration both outside and inside the nerve terminal (O'Brien et al, 1980; Connold et al, 1986; Zhu and Vrbová, 1992). Lowering the Ca^{2+} concentration has the same effect as inhibiting CANP with leupeptin, in that more neuromuscular contacts are preserved (O'Brien et al, 1980; Connold et al, 1986).
Conversely, increasing Ca^{2+} concentration increases the rate of elimination of synaptic contacts (O'Brien et al, 1984). The effects of Ca^{2+} on neuromuscular contacts were therefore thought to be caused by regulating the activity of CANP (Vrbová et al, 1988; Vrbová and Lowrie, 1989).

Leupeptin can cross muscle fibre membranes and enter the cells (Libby and Goldberg, 1978), and it could be argued that it may act on CANP within the muscle fibre. However, CANP is highly regulated in muscle by its specific endogenous inhibitor, calpastatin, levels of which are much higher in muscle than in nervous tissue (Murachi, 1983). It is therefore unlikely that leupeptin is acting on the muscle.

Leupeptin is also an inhibitor of serine proteases, and recent results show that both leupeptin and the endogenous serine protease inhibitor Protease Nexin I can prevent synapse elimination \textit{in vitro} (Liu et al, 1994; Houenou et al, 1995). Indeed, these authors argue that it is the action of leupeptin on serine proteases, not CANP, that underlies its ability to maintain neuromuscular contacts. However, in these experiments an \textit{in vitro} preparation was used where synapses were formed between nerve endings of sympathetic neurones and skeletal muscle fibres. Formation of synaptic connections between sympathetic ganglionic neurones and muscle fibres may be regulated by different mechanisms than that between motoneurones and muscle fibres. In view of the Ca^{2+} dependence of synapse formation in the neuromuscular system, the hypothesis that in the present experiments the main action of leupeptin is via the inhibition of CANP is more favourable. However, it would be interesting to examine the contribution that serine proteases may play in the regulation of synaptic
The present results show that leupeptin is able to decrease the loss of motoneurones after neonatal nerve injury or paralysis. The possible mechanism of this rescue is likely to involve its ability to maintain synaptic contacts that would otherwise be lost. After sciatic nerve crush at birth there is an extensive loss of motoneurones (Romanes, 1946; Zelená and Hník, 1963; Schmalbruch, 1984; Lowrie et al, 1987). When a peripheral nerve is crushed, the distal segment degenerates and the axons in the proximal segment begin to grow towards the denervated peripheral target. Many of the injured motoneurones die as a result of loss of interaction with the target muscle early during this critical stage of development. However, not all the motoneurones die immediately, and some survive until their axons reach the target muscle six to seven days later (Lowrie et al, 1994; Naidu et al, 1996). At this time they attempt to establish new synaptic contacts with the denervated muscle fibres. Nevertheless, a number of these motoneurones whose axons reach the muscle subsequently die (Kashihara et al, 1987; Lowrie et al, 1994). It is possible that some of these motoneurones are unable to maintain their new neuromuscular contacts and thus are once more rapidly deprived of target interaction. Therefore, by helping to maintain these new neuromuscular contacts by decreasing the activity of CANP with leupeptin, motoneurone death may be prevented.

Paralysis of the soleus muscle in newborn rats with α-BTX results in the loss of neuromuscular contacts when activity resumes a few days later (Greensmith and Vrbová, 1991) and the long term loss of soleus motoneurones in the adult (Greensmith...
and Vrbová, 1992). This loss of motoneurones is reflected by a decrease in force output of these muscles and a reduction of motor unit numbers in the adult (Greensmith et al, 1996b). In addition, those motoneurones which survive are unable to expand their peripheral field to occupy the many denervated fibres which are presumably available to them (Greensmith et al, 1996b), although as discussed previously many of these muscle fibres may die. Stabilising the neuromuscular contacts that are lost shortly after neonatal paralysis with leupeptin results in the survival of all the motoneurones in the soleus pool. After neonatal paralysis there is an initial decline in the percentage of polyneuronal innervation, after which the period for which polyneuronal innervation persists is much longer than that observed in normal muscle (Greensmith and Vrbová, 1991). A greater number of endplates are polyneuronally innervated than normal when neuromuscular interaction is blocked with α-BTX in 9 day old rats (Duxson, 1982). Thus, following muscle paralysis the innervation pattern of these muscles remains immature. Further evidence that paralysis delays the maturation of the neuromuscular system is illustrated by results which show that after nerve crush at 5 days no loss of motoneurones occurs (Lowrie et al, 1982), but when the 5 day nerve crush is preceded by paralysis a significant degree of motoneurone death is observed (Burls et al, 1991). It is possible that neonatal paralysis delays the maturation of the motoneurone, so that it remains in a state of “growth”, in which it is susceptible to nerve crush, and its development into a fully transmitting cell which is target independent is retarded.

Motoneurone death after neonatal paralysis does not occur immediately and
continues for several weeks. Those motoneurones that do survive are unable to expand their peripheral field to occupy the many denervated muscle fibres available to them (Greensmith et al, 1996b). In this study treatment with leupeptin did not facilitate this expansion. In fact, it seems that although after treatment with leupeptin more motoneurones survive, they have a smaller peripheral field than after paralysis alone. This is probably because leupeptin does not alter the survival of muscle fibres following α-BTX treatment, but it does rescue all the motoneurones.

The finding that only a few motoneurones are rescued by leupeptin after nerve crush at birth, whereas all of the motoneurones are rescued by leupeptin after neonatal muscle paralysis is very interesting. As previously stated, after nerve crush in neonatal rats it was found that most of the cell death occurred within 6 days after the initial injury, and that these cells were mainly from the lower, presumably less mature part of the motor column (Lowrie et al, 1994). Motoneurones from the upper, more mature part of the spinal cord, which represent around 20% of the motoneurone pool, died later. It is these cells which are likely to reinnervate the muscle and die thereafter, that could be rescued by the stabilisation of their newly formed neuromuscular contacts with leupeptin. In this study, the number of motoneurones which were rescued by leupeptin constituted about 15% of the soleus motoneurone pool, and this indicates that the motoneurones previously shown to die after reinnervation were rescued by leupeptin. This also indicates that the majority of those motoneurones which die between 6-12 days after nerve crush injury do reinnervate the target muscle, but are unable to maintain contact and cannot functionally reinnervate the muscle.
Thus it appears that following neonatal nerve injury there is a proportion of motoneurones which are unable to reinnervate the target, although others are able to do so. This may be due to the differing degrees of maturity of the motoneurones at the time of injury. Some motoneurones are sufficiently mature to sustain themselves long enough to successfully reinnervate the target, and these cells survive nerve crush injury. Others are less mature, but can sustain themselves sufficiently to reinnervate the target, although their newly formed contacts are weak and are easily broken down, following which they die. It is these cells which may be rescued by inhibiting CANP with leupeptin. The remaining motoneurones represent the least mature group which are unable to extend regenerating axons as far as the target muscle, and these cells die in the first few days after neonatal nerve injury.

Motoneurone death following neonatal nerve injury has recently been examined, and it was shown that those motoneurones which die rapidly within the first 6 days of injury do so by apoptosis, and those cells which died later did not appear to die by apoptosis (Lawson and Lowrie, 1997). This indicates that the mechanism of cell death is different in motoneurones which die rapidly after nerve injury, and those which survive long enough to reinnervate the target muscle and then subsequently die. The results presented in this Chapter indicate that only those motoneurones which do not appear to die by apoptosis can be rescued by the application of leupeptin. In addition it has also been shown that there is no evidence of apoptotic motoneurone death after neonatal muscle paralysis (Lawson and Lowrie, 1997). After neonatal muscle paralysis, all of the motoneurones which would otherwise die can be rescued.
by the application of leupeptin. In this case the motoneurones are already connected
to their target muscle, and the paralysis causes a loss of functional neuromuscular
interaction which results in long term motoneurone death. Those motoneurones which
reinnervate the target or are already connected do not appear to die by apoptosis, and
can potentially be rescued. Thus, there seems to be a similarity between those
motoneurones which die upon reinnervation of the target after neonatal nerve injury,
and those motoneurones which die after neonatal muscle paralysis.

However, the time-course of motoneurone death following nerve injury and
muscle paralysis is different. Whereas motoneurone death after neonatal nerve
injury is complete by 12 days (Lowrie et al, 1994), it has previously been reported that
motoneurone death following a transient period of muscle paralysis was not observed
until about 8-10 weeks after the original insult (Greensmith and Vrbová, 1992). In the
present study, after a prolonged period of muscle paralysis motoneurone death was
observed within 3 weeks, and no more motoneurones appeared to die after this time.
The application of α-BTX at birth and 3 days of age renders the muscle inactive for
a similar period of time to that seen after nerve crush at birth, and so it is with
repeated application of α-BTX that a valid comparison can be drawn between the two
methods of inhibiting neuromuscular interaction during the neonatal period. It could
be that those motoneurones which die after prolonged neonatal muscle paralysis are
similar to those motoneurones which die 6-12 days after nerve injury, ie, they can be
rescued by leupeptin and do not appear to die by apoptosis (Lawson and Lowrie,
1997). However, the process of motoneurone death after muscle paralysis is much

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slower than after nerve injury.

This Chapter also examined the effects of disrupting target interaction by either nerve injury or muscle paralysis on the structure of the nerve to soleus. These two insults had a different effect on the soleus nerve. After sciatic nerve crush at birth the nerve to soleus in the adult animal was very small and a loss of both myelinated motor and myelinated sensory axons was apparent. The nerve trunk was arranged into perineurial compartments. Following neonatal muscle paralysis only myelinated motor axons were lost, and the myelinated sensory fibres were unaffected. This is understandable since muscle paralysis by α-BTX is unlikely to affect sensory fibres, so only the motor fibres are affected, while nerve injury affects both the afferent and efferent fibres. However, there was a significant loss of unmyelinated sensory and/or sympathetic fibres after neonatal muscle paralysis. There was no reduction in the number of unmyelinated fibres after nerve crush at birth.

The nerve to soleus is not fully myelinated during the neonatal period. This indicates that the axons of motoneurones to the soleus muscle are at different stages of maturity at this time. Perhaps it is those motoneurones with axons that are already myelinated that can survive target deprivation during the neonatal period.

Perhaps the most significant finding of this Chapter is that a number of motoneurones that would otherwise die following loss of neuromuscular interaction can be rescued by stabilising neuromuscular contacts with leupeptin. These are motoneurones which reinnervate the muscle following nerve injury, or are already in contact with the muscle after paralysis, but for some reason are unable to maintain
contact and subsequently die. By stabilising these neuromuscular contacts for a brief period with leupeptin, long-term improvement in muscle function and motoneurone survival can be achieved.
Chapter 3. Uninjured motoneurones supplying partially denervated rat hindlimb muscles are susceptible to nerve injury during later postnatal development.

3.1. Introduction

Motoneurones of newborn rats die following injury to their axons. Sciatic nerve injury at birth leads to the death of 80-90% of motoneurones (Romanes, 1946; Zelená and Hník, 1963; Lowrie et al, 1982; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987; Crews and Wigston, 1990; Greensmith et al, 1994b), while most sciatic motoneurones survive the same insult when the rats are 5 days old (Lowrie et al, 1982; Greensmith et al, 1994a). Thus, during the first 5 days of life the motoneurone when in contact with the muscle undergoes some fundamental changes that enable it to withstand nerve injury. There is some evidence to suggest that this transition from a motoneurone that is unable to withstand separation from its target to one that can survive such a separation, is induced by functional interaction with the muscle during this critical developmental period. For example, motoneurone death in adult animals was observed in experiments where active interaction of the nerve terminal with the muscle fibre was prevented neonatally by paralysing the muscle with α-BTX (Greensmith and Vrbová, 1992). This toxin produces muscle paralysis by binding irreversibly to the AChR of the postsynaptic membrane and in this way preventing its response. In this case no nerve injury was inflicted, but nevertheless a substantial loss
of motoneurones occurred by 10 weeks (Greensmith et al, 1992). If the soleus muscle is paralysed at birth with α-BTX, then subsequent sciatic nerve crush at 5 days of age does in this case lead to considerable motoneurone death, as though the paralysis had delayed the maturation of the motoneurone (Burles et al, 1991). These results indicate that some event that is related to the interaction between the nerve terminals and their muscle fibres is involved in the developmental changes of the motoneurone that enable it to survive target deprivation.

Several proposals have been made as to the role of the target in the survival of motoneurones, the most popular being the possibility that the target provides trophic factors that are essential for motoneurone survival (Oppenheim, 1991; Korsching, 1993; Theonen et al, 1993). However, this idea has recently been challenged, mainly by results obtained on animals that had various trophic factors or their receptors removed by genetic manipulation, with little effect on motoneurone survival (Klein et al, 1993; Ernfors et al, 1994; Farinas et al, 1994; Klein et al, 1994; see also Greensmith and Vrbová, 1996). Thus, a clear understanding of the nature of the target influence on motoneurone survival has yet to be established.

The early postnatal period marks a time when motoneurones are not only dependent upon their target for survival, but also a time when both the developing nerve terminals and the postsynaptic membrane are undergoing radical changes as they mature. One of these developmental changes is the dramatic increase of transmitter release from nerve terminals which is triggered by its encounter with muscle fibres (Frank and Fishbach, 1979; Xie and Poo, 1986). This change in the
nerve terminal from a growing into a transmitting structure is reflected within the cell body. It has been proposed that the motoneurone must undergo this transition within a specific period of development if it is to mature appropriately and become competent to withstand target separation (Lowrie and Vrbová, 1992; Greensmith et al, 1996a; Greensmith and Vrbová, 1996).

According to this hypothesis, motoneurones that are maintained in a growing mode for an inappropriate time during their development should fail to mature and will remain dependent upon contact with their target for survival. Several observations indicate that this is indeed the case, since maintaining the motoneurone in a growing rather than transmitting state, for example by nerve injury, when the axons attempt to grow to replace their lost peripheral stump, results in motoneurone death (Romanes, 1946; Lowrie et al, 1987). When neuromuscular transmission is blocked by α-BTX sprouting of axons is induced (Holland and Brown, 1980), and this also results in motoneurone death (Greensmith and Vrbová, 1992). The possibility that a growing motoneurone is more vulnerable to the ever increasing excitatory synaptic inputs than a transmitting cell was supported by recent results, where nerve injury was preceded by premature induction of transmitter release in neonatal rats (Greensmith et al, 1996a). This precocious induction of transmitter release allowed a much greater proportion of motoneurones to survive nerve injury, indicating that the transition from the growing into the transmitting mode is an important step in the maturation of the motoneurone (Greensmith et al, 1996a).
The muscle fibres of young neonates are polyneuronally innervated (Redfem, 1970; Brown et al, 1976; O'Brien et al, 1978), so individual motoneurones supply more muscle fibres in new-born animals than in adults, and each neonatal muscle fibre contributes to more than one motor unit. Consequently, the motor unit size of neonatal rats is relatively large. During the second and third weeks after birth the superfluous neuromuscular contacts are eliminated, and the size of the individual motor unit is reduced to that seen in the adult, where each muscle fibre receives innervation from only one axon. The final size of the adult motor unit is dependant upon the number of contacts that the motoneurone maintains with individual muscle fibres.

When the soleus muscle in neonatal rats is partially denervated, the surviving motoneurones retain their relatively large peripheral field, so that the remaining motor units are larger than those of normal soleus muscle (Brown et al, 1976; Thompson and Jansen, 1977; Lowrie et al, 1985; Fisher et al, 1989; Jansen and Fladby, 1990). Following partial denervation of the soleus muscle in adult rats, the remaining motor units increase their peripheral field by sprouting (Thompson and Jansen, 1977; Brown and Ironton, 1978; Lowrie et al, 1985; Fisher et al, 1989; for review see Brown et al, 1981a). Thus, remaining innervation of the partially denervated soleus muscle can partly redress the loss of a proportion of its innervation, and bring about some recovery of muscle function.

However, in previous experiments it has been shown that unlike neonatal soleus muscle, when EDL muscle was partially denervated in 5 day old rats, 2-6 months later the remaining EDL motor units were no bigger than those in the control
Moreover, when the EDL muscle was partially denervated in 3 day old rats the remaining EDL motor units were significantly smaller 2-3 months later than those in the contralateral control muscle, illustrating the lack of capacity of the EDL muscle to compensate for a partial loss of its innervation at this young age (Tyč and Vrbová, 1995a). It is not clear why the surviving motor units in neonatal partially denervated EDL muscle are unable to maintain their expanded peripheral field. One possibility is that the increased activity of the remaining EDL motor units causes them to reduce the size of their peripheral field (Tyč and Vrbová, 1995a). When the EDL muscle was treated with leupeptin two days after partial denervation at 3 days of age, the EDL motor units were expanded and the force production of the muscle was significantly better than after partial denervation alone (Tyč and Vrbová, 1995b). Stabilising the expanded peripheral field of the partially denervated EDL motor units for a short time after the insult results in a permanent increase in motor unit size. The undamaged axons to EDL muscles of 18-20 day old rats were able to increase their territory, since after partial denervation at this time the remaining motor units were almost doubled in size (Connold et al, 1992), and the effect of leupeptin to the size of motor units in 18 day old rats was minimal (Tyč and Vrbová, 1995b).

Following partial denervation of neonatal muscles, the retraction of the expanded neonatal peripheral field proceeds more slowly (Thompson and Jansen, 1977; Brown et al, 1981a; Fladby and Jansen, 1987; Fisher et al, 1989), and possibly some axon terminals are maintained in a “growing” rather than “transmitting” state.
In this case some motoneurones that were uninjured, but their axons remained in the partially denervated muscle, also died (Tyč and Vrbová, 1995a; White and Vrbová, 1996, unpublished observation).

It is possible that when axons in a muscle are maintained in a “growing state”, such as after partial denervation, their cell bodies may be more likely to die.

The present study is designed to examine this possibility.

EDL and TA muscles were partially denervated in 3 day old rats. This procedure would have reduced the rate of nerve terminal withdrawal of the remaining axon terminals from the neuromuscular junction, and therefore probably the maturation of the remaining uninjured motoneurones. Such neurones are likely to mature more slowly, since they are forced to maintain a larger expansion of their axonal branches for a prolonged period. In this study, these motoneurones were subsequently further challenged by sciatic nerve crush injury in 9 day old rats, a time when normally motoneurones no longer die after injury to their axon (Lowrie et al, 1982). Thus, in this study the possibility that when axons are kept in a growing mode their cell bodies will die after nerve injury was examined.
3.2. Methods

Sprague Dawley rats were used in these experiments. The rats were divided into 4 groups. In Group 1 the EDL and TA muscles were partially denervated in 3 day old rats, and the L5 spinal nerve (SN) was removed and examined under an electron microscope when the rats were 9 days old. In Groups 2 and 3 the rats were partially denervated at 3 days of age, and the motoneurones to the EDL and TA muscles were retrogradely labelled 3 days later when the rats were 6 days old. In Group 2 these animals were examined when they were 9 days old, in order to ensure that the retrograde label had reached the cell body within 3 days. In Group 3 the animals were allowed to survive until they were 21 days old. In Group 4 in addition to partial denervation and retrograde labelling of the motoneurones, these animals received a sciatic nerve injury on the same side as the partial denervation at 9 days of age, and were examined at 21 days.

1) Surgery

a) Partial denervation

The EDL and TA muscles receive their innervation from motor axons that exit the spinal cord in 2 ventral roots, the L4 and L5. The major neural input to these muscle comes from the L4 which supplies 70-80% of the innervation. Under halothane anaesthesia (2% in oxygen) and using sterile precautions, the L4 Sp.N. was exposed on one side of 3 day old rat pups at its exit from the vertebral column and cut.
prevent reinnervation, 2-4mm of the L4 Sp.N. was excised. The L5 Sp.N., which provides the rest of the innervation to these muscles, was left intact. The skin was closed by sutures using fine silk thread (Ethicon, 0.7 gauge), and the animal was allowed to recover from the anaesthetic before it was returned to its mother. In all experiments the contralateral unoperated side was used as a control. In one group of rats (Group 1) the early effects of this partial denervation on the remaining uninjured L5 spinal nerve was examined under the electron microscope when the rats were 9 days old.

b) Sciatic nerve crush

In one group of rats (Group 4), when the rats were 9 days old, they were reanaesthetised and the sciatic nerve was exposed in one hindlimb on the same side as the partial denervation and crushed proximal to the division of the tibial and common peroneal nerves, with a pair of fine watchmaker's forceps. The nerve was then examined to ensure that the epineurial sheath was intact and the nerve translucent. The incision was then closed by sutures, and after recovery from the anaesthetic the animals were returned to their mothers. These animals were examined when they were 21 days old.

2) Electron microscopy

Those animals in which the L5 spinal nerve (Sp.N.) was to be examined under the electron microscope when the rats were 9 days old (Group 1) were perfused
transcardially under terminal anaesthesia (4.5% chloral hydrate, 1ml/100g body weight, i.p.) with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.3). The L5 Sp.N. was dissected from both the operated and contralateral control sides of the rat and post-fixed overnight at 4°C. The nerves were washed with 0.1M phosphate buffer, and fixed with 1% OsO₄ in 0.1M phosphate buffer. They were stained with uranyl acetate (2%), dehydrated and embedded in Araldite. Semi-thin sections (1μm) were cut with a glass knife on a microtome (Reichert Ultracut) and stained with toluidine blue for examination under a light microscope (Zeiss), and adjacent ultra-thin sections (70-90nm) were then cut with a diamond knife and collected on mesh grids and single slots coated with formvar plastic film. The sections were counterstained with lead citrate and viewed in a transmission electron microscope (Joel EM1010).

3) Fluorescent labelling

Motoneurones supplying the EDL and TA muscles were retrogradely labelled with the fluorescent dyes diamidino yellow (DiY) and fast blue (FB) (Illing, Germany), made by sonication of the solids in distilled water. The fluorescent dyes were injected into the muscle to be retrogradely transported to the motoneurone cell body in the spinal cord. DiY labels the nucleus of the cell and FB is a cytoplasmic label.

Following partial denervation at 3 days of age, the rats were reanaesthetised 3 days later and under halothane anaesthesia the EDL and TA muscles were injected with DiY (2% in sterile distilled water) in both the operated and control hindlimbs.
using a fine 5μl Hamilton syringe. In this way, those motoneurones that remained and innervated the EDL and TA muscles after transection of the L4 Sp.N. were labelled with DiY.

To ensure that the label reached the motoneurone cell body within 3 days the animals in one group (Group 2) were assessed when they were 9 days old, when the number of labelled motoneurones on the operated and control sides of the spinal cord was counted.

The remaining rats (Groups 3 and 4) were reanaesthetised when they were 18 days old, and FB (2% in sterile distilled water) was injected into the EDL and TA muscles of both legs. FB was used to double label the EDL/TA motoneurones. FB is known to spread to adjacent structures more readily than DiY (Conde, 1987), and to minimise the spread only a small amount of FB was injected. Thus, only a proportion of the motoneurones were double labelled. The size of the EDL/TA motoneurone pool was then estimated when the rats were 21 days old by counting DiY labelled nuclei.

When the rats were either 9 days (Group 2) or 21 days old (Groups 3 and 4) they were perfused transcardially with 4% paraformaldehyde in phosphate buffered saline (PBS) and the spinal cords removed. The spinal cords were post-fixed for 2-4 hours at 4°C and then transferred into a 30% sucrose solution in PBS. The lumbar region of the spinal cord was dissected, and serial sections (30μm) were cut on a freezing microtome and mounted onto gelatinised slides. These sections were then examined under a fluorescent microscope.

The number of retrogradely DiY labelled fluorescent motoneurones within the
EDL/TA motor pool was counted, and an unbiased correction factor was used to allow for sectioned particles being counted more than once (Clarke, 1993).

A summary of the methods used in this study is shown in Table 1.
Table 1. Summary of the surgical and retrograde tracing methods.

This Table shows the times at which surgical and retrograde tracing procedures were carried out. It shows 4 groups of experimental animals: Group 1 - Partially denervated (PD) at 3 days (examined at 9 days), animals in which the L5 Sp.N. was examined under the electron microscope; Group 2 - PD at 3 days (examined at 9 days), the group of animals used to test the uptake of the diamidino yellow (DiY); Group 3 - PD at 3 days (examined at 21 days); and Group 4 - PD at 3 days followed by sciatic nerve crush at 9 days (examined at 21 days).
<table>
<thead>
<tr>
<th>Age of animal</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>L4 Sp.N. section</td>
<td>L4 Sp.N. section</td>
<td>L4 Sp.N. section</td>
<td>L4 Sp.N. section</td>
</tr>
<tr>
<td>6 days</td>
<td>-</td>
<td>Injection of DiY into TA/EDL</td>
<td>Injection of DiY into TA/EDL</td>
<td>Injection of DiY into TA/EDL</td>
</tr>
<tr>
<td>9 days</td>
<td>Perfusion</td>
<td>Perfusion</td>
<td>-</td>
<td>Sciatic nerve crush</td>
</tr>
<tr>
<td>18 days</td>
<td>-</td>
<td>-</td>
<td>Injection of FB into TA/EDL</td>
<td>Injection of FB into TA/EDL</td>
</tr>
<tr>
<td>21 days</td>
<td>-</td>
<td>-</td>
<td>Perfusion</td>
<td>Perfusion</td>
</tr>
</tbody>
</table>
3.3. Results

1) Structural changes of axons in the L5 spinal nerve (Sp.N.) after removal of the L4 Sp.N. at 3 days of age.

In a group of 3 day old rat pups (Group 1) the L4 Sp.N. was sectioned on one side. Six days later the rats were terminally anaesthetised and the undamaged L5 Sp.N., that contained the axons to the partially denervated muscles, and the contralateral L5 Sp.N. were removed and processed for light microscopical and ultrastructural analysis.

The ultrastructural analysis of the control L5 Sp.N. revealed the presence of a large number of myelinated and unmyelinated axons. These were intermingled and distributed throughout the cross-section of the nerve. Figure 1 shows a typical example taken from such a 9 day old animal. In contrast, the undamaged L5 Sp.N. from the operated side where the L4 Sp.N. was sectioned 3 days earlier had some areas of unmyelinated axons, with a conspicuous absence of myelinated fibres. Figure 2 shows an example of part of a transverse section through such an uninjured L5 Sp.N. on the partially denervated side. The Figure shows an area of the nerve with some normal looking myelinated and unmyelinated fibres, together with an adjacent area that contains only small unmyelinated fibres with no myelinated fibres. These "unmyelinated areas" were present in about 10% of the total cross sectional area of the nerve. In the control nerves such very small unmyelinated fibres with no accompanying myelinated fibres were never seen. Some areas of the L5 Sp.N. on the partially denervated side were similar to the control, with myelinated fibres
Figure 1. Electron micrograph of the L5 spinal nerve on the contralateral control side after section of the L4 spinal nerve.

Electron micrograph showing part of a transverse section through the L5 spinal nerve from a 9 day old rat. The L4 spinal nerve on the contralateral side was sectioned at 3 days of age. Notice large numbers of myelinated axons (large arrows) intermingled with bundles of unmyelinated axons (small arrows).
Figure 2. Electron micrograph of the L5 spinal nerve on the operated side after section of the L4 spinal nerve.

Electron micrograph showing part of a transverse section of the L5 spinal nerve taken from the same 9 day old animal illustrated in Figure 2. The ipsilateral L4 spinal nerve was sectioned at 3 days of age. This micrograph shows an area with myelinated axons (large arrows) intermingling with unmyelinated axons (small arrows) as in the contralateral nerve, and a large area that contains only unmyelinated axons.
punctuated by bundles of unmyelinated fibres. Furthermore, in these normal looking areas of the nerve trunk the myelinated axons were of a similar diameter, and the myelin was of a similar thickness to those in the control L5 Sp.N. The high power (X15K) electron micrograph in Figure 3 shows an example of axons in the L5 Sp.N. on the partially denervated side taken from an area of the section which contained unmyelinated fibres with no accompanying myelinated fibres. The presence of many small diameter morphologically immature axons can be detected, many of which are not separated by intervening Schwann cell cytoplasm.

Thus the general appearance of the L5 Sp.N., part of which supplies the partially denervated EDL and TA muscles, indicated that it contained a proportion of axons which were morphologically immature.

These results indicate that removal of a proportion of innervation to the EDL and TA muscles delayed the maturation of the remaining undamaged axons in the L5 Sp.N. Therefore, in the next experiments the ability of the motoneurones that had their axons in the L5 Sp.N. to withstand nerve injury was tested at a time when normal motoneurones no longer die after such an insult. If the large areas of unmyelinated fibres in the undamaged L5 Sp.N. on the partially denervated side seen at 9 days of age are developmentally immature, then it is likely that the motoneurones of these axons are also immature. If this was the case these motoneurones should be more susceptible to death after sciatic nerve injury.
Figure 3. Electron micrograph showing an unmyelinated region of the L5 spinal nerve on the operated side after section of the L4 spinal nerve.

Higher power electron micrograph from an L5 spinal nerve of a 9 day old rat 6 days after the ipsilateral L4 spinal nerve was severed. In the areas containing unmyelinated axons many small diameter immature looking fibres are present (small arrows). Many of these axons are not separated by intervening Schwann cell cytoplasm (Sc).
2) Motoneurone survival after partial denervation and subsequent nerve injury.

The number of motoneurones that contributed to the innervation of the TA and EDL muscles after partial denervation was established first. Rat pups were partially denervated at 3 days of age. Three days later DiY was injected into TA and EDL muscles on both sides.

In order to ensure that the removal of the L4 Sp.N. had no effect on the rate of transport of DiY by the axons in the L5 Sp.N., the rats in Group 2 were partially denervated at 3 days, had the dye injected into their TA and EDL muscles at 6 days and were examined at 9 days of age, i.e. the time when the other group received a sciatic nerve injury. Table 2 shows that in these 9 day old animals the number of L5 motoneurones retrogradely labelled was similar to that seen in the partially denervated rats examined at 21 days. Thus, the axons of the motoneurones to partially denervated muscles were able to retrogradely transport the dye within the 3 day period between the injection of the dye and the nerve injury.

In the next group of rats (Group 3), the number of motoneurones that survive following removal of the L4 Sp.N. was assessed in older animals of 21 days of age. Examples of the spinal cord with labelled motoneurones on the operated and control sides following partial denervation are shown in Figure 4, and the data is summarised in Table 2. The number of labelled motoneurones on the operated side of the spinal cord was 34 (±4.3 S.E.M., n=4), and that on the control side was 166.7 (±11.4 S.E.M., n=4). When expressed as a percentage of the control side, 20.7% (±3.0 S.E.M., n=4) of motoneurones remained on the operated side of the spinal cord. This difference is
Table 2. Number of retrogradely labelled cells after section of the L4 spinal nerve alone and followed by subsequent sciatic nerve injury.

The L4 Sp.N. was cut in 3 day old rats, and 3 days later when the rats were 6 days old DiY was injected into the EDL and TA muscles. One group of rats was examined 3 days later, when the rats were 9 days old (Group 2), and another group of rats was examined at 21 days of age (Group 3). In the final group of rats the sciatic nerve was crushed at 9 days of age on the partially denervated side, and they were examined at 21 days of age (Group 4).

The Table shows that in Group 4 there are very few retrogradely labelled cells on the operated side. In Group 2, labelled at 6 days of age and examined at 9 days of age, the number of labelled cells is similar to that in Group 3, examined at 21 days of age. The Table shows the mean ± S.E.M.
Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Control side</th>
<th>Operated side</th>
<th>%op/con</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 2</strong> (PD only)</td>
<td>131.6 ± 14.8</td>
<td>33.1 ± 4.0</td>
<td>26.1 ± 5.5</td>
</tr>
<tr>
<td>9 day old</td>
<td>(n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 3</strong> (PD only)</td>
<td>166.7 ± 11.4</td>
<td>34 ± 4.3</td>
<td>20.7 ± 3.0</td>
</tr>
<tr>
<td>21 day old</td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 4</strong> (PD + nerve crush)</td>
<td>170 ± 11.8</td>
<td>8.2 ± 3.4</td>
<td>4.9 ± 2.0</td>
</tr>
<tr>
<td>21 day old</td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Examples of retrogradely labelled motoneurones on the control and operated sides of the spinal cord after section of the L4 spinal nerve.

The L4 Sp.N. was cut in 3 day old rats, and DiY was injected into the EDL and TA muscles 3 days later when the rats were 6 days old. FB was injected into the EDL and TA muscles when the rats were 18 days old, and the animals were transcardially perfused at 21 days of age. The Figure shows fluorescent retrogradely labelled motoneurones on a) the control side and b) the partially denervated side of the spinal cord.
Figure 4

Control side

Partially denervated side

50μm
statistically significant (Mann Whitney U-test, p<0.02), and agrees well with other studies using physiological methods to establish the number of motor units in the adult EDL muscle following partial denervation at 3 days of age (Týč and Vrbová, 1995a).

Whether those motoneurones innervating the EDL and TA muscles after removal of the L4 Sp.N. remained vulnerable to axon injury was tested next. Some animals were partially denervated at 3 days of age and like the previous group had the retrograde label injected at 6 days of age. Three days later, when the animals were 9 days old, they had their sciatic nerve crushed on the partially denervated side (See Group 4 in Table 1). Previous results show that nerve crush injury to the sciatic nerve in animals older than 5 days of age no longer leads to motoneurone death (Lowrie et al, 1982; Greensmith et al, 1994a). However, in the present experiments where nerve crush was preceded by partial denervation, the number of remaining motoneurones on the operated side of the spinal cord was only 8.2 (±3.4 S.E.M., n=4) and that on the control side was 170 (±11.8 S.E.M., n=4). When expressed as a percentage of the control side only 4.9% (±2.0 S.E.M., n=4) of motoneurones survived on the operated side of the spinal cord. This decrease is statistically significant (Mann Whitney U-test, p<0.02). Furthermore, the loss of motoneurones after partial denervation and subsequent nerve injury is significantly greater than the loss of motoneurones after partial denervation alone (Mann Whitney U-test, p<0.02). The results are shown in Table 2. Examples of the retrogradely labelled cells on the control and operated side of the spinal cords from such experiments are shown in Figure 5, and the summary of this experiment is illustrated in Figure 6.
Figure 5. Retrogradely labelled motoneurones on the control and operated sides of the spinal cord after section of the L4 spinal nerve followed by a subsequent sciatic nerve crush.

The L4 Sp.N. was cut in 3 day old rats, and DiY was injected into the EDL and TA muscles 3 days later when the rats were 6 days old. Three days later, when the rats were 9 days old, the sciatic nerve was crushed on the partially denervated side. FB was injected into the EDL and TA muscles when the rats were 18 days old, and the animals were transcardially perfused at 21 days of age. The Figure shows fluorescent retrogradely labelled motoneurones on a) the control side and b) the partially denervated side of the spinal cord. Most of the motoneurones that supplied the EDL and TA muscles after partial denervation were absent after the sciatic nerve was crushed.
Figure 5

Control side

Partially denervated side after nerve crush
Figure 6. Numbers of retrogradely labelled motoneurones after section of the L4 spinal nerve followed by subsequent sciatic nerve injury.

The L4 Sp.N. was cut in 3 day old rats, and 3 days later when the rats were 6 days old diamidino yellow was injected into the EDL and TA muscles. One group of rats were examined 3 days later, when the rats were 9 days old (Group 2), and another group of rats were examined at 21 days of age (Group 3). In the final group of rats the sciatic nerve was crushed at 9 days of age on the partially denervated side, and they were examined at 21 days of age (Group 4).

The block diagram in this Figure shows that a large proportion of the motoneurones supplying partially denervated EDL and TA muscles die after the sciatic nerve crush. The Figure also shows that the DiY is indeed transported back to the spinal cord at the time of sciatic nerve crush at 9 days of age. The error bars show the S.E.M.
Taken together these results indicate that developing uninjured motoneurones that have their axons in a partially denervated muscle remain vulnerable to nerve injury for longer than normal and die as a result of such an insult.
3.4. Discussion

The present study examined the response of intact motor axons and motoneurones that had their terminals in a muscle that has lost a proportion of its innervation as a result of partial denervation. Partial denervation of EDL and TA muscles was carried out at 3 days of age, a time when in the EDL muscle many terminals are beginning to retract and polyneuronal innervation is gradually disappearing (Balice-Gordon and Thompson, 1988). Removal of the main source of innervation to these muscles renders the remaining uninjured axons susceptible to nerve injury. Following section of the L4 axons, a large proportion of motoneurones in the L5 motor pool died following sciatic nerve injury at a time when they are normally resistant to such an insult.

At the same time that the normal elimination of nerve terminals is occurring from the neuromuscular junction, the formation of new branches of axons in the muscle is arrested and the synthesis of tubulin alpha 1 (tubulin α-1), which is associated with longitudinal growth of axons, is suppressed (see Hoffman, 1988). Coincident with this down-regulation of tubulin α-1, the synthesis of neurofilaments, which are associated with radial growth of axons, is enhanced (see Hoffman, 1988). Removing a large part of the muscle's innervation has been shown to arrest, at least temporarily, the retraction of the axon terminals of the remaining innervation (Thompson and Jansen, 1977; Brown et al, 1981a; Fladby and Jansen, 1987; Fisher et al, 1989), and possibly stimulates the growth of new branches. Therefore, it can be
expected that under these conditions the motoneurone will not down-regulate the synthesis of tubulin α-1 nor will it up-regulate the synthesis of neurofilaments. In other situations when axonal growth is observed, such as after nerve injury or during axonal outgrowth from neurones in culture, tubulin levels have been shown to increase (Neumann et al, 1983; Hoffman et al, 1987; Fine and Bray, 1971). In addition, it has been found that if axons are encouraged to sprout during early development by treating hindlimb muscles of immature mice with botulinum toxin, the down-regulation of growth associated proteins such as GAP-43 and tubulin α-1 fails to occur in motoneurones to these muscles, and these cells appear to maintain a growing phenotype (Caroni and Becker, 1992). It is possible that partial denervation may affect motoneurones in a similar manner. For example, an up-regulation of GAP-43 has been reported following partial denervation even in adult animals (Bisby et al, 1996). If nerve terminals fail to retract, the induction of an increased synthesis of neurofilaments may be prevented. The up-regulation of neurofilaments is necessary for the normal radial growth of the axons (see Hoffman, 1988). Indeed, our present results on the changes of the ultrastructure of the undamaged axons in the L5 Sp.N. that supply the partially denervated muscle, indicate that these axons fail to undergo the radial growth which is typical of axons to normal muscles. It is clear that the diameters of many of the axons in this uninjured Sp.N. remain remarkably small. It therefore appears that the retraction of nerve terminals and cessation of their longitudinal growth may be an essential stimulus for the maturation of the axon and motoneurone. It is interesting in this context that following nerve crush in young
animals where many of the motoneurones that had their axons injured die (Romanes, 1946; Schmalbruch, 1984; Lowrie et, 1987), the remaining axons of the surviving motoneurones are extremely small and are unable to achieve their normal diameter even after long periods of recovery (Zelená and Hník, 1963; see results in Chapter 2). However, the axons examined in the present study have not been injured, and have been influenced only by events associated with the different conditions of their nerve terminals in the muscle that exist after partial denervation. The results of this study indicate that the changes that normally occur in the motoneurone as a consequence of target interaction have failed to take place in motoneurones to partially denervated muscles, so that there is a reduction or a delay in the degree of radial growth of their axons.

It is well known that immature motoneurones die when their axons are injured (Romanes, 1946; Schmalbruch, 1984; Lowrie et al, 1987; Greensmith et al, 1994b). However, the response of motoneurones to axotomy changes dramatically within the first postnatal week of life. Injury to the sciatic nerve in 5 day old rats no longer causes motoneurones to die, indicating that the maturation of these cells is well under way by this time (Lowrie et al, 1982; Greensmith et al, 1994a). This period of development also marks the time when nerve terminals are no longer elongating, superfluous terminals are retracting and those which remain are increasing in volume (O'Brien et al, 1978; Duxson, 1982). In addition, the synthesis and release of transmitter from these remaining terminals rapidly increases (O'Brien and Vrbová, 1978) and levels of choline acetyltransferase, the enzyme involved in ACh synthesis,
increase in the cell body (Burt, 1975; Phelps et al, 1984). In the partially denervated muscle, these changes may be delayed and the immature morphological appearance of the axons illustrated in this study indicate a delayed maturation of the intact motoneurones left to innervate the muscles after removal of part of their innervation.

The likelihood that these intact motoneurones to the partially denervated muscles are indeed immature is also indicated by the finding that these motoneurones remain vulnerable to nerve injury. Previous results have shown that the target dependence of motoneurones decreases rapidly within the first week of life, so that by 5 days of age nerve injury no longer results in motoneurone death (Romanes, 1946; Lowrie et al, 1982; Greensmith et al, 1994a). However, in the present study the majority of motoneurones in the L5 Sp.N. that had their axons in muscles partially denervated at 3 days of age, died after nerve injury at 9 days. Thus, the removal of part of the innervation from the muscle prevented, or slowed the maturation of the remaining intact motoneurones so that they responded to axon injury in a similar way as motoneurones of neonatal rats.

What is the explanation of this reduced rate of maturation of axons and motoneurones that have their terminals in a muscle that has been deprived of part of its innervation? It is likely that interaction with the muscle fibre is necessary for the transition from a growing to a transmitting terminal. It is known that as a result of contact with a muscle cell a growth cone stops growing and transmitter release is enhanced (Frank and Fischbach, 1979; Xie and Poo, 1986). After partial denervation
of EDL and TA muscles at 3 days the terminals of the remaining axons will be at endplates that have lost most of their inputs, leaving many denervated muscle fibres. This is likely to lead to a reduction in muscle activity, and it is known that inactive muscles provide an ideal environment for the growth of axons (Duchen and Strich, 1968; Brown and Ironton, 1977; Brown et al, 1980). Thus, it is possible that many of the remaining uninjured axon terminals in these partially denervated muscles will either revert to or remain as growing structures. This delay in transforming into fully transmitting cells may retard their overall development and may account for the prolonged period for which they are dependent on target contact for their survival. Whether these motoneurones that have their axon terminals in partially denervated muscles will remain permanently more susceptible to nerve injury is not clear from the present study. It would be interesting to test this possibility and to relate more precisely the changes of the motoneurone maturation to the fate of its branches and terminals in the muscle.
Chapter 4. General Discussion

This thesis investigated the changes which are induced by loss of neuromuscular interaction during early postnatal life. The results suggest that motoneurones which are deprived of interaction with the muscle during this critical period fail to mature and are more likely to die.

1) Factors that influence motoneurone survival

During early postnatal development motoneurones remain dependent upon contact with their target for their survival. If neuromuscular interactions are prevented at this time, for example by nerve injury or muscle paralysis, many motoneurones die (Romanes, 1946; Zelená and Hník, 1963; Lowrie et al, 1982; Schmalbruch, 1984; Kashihara et al, 1987; Lowrie et al, 1987; Crews and Wigston, 1990; Greensmith and Vrbová, 1992) and the recovery of muscle function is severely impaired (Bueker and Meyers, 1951; Zelená and Hník, 1963; McArdle and Sansone, 1977; Greensmith et al, 1996b). The degree of motoneurone death induced by the loss of neuromuscular interactions during this critical period of development depends on several factors, including the age of the animal and the duration for which neuromuscular interaction is prevented.

Sciatic nerve crush injury at birth results in the loss of about 90% of sciatic motoneurones (Lowrie et al, 1987; Greensmith et al, 1994b) and injury at 3 days of age still results in the death of the majority of motoneurones (Greensmith et al,
1996a). This is not due to injury of the axon \textit{per se}, for if functional neuromuscular interactions are prevented during early postnatal development by muscle paralysis, then many motoneurones also die (Greensmith and Vrbová, 1992). The target dependence of motoneurones for their survival declines with development, and if target contact is maintained for at least 5 days after birth then motoneurones are able to survive nerve injury (Lowrie et al, 1982; Greensmith et al, 1994a).

\textit{a) The importance of motoneurone-target contact}

In the first part of this thesis, neuromuscular interaction was prevented by either sciatic nerve crush at birth, or a prolonged period of neonatal muscle paralysis, and both these insults caused motoneurone loss. When neuromuscular contacts were stabilised by inhibiting CANP with leupeptin in either situation, there was a significant increase in the number of motoneurones that survived these insults, and in the case of nerve injury this was reflected in a dramatic improvement of muscle recovery. Muscle recovery was not improved by treatment with leupeptin following neonatal muscle paralysis, even though all the motoneurones were rescued in this case. This could be due to the death of a proportion of muscle fibres following paralysis. Indeed, there are significantly fewer muscle fibres in adult soleus muscle following a period of prolonged neonatal muscle paralysis (Greensmith et al, 1996b).

After neonatal nerve injury not all of the motoneurones die immediately. Some of them reinnervate the target and then subsequently die (Lowrie et al, 1994). The results of this study show that these motoneurones may survive when their neuromuscular
contacts are stabilised for a brief period with leupeptin. After prolonged neonatal muscle paralysis, all the motoneurones which would be otherwise destined to die are rescued by leupeptin. In this case, the nerve terminals are in close proximity to the muscle fibres. Upon recovery from the paralysis some contacts between these terminals and muscle fibres are lost, and it is likely that this loss of contacts causes motoneurones to die. Perhaps the loss of contacts induces the remaining motoneurones to maintain their expanded peripheral field for longer than normal, thus slowing the rate of maturation of these motoneurones. The loss of polyneuronal innervation is much slower, after this initial loss of contacts, following neonatal muscle paralysis (Greensmith and Vrbová, 1991). It is possible that if they were immature these motoneurones would not be able to cope with the increase in afferent input, and may die by excitotoxic cell death. Indeed, it has been shown that if the NMDA receptor is blocked during the neonatal period, some motoneurones which would otherwise die after neonatal muscle paralysis are rescued (Greensmith et al, 1995).

Thus, the motoneurones which following nerve crush die later, after reinnervating the target, and those which die following muscle paralysis can be rescued with leupeptin. Additionally, motoneurones which are lost both after reinnervation following nerve injury, and also after muscle paralysis do not appear to die by apoptosis, whereas the motoneurones that die soon after neonatal nerve injury, and never get the opportunity to re-establish contact with the target muscle die by apoptosis (Lawson and Lowrie, 1997). However, it should be considered that motoneurones that die following reinnervation after nerve crush or following paralysis
may do so by apoptosis, but this is not detected because the level of cell death is not sufficiently high.

The nerve to soleus was examined in adult rats after neuromuscular interaction was blocked during the early neonatal period by either nerve crush injury or muscle paralysis. Both of these insults result in the significant loss of motoneurones, but the appearance of the nerve to soleus was quite different in these two cases. After nerve injury there was a loss of motor and sensory myelinated axons, and the remaining axons were much smaller than those in the normal nerve. However, following neonatal muscle paralysis the nerve to soleus looked remarkably unscathed. There was a loss of myelinated motor axons, but many myelinated, probably sensory axons still remained. This is not surprising since paralysis is a purely efferent insult, whereas nerve injury physically disrupts afferent and efferent fibres. Perhaps the differences of appearance of the nerve to soleus after neonatal nerve injury or muscle paralysis is due to the nature of the insult itself.

The results of this study show that there is a potential for the rescue of motoneurones following loss of neuromuscular interactions if they can reinnervate or are already in contact with the target, by stabilising their neuromuscular contacts. This could be due to maintenance of nerve-muscle interactions for a sufficient period of time to allow the motoneurone to mature into a target independent cell.

It is clear from these results that the loss of neuromuscular contacts, on reinnervation after nerve injury and following muscle paralysis, is an important factor in determining motoneurone death. However, the present study does not provide
adequate information about the specific events that occur at the nerve endings or in the motoneurone itself which lead to the rescue of motoneurones following leupeptin-treatment. From these results it seems quite clear that the motor nerve terminal plays an important role in determining the fate of the cell.

b) The effect of the duration of loss of target contact on motoneurone survival

The duration for which motoneurones are deprived of target contact is a crucial factor in determining the extent of motoneurone survival. There is a much greater chance for motoneurone survival following a brief period of target deprivation, than after a prolonged period of target separation. After section rather than crush of the nerve at birth, when reinnervation of the target muscle is impossible, almost no motoneurones survive (Schmalbruch, 1984). After nerve crush at birth when reinnervation of the target muscle is permitted, the chances of motoneurone survival are slightly improved (Lowrie et al, 1987; Greensmith et al, 1994a). If a nerve is injured further away from the muscle, more motoneurones die than when the nerve is injured close to the muscle (Lieberman, 1974; Greensmith et al, 1994b). When the site of injury is further from the muscle the period before target contact is re-established is longer than if the injury is closer to the muscle. Further evidence for the importance of the duration of target separation on motoneurone survival is provided from experiments where innervation was delayed by inflicting a second injury at the same site (Lowrie, 1990). In this case, motoneurone death was greater than when the nerve was injured only once. The distance of the injury site from the cell body did not change in this experiment, but the
period of separation of the motoneurone from the muscle was prolonged. After a transient period of neonatal muscle paralysis motoneurones die, and many more motoneurones die if the period of paralysis, and thus that of functional target deprivation, is prolonged (Greensmith and Vrbová, 1992). Therefore, it seems that the extent of motoneurone death after nerve injury is indeed influenced by the duration for which the interaction between the motoneurone and the target is prevented.

The present study compared the time-course of motoneurone death after nerve injury at birth and prolonged neonatal muscle paralysis, and found that motoneurones took longer to die after muscle paralysis than after nerve injury. Moreover, the motoneurone loss after muscle paralysis was not as severe as that after nerve crush. The time during which target interaction is lost is critical in determining the survival of motoneurones, and this period is shorter with muscle paralysis when the terminals are already in place, than with nerve crush. Therefore, the less severe loss of motoneurones after muscle paralysis, as compared to that after nerve injury, could be due to the shorter period of loss of neuromuscular interaction.

The present results show that interaction between the motoneurone and muscle is crucial for the normal development of motoneurones into target independent cells. Disruption of this interaction renders the motoneurones more susceptible to death. This susceptibility is in part determined by changes at the nerve terminal, as stabilisation of neuromuscular contacts following loss of neuromuscular interactions results in increased motoneurone survival. The nature of the “signal” that the nerve
terminal receives from the muscle, that is essential for the normal maturation of the
motoneurone into a target independent cell, is discussed next.

2) Why are motoneurones dependent upon target contact?

There are several possibilities as to the way that the target might induce the
motoneurone to mature and become fully functional. One such possibility is that the
target supplies trophic factors essential for motoneurone survival, and this theory is
greatly favoured by many investigators.

a) Neurotrophic factors

During early development and following neonatal axotomy, application of BDNF,
NT-3 and the cytokine CNTF can rescue motoneurones for a short time (Sendtner et
al, 1990, 1992; Yan et al, 1992; Henderson et al, 1993; Koliatsos et al, 1993; Li et al,
1994), but by 3 weeks following axotomy motoneurones die in spite of previous
treatment (Eriksson et al, 1994; Vejsada et al, 1995). As the above mentioned factors,
glial-derived neurotrophic factor (GDNF) prevents the naturally occurring death of
motoneurones (Oppenheim et al, 1995) and delays death induced by axotomy
(Oppenheim et al, 1995; Zurn et al, 1994; Yan et al, 1995; Henderson, 1995; Vejsada
et al, 1995). If neurotrophic factors were essential for the survival of motoneurones,
then removal of the genes coding for these factors should result in motoneurone death.
Transgenic mice have been used in experiments where the genes coding for either the
potential trophic factor or its Trk receptor were missing, and in such studies

Thus, although the action of neurotrophic factors in transiently promoting motoneurone survival is interesting, their role as a retrograde signal essential for motoneurone survival is unclear, and further long term studies are needed.

The results of this thesis could be considered in terms of the theory that the target supplies trophic factors essential for motoneurone survival. Following nerve crush at birth or prolonged neonatal muscle paralysis, treatment with leupeptin results in the stabilisation of neuromuscular contacts and consequently a reduction in the loss of motoneurones. It is possible that the increase in the number of synaptic contacts following leupeptin treatment results in an increase in the uptake of a target supplied trophic factor. However, subsequent experiments in this thesis argue against the possibility that simply increasing the number of contacts is sufficient for the survival of motoneurones. Following partial denervation the remaining motoneurones maintain more synapses and yet many of them die. Furthermore, it has been shown that some motoneurones that are uninjured, but their axons remain in a partially denervated muscle, die (Tyč and Vrbová, 1995a; White and Vrbová, 1996, unpublished observation). These experiments argue against the idea of a target supplied trophic factor, for after partial denervation the remaining terminals are exposed to many muscle fibres and had there been a competition for a trophic factor this is no longer present. Consequently, all the remaining motoneurones should have an abundant
supply of trophic factors and should not be susceptible to death, but our results show that this is not the case.

b) The role of transmitter release in motoneurone development

Therefore, other possibilities by which the target might induce the motoneurone to mature and become fully functional need to be considered. It has been proposed that as a result of continued interaction with the target the phenotype of the developing motoneurone changes, rendering it less target dependent. This change may be a consequence of alterations of the early motor nerve terminals. It has been shown for a long time that during postnatal development the amount of transmitter released at the neuromuscular junction increases. More recently, it was shown that when a growth cone contacts a target muscle fibre it is changed from a growing into a secreting structure, neurite elongation stops, and transmitter release from the growth cone is rapidly increased (Frank and Fischbach, 1979; Xie and Poo, 1986). This is shown by a dramatic increase in the frequency of miniature endplate potentials, as well as the appearance of quantal release of ACh. With continued contact with the muscle the quantal content of the endplate potential, and the activity of ChAT in the motor nerve endings rapidly increases (Diamond and Miledi, 1962; Kelly, 1978; O’Brien and Vrbová, 1978; Lowrie et al, 1985). At the same time, many nerve branches and synaptic contacts are eliminated, thus reducing the size of the peripheral field of the motoneurone. These changes in the motoneurone’s terminal are reflected in the motoneurone cell body by an increase in the proteins associated with the secretion of
ACh, and down-regulation of growth-associated proteins such as GAP-43 and tubulin. These changes in the cell body depend on the state of the terminal, for if neuromuscular transmission is blocked then the growth-associated proteins are not down-regulated (Caroni and Becker, 1992). Therefore, the motoneurone cell body is greatly influenced by changes at its terminals, and as the terminals change from growing to transmitting structures the cell body responds appropriately. This transition of the motoneurone from a growing cell to a transmitting one may affect some features that are important for its survival, and these associated changes are likely to be essential for the motoneurone’s development into a target-independent structure able to function in the mature CNS. For example, it could be necessary for the motoneurone to redistribute or down regulate its glutamate receptors, in order to withstand the increased afferent excitatory input which occurs in the developing spinal cord (Lowrie and Vrbová, 1992). The expression of one of the glutamate receptors, the NMDA receptor, has been shown to change during development (Kalb et al, 1992; Piehl et al, 1995), as do the types of NMDA-receptor subunits which are expressed, so that immature channels are replaced by their more mature counterparts (Monyer et al, 1994). The target’s role in this developmental change in the motoneurone, is suggested by results that show that if neuromuscular contact is denied during the first week of life motoneurones are susceptible to the excitotoxic effects of exogenously applied NMDA (Greensmith et al, 1994a). Furthermore, motoneurones that are destined to die can be rescued if their NMDA receptors are blocked (Mentis et al., 1993; Greensmith et al, 1994b). Target deprivation may also affect other membrane
characteristics of the motoneurone, especially those associated with its excitability. Motoneurones destined to die following axotomy are also rescued if their Na^+ channels are blocked (Casanovas et al, 1996), indicating a complex change in the motoneurone phenotype when deprived of target contact.

Results in support of the hypothesis that the transition of the motoneurone from a growing to transmitting phase is important for survival are supported by a recent study of Greensmith et al, 1996. This study shows that premature induction of transmitter release from developing nerve terminals rendered many motoneurones resistant to death following nerve injury (Greensmith et al, 1996a). It was suggested that enhancing transmitter release from nerve endings in neonatal animals increased the rate of maturation of motoneurones, and thus induced the motoneurones to become more target independent. Conversely, results from this laboratory have recently shown that preventing transmitter release during the neonatal period renders motoneurones more susceptible to nerve injury at a time when they are usually resistant to it (Greensmith and Vrbova, 1996, unpublished observation).

All the neurotrophins and cytokines that have some effect on motoneurone survival enhance expression of the cholinergic phenotype of motoneurones, and it is perhaps this ability of these compounds which is responsible for their ability to transiently rescue motoneurones. Neurotrophins have been shown to increase the activity of choline acetyltransferase (ChAT) in the motoneurone cell body both in vitro and in vivo (Martinou et al, 1992; Wong et al, 1993; Zurn et al, 1994; Wong et al, 1995), and also prevent the injury-induced decrease in ChAT in motoneurones after
axotomy (Yan et al, 1995). Furthermore, transmitter release at immature neuromuscular junctions is enhanced by BDNF or NT-3 in culture (Lohof et al, 1993) and from synaptosomes isolated from the hippocampus (Knipper et al, 1994). It is thus possible that the site of the action of neurotrophic factors and cytokines is at the nerve terminal or growth cone where they enhance transmitter release, and this could lead to the increase of ChAT and may underlie their ability to promote motoneurone survival. Therefore, trophic factors may affect nerve endings or growth cones of motoneurones in a similar way that muscle does, resulting in the enhanced expression of the cholinergic phenotype of motoneurones. Whether these survival factors have a physiological role in the induction of transmitter release, or just do so pharmacologically needs to be established. However, muscles do not necessarily release trophic factors, and could induce the cholinergic phenotype of motoneurones and thus render it target independent, in other ways. For example, K^+-induced depolarisation of neurones is likely to lead to increased transmitter release, and in culture this has been shown to increase neuronal survival (Franklin and Johnson, 1992).

Therefore we propose that the target influences the nerve-terminal to change from a growing to a transmitting structure, and this represents a crucial step in the development of the motoneurone. Without this target induced change the motoneurone would remain immature and would not transform from a “growing” cell to a “transmitting” cell within a critical period of development. This possibility is based on the idea that the physiological function of a cell has an important epigenetic
The final result of this thesis is consistent with this hypothesis. Here it was shown that undamaged motoneurones to partially denervated muscles remain susceptible to nerve injury at a time when they would normally be resistant to such an insult. The study shows that the uninjured motoneurones to partially denervated muscles are altered in some way, rendering them susceptible to nerve injury. The appearance of some of the axons in the remaining spinal nerve which provides the innervation of the partially denervated muscles, indicates that they are immature, i.e. the axons are very small and are not separated by Schwann cell processes. A possible explanation of these results is that these motoneurones are in a prolonged state of “growth”, as they are encouraged to maintain their expanded peripheral field due to the partial denervation. Indeed, following partial denervation of neonatal muscles the retraction of the expanded peripheral field proceeds more slowly (Thompson and Jansen, 1977; Brown et al, 1981a; Fladby and Jansen, 1987; Fisher et al, 1989; Gates and Ridge, 1992), indicating that perhaps some axon terminals are maintained in a “growing” rather than “transmitting” state. Whether or not motoneurones to partially denervated muscle are maintained in “growing mode” remains to be further studied, but if so, then this delay in the transformation into fully transmitting cells may retard their overall development, and could account for the prolonged period for which the motoneurones are dependent on target contact for their survival.
3) Conclusion

The results in this thesis illustrate the importance of the time at which motoneurones achieve maturity following an early post-natal period of target deprivation. If contact between nerve terminals and muscle fibres can be maintained during a critical period of development, by stabilising the terminals with leupeptin, then a significant number of remaining motoneurones destined to die can be rescued. This is true for those motoneurones which die after reinnervating the target following nerve crush, and those that die after muscle paralysis. This indicates that the nerve terminal is an important factor involved in the fate of the motoneurone.

This thesis then shows that motoneurones to partially denervated muscle remain susceptible to nerve injury in later development. It suggests that this could be due to the maintenance of the remaining motoneurones to these muscles, and their axons, in a developmentally immature “growing” state. Whether indeed these motoneurones do remain in a “growing mode” needs to be established.

The results presented in this thesis indicate that immature “growing” motoneurones are susceptible to death, and if neuromuscular contacts are stabilised so that the maturation of the motoneurone is allowed, many motoneurones can be rescued.
Appendix I

**Modified Hanker-Yates method for the demonstration of HRP.**

*A. Method*

1. Cut the spinal cord just below the L2 and L6 roots, and penetrate the control dorsal horn along the length of the block of spinal cord with a fine micropin for identification. Mount the block of spinal cord onto a freezing microtome (Pelcool) and pack with dry ice to ensure it is fully frozen.

2. Cut frozen transverse sections, 50μm thick, and collect in Millonig’s phosphate buffer in washing trays containing individual wells.

3. Wash the sections briefly (60 seconds) in water.

4. Incubate the sections in cobalt/nickel solution for 15 minutes.

5. Wash in distilled water and rinse in 2 changes of Millonig’s buffer for 10 minutes each.

6. React the sections in Hanker-Yates solution for 10-25 minutes, stopping before the background staining darkens.

7. Rinse in 2 changes of Millonig’s buffer, 5 minutes each, and then place in distilled water.

8. Mount the sections onto gelatinised glass slides (0.5%) and dry overnight at 37°C.

9. Counterstain with gallocyanin.
B. Solutions for the Hanker-Yates method

1. Millonig’s phosphate buffer.

\[
\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O} \quad 19.08\text{g}
\]

1M NaOH \quad 96.3\text{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 \quad 30\text{ml}

1% ammonium nickel sulphate \quad 20\text{ml}

Mix together just before use.

3. Cacodylate buffer.

0.1M sodium cacodylate \quad 500\text{ml}

(21.4g/500ml distilled water)

0.2M HCl

(17.22ml concentrated HCl/litre distilled water)

Adjust to pH 5.1-5.2. Make up 2 litres with distilled water. Store at 4°C.


Hanker-Yates combined reagent (Sigma VI) \quad 150\text{mg}

Cacodylate buffer \quad 100\text{ml}

30 H\text{H}_2\text{O}_2 \quad 1 \text{ drop}

Make up just before use and discard after 1 hour.
C. *Counterstaining.*

Preparation of the gallicyanin stain.

Gallocyanin  0.3g  
Chromalum  10g  
Distilled water to make 100mls.

The chromalum is dissolved in water by heating. The gallicyanin is added and the mixture is brought to the boil, covered, and allowed to simmer for 20-30 minutes. The gallicyanin solution is then filtered and kept at room temperature.

1. Wash the sections briefly (60 seconds) in distilled water.
2. Place the sections in the gallicyanin counterstain for 10-25 minutes, depending on the age of the stain.
3. Rinse the sections in distilled water and then dehydrate in 2 changes of absolute alcohol for 2 minutes each.
4. Clear the sections in 2 changes of histoclear, 2 minutes each, and then coverslip using permount as a mounting medium.
Appendix II

Standard transmission electron microscopy tissue processing

Following primary fixation with 2% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, the specimen is post-fixed in osmium tetroxide stained with uranyl acetate, dehydrated in a series of graded alcohol, and finally embedded in Araldite resin.

1. Wash specimen in 0.1M phosphate buffer, 2 changes 10 minutes each.
2. Osmicate tissue in 1% osmium tetroxide (OsO₄) made up in 0.1M phosphate buffer, and leave in fridge at 4°C for 45 minutes.
3. Wash specimen in 0.1M phosphate buffer for 5 minutes.
4. Wash specimen in 0.1M sodium acetate, 2 changes of 10 minutes each.
5. En-bloc stain in 2% uranyl acetate made up in 0.1M sodium acetate at 4°C.
6. Wash specimen in 0.1M sodium acetate for 10 minutes.
7. Wash specimen in distilled water for 5 minutes.
8. The specimen is dehydrated according to the following schedule:
   - 25% ethanol in distilled water for 5 minutes
   - 50% ethanol in distilled water for 5 minutes
   - 70% ethanol in distilled water for 5 minutes
   - 90% ethanol in distilled water for 5 minutes
   - Absolute alcohol, 4 changes 10 minutes each
9. After dehydration the specimens are soaked in propylene oxide for 3 changes of 10 minutes each at room temperature.

10. Place tissue in a 50:50 mixture of Araldite resin (see below for composition) and propylene oxide. Leave at room temperature for 45 minutes.

11. The mixture of propylene oxide is removed and the specimen is soaked in fresh Araldite resin for 24 hours at room temperature on a rotator.

12. Block out (polymerize) in the oven at 60°C.

*Composition of resin mixture*

10g DDSA (dodecenyl succinic anhydride)

10g Araldite (CY212)

0.8g Plasticizer (dibutyl pathalate)

Add 0.4ml BDMA (benzyldimethylamine), when mixed and heated.
References


Kidokoro, Y. (1975) Sodium and calcium components of the action potential in a developing muscle cell line. J. Physiol 244, 145-159.


STABILIZING NEUROMUSCULAR CONTACTS INCREASES
MOTONEURON SURVIVAL AFTER NEONATAL NERVE
INJURY IN RATS

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Abstract—Following sciatic nerve crush at birth the rat soleus muscle is rendered permanently weak. This reduction in muscle force is caused by the loss of a proportion of its motoneurons. Furthermore, motoneurons that survive and reach the muscle fail to reoccupy a sufficient number of denervated muscle fibres to compensate for the loss of neurons. Both the loss of motoneurons and poor reinnervation may be due to the inability of the regenerating axons to establish and maintain neuromuscular contacts. Application of leupeptin, an inhibitor of a calcium-activated neutral protease and some serine proteases, is known to help in the maintenance of neuromuscular contacts during development and axonal sprouting. Here we examined whether protecting new neuromuscular contacts formed between regenerating axons and denervated muscle fibres after nerve injury, would influence the survival of motoneurons and improve muscle recovery. This study shows that in muscles treated with leupeptin the reduction in weight and force output after nerve crush at birth was significantly less than in those that were untreated. Moreover, the number of motor units in the leupeptin-treated muscles was significantly higher than in untreated muscles. Thus, treating regenerating nerve terminals with leupeptin during early stages of reinnervation rescues motoneurons and improves muscle recovery.

Key words: motor units, leupeptin, calcium-activated neutral protease, muscle force.

Young motoneurons are critically dependent upon functional interaction with their target muscle for their normal development and if deprived of target contact a large proportion die.10,21,28,35,39,44 After injury to the sciatic nerve in newborn rats there is a loss of motoneurons and consequently a severe impairment of muscle function.5,30,44 Although some motoneurons survive neonatal nerve injury and their axons find their way back to the muscle, the recovery of the reinnervated muscles is very poor since the injured axons are unable to expand their peripheral field.12,27,28 Following nerve injury during early postnatal development the muscle is isolated from its nerve at a time when it is differentiating and is therefore deprived of those influences that normally bring about its maturation.3,5,6 Thus, motoneurons and the muscle fibres they innervate are co-dependent; motoneurons that are deprived of their target during a critical period of development will die, and muscle fibres that are not functionally innervated fail to differentiate.16,27-29

The extent of motoneuron death induced by neonatal nerve injury depends on several factors, including the age of the animal at which the injury is inflicted,27,28,35,39,44 the site and type of injury and the time during which nerve–muscle contact is disrupted.21,25,26 The importance of the type of injury is probably associated with the opportunity that the injured motoneuron has to re-establish contact with the target muscle. Fewer motoneurons die if they are permitted to reinnervate their target muscle than when reinnervation is prevented.21 This is consistent with findings which show that limb amputation and nerve section result in motoneuron death but that most motoneurons survive following a nerve crush injury inflicted close to the muscle.10 Another critical factor for the survival of motoneurons, even when reinnervation is permitted, is the duration for which the motoneuron is separated from its target. More motoneurons die when reinnervation is delayed either by repeated nerve injury25 or by injuring the axons further away from the muscle.15,26 When the injury site is far from the muscle, the regenerating axons have further to grow before they can reinnervate the target muscle and so there is a longer delay before neuromuscular interaction is resumed.26

It is not the nerve injury itself that results in motoneuron death, but the prevention of nerve–muscle interaction. Blocking the postsynaptic acetylcholine receptor with α-bungarotoxin at a critical stage of development also results in the loss of a large proportion of motoneurons,16 and the degree of motoneuron death is dependent upon the length of
time that neuromuscular interaction is disrupted, so that more motoneurons die after a prolonged paralysis.\(^{16}\)

Most of the available evidence therefore indicates that for developing motoneurons to survive nerve injury they must, within a certain time, resume interaction with their target. This can only occur if their axons reinnervate the muscle and their terminals are able to establish and maintain contact with the muscle fibres. Some regenerating motoneurons die even after their axons have reached the muscle,\(^{21}\) possibly because their terminals are unable to maintain contact with the muscle fibres. In this study we examined the possibility that these motoneurons could be rescued if the newly formed neuromuscular contacts made by the reinnervating axons could be maintained.

We have previously shown that a calcium-activated neutral protease (CANP) is likely to be involved in the withdrawal of synaptic terminals during both normal development and sprouting of nerve terminals after partial denervation.\(^{19,24}\) More nerve–muscle contacts are maintained when this protease is inhibited by leupeptin,\(^{19}\) or if Ca\(^{2+}\) levels are manipulated so as to prevent them reaching high enough levels to activate CANP.\(^{2}\) Since the activity of CANP is Ca\(^{2+}\) dependent these results lead to the proposal that leupeptin acts by preventing the breakdown of neurofilaments by CANP.\(^{43}\) However, leupeptin also inhibits serine proteases and recent results indicate that serine proteases, such as thrombin, may be rally occurring Protease Nexin I have been shown to maintain neuromuscular contacts that would otherwise have been lost.\(^{18}\) Inhibition of CANP, or serine proteases at newly formed neuromuscular junctions by leupeptin may allow such contacts to be maintained. This study examined the possibility that following nerve injury at birth, treatment of regenerating nerve terminals in the soleus muscle with leupeptin may lead to an increased survival of motoneurons and a better recovery of the reinnervated muscle.

**EXPERIMENTAL PROCEDURES**

**Surgery**

New-born rats (Sprague-Dawley, Biological Services, UCL, U.K.) were anaesthetized with halothane and under sterile conditions the sciatic nerve was exposed in the right hindlimb. The nerve was crushed just proximal to the division of the sciatic nerve into the tibial and common peroneal nerves, with a fine pair of watchmaker's forceps. Using a dissecting microscope the nerve was examined after the crush to ensure that the epineurial sheath was intact and the nerve translucent. The incision was then closed and after recovery from the anaesthetic the animals were returned to their mother.

Six to seven days later, when the regenerating axons reached the muscle, the rats were re-anaesthetized with halothane and a silicone strip weighing 1 mg and containing approximately 70 \(\mu\)g of leupeptin (Sigma) was inserted between the soleus and flexor hallucis longus muscles. These implants usually remained in position and so there was no need to secure them. Leupeptin is a small peptide (N-acetyl-L-leu-L-leu-arginal) which is known to inhibit the activity of CANP\(^{19}\) and that of thrombin.\(^{24}\) The procedure was repeated three days later, when the animals were nine to 10 days old. During this operation the location of the first implant was checked and it was then removed. The control group of animals received a 1 mg silicone strip containing sodium chloride (NaCl) at the same ages.

**Tension recording experiments**

Two to four months later the rats were prepared for isometric tension recordings.\(^{27}\) The animals were anaesthetized with 4.5% chloral hydrate (1 ml/100 g body wt, i.p.) and the soleus muscles on both the operated and contralateral sides exposed. The distal tendons were attached to tension transducers (PYE UNICAM) and the leg was rigidly secured to the table. Isometric contractions were elicited by square-wave pulses of 0.02 ms duration and supramaximal intensity applied to the soleus nerve, via silver-wire electrodes. The length of the muscle was adjusted for optimum twitch tension. The number of motor units in the operated and in some cases the contralateral, unoperated control muscle, was determined by grading the stimulus intensity to the nerve and recording step-wise increments in twitch tension due to successive recruitment of motor units.\(^{8,13}\) Twitch tensions were used to determine motor unit number to avoid the fatigue which may develop during repeated tetanic concentrations.

Tetanic contractions of the muscle were then recorded to determine muscle force. Contractions were elicited by trains of stimuli lasting 700–900 ms at a frequency of 10, 20, 40 and 80 Hz. The mean motor unit tetanic force was calculated by dividing the number of motor units found in each operated muscle into the maximum tetanic tension for that muscle. This was expressed as a percentage of the mean of motor unit force calculated from the contralateral muscle, using the same method.

Variations in body weight will influence muscle weight and force, and since individual motor unit force is calculated by dividing total muscle force by the number of motor units present in the muscle, only female rats were used to assess motor unit force. The variation in body weights of female rats between the ages of two to four months is significantly less than that of males, and therefore motor unit force was assessed in this single sex group only.

**RESULTS**

**The effect of leupeptin on recovery of muscle function after nerve injury at birth**

Twenty Sprague–Dawley rats had their sciatic nerves crushed in one hindlimb at birth. Eight of these animals were subsequently treated on the injured side with an implant containing leupeptin. The remaining 12 rats received treatment with NaCl-containing silicon strips.

**Changes in muscle tension and weight**

When the animals were at least eight weeks old they were anaesthetized and tension recording experiments were carried out. In both groups of animals maximum tetanic tensions of the operated and contralateral soleus muscles elicited by stimulation of
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their motor nerves were established. Figure 1 shows examples of records of twitch and tetanic contractions from the operated and control soleus muscles taken from rats from the same litter. The tension records were obtained from (A) reinnervated NaCl-treated muscle along with (B) the contralateral unoperated control muscle, and (C) reinnervated leupeptin-treated muscle along with (D) the contralateral unoperated control muscle. Note the different scale bars for records (A) and (C). Figure 1 indicates that the force output of the leupeptin-treated muscle was greater than that of the NaCl-treated muscle. The mean values for both groups are summarized in Table 1. The absolute values of muscle weight, twitch and tetanic tension are influenced by the weight of the animals, and these were slightly different in the NaCl- and leupeptin-treated groups. In order to avoid body weight from interfering with the results, the values for each of the parameters measured were calculated as a percentage of the contralateral control muscle in each animal, and are shown in Table 1 (% op/con). Table 1 shows that following nerve injury at birth those muscles treated with leupeptin weighed twice as much as the muscles treated with NaCl, and were able to generate twice the force (Mann-Whitney U-test, \( P < 0.01 \) for both).

**Motor unit number**

The question whether the observed difference in muscle tensions between the two groups of rats was due to a difference in the number of motoneurons supplying the muscles or to the size of their motor units was addressed next. The number of motor units in soleus muscles of adult rats that had their nerves injured at birth and were treated with either leupeptin or NaCl was assessed. Motor unit numbers were estimated by counting the increments in twitch tension in response to stimulation of the soleus nerve by gradually increasing the stimulus intensity. Figure 2 shows examples of such recordings obtained from a soleus muscle denervated at birth and subsequently treated with either NaCl (A) or leupeptin (B). The results are summarized in Table 2. In the leupeptin-treated animals the number of remaining motor units in the soleus muscle was 7.6 (± 0.7 S.E.M., \( n = 8 \)), whereas the NaCl-treated soleus had only 3.4 (± 0.4 S.E.M., \( n = 10 \)) motor units. This difference is significant (Mann–Whitney, U-test, \( P < 0.002 \)). The value of 3.4 motor units in the NaCl-treated muscles is in good agreement with that reported in a previous study after nerve crush at birth. Thus it appears that application of leupeptin to the soleus muscle at the time of reinnervation rescues some motoneurons.

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![Diagram](image_url)  
**Fig. 1.** Twitch and tetanic contractions from adult soleus muscles after nerve injury at birth are shown: (A) reinnervated NaCl-treated muscle with (B) the contralateral unoperated control muscle, and (C) reinnervated leupeptin-treated muscle with (D) the contralateral unoperated control muscle. Note the different scale bars for (A) and (C). The tetanic contractions were elicited at 20, 40 and 80 Hz. The leupeptin-treated muscle in this example developed a maximum tetanic tension of 40 g after nerve injury at birth, compared with that of 6.9 g in the NaCl-treated muscle.
Table 1. Summary of the changes in muscle weight, twitch tension and maximal tetanic tension after sciatic nerve crush at birth with NaCl-treated and leupeptin-treated animals

<table>
<thead>
<tr>
<th></th>
<th>Mean body weight (g)</th>
<th>Muscle weight op. (g)</th>
<th>%op./con.</th>
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<tbody>
<tr>
<td>NaCl-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±S.E.M. (n = 12)</td>
<td>301 ± 20</td>
<td>0.1538 ± 0.01</td>
<td>28.6 ± 3.9</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>±S.E.M. (n = 8)</td>
<td>424 ± 48</td>
<td>0.2122 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Twitch tension con. (g)</th>
<th>%op./con.</th>
<th>Tetanic tension op. (g)</th>
<th>%op./con.</th>
</tr>
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<tbody>
<tr>
<td>NaCl-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±S.E.M. (n = 12)</td>
<td>27.2 ± 2.1</td>
<td>13.7 ± 3.5</td>
<td>146 ± 9.5</td>
<td>9.5 ± 1.6</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>±S.E.M. (n = 8)</td>
<td>34.9 ± 3.7</td>
<td>26.6 ± 4.4*</td>
<td>23.8 ± 4.7*</td>
</tr>
</tbody>
</table>

*Significantly different from NaCl-treated; con., control; op., operated.

The mean motor unit force was calculated by dividing the maximum tetanic tension by the number of motor units. The number of motor units in the control soleus muscles was taken to be 30. This estimate is based on our previous results and agrees well with values reported by others for the rat soleus. The results are summarized in Table 2. The table shows that the mean motor unit tension in the leupeptin-treated muscles was 123.7% (±17 S.E.M., n = 4) of the contralateral control muscles, whereas the motor unit tension in those muscles treated with NaCl was 89.9% (±16 S.E.M., n = 9) of control. The difference in mean motor unit force between the leupeptin- and NaCl-treated groups, however, is not significant (Mann–Whitney U-test). Thus, the increase in force output in the leupeptin-treated group is mainly due to an increased motoneuron survival.

DISCUSSION

The results of the present study show that after sciatic nerve injury at birth, some motoneurons to the soleus muscle are rescued by application of leupeptin to the muscle at the time of reinnervation. In addition to, and possibly as a consequence of this increase in motoneuron survival, the leupeptin-treated soleus muscles lose less weight and suffer a smaller reduction of force output after neonatal injury than muscles treated with NaCl. It therefore appears that the maintenance of neuromuscular contacts achieved by leupeptin treatment rescues some motoneurons destined to die after nerve injury and improves recovery of muscle function.

Sciatic nerve crush at birth produces extensive motoneuron death. When a peripheral nerve is crushed, the distal segment degenerates and the axons in the proximal segment begin to grow towards the denervated peripheral target. Many of the injured motoneurons die as a result of this loss of interaction with the target muscle during this...
Motoneuron death may be prevented. Previous results show that during both normal development to maintain these new neuromuscular contacts critical stage of development. However, not all motor unit territory which was over and above contacts can be rescued by treatment with leupeptin, and regeneration, newly formed neuromuscular deprived of target interaction. Therefore, by helping consequently complete their development to maintain contact with the muscle fibres and to avoid being deprived of target interaction. Consequently, help to maintain these new neuromuscular contacts motoneuron death may be prevented. Previous results show that during both normal development and regeneration, newly formed neuromuscular contacts can be rescued by treatment with leupeptin, an inhibitor of CANP and serine proteases. Leupeptin locally applied to soleus muscles of rats that had been partially denervated at four to six days of age induced an expansion of the increase in size that would be due to partial denervation alone. At four to six days of age individual motor units are expanded and achieve their smaller adult size by 18 days. It was therefore thought to be caused by regulating the activity of motoneurons after neonatal nerve injury.

Table 2. Summary of the changes in motor unit numbers and mean motor unit force after sciatic nerve crush at birth with NaCl-treated and leupeptin-treated animals

<table>
<thead>
<tr>
<th></th>
<th>Mean motor unit number</th>
<th>Mean motor unit tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con.</td>
<td>op.</td>
</tr>
<tr>
<td>NaCl-treated</td>
<td>30</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>± S.E.M.</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Leupeptin-treated</td>
<td>30</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>± S.E.M.</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
</tr>
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</table>

*Only female animals used.
†Significantly different from NaCl-treated (P < 0.002).
‡Not significantly different.

The present results indicate that by maintaining neuromuscular contacts with leupeptin at the time of reinnervation some motoneurons otherwise destined to die after nerve injury will survive. Leupeptin is known to be a potent inhibitor of both CANP and serine proteases and can enter nerve terminals. CANP is an enzyme found in the peripheral and central nervous system, axons and in nerve terminals where it is bound to the cytoskeleton. It has a high affinity for cytoskeletal elements, e.g., axonal neurofilament proteins, and may be involved in the turnover and degradation of these proteins. The activity of CANP is Ca2+-dependent and it is known that the maintenance of neuromuscular contacts can be influenced by changes in Ca2+ concentration both outside and inside the nerve terminal. Lowering the Ca2+ concentration has the same effect as inhibiting CANP with leupeptin, in that more neuromuscular contacts are preserved. Conversely, increasing Ca2+ concentration increases the rate of elimination of synaptic contacts. The effects of Ca2+ on neuromuscular contacts were therefore thought to be caused by regulating the activity of CANP.

Leupeptin is also an inhibitor of serine proteases, and recent results show that both leupeptin and the endogenous serine protease inhibitor Protease Nexin I can prevent synapse elimination in vitro. However, in these experiments an in vitro preparation was used where synapses were formed between nerve endings of sympathetic neurons and skeletal muscle fibres. Formation of synaptic connections between sympathetic ganglionic neurons and skeletal muscle fibres may be regulated by different mechanisms than that between motoneurons and muscle fibres. In view of the Ca2+ dependence of synapse formation in the neuromuscular system, we therefore favour the hypothesis that in the present experiments the main action of leupeptin is via the inhibition of CANP. However, the contribution that serine proteases such as thrombin may play in the regulation of synaptic contacts in vivo needs to be explored.

CONCLUSION

The stabilization of neuromuscular contacts at the time of reinnervation after nerve crush at birth increases motoneuron survival and improves muscle recovery.

Acknowledgements—We are grateful to the Medical Research Council, the BBSRC, the Wellcome Trust and the Commission of the European Communities for their support.
REFERENCES


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(Accepted 4 September 1995)