THE POSSIBLE ROLE OF AFFERENT ACTIVITY IN MOTONEURONE
SURVIVAL AFTER NEONATAL TARGET-DEPRIVATION

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ABSTRACT

It has previously been shown that motoneurones die when neuromuscular interaction is disrupted during the early postnatal period. It is possible that as a consequence of the disruption, these motoneurones become susceptible to the excitatory inputs they receive within the maturing central nervous system. In this thesis, the role of afferent activity in the survival of motoneurones after target-deprivation in neonatal rats was examined.

In the first part of the thesis, the effect of increasing the level of afferent input to motoneurones during the early postnatal period was investigated. Inducing premature locomotor activity in the neonate resulted in the death of a proportion of otherwise normal motoneurones to the slow, soleus muscle but not of those to the fast, TA and EDL muscles. The effect of increased afferent activity on the survival of motoneurones that had been deprived of target contact was also investigated. Inducing locomotor activity in these rats resulted in a further decrease of motoneurone survival.
In the second part of the thesis, the effect of decreasing the level of afferent input to motoneurones that were destined to die after neonatal target-deprivation was studied. Several approaches aimed at decreasing the level of afferent input to motoneurones were used. Decreasing the segmental afferent activity did not affect the survival of these motoneurones. However, reducing the effects of glutamate, the main excitatory neurotransmitter in the spinal cord, by blocking the NMDA receptor with MK-801 resulted in greater survival of the target-deprived motoneurones.

The results in this study support the proposal that afferent activity plays an important role in determining the survival of motoneurones deprived of target interaction during a critical period of their development.
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CHAPTER 1 - GENERAL INTRODUCTION

It is well established now that motoneurones and the muscle fibres they innervate are interdependent on each other for their normal development and functional integrity. The interaction between the motoneurone and muscle is critical during certain stages of development where the absence of one partner would affect the other.

After nerve injury during the early postnatal period a massive number of motoneurones that supply the hind limb muscles die. The motoneurones that survive this insult exhibit a greater overall activity and this may be due to changes either in the synaptic inputs to these surviving motoneurones or their excitability. Therefore, the possible role of the afferent activity on the survival of these motoneurones after injury during the neonatal period was examined in this study.

In the first part of the introduction, a brief review of the development of motoneurones and muscles will be given including some events that occur during the postnatal maturation of the neuromuscular system. This is followed by the effects of interruption of the interaction between
nerve and muscle. Lastly, the current theories that explain why motoneurones die when disconnected from their target are explored.

1.1. Early development of the neuromuscular system

During embryogenesis, motoneurones and muscles develop independently of each other but at a particular stage of the embryonic development, they become critically dependent on each other. This happens after they have acquired certain characteristics that allow them to interact with each other, the nerve terminals synthesising and releasing acetylcholine and the muscle being able to respond to acetylcholine.

1.1.1. Early motoneurone and muscle development

During the early development, motoneurones and muscle fibres develop independently of each other. The proliferation of neuro-epithelial germinal cells and early differentiation are not affected by removal of their peripheral target organs (Hamburger, 1958; Prestige, 1967).
Neither is the formation of myoblasts from mesenchymal tissue influenced by the absence of innervation as shown by observations on the chick embryos (Hamburger, 1939) and amphibian larvae (Harrison, 1904).

After the final mitosis, the neuroblasts migrate to their final location in the motor columns of the ventral horn. This migration is believed to occur along pathways provided by radial glial cells (Leber et al., 1990). These neurone precursors are multipotential and epigenetic factors may be involved in determining their final phenotype as indicated by the finding of Leber et al. (1990). They show that clones derived from single labelled neuroblasts contained different types of neurones, motoneurones, interneurones and glial and ependymal cells. The differentiated cells take up position opposite the dorsal and ventral halves of the central canal and begin to send out processes (Jacobson, 1978). Motor nerve axons are the first to be formed by outgrowths of ventral horn cells within the grey matter. The appearance of neurotransmitter related enzymes, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in motoneurones soon after their final mitosis indicates that this event is target independent as at this
time the axons of motoneurones have not yet reached the
target muscles (Phelps et. al., 1991).

Muscle cell develop from mesoderm during the early
embryonic development. This layer contains undifferentiated
cells which after rapid proliferation gives rise to the
primitive muscle cell, the myoblast. The myoblasts has the
ability to synthesise contractile proteins such as actin,
myosin and topomyosin which will be assembled into
myofibrils later. At this time acetylcholine receptors are
synthesised and incorporated into the cell membrane
(Fambrough and Rash, 1971). Before fusion of these
myoblasts, they become elongated and several metabolic
changes take place which will prepare these myoblasts for
contractile activity including an increase in the number of
mitochondria, production of enzymes required for the
metabolism of glucose and glycogen, and the development of
rough endoplasmic reticulum and the sarcoplasmic reticulum
(Shainberg et. al., 1971; Schudt et. al., 1975; Holland and
MacLennan, 1976). Finally, the myoblasts align and fuse to
form multinucleated cross-striated myotubes which later
will differentiate to be muscle fibres (see Wakelam (1985)
for review). The centrally placed nuclei will migrate to the periphery of the myotubes. The synthesis of contractile proteins and the production of acetylcholine receptors is much increased after cell fusion when myotubes start contracting (Fambrough and Rash, 1971).

Thus, up to a certain point during the early embryonic development, motoneurones and muscle fibers are not dependent on each other.

1.1.2. Interdependence of motoneurones and target during embryonic development

When the motoneurones extend their growth cones into the periphery, further maturation of the cell body, axons and their terminals depends on the formation of functional connections with muscle fibres. The axons leave through the ventral roots at the same time that the limb buds start to develop. The nerves are present within the limb buds as soon as they are formed (Harrison, 1910). Branches or sprouts appear along intramuscular nerves, acetylcholine receptor (AChR) clusters occur on adjacent myotubes and immature synapses begin to form (Dahm and Landmesser,
Once the nerve and muscle first make contact, they become critically dependent on each other such that the development of one or the other will be retarded should the nerve terminals fail to make contact with the muscle. This dependence may be due to the interdependence of events occurring during the maturation of the muscle and those that occur during the maturation of the motoneurones. For example, once the cholinergic characteristics of motoneurones are established, interaction with the target muscle is necessary in order to increase and maintain a high level of expression of the enzymes involved in ACh synthesis (Giacobini-Robecchi et. al., 1975; for review see Vaca, 1988).

While axons are establishing muscle contacts, changes in the spinal cord that influence the motoneurones also take place. Spinal motoneurones are among the first neurones in the developing nervous system to undergo differentiation (Hamburger, 1977). The interneurones also differentiate from the neuroblasts and later, the other components of the spinal cord which forms the supporting glial cells start to develop and differentiate. The cells of the neural crest differentiate to form the sensory ganglia of the spinal
cord and the Schwann cells which form the myelin sheaths of axons.

Synaptic activation of motoneurones is mediated by neurotransmitter-gated ion channels located in their somatodendritic membrane. During embryonic development, there is a tremendous increase in afferent inputs to motoneurones. For example, in the spinal cord of mouse, synapses upon dendrites of motoneurones increase from 20% of total number of synapses at E13 to 65% of the synaptic population at E16 (Vaughn et al., 1974).

Naturally occurring cell death

An event that takes place during this time in the early development is a naturally occurring cell death. This event occurs in many populations of developing neurones which involves the loss of about 50% of the cells. It has been observed in the dorsal root ganglion (Hamburger and Levi-Montalcini, 1949) and the cervical motoneurones of the chick embryo (Levi-Montalcini, 1950) and in the frog (Hughes, 1968). This phenomenon occurs following the growth and projection of an axon to postsynaptic targets and the
neurogenic movements initiated by afferent synaptic inputs (Hamburger, 1975) (see Oppenheim, 1985 for review). In the human foetus, there is a peak of motoneuronal degeneration that occurs during the 12th and 16th week of gestation. This normal period of motoneurone death coincide with the initiation of functional neuromuscular contacts (Forger and Breedlove, 1987). The timing of this normally occurring event shows that for this event to take place, it may depend on the interdependence of motoneurones and muscle during development.

The number of motoneurones that die during this period of naturally occurring cell death can be made to increase if the target was reduced (Hamburger, 1934). Thus it was originally thought that the proportion of surviving motoneurones roughly corresponded to the amount of target available (Hamburger, 1977). On the other hand, a substantial number of motoneurones still die in favourable conditions of target availability (for review see Oppenheim, 1991). Although there seem to be quantitative discrepancies, there is substantial evidence to show that some kind of interaction with the target is important for regulating motoneurone survival during the embryonic development.
Therefore, interaction between nerve and muscle is not only necessary in this stage of development but critical for the survival of the two tissues.

1.2. Maturation of the neuromuscular system during postnatal development

The dependence of the nerve and muscle on each other continue during early the postnatal development until such time that they reach the mature adult state.

1.2.1. Interdependence between nerve and muscle

The interdependence of nerve and muscle that had begun during the embryonic development continues after birth. Further maturation of motoneurone and muscle requires normal neuromuscular interaction. For example, nerve-induced activity plays a crucial role in regulating the distribution of AChRs in muscle. AChR clusters at developing synapses have been shown to disperse within hours or days after denervation (Slater, 1982). The AChR channel opening time is also regulated by the nerve and its
activity (see Changeux, (1991) for review). After birth, slow channels predominate at the endplate and denervation at this time will block the normal developmental appearance of fast channels.

The growth of dendritic arborisation of motoneurones is also dependent on normal target interaction. When this interaction was disrupted by a nerve crush at birth, the distribution of the dendrites was altered such that there was a decrease in dendritic length in the transverse plane and a reorientation of dendrites in favour of the rostro-dorsal regions (O'Hanlon and Lowrie, 1993).

In the mouse, an average motoneurone contacts 36% of the fibres in the soleus muscle at birth and over the next two weeks, the motor unit size is reduced to the adult value of 5% of the fibres in the muscle (Fladby, 1987). When a muscle is tenotomised during the early postnatal period, the elimination of excess axonal terminals per endplate is retarded and also produces muscles composed of only small fibres (Riley, 1978). The contraction speeds of the EDL and soleus muscles are already differentiated at birth (Close, 1964) and increase rapidly during the first 3 weeks of life. But when these muscles were denervated at birth,
their contraction times became similar and this change was due to the failure of the EDL muscle to complete its differentiation (Brown, 1973). The author went on to show that the inability of the fast muscle to differentiate may be due to lack of activity that follows denervation. Thus, motor nerves maintain the structural and functional integrity of the muscle.

Therefore, target interaction and activity is very important for the postnatal development and maturation of both motoneurones and muscle. The exact nature of the retrograde influence of target on the development of motoneurones remains unclear.

1.2.2. Development of the motor unit

The motor unit is composed of one motoneurone and all the muscle fibres that this motoneurone innervates. In the adult, a muscle fibre can belong only to a single motoneurone and this arrangement is necessary to enable the motoneurone and the muscle fibres it supplies to function as a unit.
Early in embryonic development, all axons of a particular motor pool are already present among the few myogenic cells that will form a particular muscle. Thus, the number of muscle fibers available for the axons to innervate is relatively small. The initial number of primary myotubes present at this time may be one eighth or one fifth of the total number of fibers in the adult muscle (Ross et. al., 1987; Sheard et. al., 1991). Secondary myotubes appear later than these primary myotubes and develop in close contact with them, often sharing the same axon terminal profiles (Duxson et. al., 1986). With time, some nerve endings are transferred from the primary to the secondary myotubes. The selection of which terminal is to be transferred depends on the compatibility of the secondary myotube with a particular terminal. The least mature nerve ending is better matched to the relatively immature secondary myotube and thus will be transferred. This matching based on maturity may be the earliest event that helps establish the anatomical distribution of motor unit territories.

The adult pattern of mononeuronal innervation is established during the latter part of the second postnatal week (Jansen and Fladby, 1990). The early muscle
innervation is polyneuronal while the adult pattern of innervation is one axon to one endplate. This loss of polyneuronal innervation follows a rapid time course and depends on neuromuscular activity; when activity is reduced synapse elimination is delayed (Thompson et. al., 1979; Duxson, 1982; Benoit and Changeux, 1975) and when activity is increased synapse elimination is accelerated (Zelená et. al., 1979; O'Brien et. al., 1978). Therefore, in the young animal the motor unit size is big and when the adult pattern of innervation has been achieved, the motor unit size is reduced (Bagust et. al., 1973; Brown et. al., 1976).

The force developed by a motor unit depends on the number of muscle fibers that the motoneurone innervates and their size. Thus, the most active motor units which develop a smaller force compared to the stronger less active motor units, contain the smaller number and size of muscle fibers which are also the most fatigue resistant.

1.2.3. Motoneurone properties

The excitability and thus, the function of adult motoneurones is partly determined by their size (Henneman
et. al. 1965) and other factors such as the intrinsic membrane properties and synaptic inputs.

The lateral and ventral parts of the ventral horn of the spinal cord contains a population of large and small multipolar cells which have abundant Nissl substance. These cells are motoneurones, with several branching dendrites and heavily mylinated axons which leave through the ventral roots. The sizes of these motoneurone have a bimodal distribution with the smaller group described as the gamma motoneurones which innervate the intrafusal muscle fibres while the larger group of motoneurones are the $\alpha$-motoneurones which innervates the extrafusal muscle fibres (Conradi, 1976; McHanwell and Biscoe, 1981; Burke et. al., 1977).

The firing pattern of motoneurones is determined largely by the duration of after-hyperpolarisation (AHP) of the action potential. In young kittens the duration of AHP was short in motoneurones to both 'slow' and 'fast' muscles (Huizar et. al., 1975). With further development, this duration increased in motoneurones to the slow muscles and remained short in that to the fast muscles. Thus, motoneurones to
the slow muscles have a much longer AHP and are recruited more readily (Eccles et. al., 1958). There are also indications that motoneurones innervating the slow muscles are relatively smaller than those that innervate the fast muscles (Sato et. al., 1977). This finding supports Henneman's principle that smaller motoneurones will be recruited first as they have a lower threshold for excitation (Henneman et. al., 1965).

The nature of the synaptic input that activates a motoneurone also plays a role in the development of motoneurone firing pattern. At birth, motoneurones possess afferent inputs from a number of sources including segmental afferents and supraspinal inputs. These are situated primarily on the dendrites (Vaughn et. al., 1977; see Vaughn, 1989 for review). There is evidence that synaptic inputs are continuously reorganised during the first few weeks after birth (Conradi and Ronnevi, 1975; Gilbert and Stelzner, 1979). As will be elaborated in section 1.2.5., the period of maturation of the descending noradrenargic and serotonic inputs corresponds to the onset of antigravity functions and locomotor activity in the hindlimbs. At this time the postural tonic EMG activity can
first be recorded in the slow soleus muscle (Navarrete and Vrbová, 1983). Thus, changes in the afferent synaptic activity associated with the maturation of the descending and interneuronal inputs to motoneurone may regulate its functional specialisation (Vrbová et al., 1995).

1.2.4. Differentiation of fast and slow muscles

Two main types of mammalian twitch muscle can be distinguished i.e. those that produce force rapidly, for example the TA and EDL muscles, and those that contract slowly, for example the soleus muscle. The different fibre types present in the adult animal are the fast and the slow muscle fibres (Kuffler and Vaughan-Williams, 1953a,b). Each fibre type is innervated by only one nerve type: the large nerve fibres supply the fast twitch fibres and the small nerve fibres supply the slow tonic fibres. These fast and slow fibre types are specialised for different types of contraction: the fast transient and the slow maintained respectively. These differences in the functioning of the two fibre types are reflected in the differences in their structure and metabolism.
Although the early diversification of muscle fibres containing different isoforms of myosin heavy chain (MHC) can proceed even in the absence of innervation (Phillips and Bennet, 1984; Condon et. al., 1990), further differentiation requires the influence of nerves imposing a particular pattern of activity on the muscle. There is a much greater heterogeneity of muscle fibres belonging to the same motor unit in younger animals than in later postnatal life, indicating that some muscle fibres became gradually transformed under the influence of innervation (Gates and Ridge, 1992).

During the second and third postnatal week, major changes of histochemical and physiological properties of muscle fibres occurs. The oxidative capacities of neonatal muscles are low at birth and increase during postnatal development (Sieck and Blanco, 1991). In the mice EDL muscle fibres, this low SDH activity is more or less uniform up to 10 days after birth after which time the fibres become more heterogeneous with age (Dangain et. al., 1987). In the rat soleus muscle, the number of fibres containing slow MHC increases after birth and a marked increase corresponds to the time when the tonic EMG activity of the muscle is first apparent.
Differences in the time to peak of the twitch contraction are due to differing rates of force development. After birth, both slow and fast muscles increase their speed of contraction with age before their contractile speeds diverge (Close, 1964). To some extent, the speed of a muscle varies as a function of the relative amount of fast and slow MHC (Reiser et. al., 1985). Fast and slow twitch muscles can also be distinguished by their tetanic response to repetitive stimulation. Slow twitch muscles achieve maximum force production at lower rates of stimulation while fast twitch muscles require higher frequencies of activation.

Thus, slow twitch muscles which have a high content of mitochondria have a high oxidative capacity and high fatigue resistance and their time course of contraction is visibly slower than that of the fast twitch muscles.

1.2.5. Development of locomotion

During the early postnatal period, rat pups are quiet and when they do move, it is by crawling until the 10-17 days after birth when quadrupedal walking starts. These changes
in the development of locomotion correspond with changes in the structures in the central nervous system which may play a role in the control of locomotion. The first corticospinal tract fibres reach lumbar segments by the fifth postnatal day. Growth of corticospinal axons into the dorsal horn adjacent to the main tract begins 2-3 days after the initial arrival of the tract at a given segment i.e. only after the cells of origin of corticospinal axons have received a substantial afferent input (Schreyer and Jones, 1982). This growth of corticospinal axons into the dorsal horn (spinal grey) of the appropriate levels of the cord has a close temporal relationship with the appearance of placing responses (Donatelle, 1977). The rubrospinal tract develops earlier and extend to the lumbosacral cord at birth. The functional development of the cerebellum correlates well with the development of free walking. Mature Purkinje cell firing characteristics appear between 10-17 days after birth (Woodward et. al., 1969). Bekoff and Trainer (1979) found that rat pups are able to show interhindlimb coordination patterns with consistent phase relationships when swimming during the first week after birth and this suggest that the spinal circuitry is already capable of generating coordinated activity long before the onset of free walking. At postnatal day 11, quadrupedal
walking with the ventral surface off the floor was first observed (Westerga and Gramsbergen, 1990) and by P15 a rapid transformation into the mature pattern of locomotion occurred (Westerga and Gramsbergen, 1990; Altman and Sudarshan, 1975; Bolles and Woods, 1964).

Rhythmic stereotyped movements, particularly locomotor activity are generated by neuronal networks that have been called central pattern generators (CPG) (Grillner and Wallen, 1985; Lydic, 1989). These networks are located in the cervical and lumbar enlargements of the cord and are present and active at birth (Smith et. al., 1986; Atsuta et. al., 1988). Grillner (1986) found that in spinal cord preparations from which the descending inputs and sensory feedback had been removed, fictive locomotion could be elicited in various ways i.e. by means of chemical activation with L-Dopa or sensory stimulation. Although the spinal cord itself contain neural circuits that enable it to generate step cycles and coordination between the limbs, there are supraspinal influences that control locomotion (for review see Armstrong, 1988). The cerebellum, brain stem and the motor cortex exert their influence on locomotion through the main descending pathways which make excitatory synaptic connections with motoneurones either
directly or via interneurones of the CPG. Wang et. al., (1991) found that a marker for descending systems, serotonin (5-HT), was present at birth and reached adult levels by the end of the second postnatal week which correspond to the timing of the onset of the adult pattern of locomotion.

1.3. Consequence of disruption of neuromuscular interaction during the early postnatal life

1.3.1. Effects on motoneurone

Bennett et. al. (1986) show that after birth, there is normally no further loss of motoneurones in the lateral motor column. Therefore the motoneurone loss seen during development takes place normally only in the embryo. But when the neuromuscular interaction is disrupted during the early postnatal period, the number of motoneurones that survive the interruption is decreased. Romanes (1946) reported that there was a loss of over 50% of motoneurones after nerve lesion in new-born mice. Schmalbruch (1984)
showed that motoneurones die after sciatic nerve cut in newborn rats.

Susceptibility to injury decreases with age such that injury after 5 days of age causes no motoneurone death although the same injury inflicted at birth causes profound motoneurone loss to both the fast and slow muscles (Lowrie et. al., 1987).

Kashihara et. al. (1987) reported that a large proportion of motoneurones axotomised 4 days after birth could survive if peripheral innervation was allowed to proceed unhindered. On the other hand, if target contact is prevented, only 18 percent of the axotomised motoneurones survived. This suggests that death of axotomised motoneurones was not due to the nerve injury itself but due to the period of target deprivation. This proposal was supported by results of experiments in which the neuromuscular interaction was disrupted by paralysing the muscle with implants containing α-Bungarotoxin (α-BTX). Paralysis of the soleus muscle during the early postnatal period resulted in the death of a significant number of motoneurones to the muscle (Greensmith and Vrbová, 1992).
The size of surviving motoneurones is also affected by the disruption of neuromuscular interaction during the early postnatal period. After a sciatic nerve crush at birth, the size of the large motoneurones were smaller compared to that on the contralateral side (Lowrie et. al., 1987). The authors proposed that the decrease in size of the largest motoneurones was due to the temporary disconnection of these cells from their targets during the time when their growth was heavily dependent on connections with the muscle.

Thus the target seems to have an important role in the normal development of motoneurones but the nature of this role and the mechanism by which it affects the developing motoneurone is not well elucidated.

1.3.2. Changes in muscle properties

Adult mammalian muscle recover completely from temporary denervation provided that reinnervation is allowed to proceed unhindered (Gutmann and Young, 1944). However, after sciatic nerve crush in newborn rats, the weight of reinnervated soleus was reduced considerably (Zelená and
Hník, 1963). Lowrie et. al. (1982) reported that a nerve crush injury inflicted after the age of 5 days still resulted in a reduction of TA and EDL muscle weights although a nerve injury at this time resulted in no motoneurone loss. An injury inflicted 12 days postnatally allowed only 75 percent recovery of muscle weight. Thus, temporary denervation during the first two weeks after birth prevented full development of muscle weight of the fast muscles and this loss of muscle weight was not due to loss of motoneurones.

The reduction in muscle weight was due to a reduction in the number of mature extrafusal fibres which were also reduced in size (McArdle and Sansone, 1977). A nerve injury during the early postnatal period may therefore also result in a reduction of the motor unit size which may be due to the failure of the motoneurones to completely reoccupy their peripheral field (Zelená and Hník, 1963). Lowrie et. al. (1982) reported a similar reduction in muscle fibre numbers and found that the fibre composition was also altered, with increased levels of oxidative enzymes in the fast muscle. The muscles were more fatigue resistant and the maximal tetanic tension was only about half of that generated by controls. These changes were greater the
earlier during postnatal development that the nerve was injured.

It was proposed that the muscle fibre loss occurred after reinnervation and apparently as a consequence of this process (Lowrie and Vrbová, 1984). After an early postnatal injury, the number of fibres in the reinnervated EDL was near normal at 18 days but declined sharply to about one third at 35 days. Thus, muscle fibre loss may be due to the nerves failing to reach all of the muscle fibres or the muscle fibres failing to respond appropriately to the incoming motor nerve.

Brown et. al., (1976) found that the soleus muscle could recover completely after a neonatal nerve crush close to the muscle which suggested that it was the period of denervation that influenced the regeneration of the muscles and not the injury itself. Lowrie et. al. (1990) confirmed that delaying reinnervation impaired the recovery of the muscles that were denervated at 5 days of age. The authors concluded that the permanent impairment of fast muscles seen after neonatal nerve injury depends on the length of time that the muscles were separated from their motoneurones.
1.4. Why do motoneurones die?

Motoneurones depend on the their target for survival during the early postnatal period. There are several theories that may explain why motoneurones die when deprived from interaction with their target during this time.

1.4.1. The Neurotrophic Theory

The most popular theory to explain why neurones die is that the target provides some trophic factors which are essential to the survival of these developing neurones (Snider et. al., 1992). Therefore when motoneurones are axotomised, these trophic factors are denied to motoneurones so injured and they therefore die.

Neurotrophins are a family of polypeptides and at present, many members of this family have been identified and proposed to act as survival factors: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins-3,4,5 (NT3/4/5), ciliary neurotrophic factor (CNTF), fibroblast growth factor (FBF), glial-derived
trophic factor (GDNF) and the more recent ones are the insulin-like growth factors I and II (IGF-I/II).

Neurotrophins have been shown to be essential for the normal development of sensory neurones. The trophic factor NGF (Yip et. al., 1984) and BDNF and NT3 (Eriksson et. al., 1994), have been shown to affect the postnatal development and the transient survival after injury of the sensory neurones.

The same requirement for neurotrophins by motoneurones is not so clear cut. Neurotrophins were shown not to have much effect on the survival of chick motoneurones in vitro (Arakawa et. al., 1990). In fact, in some instances, when NGF was administered to neonatal rats after nerve injury, the number of motoneurones that die increased (Miyata et. al., 1986).

More recently, Oppenheim et. al. (1992) indicated that some members of the neurotrophic family may have a role in the survival of developing avian motoneurones. BDNF was found to rescue chick embryonic motoneurones from naturally occurring cell death while treatment with BDNF and NGF prevented the induced motoneurone death following removal
of the descending afferent input.

BDNF, NT-3 and NT-4/5 have also been shown to promote motoneurone survival during development in the rat (Henderson et. al., 1993). In addition, the motoneurones also express receptors for these neurotrophins.

More pertinent to this study, some neurotrophins rescued motoneurones that would have died following injury during the postnatal period. Yan et. al. (1992) found that BDNF rescued motoneurones following lesion of the sciatic nerve in the rat and Sendtner et. al. (1992) found that BDNF did the same for motoneurones of the facial nerve in the rat while NT-3 had a lesser effect. On the other hand, Eriksson et. al. (1994) found that these same neurotrophins did not rescue motoneurones after axotomy of the sciatic nerve during the postnatal development.

Receptors on which the neurotrophins act on as ligands are known as the Trk family of receptor tyrosine kinases (see Bothwell, 1995). Of these receptors, TrkB acts as the receptor for BDNF, NT4 and to a lesser extent for NT3. At the present time, it is possible to 'knockout' genes for the neurotrophins or for the receptor itself through gene
targeting. When transgenic mice deficient for TrkB were examined, the number of facial and lumbar motoneurons was reduced although no loss was observed when trkA was knocked out (Klein et al. 1993). Therefore this finding seem to show that BDNF and NT4 may be essential for the development of motoneurones.

Very recently, however, it was shown that in mice lacking both the NT4 and BDNF neurotrophins, the motoneurones remain unaffected while the sensory neurones were severely affected (Conover et al., 1995; Xin Liu et al., 1995).

Therefore, the role of neurotrophins on the survival of motoneurones is uncertain. Although there have been many publications that supported the proposal that neurotrophins are essential for the survival of motoneurones, recent findings seem to indicate otherwise.

1.4.2. The Excitotoxic Theory

A complementary explanation as to why motoneurones die is that these injured motoneurones become excessively excitable and overactivated by the ever increasing afferent
inputs they would normally receive within the maturing nervous system.

Olney (1971) suggested the hypothesis that endogenous excitatory amino acids, particularly glutamate and aspartate can cause excessive excitation and neuronal death. The discovery that exogenous glutamate was excitotoxic was first made by Lucas and Newhouse (1957) when they reported that injection of glutamate could destroy the inner neural layer of mouse retina.

The amino acid glutamate, one of the main excitatory neurotransmitters found in the mammalian central nervous system, is released when specific neural pathways are activated (Nadler et. al., 1976). Uptake systems are present to remove glutamate from the extracellular space. Enzymes necessary for the synthesis of glutamate and its receptors have been identified in both the pre- and postsynaptic membranes (Greenamyre, 1986). In the monkey, glutamate serves as the excitatory transmitter of spinal cord interneurones and the cortico-spinal pathways (Young et. al., 1983). The sensitivity to glutamate was apparently modulated by the presence of interneurones since it was greater in cultures that are composed of several cell types
non-NMDA receptors are formed by combinations of the receptor subunits GluR1-7 (for review see Bochet and Rossier, 1993). GluR1-4 have a higher affinity for AMPA than for kainate while GluR5-7 have a higher affinity for kainate than GluR1-4 do (see Wisden and Seeburg, 1993).

Glutamatergic neurotransmission is also mediated by metabotrophic receptors (mGluR) which are linked to intracellular second messenger systems. Of the metabotrophic receptors, mGluR1 and mGluR5 are coupled to phosphoinositide metabolism while mGluR2-4 regulate cAMP level (Abe et. al., 1992).
than in those composed exclusively of motoneurones (O'Brien and Fischbach, 1986a).

Glutamate receptors are activated during excitatory transmission between interneurones and motoneurones (O'Brien and Fischbach, 1986b; Ziskind-Conhaim, 1990). On the basis of their pharmacological, physiological and agonist binding properties, the ionotrophic receptors which mediate their effects through cation-selective channels, are classified into the NMDA, AMPA and kainate receptor subtypes (for review see Wisden and Seeburg, 1993).

The receptor subunits for NMDA receptors are subunits NR1 and NR2A-D. The NMDA receptor have been shown to be an important determinant of neuronal survival and this influence is likely to be calcium mediated (Brenneman et. al., 1990). The following NMDA receptor subunits are expressed in spinal motoneurones: the NR1, 2B and 2D subunits (Monyer et. al., 1994; Tolle et. al., 1994). The

Excitotoxicity may also result when the uptake mechanism for glutamate is not able to cope and thus motoneurones are exposed to increased levels of glutamate (Rothstein et. al., 1992). Excess glutamate has been found after traumatic lesions (Liu et. al., 1991). This excess glutamate or the overstimulation of NMDA receptors have been shown to be
lethal for some neurones both in vivo (Coyle et. al, 1981) and in vitro (Choi, 1991).

It has been suggested that glutamate is involved in the pathogenesis of ALS (Choi, 1988; Plaitakis at. al., 1988; Young, 1990). The abnormal glutamate metabolism observed in patients with ALS (Plaitakis and Constantakakis, 1993) may result in abnormal potentiation of excitatory transmission mediated by glutamate receptors and thus, to neurodegeneration. Exposure of cultured neurones to cerebrospinal fluid from ALS patients which have been shown to contain abnormally high levels of glutamate (Rothstein et. al., 1990) result in a significant death of the neurones in culture (Couratier et. al., 1993).

The mechanisms of excitotoxicity that result in death of neurones are not fully established (for review see Choi, 1992). The first possibility is that osmotic swelling which follows exposure to glutamate can result in immediate necrosis. Secondly, increased concentrations of calcium in the cell due to Ca$^{++}$ influx when the NMDA receptor associated channel is open (Ascher and Nowak, 1986; Dingledine, 1983), may activate proteases which will result in cell death (Choi, 1985).
The excitotoxic hypothesis also implies that the neurotoxic properties of the excitatory amino acid neurotransmitter, glutamate can be attenuated by blocking the NMDA receptor. Drugs that block NMDA receptors, including 2-amino-5-phosphonovalerate (APV), 2-amino-7-phosphonoheptanoate (APH) and kynurenate have been shown to attenuate the cytotoxicity of NMDA (Choi et. al., 1988). Other noncompetitive antagonists of the NMDA receptors, in particular MK-801 (Foster et.al., 1987) which specifically act on the associated ion channel, also reduce the neurotoxicity of NMDA.

The excitatory amino acids (EAAs) plays a crucial role in the activation of the locomotor CPGs of vertebrates (Dale and Roberts, 1984; Brodin and Grillner, 1985). Excitatory amino acids are known to be released by primary afferent inputs and interneurones (Ziskin-Conhaim, 1990). Siilar and Roberts (1988) have shown that cutaneous afferents release EAAs onto interneurones at the central synapses. O'Donovan and Landmesser (1987) found that during locomotion, glutamate receptors are activated. Recently, Cazalets et. al. (1992) also showed that NMDA receptors participate in the activation of locomotor networks.
Bath application of N-methyl-D,L-aspartate could evoke locomotor activity in hindlimb muscles (Kudo and Yamada, 1987). The endogenous EAAs, glutamate and aspartate have been shown to trigger an alternating rhythmic pattern in isolated brainstem-spinal cord preparation from the newborn rat, and this activity could be prevented by blocking the NMDA receptor with AP-5 (Cazalets et al., 1992). This supports the proposal that NMDA receptors and glutamate are involved in the central pattern generation of locomotion.

Therefore, it may be possible that when motoneurones are deprived of functional interaction with their target during a critical period during the early development, they become susceptible to the excitotoxic effects of glutamate.

1.5. Aim of Study

The aim of the study is to examine the possible role of afferent activity in motoneurone survival after neonatal injury. Normal neuromuscular interaction during the early postnatal period have been proposed to be essential for the
maturation of motoneurones. Some motoneurones will die when deprived of this interaction and those that survived exhibit increased activity. It may be that afferent activity plays a role in motoneurone death after neonatal injury.

The following questions are studied here:

1. What are the effects of exposure to increased afferent inputs during the early postnatal period on normal, uninjured motoneurones?

2. Would these responses be similar if the neuromuscular interaction was disrupted prior to the exposure to increased afferent inputs?

3. If so, could these responses be reversed by decreasing the afferent inputs?

In the present study, the neuromuscular interaction was disrupted during a critical period of development which would predictively result in a decrease in the number of surviving motoneurones. The motoneurones to the slow, soleus and the fast, TA and EDL muscles were chosen for
this study as their responses to disruption of neuromuscular interaction during the early postnatal period are well established in this laboratory.

The afferent inputs to these motoneurones were then manipulated resulting in an increase or a decrease in the supraspinal and segmental afferent activity and the effects of these manipulations on motoneurone survival were studied.
2.1. Animals

Neonatal Wistar albino rats of both sexes were used in the experiments. They were of various ages at the time of the experiments and the specific ages are detailed later for each experiment. The final experiments were done when the rats were 4 weeks old or when they were adults i.e. when they were at least 8 weeks old.

2.2. Surgical Procedures

2.2.1. Nerve Crush

Neonatal rats were anaesthetised with halothane by inhalation or with ether. An incision was made at the midpoint between the knee and the thigh bone. The muscles underneath were then separated with a pair of forceps without cutting the fibres. The sciatic nerve which will be visible just underneath the first layer of muscle was crushed with a pair of fine Watchmaker's forceps. The nerve
was checked to see that the epineurium was intact and that the nerve was completely crushed. The incision was then closed by sutures and after recovery from the anaesthetic the pups were returned to their mother.

This procedure was done on Wistar rat pups of various ages according to the study being carried out. In some cases, a second nerve crush was carried to extend the period of separation of the target muscle from its innervation (Lowrie et. al., 1990). To ensure that the nerve crush was successful, i.e., the nerve was crushed but not axotomised, the pups were observed daily after the procedure and signs of functional recovery were noted (Gutmann, 1942).

2.2.2. **Implantation of α-BTX Strips**

The soleus muscle of the experimental rats was paralysed by applying the snake toxin, α-bungarotoxin (α-BTX) which was obtained from Sigma. α-BTX binds irreversibly to the postsynaptic nicotinic acetylcholine receptor (AChR) and so interrupts the response of the postsynaptic membrane. It is incorporated into inert silicon rubber which allows a steady release of the toxin into the surrounding tissues.
Long term paralysis i.e. for up to 11-13 days, was achieved by implanting these silicone strips twice i.e. on the day of birth (P0) and again at postnatal day 3 (P3). At 8 days of age, the soleus muscles were still partially paralysed (see Greensmith and Vrbová, 1992).

Preparation of silicon strips was according to Greensmith and Vrbová (1991) and is detailed in Appendix I. Strips weighing 1 mg were cut and kept for implantation at which time the strips were further cut into smaller strips according to the amount of α-BTX tolerated at the different ages.

Rats underwent surgery on the day of birth, usually within 6 hours of birth. Under sterile conditions and halothane anaesthesia (by inhalation), a small strip of silicon rubber containing α-BTX was implanted alongside the soleus muscle of the right hindleg. A small longitudinal incision was made in the skin and fascia to reveal the underlying muscles. The silicon rubber strip containing α-BTX was implanted between the soleus and flexor hallucis longus muscles, at the midpoint of the soleus and away from the point of entry of the nerve to soleus. The skin was sutured with 0.4 silk thread (Ethicon) and the animal left to
recover from the anaesthesia before being returned to the mother.

The procedure was repeated 3 days later. The rats were reanaesthetised and under sterile conditions, the first silicon strip was removed and a second implant inserted alongside soleus.

The size of the implants used and therefore the concentration of α-BTX, was according to the dose tolerated by the animals without compromising the paralysis. The size of the implant was 0.25mg on the day of birth, and the animals were checked over the next three days to ensure that the operated hindlimb was indeed paralysed before the second implant was put in. The second implant weighed 0.3-0.35mg and the rats were checked to ensure that the operated hindlimb were paralysed for a total of at least 6 days since birth. The size of the implants could not be any bigger than that used in this study because in addition to the paralysis, these rats were also injected with either L-Dopa or MK-801 which in combination with the α-BTX, severely affected the mortality rate of these rats. The amount of α-BTX in these implants were 5μg at birth and 6-8μg at P3. These amounts of α-BTX used in this study
resulted in paralysis for a total of at least 6 days. Paralysis was confirmed behaviourally according to criteria described by Duxson (1982).

2.2.3. Dorsal Root Section

The segmental afferent input onto the motoneurones was decreased by sectioning the dorsal roots of the L4 and L5 lumbar nerves. Wistar rat pups were anaesthetised with halothane at P4 and the body is cooled by laying the body in a supine position on an ice pack. When the rat pup have stopped breathing and suitably cooled down, the pup is turned over and an incision is made on the skin along the right side of the vertebral column. The position of the incision is guided by a pad of fat which is visible through the skin. The skin was reflected back and the muscles along the vertebral column was cut with a scalpel. A line along the right side of the vertebral column was cut or pinched with a pair of forceps and the cartilage down the side was 'peeled' with a 'flicking over' movement.

Once a ganglion is visible, its position is compared with the angle of the pad of fat referred to before. If the one
visible is just below or right on an imaginary line drawn between the angles of the right and left pads of fat, it is identified as the L5 ganglion. Then, the vertebral column is opened up accordingly (e.g. go rostral for L4). To confirm the identity of the ganglion, compare the size and shape of the ganglia and the distance between them. L5 is more ellipsoid than L4 or L3 which are more circular. L6 and the ganglia caudal to it are very much smaller than L3 or L4 while L5 is in between the size of L4 and L6. L3 and L4 are closer together than L4 and L5. L5 is within the pelvic bone while L4 is above it.

When the L4 and L5 dorsal root ganglia have been identified, the dorsal roots were then sectioned with a pair of iris scissors at two different points and the piece sectioned thus was removed. Great care was taken not to section the ventral roots at this time as the ventral roots lie alongside the dorsal roots.

Pieces of Spongostan were put onto the site of surgery to speed up the process of healing. The skin was approximated together and sutured carefully with the knots hidden so that there was less probability that the mother will bite the sutures off. The wound was dusted with an antibiotic
powder, Cicatrin, which contains Neomycin Sulphate and Bacitracin Zinc. The pup was then warmed under a light. The wound was washed with hibitane and dusted with the antibiotic powder to prevent infection when the rats were inspected daily.

2.2.4. **Injection of L-Dopa**

L-Dopa was injected intraperitoneally for 12 consecutive days. In one group of rats, the treatment started on the day of birth. In another study, the treatment was started on P5 until P17. When the rats were about 9-10 days of age, the effects of the L-Dopa seemed to wear off after a shorter time period. Therefore, a decarboxylase inhibitor, Carbidopa (10mg/kg b.w.) was added to the solution containing L-Dopa. This Carbidopa containing solution was injected into rats aged P10 onwards. After a latent period of 10-15 minutes, the pups will start moving in a stereotypic manner which resembles the locomotion of more mature pups. A dose of 100mg/kg b.w. of L-Dopa will keep the pups moving for 1-1 1/2 hours each time. The nature of the movements elicited by the L-Dopa treatment changes as the rats matures. In the newborns, the forelimbs move while
the hindlimbs remain extended. When the pups are 2-3 days old the hindlimbs also move in tandem with the forelimbs with the abdomen on the ground. The pups pause briefly in between bouts of 'walking'. When they are about 13 days old they run with their bodies above the ground with longer pauses in between the running.

2.2.5. Injection of MK-801

Dizocilpine maleate or MK-801 has previously been used to block the NMDA receptor in the rat hippocampus (Bagetta et al., 1990) and in the spinal cord (Sanner and Goldberger, 1991).

In this study, MK-801 was injected intraperitoneally at a dose of 1mg/kg b.w. for a period of 12 days. This treatment started at birth and the pups were quiet and drowsy after the injections. The pups were started off with a dose of 0.5 mg/kg b.w. at P0, 0.75mg/kg b.w. at P1 and 1mg/kg b.w. at P2 onwards. This dose was lower than that used previously in this lab (Mentis et al. (1993) and Greensmith et al. (1994). This low dose was compatible with a lower mortality rate because in addition to the MK-
801 treatment, these pups had also had α-BTX containing silicon strips put alongside their soleus muscles.

2.3. Retrograde Labelling of Motoneurones

Horseradish peroxidase (HRP) is taken up by motor nerve endings and is retrogradely transported along axons into the cell body where it can be identified. In order to label motoneurones, HRP can be applied in 2 ways either by injecting directly into the muscle or by applying it to the proximal stump of a transacted motor nerve. There are certain problems associated with each method. When HRP is injected into a muscle there is a possibility that the HRP will spread into the surrounding muscles and so the motoneurones of these muscles will also be labelled with HRP. Therefore, the clear labelling of a single motoneurone pool can be difficult. When HRP is applied to the cut end of a nerve, the labelling of the motoneurones can be faint, with variable amounts of reaction product present (McHanwell and Biscoe, 1981a). In this study HRP was injected directly into the Soleus or the TA and EDL muscles of each hindlimb. In this laboratory, this method is routinely used and the motoneurone pools of the muscles
investigated have been identified (see Lowrie et. al., 1987; Greensmith and Vrbová, 1992). Thus, if any spread of HRP does occur this can be immediately noticed. Spread of HRP in adult animals can be limited by carefully injecting the HRP with a Hamilton microsyringe very slowly and holding the syringe in place for a few seconds after finishing, and by immediately mopping up any HRP that leaks from the muscle.

2.3.1. Injection of HRP

The number of motoneurones was assessed when the animals have reached adult age i.e. at least 8 weeks of age with a body weight ranging between 175-500g.

The rats were anaesthetised with Halothane or Chloral Hydrate (4% sterile chloral hydrate at 1 ml/100g b.w., i.p.). Under sterile conditions, a small longitudinal incision was made in the skin and fascia in esch hindlimb, to reveal the underlying muscles. The muscles to be injected were identified. Using a fine Hamilton syringe, the muscle was injected with a 10% solution of HRP (Sigma Type V) diluted in sterile saline (0.9%) (Lowrie at. al.,
This concentration resulted in labelling the cell body without masking the nucleolus. The HRP was administered into several parts of the muscle so that it was evenly distributed throughout the muscle. The total volume of HRP injected into the individual muscle varied according to the body weight of the animal from which one can estimate the weights of the muscle to be injected. It is usually 2μl/100g b.w. for the soleus, 2μl/100g b.w. for the EDL and 8μl/100g b.w. for the TA. The incision was then sutured with 0.7 silk thread (Ethicon) and the animal left to recover from the anaesthetic before being returned to the animal house.

2.3.2. Perfusion

The animals were left for at least 24 hours for the axons to retrogradely transport the HRP back to the cell body before they were anaesthetised with chloral hydrate (4.5%, 1ml/100g b.w., i.p.) and prepared for cardiac perfusion.

The heart was quickly exposed and the apex of the ventricles removed. A blunt needle was carefully advanced through the left ventricle and secured in place with a pair
of artery forceps. An incision was made in the right atrium to allow the perfusate to escape. The animal was perfused briefly with a 0.9% saline solution, using a Watson-Marlow perfusion pump until the perfusate was clear or the liver appeared pale, and then perfused with 2.5% gluteraldehyde in Millonig's phosphate buffer, pH 7.3, for approximately 20 minutes so that 1ml of fixative solution was used per gram of body weight.

2.3.3. **Removal and Preparation of Spinal Cord**

The lumbar region of the spinal cord was carefully dissected out. This region is easily identified as it appears as an obvious enlargement. Each vertebra was carefully removed to reveal the underlying spinal cord. A large proportion of the cord containing the lumbar region was removed and placed in a small volume of the 2.5% gluteraldehyde for postfixing for 2-4 hours at 4°C. The cord was then transferred to a solution of 30% sucrose in Millonig's phosphate buffer, pH 7.3, and kept overnight at 4°C in order to wash out all traces of the fixative and to cryoprotect it.
Using a dissecting microscope, the lumbar region was excised from the rest of the cord. This can be done in an exact manner because the L6 ventral and dorsal roots are easy to identify as they are very much thinner than the preceding L5 roots. The spinal cord was cut with a sharp scalpel blade just below L2 and L6. In order to mark the control side of the cord, a nick was made on the dorsal surface of the left dorsal horn. The block of spinal cord tissue was then cut into transverse sections. It was mounted onto a freezing microtome (Pelcool) and surrounded by dry ice to keep it fully frozen. Serial transverse sections were cut at 50 μm and collected into individual wells in a staining tray containing Millonig's phosphate buffer and then processed for HRP histochemistry.

### 2.3.4. HRP-Histochemistry

A widely used technique for HRP-histochemistry is the Hanker-Yates method (Hanker et al., 1977). Using this method, the staining is less intense than that obtained by other methods, for example, the Tetramethylbenzidine Method (TMB) (Mesulam, 1978), which is so sensitive that the
reaction product tends to mask the nucleolus and nucleus. Thus, using the Hanker-Yates method it was possible to visualise the nucleolus and the Nissl substance by counterstaining and this allowed accurate cell counting and morphometry. A detailed schedule of the modified Hanker-Yates method used in these experiments is described in Appendix II. After processing for HRP the sections were carefully mounted onto gelatinised slides (0.5%) and allowed to dry overnight at 37°C.

2.3.5. Counterstaining

When the sections had fully dried, the RNA and Nissl substance was counterstained with Gallocyanin (Culling, 1963) (see Appendix II for the protocol and preparation of the stain). The sections were stained until the nucleolus and the Nissl substance were clearly visible. The sections were then dehydrated, cleared and coverslipped using DPX as a mounting medium. The slides were left to dry overnight at 37°C before examination under the microscope.
2.4. Microscopy

The sections were examined under a light microscope (Carl Zeiss) for HRP-labelled motoneurone on both the operated and control sides of the spinal cord. The control side of the spinal cord was identified by the nick on the dorsal horn made prior to sectioning. The microscope was fitted with a camera lucida, which allowed cell profiles to be drawn.

2.4.1. Cell Counts

Each section was first carefully scanned under a low power objective (x2.5) to locate the motoneurone pool under study and to ascertain whether there had been any obvious spread of the HRP to motor pools other than that under study. The labelled cells were then examined under a high power objective (x40). Only those motoneurones containing a nucleolus were counted in order to prevent the same cell in consecutive sections being counted twice. The number of labelled cells on each side of the cord were noted separately. When all the motoneurones which were labelled with HRP had been recorded, the total for each side was
added, and the number of motoneurones on the operated side was expressed as a percentage of the number on the control side where possible.

2.4.2. Cell Morphometry

i. Drawing of Cell Profiles

After the number of labelled cells present in each side of the spinal cord had been counted, the cells were then scanned for a second time and the outline of each labelled cell was drawn with the aid of a camera lucida. This was done without any reference to the cell counts already made, and once all the labelled cells have been drawn, the total number of cells drawn were counted. This provided a backup to the initial counts, and if a discrepancy appeared, the section in question was reexamined to assess whether the nucleolus was present, and thus whether the cell should be included in the counts or not.

The outline of each positively stained cell was brought into focus under a x40 objective. If the nucleolus was present the cell outline was traced onto paper using the camera lucida which was attached to the microscope. Each
cell was magnified by around 800x. A rather subjective decision was made about the boundary of the soma as the cellular processes were not included. Each cell was numbered as it was drawn, to allow back reference at a later stage if necessary.

ii. Computer Analysis

After the cell profiles have been traced onto paper, the area of the cell body was measured by transferring the camera lucida drawings onto a Summasketch III (Summagraphics, Newbury, UK) graphics tablet interfaced with a personal computer. Using a stereometry program (Tablyt, designed by Dr. J.E. Cook, UCL), the areas of all the labelled cells from the operated and control side were measured and the accompanying statistical analysis program sorted the data into a frequency distribution histogram for each side. The histogram of the operated and control sides for individual animals was superimposed (FreeLance Graphics version 4) and thus, any shift in the distribution of the areas of the motoneurones could be seen.
2.5. Electrophysiology

2.5.1. Contractile Properties

Isometric tension recording of muscle contraction

Rats aged 4 weeks and 8-10 weeks were anaesthetised with chloral hydrate (4% aqueous solution, 1ml/100g b.w.) and prepared for tension recording of their muscles (Lowrie et. al., 1982). The soleus muscle was freed from the surrounding musculature and the Achilles tendon connected to a strain gauge (Dynamometer UFI) via a silk thread. The tendons of the Tibialis Anterior (TA) and Extensor Digitorum Longus (EDL) muscles were dissected out and connected to strain gauges. Muscle contractions were elicited by stimulating the appropriate motor nerve with a pair of silver electrodes using a pulse width of 0.02-0.05 msec and the contractions were displayed on an oscilloscope screen (Tetronix R5113) or recorded on to a personal computer. Contractions of the soleus was elicited by stimulating its motor nerve while that of the fast TA and EDL was elicited by stimulating the common peroneal nerve. The muscle length was adjusted to obtain maximum twitch tension at supramaximal stimulus strength. Maximum tetanic tension was obtained by stimulating the muscles with a series of pulses.
at different frequencies (10, 20, 40 and 80Hz for soleus and 20, 40, 80 and 100Hz for TA and EDL for 0.5secs). The absolute values of the tensions recorded were expressed in grams. For comparing the effects of the experimental treatments, these absolute values were expressed in grams per 100grams body weight to normalise the body weights of the rats which varied according to its age, sex and the treatment that it had undergone. The muscles and nerves were kept moist throughout the experiment with saline solution and all the experiments were carried out at room temperature (23°C).

Changes in time course of contraction

Two other parameters were measured to assess any changes in the speed of contraction or the relaxation time after contraction. The measurements were made from the traces of the single twitch tensions. 'Time to peak' (TTP) was calculated by measuring the time taken (msec) for the muscle to contract to produce its highest tension which is represented by the peak of the curve, and 'half relaxation time' (1/2RT) was calculated as the time taken (msec) for this maximum tension developed to drop to half its original value.
Fatigue Index (FI)

At the end of the tension recording experiments, the EDL muscles were tested to see how fatigueable they were. The muscles were stimulated repeatedly at 40Hz frequency for 250msecs every second, for a total period of 3 minutes. The calculated fatigue index gives a measure of the strength of the muscle contraction.

2.5.2. Motor Unit Number and Size

To estimate the number of motor units in the muscles studied as previously described by Fisher et. al. (1989), the motor nerve to the muscle was stimulated by single pulses every 4 seconds. The stimulus strength was gradually increased to obtain stepwise increments of twitch tensions, as individual motor units become recruited. These were displayed and saved on an oscilloscope screen. This was continued until a plateau was reached when all motor axons were recruited and tension could no longer be increased. The number of stepwise increments were counted and taken to indicate the number of α-motor axons present in the nerve.

The motor unit size was obtained by dividing the maximum tetanic tension of each muscle by the number of motor units.
identified in it. The motor unit size is the tension from the muscle fibres that an individual motoneurone innervates.

### 2.6. Histology and Histochemistry

#### 2.6.1. Cholinesterase-Silver Stain

Normal, control and experimental animals were anaesthetised with chloral hydrate (4.5%, in saline, i.p.). The soleus and the EDL muscles were quickly removed and pinned in a slightly stretched position on a Sylgard coated Petri-dish. They were covered in fixative (4% formaldehyde) for 2 hours and then washed for 30 minutes. Longitudinal sections were cut on a freezing microtome (Pelcool) at 50 μm, and the sections were collected into individual wells of a collecting tray.

The endplates and axons were then visualised using a modified, combined cholinesterase-silver stain (Namba et. al., 1967; O'Brien et. al., 1978) which allows simultaneous viewing of endplates and axons (see Appendix III for method). The stained sections were mounted with DPX (BDH)
and examined under a light microscope (Zeiss). The endplate region was shown up with a brown deposit and the nerve axons were stained black due to the impregnation with silver. The muscles were examined to determine whether the endplates appeared normal or not.

2.6.2. Freezing and Storage of Muscles

In those muscles examined by histochemistry, the animal was anaesthetised with chloral hydrate (4% in saline, i.p.) and the muscles removed and mounted on a piece of cork. Each muscle from experimental animals were mounted together with the corresponding muscle from control animals. This made it possible to examine, for example, the SDH activities in fibres of two different muscles under identical conditions (Reichmann and Pette, 1982). The muscles were coated with embedding medium (Tissuetek) and quickly frozen in melting isopentane. The block was stored briefly in liquid nitrogen before being transferred to a freezer at -70°C. Muscles were stored for a period of up to two months before being sectioned. Transverse sections were cut at 10 μm at -25°C using a cryostat (Bright) and collected onto gelatinised glass slides. They were left to dry before staining.
2.6.3. **Succinic-dehydrogenase Stain**

This stain visualises the oxidative enzyme, succinic dehydrogenase (SDH). The enzyme is bound to the inner mitochondrial membrane and is an indication of the oxidative capacity of the muscle (Nachlas et. al., 1957). Thus it was possible to assess whether the experimental treatment had altered the oxidative capacity of the muscle (see Appendix IV, for method).

2.6.4. **Slow myosin**

The phenotypic expression of slow myosin in the developing soleus and EDL muscle was examined. Immunocytochemistry was performed on frozen sections using a specific antibody directed against slow myosin (Dhoot et. al., 1986). The antibody binding was detected by diaminobenzidine (DAB) in tris buffer with hydrogen peroxide (see Appendix V for a detailed protocol). Positively stained fibres appear dark brown.
CHAPTER 3 - THE EFFECTS OF INCREASING AFFERENT ACTIVITY TO DEVELOPING NORMAL AND INJURED MOTONEURONES

3.1 INTRODUCTION

During the early postnatal period, motoneurones are known to be dependent on their target for survival. If motoneurones are denied contact with their target during the first 5 days of their life, a large proportion of them would die. Those motoneurone that do survive target deprivation have been shown to exhibit increased levels of activity (Navarrete and Vrbová, 1984). These observations have led to the proposal that inappropriate levels of activity may play an important role in the death of target-deprived motoneurones.

In this study it was assessed whether inducing an increase of afferent activity during the early postnatal period to developing normal and injured motoneurones will have any effect on the number of motoneurones to the slow, soleus or fast, TA and EDL muscles.

Increased afferent input was achieved by inducing precocious locomotor activity in the neonatal rats by
repeated treatment with L, dihydroxyphenylalanine (L-Dopa). L-Dopa is a precursor of the catecholamine transmitters, dopamine (DA) and norepinephrine (NA). It is used therapeutically in Parkinson's Disease to replace dopamine in the nigro-striatal tract (Hornykiewicz, 1974). Decrease in locomotor activity in animals that had dopamine depletion in the brain due to loss of dopaminergic neurone can be reversed by treatment with L-Dopa (Kanthasamy et. al., 1994). L-Dopa administration have been shown to induce locomotor activity in the neonatal rat (Van Hartesveldt et. al., 1991; Kellogg and Lundborg, 1972) and this locomotor activity elicited by L-Dopa is a highly robust phenomenon and easily recognised (Van Hartesveldt et. al. 1991; Iwahara et. al., 1991).

In studies on the development or control of locomotion, L-Dopa can evoke stereotyped behaviour in the decerebrate or spinal animal (Grillner and Zanger, 1979; Barbeau et. al., 1993). Barbeau and Rossignol (1991) reported that the movement pattern and the electromyographic (emg) activity in spinal cat given L-Dopa resemble those in the intact cat in many respects. In lamprey, adding D-glutamate or L-Dopa readily induced the electrical equivalent of swimming activity (Poon, 1980; Cohen and Wallen, 1980).
Therefore, dopaminergic neurotransmission is of basic importance in locomotor activity. Dopaminergic descending pathways play a role in activating the spinal networks involved in the generation of rhythmic locomotion. How exactly L-Dopa induce locomotor activity is not clear, and a detailed discussion is beyond the scope of this study.

Many studies carried out to analyse the central effects of L-Dopa administration in animals have shown that both NA and DA produced in the brain from the L-Dopa are involved in inducing the increased locomotor activity (Anden, 1970; Carlsson, 1970; Svensson and Waldeck, 1970; Kellogg and Lundborg, 1972). Both dopaminergic and noradrenergic receptors in the brain are stimulated by L-Dopa treatment (Hornykiewicz, 1973; Corrodi et. al., 1970). Grillner (1973) and Anden et. al. (1966) hypothesised that L-Dopa excite the motor pattern generator indirectly by stimulating the synthesis and release of noradrenaline from the terminals of a descending noradrenergic pathway. Noradrenergic terminals are present in the spinal cord and originate from descending neurones in the brain stem (Carlsson et. al., 1964).
Dolphin et. al. (1976) found that increased noradrenaline synthesis occurs following L-Dopa administration which correlated with the observed elevation of locomotor activity. They suggest that L-Dopa increased turnover of NA by the mechanism of granular displacement of NA by DA and that this increased NA turnover may be responsible for the locomotor response to L-Dopa. Exogenously applied L-Dopa potentiate D2 receptor mediated locomotor activities and this effect is similar to that of endogenously released L-Dopa (Nakamura et. al., 1994; Yue et. al., 1994). Therefore L-Dopa administration may elicit locomotor activity in rats in a similar way that locomotion is normally produced. The patterns of motoneurone activity during the L-Dopa evoked locomotion resemble those found in more mature animals (Navarrete and Vrbová, 1985).

Since there are indications that the motoneurones that die when the neuro-muscular interaction was interrupted during the early postnatal period may do so due to excitotoxicity (Greensmith et. al., 1994), the first question asked in this chapter was whether inducing an increase of afferent activity during the early postnatal period to normal soleus, TA and EDL motoneurones will affect the number of these motoneurones in the adult animal.
During early postnatal development, as the neonatal rat starts to move about, the amount of afferent activity in the central nervous system increases. Most neurones express NMDA receptors and there is evidence to show that generation of locomotor activity can be elicited by activating these cells (McClellan and Farel, 1985; Dale and Roberts, 1984). Since glutamate, an excitatory neurotransmitter is thought to be neurotoxic, motoneurones must, during its development acquire the ability to withstand this toxic effect of the neurotransmitter. Inducing precocious locomotor activity may destroy some motoneurones that have not achieved a sufficient degree of maturity to withstand such vigorous activity. Whether the increase in afferent activity to motoneurones brought about by the precocious locomotor activity may influence death of normal developing motoneurones was examined in this study.

The second question asked in this study was whether the afferent inputs onto motoneurones have any role on the survival of these motoneurones when the neuromuscular interaction was interfered with. In this study, the 'injured nerve' paradigm was achieved by disrupting the neuromuscular interaction for an extended period of time during development.
Following injury to motoneurones during a critical period during postnatal development, a significant number of motoneurones die. But if the injury was inflicted after the critical period, the motoneurones survive to adulthood. However, although the motoneurones survive there were changes in the motoneurones that may have contributed to changes in the muscles that these motoneurones innervate. Lowrie (1990) has shown that a second injury inflicted on motoneurones that had been injured previously after the critical period did result in motoneurone death. This shows that an extended period of interruption still renders the motoneurones to be susceptible to the injury even though the period of interruption was after the critical period. In this study, the possibility that increased motoneurone activity would have an effect on the number of motoneurones that survive such an extended period of neuromuscular disruption was investigated.

The third question asked in this chapter was whether there were any effects of increased afferent activity on the survival of uninjured motoneurones prevented from target interaction. In this experiment, the soleus muscle was paralysed with the implantation of a silicon strip that contained α-BTX at birth and at P3. Preventing
neuromuscular interaction for a longer period of time resulted in a longer period of paralysis which in turn caused a bigger amount of motoneurone death (Greensmith and Vrbová, 1992). Paralysis interrupts the neuromuscular interaction while at the same time preserving the afferents to the motoneurones. This is different from nerve crush injury which has been shown to result in sensory neurone death (Himes and Tessler, 1989; Devor et. al., 1985) in addition to the motoneurone death. Preserving sensory afferents may result in a maximal increase of afferent input to the motoneurones when locomotor activity is induced precociously in the neonatal rats.

In summary, the questions asked in this chapter are:

1. Whether precocious increase in afferent activity will influence the survival of normal motoneurones to the slow, soleus and fast, TA and EDL muscles.

2. Whether the number of motoneurones that die after an extended period of neuromuscular interruption (target-deprivation) would increase if the afferent inputs were increased.
3. Whether the extent of motoneurone death due to deprivation of motoneurone-target interaction by paralysis is enhanced by increased afferent activity.
3.2. MATERIALS AND METHODS

Neonatal Wistar rats of both sexes were used in the experiments in this chapter. They were at various ages when the first experiments were carried. The final experiments were carried out when these rats have reached adult age except in the first group where some rats were assessed when they were 4 weeks old.

The specific procedures used in this chapter are described in detail in Chapter 2.

The experiments described in this chapter are divided into 3 different groups, and are described below;

3.2.1. Neonatal rats were injected with L-Dopa or saline from the day of birth (P0) for 12 days. Treatment with L-Dopa induced precocious locomotor activity in the pups which would otherwise remain very quiet. The saline treatment served as control. When the rats were at least 8 weeks old the motoneurones to the soleus and TA and EDL muscles were examined by retrograde labelling with HRP. Changes of physiological properties of skeletal muscles
usually reflect altered motoneurone activities. In another group of rats, the soleus, TA and EDL muscles were assessed with tension recording experiments when the rats were 4 weeks old or were least 8 weeks old. Some of these muscles were examined histologically. Adult muscles were sectioned and stained for cholinesterase and with silver to visualise the endplates and the axons innervating them. To determine whether L-Dopa treatment affected the oxidative metabolism of the muscles, sections of soleus, TA and EDL muscles of P12 pups treated with saline or L-Dopa were stained for SDH. Immunocytochemistry was also performed on sections from soleus and EDL muscles of P12 pups to determine whether the L-Dopa treatment had any effects on the induction of slow myosin during the period of treatment.

3.2.2. The sciatic nerve in the right hindlimb of neonatal rats of both sexes was crushed with a watchmaker's forceps when the rats were 5 days old (P5) and again when they were 10 days old (P10). The rats were observed to ensure that the crushes resulted in no toe spreading reflex for a certain period of time after which the rats should regain the normal use of their lower legs. In addition, these rats were injected with either L-Dopa or saline for 12 days
starting on P5. When the rats were at least 8 weeks old, the motoneurones to the TA and EDL muscles were examined by retrograde labelling with HRP.

3.2.3. The soleus muscle in the right hindlimb of neonatal rats was paralysed with a silicon implant containing α-BTX within 6 hours of birth (P0). This operation was repeated when the rats were 3 days old (P3). The rats were observed daily to ensure that the paralysis had indeed occurred by observing the spontaneous and reflexly elicited movements of the treated hindlimb. This indication of the effect of the α-BTX containing implant on the use of the lower leg gives an indirect indication of paralysis of the soleus muscle itself. In addition, these rats were injected with L-Dopa or saline for 12 days starting from P0. When the rats were at least 8 weeks old the motoneurones to the soleus were examined by retrograde labelling with HRP.
3.3. RESULTS

3.3.1. EFFECTS OF INCREASING THE ACTIVITY OF DEVELOPING MOTONEURONES

i) Changes In The Numbers of Motoneurones to the Soleus Muscle in Animals Treated with L-DOPA

Following nerve injury at birth a large proportion of motoneurones die. Blocking NMDA receptors which is likely to decrease excitatory influences to the motoneurones protects a considerable proportion of motoneurones destined to die. Here the possibility was examined whether increasing excitatory influences to motoneurones during early postnatal life will affect the survival of motoneurones to soleus. Precocious locomotor activity can be expected to increase excitation of the appropriate motoneurone pools at an age when activity of these neurones is elicited only rarely. Therefore, in this study excessive locomotor activity of newborn pups was elicited by repeated treatment with L-Dopa. Intraperitoneal injection of L-Dopa to rat pups aged P0 to P11 induced excessive locomotor activity after a latent period of 10-15 minutes. The pups were actively moving, paddling their forelimbs and
hindlimbs; they seemed to be trying to support themselves on their hindlimbs. This swimming-like movement has been previously described (Bolles and Woods, 1964; Altman and Sudarshan, 1975) and in the present experiment this stereotyped, premature activity lasted for at least 1 1/2 hours after each injection of L-Dopa. When the effects of the L-Dopa have receded and the pups have returned to their pre-injection quiet state, they were taken back to their mothers. This treatment started on the day of birth and continued for 12 days.

Littermates of this group of rats were given daily i.p. injections of saline for 12 days after birth instead of the L-Dopa. The saline injections did not change the behaviour of the pups. They remained quiet, dormant and did not move very much which is the typical behaviour of neonatal rats. They were kept away from their mother for the same period of time as the L-Dopa treated pups. The difference in behaviour between these two groups of animals was obvious and easily recognised (Van Hartesveldt et. al., 1991). It can be observed clearly that the L-Dopa treated rats moved about more in comparison to the saline treated pups.
As another indicator of the difference in the amount of activity exhibited by the neonatal rats during the period of treatment, the body weights of the pups were monitored and compared. The L-Dopa treated rats always weighed less than the saline treated pups during the treatment period and this was possibly due to the increase in their energy consumption due to the excessive locomotor activity. An example showing the difference in body weights due to the different treatments is seen in Figure 1. It shows a graph in which the body weights of pups from the same litter were plotted against age. Values were taken from just one litter since the birth weight of pups vary according to the number of pups born in the litter. The number of L-Dopa treated pups in this litter was three while the number of saline treated pups was one. As can be seen from the figure, the difference in the body weights between the two groups of animals increased with time during the treatment.

Two to 3 months later, the number of motoneurones to soleus was assessed by retrograde labelling with HRP. The soleus muscle on both sides were injected with HRP. After the animal had been perfused, the spinal cord was taken out and the sections cut from the spinal cord were then reacted for HRP histochemistry. The sections were counterstained to
Figure 1. Body weight increase of rats during the period of treatment with L-Dopa or saline.

Graph of age of animals from one litter plotted against the weight increase of the animals during the period of treatment with saline (n=1) or L-Dopa (n=3). As the animals increased in age the difference in the weight increase became more apparent.
FIGURE 1
show the nucleus and nucleolus of all cells. The location of the different motoneurone pools can then be discerned more easily and the soleus motoneurone pool identified.

Figure 2 shows photomicrographs of a transverse section taken from a spinal cord at the level of L4. The neurones that contain the dark granules are motoneurones which had been retrogradely labelled with HRP and show the product of the histochemical reaction. Figure 2A shows the position of the soleus motoneurone pool in the ventral horn. This pool is located dorsomedially in comparison to the other motoneurone pools present in the spinal cord at the region examined and is found in the L4 and L5 spinal segments. This finding concurs with the findings of Lowrie et al., 1987; Greensmith and Vrbová, 1992; and Nicolopoulos-Stournaras and Iles, 1983. To avoid counting a cell more than once only those neurones where a clearly defined nucleolus was seen as shown in Figure 2B were counted.

The results obtained from these experiments are summarised in Table 1A and 1B which show the numbers of HRP-labelled motoneurones innervating the soleus muscle of both legs of rats treated with either L-Dopa or saline. There was no
Figure 2. Motoneurone pool of soleus muscle.

Microphotographs of sections taken from a spinal cord at the level of L4 of a rat to show HRP retrogradely labelled motoneurones. Figure 2A shows the position of the motoneurone pool of the soleus muscle in the ventral horn of the spinal cord. Figure 2B shows the different intensity of HRP labelling that is possible. Only motoneurones with a clearly defined nucleolus as seen in cell body 'a' were counted.

The scale bar in Figure 2A = 500µm
The scale bar in Figure 2B = 50µm
FIGURE 2

A

B

a
Table 1A. The effect of treatment with L-Dopa on the number of motoneurones to soleus muscle.

Table 1B. The effect of treatment with saline on the number of motoneurones to soleus muscle.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after treatment with L-Dopa (Table 1A) or saline (Table 1B) was assessed in adult rats. The number of labelled motoneurones on the right side was expressed as a percentage of the number of labelled motoneurones on the left side. The number of labelled motoneurones on the right side is similar to that in the left side and so the mean number of motoneurones to the soleus muscle in each group of animals was calculated.
**TABLE 1A: L-DOPA TREATED**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
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<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
<td>% Rt/Lt</td>
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<td>49</td>
<td>31</td>
<td>63.27</td>
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Mean ±S.E.M = 35 ± 1.8

**TABLE 1B: NORMAL, SALINE-TREATED**

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Mean ±S.E.M = 55 ± 1.8
significant difference between the numbers on the left and right sides of the spinal cord.

In the L-Dopa treated animals, the mean number of motoneurones on the left side was 36(±3.2 S.E.M, n=7) and on the right was 34(±1.9 S.E.M, n=7). Taking both sides together, the mean number of motoneurones innervating the soleus muscle of the L-Dopa treated rats was 35(±1.8 S.E.M, n=14).

Table 1B shows the number of retrogradely labelled motoneurones on the left and right side from control rats that were treated with saline during the first 12 days of life. The mean motoneurone number for the left side was 56(±3.7 S.E.M., n=9) and the right side was 55(±2.8 S.E.M., n=9). The mean number of motoneurones innervating the soleus in the saline-treated rats was calculated by pooling the data from both the left and the right side. It was 55(±1.8 S.E.M, n=18) and this finding is consistent with previous findings for the number of motoneurones to the soleus muscle.

A bar diagram of the mean (±S.E.M) numbers of motoneurones to the soleus muscle from animals treated with L-Dopa or
saline is shown in Figure 3. A comparison of the numbers of motoneurone of the two groups of animals showed that the L-Dopa treated rats had significantly fewer motoneurones than the saline treated group (Mann-Whitney U Test, p<0.001). Thus, L-Dopa treatment reduced the number of motoneurones to the soleus muscle.

ii) The Effects of L-Dopa Treatment on Motoneurones to the Tibialis Anterior and Extensor Digitorum Longus (TA/EDL) Muscles

L-Dopa treatment had significantly reduced the number of motoneurone to soleus. Previous studies show that the increase of EMG activity induced by L-Dopa is greater in the slow soleus than in the fast EDL muscle (Navarrete and Vrbová, 1985). Therefore it could be that motoneurones to the fast TA and EDL muscles may respond differently to L-Dopa treatment. A group of newborn Wistar rats was treated daily with L-Dopa for 12 days as described previously. This treatment induced an increase in the locomotor activity of the pups which lasted for at least 1 1/2 hours. Littermates of this group of animals were injected with saline instead
Figure 3. The effect of treatment with saline or L-Dopa on the mean number of motoneurones to the soleus muscle.

The bar diagram shows that the mean motoneurone number to the soleus muscle in the saline treated animals was 55 ±1.8, n=18, while that in the L-Dopa treated rats was 35 ±1.8, n=14.

It can be seen that L-Dopa treatment reduced the number of motoneurones to the soleus muscle.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 3

Mean Motoneurone Number

SALINE
L-DOPA
of the L-Dopa from birth until they were 12 days old. They were separated from their mother for the same length of time as the L-Dopa injected pups. Observations regarding the behaviour and the weight gain of the pups in response to the treatments are similar to that of the previous group (see Figure 1).

Two-three months later the effect of this treatment on the motoneurones to the TA and EDL was assessed. The same method of HRP retrograde labelling was used as in the previous experiment except here the TA and EDL muscles were injected.

The position of the motoneurone pool of the TA and EDL muscles in the ventral horn is shown in Figure 4. The TA and EDL motoneurone pool is more dorsolateral to the soleus pool and extend from the L3 to L5 segment of the lumbar region in the spinal cord. The position of the motor pool found is consistent with that documented by Lowrie et. al., (1987).

The number of motoneurones innervating the TA and EDL muscles in the L-DOPA treated rats is shown in Table 2A. The mean number of motoneurones on the left side was
Figure 4. Motoneurone pool of TA and EDL muscles.

The figure shows the position of the motoneurone pool of the TA and EDL muscles in the ventral horn of the spinal cord.

The scale bar = 500μm
Table 2A. The effect of treatment with L-Dopa on the number of motoneurones to the TA and EDL muscles.

Table 2B. The effect of treatment with saline on the number of motoneurones to the TA and EDL muscles.

The number of motoneurones to the TA and EDL muscles labelled with HRP on each side of the spinal cord after treatment with L-Dopa (Table 2A) or saline (Table 2B) was assessed in adult rats. The number of labelled motoneurones on the right side was expressed as a percentage of the number of labelled motoneurones on the left side. The number of labelled motoneurones on the right side is similar to that in the left side and so the mean number of motoneurones to the soleus muscle in each group of animals was calculated. Treatment with L-Dopa did not seem to affect the number of motoneurones to the TA and EDL muscles.
### TABLE 2A: L-DOPA TREATED

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Mean ±S.E.M = 167 ±5.5

### TABLE 2B: NORMAL, SALINE TREATED

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Mean ±S.E.M = 160.6 ±6.7
166(±7.2 S.E.M, n=5) and 168(±9.2 S.E.M, n=5) on the right side. These two groups of data are not significantly different from each other (p=0.84, Mann-Whitney U Test). The mean number of motoneurones in the TA and EDL pool was calculated by pooling the values on the left and the right side and found to be 167(±5.5 S.E.M, n=10). This value is similar to that reported by Lowrie et. al. (1982; 1987).

Table 2B summarises the results obtained from saline treated animals. It shows that the mean number of motoneurones of the TA and EDL muscles on the right side was 164(±11 S.E.M, n=5) and on the left side was 158(±8.7 S.E.M, n=5). The mean numbers of motoneurones from the two sides were not significantly different. The mean number of motoneurones innervating the TA and EDL was calculated as before and found to be 161(±6.7 S.E.M., n=10).

When comparing the numbers of motoneurones innervating the TA and EDL muscles of rats that were treated with saline with those of rats that were treated with L-Dopa during the early postnatal development, they were found to be similar (p=0.63, Mann-Whitney U Test). Thus, L-Dopa treatment had no effect on the number of motoneurones to the TA and EDL muscles.
To compare directly the different effects of increased locomotor activity on the two types of muscle (i.e. slow and fast), the results from both experiments are shown in the bar diagram of Figure 5. The bars show the mean (±S.E.M) numbers of motoneurones from each experimental group supplying soleus and TA and EDL. As can be seen, L-Dopa treatment lead to a significant reduction of the number of motoneurones to soleus, but had no effects on the number of motoneurones of the TA and EDL pool. This result is consistent with the finding that L-Dopa treatment increased the EMG activity in the slow soleus to a greater extent than in the fast EDL muscle. It is however, possible that soleus motoneurones of neonates are more susceptible to damage caused by excess activity.

iii) The Effects of Increased Activity on the Number of Motor Units of Soleus

In the next part of this study the possibility was investigated that the reduction of retrogradely labelled cells found in the soleus motoneurone pool after increased activity shortly after birth was also reflected in the
Figure 5. The effect of treatment with saline or L-Dopa on the mean number of motoneurones to the soleus and to the TA and EDL muscles.

The bar diagram illustrates that the mean number of motoneurones to the soleus muscle was reduced after treatment with L-Dopa while that to the fast, TA and EDL muscles was not affected.

The error bars represent the Standard Error of the Mean (S.E.M.)
FIGURE 5

Mean Motoneurone Number

SOLEUS

TA/EDL

Saline  L-Dopa
number of α-motoneurone to the soleus muscle. Therefore, the number of motor units of the soleus muscle was assessed physiologically. This was done by recording stepwise increments of twitch tensions elicited by stimulation of the motor nerve with stimuli of increasing intensity. These final experiments were carried out on animals 4 or 8 weeks after they were treated either with L-Dopa or saline. Figure 6 shows an example from an experiment in which the number of motor unit to the soleus muscle was assessed. Fig. 6A is from a saline treated 4 weeks old rat while Fig. 6B is from an L-Dopa treated 4 week old rat.

The numbers of motor units in the soleus muscle in the L-Dopa treated rats were compared with those in the saline treated rats and the results are summarised in Table 3 and shown graphically in Figure 7A. At 4 weeks of age, the number of motor units of the soleus muscle in rats that had been treated with L-Dopa was significantly less (Mann-Whitney U Test, p<0.001) than that in rats which had been treated with saline. It was 23.88 (±0.72 S.E.M., n=8) in the L-Dopa treated rats and 31.5 (±1.09 S.E.M., n=6) in the saline treated rats. Another group of animals was assessed when they were 8 weeks old. The mean motor unit number in the L-Dopa rats was 26.71 (±0.47 S.E.M., n=7) while that in
Figure 6. The number of motor units in the soleus muscle of 4 weeks old rats that had been treated with saline or L-Dopa.

The figure shows examples of experiments in which the number of motor units to the soleus muscle was assessed. The stimulus to the motor nerve was increased slowly and the stepwise increase in the twitch tension was counted. Fig. 6A is from a saline treated 4 weeks old rat and shows that there are 28-30 motor units while Fig. 6B is from an L-Dopa treated 4 weeks old rat and shows that there are 19-20 motor units. Tension is expressed in grams (g) and time in milliseconds (ms).
FIGURE 6
The effects of treatment with L-Dopa or saline during the early postnatal development on the number of soleus motor unit and their size was assessed at two different ages, 4 and 8 weeks. The values shown are mean values ± S.E.M. The pairs of symbols denotes that the two values having the same symbols were significantly different from each other.
Figure 7. The effect of treatment with saline or L-Dopa on the number and size (g/100g b.w.) of soleus motor units.

The effect of treatment with saline or L-Dopa on the number of motor units of the soleus muscle (Fig. 7A) and their size (g/100g b.w.) (Fig. 7B). The values shown are the mean values ±S.E.M. The animals were assessed at 2 different time points, at 4 weeks and at 8 weeks.

The error bars represent the Standard Error of the Mean (S.E.M.)
FIGURE 7

A

Soleus

Motor Unit Number

4 Weeks 8 Weeks

Saline □ L-Dopa

B

Soleus

Motor Unit Size (g/100g b.w.)

4 Weeks 8 Weeks

Saline □ L-Dopa
the saline treated rats was 30.8 (±0.49 S.E.M., n=5). This difference was significant as assessed by the Mann-Whitney U Test (p<0.01). The number of motor units of the soleus muscle in the saline treated rats assessed at the two time points is similar to that reported previously (Zelená and Hník, 1963; Brown et. al., 1976; Close, 1967; Connold et. al., 1992).

The motor unit size was then calculated by dividing the maximum tetanic tension which was expressed in grams per 100 grams body weight (g/100g b.w.) by the number of motor units identified in the soleus muscle. The tetanic tension was recorded in grams and was expressed in g/100g b.w. to standardise the body weights of the animals which varied considerably between 4 and 8 weeks of age. The absolute values and the values calculated per 100g b.w. for the tetanic tension are presented in Table 4. As can be seen in Table 3, at 4 weeks of age, the mean motor unit size in the L-Dopa treated animals was 2.1g/100g b.w.(±0.11 S.E.M., n=8) while that in the saline treated animals was 1.27g/100g b.w.(±0.19 S.E.M., n=6). This difference was significant as assessed by the Mann-Whitney U Test (p<0.01). On the other hand, as shown in Figure 7B, the
Table 4. The effect of treatment with saline or L-Dopa on the mean body weight of the animal, mean muscle weight and contractile properties of soleus, and the number of motor units and motor unit size of the soleus muscle.

These values were taken at 2 time points: when the animals were 4 weeks old and 8 weeks old.

SEM = standard error of the mean,
\( n \) = the number of muscles assessed,
TTP = time to peak
\( 1/2RT \) = half relaxation time
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<th>Muscle Weight</th>
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<th>Motor Unit</th>
<th>TTP</th>
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**TABLE 4**
motor unit size proved to be not significantly different in the two groups of rats when assessed at 8 weeks of age.

These experiments show that excessive afferent activity during the early postnatal period reduced the number of motor units when assessed at 4 weeks and the motor units present at this time were able to develop significantly larger tensions. Assessed at 8 weeks of age, the same treatment during the early postnatal period caused a reduction in the number of motor units of the soleus muscle but it did not significantly affect the motor unit size.

**Effects Of Increasing Afferent Activity On Motor Unit Numbers And Sizes Looked at Two Different Ages**

L-Dopa treatment had significantly decreased the motor unit number of soleus when assessed at 4 weeks and at 8 weeks. The motor unit size was increased significantly when assessed at 4 weeks but not when assessed at 8 weeks. This indicated that some of the changes induced by L-Dopa treatment during the early postnatal development were not permanent. A direct comparison between results obtained at 4 and 8 weeks was therefore carried out. The results are
summarised in Table 3. The table shows that in the L-Dopa treated rats, the number of motor units of the soleus was significantly higher at 8 weeks at 26.71(±0.47 S.E.M., n=7) than at 4 weeks at 23.88(±0.72 S.E.M., n=8). The numbers of motor units were similar at 4 and 8 weeks in the saline treated rats. This can also be seen in Figure 7A.

The greater number of motor units in the 8 weeks old rats was also reflected in the size of their motor unit. As can be seen in Figure 7B, unlike in 4 weeks old rats, at 8 weeks the motor unit size was no longer expanded and was no different from that of the saline treated rats.

These results indicate that some of the changes after L-Dopa treatment were less apparent at 8 weeks.

iv) The Effects of Treatment with L-Dopa on the Contractile Characteristics of Soleus

L-Dopa treatment induced premature locomotor activity in newborn rats. Whether this premature activity had an effect on the contractile properties of the slow soleus muscle was
examined next. Newborn rats were treated with L-Dopa or saline as previously described. A group of rats was assessed at 4 weeks of age when they have not yet attained their adult muscle properties. The same experiments were also done on another group of rats at 8-10 weeks. At this time the rats were supposed to have reached their adult muscle physiological properties.

The mean absolute values in grams recorded for the soleus muscle weight, maximum twitch tension and maximum tetanus tension and the same values expressed per 100 grams body weight (b.w.) are summarised in Table 4. The table also show the mean body weight of the animals, the time to peak (TTP) and the half relaxation time (1/2RT). Control values were recorded from rats that had been treated with saline. The values expressed in g/100 g b.w. were used when comparing the results obtained from the L-Dopa and saline treated group.

**Twitch and Tetanic Tensions**

The effect of excessive locomotor activity induced by the L-Dopa treatment on the tensions developed by the soleus
was assessed. Twitch and tetanic tensions were recorded in vivo from the soleus muscle of rats that had been treated with L-Dopa. Control values were taken from saline treated animals. Figure 8 shows an example of the trace of single twitch and tetanic contractions developed by the soleus muscle of a 4 weeks old rat. Table 5 summarises the mean values(±S.E.M.) of the twitch and maximum tetanic tensions for soleus recorded at 4 and 8 weeks. The mean twitch tension developed by the soleus muscle in the L-Dopa treated rats was higher than that developed in the saline treated rats at 4 weeks but not at 8 weeks. These differences in the mean twitch tensions are not significant and this can be seen in Figure 9B. The soleus muscles in the L-Dopa treated rats also developed higher tetanic tensions at 4 and 8 weeks as shown in Figure 9C but again, the differences are not significant.

Therefore, at 4 and at 8 weeks of age, increasing the locomotor activity by treatment with L-Dopa during the postnatal period did not affect the twitch and tetanic tensions developed by soleus although there was a tendency for the tensions to be higher in the L-Dopa treated rats.
Figure 8. Twitch and tetanic contractions from soleus muscles of 4 weeks old rats that had been treated with saline or L-Dopa.

Each trace shows single twitch (st) and tetanic contractions (20, 40 and 80 Hz) from the soleus muscles elicited by stimulation of their motor nerves. Trace 8A is from an animal that had been treated with saline from P0 to P12. Trace 8B is from an animal that had been treated with L-Dopa from P0 to P12. Tension is expressed in grams (g) and time in milliseconds (ms).

It can be seen that in this example the maximum tetanic tension developed by the L-Dopa-treated rat was bigger although the body weights of the two rats were similar. However, the mean value of the maximum tetanic tension developed by the L-Dopa-treated animals was not significantly different from that developed by the saline-treated animals.
FIGURE 8
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</table>

Newborn rats were treated with L-Dopa or saline for 12 days. The muscle weight and twitch and maximum tetanic tension developed by the soleus were assessed at 4 or 8 weeks of age. The values shown are the means ±S.E.M. The pairs of symbols denote that the values with the same symbols are significantly different from each other.
Figure 9. The effect of treatment with saline or L-Dopa on muscle weight of soleus, the maximum twitch tension and tetanic tension developed by the soleus.

The effect of treatment with saline or L-Dopa on muscle weight of soleus (Fig. 9A), the maximum twitch tension (Fig. 9B) and tetanic tension (Fig. 9C) developed by the soleus. The values shown are the mean values ±S.E.M. (g/100g b.w.). The animals were assessed at 2 different time points, at 4 weeks and at 8 weeks.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 9
Changes in Tensions Developed by Soleus due to Age

The muscle contractile characteristics might change due to further maturation and this would be seen by comparing the tensions developed at 4 weeks of age directly to the tensions developed at 8-10 weeks of age. Whether increasing locomotor activity during the early postnatal period would result in further changes of contractile characteristics of the slow soleus muscle was ascertained next.

As the rats mature further, the mean twitch tensions (g/100g b.w.) developed by soleus was smaller although the reduction was not significant as seen in the saline treated rats (see Table 5 and Figure 9B). An example of the traces showing the effects of age on twitch and tetanic contraction developed by the soleus muscle is shown in Figure 10. Interestingly, this reduction is significant in the L-Dopa treated rats. The tetanic tensions developed by the slow soleus in the saline treated rats were not different at 4 and 8 weeks but in the L-Dopa treated group the tetanic tensions developed at the two ages were significantly different (see Table 5 and Figure 9C).
Figure 10. Twitch and tetanic contractions from soleus muscles of 4 and 8 weeks old rats that had been treated with saline during the neonatal period.

Each trace shows single twitch (st) and tetanic contractions (20, 40 and 80 Hz) from the soleus muscles elicited by stimulation of their motor nerves of animals that had been treated with saline from P0 to P12. Trace A is from a 4 week old rat. Trace B is from an 8 week old rat. Tension is expressed in grams (g) and time in milliseconds (ms).

Although the tensions developed by the older and thus, bigger rat are bigger than that developed by the 4 week old rat, when these values are expressed in g/100g b.w., the tensions developed are found to be similar. In the 4 weeks old rat, the twitch tension developed by the soleus is 5.5g/100g b.w. while the maximum tetanic tension is 50g/100g b.w. In the 8 weeks old rat, these values are 5.6g/100g b.w. and 45.7g/100g b.w. respectively.
FIGURE 10

A

\[ 5g \]

\[ 80 \text{ Hz} \]

\[ 40 \]

\[ 20 \]

\[ \text{st} \]

B

\[ 50g \]

\[ 80 \text{ Hz} \]

\[ 40 \]

\[ 20 \]

\[ \text{st} \]

\[ 200 \text{ ms} \]
Therefore, increasing activity by L-Dopa treatment caused significant changes in the differences due to age in the twitch and tetanic tensions developed by soleus muscle.

**Changes in Soleus Muscle Weights**

The muscle weights of soleus were examined to see if there was any correlation with the tension developed. The results are presented in Table 5 and shows that at 4 weeks of age, soleus muscle from L-Dopa treated rats weighed significantly (p<0.01, Mann-Whitney Test) more than that from saline treated rats. This effect however, did not persist because at 8 weeks of age the muscle weights were similar to each other. These results do correlate with the tension results as can be seen by comparing Figure 9A to Figure 9B and 9C.

When examined across time, the weight of soleus muscle per 100g b.w. was significantly increased in the 8 weeks old saline treated rats. However, in the L-Dopa treated rats, the muscle weight of soleus was similar at both 4 and 8 weeks. This shows that increasing locomotor activity during the early postnatal period resulted in temporary changes in
the muscle weights that were no longer discerned when the rats reached adulthood.

**Time Course of Contraction**

The Time To Peak (TTP) and 1/2 Relaxation Time (1/2RT) of the soleus muscle of both the L-Dopa and saline treated rats were examined at 4 and 8-10 weeks of age. The results are summarised in Table 6. In all instances the mean values of the TTP and 1/2RT of the L-Dopa treated rats were higher than the values of the saline treated rats. At 4 weeks of age these differences proved to be not significant and therefore suggests that there was a tendency for the slow soleus muscles in the L-Dopa treated rats to be slower in their time course of contraction. At 8 weeks of age, these differences in the TTP and 1/2RT proved to be significant and this can be seen clearly in Figures 11A and 11B.

These results suggest that increasing locomotor activity during the early postnatal period caused a change in the time course of contraction of the soleus muscle and this persisted until the rats were 8 weeks old.
Newborn rats were treated with L-Dopa or saline for 12 days. The muscle weight and twitch and maximum tetanic tension developed by the soleus were assessed at 4 or 8 weeks of age. The values shown are the means ±S.E.M. The pairs of symbols denote that the values with the same symbols are significantly different from each other.
Figure 11. The effect of treatment with saline or L-Dopa on the time to peak (TTP) and 1/2 relaxation time (1/2RT) of soleus muscle at 4 weeks and 8 weeks.

The effect of treatment with saline or L-Dopa on the time to peak (TTP) (Fig. 11A) and 1/2 relaxation time (1/2RT) (Fig. 11B) of soleus muscle at 4 weeks and 8 weeks. The values shown are mean values ±S.E.M. (ms).

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 11
Changes in the Time Course of Contraction due to Age

L-Dopa treatment had resulted in the soleus muscle to be slower in its' time course of contraction and the increases in the mean TTP and 1/2RT were significant at 8 weeks of age. Whether these results were different when compared across time was analysed next. Table 6 and Figure 11A show that the mean TTP of soleus was increased at 8 weeks when compared to the mean TTP at 4 weeks after both L-Dopa and saline treatment. In the saline treated rats, the difference was not significant. This shows that the older rats have soleus muscles which tend to be slower. In the L-Dopa treated animals the increase in mean TTP was significant. Nevertheless, this confirms earlier findings that L-Dopa treatment had affected the difference due to maturation in the TTP of soleus so that the soleus had a significantly bigger TTP.

The mean 1/2RT of soleus at 4 weeks and 8 weeks in the L-Dopa or saline treated rats did not differ significantly although in the L-Dopa treated rats it appeared to be bigger (see Table 6 and Figure 11B). Therefore, further maturation of the soleus muscle did not affect their 1/2RT.
Increasing locomotor activity seemed to have made the difference in 1/2RT at the two time points more obvious.

v) The Effects of Treatment with L-Dopa on the Contractile Characteristics of the Fast TA and EDL Muscles

Although the number of retrogradely labelled motoneurone to the fast TA and EDL motoneurones were not affected by increasing locomotor activity, there may be an effect on the contractile properties of the muscles. Therefore the effects of increased locomotor activity during early postnatal development on the contractile characteristics of TA and EDL were examined.

After L-Dopa or saline treatment during the early postnatal age, a group of rats was assessed at 4 weeks of age when they have not yet attained their adult muscle properties and at 8 weeks of age as in section iv). The results were tabulated and the mean values of muscle weight, twitch and tetanic tensions were also expressed in g/100g b.w. as in section iv). The mean values for the TA and EDL muscles are presented in Tables 7 and 8 respectively. The tables also
Table 7. The effect of treatment with saline or L-Dopa on the mean body weight of the animal, mean muscle weight and contractile properties of the TA muscle.

These values were taken at 2 time points: when the animals were 4 weeks old and 8 weeks old.

SEM = standard error of the mean
n = the number of muscles assessed
TTP = time to peak
1/2RT = half relaxation time
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<th>Muscle Weight</th>
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**TABLE 7**
Table 8. The effect of treatment with saline or L-Dopa on the mean body weight of the animal, mean muscle weight and contractile properties of the EDL muscle.

These values were taken at 2 time points: when the animals were 4 weeks old and 8 weeks old.

SEM = standard error of the mean  
n = the number of muscles assessed  
TTP = time to peak  
1/2RT = half relaxation time  
FI = fatigue index
### TABLE 8

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show the mean body weight of the animals, the time to peak (TTP) and the half relaxation time (1/2RT). Control values were recorded from rats that had been treated with saline. The values expressed in g/100g b.w. were used when comparing the results obtained from the L-Dopa and saline treated group.

**Twitch and Tetanic Tensions**

In this study the effect of excessive locomotor activity induced by the L-Dopa treatment during early postnatal development on the tensions developed by the fast TA and EDL muscles was assessed. Twitch and tetanic tensions developed by the TA and EDL muscles of control rats and those that had been treated with L-Dopa were recorded. The rats were assessed at 4 weeks of age when the muscles would not have fully acquired their adult muscle properties. To determine if further changes would occur by the time the muscles attain their adult physiological characteristics, another group of animals which had undergone L-Dopa or saline treatment were assessed when they were at least 8 weeks old.
Tables 9A and 9B summarise the mean values (±S.E.M.) of the twitch tensions for TA and EDL respectively. They are also presented graphically in Figures 12A and 12B. At 4 weeks, the mean twitch tensions developed by these muscles in the L-Dopa treated rats appear bigger than those developed in the saline treated rats, but the differences are not significant. When assessed at 8 weeks, the mean twitch tension of both muscles in the L-Dopa treated rats were smaller compared to those in the saline treated rats although the difference is significant for the TA muscle only. In the TA muscle, the mean twitch tension developed in the L-Dopa treated rats were smaller at 68.28g/100g b.w. (±3.79 S.E.M., n=7) than that developed in the saline treated rats which was 83.2g/100g b.w. (±2.14 S.E.M., n=9).

The mean maximum tetanic tensions of the fast TA and EDL muscles are shown in Tables 9A and 9B respectively. At 4 weeks, the muscles in the L-Dopa treated rats seemed to have developed bigger tensions but the differences are not significant. On the other hand, when assessed at 8 weeks of age, the tetanic tensions developed by TA and EDL of the L-Dopa treated rats were smaller compared to those in the saline treated rats but again the differences are not significant. Figures 12C and 12D illustrate these changes.
Newborn rats were treated with L-Dopa or saline for 12 days. The muscle weight and twitch and maximum tetanic tension developed by the TA (Table 9A) or EDL (Table 9B) were assessed at 4 or 8 weeks of age. The values shown are the means ± S.E.M. The pairs of symbols denote that the values with the same symbols are significantly different from each other.
Figure 12. The effect of treatment with saline or L-Dopa on the maximum twitch and tetanic tension developed by TA and EDL muscles.

The effect of treatment with saline or L-Dopa on the maximum twitch tension developed by TA (Fig. 12A) and EDL (Fig. 12B) and the maximum tetanic tension developed by TA (Fig. 12C) and EDL (Fig. 12D). The values shown are the mean values ±S.E.M. (g/100g b.w.). The animals were assessed at 2 different time points, at 4 weeks and at 8 weeks.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 12
Therefore, at 4 weeks of age, increasing the locomotor activity by treatment with L-Dopa during the postnatal period did not significantly affect the twitch and tetanic tensions developed by both fast muscles although there was a tendency for the tensions to be bigger in the L-Dopa treated rats. But at 8 weeks of age the twitch and tetanic tensions developed by these same muscles tend to be smaller in the L-Dopa treated rats.

Changes in Tensions Developed Due to Age

Any changes in the contractile characteristics due to further maturation would be seen by comparing the tensions developed at 4 weeks of age to the tensions developed at 8-10 weeks of age. Whether increasing locomotor activity during the early postnatal period would result in further changes of contractile characteristics of the fast, TA and EDL was examined next.

The mean twitch tensions developed by TA and EDL at 4 and 8 weeks after L-Dopa or saline treatment are summarised in Tables 9A and 9B respectively. The mean twitch tension developed at 8 weeks is significantly smaller than that
developed at 4 weeks by the TA muscle in the saline treated animals. A similar change is seen in the L-Dopa treated rats. On the other hand, the mean twitch tensions developed by the EDL muscle were not significantly different at 4 and 8 weeks in both groups of animals. Therefore, increased activity did not seem to affect the difference in the twitch tensions developed by the fast muscles. They developed similar changes as they mature in both the L-Dopa and saline treated groups and this is shown in Figures 12A and 12B.

The maximum tetanic tensions developed by the TA and EDL muscles were affected in a similar way as the twitch tensions. Tables 9A and 9B shows the mean tetanic tensions developed at 4 and 8 weeks after the L-Dopa or saline treatments by the TA and EDL muscles respectively. For the TA, the differences were not significant in both groups of rats as can be seen in Figure 12C. The differences in the tensions due to age for the EDL were significant in both the L-Dopa and saline treated rats and this is shown in Figure 12D.
Therefore, increasing activity by L-Dopa treatment did not affect the differences due to age in the twitch and tetanic tensions developed by the fast TA and EDL muscles.

Changes in Muscle Weights

The muscle weights of TA and EDL were examined to see whether increasing locomotor activity at a time when newborn rats were normally inactive had any effects that might correlate with that on the development of tensions by these fast muscles. Tables 9A and 9B show that the muscle weight of TA was affected by this experimental procedure when examined at 4 weeks while that of EDL was affected only when examined at 8 weeks. There was generally no changes in the muscle weights per 100 grams body weight in the fast muscles due to age but L-Dopa treatment did result in the EDL being bigger at 8 weeks than it was at 4 weeks (see Figures 13A and 13B).
Figure 13. The effect of treatment with saline or L-Dopa during the neonatal period on muscle weight of TA and EDL of 4 and 8 weeks rats.

The effect of treatment with saline or L-Dopa on muscle weight of TA (Fig. 14A) and EDL (Fig. 14B). The values shown are the mean values ±S.E.M. (g/100g b.w.). The animals were assessed at 2 different time points, at 4 weeks and at 8 weeks.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 13
Time Course of Contraction

The Time To Peak (TTP) and 1/2 Relaxation Time (1/2RT) of the TA and EDL muscles of both the L-Dopa and saline treated rats were assessed when the rats were 4 and 8-10 weeks old. The TTP results are summarised in Tables 10A and 10B for the TA and EDL muscles respectively. The mean TTP of both muscles in the L-Dopa treated rats were longer than that in the saline treated rats at 4 and 8 weeks. This difference is significant only for the EDL at 8 weeks. Figures 14A and 14B illustrates the tendency for the TTP to be bigger in the L-Dopa treated rats.

As for the 1/2RT, the mean values for both TA and EDL were higher in the L-Dopa treated rats when assessed at 4 and 8 weeks as shown in Tables 10A and 10B and Figures 14C and 14D but the differences were not significantly different.

These results show that inducing excessive premature locomotor activity influenced the time course of contraction for both the fast TA and EDL muscles.
Newborn rats were treated with L-Dopa or saline for 12 days. The muscle weight and twitch and maximum tetanic tension developed by the soleus were assessed at 4 or 8 weeks of age. The values shown are the means ±S.E.M. The pairs of symbols denote that the values with the same symbols are significantly different from each other.
Figure 14. The effect of treatment with saline or L-Dopa during the neonatal period on the time to peak (TTP) and 1/2 relaxation time (1/2RT) by the TA and the EDL muscle at 4 and 8 weeks.

The effect of treatment with saline or L-Dopa on the time to peak (TTP) by the TA muscle (Fig. 14A) and by the EDL muscle (Fig. 14B) and 1/2 relaxation time (1/2RT) by the TA (Fig. 14C) and by the EDL (Fig. 14D) at 4 weeks and 8 weeks. The values shown are mean values ±S.E.M. (ms.).

The error bars represent the Standard Error of the Means (S.E.M.)
Changes in the Time Course of Contraction due to Age

The mean time course of contraction was increased in the L-Dopa treated rats for the TA and EDL muscles when examined at 4 and 8 weeks although the increase were significant only for the TTP of EDL at 8 weeks. Whether these results were different when compared across time was analysed next. Tables 10A and 10B and Figures 14A and 14B show that the mean TTP of TA and EDL were increased at 8 weeks when compared to the mean TTP at 4 weeks after both L-Dopa and saline treatment. Therefore, the mean TTP was increased in the older rats due to maturation of the muscles. In the TA, L-Dopa treatment and saline treatment resulted in similar significant increases. This was not the case for EDL where the difference was significant in the L-Dopa treated rats, but not significant in the saline treated rats. This shows that L-Dopa treatment affected the differences due to maturation in the TTP of EDL and not that of TA.

Tables 10A and 10B also compares the 1/2RT at 4 weeks and 8 weeks in the L-Dopa or saline treated rats for the TA and EDL respectively. In both groups of rats the 1/2RT at 8 weeks did not significantly differ from that at 4 weeks although the mean values at 8 weeks were always smaller
than that at 4 weeks. Figures 14C and 14D also illustrate these differences. Therefore, further maturation of the TA and EDL muscles resulted in a tendency for the fast muscles to be faster in their 1/2RT. L-Dopa treatment in early life did not affect this change.

Changes in the time course of contraction due to further maturity of the fast muscles as assessed in this experiment can be summarised as follows; The TTP increased due to age while the 1/2RT was decreased. Increased locomotor activity during the early postnatal period generally had no effects on these changes due to age with the exception of the TTP in EDL.

Fatigue Index

The Fatigue Index (FI) of the fast, EDL muscle was examined to see if it will be affected by the increase in locomotor activity. The FI is a measure of the fatigue resistance of a muscle and was calculated here by expressing the residual tension after the period of stimulation as a percentage of the initial tension. The fatigue resistance of the EDL
muscle in the two groups of rats was examined when the rats were 4 or 8-10 weeks old. The results are summarised in Table 11. This table shows that the mean FI at 4 weeks is 29.61 (±1.45 S.E.M., n=8) in the L-Dopa treated rats and 43.80 (±6.22 S.E.M., n=7) in the saline treated rats. Although the fatigue resistance of the EDL in the L-Dopa treated rats appear to be decreased compared to the fatigue resistance of the saline treated rats, this difference is not significant (Mann-Whitney U Test). This is probably due to the variability in the results.

At 8-10 weeks of age, the FI of the EDL muscle in the L-Dopa treated rats was no different from that in the saline treated rats.

Changes in FI due to Age

To assess if there was any difference in the FI of EDL due to further maturation of this muscle, the FI values were compared across time. The mean fatigue index of the EDL muscle at 4 weeks were not different from that at 8 weeks in both the saline and L-Dopa treated rats as can be seen in Table 11. Therefore between 4 and 8 weeks there was not
The fatigue index (FI) of the EDL muscle of rats that had been treated with L-Dopa or saline during the early postnatal period was assessed at two different ages, at 4 and 8 weeks.

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<td>8 Weeks</td>
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TABLE 11: FATIGUE INDEX OF EDL MUSCLE AFTER L-DOPA OR SALINE TREATMENT
a significant change in the FI and increasing locomotor activity during the early postnatal period did not have any further effect.

iv) Histology

Examining the histology of muscles from the L-Dopa treated animals may provide some insight to the changes that may have occurred as a result of the treatment.

A. Pattern of Innervation.

The endplates of the soleus and EDL muscles of L-Dopa treated rats were examined for any changes that may help explain the differences found in the survival of motoneurones to the soleus and TA and EDL muscles, and in the number of motor units of soleus after treatment with L-Dopa. Although there was loss of soleus motor units, the muscles seemed to develop normal or bigger tensions. The soleus and EDL muscles of normal and L-Dopa treated adult rats were fixed, sectioned and stained using the cholinesterase and silver staining method so that the endplates and the axons would be visualised simultaneously.
The results show that the endplates of the EDL muscles of the L-Dopa treated rats appear similar to those of normal rats in that one endplate is innervated by one axon terminal. But in the soleus muscle of the L-Dopa treated rats, there appear to be more abnormal endplates than would be expected in an adult rat. There appears to be both collateral and terminal sprouting of axons and in some muscle fibres there were more than one endplate which were innervated either by sprouts from the same axon or by two axons. The percentage of abnormally innervated endplates in the soleus and EDL muscle was thus determined. 400-600 endplates were examined in each muscle. Preliminary results from just one animal indicated that in the soleus the percentage of abnormal innervation of endplates was 8.7% while that in EDL was 2%. In normal rats, the percentage of abnormal innervation of endplates in the soleus muscle is 3% (Connold and Vrbová, 1991). Figure 15 shows an example of the normal innervation of the soleus muscle of a 2 month old rat where each endplate is innervated by one axon. In contrast, Figure 16 shows some examples of the abnormal endplates observed in the soleus muscle of an L-Dopa treated rat. Therefore the normal force developed by fewer motor units may be due to the sprouting observed in the soleus muscle.
Figure 15. Combined cholinesterase and silver stain of soleus muscle from a normal rat.

Longitudinal sections (50µm) of soleus muscles from adult (2 months old) rats were stained for cholinesterase activity (brown) and silver (black) to visualise endplates and nerve axons simultaneously. The photograph shows the normal innervation of one axon to one endplate of adult muscle.

Scale bar = 50µm.
Figure 16. Combined cholinesterase and silver stain of soleus muscle from a rat treated with L-Dopa.

Longitudinal sections (50µm) of soleus muscles from adult (2 months old) rats that had been treated with L-Dopa during the neonatal period were cut and stained for cholinesterase activity (brown) and silver (black) to visualise endplates and axons simultaneously. The microphotographs show examples of the abnormal innervation of endplates of the adult soleus muscle. The arrows in the figure show the various sprouting described below.

A. Sprouting

B. Preterminal sprouting
   Two endplates on a single muscle fiber

C. Terminal sprouting

D. Retraction bulb of terminal sprout

E. Ultraterminal sprouting

F. Numerous sprouting

G. Polyneuronal innervation of endplates

H. Collateral sprouting

Scale bar = 50µm.
B. Oxidative Capacity of Muscle Fibres

As the level of locomotor activity in the L-Dopa treated pups increased tremendously, there may be changes in the oxidative metabolic activity of the soleus and EDL muscles in these pups. Immediately after the end of the treatment period i.e. at P12, the muscles from a saline treated pup were mounted next to corresponding muscles from an L-Dopa treated pup. Therefore, the pair of muscles underwent the same processing, i.e. sectioning and staining for succinic dehydrogenase (SDH) which reflects the oxidative capacity of the muscle fibres. It was found that at P12 there was no discernible difference in the intensity of the SDH stain between the soleus muscles from the saline and L-Dopa treated pups. However, the EDL muscle of the L-Dopa treated pup showed a more intense SDH stain than that of the saline treated pup (see Figure 17). Therefore at P12, the oxidative capacity of the soleus muscles of the L-Dopa treated pups was no different from that of the saline treated pups while the EDL muscles showed a higher oxidative capacity after L-Dopa treatment. This may mean that the treatment with L-Dopa increased the activity of the animal such that it caused an increase in the oxidative capacity of the EDL muscle. However, when examined at 4
Figure 17. Transverse sections of EDL muscle from 12 day old, normal and L-Dopa-treated rats stained for SDH enzyme activity.

The photomicrographs are from cross sections of EDL muscle from normal (A) and L-Dopa treated (B) 12 day old rats stained for SDH activity. The staining of the sections from the L-Dopa treated rat seemed more intense than that of those from the saline treated rat.

Scale bar = 50 μm
FIGURE 17
weeks of age, the degree of intensity of the SDH stain and the degree of heterogeneity of the fibre population in muscle from L-Dopa treated rats appear similar to that in muscle from saline treated rats for both the soleus and EDL muscles.

C. Slow myosin

During postnatal development, soleus fast fibres convert into slow ones. Whether treatment with L-Dopa will have any effect on this conversion was examined. The distribution of slow myosin containing muscle fibres in the soleus and EDL muscles of saline and L-Dopa treated P12 rat pups were determined by immunocytochemistry using a specific antibody directed against slow myosin. Muscles from saline treated pups were mounted next to corresponding ones from L-Dopa treated pups. Examples of the pairs of sections of soleus and EDL muscles are shown in Figure 18. There appear to be no detectable difference in the induction of slow myosin isoform in the developing soleus muscle nor was there a change in the distribution of slow myosin in the EDL muscle.
Figure 18. Transverse sections of Soleus and EDL muscles from normal and L-Dopa-treated 12 day old rats reacted for slow myosin.

The photomicrographs are from cross sections of soleus muscles from normal (A) and L-Dopa treated (B) 12 day old rats. Photographs C and D are from cross sections of EDL muscles from normal (C) and L-Dopa treated (D) 12 day old rats.

Scale bar = 50 μm
FIGURE 18
3.3.2 EFFECTS OF INCREASED MOTOR ACTIVITY ON INJURED MOTONEURONES

In the previous section it has been shown that increased locomotor activity during the early postnatal period had an effect on the number of motoneurones to the normal slow soleus muscle but not on that to the normal fast, TA and EDL muscles. It is possible that injuring the motor nerve to these fast muscles might render their motoneurones susceptible to the increased motor activity.

In this study, 5 day old (P5) Wistar rats were anaesthetised and the sciatic nerve in the right hindlimb was crushed. After recovery from the anaesthesia, the pups were returned to their mothers. To extend the period of separation of the motoneurones from the target muscles, the injury to the sciatic nerve was repeated 5 days later (P10). The rats were observed daily to ensure that the nerve crush was successful and that the rats recovered the use of their hindlimb after a period of time (Gutmann, 1942). Increased motoneurone activity during this period of separation from the target muscles was elicited by intraperitoneal injections of L-Dopa from P5 till P17.
Control animals were injected with saline. The behavioural changes of these L-Dopa treated rats were similar to those previously described in section 3.3.1.i. In addition, in these older pups, the types of locomotion observed reflected the locomotor activity that the pups become capable of as they matured. 9-11 day old pups walked with their ventral surface off the floor and 14-17 day old pups run in a manner similar to that of mature rats (Westerga and Gramsbergen, 1990). The P14-P17 rats were also observed to run sporadically with intermittent periods in which they appear quiet as previously described by Kellogg and Lundberg (1972).

i) The Effects of Prolonging the Period of Separation from the Target TA and EDL muscles

Previous reports show that following sciatic nerve crush at P5, the number of motoneurones to the slow soleus muscle is not affected. However, if the period of separation from the target was extended by crushing the sciatic nerve a second time 5 days after the first sciatic nerve crush, the number of surviving motoneurones is reduced to 59% of the control
side (Lowrie, 1990). Whether a similar effect can be seen on the motoneurones to the fast TA and EDL muscles which were only slightly affected by a single sciatic crush at P5 was examined.

The number of motoneurones innervating the TA and EDL muscles of rats which had their right sciatic nerve crushed at P5 and P10 and had i.p. injections of saline is shown in Table 12A. The mean number of HRP labelled motoneurones in the left (control) ventral horn was 162(±2.14 S.E.M., n=6) and in the right (operated) ventral horn was 132(±10.6 S.E.M., n=6). The number of motoneurones on the control side is within the range found in normal rats. It also confirms the number found in the TA and EDL pool in the saline treated only animals in section 3.3.1.i. The number of surviving motoneurones on the operated side was expressed as a percentage of the control number of motoneurones and was found to be 81% (±6.5 S.E.M., n=6). The reduction of 19% in the number of surviving motoneurones due to the double sciatic crushes is significant (p<0.01, Mann-Whitney U Test).

To confirm that nerve injury at P5 does not affect the number of motoneurones to the TA and EDL, some 5 day old
Table 12A. The effect of treatment with saline on the survival of motoneurones to the TA and EDL muscles after nerve crush at P5 and a second nerve crush at P10.

The number of motoneurones to the TA and EDL muscles labelled with HRP on each side of the spinal cord after sciatic nerve crush at P5 and P10 and treatment with saline was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.

Table 12B. The survival of motoneurones to the TA and EDL muscles after a nerve crush at P5.

The number of motoneurones to the TA and EDL muscles labelled with HRP on each side of the spinal cord after a sciatic nerve crush at P5 was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.
### TABLE 12A: NERVE CRUSH AT P5 AND P10 + SALINE

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>%Op/Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Op</td>
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<tr>
<td>1</td>
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<td>6</td>
<td>158</td>
<td>132</td>
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<td>Mean</td>
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<td>131.67</td>
</tr>
<tr>
<td>±S.E.M</td>
<td>2.14</td>
<td>10.63</td>
</tr>
</tbody>
</table>

### TABLE 12B: NERVE CRUSH AT P5

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>%Op/Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
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<td>7</td>
<td>161</td>
<td>175</td>
</tr>
<tr>
<td>Mean</td>
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<td>151.8</td>
</tr>
<tr>
<td>±S.E.M</td>
<td>3.9</td>
<td>7.1</td>
</tr>
</tbody>
</table>
pups were anaesthetised and had their right sciatic nerve crushed. The results of this experiment are summarised in Table 12B. The mean motoneurone number labelled on the control side was 171.4 (±3.9 S.E.M., n=7) while that on the operated side was 151.8 (±7.1 S.E.M., n=7). The survival of motoneurones after the sciatic nerve crush at P5 found in this study was 88.39% (±4.2 S.E.M., n=7) and this reduction is not significant. The percentage of surviving motoneurones in the present study is however slightly less than that previously reported by Lowrie et al., (1982).

Thus, prolonging the period of separation from the target fast muscles resulted in a 19% death of motoneurones to the fast muscles. The extent of motoneurone death for fast muscles seen in this study after repeated nerve injury is less than that seen in motoneurones to the slow, soleus muscle which was 41% (Lowrie, 1990).

ii) Effects of Increasing Activity On The Survival of Injured Motoneurones to TA and EDL Muscles

Whether increasing motoneurone activity would modify the survival rate of the motoneurones to TA and EDL which had
undergone the double sciatic nerve crushes was examined next.

Table 13 shows the number of motoneurones innervating the TA and EDL muscles of rats that had undergone the same surgical procedure as in the previous experiment but received daily injections of L-Dopa instead of saline. The mean number of HRP-labelled motoneurones in the control ventral horn was $164 (±2.7 \text{ S.E.M.}, n=6)$ and that in the operated ventral horn was $105 (±12.9 \text{ S.E.M.}, n=6)$. The number of motoneurones on the control side was within the range of the normal number of motoneurones found in the TA and EDL pool. This confirms the results reported in section 3.3.1.ii. that L-Dopa treatment did not affect the number of normal, uninjured TA and EDL motoneurones. The number of motoneurones on the operated side was then expressed as a percentage of the number in the control side and this revealed that only $64\% (±7.5 \text{ S.E.M.}, n=6)$ of motoneurones survived whereas in the saline treated group $81\% (±6.5 \text{ S.E.M.}, n=6)$ of motoneurones survived. This reduction in the number of motoneurones in the L-Dopa treated animals was found to be significant ($p<0.01$, Mann-Whitney U Test) and was greater than that seen in the saline treated animals subjected to the same sciatic nerve injury.
Table 13. The effect of treatment with L-Dopa on the survival of motoneurones to the TA and EDL muscles after nerve crush at P5 and a second nerve crush at P10.

The number of motoneurones to the TA and EDL muscles labelled with HRP on each side of the spinal cord after sciatic nerve crush at P5 and P10 and treatment with L-Dopa was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.
### TABLE 13: NERVE CRUSH AT P5 AND P10 + L-DOPA

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>%Op/Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Op</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>4</td>
<td>168</td>
<td>103</td>
</tr>
<tr>
<td>5</td>
<td>175</td>
<td>140</td>
</tr>
<tr>
<td>6</td>
<td>163</td>
<td>88</td>
</tr>
<tr>
<td>Mean</td>
<td>164.50</td>
<td>105.17</td>
</tr>
<tr>
<td>±S.E.M</td>
<td>2.74</td>
<td>12.93</td>
</tr>
</tbody>
</table>
Figure 19 shows the percentage survival of motoneurones to the fast muscles in rats which had undergone sciatic nerve crushes at P5 or P5 and P10 and had been treated with either saline or L-Dopa. The percentage survival of motoneurones to the slow, soleus muscle in rats which has undergone sciatic nerve crushes at P5 and P10 reported by Lowrie (1990) is also shown. The figure clearly shows that prolonging the period of separation of the muscle from its innervation reduce the survival of motoneurones to the muscle. The reduction in the survival of motoneurones of the fast TA and EDL muscles is however, smaller than that seen for the slow, soleus muscle. The survival of the motoneurones to the fast muscles is further reduced in rats that had been treated with L-Dopa.

Therefore, increasing motoneurone activity with treatment with L-Dopa further reduced the survival of motoneurones to the injured fast, TA and EDL muscles.
Figure 19. The percentage survival of motoneurones to the TA and EDL muscles and the soleus muscle after various experimental procedures.

The error bars represent the Standard Error of the Means (S.E.M.).

The value for the percentage survival of motoneurones to soleus muscle was taken from Lowrie (1990).
FIGURE 19

% Survival Of Motoneurons

Experimental Procedure

- TA + EDL
- SOLEUS

- P5 CRUSH
- P5 + P10 CRUSH + SALINE
- P5 + P10 CRUSH + L-DOPA
- SOLEUS
- P5 + P10 CRUSH
iii) Effects Of Increasing Activity On The Motoneurone Area Of Injured Motoneurones to TA and EDL Muscles

Previous results show that the size of motoneurones that survive injury may be affected by the injury and whether increasing motoneurone activity would further modify these changes was ascertained here.

Table 14A summarises the mean area of the motoneurones to the TA and EDL muscles on the control and operated side of each of the rats which had undergone right sciatic nerve crushes at P5 and P10 and had saline injections starting at P5 for 12 consecutive days. The mean values of the mean motoneurone areas of each side of the spinal cord are also shown. The mean value of the motoneurone area on the control side was $1078 \mu m^2$ (±72.1 S.E.M., n=6) and on the operated side was $816 \mu m^2$ (±69 S.E.M., n=6). This differs slightly from the mean motoneurone area calculated when all the data of each side of the 6 rats were pooled together. Calculated this way, the mean motoneurone area on the control side was $1078 \mu m^2$ (±12.8 S.E.M., n=6) and on the operated side was $812 \mu m^2$ (±11.1 S.E.M., n=6). The area of motoneurones on the control side was significantly
Table 14A. The effect of treatment with saline on the mean area of motoneurones to the TA and EDL muscles after sciatic nerve crush at P5 and P10.

Table 14B. The effect of treatment with L-Dopa on the mean area of motoneurones to the TA and EDL muscles after sciatic nerve crush at P5 and P10.

The sciatic nerve was crushed on one side of the animal at P5 and P10 and the animal was treated with saline (Table 14A) or L-Dopa (Table 14B) for 12 days starting on P5. 8-10 weeks later the motoneurones to the TA and EDL muscles were retrogradely labelled with HRP. The outline of HRP labelled motoneurones was traced with the aid of a camera lucida onto a graphics tablet attached to a microcomputer, which calculated the area of the motoneurones. The table shows the mean motoneurone areas of the operated and control sides of individual spinal cords. The mean motoneurone area on the operated side was expressed as a percentage of that on the control side. The mean motoneurone areas was also calculated by pooling together all the data and shown in the tables.
### TABLE 14A: NERVE CRUSH AT P5 AND P10 + SALINE

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mean Motoneurone Area (μm²)</th>
<th>%Op/Con</th>
<th>K-S Test</th>
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</thead>
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<td></td>
<td>Con</td>
<td>Op</td>
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<td>863.437</td>
<td>71.49</td>
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<td>5</td>
<td>997.843</td>
<td>717.61</td>
<td>71.92</td>
</tr>
<tr>
<td>6</td>
<td>1124.46</td>
<td>750.90</td>
<td>66.78</td>
</tr>
<tr>
<td>Mean ±S.E.M</td>
<td>1077.99 ±72.06</td>
<td>815.61 ±69.14</td>
<td>75.46 ±2.83</td>
</tr>
</tbody>
</table>

Pooled Data Mean ±S.E.M | 1078.44 ±12.80 | 811.80 ±11.12 | p<0.001 |

### TABLE 14B: NERVE CRUSH AT P5 AND P10 + L-DOPA

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mean Motoneurone Area (μm²)</th>
<th>%Op/Con</th>
<th>K-S Test</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Op</td>
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<tr>
<td>1</td>
<td>933.91</td>
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<td>6</td>
<td>1048.53</td>
<td>649.73</td>
<td>61.97</td>
</tr>
<tr>
<td>Mean ±S.E.M</td>
<td>1073.44 ±43.43</td>
<td>897.81 ±73.23</td>
<td>83.78 ±6.29</td>
</tr>
</tbody>
</table>

Pooled Data Mean ±S.E.M | 1074.9 ±12.37 | 910.29 ±12.45 | p<0.001 |
different from the area of motoneurones on the operated side (p<0.001, T-Test).

For each animal, the Kolmogorov-Smirnov Test was done and the distribution of the motoneurone sizes on the operated side was found to be significantly different from that on the control side in all 6 animals used in this study. When the data was pooled, the distribution of the motoneurone area on the control side was significantly different from that on the operated side (p<0.001, Kolmogorov-Smirnov Test). This can be seen in Figure 20A where the distribution of areas on the operated side is skewed to the left. It shows that the surgical procedure had affected a particular group of cells i.e. there were fewer large cells.

To assess if increasing the afferent activity had any effects on the area of the surviving motoneurones of rats which had undergone the double sciatic nerve crush procedure, L-Dopa was injected instead of saline for 12 days. The mean motoneurone areas of the control side and the operated side of each experimental animal are shown in Table 14B. The mean value of the mean motoneurone areas on the control side was 1073 μm² (±43.4 S.E.M., n=6) while that
Figure 20. The effect of treatment with saline (Fig. 20A) or L-Dopa (Fig. 20B) on the distribution of the area of motoneurones to the TA and EDL muscles after nerve crush at P5 and P10.

The histogram of the distribution of the motoneurone sizes on the operated side of the spinal cord was superimposed onto that of the motoneurones on the control side.
FIGURE 20

A: NERVE CRUSH + SALINE

B: NERVE CRUSH + L-DOPA
on the operated side was 898 \( \mu m^2 \) (±73 S.E.M., n=6). When all
the data on the control side of the 6 rats were pooled
together, the mean motoneurone area was found to be 1075\( \mu m^2 \)
(±12.4 S.E.M., n=6) while the mean of the pooled data on
the operated side was 910\( \mu m^2 \) (±12.4 S.E.M., n=6). The
reduction in the mean motoneurone area on the operated side
compared to that on the control side is significant
(p<0.001, T-Test).

In 4 of the 6 L-Dopa treated rats, the distribution of the
motoneurone areas of the operated side was significantly
different from that of the control side (Kolmogorov-Smirnov
Test). The distribution of the pooled data on the control
side was significantly different from that of the operated
side (p<0.001, Kolmogorov-Smirnov Test) and this is shown
graphically in Figure 20B. It also shows that the large
motoneurones were affected.

The pooled data on the control side of the saline treated
rats were compared to the pooled data of the L-Dopa treated
rats. The mean size and also the distribution of the sizes
of the surviving motoneurones between these two groups of
rats were found to be similar. To evaluate the effects of
L-Dopa on the area of surviving motoneurones after the sciatic nerve injury, the pooled data on the operated side of the saline treated rats were compared with that of the L-Dopa treated rats. The mean areas of motoneurones on the operated side of the L-Dopa treated rats was reduced less than that of the saline treated rats (p<0.001, T-Test).

Figure 21 shows the distribution of the pooled motoneurone sizes on the operated side of the saline injected rats superimposed on to the distribution of that on the operated side of the L-Dopa treated rats. The difference in the distribution is significant (p<0.001, Kolmogorov-Smirnov Test).

Therefore, the area of the motoneurones to the fast, TA and EDL muscles that had survived the sciatic nerve injury was affected by increasing the motoneurone activity. As assessed in this study, the mean motoneurone size was decreased less in the L-Dopa treated animals and the distribution of the sizes of motoneurones was also less affected than in motoneurones of those animals that had been treated with saline.
Figure 21. The effect of treatment with L-Dopa on the distribution of the area of motoneurones to the TA and EDL muscles after nerve crush at P5 and P10.

The histogram of the distribution of the motoneurone sizes on the operated side of the spinal cord of saline treated animals was superimposed onto that of the operated side of the spinal cord of L-Dopa treated animals.
FIGURE 21

NERVE CRUSH OPERATED SIDE

No. of Motoneurones

L-Dopa
Saline

Motoneurone Size (100 μm²)
iv) Effects of Increasing Activity on the Muscle Weights of TA, EDL and Soleus Muscles

The fast TA and EDL and slow, soleus muscles of rats which had undergone sciatic nerve crushes at P5 and P10 and had been treated with either saline or L-Dopa were dissected out and weighed. The muscle weights on the operated side expressed as a percentage of the weights on the control side are summarised in Table 15. The mean percentage value for each group is also shown. The L-Dopa treatment resulted in a smaller decrease of muscle weight for all three muscles, although the difference was significant only for the TA muscle (p<0.02, Mann-Whitney U Test).
TABLE 15: MUSCLE WEIGHT (%OP/CON) AFTER SCIATIC NERVE CRUSH AT P5 AND P10 AND L-DOPA OR SALINE TREATMENT

<table>
<thead>
<tr>
<th>Animal</th>
<th>TA</th>
<th></th>
<th>EDL</th>
<th></th>
<th>Soleus</th>
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<td>Saline</td>
<td>L-Dopa</td>
<td>Saline</td>
<td>L-Dopa</td>
<td>Saline</td>
</tr>
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<td>41.4</td>
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<td>37</td>
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<td>4</td>
<td>62.8</td>
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<td>62.7</td>
<td>29.5</td>
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<td>6</td>
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<td>52.9</td>
<td>89.5</td>
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<td>41.05</td>
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<td>3.88</td>
<td>4.08</td>
<td>6.81</td>
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</tbody>
</table>

The sciatic nerve of neonatal rats was crushed at P5 and again at P10. In addition, they were treated with L-Dopa or saline for 12 days starting on P5. The muscle weights of the TA, EDL and soleus was assessed when the rats were 8-10 weeks old.
3.3.3. EFFECTS OF INCREASING MOTOR ACTIVITY BY TREATMENT WITH L-DOPA ON MOTONEURONES DEPRIVED OF TARGET INTERACTION

Motoneurones are known to die when deprived of target interaction during the first five days after birth. In this study, target interaction was prevented by paralysis at birth. The effects of increased activity on the survival of such deprived motoneurones were studied. In a group of rats pups, implants containing α-BTX were inserted next to the soleus muscle in one hindlimb at birth and at P3. This treatment can be expected to paralyse the soleus muscle for at least 8 days. In addition, some of these animals received daily i.p. injections of L-Dopa while a control group received saline injections during the same period. The pups were observed daily to ensure that paralysis was achieved after the α-BTX implantations. Paralysis was monitored behaviourally as described by Duxson (1982), i.e. by observing the toe spreading reflex on the operated side and comparing it to that on the contralateral side when the pup was picked up by its tail, and by the appearance of the toes on the operated side. Only pups whose hindlimbs were seen to be paralysed for at least 7 days were used in this study. On receiving the L-Dopa injections the pups behaved...
as previously described in section 3.3.1.i. Two to 3 months later, the numbers and areas of motoneurones to the contralateral and experimental soleus muscles were assessed from sections where motoneurones were retrogradely labelled with HRP.

i) The Effects of Treatment with L-Dopa on the Number of Motoneurones to α-BTX-treated Soleus Muscle

The number of motoneurones innervating the soleus muscle of animals which had been paralysed with two α-BTX implants and treated with saline shortly after birth was examined 10 weeks later. Table 16A shows the number of HRP-labelled motoneurones in the soleus pool on the paralysed and the contralateral side of the spinal cord. The mean number of motoneurones on the contralateral side was 54 (± 2.3 S.E.M., n=10) while that on the paralysed side was 27 (±1.5 S.E.M., n=10). The number of labelled motoneurones on the paralysed side expressed as a percentage of the number of labelled motoneurones on the control side was 51.8% (±3 S.E.M.). The reduction in the number of motoneurones to the paralysed soleus was significant (p<0.001, Mann-Whitney U Test) and
### Table 16A. The survival of motoneurones to the soleus muscle after paralysis.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after paralysis with 2 α-BTX containing implants and treatment with saline was assessed in adult rats. The number of labelled motoneurones on the operated side was also expressed as a percentage of the number of labelled motoneurones on the control side.

### Table 16B. The effect of L-Dopa on the survival of motoneurones to the soleus muscle after paralysis.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after paralysis with 2 α-BTX containing implants and treatment with L-Dopa was assessed in adult rats. The number of labelled motoneurones on the operated side was also expressed as a percentage of the number of labelled motoneurones on the control side.

N.B. The number of motoneurones on the control side was also affected by the L-Dopa treatment.
### TABLE 16A: α-BTX + SALINE

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone number</th>
<th>%Op/Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Op</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
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</tr>
<tr>
<td>Mean</td>
<td>53.50</td>
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</tr>
<tr>
<td>±S.E.M</td>
<td>2.31</td>
<td>1.47</td>
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</table>

### TABLE 16B: α-BTX + L-DOPA

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>%Op/Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>7</td>
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<td>31</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
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</tr>
<tr>
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</tr>
<tr>
<td>±S.E.M</td>
<td>3.02</td>
<td>2.41</td>
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</table>
this result is consistent, though not identical with that described previously (Greensmith and Vrbová, 1992).

In this study, 51.8% of motoneurones to the soleus survived and this value is higher than that attained in the 1992 study where only 34.7% of the motoneurones survived. This difference is significant (p<0.001, Mann-Whitney U Test) and could be explained by the fact that the size of the α-BTX containing silicon strip that was implanted in this study was smaller compared to that used in the 1992 study. The dose of α-BTX was therefore smaller and this was necessary because the additional administration of L-Dopa to the α-BTX treated animal lead to a high mortality. In the present study, silicon strips weighing 0.2mg and 0.3mg were used at birth and P3 respectively while in the previous study 0.3mg and 0.6mg strips were used. The amount of α-BTX used in this study was therefore 4μg at birth and 7μg at P3 whereas in the previous study it was 7μg at birth and 14μg at P3. Figure 22 shows graphically the results obtained in this study compared to that from the 1992 study. Results from a second group of rats that had the soleus muscles paralysed with just one application of the α-BTX silicon strip in the 1992 study are also shown. 63.7%
Figure 22. The dose dependent effect of α-BTX on the number of motoneurones to the soleus muscle.

The error bars represent the Standard Error of the Means (S.E.M.)

The values of the first and second bar are from Greensmith and Vrbová (1992).
FIGURE 22: Dose Dependent Effects of BTX on Motoneurone Numbers
of motoneurones to the soleus survived in that study and this is significantly higher than the survival seen here (p<0.05, Mann-Whitney U Test).

Therefore, it appears that the effect of treatment with α-BT X on the motoneurone survival is dose dependent, possibly because the extent and duration of paralysis achieved with a smaller dose of α-BTX is less.

Whether the survival of motoneurones to paralysed soleus could be modified by L-Dopa treatment was examined next. Table 16B shows the number of HRP-labelled motoneurones in the soleus pool of rats which had their soleus muscle paralysed on one side and had been treated with L-Dopa from P0 for 12 days. The mean number of motoneurones on the side without α-BTX (contralateral side) was 36(±3 S.E.M., n=8) and that on the paralysed side was 22(±2.4 S.E.M., n=8).

Since the number of motoneurones on the contralateral side was not comparable to the normal number of motoneurones in the soleus pool, the survival of motoneurones on the paralysed side was not expressed as a percentage of the number of motoneurones on the contralateral side. Comparing
the number of motoneurones between the paralysed and contralateral sides in these L-Dopa treated rats, it was found to be significantly different (p<0.01, Mann-Whitney U Test). This difference was due to the paralysis induced after birth since motoneurones in both ventral horns were exposed to the L-Dopa.

The number of motoneurones on the side that had not been paralysed (contralateral side) is similar to that in the L-Dopa treated only rats (see section 3.3.1.i). The difference in the number of motoneurones in the contralateral sides of the saline injected animals i.e. 54 (± 2.3 S.E.M., n=10) and the L-Dopa injected animals i.e. 36 (± 3 S.E.M., n=8) was significant (p<0.001, Mann-Whitney U Test). This was a very interesting finding because this shows that L-Dopa reduced the number of normal, uninjured soleus motoneurones when exposed to it from P0 for 12 days and confirms the results described in section 3.3.1.i.

To assess the effects of L-Dopa administration on the survival of motoneurones to paralysed soleus, comparisons have to be made between the number of HRP-labelled motoneurones in the paralysed sides of the saline and L-Dopa treated groups of animals. The results from these two
groups of animals together with that from the previous experiment where rats were treated with L-Dopa or saline only (section 3.3.1.) were compiled and shown graphically in Figure 23. The difference between the number of motoneurones on the paralysed side of the saline injected rats and the paralysed side of the L-Dopa injected rats was just significant (p=0.05, one-tailed Mann-Whitney U Test). Thus L-Dopa has a small if any, added effect to that induced by the paralysis in further reducing the number of surviving motoneurones to the paralysed soleus.

ii) The Effects of Treatment with L-Dopa on the Area of Motoneurones to Paralysed Soleus Muscle

Previous studies have shown that the areas of motoneurones which had been injured or which innervated paralysed muscle are smaller. Therefore, the areas of motoneurones to the paralysed soleus muscles were examined in the two groups of animals.

The mean area of the motoneurones in the soleus motoneurone pool on each side of each rat that had been paralysed and injected with saline are shown in Table 17A which also
Figure 23. The effect of various experimental procedures on the mean number of motoneurones to the soleus muscle.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 23

Experimental Procedure

Mean Motoneurone Number

NORMAL  L-DOPA  BTX + SALINE  BTX + L-DOPA

Con  Op
Table 17A. The mean area of motoneurones to the soleus muscle after paralysis.

Table 17B. The effect of treatment with L-Dopa on the mean area of motoneurones to the soleus after paralysis.

The soleus muscle was paralysed by the implantation of 2 α-BTX containing silicon strips at P0 and P3. The animal was treated with saline (Table 17A) or L-Dopa (Table 17B) for 12 days starting on P0. 8-10 weeks later the motoneurones to the soleus muscles were retrogradely labelled with HRP. The outline of HRP labelled motoneurones was traced with the aid of a camera lucida onto a graphics tablet attached to a microcomputer, which calculated the area of the motoneurones. The table shows the mean motoneurone areas of the operated and control sides of individual spinal cords. The mean motoneurone area on the operated side was expressed as a percentage of that on the control side. The mean motoneurone areas was also calculated by pooling together all the data and shown in the tables.
### TABLE 17A: \( \alpha \)-BTX + SALINE

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mean Motoneurone Area ((\mu m^2))</th>
<th>%Op/Con</th>
<th>K-S Test</th>
</tr>
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<td>Con</td>
<td>Op</td>
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<td>88.25</td>
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<tr>
<td>±S.E.M</td>
<td>±64.87</td>
<td>±53.10</td>
<td>±6.95</td>
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</table>

**Pooled Data**

| Mean ±S.E.M | 1,077.85 | ±17.48 | p<0.01 |

### TABLE 17B: \( \alpha \)-BTX + L-DOPA

<table>
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<tr>
<th>Animal</th>
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<th>K-S Test</th>
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<td>94.79</td>
</tr>
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<td>±S.E.M</td>
<td>±65.1</td>
<td>±50.3</td>
<td>±6.76</td>
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</table>

**Pooled Data**

| Mean ±S.E.M | 1,065.62 | ±22 | p<0.05 |
shows the mean value of the mean motoneurone areas. The data of all the contralateral sides were then pooled together and compared with the pooled data of the paralysed side. Paralysis with two $\alpha$-BTX implants resulted in a decrease in the motoneurone area as evaluated by the T-Test ($p<0.001$). The mean motoneurone area on the contralateral side was $1077.9\mu m^2$ ($\pm17.5$ S.E.M., $n=8$) and on the paralysed side it was $934\mu m^2$ ($\pm20.4$ S.E.M., $n=8$). This result is consistent with that reported by Greensmith and Vrbová, 1992 where the larger $\alpha$-BTX dose used in that study resulted in a bigger difference in the motoneurone sizes between the contralateral and paralysed side. The motoneurone size on the paralysed side was expressed as a percentage of that on the contralateral side and this percentage was used to compare the results obtained in this study with those in the 1992 study. This is shown in Figure 24. In the present study, the motoneurone size on the paralysed side was reduced to $88.3\%$ ($\pm6.95$ S.E.M) of that on the contralateral side while that from the 1992 study was reduced to $69.4\%$ ($\pm6.95$ S.E.M) and this is significantly different ($p<0.05$, Mann-Whitney U Test). On the other hand, the present result is no different from
Figure 24. The dose dependent effect of α-BTX on the size of motoneurones to the soleus muscle.

The error bars represent the Standard Error of the Means (S.E.M.).

The values of the first and second bar are from Greensmith and Vrbová (1992).
FIGURE 24: Dose Dependent Effects of BTX on Motoneurone Size

% Op/Con

7µg BTX  7µg + 14µg BTX  4µg + 7µg BTX
that in the 1992 study where only one α-BTX containing silicon strip was used.

For each individual animal, the Kolmogorov-Smirnov Test was done to see if the paralysis had affected the distribution of the motoneurone sizes. As shown in Table 17A, the distribution of the motoneurones sizes was significantly different between the contralateral and the paralysed sides in 5 of the 8 rats used in this study. When pooled, the difference between the distribution of the motoneurone areas on the paralysed side and that on the contralateral side was also significant (p<0.01, Kolmogorov-Smirnov Test). The distribution of the motoneurone areas is shown graphically in Figure 25A to see if paralysis had affected a particular group of cells. It shows that paralysis resulted in a reduction in the number of the larger cells. There are two possible explanations i.e. paralysis resulted in the death of the larger cells or alternatively, paralysis resulted in the death of the smaller cells and the larger cells have become smaller in size. These results are similar to those in the 1992 study and thus confirms that paralysis affects the distribution of the sizes of the motoneurones to the muscle.
Figure 25A. The distribution of sizes of motoneurones to the soleus after paralysis.

The histogram of the distribution of the motoneurone sizes on the operated side of the spinal cord was superimposed onto that of the contralateral side of the spinal cord of saline treated animals.

Figure 25B. The effect of treatment with L-Dopa on the distribution of motoneurone sizes after paralysis.

The histogram of the distribution of the motoneurone sizes on the operated side of the spinal cord was superimposed onto that of the operated side of the spinal cord of L-Dopa treated animals.
FIGURE 25

A: BTX + SALINE

B: BTX + L-DOPA
Therefore, paralysis of the soleus muscle during the early postnatal period affects both the mean motoneurone size and the distribution of the sizes. This effect on the mean motoneurone size is dependent on the dose of $\alpha$-BTX used which determined the extent of paralysis achieved.

Table 17B summarises the results of the study in which the soleus muscles of rats were paralysed with $\alpha$-BTX and the rats were injected (i.p.) with L-Dopa from P0 for 12 days. The mean motoneurone areas for each rat are shown. The mean values of the mean motoneurone areas for each side are also shown. Statistical tests done on the pooled data of the contralateral and paralysed sides showed that the motoneurone area was significantly reduced on the paralysed side ($p<0.05$, T-Test). The mean motoneurone area on the contralateral side was $1065.6\mu m^2$ ($\pm 22$ S.E.M., n=8) while that for the paralysed side was $981.2\mu m^2$ ($\pm 28$ S.E.M., n=8).

Kolmogorov-Smirnov Tests done to evaluate whether there was any differences in the distribution of the areas of motoneurones in the contralateral side compared to the paralysed side proved to be significant in 5 of the 8 rats. When the data was pooled, the distribution of the areas of
the motoneurones on the contralateral side was significantly different from that of the areas of motoneurones on the paralysed side (p<0.05, Kolmogorov-Smirnov Test). Figure 25B shows the differences between the distribution of the motoneurone area of the contralateral side compared to the paralysed side. Although there was not an obvious shift to the left, the distribution shows that the reduction in the number of motoneurones was mainly in the larger group of cells. As explained before, this means either that the larger cells were more susceptible to the treatment received or that the smaller cells were more susceptible and the surviving cells become smaller as a result of the treatment received.

To see if the differences between the two sides can be attributed by the L-Dopa treatment, comparisons were made on the pooled data between the two experimental groups of rats. The mean motoneurone areas on the contralateral side of the saline treated rats proved to be similar to that of the contralateral side of the L-Dopa treated rats (T-Test). Therefore, comparisons can be made between the paralysed sides of the saline and L-Dopa treated rats and they were found to be similar.
The distribution of the pooled data of motoneurone sizes on the contralateral side of the saline treated rats compared to that of the L-Dopa-treated rats proved to be not different (Kolmogorov-Smirnov Test). Since the distribution of the motoneurone areas of the contralateral sides were similar, the distribution of motoneurone areas on the paralysed sides of the saline and L-Dopa treated rats was compared and was found to be no different.

Therefore, increasing locomotor activity during the early postnatal period did not further affect the mean motoneurone areas which had been reduced due to paralysis of the soleus muscle. Also, this treatment did not further affect the distribution of the motoneurone sizes which had changed due to paralysis of the soleus muscle.
3.4. DISCUSSION

Following nerve injury at birth motoneurones become excessively active. It is therefore possible that their death is caused by increased excitatory response and the excitotoxic effects of such an event. This hypothesis was tested here and the results provide some evidence in support of this idea.

Increasing afferent activity to normal motoneurones

In the first part of the study, attempts were made to increase the overall activity of motoneurones prematurely. This was achieved by systemic injections of L-Dopa during the early stages of development. This treatment resulted in precocious locomotor activity. The results show that a proportion of motoneurones to the soleus muscle died after the L-Dopa treatment, while there was no loss in the number of motoneurones to the fast, TA and EDL muscles. The death of motoneurones to the soleus muscle may be due to the increased afferent input onto the motoneurones as a result of enhanced locomotor activity. It is likely that the L-Dopa acts in the central nervous system and activates the
central pattern generator which results in the generation of locomotor activity. This precocious increase in locomotor activity presumably resulted in an increase of afferent activity onto the motoneurones. The increased amounts of afferent activity, both supraspinal and segmental, impinging onto the motoneurones may have been too high for some of the motoneurones to the soleus muscle to cope with and caused them to die. Soleus motoneurones have been shown to be resistant to overactivation to a certain degree where injection of a low dose of NMDA after a nerve crush at P5 did not cause motoneurone death while a higher dose of NMDA did result in motoneurone death (Greensmith et. al., 1995). Therefore, it is likely that as a result of the L-Dopa treatment carried out in this study, the afferent inputs were increased to such a degree as to bring about excitotoxicity and death of motoneurones.

The present study shows that unlike motoneurones to the soleus, motoneurones to the TA and EDL did not seem to be killed by the increased activity achieved by treatment with L-Dopa. This difference could be due to several possibilities; 1) the activity of motoneurones to the slow, soleus increased more after L-Dopa treatment or 2) the
motoneurones to the soleus were more immature and therefore were more susceptible to the increased activity.

Navarette and Vrbova (1983) found that during the first week of postnatal development, the overall amount of EMG activity in soleus and EDL muscle is similar. However, treatment with L-Dopa has been shown to increase the level of activity in soleus to a greater extent than in EDL. After just one administration of L-Dopa, the overall EMG activity in soleus muscle increased 24 fold while in EDL it increased 17 fold compared to its activity before treatment (Navarrete and Vrbova, 1985). Nevertheless, this difference may not account for the present results.

There are indications that motoneurones to the soleus muscle are less mature than motoneurones to the fast TA and EDL muscles at the same point in time during the early postnatal period. After a nerve crush at birth, motoneurones to the soleus were found to be more susceptible to this procedure than the motoneurones to the fast, TA and EDL muscles. This is reflected in the number of motor units that remain in these muscles after nerve injury during the neonatal period. There is a reduction of 88% of motor units in the soleus muscle while in the EDL
muscle there was a 76% reduction of motor units after nerve injury at birth (Dick et. al., 1995). A nerve injury at P3 results in a reduction of 82% of motor units in the soleus muscle and 71% of motor units in the EDL muscle (Greensmith et. al., 1996). This difference in the extent of motoneurone loss in the different motor pools may indicate different levels of maturity of these motoneurones. Furthermore, younger motoneurones have also been shown to be more vulnerable to the excitotoxic effects of glutamate agonists than mature motoneurones (Olney, 1974) and the finding that motoneurones to the soleus muscle were vulnerable to the increased afferent activity while those to the TA and EDL muscles were not may indicate the immaturity of the motoneurones to the soleus muscle.

The finding that the number of motor units in the soleus muscle is reduced after inducing precocious locomotor activity confirms the finding that some motoneurones to soleus were susceptible to this induced locomotor activity. The size of these motor units was also bigger. In this study, soleus muscle in the adult animals that were treated with L-Dopa have more abnormal endplates than normal, i.e., there appears to be more sprouting of the axons that innervate these endplates. The presence of sprouts in adult
muscles have been described previously in the soleus (Connold and Vrbová, 1991) and EDL (Connold et. al., 1992) muscle which resulted when the neuromuscular interaction was disrupted.

Increasing afferent activity to injured motoneurones to the TA and EDL muscle

As mentioned previously, normal, uninjured motoneurones to the fast TA and EDL muscles were not affected by the precocious activity induced by treatment with L-Dopa. Nevertheless, when motoneurones to the TA and EDL were injured and increased locomotor activity was then induced by repeated treatment with L-Dopa, a proportion of these motoneurones die.

Possibly, injured motoneurones may be more vulnerable and the increased afferent activity causes them to die. It has been shown previously that even those motoneurones that survive neonatal nerve injury are more active (Navarrete and Vrbová, 1984). This increased overall activity was also exhibited by motoneurones that had been injured at a time when the injury would not have resulted in motoneurone death i.e. at P5 (see Navarrete and Vrbova, 1984). Lowrie et.al.
(1982) showed that nerve injury at this same time caused changes in the muscle which may reflect permanent changes in the activity pattern of motoneurones (Pette and Vrbová, 1985). It has been shown that the distance of the lesion from the cell body determines the severity of the lesion (Lieberman, 1971; Brown et al., 1976; Zelená and Hník, 1963). Since the distance of the lesion from the cell body determined the period of target-deprivation, it may be that the period of target-deprivation, and not the injury itself, contributed to these differences.

In the present study, the motoneurones that survived having their axons injured twice had a longer period of target-deprivation. Therefore, since their maturation was further delayed, they may be more susceptible to excitotoxicity. Since the amount of activity of motoneurones to the hindlimb muscles increases during the first 3 weeks of life towards the adult values (Navarrete and Vrbová, 1983), these motoneurones may succumb to the increased afferent activity that result during normal development. This may explain the death of motoneurones to the fast TA and EDL muscles seen in this study after the sciatic nerve was injured twice. Lowrie, (1990) has shown that the motoneurones to the slow, soleus muscle were similarly more
affected when their axons were injured twice. This result agrees with that found by Kashihara et. al. (1987) where the number of surviving motoneurones was significantly reduced when reinnervation was prevented after neonatal nerve section.

Thus, it was only when the motoneurones were deprived of target interaction for a longer period of time that they became susceptible to the increased afferent activity. This study shows that there was a further significant decrease in the number of surviving motoneurones after L-Dopa treatment which lends support to the proposal that when developing motoneurones are deprived of neuro-muscular interaction they fail to acquire the characteristics needed to withstand increasing excitatory activity (Lowrie and Vrbová, 1992).

Timing of injury

A nerve injury done after 5-6 days of age usually did not cause motoneurone death (Lowrie et. al., 1982). The motoneurones would have acquired the ability to withstand the normal increases in afferent activity during
development by this time. There are several possible explanations for the discrepancy found in the present study after nerve crush at P5 where there was a non significant 12% motoneurone death and that of (Lowrie et. al., 1982). The first reason may be that there were differences in the site of injury. The further away a crush is to the muscle, the longer it will take to reinnervate the muscle (Lowrie et. al., 1990; Naidu, 1994) and this may result in differences in the number of motoneurones that survive the injury. The second reason may be that the variability in the maturation of the nervous system of the individual pups resulted in differences in actual time after birth when the procedure was done. Although care was taken to ensure the actual time of birth, the age of the pups may be ± 1/2 day. Therefore in the present study some pups may be as young as 4 1/2 day when the sciatic nerve crush was done.

**Increasing afferent activity to motoneurones to paralysed soleus muscle**

The next question asked in this series of experiments was whether motoneurones to paralysed slow, soleus will be susceptible to increased afferent activity. In this study,
it was found that paralysis resulted in motoneurone death and that increased afferent activity caused further motoneurone death.

Greensmith et. al., (1993) proposed that an increased susceptibility to glutamate over an extended period of time may explain the eventual death of motoneurones to paralysed muscles. The transient paralysis of the soleus muscle shortly after birth also appears to affect the ability of motoneurones to survive the excitotoxic effects of an exogenously applied glutamate agonist (Greensmith et. al., 1995). The same explanation is probably applicable in this study although it was the effects of putative glutamate that was seen which the motoneurones could not cope with when the locomotor activity was increased precociously. The increase of afferent activity to motoneurones when the animals were treated with L-Dopa probably came from the supraspinal inputs (Grillner, 1973; Anden et. al., 1966; Armstrong, 1988). In addition, in this case, by paralysing the postsynaptic membrane of the soleus muscle, the afferents were not injured and therefore whatever increases in afferent activity from the periphery that would result from the L-Dopa treatment would reach the motoneurones.
The results from this experiment add support to the proposal that nerve muscle interaction plays a very important role in survival of developing motoneurones (Greensmith and Vrbová, 1992). The extent of motoneurone death and decrease in soma size found in this study seem to correlate well with the amount of α-BTX present in the silicon implants which determined the length of time that the muscle remain paralysed.

It is interesting that normal motoneurones to soleus were susceptible to increased activity but when the motoneurones were injured before the induction of increased activity, this same increase in activity did not further increase death substantially. Why is it not possible to kill off much more motoneurones to the slow, soleus muscle by the increase in activity after the neuromuscular interaction was disrupted?

It may be that the paralysis has resulted in the maximum death of motoneurones that could have occurred in this system at the particular point in development. Another possibility is that there may be a population of motoneurones that is resistant to excitotoxicity. Thus, the increase in afferent input due to the increase in locomotor
activity may result in a non significant death of motoneurones.

Changes in Motoneurone Sizes

Examining the effects of increased afferent activity on the size of the motoneurones that survive the injuries may lead to a better understanding of the different effects of this increase of afferent activity on the survival of the motoneurones.

It appears that the mean size and the distribution of the sizes of normal, uninjured motoneurones to both the slow and fast muscles were not affected by the increase in locomotor activity as induced in the series of experiments in this chapter. But the effects of increased activity seem to be different on the motoneurones to the different muscle types when the neuromuscular interaction was interrupted prior to this induction of locomotor activity. In motoneurones to the fast TA and EDL muscles, decrease in the soma size seen after nerve injury was significantly smaller in the animals treated with L-Dopa although the number of surviving motoneurones was reduced. This does not
agree with previous findings where a reduction in the number of motoneurones after injury was accompanied by the reduction of the mean soma size of the surviving motoneurones (Lowrie et. al., 1987; Greensmith and Vrbová, 1992). This discrepancy in the effects of increased activity on the size of normal and injured motoneurones to the fast muscles may explain why normal motoneurones to the fast muscles were not susceptible to the excitotoxic effects of increased afferent activity.

On the other hand, the mean size and the distribution of sizes were not further changed after the induced increased activity of motoneurones to the soleus muscle. This may indicate that the increase in activity was not sufficient to induce any change in the soma of the injured motoneurone and may in turn explain why the number of surviving motoneurones to soleus was not much further reduced. Burls et. al., (1991) and lately Kerai et. al. (1995) found that after neonatal paralysis there was no change in the soma size during the first 3 weeks of life although by 10 weeks of age, the mean size was reduced (Greensmith and Vrbová, 1992). Therefore, it may be that neonatal paralysis may alter the motoneurone phenotype in ways other than just soma size. In the present study the mean motoneurone area
and the distribution of the motoneurone sizes did change due to the paralysis when assessed at 8 weeks of age.

Locomotion as induced in this study presumably result in increased activation of the motoneurones under study. Locomotion is the result of complex processes that take place in many systems. Increasing the overall locomotor activity of the animal may result in events that may 'dampen' the excitotoxic effects. For example, although L-Dopa may mediate motoneurone death by increasing the activation of the motoneurones, at the same time the increase in locomotor activity may result in increased transmitter release, or rate of turnover of transmitter. Enhancing transmitter release at the neuromuscular junction may increase the rate of maturation of motoneurones such that they were able to survive nerve injury better (Greensmith et. al., 1996). More mature motoneurones may not succumb as readily to the bombardment of the increased afferent inputs. Therefore, it may be that motoneurone death induced by increased locomotor activity reflect the final balance between the excitotoxic effects of afferent inputs to motoneurones and the increase of maturity that this activity afford these motoneurones. That the muscle weights on the operated side of animals in the L-Dopa
treated groups recovered better after being injured twice as found in this study may mean that the muscles were better reinnervated.

Conclusion

The results in this chapter confirm previous results that suggest motoneurones, when deprived of the normal neuromuscular interaction during the early postnatal period, will remain susceptible to the increases of afferent activity as the animal becomes more active. Inducing locomotor activity at a time when the animal has not yet increased its own locomotor activity resulted in further motoneurone death. The results found in this study support the proposal that motoneurones die because they were susceptible to excitotoxicity due to increased afferent inputs. The differences seen in the effects of increased afferent activity on the motoneurone to the different muscle phenotypes may indicate that these motoneurones differ in their rate of maturation during development.
CHAPTER 4 - THE EFFECTS OF DECREASING AFFERENT ACTIVITY TO INJURED MOTONEURONES

4.1. INTRODUCTION

Motoneurones to the soleus muscle are known to die when injured during a critical period of postnatal development. Paralysing the muscle by blocking the acetylcholine (ACh) receptor during this critical period also causes motoneurones to die. Recent results show that a proportion of motoneurones to the soleus muscles die (Greensmith and Vrbová, 1992) when neuromuscular transmission is blocked by α-BTX, a compound that binds irreversibly to the nicotinic ACh receptor (Giacobini et. al., 1973). This finding has led to the proposal that motoneurone death induced during the critical period in postnatal development was due to the interruption of neuromuscular interaction and not to the injury to the axon itself. It was suggested that motoneurone death is caused by a relatively high susceptibility of these target-deprived cells to the excitotoxic effects of glutamate (Choi, 1988). It may be possible that preventing motoneurone target interaction interfered with the acquisition of the characteristics that
allows motoneurones to withstand the increasing afferent inputs within the normal developing spinal cord (Lowrie and Vrbová, 1992). If this was the case, then a reduction of afferent inputs to developing motoneurones that had been deprived of target interaction should allow the survival of a larger number of cells.

In this chapter, the effects of decreased afferent activity on the motoneurone death induced by the interruption of neuromuscular interaction during the critical period were examined.

The first question asked in this study was whether decreasing afferent input by performing dorsal root sections will affect the number of surviving motoneurones after a sciatic nerve crush. As explained in the previous chapter, a nerve injury performed during the critical period will cause a considerable number of motoneurones to die. This particular method of interrupting the neuromuscular interaction when done at P3 still result in a massive amount of motoneurone death i.e. 80% of the motoneurones to soleus die (Greensmith et. al., 1996) but a sciatic nerve crush at P5 would result in little or no motoneurone death (Lowrie et. al., 1982). This may indicate
that between the ages of P3 and P5, motoneurones have acquired the ability to withstand the increasing afferent activity in the normally developing spinal cord (Lowrie and Vrbová, 1992). Therefore, in this study the sciatic nerve was crushed at P4 to determine if the motoneurones at this point in development are still susceptible to interruption of the neuromuscular interaction. Furthermore, the effect of manipulating the afferent inputs to these motoneurones was examined. The segmental afferent input to the soleus motoneurones are from several segments of the spinal cord. Dorsal root section was performed on the L4 and L5 dorsal roots on the same side of the sciatic nerve crush to reduce the afferent input onto the injured motoneurones in this study.

The second question asked in this chapter was whether decreased afferent activity will affect the motoneurone death seen after target-deprivation by paralysis. As shown in the previous chapter, paralysis of the soleus muscle during the early post-natal period results in about 48% death of motoneurones when examined at 8-10 weeks of age. Since it has been proposed that motoneurones die by excitotoxicity when deprived of normal neuromuscular interaction during the early postnatal period, reducing the
afferent inputs should protect some motoneurones from succumbing to the excitotoxicity. The method used to reduce the afferent inputs onto these target-deprived motoneurones was by blocking the receptors of the excitatory neurotransmitter, glutamate by treating the animals with MK-801 (dizocilpine maleate), an NMDA receptor antagonist. This non-surgical method of reducing the afferent inputs onto motoneurones have been used previously (Sanner and Goldberger, 1991). Non-competitive NMDA antagonists act by blocking the glutamate-gated calcium channel in a use-dependent manner. They are lipophilic and can be given systemically (Buchan, 1990). MK-801 is able to cross the blood brain barrier after systemic administration. Bagetta et. al. (1990) and Sanner and Goldberger (1991) have shown that treatment with MK-801 could prevent cell death after axotomy.

In summary, in this chapter the following questions are examined:

1. Whether the motoneurones destined to die after their axons were injured during the critical period could be saved by surgically reducing the segmental afferent input via the dorsal roots.
2. Whether there is a possibility that some of the motoneurones destined to die might be rescued by blocking the NMDA receptors with MK-801.
4.2. MATERIALS AND METHODS

Neonatal Wistar rats of both sexes were used in the experiments described in this chapter. They were within the critical period i.e. during the first 5 days of life, when the first experiments were carried out. The final experiments were carried out when these rats have reached adult age i.e. when they were at least 8 weeks old.

The specific procedures used in this chapter are described in detail in Chapter 2.

The experiments in this chapter are divided into 2 different groups as described below:

4.2.1. The sciatic nerve in the right hindlimb of neonatal rats of both sexes was crushed with a watchmaker's forceps when the rats were 4 days old (P4). The neuromuscular interaction was thus interrupted for a period of time. In one group of rats, another operation was carried out at the same time where the dorsal roots of the L4 and L5 nerves were sectioned. When the rats were at least 8 weeks old,
the motoneurones to the soleus muscle were examined by retrograde labelling with HRP.

4.2.2. To interrupt the neuromuscular interaction without affecting the afferent input onto the motoneurones, the soleus muscle in the right hindlimb of neonatal rats was paralysed with an implant containing α-BTX within 6 hours of birth (P0). This operation was repeated when the rats were 3 days old (P3) when a second implant was put into the same position as the first. In addition, these rats were injected with MK-801 or saline for 12 days starting from P0. When the rats were at least 8 weeks old the motoneurones to the soleus were examined by retrograde labelling with HRP.
4.3 RESULTS

4.3.1 EFFECTS OF DECREASED SEGMENTAL AFFERENT ACTIVITY ON INJURED MOTONEURONES

Most motoneurones to hind leg muscles are known to survive nerve injury when this injury is inflicted on them at P5 or later. When the injury was inflicted at P0 or P3, a massive proportion of motoneurones die. Therefore within 2 days time, the motoneurones have undergone changes that made them able to withstand the injury inflicted upon them. In this study the survival of motoneurones to the soleus after nerve crush at P4 was determined. Whether this survival rate can be modified by decreasing the afferent activity to the motoneurones was determined next.

In a group of rat pups, the sciatic nerve on the right side was crushed at P4. In addition to this procedure, some of these rats underwent sections of the L4 and L5 dorsal roots on the side of the nerve injury at the same time. There was a lower mortality among the pups when the two procedures were done together. The dorsal root sections were done at P4 because at this time the dorsal and ventral roots were
distinguishable. Two to 3 months later the survival of the motoneurones to the soleus was determined by retrograde labelling with HRP.

The rats were observed daily to ensure that the sciatic nerve crush was done properly and that the rats recovered the use of their legs after a determined period of time. There was no toe spreading reflex in rats that had the sciatic nerve crush at P4 but this reflex should return by 2 weeks after the crush when reinnervation would have taken place (Gutmann, 1942). In rats that had undergone the dorsal root sections, there was no withdrawal reflex when the skin of the foot was pinched.

i) Effects of Sciatic Nerve Crush at P4 on the Number of Motoneurones to the Soleus Muscle

The numbers of HRP-labelled motoneurones innervating the soleus muscle in rats that had their right sciatic nerve crushed at P4 are shown in Table 18A. The mean number of motoneurones on the contralateral side was 55(±1.6 S.E.M., n=9) while that on the operated side was 23(±1.3 S.E.M., n=9). Since the mean number of motoneurones on the
Table 18A. The survival of motoneurones to the soleus muscle after nerve crush at P4.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after a sciatic nerve crush at P4 was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.

Table 18B. The effect of dorsal root section on the survival of motoneurones to the soleus muscle after nerve crush at P4.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after a sciatic nerve crush and dorsal root section at P4 was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.
### TABLE 18A: NERVE CRUSH AT P4

<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>% Op/Con</th>
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<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Op</td>
</tr>
<tr>
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<td>8</td>
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<td>24</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>23</td>
</tr>
<tr>
<td>Mean</td>
<td>55.22</td>
<td>23.44</td>
</tr>
<tr>
<td>±S.E.M</td>
<td>1.61</td>
<td>1.30</td>
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</table>

### TABLE 18B: NERVE CRUSH AT P4 + DORSAL ROOT SECTION

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<th>Animal</th>
<th>Motoneurone Number</th>
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</thead>
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<td>Op</td>
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<td>1.84</td>
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contralateral side is similar to the normal number of motoneurones to the soleus muscle, the number of motoneurones on the operated side was expressed as a percentage of that found on the contralateral side. This means that 42.9% (±3.0 S.E.M.) of motoneurones to the soleus muscle survived the injury inflicted on them at P4. The reduction of about 57% of the motoneurones to the soleus muscle is significant (p<0.0001, Mann-Whitney U Test). Figure 26 shows this result plotted on the curve drawn for the survival of motoneurones to the soleus muscle after nerve crush at P0, P3 and P5 of previous published studies. The result from this study shows that a massive proportion of motoneurones still dies due to the sciatic nerve injury inflicted at P4. This means that the motoneurones have not yet acquired the ability to withstand injury so that nerve injury at P4 still results in the death of a great proportion of motoneurones to the soleus.

ii) Effects of Decreasing Segmental Afferent Activity on the Number of Injured Motoneurones

To assess whether decreasing the afferent activity of the soleus motoneurones will reduce death of the motoneurones
Figure 26. Graph of the percentage of survival of motoneurones after nerve crush at various ages during the critical period of postnatal development.

The values of percentage survival of motoneurones after nerve crush at P0 were taken from Mentis et al. (1993), at P3 from Greensmith et al. (1996) and at P5 from Greensmith et al. (1994) and Lowrie (1990).
FIGURE 26

% Survival of Motoneurones

Age of Nerve Crush (Days)
due to the nerve crush at P4, dorsal root sections were carried out on a group of rats which also had their sciatic nerve crushed at the same time.

Table 18B shows the number of motoneurones innervating the soleus muscle in rats which had their right sciatic nerve crushed and the L4 and L5 dorsal roots sectioned at P4. The mean number of motoneurone on the control side was 60 (±2.7 S.E.M., n=9) while that on the operated side was 25 (±1.8 S.E.M., n=9). This result represents a 42% (±3.3 S.E.M.) survival of the motoneurone to the soleus. The difference in the motoneurone number between the two sides was significant as evaluated by the Mann-Whitney U Test (p<0.0001).

The numbers of motoneurones on the contralateral sides of the two groups of rats which had the nerve crush only or the nerve crush and dorsal root section were similar to each other (p=0.17, Mann-Whitney U Test). Therefore, the percentage survival of motoneurones to the soleus muscle in the two groups can be compared to ascertain whether the dorsal root sections had any effect on the survival of motoneurones. The survival of soleus motoneurones in the two groups of rats appear similar to each other with
42.9% (±3.0 S.E.M.) survival for the nerve crush only group and 42% (±3.3 S.E.M) survival for the nerve crush plus dorsal root section group. In fact, the Mann-Whitney U Test indicated that the two groups of results were from identical population (p=1.00).

Therefore, according to this study, attempts to decrease the segmental afferent activity to the injured motoneurones by L4 and L5 dorsal root sections seemed to have no effect on the number of motoneurones that survived nerve crush at P4.

iii) Effects of Sciatic Nerve Crush at P4 on the Area of Motoneurones to the Soleus Muscle

Previous studies have shown that the area of motoneurones that survived injury are smaller than normal. Whether motoneurones that survive sciatic nerve crush at P4 will be similarly affected was examined.

The mean motoneurone areas on the contralateral and operated side of each rat that had their right sciatic nerve crushed at P4 are shown in Table 19A. The mean value of the mean motoneurone area on the control side was 1089\(\mu m^2\)
Table 19A. The mean area of surviving motoneurones to the soleus muscle after a nerve crush at P4.

Table 19B. The effect of dorsal root section on the mean area of surviving motoneurones to the soleus muscle after a nerve crush at P4.

The sciatic nerve was crushed at P4 on one side of the animal (Table 19A) and in some of the animals dorsal root section were also carried out (Table 19B). 8-10 weeks later the motoneurones to the soleus muscles were retrogradely labelled with HRP. The outline of HRP labelled motoneurones was traced with the aid of a camera lucida onto a graphics tablet attached to a microcomputer, which calculated the area of the motoneurones. The table shows the mean motoneurone areas of the operated and control sides of individual spinal cords. The mean motoneurone area on the operated side was expressed as a percentage of that on the control side. The mean motoneurone areas was also calculated by pooling together all the data and shown in the tables.
### TABLE 19A: NERVE CRUSH AT P4

<table>
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<tr>
<th>Animal</th>
<th>Mean Motoneurone Area ((\mu m^2))</th>
<th>%Op/Con</th>
<th>K-S Test</th>
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<td>±34.87</td>
<td>±3.05</td>
</tr>
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</table>

### TABLE 19B: NERVE CRUSH AT P4 + DORSAL ROOT SECTION

<table>
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<tr>
<th>Animal</th>
<th>Mean Motoneurone Area ((\mu m^2))</th>
<th>%Op/Con</th>
<th>K-S Test</th>
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<td>1161.75</td>
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<td>3</td>
<td>967.37</td>
<td>885.97</td>
<td>91.59</td>
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<td>82.17</td>
</tr>
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<td>966.56</td>
<td>913.28</td>
<td>94.49</td>
</tr>
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<td>1392.22</td>
<td>1159.31</td>
<td>83.27</td>
</tr>
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<td>1084.22</td>
<td>924.46</td>
<td>85.26</td>
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<td>8</td>
<td>1203.85</td>
<td>955.51</td>
<td>79.37</td>
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<tr>
<td>9</td>
<td>867.29</td>
<td>744.61</td>
<td>85.85</td>
</tr>
<tr>
<td>Mean</td>
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<td>884.07</td>
<td>85.21</td>
</tr>
<tr>
<td>±S.E.M</td>
<td>±61.16</td>
<td>±42.56</td>
<td>±2.60</td>
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### Pooled Data

<table>
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<th>Op</th>
<th>K-S Test</th>
</tr>
</thead>
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<td>1086.23</td>
<td>852.28</td>
<td>n.s.</td>
<td></td>
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<tr>
<td>±18.08</td>
<td>±16.93</td>
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<tr>
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<td>p &lt; 0.001</td>
<td></td>
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</tr>
</tbody>
</table>
(±57.3 S.E.M., n=9) and on the operated side was 846\mu m^2 (±34.9 S.E.M., n=9). When the mean motoneurone area on the operated side was expressed as a percentage of that on the control side, it was found to be 78% (±3.1 S.E.M.). The mean motoneurone area on the operated side compared to the mean motoneurone area on the control side was found to be significantly different (p<0.01, Mann-Whitney U Test).

This reduction in the soleus motoneurone area due to the sciatic nerve crush at P4 was also found to be significant when tested on the pooled data (p<0.001, T-Test). The mean motoneurone area calculated on the pooled data on the operated side was 852.3\mu m^2 (±16.9 S.E.M.) while that on the control side was 1086.2\mu m^2 (±18.1 S.E.M.).

Table 19A also shows the significance level of the differences in the distribution of the soleus motoneurone areas due to the nerve crush at P4 for each animal. In 7 of the 9 rats, the difference between the operated side and the control side was significant as evaluated by the Kolmogorov-Smirnov Test. The distribution of the pooled motoneurone area of the control and operated side in rats which had their sciatic nerve crushed at P4 also proved to
be significantly different (p<0.001, Kolmogrov-Smirnov Test). Figure 27A shows the distribution of the motoneurone sizes on the operated side superimposed onto that on the control side. It shows that there was a bimodal distribution of the motoneurone sizes on the control side. On the other hand, the distribution of motoneurone sizes on the operated side does not show a bimodal distribution. The figure also shows that the decrease in the mean motoneurone size appears to be due to the absence of large motoneurones.

Therefore, nerve injury at P4 reduced the mean size of surviving motoneurone as assessed in this study. The size distribution of the motoneurones was also significantly different from that of the contralateral side.

iv) Effects of Decreased Afferent Activity on the Area of Injured Motoneurones

Table 19B shows the mean area of motoneurones to the soleus of the control and operated sides of rats which had their right sciatic nerve crushed at P4 and had the L4 and L5 dorsal roots of the right side sectioned at the same time.
Figure 27A. The distribution of the sizes of surviving motoneurones to the soleus muscle after a nerve crush at P4.

The histogram of the distribution of the motoneurone sizes on the operated side was superimposed onto that of the contralateral side of the spinal cord of animals that had a sciatic nerve crush at P4.

Figure 27B. The effect of dorsal root section on the distribution of the sizes of surviving motoneurones to the soleus muscle after a nerve crush at P4.

The histogram of the distribution of the motoneurone sizes on the operated side was superimposed onto that of the contralateral side of the spinal cord of animals that had undergone dorsal root sections in addition to a sciatic nerve crush at P4.
FIGURE 27

A: NERVE CRUSH AT P4

B: NERVE CRUSH AT P4 + DORSAL ROOT SECTION
The mean motoneurone area on the operated side was also expressed as a percentage of the mean motoneurone area on the control side.

The mean value of the mean motoneurone area on the control side was 1046.4 \( \mu \text{m}^2 \) (±61.2 S.E.M., n=9) and on the operated side was 884 \( \mu \text{m}^2 \) (±42.6 S.E.M., n=9). The mean percentage of the operated over the control motoneurone area was 85% (±2.6 S.E.M.). This reduction in the mean motoneurone area proved to be significant (p<0.05, Mann-Whitney U Test). When the data was pooled together, the reduction in size was significant as evaluated by the T-Test (p<0.001). The mean size of pooled data on the operated side was 897.6\( \mu \text{m}^2 \) (±20.4 S.E.M.) while that on the control side was 1044\( \mu \text{m}^2 \) (±16 S.E.M.).

Table 19B also indicates the significance levels of the difference in the distribution of motoneurone areas between the operated side and the control side of each animal. The difference was significant in 5 of the 9 rats. When pooled, the distribution of the data on the operated side was different from that on the control side (p<0.001, Kolmogorov-Smirnov Test). Figure 27B shows the distribution
of the pooled data of the motoneurone sizes on the operated side superimposed onto that on the control side of these rats. Again the reduction in the mean motoneurone size seem to be due to the absence of the large cells.

The data between the two experimental groups of rats were compared to see if there was any differences due to the dorsal root sections that were done after the nerve crushes. The mean motoneurone area of the two control sides were not significantly different but the distribution of the motoneurone sizes were significantly different ($p<0.05$, Kolmogorov-Smirnov Test). This difference could be explained by the fact that this test is very sensitive and would be significantly different even if only one of the factors tested in the distribution was found to be different.

When the pooled data of motoneurone areas on the operated side of the two groups of animals were compared, they proved to be similar in the motoneurone areas and in the distribution of the motoneurone sizes. Therefore, the dorsal root sections did not make any difference on the reduction in the motoneurone areas due to the nerve crush at P4 as tested in this study.
4.3.2 EFFECTS OF MK-801 ON MOTONEURONES TO PARALYSED MUSCLES

In section 3.3.3., it has been shown that treatment with two α-BTX containing silicon strips during the early postnatal period resulted in a reduction in the number of motoneurones to the soleus muscle such that only 51.8% of the motoneurones survived. Although this result is less than that previously reported by Greensmith and Vrbová (1992), it was concluded that the effect of the paralysis induced by such a treatment depended on the actual amount of α-BTX present. Increasing the motor activity resulted in a further reduction in the number of surviving motoneurones. In this study, we tested the possibility that decreasing the motoneurone activity by treatment with the NMDA receptor antagonist MK-801 will affect the survival of motoneurones to paralysed soleus muscle.

In newborn Wistar rats silicon strips containing α-BTX were implanted close to the soleus muscle on P0 and again at P3. In addition, the rats were given i.p. injections of either 1mg/kg b.w. MK-801 or saline for 12 days starting at birth.
The rats were checked for paralysis as described in section 3.3.3. The MK-801 injected rats were drowsy and quiet for a few hours after the injection. The dose given in this study was less than that previously given as the combination of the effects of α-BTX and MK-801 was affecting the mortality of these neonatal rats. After two to 3 months, the number and size of motoneurones to the soleus muscle was assessed by retrograde labelling with HRP.

i) The Effects of Treatment with MK-801 on the Number of Motoneurones to α-BTX Treated Soleus Muscle

Table 20 summarises the results from the experiment in which α-BTX containing silicon strips were implanted alongside the soleus muscle and the rats were given i.p. injections of MK-801. The mean number of HRP labelled motoneurones on the operated (paralysed) side was 37.3(±2.1 S.E.M, n=10) while that on the contralateral side was 56.6(±1.6 S.E.M, n=10). The mean number of motoneurones on the contralateral side is no different from the normal number of motoneurones to the soleus muscle reported previously (Greensmith and Vrbová, 1992; Burls et. al.,
Table 20. The effect of treatment with MK-801 on the survival of motoneurones to the soleus muscle after paralysis.

The number of motoneurones to the soleus muscle labelled with HRP on each side of the spinal cord after paralysis with two α-BTX containing implants and treatment with MK-801 was assessed in adult rats. The number of labelled motoneurones on the operated side was expressed as a percentage of the number of labelled motoneurones on the control side.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Motoneurone Number</th>
<th>% Op/Con</th>
</tr>
</thead>
<tbody>
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<td>Op</td>
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<td>1</td>
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<td>10</td>
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</tr>
<tr>
<td>Mean</td>
<td>56.60</td>
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</tr>
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<td>±S.E.M</td>
<td>1.66</td>
<td>2.12</td>
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</table>
The reduction of the number of motoneurones on the paralysed side was significant (p<0.0001, Mann-Whitney U Test) where the survival of motoneurones on the paralysed side was 65.6% (±2.9 S.E.M, n=10). This result is higher than that in which rats were treated with saline in addition to the paralysis where the survival was 51.8% (see Table 16A, section 3.3.3.i). Figure 28 shows the mean motoneurone survival on the right side of the spinal cord in three groups of experimental rats. The value for the saline treated only is taken from section 3.3.1.i. It shows that treatment with MK-801 resulted in a smaller reduction in the number of motoneurones i.e. only 35% of motoneurones died in the MK-801 treated rats compared with 48% that died in the saline treated rat (p<0.01, Mann-Whitney U Test).

Therefore, these results show that a proportion of motoneurones that would have died following paralysis at birth were rescued by treatment with MK-801.

Greensmith et. al. (1994) found that MK-801 caused reduction in death of motoneurones following nerve injury after birth that was dose-dependent. A higher dose of 2mg/kg b.w. resulted in a greater saving of motoneurones destined to die. It is very likely that a greater number of
Figure 28. The mean percentage of survival of motoneurones to the soleus muscle after paralysis: comparison of the effect of treatment with saline and L-Dopa.

The error bars represent the Standard Error of the Means (S.E.M.)
FIGURE 28

Experimental Procedure

Mean % MN. Survival

NORMAL + SALINE  BTX + SALINE  BTX + MK-801
motoneurones would have been saved had it been possible to give a bigger dose of MK-801 to the rats that had their soleus muscle paralysed in the present study.

ii) The Effects of Treatment with MK-801 on the Area of Motoneurones to Paralysed Muscles

As reported in section 3.3.3., the mean area of motoneurones to the α-BTX treated muscles was reduced to 88% of that on the contralateral side. Whether decreasing the motor activity will have any effect on the reduction of the motoneurone area due to the paralysis was examined next.

The results of the experiment in which neonatal Wistar rats had their right soleus muscles paralysed and had been given i.p. injections of MK-801 are summarised in Table 21. It shows the mean motoneurone area on both sides of the spinal cord of each rat. The mean value of the mean motoneurone area on the paralysed side was 914µm² (±46.8 S.E.M., n=10) compared to 964.5µm² (±53.5 S.E.M., n=10) on the contralateral side.
Table 21. The effect of treatment with MK-801 on the mean area of motoneurones to the soleus muscle after paralysis.

The soleus muscle was paralysed with the implantation of 2 α-BTX containing silicon strips. The animal was treated with MK-801 for 12 days starting on P0. 8-10 weeks later the motoneurones to the soleus muscles were retrogradely labelled with HRP. The outline of HRP labelled motoneurones was traced with the aid of a camera lucida onto a graphics tablet attached to a microcomputer, which calculated the area of the motoneurones. The table shows the mean motoneurone areas of the operated and control sides of individual spinal cords. The mean motoneurone area on the operated side was expressed as a percentage of that on the control side. The mean motoneurone areas was also calculated by pooling together all the data and shown in the table.
### TABLE 21: α-BTX + MK-801

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mean Motoneurone Area (μm^2)</th>
<th>%OP/CON</th>
<th>K-S Test</th>
</tr>
</thead>
<tbody>
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<td>Con</td>
<td>Op</td>
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<td>1034.31</td>
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<td>934.96</td>
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<td>92.54</td>
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<td>95.96</td>
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<td>95.65</td>
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<td>964.63</td>
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<td>Mean ±S.E.M</td>
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<td>±16.28</td>
<td></td>
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</table>
The mean value of the motoneurone area on the contralateral side in these MK-801 treated rats was smaller than that in rats that had been injected with saline (p<0.001, T-Test, see Table 17A, section 3.3.3.ii). This could be due to the smaller body size of the MK-801 treated rats compared to the saline treated rats. To examine this possibility, the body weights of the MK-801 treated rats were compared with that of the saline treated rats. Since body weights of rats vary according to their age, sex and litter, only rats that have these three factors controlled for were taken into consideration. The mean body weight of the MK-801 treated rats calculated thus was 186.7g (±11.7 S.E.M., n=6) which was significantly less compared to 243g (±16.4 S.E.M., n=5) of the saline injected rats (p<0.05, Mann-Whitney U Test). Another possibility is that the rate of growth of these MK-801 treated rats were severely affected during the period of treatment. Figure 29 shows a graph of the body weight plotted against age for the MK-801 and saline treated pups from one litter during this period. It shows clearly that the body weight increase of the MK-801 treated rats was severely retarded compared to that of the saline treated rats. This retarded body weight might account for the
Figure 29. Graph of the body weight of animals plotted against their age during treatment with either MK-801 or saline.

Graph of age of animals plotted against the weight increase of the animals from one litter during the period of treatment with saline or MK-801. The animals were injected (i.p.) with saline or MK-801 for the first 12 days after birth. As they increased in age the difference in the weight increase became more apparent.
smaller sized motoneurones observed on the contralateral side of these MK-801 treated rats.

To determine the effect of decreasing motor activity on the reduction in area of motoneurones due to paralysis of the soleus muscle, the mean area of motoneurones on the paralysed side was expressed as a percentage of that on the contralateral side as shown in Table 21. The mean motoneurone size on the paralysed side was 95.7% (±3.7 S.E.M., n=10) of that on the contralateral side. Thus, there was a small reduction in the mean area of motoneurones to the paralysed soleus muscle. The data from both sides of the spinal cord were pooled and the mean motoneurone area on the paralysed side calculated thus was 923.9μm² (±16.3 S.E.M., n=10) while that on the contralateral side was 964μm² (±14.2 S.E.M., n=10). This small reduction in the mean motoneurone area of the paralysed side was not significant (T-Test). In the saline treated rats which had their right soleus muscle paralysed, the difference in the mean motoneurone area between the paralysed and the contralateral side was significant (p<0.01, T-Test, see Table 17A, section 3.3.3.ii) where the size of motoneurones on the paralysed side was 88% of that on the contralateral side. These results show that
treatment with an NMDA receptor antagonist lessened the reduction in motoneurone size such that the difference between the paralysed and contralateral side was no longer significant.

Table 21 also shows that the size distribution of the motoneurones on the paralysed side was significantly different from that on the contralateral side in 5 of the 10 MK-801 treated rats used in this study as assessed by the Kolmogrov-Smirnov Test. To assess the effects of the MK-801 treatment on the distribution of the motoneurone sizes, the results of this study was compared to that of the study in which the rats received saline treatment instead of the MK-801. In the saline treated group, this difference was significant in 5 of the 8 rats used in the study (see Table 17A, section 3.3.3.ii). In both groups of animals, the difference in the distribution of the sizes of motoneurones on the paralysed side compared to that on the contralateral side proved significant when tested on the pooled data where \( p<0.05 \) in the MK-801 treated animals and \( p<0.01 \) in the saline treated animals. Thus, although the difference was significant for both groups of animals, it can be seen that treatment with MK-801 had reduced the
difference in the distribution of the motoneurone sizes to some extent.

A graphic way to show that the MK-801 treatment had decreased the effect of the paralysis on any particular group of cells is shown in Figure 30. Here, the distribution of the motoneurone sizes on the paralysed side was superimposed onto that on the contralateral side of the MK-801 treated rats. The figure shows that no particular group of cells was affected by the paralysis induced by the α-BTX. This result is different from that in which the rats were treated with saline (see Figure 25A, section 3.3.3.ii) where paralysis resulted in a reduction in the number of the larger sized motoneurones. An interesting finding mentioned before was that the mean motoneurone size on the contralateral side of the MK-801 treated rats was smaller than that on the contralateral side of the saline treated rats. Figure 31 shows the distribution of the motoneurone sizes on the contralateral side of the MK-801 treated rats superimposed onto that of the saline treated rats. It shows that there was a bigger number of smaller sized motoneurones in the MK-801 treated rats.
Figure 30. The effect of treatment with MK-801 on the distribution of sizes of motoneurones to the soleus muscle after paralysis.

The histogram of the distribution of the motoneurone sizes on the operated side of the spinal cord was superimposed onto that of the motoneurone sizes on the contralateral side.
FIGURE 30

BTX + MK-801

Motoneurone Size (100 μm²)
Figure 31. The effect of treatment with MK-801 on the distribution of sizes of motoneurones to the soleus muscle.

The histograph of the distribution of motoneurone sizes on the control side of the spinal cord of animals treated with MK-801 was superimposed onto that of the control side of the spinal cord of animals treated with saline.
FIGURE 31

CONTROL SIDE

MK-801
Saline

No. of Motoneurones

Motoneurone Size (100 μm²)
Therefore, the results of this study show that treatment with MK-801 prevented the reduction in motoneurone size that would have been caused by the paralysis due to the α-BTX.
4.4. DISCUSSION

Recent results support the possibility that motoneurones that die after being deprived of target interaction may do so due to excitotoxicity. The maturity of these motoneurones may have been delayed and therefore they would have failed to acquire the ability to withstand the increasing afferent inputs that the motoneurones would receive as the animal matures. The results found in this study provide some evidence for this proposal.

Survival of motoneurones after nerve injury at P4

Nerve injury at P4 still resulted in a considerable death of motoneurones supplying the slow, soleus muscle. Interestingly previous studies have shown that injury at P5 causes no death of motoneurones to the soleus muscle (Lowrie, 1990). This means that within a period of 24 hours these motoneurones to soleus would have acquired the ability to withstand such injuries. That motoneurones die when injured at four days after birth is in accord with previous findings that there exist a critical period of about five days after birth when motoneurones remain
susceptible to injury (Lowrie et. al., 1987; Vejsada et. al., 1991; Mentis et. al., 1993; Greensmith et. al., 1995, 1996).

**Decreasing afferent activity to injured motoneurones**

The extent of motoneurone death after nerve injury at P4 remained unchanged when in addition to nerve injury, the segmental afferent input was reduced by dorsal root sections. The findings in this particular study is in apparent contrast with that reported by Sanner et. al., (1993). In their study, removal of dorsal root afferents prevented death of axotomised Clarke's Nucleus neurones. Thus, in the present study removing the afferent inputs from segmental afferents that reach the central nervous system through the L4 and L5 Lumbar dorsal roots had no effect on the overall loss of motoneurones. This could mean that sectioning just the 2 dorsal roots was not sufficient to result in a big enough decrease of the afferent activity. In the study by Sanner et. al. (1993), a much more extensive removal of segmental afferents was carried out. They sectioned dorsal roots L1 to S2 and prevented death of neurones in the L3 segment. Although
afferent projections may reach motoneurones only in their entry segments and the adjacent segments just above and below their entry (Culberson and Brown, 1984), Rivero-Melián and Grant (1990) have shown that fibres and terminal-like structures were found 8-13 segments rostral and 1-5 segments caudal to a dorsal root ganglion. Wall and Shortland (1991) proposed that these projections may represent a potential substrate for the development of functional connections under conditions of denervation.

Another possibility why the dorsal root sections in the present study had no effect on the survival of motoneurones may be due to sprouting. It is known that neonatal lesions induce sprouting in several sensory systems (Barr et. al., 1987; Schneider and Nauta, 1969). In this study, the terminals of the sensory neurones through neighbouring dorsal roots may have sprouted into the terminal fields of sensory neurones which were deprived of their own afferent input (Hulsebosch and Coggeshall, 1983; Stelzner et. al., 1979). Sprouting after neonatal lesions have been shown to be extensive when the afferents were sectioned early after birth compared to sectioning at P10 where no sprouting was observed (Fitzgerald, 1985). Sectioning at P5 resulted in weak sprouting. Therefore, there seem to be a defined
period during the early postnatal development when sectioning would result in sprouting. In the present study the dorsal root sections were carried out at P4 which would presumably result in sprouting. Fitzgerald et. al. (1990) proposed that such plasticity of the central terminals of sensory afferents to sprout into denervated areas of neonatal spinal cord may be to provide compensation following neonatal injury.

Blocking NMDA receptors of motoneurones to paralysed soleus muscle

Choi et. al., (1988) suggested that glutamate neurotoxicity may be predominantly mediated by the activation of the NMDA subclass of glutamate receptors which occur both directly during exposure to exogenous compounds and indirectly due to subsequent release of endogenous NMDA agonists. The motoneurone death seen after paralysis of the soleus muscle have been proposed to be a result of excitotoxicity (Vrbová and Lowrie, 1992). Therefore, blocking the receptor with MK-801 should attenuate this motoneurone death. The findings in this study supports this proposal. Blocking the NMDA receptors with MK-801 resulted in the saving of a
substantial number of motoneurones to the soleus that would have died due to the interruption of neuromuscular interaction by paralysis.

Mentis et. al. (1993) and Greensmith et. al (1994) similarly found that treatment with MK-801 rescued motoneurones destined to die after nerve crush injury at birth. The dose of MK-801 used in their study was higher than that used in the present study and accordingly more motoneurones were rescued from death. Thus, had a higher dose been used in the present study, a bigger number of motoneurones may have been rescued from death. It was not possible to use high doses of MK-801 in the present study because of the high mortality rate observed in the treated neonatal rats. Another method of delivery of the blocking agent might make it possible to deliver a bigger dose, for example a local application directly to the spinal cord may lessen the systemic side effects. The finding in this study concurs with that by Sanner and Goldberger (1991) who found that treatment with MK-801 prevented axotomy induced death in the Clarke's Nucleus. They suggested that the induced cell death in Clarke's Nucleus may be partially mediated by glutamate and its effects on NMDA receptors. Recent results found in this lab support the proposal that interruption of
neuromuscular interaction during the early postnatal periods will render motoneurones susceptible to glutamate excitotoxicity (Vrbová and Lowrie, 1992). An injury at a time when motoneurones were no longer dependent on neuromuscular interaction would still result in motoneurone death if the injury was followed by NMDA injections (Greensmith et. al., 1994). Paralysis of the soleus muscle during the early postnatal period would not result in motoneurone death at 3 weeks of age but when paralysis was followed by NMDA applications, motoneurones died by this age (Greensmith et. al., 1995). This may indicate that motoneurones have not yet completed their maturation by 5 days after birth as it was still possible to induce motoneurone death.

The results found in this particular experiment provide evidence that decreasing the afferent inputs with NMDA receptor blockers protected vulnerable motoneurones from succumbing to the excitotoxic effects of glutamate. Thus, this further supports the proposal that glutamate plays an important role in the death of motoneurones which have been denied contact with their target during the early postnatal period.
Changes in soma size

In this study, the soma size of the surviving motoneurones after nerve injury at P4 was found to decrease compared to that found on the contralateral side which concurs with previous studies (Lowrie et. al., 1987: Greensmith and Vrbová, 1992). When dorsal root sections were carried out in addition to the nerve crush, the size of the surviving motoneurones did not change further. Therefore, decreasing the afferent input by sectioning the L4 and L5 dorsal roots did not affect the size changes that resulted after a nerve crush injury at P4. Although Sanner et. al. (1993) found that deafferentation rescued Clarke's Nucleus neurones from dying after axotomy, it still did not prevent the decrease in size of the surviving neurones. The finding that the soma area of the surviving motoneurones was not affected by manipulating the segmental afferent activity as done in the present study was not surprising considering the possibilities discussed in the section above.

On the other hand, blocking the NMDA receptors with MK-801 successfully prevented the decrease in size of surviving motoneurones after paralysis of the soleus muscle during the early postnatal period. In the present study paralysis
resulted in a decrease in the size of surviving motoneurones. This has been previously shown by Greensmith and Vrbová (1992). Therefore, decreasing the afferent input by blocking NMDA receptors prevented decrease in motoneurone size which would have occurred as one of the typical effects of neonatal injury. This finding supports the proposal that glutamate excitotoxicity may be mediated by the NMDA receptors.

Conclusion

The results of the present experiments described in this chapter indicates that similar to nerve injury, interfering with the normal neuromuscular interactions during the early postnatal period renders some motoneurones unable to withstand the increasing activity in the developing spinal cord. A nerve crush inflicted at P4 still causes many motoneurones to die as does target deprivation by paralysis of the muscle. Decreasing the afferent inputs to the motoneurones by blocking NMDA receptors during this period saves a considerable number of motoneurones that would have died after their neuromuscular interaction was interfered with. This finding supports the proposal that motoneurones die due to excitotoxicity when they fail to achieve the maturity required to withstand increased afferent inputs.
CHAPTER 5 - GENERAL DISCUSSION

The present results show that induced activity causes some motoneurone to the soleus muscle to die. This is consistent with the proposal that motoneurones have to achieve a certain degree of maturity to enable them to accept the increased amount of afferent activation that they are subjected to as the circuitry of the spinal cord matures. This proposal was based on results obtained from experiments on target deprived motoneurones where NMDA injection resulted in death of motoneurones (Greensmith et al., 1994) even when the injury was inflicted after P5 when such injuries at this time normally would not result in motoneurone death (Lowrie et al., 1982; Lowrie, 1990). However in all the previous experiments the motoneurones were altered by target deprivation whereas in the present study motoneurones were not interfered with, but the activity of the CNS was increased by inducing precocious locomotor activity. It is interesting that it is possible to achieve a level of activity in the CNS that is high enough to destroy motoneurones and possibly other cells. This indicates that the basic mechanisms that allow activation of synaptic connections in the CNS are already mature before they are fully used.
The study shows that only some motoneurones are destroyed. This may be due to differences in either their maturity or their connectivity. Indeed, motoneurones to some muscles such as the fast TA and EDL were completely spared from the ill effects of the increased afferent activity. Nevertheless, the results of this study show that it was possible to increase the susceptibility of these motoneurones to activity after target deprivation. The suggestion that motoneurone death is caused by activity was based on findings that surviving motoneurones were more active than normal (Navarrete and Vrbová, 1984). It is possible to enhance motoneurone death by an additional increase of activity. The present results show that induced increase in afferent activity to the injured motoneurones caused a greater proportion of them to die. Thus, events associated with activity appear to be directly responsible for the loss of injured developing motoneurones.

Previous findings that NGF treatment resulted in the formation of additional muscle spindles after nerve lesion (Sekiya et. al., 1986) and in enhanced death of motoneurones after nerve crush (Miyata et. al., 1986) led to the suggestion that death of the motoneurones may be due
to the enhancement of the development of sensory inputs (Lowrie and Vrbova, 1992).

However, results in this study on the deafferented animals are in apparent disagreement with this proposal. Segmental afferent removal had no effect on the death of motoneurones after sciatic nerve injury. It may be that the procedure used in the present study was not radical enough to cause a sufficient decrease in the afferent inputs to motoneurones or that segmental afferents play a small role in death of motoneurones by excitotoxicity. Moreover, only a small part of synaptic input to motoneurones is monosynaptic and the vast majority is mediated through interneurones (see Baldissera et. al., 1981 for review).

The study shows that it is possible to decrease the number of motoneurones that die after being target-deprived during the neonatal period. Blocking NMDA receptors with MK-801 rescued a substantial number of motoneurones from dying. This result supports the proposal that afferent inputs of the maturing CNS may have been too much for the developing motoneurones to cope with if the normal neuromuscular interaction was interfered with. This finding which supports the proposal that motoneurone death may be due to excitotoxicity mediated by NMDA receptors seemed at contrast with that found by Piehl et. al. (1994). They
found that the expression of mRNAs for the NMDA receptor subunits was down-regulated after axotomy with the strongest down-regulation occurring after the procedure that resulted in the largest motoneurone loss. Choi et. al. (1987) found that some immature neurones that were resistant to excitotoxins were still chemosensitive to glutamate, so that in this case resistance to excitotoxicity was not due to a smaller number of glutamate receptors. It is possible that the increased activity may cause motoneurone death even in the absence of upregulation of the NMDA receptor numbers. Kuno and Llinás, (1970) found that the efficacy of synaptic excitation was increased in injured motoneurones. Thus, the excitability of the motoneurones itself might be increased. The finding of Navarrete et. al. (1990) supports the proposal that the intrinsic excitability of the motoneurone may be affected after a neonatal nerve injury.

The question that arises in this study is how does the target prepare motoneurones for accepting activity? A possibility is that the target derived trophic factors play a role in preparing the motoneurones to accept the increasing activity in the maturing CNS. But the finding in the present study that normal motoneurones that have a
supply of these factors still die argues against this. Furthermore, although the various trophic factors in different combinations have been shown to rescue mouse lumbar motoneurones after axotomy (Li et. al., 1994), this rescue seem to be short-lived (Vejsada et. al., 1995). The knockout experiments in which the genes for the neurotrophic factors or for their receptors have been removed seemed to indicate that trophic factors may not be essential in regulating the survival of motoneurones as no or little loss of motoneurones was observed in these animals (Klein et. al., 1993; Conover et. al., 1995; Jones et. al., 1994; Xin Liu et. al., 1995).

The present results supports the proposal that normal neuromuscular interaction during the early postnatal development is essential for motoneurones to mature and acquire the characteristics which enable them to withstand the increasing activity in the maturing spinal cord. It has been proposed that this interaction results in a change in the phenotype of the motoneurone such that the motoneurone is transformed from a growing into a secreting cell and thus become integrated in the circuitry of the spinal cord (Lowrie and Vrbová, 1992). Xie and Poo (1986) showed that when a growth cone encounters a muscle cell, there is a
large increase in transmitter release. Trophic factors are known to increase the release of transmitter at developing neuromuscular junctions in culture (Lohof et. al., 1993). Thus, although neurotrophic factors may not be essential in the survival of motoneurones, they may play a role in the maturation of motoneurones.

**Concluding remarks**

It has been demonstrated here that uninjured motoneurones that are allowed to interact with their target muscles can be induced to die. This indicates that the coordination of the timing of developmental events in the CNS and at the neuromuscular junction is essential for normal development. Injury or target deprivation probably slows maturation of motoneurones while the CNS matures, thus the developmental age of the motoneurones is out of phase with the degree of maturation of the CNS. The immature motoneurones are unable to cope with the increasing activity in the CNS since they still have a relatively high susceptibility to excitotoxicity. Thus, the findings in this study support the proposal that by disrupting the normal neuromuscular interaction, the maturation of motoneurones is delayed such that they become vulnerable to the excitotoxic effects of the neurotransmitter, glutamate.
APPENDIX I

SILICONE IMPLANT CONTAINING $\alpha$-BUNGAROTOXIN

2mg. $\alpha$-bungarotoxin (Sigma T3019).

Ground NaCl with pestle and mortar.

Add 28mg. NaCl into the container of $\alpha$-bungarotoxin (in fume cupboard, wearing face mask and gloves). Mix with spatula.

Weigh out 70mg. silicone rubber solution onto a Petri dish. NB. 3140 Dow Corning Silicone Rubber Solution 1ml. weighs 1mg.

Add dry mixture onto the blob of silicone rubber (fume cupboard switched off). Mix with spatula. Switch fume cupboard on as soon as dry mixture is safely enfolded into the silicone rubber.

Scrape mixture onto a fresh Petri dish and spread out evenly.

Dry overnight.

Each 1mg. strip will contain 20$\mu$g of $\alpha$-BTX and 280$\mu$g NaCl.

SILICONE IMPLANT CONTAINING NaCl
(Control) (0.9% Saline)

Mix 28mg. NaCl (ground) into 72mg. silicone rubber solution.

Each 1mg. strip will contain the same amount of NaCl as in $\alpha$-BTX plug.
APPENDIX II

MODIFIED HANKER-YATES METHOD FOR THE DEMONSTRATION OF HORSE RADISH PEROXIDASE

A. METHOD

1. Cut the spinal cord just below the L2 and L6 roots, and mark the control side of the cord by making a nick along the surface of the dorsal horn 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, 50 μm thick, and collect in Millonig's phosphate buffer in washing trays containing individual wells.

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

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

5. Wash in distilled water and rinse in two changes of Millonig's phosphate buffer for 10 minutes each.

6. React the sections in Hanker-Yates solution for 10-20 minutes, stopping before the background staining darkens.

7. Rinse in 2 changes of Millonig's phosphate buffer for 5 minutes each.

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

9. Counterstain with galloxyanin.

B. SOLUTIONS FOR THE HANKER-YATES METHOD

1. Millonig's phosphate buffer.

\[
\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O} \quad 19.08\text{g} \\
1\text{M NaOH} \quad 96.3\text{ml}
\]

Make up to 1 litre with distilled water.
Adjust to pH 7.3 and store at 4°C.
2. Cobalt/Nickel solution.

\[
\begin{align*}
1\% \text{ cobalt chloride} & \quad 300\text{ml} \\
1\% \text{ ammonium nickel sulphate} & \quad 200\text{ml}
\end{align*}
\]
Mix together just before use

3. Cacodylate buffer.

\[
\begin{align*}
0.1\text{M sodium cacodylate} & \quad 500\text{ml} \\
(21.4\text{g/500ml distilled water}) \\
0.2\text{M HCL} & \quad 440\text{ml} \\
(17.22\text{ml conc. HCL/litre distilled water})
\end{align*}
\]
Adjust to pH 5.1-5.2. Make up to 2 litres with distilled water. Store at 4°C


\[
\begin{align*}
\text{Hanker-Yates combined reagent (Sigma VI)} & \quad 150\text{mg} \\
\text{Cacodylate buffer (pH 5.1-5.2)} & \quad 100\text{ml} \\
30\% \text{ H}_2\text{O}_2 & \quad 1 \text{ drop}
\end{align*}
\]
Mix up just before use and discard after 1 hour.

C. COUNTERSTAINING

Preparation of Gallocyanin stain.

\[
\begin{align*}
\text{Gallocyanin} & \quad 0.3\text{g} \\
\text{Chromalum} & \quad 10\text{g} \\
\text{Distilled water to make} & \quad 100\text{ml}
\end{align*}
\]
Dissolve the chromalum in water by heating. Add the gallocyanin and simmer for 20-30 minutes. Cool, then add distilled water up to the original volume of 100ml and filter. Keep at room temperature.

Method

1. Wash the oven dried slides in distilled water briefly.

2. Place the slides in the gallocyanin stain for 10-25 minutes, depending on the freshness of the stain.

3. Rinse the sections in distilled water and then dehydrate in a graded series of ethanols, 2 minutes each.

4. Clear the sections in 2 changes of Histoclear, 2 minutes each. Mount in DPX.
APPENDIX III

ACETYLCHOLINESTERASE AND SILVER STAIN

1. Fix slightly stretched muscles in buffered formol-calcium at 4 °C for a min. of 6 hours.

   **Veronal-acetate buffer**

   **Stock solution:**
   Sodium acetate.\(3H_2O\) (MW=136) 9.714g
   Sodium barbitone (MW=206.18) 14.714g
   Dissolve in CO\(_2\) free distilled water, make up volume to 500ml.

   **Working solution:**
   V-A stock solution 20ml
   8.5% sodium chloride 8ml
   0.1M HCl added until meter reads pH 6.45 (~25ml HCl)
   Distilled water (CO\(_2\) free) added to make up 100ml.

   **Fixative**
   40% Formaldehyde 10ml
   CaCl\(_2\).2H\(_2\)O 1.0g
   MgCl\(_2\).6H\(_2\)O 0.5g
   CdCl\(_2\).2 1/2H\(_2\)O 0.1g
   V-A buffer (working soln.) to make up to 100ml.

2. Transfer tissue to 10% sucrose at 4 °C for a min. of 1 hour (or overnight).

3. Cut frozen sections at 50μm into distilled water and hold for 10 min. on ice.

4. Incubate sections for 20 minutes on ice:-

   **Incubation Medium:**
   Acetylthiocholine iodide . . 10mg
   Maleate Buffer ** . . 13ml
   100mM tri-sodium citrate . . 1ml
   30mM Copper Sulphate . . 2ml
   Double distilled water . . 2ml
   5mM Potassium Ferricyanide . 2ml
   Sucrose . . . . 3g

   N.B. Add solutions in order and mix well.
   Adjust solution to pH 6.0 if necessary.
** Maleate Buffer (0.1M Sodium hydrogen maleate)**

Maleic acid 1.16g
Sodium hydroxide 0.62g
Distilled water to make up to 200ml.

5. Rinse sections in distilled water for 30 seconds (or hold overnight).

6. Immerse in potassium ferricyanide (0.25g K₃Fe(CN)₆ /100ml H₂O) for 5 minutes at room temperature.

7. Rinse in distilled water, 3 washes of 5 minutes each.

8. Place in absolute ethanol, 2 washes of 30 min. each (sections tend to curl, handle individually, allow to spin flat).

9. Rinse in distilled water, 2 washes of 15 minutes each (may be left overnight at 4°C).

10. Transfer sections with glass rod to silver solution. Incubate at 37°C for between 20 minutes and 1 hour. (Preferably nearer 1 hour since only one side of the section is exposed to the silver stain).

    **Silver solution**
    Distilled water 50ml
    CaCO₃ 0.05g (double filter)
    CuSO₄.5H₂O 0.025g
    AgNO₃ 5g


12. Immerse in reducer solution and control microscopically. When nerves and endplates are visible, transfer to distilled water. Background comes up if left too long in the reducer.

    **Reducer solution**
    Hydroquinone 1g
    Na₂SO₃ 10g
    Distilled water 100ml

13. Wash in distilled water with 2 washes of 5 min. each.

14. Mount onto gelatinised slides and dry in 37°C oven. Dehydrate, clear and mount with DPX.
APPENDIX IV

PETTE'S MIRACLE STAIN FOR SUCCINIC DEHYDROGENASE

Stock Solutions

0.1M Phosphate Buffer pH 7.6
a) Dissolve 1.42g Na$_2$HPO$_4$ in 100 ml distilled water.
b) Dissolve 0.468g NaH$_2$PO$_4$ in 30ml distilled water.
Add b) to a) until the pH reaches 7.6

1M Sodium Succinate
1.62g in 10ml of the phosphate buffer.

15mM nitro-blue tetrazolium
246mg in 20 ml of distilled water (may need to be heated to 45°C to dissolve)

0.1M KCN
13mg KCN in 2ml of the phosphate buffer (make freshly)

10mM phenazine methosulphate
12.2mg in 4ml distilled water. Make up freshly. Very sensitive to light so protect whilst making up the working solution. If colour changes from yellow to green, discard.

To make up 40 ml of the working solution
0.1M Phosphate buffer 32.8ml
1M Sodium succinate 2ml
15mM Nitroblue tetrazolium 4ml
0.1M KCN 0.4ml
10mM Phenazine methosulphate 0.8ml
Filter and keep in dark bottle in the fridge at 4°C

Method
1) Put a few drops of working solution over sections on the slide. Incubate at 37°C for 5 minutes.
2) Wash in 0.9% saline for 1 minute, 70% acetone, 90% acetone, twice in absolute alcohol and two changes of histoclear/xylene for 2 minutes each. Mount in DPX.

Results
SDH stains blue whilst the background remains clear.
APPENDIX V

SLOW MYOSIN ANTIBODY STAIN

1. Dry sections for 30 mins. at room temperature.

2. Fix in absolute alcohol for 10 mins. at room temperature.

3. Rinse in PBS+0.1% BSA (Bovine Serum Albumin).

4. Block endogenous peroxidase activity. Incubate sections in 0.3% H₂O₂ in PBS for 30 mins.
   (1ml. of 30% w/v solution of H₂O₂ in 100ml. of PBS)

5. Rinse in PBS.

6. Background blocking. Block non-specific binding sites with Normal Horse Serum for 30 mins.
   Dilute NHS with PBS+Triton in 1:30

7. Shake off serum.

8. Rinse in PBS for 5 mins.

9. Dry around sections. Incubate in primary antibody 96J (Slow Myosin Antibody from Tej Dhoot) for 1 hr.
   1:100 Dilution in PBS+Triton

10. Rinse in PBS for 3x 5 mins.

11. Dry around sections. Incubate in B-H/M (Biotinylated Anti-Mouse produced in Horse) for 2 hrs.
    1:200 dilution in PBS+Triton
    (Prep. solution for step 13)

12. Shake off secondary layer antibody. Rinse in PBS 3x 5 mins.

13. HRP-Stain Procedure
    a. Put in Avidin-HRP Complex for 1 hr.
       Avidin-HRP Complex: Mix Sol. A (10µl) and Sol. B (10µl) in 980µl PBS+Triton 30 mins. before use
    b. Wash in PBS 5 mins., in Tris Buffer (pH=7.6) 2x 5 mins.
c. Incubate in DAB solution for 10 mins.
Make up a 0.05% DAB (Diamino Benzidine) solution in 5 mM Tris Buffer (pH=7.6)
Take 1x1ml. Ependorf aliquot from freezer and add 80mls. of Tris Buffer (pH=7.6)

d. Add H$_2$O$_2$: 10µl of 30% w/v solution H$_2$O$_2$ to 80 mls DAB
Incubate sections for 5-10 mins.

e. Wash in Tris Buffer (pH=7.6) 3x 5 mins.

14. Dehydrate and mount in DPX.


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TRANSIENT MUSCLE PARALYSIS IN NEONATAL RATS
RENDERS MOTONEURONS SUSCEPTIBLE TO
N-METHYL-D-ASPARTATE-INDUCED NEUROTOXICITY

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Abstract—Paralysis of the soleus muscle in newborn rats causes a large proportion of motoneurons to
die by 10 weeks of age. However, all of these neurons are still present at three to four weeks of age. We
have previously shown that although nerve injury at five days does not result in any motoneuron death,
it does render these neurons susceptible to the toxic effects of the glutamate agonist N-methyl-D-aspartate.
Using retrograde labelling of soleus motoneurons, in this study we show that an increased susceptibility
to glutamate also plays a role in the eventual death of those motoneurons which survive for three weeks
after interruption of neuromuscular transmission at birth but die by 10 weeks. Treatment with dizocilpine
maleate an antagonist of the N-methyl-D-aspartate receptor increased the survival of motoneurons
to alpha-bungarotoxin-treated soleus muscles. By 10 weeks of age the size of motoneurons to alpha-
bungarotoxin-treated soleus muscles is smaller than that of controls, but after treatment with dizocilpine
maleate the sizes of motoneurons to control and treated muscles are similar. Moreover, only 55 ± 2.7%
of motoneurons to the soleus muscle paralysed at birth with alpha-bungarotoxin survive for three weeks
after a single injection of N-methyl-D-aspartate at 12 days of age. This motoneuron death is due to the
application of N-methyl-D-aspartate since treatment with alpha-bungarotoxin alone causes no loss of
neurons at this age. These results provide support for the proposal that motoneurons deprived of functional interaction with
their target during a critical period in development, exhibit an increased sensitivity to the toxic effects of
 glutamate, and indicate that such sensitivity may be involved in the long term death of these neurons.

During early postnatal development, motoneurons remain dependent upon target contact for their sur-
vival. Following injury to the sciatic nerve in neonatal
rats, a large proportion of motoneurons dies.29,36,32,14,15
This dependence on target contact decreases rapidly
within the first week of postnatal life, so that nerve
crush at five days of age results in little motoneuron
death,10 although motor function is severely im-
paired. This impairment is due to a loss of muscle
fibres. The surviving motoneurons not only have a
small motor unit territory, but their dendritic tree is
permanently altered.15,25,26 These motoneurons are
also more active and exhibit altered reflex responses
such as inappropriate activation during both volun-
tary and reflexly elicited movement.20,21 Furthermore,
although motoneurons survive nerve injury at five
days, they become sensitive to the toxic effects of
the excitatory neurotransmitter glutamate. Following
sciatic nerve crush at five days and treatment with the
 glutamate agonist N-methyl-D-aspartate (NMDA)
one week later, 65% of soleus motoneurons die.6
Thus, for the motoneuron to survive and develop
normally it is essential to remain in contact with
the muscle during this critical period of postnatal
development.

The mechanism by which interaction with the
target promotes motoneuron survival and normal
development is not fully understood. However, we do
know that it is not just physical contact between the
motoneuron and its target that is the critical factor in
the maintenance of developing motoneurons. In a
previous study we examined the effects of preventing
neuromuscular interaction in neonatal rats on the
survival of motoneurons.10 Following paralysis of the
soleus muscle, the motoneurons supplying this muscle
survived for up to four weeks after the period of
muscle paralysis, although 10 weeks later 36% of
these motoneurons died. In addition, as with nerve
injury, the soma area of the surviving motoneurons
was smaller than that of control soleus moto-
neurons.10 Thus, the initial paralysis lasting for just
over a week, has long-lasting effects on these cells. It
is interesting that following both nerve injury and
muscle paralysis motoneurons can survive, at least
for four weeks, even though they exhibit altered
characteristics.

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Abbreviations: BDNF, brain-derived neurotrophic factor;
alpha-BTX, alpha-bungarotoxin; CNTF, ciliary neuro-
trophic factor; HRP, horseradish peroxidase; MBP,
Millonig's phosphate buffer; MK-801, dizocilpine
maleate; NGF, nerve growth factor; NMDA, N-methyl-
d-aspartate.
The mechanism of this delayed cell death is not known. In this study we investigated the possibility that motoneurons, prevented from functionally interacting with their target during a critical period in their postnatal development, fail to develop normally. It has been proposed that the role of the target in early postnatal development is to induce the motoneuron to acquire characteristics which enable it to withstand the level of activity present in the developing spinal cord, which is mediated by the excitatory transmitter glutamate. Whether motoneurons denied functional interaction with their target exhibit an inappropriate sensitivity to glutamate was tested here. In addition we examined whether some of the motoneurons destined to die by 10 weeks of age after neonatal muscle paralysis could be rescued by blocking the NMDA receptor with MK-801 (dizocilpine maleate). A preliminary report of some of these results was given to the Physiological Society of Great Britain.

**EXPERIMENTAL PROCEDURES**

**Surgery**

Neonatal Wistar albino rats of both sexes were used. Under halothane anaesthesia and sterile conditions, a small strip of silicon rubber (Dow Corning, 3140 RTV) containing alpha-bungarotoxin (z-BTX; Sigma) was implanted between the soleus and flexor hallucus longus muscles in one leg of newborn rats, within 3–6 h of birth. This treatment caused complete paralysis of the soleus muscle for the first 24 h. The muscles gradually recovered from the paralysis and by eight to nine days postnatally neuromuscular transmission was restored (see Ref. 9). Control animals, usually littermates, were implanted with silicon strips containing only NaCl. After recovery from the anaesthesia the pups were returned to their mother.

In another group of animals, a second z-BTX strip was implanted alongside the soleus muscle at three days of age in order to prolong the period of paralysis and thereby increase the degree of motoneuron death observed at 10 weeks of age. Such treatment with z-BTX has previously been shown to prolong the period of muscle paralysis by three to four days, so that at eight days of age the muscle is still 50% paralysed, resulting in the death of approximately 65% of soleus motoneurons by 10 weeks of age.

**Preparation of implants**

The implants were prepared in the following manner: ground NaCl was added to the z-BTX powder, mixed thoroughly with the silicon rubber solution and allowed to dry (for details see Refs 4, 9). Small strips weighing approximately 0.3 mg were cut from the silicon rubber for implantation into the newborn rats. In those animals treated with a second z-BTX implant at three days of age the size of the implant was doubled for the second implantation. The final amount of z-BTX in each implant was 7 µg for newborn rats and 14 µg for the second implantation. These implants caused no direct physical damage to the muscle fibres.

**Administration of N-methyl-D-aspartate**

At 12 days of age the rats were treated with either the glutamate agonist NMDA or control saline (see Ref. 6). Under halothane anaesthesia and sterile conditions, a partial laminectomy was performed on the same side as the previous treatment with z-BTX. Using a Hamilton microsyringe and a micromanipulator, a stereotactic injection was made slowly into the ventral horn on one side of the spinal cord at the level of L4-L5. The injection site was rinsed with sterile saline, and the area of laminectomy covered with gelfoam (Spongostan) to aid wound repair. The animals were allowed to recover from anaesthesia before being returned to their mother. The 12-day-old rats weighed approximately 30 g, and they received an injection of either 1 µl 50 mM NMDA, 2 µl 50 mM NMDA or an equivalent volume of sterile saline. The maximum dose of NMDA that was tolerated by these animals was 2 µl of 50 mM NMDA, a similar dose to that used in another study on retinal ganglion cells.

**Treatment with dizocilpine maleate**

In the group of rats which received two z-BTX implants, one at birth and another three days later, the pups were treated with MK-801, an antagonist of the NMDA receptor. The rats received daily i.p. injections from birth until 12 days of age. Control animals were injected with sterile saline. In previous experiments in which we have shown that MK-801 can rescue motoneurons destined to die following neonatal nerve injury we have used a dose of 2 mg/kg MK-801 for 12 days, which was found to be the maximum dose tolerated by neonatal rats after nerve injury.

However, treatment with z-BTX lowered the tolerance to MK-801, so that in the present experiments the dose of MK-801 injected was reduced to 1 mg/kg.

**Retrograde labelling of motoneurons**

When the rats were four weeks old the number of surviving motoneurons was assessed using the retrograde tracer horseradish peroxidase (HRP). Under halothane anaesthesia and sterile conditions, HRP (Type VI; Sigma) was injected into the soleus muscles of both hindlimbs using a Hamilton microsyringe. Twenty-four hours later the animals were re-anaesthetized (4.5% chloral hydrate; 1 ml/100 g body weight, i.p.), the soleus muscles removed and weighed, and the animals perfused transcardially with a fixative containing glutaraldehyde (2.5% in Millong's phosphate buffer (MPB) pH 7.3). The spinal cords were removed and the lumbar region postfixed for 2 h in the same fixative, cryoprotected in sucrose (30% in MPB) and frozen sections cut at 50 µm. The free-floating sections were then processed for HRP histochemistry using a modified Hanks-Yates method and lightly counterstained with a Nissl stain (gallocoyanin). The number of HRP-labelled motoneurons in each ventral horn was counted under a light microscope. In order to avoid counting the same cell twice in consecutive sections, only those neurons in which the nucleolus was clearly visible at high magnification were included in the counts. As an index of motoneuron survival, the number of labelled cells on the operated side of each spinal cord was expressed as a percentage of the number on the control side. The cross-sectional area of the motoneuron perikarya was measured using a digitizing tablet linked to a computer.

**RESULTS**

**Rescue of alpha-bungarotoxin-treated motoneurons with dizocilpine maleate**

Previous results have shown that 10 weeks after treatment of the soleus muscle with z-BTX shortly after birth, a large number of motoneurons die. Whether these motoneurons destined to die can be rescued was tested. A group of rat pups treated with z-BTX was injected with an NMDA antagonist, MK-801. The rats received an i.p. injection (1 mg/kg) immediately following implantation of z-BTX, and every day thereafter until they were 12 days old. On the third day they were treated with a second z-BTX dose tolerated by neonatal rats after nerve injury.
implant. The effect of the muscle paralysis and blockade of NMDA receptors on motoneuron survival was assessed when the rats were 10 weeks old, by retrograde labelling with HRP.

Table 1 summarizes the results from this group of experiments. These confirm our previous findings that 10 weeks after an extended period of muscle paralysis only 51% (± 3.02 S.E.M., n = 8) of soleus motoneurons survive. Treatment with MK-801, an NMDA receptor antagonist rescues a proportion of such motoneurons destined to die after z-BTX treatment of soleus, so that as many as 65% (± 2.86 S.E.M., n = 10) survive by 10 weeks. This result represents a significant increase in motoneuron survival (P < 0.01, Mann-Whitney U-test). It is possible that more motoneurons could have been rescued had it been possible to use a higher dose of MK-801. Our previous study found that a higher dose of MK-801 (2 mg/kg) caused a greater reduction in motoneuron death following nerve injury at birth than the dose used in this study.

We have previously found that treatment of the soleus muscle with two z-BTX implants not only causes the death of motoneurons but significantly reduces the soma size of the surviving neurons. Table 1 also illustrates the results of motoneuron survival following nerve injury at birth and the dose used in this study.

The results also indicate that a proportion of motoneurons which would be expected to die by 10 weeks of age following a period of muscle paralysis during early postnatal development, were saved by treatment with MK-801, an antagonist of the NMDA receptor. The results also indicate that z-BTX treatment of soleus muscles renders a proportion of motoneurons susceptible to the excitotoxic effects of NMDA. The next set of experiments were carried out to test this possibility.

Table 1. The effect of neonatal muscle paralysis and treatment with dizocilpine maleate on motoneuron survival and soma area in adult rats

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Number of motoneurons</th>
<th>Mean motoneuron area % Op/Con</th>
<th>S.E.M.</th>
<th>n =</th>
</tr>
</thead>
<tbody>
<tr>
<td>z-BTX: saline</td>
<td>51.8 ± 3.02</td>
<td>88.2 ± 6.95</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>MK-801: 10 weeks</td>
<td>65.3 ± 2.86</td>
<td>95.7 ± 3.71</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 summarizes results from experiments in which rat pups were treated with z-BTX at birth and three days of age. One group of rats also received daily i.p. injections of saline from birth to 12 days of age, whilst the experimental group was treated with MK-801 (1 mg/kg body weight). The number of retrogradely-labelled soleus motoneurons in the operated ventral horn of each spinal cord was counted and expressed as a percentage of the number in the control (Con) ventral horn. The soma area of HRP-labelled motoneurons was measured with a digitizing tablet and, in each animal, the mean area of those motoneurons in the operated (Op) ventral horn is expressed as a percentage of the mean area of the control motoneurons. The table gives the mean ± S.E.M. for all experiments and n is the number of animals in each group.

Treatment with MK-801 for 12 days after a period of muscle paralysis rescues a significant number of soleus motoneurons which would otherwise die by 10 weeks of age (P < 0.01, Mann-Whitney U-test). Furthermore, there is no significant reduction in motoneuron area after MK-801 treatment, whereas treatment with saline after z-BTX causes a significant reduction in soma area (P < 0.001, t-test).
Table 2. The effect of N-methyl-D-aspartate after transient neonatal muscle paralysis on the survival of soleus motoneurons

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Number of motoneurons</th>
<th>% Op/Con motoneurons</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle: Spinal cord</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-BTX: NMDA (1 μl) mean ± S.E.M. n = 3</td>
<td>61 ± 3.5</td>
<td>62 ± 2.3</td>
</tr>
<tr>
<td>a-BTX: NMDA (2 μl) mean ± S.E.M. n = 13</td>
<td>59 ± 2.0</td>
<td>32 ± 1.6</td>
</tr>
<tr>
<td>a-BTX: Saline mean ± S.E.M. n = 5</td>
<td>57 ± 4.7</td>
<td>57 ± 3.8</td>
</tr>
<tr>
<td>NaCl: NMDA (2 μl) mean ± S.E.M. n = 7</td>
<td>51 ± 2.6</td>
<td>53 ± 2.5</td>
</tr>
<tr>
<td>NaCl: Saline mean ± S.E.M. n = 9</td>
<td>52 ± 2.3</td>
<td>52 ± 1.6</td>
</tr>
<tr>
<td>a-BTX only four weeks mean ± S.E.M. n = 3</td>
<td>51 ± 2.7</td>
<td>58 ± 3.5</td>
</tr>
<tr>
<td>a-BTX only ten weeks mean ± S.E.M. n = 6</td>
<td>56 ± 1.5</td>
<td>35 ± 2.8</td>
</tr>
</tbody>
</table>

Table 2 summarizes results from experiments in which the soleus muscle in one hindlimb of newborn rats was paralysed with a-BTX at birth, followed by an injection of NMDA into the spinal cord at 12 days of age. The mean number of HRP-labelled soleus motoneurons in the control and operated ventral horn is given for each experimental group, and the number of cells present in the operated side is expressed as a percentage of the number in the control side; n is the number of animals in each experimental group.

Statistical comparisons were made between the number of labelled motoneurons in the control and operated sides of spinal cords for each experimental group (Wilcoxon rank sum test). The only group in which there was a significant loss of labelled motoneurons was that in which muscle paralysis was followed by treatment with 2 μl NMDA.

This table also presents our previous findings in which the soleus muscle was treated with a-BTX at birth and examined four weeks and 10 weeks later. Although no motoneurons had died at four weeks of age a significant loss of motoneurons had occurred by 10 weeks.

DISCUSSION

The results presented here confirm our earlier findings that treatment of the soleus muscle with a-BTX at birth, does not result in motoneuron death at three to four weeks of age. However, we previously found that a proportion of these motoneurons had died by 10 weeks of age, which is also confirmed by the results of this study. Our previous results have shown that motoneurons destined to die after axotomy at birth can be rescued when animals are treated with an NMDA receptor blocker, MK-801. This result suggests that these motoneurons become excessively sensitive to the excitotoxic effects of glutamate. The present results show that many soleus motoneurons that would have died after a-BTX treatment of the muscle can be saved if the animals are treated with MK-801. It is well known that contact with the target during the neonatal period is essential for the survival of developing motoneurons, and our study indicates that functional interaction between the motoneuron and its target is a critical factor in the survival of motoneurons and the development of their resistance to the excitotoxic effects of glutamate. The abnormal development of approximately 40-50% of a-BTX-Paralyzed motoneurons supports the earlier findings. It is important to note that the muscle paralysis was followed by treatment with NMDA and that the effect of NMDA treatment is dose dependent. Therefore, the muscle paralysis is followed by treatment with NMDA that a loss of HRP-labelled motoneurons is observed. Moreover, the effect of NMDA treatment is dose dependent.

The soleus muscles in both hindlimbs were removed prior to perfusion, and weighed. The results of these muscle weights reflect the findings of motoneuron counts (see Table 3), in that the only group in which there was a significant reduction in muscle weight was that in which the soleus muscle was paralysed with a-BTX before treatment with NMDA 12 days later.
Paralysis renders motoneurons sensitive to NMDA

Motoneurons is also illustrated by the present results, which show that prevention of functional neuromuscular interaction during the first week of postnatal life renders these motoneurons susceptible to the excitotoxic effects of the glutamate agonist, NMDA. Nevertheless, these motoneurons appear to be resistant to a certain degree of over-activation, since application of a lower dose of NMDA (1 μl) fails to induce motoneuron death.

The number of motoneurons labelled with HRP in the control soleus motor pools compares well with the findings of other authors, and indicates that HRP has been taken up by axons of motoneurons in the soleus motor pool. Fewer motoneurons are labelled with HRP after treatment with α-BTX and NMDA, but this is unlikely to be due to a failure of HRP uptake since we also found a reduction in the number of counterstained motoneurons on the treated side of the spinal cords. In other studies, retrograde labelling of motoneurons has been shown to compare well with counts of Nissl stained motoneurons following nerve injury (for a review see Ref. 17).

In a recent study we have shown that nerve injury at five days of age, an insult that does not normally result in motoneuron death, nevertheless renders motoneurons susceptible to NMDA induced neurotoxicity, with the death of approximately 50% of soleus motoneurons. Glutamate and its agonist NMDA are known to be excitotoxic and cause death of both developing and mature neurons. These results show that although motoneurons deprived of target interaction either by nerve injury or muscle paralysis can survive, at least in the short term, they are rendered abnormally sensitive to glutamate. It is interesting that in the three series of experiments, i.e. the present series, those in which animals received a nerve crush at five days followed by exposure to NMDA, and those experiments where the muscle was paralysed with α-BTX at birth and the animals were examined 10 weeks later, a similar number of motoneurons is lost. It is likely that these are predominantly α-motoneurons, since changes in soleus muscle weight closely parallel the results of motoneuron number obtained by retrograde labelling. However, it is not clear why a particular population of motoneurons from the same motor pool should be more susceptible to glutamate excitotoxicity, and further investigation is needed before we can clearly understand these findings.

Several indications that NMDA mediated events are involved in the death of motoneurons after neonatal nerve injury have been recently obtained. Treatment with MK-801, a NMDA receptor antagonist, prevents the death of some motoneurons destined to die after nerve crush at birth. Not only

![Fig. 1](image)

Fig. 1. This photomicrograph shows a cross-section of the lumbar region of a four-week-old rat spinal cord after injecting the soleus muscles of both hindlimbs with HRP. For identification the control dorsal horn has been marked with a fine micropipet prior to processing. The soleus muscle in one hindlimb of this animal was paralysed with α-BTX at birth, and this was followed by an injection of NMDA into the treated side of the spinal cord 12 days later. It can be seen that there are fewer HRP-labelled motoneurons (arrows) present in the operated (Op) ventral horn compared to the control (Con) ventral horn. Furthermore, there is also a decrease in the number of counterstained motoneurons within the soleus motor pool on this side of the spinal cord. Scale bar = 500 μm.
do these motoneurons survive in the long term, but the function of these injured motoneurons and their target muscles improves significantly. The muscles are stronger, and have more motor units than after nerve crush alone.\textsuperscript{9} Recent results obtained on axotomized neurons of the Clarke's nucleus are consistent with the proposition that excessive excitation may be involved in the death of target-deprived neurons. Neurons in the Clarke's nucleus die after axotomy, but reduction of afferent inputs to these cells prevented axotomy induced cell death. Moreover, treatment with the NMDA inhibitor MK-801, prevented axotomy induced cell death.\textsuperscript{30,31} The results presented in this study provide further evidence for the role of glutamate in the death of motoneurons denied interaction with their target during a critical period of their development. Blocking NMDA receptors rescues a proportion of those motoneurons which would otherwise die following muscle paralysis. The dose of MK-801 used in this study is lower than the optimal dose used in a previous study (see Refs 8, 18) since \(\alpha\)-BTX treatment lowered the tolerance of the rat pups to MK-801.\textsuperscript{9} However, the dose used in this study (1 mg/kg body weight) has been shown to rescue motoneurons after neonatal nerve injury, but at a reduced level (see Ref. 18). It is likely therefore, that additional motoneurons could be saved by treatment with MK-801 if the dose could be increased, or if a local application of a high dose of antagonist directly to the spinal cord could be made.

NMDA excitotoxicity has also been reported to be involved in motoneuron death during embryogenesis. Blockade of neuromuscular activity in the chick embryo, together with spinal cord stimulation results in an increased loss of motoneurons.\textsuperscript{7} These authors argue that activity blockade deprives embryonic motoneurons of a muscle-derived signal, which results in an increase in their vulnerability to excessive afferent input. These results taken together indicate that target interaction during both embryogenesis and early postnatal development is essential for the motoneuron to mature and acquire those characteristics which enable it to withstand the level of excitation within the developing CNS, which leads to increased activity. Glutamate is the main excitatory neurotransmitter in the spinal cord. Developing motoneurons are known to have glutamate receptors, which are activated during locomotion.\textsuperscript{22,24} We have previously proposed that target interaction during early postnatal development may be involved in the regulation of the number, type and distribution of glutamate receptors on motoneurons.\textsuperscript{17} It may be that for the motoneuron to become successfully integrated into the circuitry of the spinal cord, the correct number and type of glutamate receptor must be expressed in the appropriate region, and this process may be regulated by target interaction.

The mechanism by which the target may influence NMDA receptors on spinal motoneurons remains unclear. It is possible that target-derived trophic factors may be involved in this interaction. The role of target-derived trophic factors in the maintenance of developing motoneurons has received much attention (for reviews see Refs 17, 27). Although nerve growth factor (NGF) does not prevent motoneuron death\textsuperscript{9,25} other NGF-related molecules, such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 have recently been shown to support injured motoneurons, at least in the short term\textsuperscript{11,33,35} as has the cytokine ciliary neurotrophic factor (CNTF).\textsuperscript{24} However, their survival-promoting effects have only been assessed in the short term, and so their role in motoneuron survival has yet to be confirmed.

**CONCLUSION**

The results of the present study clearly show that motoneurons denied interaction with their target are vulnerable to the excitotoxic effects of NMDA and may explain the long term death of motoneurons to \(\alpha\)-BTX-treated soleus muscles. Furthermore, these target-deprived motoneurons can be rescued from death by blocking NMDA receptors with MK-801. Thus motoneurons denied functional contact with their target during early postnatal development are rendered susceptible to the toxic effects of glutamate, which in the long term may result in their death. Furthermore, these findings support the view that target interaction is essential for motoneurons to mature and become resistant to increased levels of excitation within the developing spinal cord.

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Increased locomotor activity in newborn rats causes motoneurone death

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Our previous studies have indicated that excess activation of NMDA receptors is an important contributing factor in the death of neonatal axotomized motoneurones. In this study we have tested whether inducing excessive locomotor activity during early postnatal development can induce the death of intact motoneurones.

In neonatal Wistar rats premature locomotor activity was induced by treatment with L-DOPA (100 mg (kg body wt)^{-1} day^{-1}, i.p.) from birth until they were 12 days old. This treatment increases the total amount of EMG activity of the soleus by at least 20-fold (Navarrete & Vrbova, 1985). Ten weeks later, under halothane anaesthesia, horseradish peroxidase (HRP) was injected into either the slow soleus or the fast TA/EDL muscles in both hindlimbs. After 24 h the animals were terminally anaesthetized and perfused transcardially with 2-5% glutaraldehyde and the spinal cord processed for HRP-histochemistry (Hanker et al. 1977). The number of HRP-labelled motoneurones in each ventral horn was counted using a light microscope.

The survival of motoneurones in the soleus and the TA/EDL motoneurone pools was examined. Treatment of rats with L-DOPA had no effect on the fast TA/EDL motoneurone pool: the number of labelled motoneurones present in each ventral horn of control, saline-treated animals was 161 ± 67 (mean ± S.E.M., n = 10) and in those animals treated with L-DOPA was 167 ± 55 (n = 10). However, L-DOPA treatment significantly reduced the number of labelled soleus motoneurones. In treated animals an average of 35 ± 1-8 (n = 14) motoneurones were counted compared to 55 ± 1-8 (n = 18) in control, saline-treated animals. This result is consistent with previous observations showing that treatment with L-DOPA increases the level of activity in the soleus muscle to a greater extent than in EDL (Navarrete & Vrbova, 1985).

These findings support the proposal that excess activity of immature motoneurones may cause them to die.

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