THE IMPORTANCE OF EARLY NERVE-MUSCLE INTERACTIONS ON MUSCLE DEVELOPMENT

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by

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ABSTRACT

During early postnatal development, individual muscle fibres are contacted by several axons at a single endplate. The excess synaptic input is eliminated during the first 3 weeks of postnatal life, so that only a single axon remains in contact with each endplate. Neuromuscular activity is important for this event to occur. Here evidence is provided in support of the hypothesis that the process of elimination involves a calcium-activated neutral protease (CANP) which degrades the neurofilament structure of axons which are being retracted.

The second part of this thesis addresses the question of the importance of target activity in the formation and establishment of the normal distribution of innervation in the slow soleus muscle. The soleus muscle is innervated by axons emanating from the L4 and L5 spinal roots. At birth there is an overlap of innervation between the L4 and L5 axons. As polyneuronal innervation is eliminated there is a disproportionate reduction in the territory of the L4 axons relative to the L5 axons. Following temporary paralysis at birth, the adult soleus muscle demonstrates a reduction in tension output and muscle fibre numbers. This reduction is almost solely due to loss of muscle fibres innervated by L5 axons. Thus, target activity is essential in the early postnatal period for the development of the normal distribution of innervation.

The extent to which target activity affects the normal development of the fast muscles, tibialis anterior (TA) and extensor digitorum longus (EDL) was also investigated. Nerve-muscle interactions were prevented shortly after birth and the effects on muscle development
following subsequent nerve injury were investigated. Nerve crush at 5
days produced a significant loss of tension, so that TA and EDL muscles
produced 50-55% of the force of controls. If nerve crush was preceded
by α-bungarotoxin paralysis the force produced by the TA muscle was
further reduced so that it produced only 38% of tension compared to
control. EDL muscles were not affected by the paralysis and produced
approximately 50% of tension of controls. It therefore appears that the
TA muscle is more significantly affected by interruption in nerve-muscle
contacts in early development than the EDL muscle.

The results presented in this thesis illustrate the critical
interdependence of nerve and muscle in early postnatal development and
that the interaction between the motor nerve and the muscle has a major
influence on the development of the neuromuscular system.
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PUBLICATIONS

CHAPTER ONE

GENERAL INTRODUCTION: THE DEVELOPMENT OF THE MAMMALIAN NEUROMUSCULAR SYSTEM
1) EARLY EMBRYOGENESIS

During early embryogenesis of the neuromuscular system motoneurones and their associated muscle fibres develop independently of each other. At a specific point in development, when both the motoneurone and the muscle fibre have acquired the rudimentary properties which allow them to interact with each other, the continued survival of both the motoneurone and the muscle becomes dependent upon functional synaptic connections between the two. Interdependence of nerve and muscle is particularly critical during the later stages of embryonic development, an interruption of the synaptic connections can lead to devastating results. In the mammalian adult, interactions between muscle fibres and neurites maintain their importance but the consequences of a disruption of this interaction are less devastating to both participants.

This Chapter will review the processes involved in the development of nerves, muscles and the neuromuscular junction, their point of interaction, as well as their mutual dependence on each other.

I) Motoneurone Development

Motoneurones originate within the neural tube; the germinal cells of the neural epithelium giving rise to neurones by rapid proliferation (His, 1887; 1890). All of the cells undergo mitosis. The first postmitotic cells, which are incapable of further division, become neuroblasts (Fujita, 1962; 1963; 1965; 1966). These neuroblasts migrate towards the outer surface of the tube and are released from the
epithelium, migrating away from the germinal layer. The neuroblasts become the white matter of the spinal cord, where they differentiate into neurones (Fujita, 1966; Jacobson, 1970). Motoneurones are the first neurones to become established, they migrate laterally in the ventral horn of the developing spinal cord and develop from two longitudinal columns of neuroblasts lying in a ventrolateral position in the embryonic spinal cord (Fujita, 1963; Jacobson, 1970).

The axons of the motoneurones are formed by outgrowths of the ventral horn cells. The motoneurones send out processes, often whilst still migrating, which leave the neural tube along the ventral surfaces, their axons passing through the ventral roots, at the time of limb bud development (Windle & Orr, 1934; Lyser, 1964). Motoneurones of the rat ventral horn originate between days E11 and E13 (Nornes & Das, 1974). In the mouse the motoneurones from the lumbrosacral regions have extended into the hindlimb buds by E13 (Filogamo & Gabella, 1967; Platzer, 1978; Lance-Jones, 1982). The ventral horn cells form a column of motor cells throughout the spinal cord. During development there is thought to exist a cranio-caudal differentiation and maturation of motoneurones (Romanes, 1941; Hamburger, 1948; Langman & Haden, 1970) and a proximal-distal sequence of limb innervation (Romanes, 1941; 1946; Taylor, 1943; Roncali, 1970).

II) Muscle Development

Vertebrate skeletal muscle fibres develop from undifferentiated mesenchymal cells derived from the lateral plate of the somatic mesoderm. In 1917, Tello reported the rapid proliferation of these mitotically active, mononucleated, presumptive myoblasts followed by
the emergence of primitive muscle cells, myoblasts. It has been postulated that these presumptive myoblasts undergo many proliferative cell cycles producing identical progeny but must undergo a "critical" mitosis (the "quantal cell cycle") before they are transformed into myoblasts (Okazaki & Holtzer, 1966; Ishikawa et al., 1968). The "quantal division" concept assumes that all cells are differentiated and committed to a specific developmental programme. Konisberg (1971) and other workers disagree with this idea of predetermination and maintain that all cells are capable of re-entering the mitotic cycle, i.e. are undifferentiated, until they fuse. Fusion, they claim, is the signal for the production of muscle-specific proteins, characteristic of myoblasts. The process of formation of myoblasts is still contested.

Populations of mitotically active cells accumulate at certain sites destined to form muscle (limb rudiments etc.). After completing their final mitotic division the presumptive myoblasts begin to synthesize the muscle specific proteins; actin, myosin and tropomyosin (Holtzer, 1961), which are the characteristic proteins of muscle fibres; and organize them into thick and thin filaments (Okazaki & Holtzer, 1966). These filaments are loosely bundled beneath the sarcolemma (Holtzer et al., 1957). Synthesis of these muscle-specific proteins occurs after the myoblast has withdrawn from the cell cycle, but prior to fusion with another myoblast, to form the more specialized myotube. One of the first enzymes to be synthesized by the myoblast is creatine phosphokinase (Shainberg et al., 1971; Morris et al., 1972), followed by the muscle specific proteins, and subsequently the synthesis of acetylcholine receptors (AChR) and their incorporation into the membrane (Famborough & Rash, 1971; Sytkowski et al., 1973).
Prior to fusion, of the mononucleated myoblasts into multinucleated myotubes, myoblasts become elongated and changes occur in their metabolic activity, as the cell prepares for contractile activity or fusion. Mitochondria increase in number, enzymes for utilizing glucose are found, and the first signs of rough endoplasmic reticulum and sarcoplasmic reticulum appear (Schundt et al., 1975; Holland & McLennan, 1976). The myoblasts align and fuse with one another. This fusion is known to be tissue-specific i.e. different cell types from the same animal do not fuse with myoblasts, however myoblasts may fuse across species (Yaffe & Feldman, 1965). Myoblasts, therefore, have the ability to recognize one another. The fusion process is preceeded by electrical coupling, both tight and gap junctions have been observed between cells allowing the passage of small molecules and electrical currents (Kelly & Zacks, 1969a; Dennis et al., 1981; Allen, 1986). The strength of electrical coupling decreases throughout gestation, and disappears completely during the first postnatal week (Dennis et al., 1981). This coupling is thought to be important for the normal development of the muscle mass. It may enhance the low levels of activity in the early stages of development and help co-ordinate differentiation throughout the muscle.

Myoblast fusion is known to be calcium dependent, in the absence of Ca$^{2+}$ myoblasts are unable to fuse but continue to divide at a normal rate (Shainberg et al, 1969); but it is known to be independent of both functional ACh receptors (Famborough & Rash, 1971) and the presence of the nerve (Giacobini et al, 1975). The concentration of enzymes related to muscle activity increases with fusion (Shainberg et al. 1971), and the proportion of contractile proteins increases with the continued
The differentiation of myoblasts into myotubes is associated with the occurrence of specific membrane proteins, e.g. ACh receptors; application of ACh to the developing cell results in a depolarization of the membrane and subsequent contraction of the myotubes (Dryden, 1970; Famborough & Rash, 1971; Dryden et al., 1974). A rapid increase in the proteins responsible for muscle contraction has also been observed (Famborough & Rash, 1971; Giacobini et al., 1973; Whalen et al., 1976), along with a marked increase in the number of mitochondria (Allen & Pepe, 1965). Myofibrils become peripherally placed within the elongated, multinucleated myotube.

The further development of mammalian skeletal muscle occurs in two phases; the early generation of primary myotubes, followed by the formation and growth of secondary myotubes beneath the basal lamina of the primary myotube. These two generations are different in origin but become indistinguishable in the adult.

Initially the muscle is composed of a small number of primary myotube clusters consisting of a single multinucleate primary myotube and other less differentiated presumptive myoblasts and myoblasts enclosed within a common basal lamina (Kelly & Zacks, 1969b; Ontell & Dunn, 1978; Harris 1981a; 1981b). Using retroviral lineage markers, myoblasts have recently been shown to cross their surrounding basal lamina and become associated with a different primary myotube (Hughes & Blau, 1990). The authors suggest this may be a mechanism by which repair of damaged or injured fibres may occur during normal development. The primary myotube within each cluster extends from tendon to tendon in the developing muscle (Ontell & Kozeka, 1984), and appear in the rat between
embryonic days 13 and 14 (E13 - E14) (Dennis et al., 1981). Most noticeably, development of skeletal muscle up to the stage of primary myotube formation arises in the absence of the motor nerve (McLennan, 1983; Harris, 1981a). Further differentiation and development of the muscle cells into secondary myotubes is however, critically dependent upon functional innervation of the muscle by its motor nerve (Betz et al., 1980; Harris et al., 1981a; Condon et al., 1990a).

Following primary myotube development a second generation of myotubes is formed from within the initial myotube cluster. The primary myotube acts as a template from which the next generation of myotubes develops (Ontell & Dunn, 1978). The secondary myotubes form near the midpoint of the primary myotube and initially remain closely aligned with the primary myotubes. In rat hindlimb, secondary myotubes are first seen at E18 (Condon et al., 1990a). On connection with the muscle tendon the secondary myotube separates from the parent, primary myotube and acquires an independent basal lamina (Kelly & Zacks, 1969b; Ontell & Kozeka, 1984).

As development proceeds the myotubes continue to differentiate and acquire the characteristic complexities of mammalian skeletal muscle. At E14 individual muscles have not yet formed in the rat hindlimb (Condon et al., 1990a), instead developing cells exist as clusters, termed "pre-muscle masses" by various researchers (e.g. Lance-Jones, 1979). Over the next three days, E14-E17, cleavage of individual muscles occurs in the rat hindlimb, concurrent with an increase in muscle fibre size and number (Condon et al., 1990a). In the rat hindlimb formation of individual muscles from the major masses occurs with the formation of the anterior muscles, preceeding the lateral group and similarly the
deep posterior muscles preceding the superficial group (Condon et al., 1990a). Cleavage of all the rat hindlimb muscles is complete by E17, and at E18 the muscles have taken on their neonatal positions, with well developed tendons (Condon et al., 1990a). Newly formed myotubes contain thick and thin filaments which are aligned parallel to the long axis but have not yet fused into myofibrils, and have centrally placed nuclei. By E16, in the mouse, coincident with the first spontaneous movements of the embryo this fusion has occurred (Ontell & Dunn, 1978; Dennis et al., 1981). The nuclei then migrate to the periphery, occupying a sarcomemal position, and the myofibrils become more evenly distributed throughout the cytoplasm of the developing myofibre.

All primary and secondary myotubes express an embryonic myosin heavy chain isoform (MHC) throughout prenatal development (Dhoot, 1986; Narusawa et al., 1987; Harris et al., 1989; Condon et al., 1990a). The myotubes then differentiate, through distinct sequences of MHC isoform expression. By E18, all primary myotubes express, or have expressed, slow MHC myosin isoform, as well as embryonic MHC (Dhoot, 1986; Narusawa et al., 1987; Harris et al., 1989; Condon et al., 1990a). Some primary myotubes continue to express slow MHC, others, in regionalized areas of specific muscles - e.g. the deep, axial parts of TA; replace slow MHC with neonatal MHC (neonatal MHC is a precursor of fast adult MHC (Whalen et al., 1981)). A subsequent generation of secondary myotubes are formed, containing embryonic MHC and neonatal MHC. In the slow soleus muscle neonatal MHC is converted to slow MHC.

Some skeletal muscles continue to undergo myogenesis in the early postnatal period, whilst others possess the adult complement of muscle fibres by birth. Most of the muscle fibres present at birth are still
clustered. Once myogenesis is complete the further postnatal development of the muscle compliment involves the separation of these clusters (Riley, 1977; Harris, 1981a; Ontell, 1979) and a subsequent increase in fibre size (Goldspink, 1962). This increase in fibre size is predominantly due to additional synthesis and assembly of myofibrills. The number of myofibrills per fibre increases from approximately 50 in a myotube to over 1000 in an adult muscle fibre (Goldspink, 1970). Muscle fibres also undergo an increase in length during postnatal development, new sarcomeres being added to the ends of the existing myofibrills (Griffen et al., 1971). In adult muscle, a mononucleated cell lying beneath the basal lamina of an adjacent myofibre is termed a satellite cell (Mauro, 1961; Ishikawa, 1966).

III) Interdependence of nerve and muscle

The initial proliferation, migration and differentiation of both motoneurones and target skeletal muscle cell occurs in the absence of a functional interaction between the two. Thereafter, the continued survival and maturation of both the motoneurone and the muscle cell becomes dependent upon their interaction with each other. In the absence of functional connections between the two cell types both the motoneurone and the muscle cells die.

The initial stages of motoneurone differentiation are independent of the periphery and the future target of the neurone. Amputation of a limb bud in Aneuran amphibians (Prestige, 1967) or in the chick embryo (Hamburger, 1958) had no effect upon the proliferation of neuroepithelial cells or the initial stages of neural differentiation. Indeed, mutant chicks (limbless) initially produce normal lateral motor
column populations, which later in development degenerate (Lanser & Fallon, 1987). At later developmental stages those nerves deprived of a target during development will die (Hamburger, 1958; Oppenheim et al., 1978).

Early muscle fibre development can proceed independently of innervation from a motor nerve. In 1918, Harrison demonstrated this independence. By destroying frog spinal cord segments prior to ventral root outgrowth, he was able to show that muscles are capable of differentiating independently of motor innervation. Myoblasts were able to align and fuse to form multinucleated myotubes in the absence of innervation. In tissue culture myoblasts will fuse to form myotubes in the absence of motoneurones or nerve extracts (Konisberg, 1963). Fusion of myoblasts has been shown to occur without the presence of the nerve or functional ACh receptors (Harris, 1981a; Famborough & Rash, 1971). However, as with the development of motoneurones, further maturation and continued survival of the muscle is critically dependent upon functional nerve-muscle contacts.

The developing neurone usually reaches the developing muscle mass at the myotubal stage. At E16 all rat hindlimb muscle masses have nerves coursing through them (Condon, et al., 1990a). Muscle fibres can differentiate up to and including the stage of the primary myotube in the absence of the motor nerve (Harris 1981a; Harris et al., 1989). The further generation of secondary myotubes and the differentiation of the developing muscle mass into the highly specialized mammalian adult muscle is, however, dependent upon innervation (Harris, 1981a; McLennan, 1983; Harris et al., 1989); myotubes without innervation eventually degenerate and are replaced by connective tissue (Studitskji et al.,
1963). During development of the chick embryo, if innervation is prevented by extirpation of a section of the spinal cord, the denervated muscles are then found to be severely atrophied and replaced almost completely by fat (Drachman, 1968). In contrast, in rat muscle denervated at birth or shortly before, only some fibres degenerate (Zéléna, 1962), many of the fibres, in fact, continue to grow, though at a slower rate than in normal control muscles (Vrbova, 1952; Brown, 1974). For normal muscle development to proceed it is therefore essential that contact is maintained with the motor nerve.

Drachman and co-workers (Drachman & Coulombre, 1962; Drachman, 1965, 1968) demonstrated, by paralysing muscle in the chick embryo using either botulinum toxin, curare, hemicholinium or bungarotoxin, that activation of muscle by its motor nerve, and not merely the presence of innervation, is essential for the differentiation of muscle.

Differentiation of muscle fibre types is coincident with the arrival of axons in the muscle and with the formation of neuromuscular junctions (Rubinstein & Kelly, 1981; Ross et al., 1987a; Narusawa et al., 1987; Condon et al., 1990a). Muscle fibres containing isoforms of myosin heavy chain, characteristic of adult slow and fast fibre type (slow and neonatal myosin isoforms) appear within a few days of the formation of fibres (Lyons et al., 1983; Dhoot, 1986; Narusawa et al., 1987). In 1968, Engel and Karpati demonstrated, using histochemical techniques, that denervation of newborn muscle impairs fibre type differentiation. This seems to suggest that innervation is essential for the normal pattern of muscle development.

Characteristic myosin isoforms can occur in chick aneural muscles. Myosin isoforms have been observed in muscles following ablation of the
neural tube, prior to innervation of the muscle mass (Butler et al., 1982; Phillips & Bennett, 1984) as well as following paralysis with curare (Crow & Stockdale, 1986). Denervation or removal of muscle activity in rat hindlimb muscles before the time of complete primary myotube generation (E15) does not prevent the initial expression of slow MHC in the primary myotubes (Harris et al., 1989), however, this expression was lost with age. In addition, secondary myotube development is prevented. Condon et al. (1990b) demonstrated that β-bungarotoxin treatment of rat embryonic hindlimbs, on E13-E15, prevented the formation of the full compliment of individual hindlimb muscles and those formed were markedly reduced in size. The myotubes present expressed embryonic MHC as normal, however, they did not express slow MHC until later stages in development. Harris et al. (1989) demonstrated that denervation at a later stage (E16) did not affect the pattern of distribution of MHC, except for reduction in the number of secondary myotubes. The experimental evidence seems to suggest that the initial expression of myosin heavy chain isoforms is independent of innervation. Further differentiation of muscle fibre properties, such as slow myosin light chains, is however, dependent upon innervation (Rubinstein et al., 1988).

Although the development of motoneurones and muscle fibres is independent of each other over the initial stages of differentiation, subsequent development of both the nerve and muscle is interdependent. This interdependence is essential, to the extent that if connections or functional interactions between the two are prevented then both the nerve and muscle cells die.
2) **FORMATION OF THE NEUROMUSCULAR JUNCTION**

After the processes of cell division and migration the next stage in the formation of the neuromuscular system is the establishment of connections between the two participants; involving the growth of axons to their correct target, recognition of the appropriate cells and synaptogenesis.

I) **Axon and Dendrite Growth**

In order to establish and receive connections, neurones elaborate the specialized structures of dendrites and axons. Axons of the motor nerves are formed by outgrowths of the ventral horn cells within the grey matter. As the embryo develops *in utero* the distance between the end organ and the neurone cell body gets larger and so the nerves must elongate. Ramon y Cajal (1890) was the first to recognise that axon growth occurred at a terminal enlargement of the axon, which he termed the "cone of growth". Harrison (1910) subsequently noticed that nerve fibres have a tendency to grow in definite directions in tissue culture and confirmed the dynamic nature of the growth cone. Most of the interactions of the developing neurone with its surrounding environment occur at the growth cone. The growth cone is thus responsible for the direction of the pathway that the neurone takes towards a potential target and the recognition of that target. In addition, the growth cone is the region of membrane addition for neurite elongation (Bray, 1970; 1973; Spooner *et al.*, 1974).

As the neurone develops, the complex three-dimensional cytoskeletal structure of neurofilaments and microtubules within the axon begins to
take form. New material for axonal growth is transported continually
from the cell body towards the axon tip (Hoffman & Lasek, 1980;
Grafstein & Forman, 1980). Actin, tubulin and neurofilament triplet
proteins are synthesized in the cell body (on the polyribosomes of the
Nissl substance), transported in the axon (Hoffman & Lasek, 1975) and
assembled into the stable axonal cytoskeleton. Embryonic axons are thin
and their cytoskeleton consists largely of microtubules; the developing
axon expresses low levels of neurofilaments (Peters & Vaughn, 1967). In
the chick embryo neurofilament proteins (NFP) appear in the post-mitotic
neuroblast at the time of initial axon formation (Tapscott et al.,
1981). During axonal growth the total mass of the neurone increases as a
consequence of the addition of new structural material (Lasek, 1981),
thus developing and extending the axonal cytoskeleton.

Once the initial axons have grown out of the spinal cord into a
region in the periphery they themselves then provide a pathway for the
growth of subsequent fibres (Harrison, 1904) which grow along the
leading surfaces of the pathfinder nerves. Many pathfinder nerve fibres
can be seen in developing myotubes during early stages of development.

II) Axon Guidance and Target Recognition

One of the first tasks of the developing motoneurone is to seek and
grow towards an appropriate target. The innervation of limbs by
exploring axons is not a haphazard process and occurs in an orderly and
stereotyped manner (Romanes, 1946; Cruce, 1974; Landmesser, 1978a;
1978b). Guidance pathways are thought to exist in the CNS and PNS which
bring about this apparently orderly innervation of limbs. Attempts to
understand axon guidance have involved experiments in which the spatial
relationships between the spinal cord and the developing limbs have been altered. Experiments have been performed on chick embryos in which cord segments underwent anterior to posterior reversals, before axon outgrowth (Lance-Jones & Landmesser, 1980). Motoneurones consistently innervated the correct muscles by correcting the experimentally-manipulated shift within the plexus region of the limb (Lance-Jones & Landmesser, 1980). Shifting the position of the limb similarly demonstrated that the outgrowing axons can find and innervate the appropriate target. The correct target was innervated by the axons taking aberrant pathways through the limb, thus correcting for the shift (Lance-Jones & Landmesser, 1981). When motoneurones are forced to innervate a foreign limb either by anterior-posterior reversals of chick limb buds (Stirling & Summerbell, 1979) or by the addition of supernumery limbs (Hollyday et al., 1977) the motoneurones innervate the foreign muscle with which they would not normally connect, however the axons innervate the foreign muscles by tracing out a pathway appropriate for that limb, i.e. following the route taken by the normal nerve. Indeed, transplants of foreign nerves have been demonstrated to grow through foreign tissue along pathways normally taken by normal nerves (Summerbell & Stirling, 1981). It appears that outgrowth of motoneurones is controlled by guidance cues and/or pathways which may take the form of physical strata but are relatively non-specific since inappropriate axons can be guided by them.

Experiments performed by Lewis et al. (1981) in which they created limbs devoid of muscle by X-irradiation of the somatic mesoderm at an early stage, demonstrate that main nerve trunks develop according to the normal pattern, but the muscle branches consistently failed to develop.
It appears therefore that target recognition does not have a major role in the organization of innervation.

III) Synaptogenesis

Synapse formation requires the incoming axon to change from a growing structure into a stable stationary structure capable of releasing transmitter. The target cell, the skeletal muscle fibre, must also undergo changes so that it becomes capable of receiving transmitter and forming a functional unit with the nerve cell.

i) First Contacts

The first motor nerves that grow out of the spinal cord reach the developing muscle masses almost immediately, due to the relatively short distances the motor nerves have to travel. Initial synaptic contacts occur at random along the length of the elongating myotubes (Bennett & Pettigrew, 1974a), the entire length of the myotube membrane is thought to be equally receptive for initial synapse formation (Harrison, 1910). ACh sensitivity is distributed over the entire membrane (Ginetzinski & Sharamina, 1942; Diamond & Miledi, 1962; Bennett & Pettigrew, 1974a). In the rat the first signs of synaptic contact are seen morphologically on E16 in the intercostal muscle (Kelly & Zacks, 1969b) and the diaphragm muscle (Bennett & Pettigrew, 1974a), and on E17 in the rat soleus muscle (Angulo y Gonzales, 1932). Physiological recordings show that innervation of some muscle fibres had already occurred by E14 in rat intercostal muscle (Dennis et al., 1981) and by E15 in rat diaphragm muscle (Bennett & Pettigrew, 1974a).

After the initial synaptic contact is established the receptive
capacity of the muscle fibre membrane is withdrawn outside of the synaptic site so that it is made refractory to further synapse formation for some distance from the initial site of synapse formation (Bennett & Pettigrew, 1975). Further synapse formation occurs at the primary synaptic site and the muscle then becomes hyperinnervated. This state of polyneuronal innervation is however transitory (Redfern, 1970; Bagust, et al., 1973; Bennett & Pettigrew, 1974a; Benoit & Changeaux, 1975; Brown et al., 1976; Riley, 1976; O’Brien et al., 1978). Synapses appear suddenly, and rapidly increase in numbers after the initial contact has been made. The formation of additional synaptic contacts takes place at the initial site and continues for several days.

Secondary myotubes receive their first innervation by transfer of nerve terminals from the densely innervated primary myotubes, on which the secondary myotubes develop. In intermediate stages primary and secondary myotubes share terminals (Duxson et al., 1986).

ii) The Acetylcholine Receptor

In order for the motoneurone and muscle to function as a cohesive unit they must have the ability to interact. This ability is dependent upon the release of ACh from the nerve terminal and the subsequent response of the muscle. The muscle is able to respond because of the nicotinic acetylcholine receptor (AChR).

The mature AChR is a transmembrane protein constituting a ligand-gated ionic channel. It has a structure of roughly circular rosettes of five to six subunits with an overall diameter of 8-9 nm and a subunit diameter of 3-4 nm, subunits are designated alpha, beta, gamma and delta. The ion channel of the AChR extends through the lipid bilayer,
providing an aqueous channel selective for small cations, with all of the receptor subunits spanning the membrane. AChR's have two binding sites for ACh molecules with the alpha-subunit carrying the ACh-binding site (Karlin et al., 1975). The composition of a monomer has been postulated to be $\alpha_2, \beta, \gamma, \delta$ (O'Brien et al., 1979).

During embryogenesis the nerve reaches the muscle at the myotubal stage when the entire membrane of the myotube is sensitive to ACh (Ginetzinski & Shamarina, 1942; Diamond & Miledi, 1962), even so only low levels of the AChR are expressed on the myotube (Stykowski et al., 1973). Myogenic cells grown in vitro in the absence of neurones are sensitive to ACh (Dryden, 1970). Functional innervation develops at this time when the muscle cell is uniformly sensitive to ACh (Blackshaw & Warner, 1976; Bevan & Steinbach, 1977). The appearance of ACh receptors in the myoblast membrane precedes fusion of myoblasts into the more specialized myotube (Famborough & Rash, 1971). After fusion has occurred the number of receptors increases dramatically (Bevan & Steinbach, 1977; Dennis et al., 1981; Ziskind-Conheim et al., 1984), correlated with the increasing age of the muscle cells (Devreotes & Famborough, 1975). Initially, in the early myotube the AChR's are distributed over the entire myotube membrane. Chick myofibres grown in culture show an uneven distribution of acetylcholine receptors (Vogel et al., 1972; Fischbach & Cohen, 1973; Stykowski et al., 1973). As development proceeds some of the receptors aggregate into "hot spots" which are randomly distributed throughout the muscle (Fischbach & Cohen, 1973; Vogel et al., 1983). High receptor density areas also occur in tissue culture of mouse (Powel & Friedman, 1977) and rat (Axelrod et al., 1976) muscle cells. As a result of this increase in the numbers of AChR's the amount of ACh
required to depolarize the muscle cells is 1000 times less than that required before fusion (Dryden et al., 1974).

Incoming axons show no preference for making contacts with the areas of high acetylcholine receptor clustering, in cultured Xenopus muscle cells (Anderson & Cohen, 1977). Endplate formation begins with an accumulation of pre-formed AChR's at the site of neuromuscular contact (Bevan & Steinbach, 1977). Contact with the nerve causes AChR's to accumulate beneath cholinergic nerve terminals in clusters (Anderson & Cohen, 1977; Cohen & Weldon, 1980). Newly synthesized receptors are then incorporated into the clusters (Role et al., 1985). Clustering at the junctional site, in the embryo, is like that seen in the adult (Kuffler, 1943; Axelrod & Thesslef, 1959; Miledi, 1960b). If a nerve is prevented from innervating an embryonic muscle, receptor clusters still appear, aligned in the region where endplates are normally formed (Braithwaite & Harris, 1979). However, if innervation is further delayed or if the nerve is cut soon after innervation, the non-innervated clusters lose their orderly orientation (Braithwaite & Harris, 1979; Ziskind-Conheim & Bennett, 1982), and become dispersed over the muscle fibre. This clustering ensures a high post-junctional concentration of receptors essential for efficient transmission of information from the nerve to the muscle cell. Non-junctional receptor clusters do not usually occur in vivo but can form under conditions of denervation (Braithwaite & Harris, 1979; Ziskind-Conheim & Bennett, 1982).

As the neuronally-induced hot spots accumulate, partially as a result of the redistribution of existing receptors (Anderson & Cohen, 1977), and under the influence of muscle activity (Lømo et al., 1984), the more diffusely distributed AChR's are reduced, due to a suppression
of the synthesis of extra-junctional receptors (Moody-Corbett & Cohen, 1982). In rats, this recession begins in the week preceding birth and extends for one to two weeks after birth (Diamond & Miledi, 1962; Bevan & Steinbach, 1977). The decline in extra-junctional receptors is dependent, at least in part, on muscle activity, as the rate of decline can be reduced by paralysis of chick embryos with curare (Gordon et al., 1974; Burden, 1977). Denervation at birth delays the accumulation of acetylcholine receptors at the neuromuscular junction and prevents the decline of extrajunctional receptor density (Vrbová, 1970; Jones & Vrbová, 1972; Brown, 1975).

Accumulation of acetylcholine receptors at the site of nerve contact appears to occur by lateral migration of the receptors in the membrane (Anderson & Cohen, 1977; Peng et al., 1981). α-BTX labelled acetylcholine receptors have been demonstrated to move from extrajunctional regions in cultured myotubes to form clusters (Axelrod et al., 1976; Poo, 1982). If an electrical field is applied to cultured myotubes, labelled acetylcholine receptors move towards the cathode region (Poo, 1981). The exact nature of the influence of the nerve on receptor clustering is unsure. The nerve may be producing electric currents by direct interaction with the receptors or may be inducing alterations in the extracellular matrix or plasma membrane (see Poo, 1985).

The basal lamina may be an important factor in receptor clustering. McMahan and colleagues have shown that basal lamina from amphibian muscle persists after denervation and after destruction of muscle cells. This isolated basal lamina maintains its structure and can direct the formation of receptor clusters on regenerating muscle in the absence of
nerve (Burden et al., 1979; McMahan & Slater, 1984). Heparin sulfate proteoglycan (Anderson & Famborough, 1983) and laminin (Daniels et al., 1984), components associated with basal lamina, have been shown to correlate strongly with AChR clusters. Laminin has been reported to increase the number of clusters (Vogel et al., 1983).

As the development of the neuromuscular system continues, the AChR's at the neuromuscular junction mature and undergo various structural changes. Signs of this maturation include resistance to the effects of muscle activity, positional and metabolic stability, and short ionic-channel opening times.

Positional stability is one of the early signs of maturation. Receptors which have accumulated beneath the site of nerve contact have been found to be immobile (Axelrod et al., 1981). Cytoskeletal anchoring of AChR's may have an important effect on AChR clustering. Meshworks of cytoskeletal material have been demonstrated to be localized under subsynaptic muscle membrane (Heuser & Salpeter, 1979; Hirokawa & Heuser, 1982; Peng, 1983). A unique 43 Kd protein found associated with mature post-synaptic membranes containing AChR in both electric organ (Sealock et al., 1984) and muscle (Froehner et al., 1981), and in similar concentrations to the AChR, is thought to be involved in the anchoring of AChR's to cytoskeletal elements. Burden and colleagues (1983) have demonstrated a close relationship between the 43 Kd protein and the β-subunit of the AChR oligomer. The 43 Kd protein also binds to actin (Walker et al., 1984) suggesting a route through which the 43 Kd protein could link the receptor and the cytoskeleton.

Stabilization of the acetylcholine receptors may be brought about by covalent modification of the receptor molecule (Changeux & Danchin,
Acetylcholine receptors are known to undergo phosphorylation (Gordon et al., 1977) and the phosphorylated molecule is stabilized and anchored in the membrane (Cohen, 1976). Whatever the means of anchoring of the receptors, they become more resistant to disruption with embryonic maturation (Bloch & Steinbach, 1981). This newly found stability ensures the survival of the developing endplate, whilst the extra-junctional AChR's are eliminated as a result of nerve-evoked muscle activity, thus making the rest of the muscle refractory to further synapse formation (Lømo & Slater, 1980b). The stabilization of normal adult AChR's at the neuromuscular junction is dependent upon motor nerve innervation (Stanley & Drachman, 1983; Avila et al., 1989).

Along with positional stability of the maturing junctional receptors, the receptors also become metabolically more stable. Embryonic receptors have a rapid turnover rate, with a half-life of 16-24 hours. Within five days of synapse formation the receptors become more stable (Steinbach et al., 1979; Steinbach, 1981) with a half-life of 8-11 days (Berg & Hall, 1975; Chuang & Huang, 1975; Devreotes & Famborough, 1975; Shainberg et al., 1976; Reiness & Weinberg, 1981), this stability persisting into adulthood. Metabolically stable junctional receptors first appear in rat muscle before birth and continue to develop for the next 3 to 4 weeks, however, in the chick they do not stabilize until several weeks after birth. Even in adulthood, the extra-junctional receptors remain relatively unstable with a half-life of approximately 20 hours (Famborough, 1979). How exactly this stabilizing process occurs is unknown, though muscle activity is thought to be an important factor (Fumagalli et al., 1990).

Further changes, specifically in the kinetics of the AChR, modify...
the postsynaptic effects of the transmitter. Extra-junctional receptors and the earliest embryonic AChR's have an average opening time of the receptor-associated ion channel of about 4 milliseconds. Over the first few weeks after the junctions are formed, new receptors with open times of about 1 millisecond are synthesized and incorporated into the postsynaptic membrane (Brenner & Sakmann, 1983; Vicini & Schuetze, 1985) and by the second postnatal week the open times are similar to that of the adult (Sakmann & Brenner, 1978; Fischbach & Scheutze, 1980). Thus, embryonic AChR-associated ion channels are open 3-5 times longer than adult channels, and their conductance is lower. In 1986, Scheutze & Vicini suggested that this change is due to a post-translational modification of pre-existing embryonic AChR's. The recent work of Sakmann et al. (1989) investigating specific mRNAs, have shown that these changes in channel opening times are due to the substitution of one of the five subunits which constitute the AChR. This conversion from embryonic to adult opening times has been shown to be dependent upon the motoneurone. Looking at the formation of ectopic neuromuscular synapses Brenner et al., (1987) demonstrated that the conversion of gating properties of AChR's occurs in the absence of impulse activity provided that the nerve continues to be present, activity having been eliminated by the application of TTX. Levels of both Ca$^{2+}$ and cyclic GMP (Nestler et al., 1978) increase during muscle activity and Brenner et al. (1987) suggest that this might stimulate the expression of fast-gating adult channels. Denervation of soleus muscles at birth prevents the normal transformation of channel properties (Scheutze & Vicini, 1984). Direct chronic stimulation of the denervated muscle permits the development of the mature, shortened opening times (Brenner et al., 1983).
Once the AChR has accumulated, become successfully anchored and developed its mature characteristics, it is a relatively stable structure to the extent that if denervation occurs the endplate region remains more sensitive to ACh than the surrounding areas for several weeks (Lømo & Rosenthal, 1972; Bennett & Pettigrew, 1974b).

iii) Differentiation of the synaptic cleft

As the process of synaptogenesis proceeds, ACh receptors accumulate and aggregate. This is accompanied by the development of structural specializations in the pre- and post-synaptic cell membranes, constituting the neuromuscular junction (NMJ). Synaptic function appears prior to maturation of synaptic structure. In rats, the first morphologically identifiable contacts between nerve and muscle have been seen at E16 (Kelly & Zacks, 1969b), these early contacts are, however, relatively unspecialized. They appear to lack the morphological specializations characteristic of mature neuromuscular contacts; the terminals lack clustered vesicles, release sites, and mitochondria; the synaptic cleft is of irregular width with limited or no basal lamina; and there are no postsynaptic folds. It has therefore been suggested that neuromuscular transmission may play a role in the maturation of neuromuscular contacts.

In mature junctions the synaptic cleft is occupied by basal lamina, an extracellular network of collagen and glycoprotein (Sanes et al., 1978). Basal lamina surrounds the entire myofibre and projects into the secondary folds of the postjunctional membrane. In rat intercostal muscle the first rudiments of basal lamina are seen at E16, with basal lamina being deposited primarily at the endplate region, later
developing at the extrajunctional regions (Kelly & Zacks, 1969b). In chick, basal lamina has been seen at E8 and again is more fully developed at synaptic junctions (Atsumi, 1977). Basal lamina deposition appears to be under the influence of the nerve (Sanes et al., 1984; Anderson et al., 1984).

Early in the differentiation of the NMJ, at the same time as the deposition of basal lamina, the enzyme acetylcholinesterase (AChE) becomes localized (Bennett & Pettigrew, 1974a; Bevan & Steinbach, 1977; Harris, 1981b). A 16S-AChE, responsible for terminating transmitter action by catalyzing the hydrolysis of ACh, is found exclusively in synaptic basal lamina and is not detected until E15 in rat muscles (Vigney et al., 1976). AChE develops rapidly after the motor nerve has contacted the muscle (Kupfer & Koelle, 1951; Gerb tzoff, 1959). Filogamo & Gabella (1967) demonstrated a close correlation between the earliest arrival of nerve fibres and the appearance of AChE in myoblasts of every class of vertebrate. Muscle activity has been demonstrated to play an important role in the deposition of AChE. Chick embryos paralysed with curare (Gordon et al., 1974) or with α-neurotoxin (Giacobini et al., 1973) are developmentally delayed in that the postsynaptic membrane and junctional AChE fail to develop. By chronically electrically stimulating curarized muscle, the appearance of AChE was demonstrated at the neuromuscular junctions (Rudin et al., 1980). Lømo & Slater (1980a), looking at experimentally induced ectopic endplates, demonstrated that AChE did not develop in denervated endplates. If they then chronically stimulated the denervated muscle, plaques of AChE did occur (Lømo & Slater, 1980b). Muscle activity therefore appears to play an important role in the development and deposition of AChE in the developing
As the development of the neuromuscular junction proceeds, the sarcolemma thickens and the postsynaptic membrane develops extensive folding. These foldings gradually become deep and narrow and form secondary clefts, the folding continuing until the whole endplate region is covered in secondary clefts. By the second postnatal week the development of the synaptic cleft is complete but the endplate continues to increase in complexity and size with age.

3) MODIFICATIONS OF EXISTING CONNECTIONS

As synapses form there are processes occurring which considerably modify the initial pattern of connections existing between nerve and muscle. These processes include the wholesale loss of a large number of motoneurones followed by the elimination of excess synaptic connections of the remaining motoneurones. The purposes of these events, which occur during the establishment of normal adult innervation patterns, are unknown. It has been postulated that they may allow for the elimination of errors, as well as matching pre- and postsynaptic cell numbers and requirements, and may therefore facilitate the fine specific tuning that occurs between nerve and muscle in the mature adult vertebrate.

I) Naturally Occurring Motoneurone Death

Neuronal cell death is a phenomenon common to many types of neurone in the vertebrate nervous system (Cowan, 1973; Jacobson, 1970) and occurs shortly after axons reach and activate their targets (Hamburger & Oppenheim, 1982), at the time when neurogenic locomotor activity of the embryo begins. Naturally occurring cell death occurs amongst neurones
which have already migrated to their final destination and are well
differentiated. The neurones which die possess extensive dendrites and
have axons which have begun to form connections. This large scale cell
death is part of the normal developmental programme of the nervous
system, and occurs over a well defined period of a few days or weeks,
depending on the species, and the population of neurones studied.

Neuronal cell death was first reported in the developing nervous system
by Collin in 1906. More recent work has demonstrated that during this
normal developmental process, 50-70% of developing motoneurones in the
lateral motor column of the spinal cord die (Hamburger & Levi-
Montalcini, 1949; Oppenheim, 1981; 1985; Purves & Lichtman, 1985). The
extent of cell death varies between species and different parts of the
nervous system, though the timing of cell death is usually well defined
within populations of motoneurones and usually occurs at approximately
the same time that axons reach and activate their targets (Hamburger &
Oppenheim, 1982). Large scale cell death begins in chick embryos from E5
(Hamburger, 1975) and from E12 in the mouse (Lance-Jones, 1982). In
chick embryo, 50% or more of the motoneurones innervating skeletal
muscle degenerate between E5.5 and E12 (Hamburger & Oppenheim, 1982).

Prestige (1976) demonstrated that the motoneurones which die are
initially indistinguishable from those which survive. Oppenheim et al.
(1978) demonstrated that spinal motoneurones destined to die as a result
of peripheral ablation, differentiate and develop normally before they
die. The motoneurones develop normal levels of cholineacetyltransferase
(ChAT) and AChE. Neurones lost at this stage in development generally
cannot be replaced.

The purpose of such large scale cell death is unclear. Ramon y
Cajal suggested that cell death may be a mechanism by which wiring errors can be eliminated. However, this explanation seems unlikely since Landmesser (1980) and Oppenheim (1981) have demonstrated that few aberrant connections are in fact made initially. In addition, when neurones are experimentally forced to innervate foreign muscles there is no additional cell death (Whitelaw & Hollyday, 1983). This explanation of cell death in terms of a rewiring capacity cannot be ruled out however since aberrant connections within individual muscles may be regulated as a result of widescale neuronal cell death. Alternatively, large scale cell death may be a developmental process by which a quantitative balance between the neurones and target cells is achieved. As development proceeds a given population of neurones undergoes a specific quantal number of divisions. This will ultimately result in an overproduction in the number of neurones necessary to innervate a specific target. The excess number of neurones may then be removed and an accurate matching of motoneurone and muscle fibre numbers be achieved by cell death (Hamburger & Oppenheim, 1982). However, there is no direct evidence to support this idea of overproduction and subsequent rebalance. Indeed, according to this explanation of cell death up to 50% of neurones, and not more, would need to die to produce a matching of populations. This is not the case, since in some populations of cells up to 70% of motoneurones die. This wholesale overproduction in the number of neurones seems an energy-wasteful process through which a relatively simple goal is achieved; that is the matching of populations. It is possible that the overproduction in neuronal cells may confer an evolutionary advantage in that the overproduction of cells allows for an adaptability of response to the demands of the target tissue. If an
accurate quantitative matching of neurones and target cells was
initially achieved then the developmental process would be unable to
adapt and any developmental errors could not be surmounted.

The mechanism(s) involved in naturally occurring cell death is not
known. However, interaction with the target has been shown to be
important. Evidence for the critical role of the target in this process
comes from various studies manipulating neurone-target interactions and
in doing so altering motoneurone survival. During this period in
development the normal developmental processes of the neurones are
dependent upon and respond to the external environment. In 1934,
Hamburger demonstrated that the removal of a limb bud from chick embryo
prior to the onset of cell death results in the death of the majority of
motoneurones and sensory neurones that would normally innervate that
limb. This wholesale death of motoneurones occurring in the absence of
the limb, occurs at the same time as normal cell death (Hamburger &
Levi-Montalcini, 1949; Hamburger, 1975) and is an accentuation of the
natural phenomenon. The proliferation and differentiation of
motoneurones prior to the onset of cell death are unaffected by target
removal (Hamburger, 1975). The results of these and other similar
experiments lead to the suggestion that the motoneurones which die are
those which have simply failed to reach their destined target tissue.
Experiments increasing the amounts of target tissue by the grafting of
extra limbs, have resulted in less neuronal death at the level
innervating the extra limb (Hollyday & Hamburger, 1976). It has been
suggested that motoneurones are competing for either available target
tissue or for a trophic factor released from the target in limited
amounts. Therefore, extra grafted tissue would result in a greater
survival of motoneurones.

Competition between motoneurones for a "trophic" factor released by a specific target does not appear to be the only factor involved in cell death or survival. Landmesser & Pilar (1976) performed experiments in which limb buds were removed in chick embryos. They subsequently found that some cells remaining in contact with the target proceeded to die. Lamb (1981) performed experiments on *Xenopus laevis* tadpoles in which both sides of developing spinal cord were forced to innervate a single hindlimb, following amputation of a limb bud. The result was a normal number of motoneurones on both sides of the spinal cord - that is, the target tissue was supporting twice as many motoneurones as normal. Competition between neurones for a limited supply of a substance does not appear to be the only factor involved in naturally occurring cell death.

Muscle activity appears to be of importance in the process of cell death. Evidence for the involvement of the target in neuronal cell death comes from work on motor innervation of muscles in which the activity of the muscle is modulated by direct electrical stimulation. The result of such experiments was a more extensive loss of motoneurones than normal (Oppenheim & Nunez, 1982). Inactivation of postsynaptic activity with $\alpha$-bungarotoxin or curare, or inactivation of presynaptic activity with botulinum toxin, prevents cell death (Pittman & Oppenheim, 1979; Okada et al., 1989) and the rescued motoneurones are reported to differentiate normally (Oppenheim & Chu-Wang, 1983). In a recent study Oppenheim et al. (1990) reported that naturally occurring cell death is an active process requiring protein and RNA synthesis; they inhibited RNA and protein synthesis *in vivo* and reported reduced levels of naturally
Several studies have been performed attempting to establish whether motoneurone death continues into the early postnatal period of development. In 1946, Romanes reported a 20% loss of motoneurones in mouse spinal cord between PN 0-6. However, more recent studies have failed to detect postnatal cell death (Lance-Jones, 1982; Oppenheim, 1986). These authors reported a 60% loss of motoneurones during early developmental stages (E13-E18). However, at later developmental stages (E18-PN20) further death was not reported.

II) Elimination of Polyneuronal Innervation

The first axon to reach a developing myotube makes contact and a neuromuscular junction is formed between the two. Even after the first contact has been made between the motoneurone and the muscle fibre the state of innervation characteristic of an adult motor unit has yet to be formed, in that, the motoneurone supplies many more muscle fibres than it does in the adult, and each muscle fibre is polyneuronally innervated, with more than one source of innervation (Redfern, 1970; Brown et al., 1976; O'Brien et al., 1978; Bagust et al., 1983). Electron microscopic studies show that muscle fibres are innervated by terminals of various sizes (O'Brien et al., 1978; Duxson & Vrbová, 1985). This state of polyneuronal innervation is transitory and is subsequently eliminated in the weeks following birth. The exact nature of the mechanisms which bring about this modification of neural input are not fully understood.

The establishment of excess synaptic contacts and their subsequent elimination during development may be a mechanism by which a matching of
motoneurone and muscle fibres takes place. During synapse elimination motoneurones may possibly lose synapses from muscle fibres of an inappropriate type (Jones et al., 1987a).

The excess innervation of skeletal muscle begins to decline around birth and within the first three weeks of life, when the motor activity of the animals is increasing rapidly, the neonatal pattern of innervation is modified so that the adult pattern is achieved - the motoneurone limits its peripheral field and the muscle fibres become innervated by a single motoneurone (Brown et al., 1976). As this reorganization process proceeds, the size of an individual motor unit decreases until it reaches that of the adult. At a single endplate histological examination demonstrates that the smaller axons profiles are eliminated at a single endplate (O'Brien et al., 1978; Duxson, 1982), whilst the larger axons remain.

Extensive experimentation has demonstrated that neuromuscular activity is critically important in this remodelling of synaptic connections. This is illustrated in experiments where elimination of superfluous synapses, and therefore a reduction in motor unit size, is accelerated by electrical stimulation of the muscle (O'Brien et al., 1978) - inactivity of the muscle, as a consequence of tenotomy, slows down the rate of the natural elimination process (Benoit & Changeaux, 1975). Blocking nerve activity with tetrodotoxin or botulinum toxin (Thompson et al., 1979; Brown et al., 1982) or by spinal cord section (Zelená et al., 1979) similarly delays the elimination of excess terminals. The importance of neuromuscular activity in the establishment of the adult pattern of innervation may be explained in terms of a competitive process. Competition may arise between multiple nerve
terminals innervating a single muscle fibre. Motor units with a large peripheral field may loose more terminals than those with a smaller peripheral fields (Bagust et al., 1973; Brown et al., 1976). The terminals of a motoneurone with a large peripheral field may be less able to successfully compete at a single endplate. Neuromuscular activity of the preparations or experimentally-imposed activity may be affecting the release or synthesis of a factor which affects the survival or elimination of synapses at an endplate.

Alternatively, the target may be influencing the development of the motor unit and consequently its own pattern of innervation. This possibility can be addressed by experimentally manipulating the activity of the target, whilst leaving the motoneurones and therefore the presynaptic activity unaltered. Postsynaptic activity i.e. target activity, has been demonstrated to be important in the elimination of polyneuronal innervation, blocking of postsynaptic AChR's with α-bungarotoxin delays the elimination of excess synapses (Srihari & Vrbova, 1980; Duxson, 1982). Conversely, by blocking AChE, the rate of elimination of excess synaptic inputs onto a muscle can be enhanced (Duxson & Vrbova, 1985). It appears therefore that the activity of the target is influencing the attainment of the adult pattern of innervation.

The mechanisms by which the target regulates its synaptic input are unclear. Calcium ions and a calcium-activated neutral protease seem to be involved, since the inhibition of the neutral protease or reduction of the Ca$^{2+}$ concentration appear to reduce the elimination of the polyneuronal innervation (O'Brien et al., 1980; 1984; Connold et al., 1986).

The loss of redundant terminals occurs by a process of physical
retraction of the terminal from the endplate (Korneliussen & Jansen, 1976). "Retraction bulbs" have been demonstrated by light microscopy on axons withdrawing away from endplate regions (Riley, 1977; O'Brien et al., 1978). Indeed, recently Balice-Gordon & Lichtman (1987) demonstrated terminal retraction in vivo.

4) PLASTICITY OF THE NEUROMUSCULAR SYSTEM

The neuromuscular system in mammals is plastic and undergoes considerable modifications during development as the system develops and the adult patterns are achieved. Once the adult state of the neuromuscular system has been established the system is relatively stable, however plasticity remains and is apparent in an ability of both the nerve and muscle cells to respond to changes in their state or the surrounding environment. This ability to respond to changes is manifest by an altered phenotypic expression. A plasticity of phenotypic expression may also confer onto the cells an ability to adapt to, and survive injury. The ability to survive and respond to injury is however dependent upon age.

I) Effects of nerve injury on the neuromuscular system

i) Response of the cell body

Within a few hours or days of peripheral nerve injury a complex series of reactions occurs within the cell body of the injured neurone. The regeneration response of mature cell bodies to peripheral nerve injury is termed the "axon reaction" or "chromatolysis" (for a review, see Lieberman, 1971). The rate and degree of the response of the cell
body to injury depends upon the distance of the injury from the cell body. The chromatolytic response is more severe and has a more rapid onset the closer the cell body to the site of injury. In addition, the duration that the neurone is separated from the target is critical for the recovery of the neurone from the injury. The chromatolytic response of the cell body to injury involves the motoneurone cell changing its mode of function from that of a transmitting cell to a growing cell. The primary regenerative response of the cell body to nerve injury is the enhancement of both the RNA and protein metabolism. Axotomy appears to trigger the cellular biochemical machinery, enabling it to respond to nerve injury and send new material rapidly to the site of injury (Grafstein & McQuarrie, 1978). In addition, axotomized motoneurones re-express growth associated proteins (GAP's) (Bisby et al., 1988). The Nissl substance looses its characteristic shape and disintegrates as a consequence of the injury and is redistributed throughout the cell body. Morphologically, the cells swell (Cohen & Jacklett, 1965) due to an uptake of water (Marinseco, 1909), and the nucleus is displaced taking up an eccentric position close to the cell membrane (Nissl, 1892).

In axons where functional re-connection with the target proceeds, cellular metabolism is increased (Watson, 1970). Accompanying the increased second phase of cellular metabolism there is an increase in nuclear volume (La Velle & La Velle, 1958; Watson, 1965) and nucleolar RNA and protein content (Watson, 1965; 1968; 1969). The nuclei regain their normal position, at the same time the normal Nissl pattern is re-established (Ransen, 1909).

If functional regeneration does not occur the initial increased metabolic activity slows markedly, so that cellular metabolism becomes
relatively depressed. The cellular metabolism then reduces to the extent that the cell dies.

Immature motoneurones are unable to undergo chromatolysis and so cannot respond to injury by regeneration - instead the cells die in large numbers. It is possible that immature cells cannot increase their metabolism to the extent needed to produce sufficient material to ensure repair. This seems a possible explanation since immature motoneurones have overexpanded peripheral fields as a result of extensive polyneuronal innervation.

Nerve injury promotes within the motoneurone mechanisms which attempt to bring about a reunion with the disconnected target. Regeneration of the axon from the proximal stump and sprouting of new outgrowths occurs. These outgrowths are guided by the distal nerve stump and reinnervation of the target proceeds.

ii) Response of the axon to injury

Injury of nerve cells, either axonal crush injury or axotomy, disconnect the axon from its cell body, and therefore from its metabolic machinery. The supply of all metabolic and cytoskeletal material available to axonal processes is directed from the cell body down the axon by slow axonal transport. If an axon is disconnected from the cell body then the supply of nutrients is interrupted and the distal stump of the injured axon begins to degenerate. Transection of the axon leads to an immediate influx of Ca\(^{2+}\) into the severed ends of the axon, causing a local disintegration of neurofilaments and microtubules (Zelená et al., 1968), known as Wallerian degeneration. A further major influx of Ca\(^{2+}\) occurs across the axolemma, triggering widespread disintegration of neurofilaments and microtubules in the transected nerve fibres.
Following nerve injury a decrease in nerve fibre diameter and size distribution occurs in the proximal stump (Weiss & Edds, 1946). This is coincident with a reduction in the slow axonal transport of neurofilaments relative to microtubules and actin filaments (Hoffman et al., 1985; Hoffman, 1988) and a reduced expression of neurofilament protein gene expression (Hoffman, 1988). The altered supply of cytoskeletal material, coincident with the degeneration of the axonal cytoskeleton in the vicinity of the injury, results in a reduced axonal diameter in the proximal stump (Gorden & Stein, 1982; Hoffman et al., 1988).

After axotomy the proximal stump, produces sprouts regenerates and re-establishes synaptic contact with the denervated muscle (Cajal, 1928). The cell body of the injured axon changes its metabolic emphasis and alters its protein priorities in order to deal with the different and increased demands of repair and regeneration. The expression of a tubulin gene encoding a distinct beta tubulin isotype is selectively induced during axonal regeneration; tubulin is the principal cytoskeletal protein in regenerating sprouts (Berthold, 1978). In peripheral nerves the regrowth of the proximal stump of injured axons begins 1-2 days after injury. Nerve crush injury maintains the continuity of the basement membrane, and the injured axons can therefore be accurately guided back to the target. When the injury is more severe, such as axotomy, the basement membrane is damaged, and the axon becomes severed into two disparate parts. In this case the glial cells within the vicinity of the injury divide and migrate to the ends of the severed nerve, and may act as a bridge between the proximal and distal stumps,
thus aiding regrowth and reconnection with the target.

Once regeneration has occurred, the axon calibre recovers in the proximal stump (Kuno et al., 1974; Hoffman et al., 1985), which correlates with a restoration to the levels prior to axotomy, of neurofilament gene expression (Hoffman, 1988) and coincides with reinnervation of target tissue (Kuno et al., 1974).

iii) Changes at the neuromuscular junction

Axotomy results in degenerative changes in the distal stump of the injured axon, and subsequently the neuromuscular junction. The first changes to occur at the neuromuscular junction involve a failure in spontaneous and evoked release of transmitter and a decrease in synaptic efficiency. Cholinesterase at the endplate region decreases and a loss of postsynaptic thickening occurs, with the junctional folds opening. Subsequently a loss of neuromuscular contacts occurs.

iv) The sprouting response

Uninjured motoneurones will sprout to innervate a muscle which is partially denervated or inactivated (Hoffman, 1950; Edds, 1953). Growth cones give rise to nodal/collateral sprouts (emerging from the Nodes of Ranvier) or terminal sprouts (from unmyelinated terminals) from axon collaterals. In partially denervated skeletal muscles fine outgrowths develop from the motor nerve endings and collateral branches develop from the motor axons. These sprouts then make functional synapses with the denervated muscle fibres. The sprouting response of neurones increases motor unit sizes 4-5 fold (Jansen et al., 1975; Brown et al., 1976; Thompson & Jansen, 1977; Brown & Ironton, 1978). The sprouting response seems to be dependent upon activity and not solely upon the
inflicted injury. If muscles are paralysed with neurotoxins sprouting occurs; which can be partly prevented by chronically electrically stimulating the muscle. Blocking presynaptic activity with botulinum toxin causes profuse terminal sprouting (Duchen & Strich, 1968; Duchen, 1970; Pestronk & Drachman, 1978; Ironton et al., 1978; Brown et al., 1978; Duchen et al., 1980). Blocking nerve conduction with tetrodotoxin (TTX) (Pestronk & Drachman, 1978; Brown & Ironton, 1977; Betz et al., 1980) or local anaesthetics (Benoit & Changeaux, 1978) also induces terminal sprouting. The lack of activity appears to be an effective stimulus to induce sprouting. The ability of the cell body to respond to peripheral injury and to establish and maintain sprouts confers an extensive flexibilty onto the neuromuscular system, and an ability to withstand injury.

v) Effect of Nerve Injury on Muscle

When muscle cells become denervated the extrajunctional area becomes highly sensitive to ACh and AChR are incorporated into the extrajunctional membrane (for a review, see Gordon et al., 1976). Denervated muscle fibres also express glycoproteins e.g. NCAM, which are normally expressed in immature muscles (Sanes et al., 1986) and are thought to be involved, during development, in the guidance of axons to the target cells.

Muscle fibre nuclei in denervated muscle become rounded and swollen and some nuclei become centralized within the muscle fibres 2 weeks after the denervation injury. Over the long term, muscle fibres shrink and atrophy. If reinnervation is prevented then muscle fibres die and are replaced by connective tissue.
This thesis attempts to address the question of the importance of early nerve-muscle interactions on muscle development. Chapter 2 will attempt to investigate the mechanisms by which the activity of the target brings about the elimination of excess synaptic input during early postnatal development. By reducing target activity in the immature soleus muscle of the rat, Chapter 3 will investigate the influence of target activity on the development of the adult soleus muscle fibre properties and on its adult pattern of innervation. Chapter 4 will investigate the importance of the activity of the target in the development of fast muscles. In addition, the role of postsynaptic activity in the changing response of the fast hindlimb muscles of the rat to denervation injury will be studied.
CHAPTER TWO

The Role of Activity and a Calcium-Activated Neutral Protease in the Elimination of Functional Neuromuscular Contacts in the Developing Rat Diaphragm Muscle
During the normal development of the mammalian neuromuscular system there exists a period when an excess number of synapses are found on each muscle fibre. Following the initial establishment of contact between the first developing axon terminal and a developing myotube, subsequent additional axons form junctions in close proximity to the initial site of contact. This excess synaptic input is generally referred to as polyneuronal innervation, where each muscle fibre is characteristically innervated by 2 to 6 axons. Polyneuronal innervation appears to be a universal phenomenon in mammalian skeletal muscle, since it has been demonstrated in many species and various muscles. However, the average number of terminals on each muscle fibre varies to a large degree depending on the species and the muscle studied. The fast extensor digitorum longus (EDL) muscle of the rat has a maximum polyneuronal innervation of 3 axon terminals (Balice-Gordon & Thompson, 1988a), the rat diaphragm muscle (a predominantly fast muscle), 2.9 terminals (Bennett & Pettigrew, 1974a). The soleus muscle (a slow muscle) has a maximum polyneuronal innervation of 5 terminals (Brown et al., 1976), and the intercostal muscle, which has a mixed population of muscle fibres, has a maximum innervation of 6 terminals (Brown et al., 1981). In a more recent study, Callaway & Van Essen (1989) investigated the degree of polyneuronal innervation on different muscle fibres types, in contrast to previous experiments which investigated mixed populations of muscle fibres. Interestingly, this study demonstrated that within the same muscle the degree of polyneuronal innervation appears to be greater for the slow population of muscle fibres than on the population of
fast fibres, within the same rabbit soleus muscle.

After the initial establishment of contacts on the developing muscle fibre the number of axon terminals remains stable for 2-3 weeks. During, and also immediately after, this period the postsynaptic membrane undergoes many changes and becomes specialized at the points of contact (Couteux, 1963; Nystrom, 1968a; 1968b). This maturation and specialization develops over the following few weeks when the nerve terminal increases in size and complexity (Slater, 1982). In addition the ACh receptors stabilize (Changeux & Danchin, 1976; Cohen, 1976) and acquire their mature characteristics (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Brenner & Sakmann, 1983). AChE is deposited in the subsynaptic membrane (Bennett & Pettigrew, 1974a; Bevan & Steinbach, 1977; Harris, 1981b).

As the maturation of the postsynaptic component of the neuromuscular junction proceeds the presynaptic component undergoes a similar maturation process. The motoneurone begins to limit its peripheral field and the number axonal contacts converging on a single site is progressively reduced. As polyneuronal innervation is eliminated the mature adult pattern of innervation is achieved. In most skeletal muscles this reorganization of neural input begins around the time of birth. Original investigations into the elimination of polyneuronal innervation (Redfern, 1970; Bagust et al., 1973; Bennett & Pettigrew, 1974a) suggested that the apparently redundant axons were eliminated on account of the normal loss of motoneurones occurring at this stage in development (e.g. Prestige, 1967). However, it is now well documented that the period of naturally occurring cell death is completed by birth. Brown and colleagues (1976) demonstrated that the number of motoneurones
remains constant during the elimination process and the motor unit size decreases during this early postnatal remodelling period. Thus, the number of muscle fibres innervated by a single motoneurone decreases and the elimination of excess synaptic input is the consequence of the reduction of axon collaterals within each motor unit.

Early investigations into this regressive event demonstrated that the loss of redundant terminals is not a degenerative process (Kornelliussen & Jansen, 1976; Riley, 1977; Östberg & Vrbová, 1977; Zelená et al., 1979; Bixby, 1981). Specific synapses appear to be lost by a process of retraction of terminal branches into the parent axons (Balice-Gordon & Lichtman, 1987). Light microscopical examination of muscles undergoing elimination of axon terminals have observed axons which appear to end in "retraction bulbs" (Riley, 1977), which are thought to be in the process of withdrawal from the endplates (Östberg & Vrbová, 1977; Riley, 1977). Kornelliussen & Jansen (1976) suggested that the retraction of supernumery axon terminals is the most economical way of eliminating excess terminals, in that the axoplasm and membrane material can be saved and re-employed at other actively developing and/or growing sites in the neurone. At a single endplate it is the thinner axons, possessing more primitive terminals, which appear to be eliminated in preference to the larger axons with more elaborate terminals (O'Brien et al., 1980).

In most skeletal muscles loss of excess terminals is underway at the time of birth, but the time course and rate of elimination are markedly different between different muscles. The intercostal muscles of the rat develop early in embryogenesis. At the time of birth, the level of polyneuronal innervation in the intercostal muscle is reduced,
and elimination is complete by postnatal day (PN) 10-11 (Dennis et al., 1981). The elimination of supernumery terminals is complete by PN14-18 in rat diaphragm (Redfern, 1970; Rosenthal & Teraskevich, 1977), and in the fast limb muscle, EDL, the first singly innervated fibres appear at PN3 and elimination is over by PN18 (Balice-Gordon & Thompson, 1988). The fourth deep lumbrical (4DL) muscle of the rat hindlimb maintains its motor unit size for the first 10 days post-natally, mainly due to continued myogenesis, and the first singly innervated fibres appear at PN8. By PN21 elimination of excess synapses is complete (Betz et al., 1979). In contrast, the rate of elimination of terminals is more rapid in the slow soleus muscle of the rat hindlimb. The first singly innervated fibres appear as late as PN8, and elimination is complete by PN16-20 (Benoit & Changeux, 1975; O'Brien et al., 1978; Miyata & Yoshioka, 1980). Interestingly, Callaway and Van Essen (1989) demonstrated that the rate of synapse elimination appears to be greater for the slow fibres than the fast fibres of the rabbit soleus muscle. Even so, both populations of muscle fibres become mononeuronally innervated at the same time.

Redfern (1970) was the first to note the importance of activity in the elimination of polyneuronal innervation in the rat diaphragm muscle. It is now well established, through a great wealth of experimental data, that neuromuscular activity is involved in the synaptic remodelling process. Elimination of excess synaptic inputs can be delayed by a reduction in activity. Benoit & Changeux (1975) and Riley (1978) reduced activity by tenotomizing rat soleus muscles soon after birth and demonstrated a persistence of polyneuronal innervation. Neonatal cordotomy (Miyata & Yoshioka, 1980), as well as cordotomy accompanied
with deafferentation (Zelená et al., 1979) also result in a retardation in the loss of terminals to specific muscles. Preventing neuromuscular activity can delay the elimination of polyneuronal innervation. Brown et al. (1981b) applied botulinum toxin (BoTx: which blocks evoked and spontaneous quantal transmitter release (Spitzer, 1972)) to rat soleus muscles, to pharmacologically block neuromuscular transmission, whilst Srihari & Vrbová (1978) and Sohal & Holt (1980) subjected chick muscles to curare and α-bungarotoxin treatment. All of these treatments resulted in the delayed withdrawal of polyneuronal innervation. Conversely, the elimination of excess synaptic input can be accelerated by increasing neuromuscular activity. O'Brien et al. (1978) chronically electrically stimulated the sciatic nerve of newborn rats, Zelená and colleagues (1979) removed synergistic muscles in the hindlimb, thus overloading the remaining muscles, and Thompson (1983) stimulated the soleus muscle in newborn rats. The increased activity of both nerve and muscle achieved by these experimental procedures accelerated the loss of redundant synaptic terminals in the muscles studied.

Extensive experimentation has demonstrated that neuromuscular activity is of importance in the remodelling of the neonatal neuromuscular junction into the highly specialized junction of the adult. More specifically, it appears that postsynaptic activity is a major contributory factor in the withdrawal of supernumerary terminals. When target activity is blocked using postsynaptic blocking agents, there is a retardation in the elimination of superfluous nerve endings. Srihari & Vrbová (1978) paralysed chick embryos with the postsynaptic blocker curare and Duxson (1982) employed α-bungarotoxin to bind to the postsynaptic AChR and paralyse immature rat muscles; both studies
prevented the removal of extra nerve terminals for the duration of the treatment. Callaway & Van Essen (1989) treated rabbit soleus muscles for 5 days postnatally with α-bungarotoxin, blocking less than 15% of the fibres and reported a reduction in the rate of elimination of polyneuronal innervation. Conversely, by specifically increasing postsynaptic activity using DFP (di-isopropylfluorophosphate), an AChE inhibitor, Duxson & Vrbová (1985) were able to increase the elimination of polyneuronal innervation. Specific postsynaptic activity of the muscle therefore appears to be of importance in the reorganization of innervation occurring at the developing neuromuscular junction. In the normal animal, the timing of this elimination of excess synaptic contacts coincides with the increasing neurogenic locomotor activity of the developing animal (Navarrete & Vrbová, 1983). It appears, therefore, that in mammals increasing neuromuscular activity, seen as a consequence of the normal developmental programme, is closely linked to synapse elimination.

The underlying molecular mechanisms which link increased muscle activity to excess synapse elimination are, as yet, unclear. Various models have been proposed to explain the events occurring during this remodelling process. Many of the models have postulated a role for a trophic factor, which is released from muscle fibres in an activity-dependent fashion. The released trophic factor may promote the growth of overlying nerve terminals. Some experimental evidence supporting the model of a muscle-derived trophic factor comes from a study by Brown et al. (1981) investigating the sprout-inducing effects of muscle activity in partially denervated or paralysed muscle. Conversely, it is also possible that a trophic factor may have an inhibitory effect on the...
terminals, thus preventing excessive innervation. Ribchester (1988) has postulated a role for the interaction between a growth promoting and growth repressing stimulus at motor nerve terminals.

The reorganization of synapses occurring during development has been explained using a model based on the principle of electromigration. The model suggests that a factor present at the endplate has a stabilizing influence on the immature overlying nerve terminal. The distribution of the factor within the terminal is dependent upon its electromigration within the membrane. Migration of the factor is thought to occur as a result of an extracellular magnetic field set up as a consequence of synaptic activity. ACh receptors have been shown to migrate in an imposed electrical field (Poo, 1981; 1982). In a recent study Rich & Lichtman (1989) visualized multiply-innervated endplate structures, and observed the competitive interactions occurring at terminals in reinnervated muscles. These authors observed the loss of a terminal branch at an endplate and were able to establish that the loss of a branch was initiated by the loss of the post-synaptic ACh receptor at the endplate. The vacated synaptic sites were not invaded by sprouts from remaining terminals. The ACh receptor may be the stabilizing influence on the immature nerve terminals, the more stable the ACh receptor the greater the chance of survival of the overlying terminal.

O’Brien and colleagues (1980, 1984) and the work of Connold et al. (1986) have demonstrated that a calcium-dependent mechanism is involved in the elimination of polyneuronal innervation. O’Brien et al. (1980, 1984), raised the extracellular Ca\(^{2+}\) concentration around rat soleus muscle preparations and demonstrated that increased Ca\(^{2+}\) levels can accentuate the effects of ACh, or neuromuscular activity, in disrupting
neuromuscular contacts. This in vitro manipulation of polyneuronal innervation could be prevented by the addition of inhibitors of a proteolytic enzyme, the calcium-activated neutral protease (CANP). Connold et al. (1986) further investigated the possibility that a Ca²⁺-dependent proteolytic mechanism is involved in the elimination of excess terminals. Using an in vivo approach they treated perinatal rat soleus muscles with Ca²⁺-chelators and conversely, with inhibitors of the Ca²⁺-activated neutral protease, and subsequently demonstrated a reduced rate of synapse elimination during the normal developmental period. These experimental results, therefore, demonstrate the essential role of calcium and of the proteolytic enzyme, CANP, in the elimination process.

Several research groups have suggested that the muscle may release a factor which promotes nerve growth (e.g. Brown et al., 1976) and for which nerve terminals compete. Vrbová et al. (1988) postulated that K⁺ ions could fit this role of a locally released factor. For many years it has been known that K⁺ ions are released from muscle fibres during depolarization of the muscle fibre membrane (Hodgkin & Huxley, 1952) and can raise to high levels (21mM) around stimulated optic nerves of neonatal rats (Connors et al., 1982). Neuromuscular activity leads to an increase in K⁺ concentration in the synaptic cleft (Hohlfield et al., 1981). Vrbová and colleagues (1988) proposed that during the period of increased muscle activity in the neonatal animal (Navarette & Vrbová, 1983), K⁺ ions are released from the muscle fibres opening voltage-gated Ca²⁺ channels in the nerve terminals. Consequently, levels of internal Ca²⁺ increase and this may activate the CANP within the terminals, which has a proteolytic role in the elimination of excess synapses. In a study of neonatal rat soleus muscles, electrophysiological recordings
of polyneuronal innervation demonstrate that experimentally increased external K\(^+\) concentration affects the presynaptic terminal, in that raised external K\(^+\) concentration was able to reduce levels of polyneuronal innervation on neonatal rat soleus muscles compared to normal control preparations (Vrbová et al., 1989).

Neurofilaments are the prominent structural elements of neurones in most species of vertebrates and invertebrates (for a general review see Shelanski & Liem, 1979). The mammalian axonal cytoskeleton has been shown, by electron microscopy, to be composed of longitudinally orientated microtubules (composed primarily of tubulin) and neurofilaments, which are extensively cross-linked to themselves, each other and to the plasma membrane by thin fibrils (Ellisman & Porter, 1980; Hirokawa & Heuser, 1982; Metuzals & Muschynski, 1974). Mature neurofilaments have a structure which is similar to other intermediate filaments and is composed of 3 subunit proteins. Each of the neurofilament subunits is a different gene product (Czonak, 1980; Sneidman et al., 1989) and possess 3 distinct domains; including a middle "rod" domain flanked by a carboxy-terminal tailpiece (Geisler & Weber, 1981; Weber et al., 1983). Willard & Simon (1981) noted that the location of each of the 3 neurofilament subunits within the neurofilament is non-identical. They demonstrated that NF-L (68Kd) forms a helical core to the neurofilament, whereas NF-H (200Kd) is peripherally attached and is periodically arranged along the axis of the neurofilament. From this data they suggested that the NF-H functions to form cross-bridges between filaments. Hirokawa et al. (1984) localized NF-H in lateral "projections" of neurofilaments.

Calcium-activated neutral proteases are known to be present in
muscle and nerve cells and is highly specific for cytoskeletal proteins (Schlaepfer & Hasler, 1979; Kamakura et al., 1983). CANP is therefore an ideal candidate to degrade the cytoskeletal structure of axons, a process which is necessary for the elimination of supernumery axons.

The experiments performed in this chapter attempt to clarify further the mechanisms involved in the elimination of supernumery nerve terminals, mimicking the conditions thought to be responsible for the elimination process in vivo. Using in vitro tension recordings and the immunoblotting technique, these experiments investigate the possibility that due to a calcium-dependent proteolytic enzyme, a breakdown of the cytoskeletal elements of nerves within the muscle occurs, similar to that which may be occurring during the period of elimination of polyneuronal innervation.
MATERIALS AND METHODS

I) Animals

Neonatal albino Wistar rats of both sexes were used in these experiments, at 9-11 days postnatally (17-28g). Animals were supplied by either Joint Animal House, University College London or by Charles Rivers, Animal Suppliers, U.K.. Animals were watered and feed ad libitum, and were kept in a constant light/dark cycle.

II) In vitro Isometric Tension Recordings

The experimental animals were anaesthesized with ether, decapitated and exsanguinated. The diaphragm with its intact motor nerve supply, was then quickly dissected from the animal and placed in a large volume of aerated Krebs-Henseilt physiological saline:–

- 4.75mM KCl
- 1.2mM KH$_2$PO$_4$
- 1.2mM MgSO$_4$
- 1.9mM CaCl$_2$
- 120mM NaCl
- 25mM NaHCO$_3$
- 5mM glucose

The diaphragm nerve–muscle preparation was the chosen experimental preparation in this series of experiments since the diaphragm muscle is a thin, sheet-like muscle which can be successfully perfused in an organ bath. The thin nature of this muscle means that the preparation can be functionally maintained in physiological saline for long periods of time. All of the muscle fibres in the preparation have an adequate supply of oxygen and nutrients, and anoxia is unlikely to occur in any
of the fibres since none are placed deep into a muscle mass. In addition, as explained in Section IV, the thin sheet-like nature of the muscle makes the diaphragm an ideal preparation for easy visualization of the nerves coursing through the preparation. This visualization, using transillumination of the nerves, allows for easy removal of the endplate region from the rest of the muscle fibres, with as little muscle protein contamination as possible.

The diaphragm was hemisected and the rib attachment secured to a rod in a 10ml organ bath containing aerated Krebs solution. A further ligature was tied around the tendon of the diaphragm with 0.7 silk thread (Ethicon) and the tendon ligature attached to a 4oz strain gauge (Dynamometer, UFI). The muscle was then set at the optimum length for maximum twitch contractions and was stimulated with a supramaximal voltage. The preparation was allowed to equilibrate for 15 minutes. Throughout the experiment, the hemi-diaphragm preparation was kept at room temperature, which varied between 20-23°C.

Direct and indirect tetanic contractions (40Hz) were elicited from the diaphragm preparations at the beginning of the experiment. Indirect contractions were elicited by stimulating the motor nerve, via a suction electrode, using a pulse width of 0.02 msecs. Direct stimulation was achieved using an electrode running the entire length of the pole to which the diaphragm was fixed, in combination with a silver electrode placed over the muscle. In this way the current was passed along the longitudinal axis of the muscle and most of the fibres were activated. Square wave pulses of 2 msecs. and supramaximal intensity were applied. Twitch and tetanic contractions were elicited from the muscle preparation and displayed on a pen recorder (Multitrace 2, Electromed).
The amount of tension produced by the preparation was calculated (in grammes). The ratio of indirect/directly elicited maximum tetanic tension was established, and those preparations where the innervation ratio was 0.95 or higher were used for experiments. Values lower than this are an indication of damage to the preparation, and so the preparations were discarded (approximately 20% of the preparations were discarded in this series of experiments).

The nerve-muscle preparations were then incubated in 10mls of various aerated experimental solutions for a period of 2 hours (See Section III).

After the 2 hour incubation period the organ baths were thoroughly washed with a large volume of normal Krebs solution and the muscle preparations returned to normal aerated Krebs. The diaphragm length was reset, if necessary, to the optimum length for maximum twitch contractions. Indirectly and directly elicited tetanic contractions were recorded at time intervals of 0, 15, 30, 45, and 60 minutes, post-incubation.

For each time interval the ratio of indirectly/directly elicited tetanic tension was calculated and taken as an indication of the extent of innervation of the preparations.

III) Incubation Media

The nerve-muscle preparations of hemi-diaphragms were exposed to various experimental solutions for a period of 2 hours at room temperature. The solutions used were:-

Normal Krebs solution.
Normal Krebs with $10^{-2}$M ACh.

Normal Krebs with $10^{-2}$M ACh and 0.2 mg/ml leupeptin.

High Ca$^{2+}$ Krebs (containing 10mM CaCl$_2$) with $10^{-2}$M ACh.

High Ca$^{2+}$ Krebs (10mM) with $10^{-2}$M ACh and 0.2mg/ml leupeptin.

Normal Krebs with 0.2mg/ml leupeptin.

Leupeptin (N-acetyl-Leu-Leu-Arg, Sigma) was used to inhibit calcium activated neutral protease (CANP) within the preparations since it can penetrate cell membranes and act inside cells. Leupeptin has been shown to pass the sarcolemma and enter into the myofibrills (Libby & Goldberg, 1978) and act inside muscle cells (Stracher et al., 1978) as well as entering and influencing the nerve terminal (Roots, 1983). The minimum dose of leupeptin capable of preventing neurofilament degradation in rat sciatic nerve segments is 0.03mg/ml (Kamakura et al., 1983). The level of leupeptin used in these experiments, 0.2mg/ml, exceeds this minimum dose.

In some experiments ACh was included in the incubation media. The dose used in this series of experiments ($10^{-2}$M) is within the range calculated to be present in the normal animal during neuromuscular activity (Matthews-Bellinger & Salpeter, 1978).

The pH of the high Ca$^{2+}$ experimental solutions was 7.2 compared with a pH of 7.4 for normal Krebs solution. The addition of ACh or leupeptin to the experimental solutions did not affect the pH.

In some of the experimental conditions the motor nerve was stimulated continuously over the 2 hour incubation period: stimulation was achieved using a suction electrode and the nerve was stimulated at 10Hz. A similar level (8Hz) of stimulation has been used in a previous
study to continuously stimulate soleus muscle preparations for 2 hours (O'Brien et al., 1980) with negligible adverse effects on the preparations.

IV) Extraction of Neurofilaments from Samples

Diaphragms were dissected from experimental rats and pinned in 10ml organ baths, in aerated Krebs. After 15 minutes equilibration, the Krebs was changed to one of the various incubation media (as described above) and the nerve-muscle preparation incubated at room temperature for 2 hours.

At the end of the 2 hour incubation period the muscles were placed in normal Krebs plus 5mM EDTA (ethylenediamine-tetraaceitic acid), on ice, and the endplate region cut out and weighed.

The endplate region of diaphragm preparations had previously been identified in a whole mount preparation stained for cholinesterase (using the method of Namba et al. (1967), adapted by O'Brien et al (1978): see Appendix I for method). An example of a cholinesterase-stained diaphragm is illustrated in Figure 1. The endplate region can be seen within the muscle. This cholinesterase staining was used as a landmark when dissecting the endplate region from the incubated preparations and, along with transluminescence, which illuminated the nerve branches coursing through the muscle preparation, the endplate region could be dissected free from the rest of the muscle.

The endplate regions of 4 diaphragm preparations were pooled for neurofilament extraction, so that the volume of material being handled was adequate for manipulation.

The endplate region was finely minced and homogenized (10% w/v) in
A diaphragm muscle was removed from a normal 9 day old Wistar rat. The muscle was hemi-sected. The fixed, frozen tissue was then stained for cholinesterase activity to depict the endplate region, using the method of Namba et al. (1967), adapted by O'Brien et al. (1978). The preparation was mounted on 0.5% gelatine-coated glass slides and permanently mounted for viewing. The endplate region is marked on both photographs. Scale bar = 4 mm.
Figure 1

Polyacrylamide gel electrophoresis (PAGE) was performed on experimental samples followed by electrophoretic separation of PAGE-separated proteins onto nitrocellulose paper for detection by specific antibodies.

The SDS-PAGE technique employs a method by which the proteins within an experimental sample are reduced, denatured, and solubilized by the reagents within the gel-isolated matrix. The loading buffer contains the anionic detergent, sodium dodecyl sulfate (SDS), which binds to the
a solution of 20mM Tris, pH 6.7, containing:

2mM diethoerythritol
1mM EDTA (tetrasodium salt)
2% sodium dodecyl sulphate (SDS)
0.05% bromophenol blue

(modified from Schlaepfer & Bruce, 1990).

The resultant homogenate was heated to 100°C for 5 minutes and then centrifuged for 15 minutes at 15,000 revs. min\(^{-1}\). The supernatant was collected and 10% glycerol was added; the sample was then aliquoted and stored at -20°C.

Neurofilaments were also extracted from other sources, to act as controls to the diaphragm samples. The brain and spinal cord were removed from a freshly killed male adult Wistar rat. The brain stem was excised from the other unwanted brain tissues and the spinal cord was removed from the animal. Both tissues were prepared for electrophoresis in the same manner as the diaphragm samples. Supernatants of both samples were stored at -20°C.

V) SDS Polyacrylamide Electrophoresis

SDS Polyacrylamide gel electrophoresis (SDS-Page) was performed on experimental samples followed by electrotransfer of SDS-Page separated proteins onto nitrocellulose paper for detection by specific antibodies.

The SDS-Page technique employs a method by which the proteins within an experimental sample are reduced, denatured and solubilized by the reagents within the gel-loading buffer. The loading buffer contains the anionic detergent, sodium dodecyl sulphate (SDS), which binds to the
now solubilized protein and bestows upon the protein a negative charge. These protein-SDS complexes can then be separated by electrophoresis through an acrylamide matrix (the pore size of which is determined by the acrylamide concentration). The proteins are separated depending on their molecular size (smaller proteins being more mobile through the acrylamide matrix and therefore travelling further in a fixed period of time than larger proteins).

The buffers and gel system employed in this experimental series were based on those of Laemmli (1970). Stock buffers were stored at 4°C with the exception of the 20% SDS which was stored at room temperature. Stock acrylamide was filtered following preparation, and the acrylamide and gels were handled with care due to the carcinogenic nature of acrylamide. 0.1% ammonium persulphate (AMPER) was prepared freshly.

i) Preparation of slab gel

Two glass plates were cleaned with ethanol to remove all protein contamination. Disposable gloves were worn whilst handling the material to prevent further protein deposition. The cleaned plates were assembled into a sealed mould, with 1mm gap separating the two plates, by placing vaseline coated perspex spacers along both the vertical and the lower horizontal borders. The prepared, sealed mould was then fixed together in an upright position using bulldog clips.

Table 1 lays out the ingredients used to make the separating gel. The separating gel was mixed thoroughly (in the order of ingredients in the Table). Upon addition of the polymerizing agents, tetramethyethylendimine (TEMED) and ammonium persulphate (AMPER), the gel was poured into the glass mould approximately 30mm from the top of the mould. Distilled water was then carefully pipetted on top of the
Table 1: Ingredients for Separating and Stacking Gels, used for SDS-Page Electrophoresis

The ingredients shown in the table were mixed thoroughly in order of listing and poured between two glass plates and used for SDS-Page electrophoresis.
<table>
<thead>
<tr>
<th></th>
<th>7.5% SEPARATING GEL</th>
<th>3% STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%/0.8% Acrylamide/Bis</td>
<td>2.9 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2.0M Tris, pH 8.8</td>
<td>2.4 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris, pH 6.8</td>
<td>-</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.24 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.4 ml</td>
<td>3.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 μl</td>
<td>5 μl</td>
</tr>
<tr>
<td>10% AMPER</td>
<td>80 μl</td>
<td>20 μl</td>
</tr>
</tbody>
</table>
separating gel, to assist the formation of a level, horizontal interface as the gel polymerized. Care was taken to avoid any mixing of the two which would therefore dilute the gel.

Following polymerization of the separating gel, the functional layer of water was poured away and an ethanol cleaned comb was inserted between the two plates. After insertion of the comb, the stacking gel was mixed (See Table 1) and poured into the remaining space in the mould. The comb acts to form wells in the polymerized stacking gel.

After polymerization of the stacking gel the comb was carefully withdrawn and any excess gel was scraped away with a clean spatula. The horizontal perspex spacer at the bottom of the mould had fulfilled its purpose and was carefully removed. The glass mould and the prepared gel were then placed in a perspex reservoir and secured with bulldog clips, ready for electrophoresis. Figure 2 demonstrates the gel equipment used in this experimental series.

Laemmli's buffer (50mM Tris, 380mM Glycine, 0.1% SDS) was poured into the upper and lower reservoirs covering both of the electrodes. Any air bubbles trapped between the glass plates in the place originally occupied by the horizontal spacer, were flushed out using a syringe filled with buffer with a needle bent to a 90° angle. Buffer was injected into the wells to dislodge air bubbles and any loose gel. All air bubbles must be removed as they result in uneven running of the gel and therefore uneven protein gel profiles. Two vaseline covered silicone-rubber tubing lengths acted as a seal between the glass plates and the perspex reservoir therefore preventing mixing of the buffers from the two reservoirs, and thus ensuring the only route for the current was through the gel (See Figure 2).
This figure illustrates the equipment used for SDS-Page electrophoresis. The upper and lower reservoirs contain Laemmlis buffer and the rubber tubing behind the glass plates prevents the mixing of the liquid in the two reservoirs, so ensuring that the current moves through the gel only. The blue line seen half way down the equipment is the "dye front" indicating the distance the proteins have travelled through the acrylamide gel.
ii) Preparation of samples for acrylamide gel electrophoresis

Pre-extracted experimental samples were thawed, vortexed and then centrifuged for 5 minutes to separate any insoluble material. An appropriate quantity of the supernatant was loaded onto the gel, taking care to avoid the insoluble pellet.

iii) Loading samples onto polyacrylamide gels

10 µl of each sample, was carefully injected into each well through the buffer, being careful to avoid cross contamination of samples. Since the loading buffer contains glycerol the samples drop to the bottom of each well. 15 µl of a mixture of molecular weight samples (Sigma Chemical Company) was loaded into one well (See Appendix II). The end lanes of the 13-lane gels were avoided because end-lane "smiling" tends to distort the protein profiles.

iv) Running the gel

Once the samples and standards were loaded the gel was ready to run. The electrophoresis reservoir was connected, via electrodes, to a D.C. power supply (LKB, Broma 2301 macrodrive 1 power supply). A constant current of 35mA was applied to the gel for 1 hour and 15 minutes, this was usually sufficient enough for the dye front to reach the bottom of the 7.5% gel. A starting voltage of approximately 180 volts would steadily increase over this period of time to approximately 250 volts.

Upon application of the electric current, the loaded protein complexes enter into the gel and rapidly reach the interface of the stacking/separating gels. When the proteins reach this interface they have been roughly divided into two groups of high and low molecular mass

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proteins as a result of their movement through the low-acrylamide concentration stacking gel. The change in gel concentration and pH encountered on entering and moving through the separating gel allows further, finer separation of the proteins dependent upon molecular size.

Once the dye front had reached the bottom of the gel, the current was turned off and the buffers poured away. The glass mould was then carefully separated and the gel divided, using an ethanol cleaned ruler, into those lanes to be stained with Coomasie Brilliant Blue stain and those to undergo Western blotting. The exposed gel was kept wet at all times with Laemli's buffer.

v) Staining gel with Coomasie Brilliant Blue

SDS Page-separated proteins were visualized in SDS gels by incubating the gels in Coomasie Brilliant Blue stain (0.025% Coomasie Brilliant Blue, 50% methanol, 7% acetic acid) for a minimum of 20 minutes. Following destaining of the gel in several changes of gel destain (40% methanol, 7% acetic acid) over a period of 1 to 2 hours, or until complete destain of the background, the proteins could be visualized as discrete blue-stained bands.

VII) Western Blotting

The technique of Western Blotting was employed to detect specific antigenic components of protein preparations which had been previously separated according to their molecular weight by SDS polyacrylamide gel electrophoresis, essentially according to the method of Towbin et al. (1979).
i) Electrotransfer of proteins

Following the migration and separation of the proteins in the sample, the gel was cut into lanes and each gel lane placed on top of a pre-cut strip of nitrocellulose paper (pore size 0.2 μm, Schleicher and Schuell, BA83). The nitrocellulose paper was pre-soaked in transfer buffer (25mM Tris, 192mM glycine, 20% methanol; pH 8.3). All instruments coming into contact with the nitrocellulose were cleaned with ethanol to remove all protein contamination which would otherwise bind to the protein-binding nitrocellulose paper. Clean disposable gloves were used to handle the nitrocellulose paper.

Transfer buffer was poured over the layered gel-nitrocellulose paper and any air bubbles between the two eliminated, taking care not to allow the nitrocellulose to dry out. The strips of gel-nitrocellulose were then sandwiched between two pieces of wetted blotting paper, and placed into the cassette of the transfer apparatus. This complete sandwich was loaded vertically into a 4 litre tank containing transfer buffer. Any air bubbles formed around the cassette were flushed out. Electrodes were attached to the transfer chamber so that the SDS-bound proteins would travel from the gel to the nitrocellulose, towards the positive electrode (anode), and then bind to the nitrocellulose paper. Transfer was carried out at a constant voltage of 110V for 1 hour at room temperature.

After transfer of the proteins the sandwich was opened to expose the gel on top of the nitrocellulose, the gel was removed and stained with Coomassie Brilliant Blue to check the extent of transfer of the proteins out of the gel into the nitrocellulose. Very pale blue bands were visible on the gel indicating successful transfer of almost all of...
the proteins originally separated by gel electrophoresis.

ii) Blocking of non-specific binding sites

After electrotransfer of the proteins into the nitrocellulose the strips were immediately placed into 30mls of Tris-buffered saline (TBS: 200mM NaCl, 50mM Tris-HCl; pH 7.4) containing a blocking agent to saturate remaining non-specific protein-binding sites on the nitrocellulose. The blocking agents were either bovine serum albumin (3% (w/v) essentially fatty acid free, in TBS) or BLOTTO (5% (w/v) non-fat dry milk powder in TBS; Johnson et al., 1984). Strips were blocked for 3 hours at room temperature.

iii) Primary antibody incubation

Antibody dilutions were made in TBS to a minimum volume of 0.75ml for the incubation of the strips in 10ml glass test tubes. Blocked nitrocellulose strips were placed in individual test tubes containing the appropriate antibody mixture and sealed with parafilm. The test tubes were then placed on a rocking platform and the tubes gently rocked overnight at 4°C.

RT97, an antibody recognizing 155Kd and 200Kd neurofilament subunits, was kindly donated by Dr. J. Wood, Sandos Institute, University College London. RT97 was used at a dilution of 1:1000.

An antibody against the remaining neurofilament subunit, 68Kd, was purchased from Boehringer Manheim and used at a dilution of 1:100.

Following primary antibody incubation each strip was washed in 3 changes of TBS over a period of 30 minutes, on a rotating horizontal platform (30 revs. per min.), to remove all excess unbound antibody.
iv) Secondary antibody incubation

The secondary antibody, rabbit anti-mouse IgG (Nordic) was diluted in TBS to a dilution of 1:100. Secondary layer incubations were carried out at room temperature on a rotating platform, for 2 hours. After incubation, excess unbound antibody was removed by washing in TBS (3 X 10 mins.).

v) Tertiary antibody incubation

125 I-radiolabelled anti-rabbit whole IgG (Amersham International, Amersham) was diluted to 3-5 microcurries (μCi) in 30mls of TBS. Radiolabelled antibody incubations were carried out at room temperature on a rotating platform (30 revs. per mins.) and the strips incubated in the antibody for 1-2 hours. All appropriate safety precautions were observed when handling radioactive agents. Radioactively-labelled antibodies were stored in 4 μCi aliquots, at 4°C in a lead box. For maximum radioactivity of the antibody these reagents were used within 2 to 3 months of purchase. The half-life of the 125I-labelled antibody is 60 days.

After the incubation, excess unbound antibody was removed by washing for 2 x 10 minutes in TBS, containing Nonidet P40, followed by 2 X 10 minutes in TBS.

vi) Exposure of X-ray film

Washed strips were dried on blotting paper before exposing to X-ray film (Fuji RX or RX-G X-ray film). Nitrocellulose strips were placed on the X-ray film and held in place with cling-film, the film was then placed in a lightproof cassette containing an intensifying screen. The cassette was stored for 1-5 days at -20°C.
vii) Developing the film

After storage for the appropriate time, the X-ray film was developed for 3 minutes in X-ray developer diluted 1 to 4 in water (Photosol CD18 X-ray developer). The developing process was stopped by placing the film in a weak acetic acid solution for a few seconds and the film was then fixed for 3 minutes in X-ray film fixative (Photosol CF40 X-ray fixative). The film was then washed in running tap water for 20 minutes and dried.
RESULTS

I) Physiological Recordings

Nerve-muscle preparations of 9-11 day rat hemi-diaphragm muscles were exposed to various experimental conditions as described in the previous Materials and Methods sections.

Twitch and tetanic contractions of the preparations were elicited by directly stimulating the muscle, and by stimulating the phrenic nerve (indirect stimulation). Maximum tetanic tension was usually obtained at 40Hz, above which the tetanic tension produced by the muscle no longer increased. 40Hz stimulation was therefore used to establish the ratio of indirect/directly elicited maximum tetanic tension of the hemi-diaphragm preparations. Measurements of tetanic tension were recorded at the beginning of each experiment, and at 15 minute intervals after the 2 hour incubation period. All recordings of tetanic tension, both pre- and post-incubation were carried out in normal Krebs solution at room temperature.

The total time taken from the point of dissection of the nerve-muscle preparation to the end of the experiment was on average 4 hours. Occasionally, over this time period, some deterioration of the muscle itself occurred. Therefore, in any preparations where the direct tetanic tension at the end of the experimental period was less than 75% of that at the beginning, the experiment was discarded and the results not included in any calculations. This deterioration in the contractile strength of the muscle occurred in about 20% of the preparations in this experimental series; deterioration appeared to be a random phenomenon.
and could not be connected to any specific experimental procedure. This random deterioration of in vitro muscle preparations has previously been reported by O'Brien et al. (1980). These authors reported deterioration in muscle performance in 20% of their in vitro preparations of soleus muscles; and using similar experimental conditions to those used in this series of experiments, also observed the random nature of this phenomenon.

i) Effects of Exposure to Different Incubational Media on Muscle

A series of experimental approaches was planned to investigate the effects of various incubation media on the immature neuromuscular junctions of the rat diaphragm muscle. It was initially important to assess whether the muscle itself was affected by these conditions. Therefore, the changes of directly elicited tetanic tension of the muscle, after the various treatments, was established. The results are summarized in Table 2. The directly elicited tetanic tension, at various time points, is expressed as a percentage of the directly elicited tetanic tension measured before the incubation.

As a control, hemi-diaphragm nerve-muscle preparations were incubated in normal Krebs solution for 2 hours, at room temperature. Table 2 shows the increase in direct tetanic tension observed over the 60 minutes recording period. The observed increase in directly-elicited tetanic tension of the in vitro muscle preparations, as a result of incubation in normal Krebs, was not an uncommon phenomenon and has been repeatedly observed.

The results in Table 2 show that following incubation of the hemi-
Table 2: Effects of 2 Hour Incubation Conditions in Various Solutions on the Directly Elicited Maximum Tetanic Contractions of Hemi-Diaphragm Preparations

Nine to eleven day old hemi-diaphragm nerve-muscle preparations were incubated in various incubation solutions. Prior to incubation the direct tetanic tension of the preparations was elicited by stimulating the muscle at 40Hz, in normal Krebs solution at room temperature. The preparations were then incubated in one of the various solutions for 2 hours. After the incubation period the hemi-diaphragm preparations were returned to normal Krebs solution, and the direct maximum tetanic tension elicited at subsequent 15 minute intervals. The amount of tension produced by direct stimulation, at each of these time points, was expressed as a percentage of that produced by the muscle prior to incubation. In this table the amount of tension (grammes) produced at each time point is given, in addition to the percentage of that elicited prior to incubation. The values given are mean ± s.e.m.
<table>
<thead>
<tr>
<th>EXPERIMENTAL MEDIA</th>
<th>n</th>
<th>BEFORE</th>
<th>0</th>
<th>15</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tet.T</td>
<td>Tet.T</td>
<td>%</td>
<td>Tet.T</td>
<td>%</td>
<td>Tet.T</td>
<td>%</td>
</tr>
<tr>
<td>NORMAL KREBS</td>
<td>6</td>
<td>20.3</td>
<td>21.7</td>
<td>113±4.0</td>
<td>21.1</td>
<td>114±3.9</td>
<td>24.6</td>
<td>120±6.0</td>
</tr>
<tr>
<td>10⁻² MACH</td>
<td>4</td>
<td>26.2</td>
<td>16.1</td>
<td>74±11.5</td>
<td>25.8</td>
<td>89±10.6</td>
<td>26.4</td>
<td>99±4.7</td>
</tr>
<tr>
<td>10⁻² MACH+10Hz STIM</td>
<td>8</td>
<td>18.4</td>
<td>14.9</td>
<td>83±8.6</td>
<td>18.5</td>
<td>92±15</td>
<td>17.6</td>
<td>100±8.4</td>
</tr>
<tr>
<td>10⁻² MACH+10Hz STIM+LEUPEPTIN</td>
<td>5</td>
<td>24.9</td>
<td>21.3</td>
<td>85±3.9</td>
<td>24.6</td>
<td>99±2.8</td>
<td>25.9</td>
<td>104±9.0</td>
</tr>
<tr>
<td>10⁻² MACH+10Hz STIM+10mM Ca²+</td>
<td>7</td>
<td>24.9</td>
<td>18.6</td>
<td>75±9.2</td>
<td>-</td>
<td>-</td>
<td>24.9</td>
<td>101±9.0</td>
</tr>
<tr>
<td>10⁻² MACH+10Hz STIM+10mM Ca²+ + LEUPEPTIN</td>
<td>6</td>
<td>25.6</td>
<td>16.6</td>
<td>73±11.0</td>
<td>29.8</td>
<td>116±11.4</td>
<td>32.7</td>
<td>129±6.8</td>
</tr>
<tr>
<td>KREBS+LEUPEPTIN</td>
<td>7</td>
<td>15.9</td>
<td>17.3</td>
<td>109±2.2</td>
<td>18.3</td>
<td>116±5.4</td>
<td>18.4</td>
<td>116±3.6</td>
</tr>
</tbody>
</table>

**Table 2**
diaphragm preparations in ACh ($10^{-2}$M) there was consistently an initial reduction in directly elicited tetanic tension. This loss in contractile strength of the preparations was, however, only transient and subsequently recovered. Recovery was usually demonstrated by 15 minutes, post-incubation, after return to normal Krebs. It is clear from Table 2 that when these muscle was kept in normal Krebs solution for 60 minutes, the recovery of tension was complete in all cases (Mann-Whitney U-test, at the 5% level of significance).

In various experimental groups leupeptin, an inhibitor of CANP, was added to the incubation media to ascertain whether any of the observed effects were occurring via calcium-activated proteolysis by the CANP enzyme. In order to assess the effects of leupeptin on nerve-muscle preparations and to eliminate the possibility that leupeptin itself was affecting the contractile nature of the muscle in some way, hemi-diaphragms were incubated for a 2 hour period in normal Krebs containing 0.2mg/ml leupeptin. Table 2 demonstrates that the application of leupeptin to hemi-diaphragm preparations in vitro has no detrimental effects on the muscle (Mann-Whitney U-test, p=0.34; at 60 minutes, post-incubation).

An interesting observation from this series was the increase in tension output from the group of muscles incubated in solutions containing ACh, raised Ca$^{2+}$, and 0.2mg/ml leupeptin, in addition to motor nerve stimulation (see Table 2). There is a significant increase in directly elicited maximum tetanic tension as a consequence of incubation (Mann-Whitney U-test, p=0.025). Noticeably, the effect on the directly elicited maximum tetanic tension is significantly different from that seen in experiments where preparations were incubated in
solutions containing ACh, raised Ca\(^{2+}\) concentration, plus motor nerve stimulation (Mann-Whitney U-test, p=0.021). Figure 3 illustrates the effects of incubation on the directly elicited tetanic tension at various time points in the experiment. These experimental conditions appear to have long-term effects on the muscle fibre properties. It could be that by inhibiting CANP activity in the nerve-muscle preparation the leupeptin may have been influencing the normal turnover and breakdown of myofibrillar proteins, since CANP is known to be involved in the breakdown of muscle specific proteins (Reddy et al., 1975). Leupeptin inhibition of CANP activity may result in a greater supply of contractile proteins to the hemi-diaphragm preparation, thus resulting in an increased contractile strength.

In conclusion, it can be seen that some of the incubation conditions used in this experimental series affect the contractile nature of the hemi-diaphragm preparations. Figure 4 summarizes the results.

ii) Effects of Exposure to Different Incubation Media, on the Functional Innervation of Hemi-Diaphragm Preparations

Having established that incubation in the various solutions has no detrimental influence on the directly elicited tetanic contractions, the effects of the different incubation media on the indirectly elicited tetanic contractions of the preparations were investigated. The maximum indirectly elicited tetanic tension was achieved by stimulating the preparation through the motor nerve at 40Hz, prior to incubation, and following the 2 hour incubation period, at subsequent 15 minute
Figure 3: The Effects of Incubation in Solutions Containing ACh, Raised External Ca$^{2+}$ Concentration and Motor Nerve Stimulation on the Contractile Response of Hemi-Diaphragm Preparations

A group of hemi-diaphragms were incubated for 2 hours in Krebs solution containing $10^{-2}$M ACh and 10mM Ca$^{2+}$, whilst having their motor nerves stimulated (represented by open circles). A similar group of 9-11 day old hemi-diaphragms were incubated for 2 hours under the same conditions with the addition of 0.2mg/ml leupeptin to the media (represented by open squares). A control group of hemi-diaphragms were incubated for 2 hours in normal Krebs solution (represented by open triangles). Prior to incubation all hemi-diaphragms were stimulated directly to elicit the maximum tetanic contractions. The preparations were incubated for 2 hours in the various solutions and then returned to normal Krebs. At subsequent 15 minute intervals the maximum tetanic tension was elicited by direct stimulation, and expressed as a percentage of that elicited prior to incubation. The results are illustrated in this graph. All points are mean values.
Figure 3

Direct Tetanic Tension (as % of TT before incubation)

Minutes post-incubation
Figure 4: Block Diagram of the Effects of Incubation in Various Solutions on the Contractile Response of 9-11 Day Old Hemi-Diaphragm Preparations

This block diagram demonstrates the effects of incubation for 2 hours in various solutions, on the contractile response of hemi-diaphragm preparations. The maximum tetanic tension of the 9-11 day old hemi-diaphragm preparations was elicited by stimulating the muscle directly at 40Hz in Krebs solution, at room temperature. The preparations were then incubated in one of the various solutions for 2 hours. After the incubation period the muscle preparations were returned to normal Krebs solution, and the direct tetanic tension measured at 15 minute intervals. The direct tetanic tension, measured at 60 minutes post-incubation, is expressed as a percentage of that elicited from the muscle prior to incubation and the results are illustrated in this diagram. The values shown are mean ± s.e.m.
Direct Tetanic Tension (as % of DTT pre-incubation)

Figure 4

INCUBATION MEDIA
intervals. Table 3 summarizes the results and demonstrates the changes of indirectly elicited tetanic tension over time, in the different experimental conditions.

The ratios of indirect/directly elicited maximum tetanic tension at each time interval was also established. These ratios were investigated so as to give an indication of the recovery of neuromuscular transmission of the preparations, over time. Table 4 summarizes the results.

a) Effects of normal Krebs

Muscles incubated for 2 hours in normal Krebs solution demonstrated an increased tension output in response to direct stimulation (see Table 2), but the tension output in response to indirect stimulation was unaffected by the incubation (see Table 3). The ratio of indirect/directly elicited maximum tetanic tension was then calculated as an indication of the functional innervation of the preparations. At 60 minutes after incubation the ratio of indirect/direct tetanic tension of muscles incubated in normal Krebs solution was $0.82 \pm 0.05$ (+ s.e.m., n=4)(see Table 4). A ratio of indirect/direct tetanic tension of less than 1.0 is an indication of partial denervation of the preparation. Thus, some functional denervation did occur even in normal Krebs solution, over the experimental period, presumably due to the rigors of the experiment.

b) Effects of exposure to ACh

A group of hemi-diaphragm nerve-muscle preparations were exposed to solutions containing $10^{-2}$M ACh for 2 hours. Incubation in, and exposure to, $10^{-2}$M ACh had no significant effects on the directly elicited
Nine to eleven day old rat hemi-diaphragm preparations were incubated in various solutions for 2 hours. Prior to incubation the maximum tetanic tension was elicited by stimulating the phrenic nerve of the hemi-diaphragm preparations at 40Hz, in normal Krebs solution. The preparations were then incubated in one of the solutions for 2 hours. After the 2 hour incubation period the preparations were returned to normal Krebs solutions and the tetanic tension elicited at subsequent 15 minute intervals by stimulation of the phrenic nerve at 40Hz. In this table the amount of tension (grammes) produced at each time point, by indirect stimulation, is presented. In addition, this value is expressed as a percentage of that elicited prior to incubation. The values presented in this Table are mean ± s.e.m.
<table>
<thead>
<tr>
<th>EXPERIMENTAL MEDIA</th>
<th>n</th>
<th>Tet.T</th>
<th>Tet.T</th>
<th>%</th>
<th>Tet.T</th>
<th>%</th>
<th>Tet.T</th>
<th>%</th>
<th>Tet.T</th>
<th>%</th>
<th>Tet.T</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL KREBS</td>
<td>4</td>
<td>20.2</td>
<td>20.6</td>
<td>101±2.1</td>
<td>21.1</td>
<td>103±2.4</td>
<td>19.7</td>
<td>98±3.3</td>
<td>19.2</td>
<td>96±2.4</td>
<td>19.6</td>
<td>98±2.7</td>
</tr>
<tr>
<td>10^{-2} MACH</td>
<td>4</td>
<td>26.9</td>
<td>6.4</td>
<td>23±10.1</td>
<td>15.8</td>
<td>62±8.4</td>
<td>21.6</td>
<td>83±3.8</td>
<td>19.9</td>
<td>77±6.0</td>
<td>18.9</td>
<td>72±5.4</td>
</tr>
<tr>
<td>10^{-2} MACH+10Hz STIM</td>
<td>8</td>
<td>18.5</td>
<td>6.6</td>
<td>37±12.2</td>
<td>13.2</td>
<td>63±10.3</td>
<td>9.8</td>
<td>53±10.0</td>
<td>-</td>
<td>-</td>
<td>12.2</td>
<td>56±7.9</td>
</tr>
<tr>
<td>10^{-2} MACH+10Hz STIM•LEUPEPTIN</td>
<td>5</td>
<td>24.2</td>
<td>15.2</td>
<td>64±5.1</td>
<td>17.2</td>
<td>84±7.6</td>
<td>22.7</td>
<td>95±3.5</td>
<td>23.1</td>
<td>96±3.5</td>
<td>23.1</td>
<td>95±4.8</td>
</tr>
<tr>
<td>10^{-2} MACH+10Hz STIM•10mM Ca^{2+}</td>
<td>7</td>
<td>21.8</td>
<td>5.3</td>
<td>27±2.4</td>
<td>-</td>
<td>-</td>
<td>7.1</td>
<td>36±13.5</td>
<td>-</td>
<td>-</td>
<td>8.6</td>
<td>42±10.4</td>
</tr>
<tr>
<td>10^{-2} MACH+10Hz STIM•10mM Ca^{2+}•LEUPEPTIN</td>
<td>6</td>
<td>24.8</td>
<td>4.4</td>
<td>20±8.6</td>
<td>19.4</td>
<td>83±12.7</td>
<td>28.3</td>
<td>113±11.3</td>
<td>32.0</td>
<td>131±10.3</td>
<td>30.8</td>
<td>127±11.0</td>
</tr>
<tr>
<td>KREBS•LEUPEPTIN</td>
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<td>15.7</td>
<td>15.1</td>
<td>94±4.8</td>
<td>15.1</td>
<td>96±2.7</td>
<td>14.8</td>
<td>91±3.3</td>
<td>14.7</td>
<td>91±2.9</td>
<td>14.9</td>
<td>91±2.9</td>
</tr>
</tbody>
</table>
Table 4: Changes in the Ratio of Indirect/Directly Elicited Maximum Tetanic Tension Of Hemi-Diaphragm Preparations as a Result of Incubation in Various Solutions

Nine to eleven day old hemi-diaphragm muscles were incubated in various solutions for 2 hours. Maximum tetanic contractions were elicited from the preparations in the way previously described. At each time point the indirectly and directly elicited maximum tetanic tension was recorded either by direct stimulation of the muscle, with a pulse width of 2msecs., or indirect stimulation of the phrenic nerve using a pulse width of 0.2msecs. The ratio of indirectly elicited maximum tetanic tension was calculated for each time interval and each incubation condition and presented in this Table. The values presented are mean ± s.e.m.
<table>
<thead>
<tr>
<th>EXPERIMENTAL MEDIA</th>
<th>N</th>
<th>BEFORE INCUBATION</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL KREBS</td>
<td>4</td>
<td>1.00±0.02</td>
<td>0.90±0.03</td>
<td>0.89±0.04</td>
<td>0.82±0.04</td>
<td>0.77±0.06</td>
<td>0.82±0.05</td>
</tr>
<tr>
<td>10^-2 MACH</td>
<td>4</td>
<td>1.01±0.04</td>
<td>0.30±0.05</td>
<td>0.68±0.05</td>
<td>0.86±0.04</td>
<td>0.80±0.01</td>
<td>0.75±0.04</td>
</tr>
<tr>
<td>10^-2 MACH+10Hz STIM</td>
<td>8</td>
<td>1.01±0.02</td>
<td>0.46±0.01</td>
<td>0.67±0.06</td>
<td>0.46±0.01</td>
<td>0.57±0.02</td>
<td>0.52±0.07</td>
</tr>
<tr>
<td>10^-2 MACH+10Hz STIM+LEUPEPTIN</td>
<td>5</td>
<td>0.97±0.01</td>
<td>0.73±0.02</td>
<td>0.83±0.06</td>
<td>0.88±0.05</td>
<td>0.90±0.04</td>
<td>0.89±0.05</td>
</tr>
<tr>
<td>10^-2 MACH+10Hz STIM+10mM Ca2+</td>
<td>7</td>
<td>1.03±0.01</td>
<td>0.17±0.08</td>
<td>-</td>
<td>0.33±0.12</td>
<td>-</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td>10^-2 MACH+10Hz STIM+10mM Ca2+ + LEUPEPTIN</td>
<td>6</td>
<td>0.97±0.02</td>
<td>0.24±0.07</td>
<td>0.72±0.12</td>
<td>0.84±0.10</td>
<td>0.90±0.04</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>KREBS+LEUPEPTIN</td>
<td>7</td>
<td>0.99±0.01</td>
<td>0.88±0.05</td>
<td>0.84±0.05</td>
<td>0.83±0.08</td>
<td>0.81±0.08</td>
<td>0.83±0.05</td>
</tr>
</tbody>
</table>

Table 4
contractions (see Table 2). After the 2 hour incubation, when the muscle had been transferred to normal Krebs solution, the indirectly elicited contraction was not maintained. Figure 5 illustrates an example of this inability to maintain tension. Table 3 tabulates the percentage change in indirect tetanic tension, over the 60 minute recording period. Stimulation of the motor nerve produced 23% of the tension produced before the experimental incubation. The preparation then recovered to some extent, over the following 60 minutes in normal Krebs solution. This phenomenon of a transiently decreased ability to produce tension both in response to direct (see Table 2) or indirect stimulation observed in ACh-treated muscles was to be expected, and is likely to be caused by ACh-induced depolarization of the preparation, as a result of long term exposure to ACh (Katz & Thesleff, 1957).

Calculations of the ratio of indirect/directly elicited maximum tetanic tension of the preparations show that immediately after the 2 hour incubation in solutions containing ACh the ratio was only 0.30 ± 0.09 (± s.e.m., n=4). With time, in normal Krebs solution, the indirectly elicited tension recovered, so that 60 minutes after the incubation the ratio of indirect/direct tetanic tension was 0.75 ± 0.04 (± s.e.m., n=4). This ratio of ACh-treated preparations is not significantly different from that observed in control experiments where preparations were incubated for 2 hours in normal Krebs (Mann-Whitney U-test, p=0.18). Exposure to 10⁻² M ACh for 2 hours therefore, has no significant effects on the innervation ratio of hemi-diaphragm preparations.

In the next experiment hemi-diaphragm preparations were exposed to 10⁻² M ACh and in addition, had their motor nerves stimulated at 10Hz,
Figure 5: **Traces of Indirectly Elicited Maximum Tetanic Contractions from a 9 day old Hemi-Diaphragm Preparation, Following 2 Hour Incubation in Krebs containing $10^{-2}$M ACh**

Examples of traces of maximum tetanic contractions taken from a 9 day old hemi-diaphragm nerve-muscle preparation. Maximum tetanic contractions from the hemi-diaphragm preparation were elicited prior to incubation in Krebs solution containing $10^{-2}$M ACh, by stimulating the phrenic nerve at 40Hz.

The preparation was then incubated in Krebs solution containing $10^{-2}$M ACh for 2 hours. After the 2 hour incubation the preparation was returned to normal Krebs solution and the maximum tetanic contraction elicited indirectly at subsequent 15 minute intervals. Trace a) is an example of an indirectly elicited tetanic contracture elicited from the preparation immediately after return to normal Krebs. Trace b) is an example of a trace of an indirectly elicited maximum tetanic contrature of the same hemi-diaphragm preparation 15 minutes after return to normal Krebs. Trace c) is an example of an indirectly elicited tetanic contraction taken 60 minutes after the 2 hour incubation period. Tension is expressed in grammes and the time scale is in milliseconds.
Figure 5

a) 

b) 

c) 

500msecs.

10g
for the 2 hour period. At the end of the 2 hour incubation, on transfer into normal Krebs solution the preparations exhibited a similar initial reduction in indirectly-elicited tetanic tension and an inability to maintain the tetanus, as seen in muscles subjected to $10^{-2} \text{M} \text{ACh}$ alone (see Table 3). Sixty minutes after the 2 hour incubation the indirectly elicited tetanic tension was only 56% of that recorded before incubation.

The ratio of indirect/direct tetanic tension was therefore calculated. Before the incubation the ratio of indirect/direct tetanic tension was $1.01 \pm 0.02$ (± s.e.m., n=7). Immediately after the 2 hour incubation period this had decreased to $0.46 \pm 0.10$ (± s.e.m., n=7). The ratio then increased over the following 60 minutes. However, recovery was not complete and at 60 minutes post-incubation the ratio of indirect/directly elicited tetanic tension was $0.52 \pm 0.07$ (± s.e.m., n=7). This is significantly different to that observed in normal Krebs (Mann-Whitney U-test, p=0.006). Thus, stimulation of the motor nerve of the preparations, plus exposure to ACh resulted in a significant reduction in the number of functional contacts of the nerve-muscle preparations.

In a further group the hemi-diaphragm preparations were incubated in solutions containing $10^{-2} \text{M ACh}$ and 0.2mg/ml leupeptin. Their motor nerves were stimulated at 10Hz. Immediately after incubation the preparations demonstrated an initial small reduction in indirectly elicited maximum tetanic tension, as seen in other experimental groups as a result of ACh exposure, this subsequently recovered so that at 60 minutes post-incubation, the indirect tetanic tension was 95% of that recorded before incubation (see Table 3).
The ratio of indirect/directly elicited maximum tetanic tension was calculated for these preparations (see Table 4). After the 2 hour incubation period, on return to normal Krebs, the ratio was 0.73 ± 0.02 (+ s.e.m., n=5). After 60 minutes in normal Krebs solution the ratio had increased to 0.89 ± 0.05 (+ s.e.m., n=5). Noticeably this ratio, calculated 60 minutes after incubation, is not significantly different from that exhibited by preparations incubated in normal Krebs solutions for 2 hours (Mann-Whitney U-test, p=0.2).

Interestingly, the ratio of indirect/direct tetanic tension exhibited 60 minutes after incubation, by those preparations which had been incubated in ACh whilst having their motor nerve stimulated at 10Hz (0.52 ± 0.07), is significantly different from that exhibited by preparations incubated in the same conditions but with the addition of leupeptin (Mann-Whitney U-test, p=0.002). Figure 6 shows examples of isometric tetanic contractions from hemi-diaphragm preparations in each experimental group. This significant difference in the ratio seems to indicate that the thiol protease inhibitor, leupeptin, is protecting the terminals from the effects of ACh and motor nerve stimulation. Figure 7 illustrates the significant difference observed between the two experimental groups.

c) Effects of Raised External Calcium Levels

A group of nerve-muscle hemi-diaphragm preparations were incubated in solutions containing ACh (10^{-2}M) but in addition the Ca^{2+} concentration was also raised, to 10mM. The motor nerves were stimulated at 10Hz. As previously demonstrated (See section I, Effects of incubation media on the muscle) the directly elicited tetanic tension of
Figure 6: Traces of Indirectly and Directly Elicited Tetanic Contractions From Hemi-Diaphragm Preparations After a 2 Hour Incubation in Media Containing ACh, and ACh plus Leupeptin

Maximum tetanic contractions were elicited from 9 day old hemi-diaphragm preparations either by indirectly stimulating the preparation through the motor nerve, or by directly stimulating the muscle. A group of hemi-diaphragm preparations were incubated for 2 hours in Krebs containing $10^{-2}$M ACh, whilst having their motor nerves stimulated at 10Hz. A further group of hemi-diaphragm preparations were subjected to the same conditions, in addition 0.2mg/ml leupeptin was added to the media. The hemi-diaphragm preparations were incubated for 2 hours, after which the preparations were returned to normal Krebs solution. Traces a) and b) are taken from a 9 day old hemi-diaphragm preparation incubated in Krebs containing ACh plus motor nerve stimulation for 2 hours. Trace b) is an example of an indirectly elicited maximum tetanic contraction elicited from the preparation 60 minutes after the 2 hour incubation. Trace a) is taken from the same preparation also 60 minutes after the preparation and is a trace of a maximum tetanic contraction elicited by direct stimulation of the muscle. Traces c) and d) are traces of maximum tetanic contractions taken from a hemi-diaphragm preparation following a 2 hour incubation in Krebs containing ACh and leupeptin, in addition to motor nerve stimulation at 10Hz. Trace c) is an example of an indirectly elicited maximum tetanic contraction elicited from the preparation 60 minutes after the 2 hour incubation. Trace d) is an example of a directly elicited tetanic contraction from the same preparation 60 minutes after the incubation.
Figure 6

a) 

b) 

10g

3 sec.

10g

c)  

d)
Figure 7: Block Diagram of the Effects on the Ratio of Indirect/Directly Elicited Tetanic Tension of Hemi-Diaphragm Preparations, of Incubation in Krebs containing ACh, and ACh plus leupeptin

Nine to eleven day old hemi-diaphragm preparations were incubated in Krebs solutions containing $10^{-2}$M ACh plus motor nerve stimulation. A further group of hemi-diaphragm preparations were incubated in Krebs containing $10^{-2}$M ACh and 0.2mg/ml leupeptin, whilst having their motor nerves stimulated. Maximum tetanic contractions were elicited from the preparations before incubation and after the 2 hour incubation at 15 minute intervals. Maximum tetanic contractions were elicited either by stimulation of the muscle directly or by indirect stimulation of the motor nerve. The ratio of indirect/directly elicited maximum tetanic tension was calculated for each time point. The mean ± s.e.m. are presented in this diagram.
Figure 7

- $10^2$ M ACh + 10Hz
- $10^2$ M ACh + 10Hz + leupeptin

![Chart showing indirect: direct tetanic tension over time after incubation](image)

Minutes after incubation:
- BEFORE INCUBN
- TIME 0
- TIME 30
- TIME 60

Indirect: direct tetanic tension
these preparations was unaffected by the incubation media. In contrast the indirectly elicited tetanic tension was greatly affected. The preparations were incubated for 2 hours and then returned to normal Krebs solution; immediately after return to normal Krebs the indirectly elicited tetanic tension was only 27% of that produced prior to incubation. After 60 minutes in normal Krebs solution the preparation had recovered from the effects of the previous procedure. However recovery was not complete and indirect stimulation of the muscles produced only 42% of the tension by indirect stimulation as compared to that elicited prior to incubation. Since the incubation conditions had such a striking effect on the indirectly elicited tetanic contractions the ratio of indirect/directly elicited maximum tetanic tension was calculated. Table 4 demonstrates the changes in this ratio over time, as a consequence of the incubation conditions.

Immediately after the 2 hour incubation period on return to normal Krebs the ratio of indirect/direct tetanic tension was greatly reduced (0.17 ± 0.08; n=7). Although there was a slight increase in the ratio over time, this recovery was not complete, and even after 60 minutes the ratio of indirect/directly elicited tension was only 0.38 ± 0.10 (± s.e.m., n=7); indicating that 64% of the muscle fibres could not be activated. This decrease in the ratio of indirect/directly elicited maximum tetanic tension is significantly different from that seen in control experiments, where preparations were incubated in normal Krebs solution for 2 hours (Mann-Whitney U-test, p<0.001). Figure 8 shows examples of traces of direct and indirect maximum tetanic contractions recorded before incubation, and 60 minutes after incubation. The traces demonstrate the changes in the ratio of indirect/direct tetanic tension.
Figure 8: Examples of Indirectly and Directly Elicited Tetanic Contractions From Hemi-Diaphragm Preparations Before and After Incubation in Solutions Containing ACh and raised Ca\textsuperscript{2+}, plus Motor Nerve Stimulation

Nine to eleven day old hemi-diaphragm preparations were incubated in solutions containing $10^{-2}$ M ACh and 10mM Ca\textsuperscript{2+}, whilst their motor nerves were stimulated. Maximum tetanic contractions were elicited from the preparations either by direct stimulation of the muscle or by indirectly stimulating the preparation through the motor nerve. The traces presented in a) and b) are taken from a 9 day old hemi-diaphragm preparation prior to incubation in Krebs containing ACh and raised Ca\textsuperscript{2+}. Trace a) is an example of an indirectly elicited maximum tetanic contraction. Trace b) is an example of a directly elicited tetanic contraction from the same hemi-diaphragm preparation elicited prior to incubation. Traces c) and d) are taken from the same muscle after the 2 hour incubation. Trace c) is an example of an indirectly elicited tetanic contraction elicited from the preparation 60 minutes after the 2 hour incubation. Trace d) is an example of a directly elicited maximum tetanic contraction elicited from the same preparation 60 minutes after the 2 hour incubation.
induced as a result of the incubation and stimulation.

The ratio of indirect/direct tension exhibited by preparations stimulated in the presence of ACh and raised Ca\(^{2+}\) concentration, is however, not significantly different from the ratio exhibited by preparations incubated in solutions containing ACh, plus motor nerve stimulation (Mann-Whitney U-test, p=0.11). Therefore, raising external Ca\(^{2+}\) concentration has no significant further effect on the level of functional denervation than that seen as a result of treatment with ACh and motor nerve stimulation.

In a separate series of experiments leupeptin was added to incubation solutions containing ACh, and raised Ca\(^{2+}\) concentration; which, when combined with motor nerve stimulation, had resulted in a reduction in the ratio of indirect/direct tetanic tension (as demonstrated above).

The indirect tetanic tension was investigated (see Table 3). Immediately after incubation the preparation produced only 20% of the tension produced prior to incubation. This drop in indirectly elicited tetanic tension was only transient, and rapidly recovered so that 60 minutes after the incubation stimulation of the phrenic nerve produced 127% of the tension produced before incubation. As an indication of the functional innervation of the preparation the ratio of indirect/directly elicited maximum tetanic tension was calculated. Immediately after the incubation on return to normal Krebs solution the ratio demonstrated an initial drop and was 0.24 ± 0.07 (± s.e.m., n=5). However, this increased over the following 60 minutes, so that at the end of the experimental period the ratio had increased to 0.85 ± 0.05 (± s.e.m.,
n=5). This change over time can be followed in Table 4. Noticeably, the ratio of indirect/directly elicited maximum tetanic tension is not significantly different from that observed in control experiments (Mann-Whitney U-test, p=0.33).

On further statistical investigation it became apparent that the ratio of indirect/directly elicited tetanic tension is significantly different from that exhibited by preparations incubated in solutions containing ACh and raised Ca$^{2+}$ concentration, in the absence of leupeptin (Mann-Whitney U-test, p=0.002). With the addition of leupeptin to the incubation media only 15% of the muscle fibres were inactivated in ACh and raised Ca$^{2+}$ conditions compared to 64% in the absence of the CANP inhibitor. Examples of such experiments are illustrated in Figure 9. Figure 10 illustrates the changes occurring in the ratio of indirect/directly elicited maximum tetanic tension at various time intervals after the 2 hour incubation period and illustrates the protective nature of leupeptin.

In order to check that the effects of ACh incubation were not due to mechanical damage of the preparations, but are instead due to the long term exposure to the ACh, another group of hemi-diaphragm preparations where briefly exposed to the same level of ACh. Mechanical damage may have occurred as a result of the sheering forces produced during the large contracture elicited from the preparation. A single ACh-induced contracture was elicited from the preparation, the muscle was allowed to briefly relax, and then the preparations returned to normal Krebs solution. The brief exposure to 10$^{-2}$M ACh resulted in an initial reduction in the directly elicited tetanic tension. At time 0,
Figure 9: Traces of Indirectly and Directly Elicited Maximum Tetanic Contractions Elicited from Hemi-Diaphragm Preparations 60 Minutes After Incubation in Solutions Containing Either ACh and Raised Ca\textsuperscript{2+}, or ACh, Ca\textsuperscript{2+} and Leupeptin

Hemi-diaphragm preparations were incubated in solutions containing \(10^{-2}\text{M}\) ACh and 10mM Ca\textsuperscript{2+}, whilst having their motor nerves stimulated at 10Hz. Another group of hemi-diaphragms were subjected to identical conditions with the addition of 0.2mg/ml leupeptin to the media. The preparations were incubated for 2 hours and then returned to normal Krebs solution. Traces a) and b) are examples of maximum tetanic contractions elicited from preparations 60 minutes after incubation in Krebs containing ACh and raised Ca\textsuperscript{2+}. Trace b) was elicited from the preparation by stimulation of the motor nerve, whilst trace c) was elicited from the same preparation by direct stimulation of the muscle. Traces c) and d) are examples of maximum tetanic contractions elicited from preparations 60 minutes after incubation in Krebs containing ACh, raised Ca\textsuperscript{2+} and leupeptin. Trace d) was elicited from a hemi-diaphragm preparation by indirectly stimulating the motor nerve, whilst trace e) was elicited from the same preparation by direct stimulation of the muscle. Tension is measured in grammes and time in milliseconds.
Nine to eleven day old hemi-diaphragm preparations were incubated in Krebs containing $10^{-2}\text{M ACh}$ and $10\text{mM Ca}^{2+}$, whilst having their motor nerves stimulated at 10Hz. Another group of hemi-diaphragms were subjected to the same conditions with the addition of leupeptin to the incubation media. Maximum tetanic contractions were elicited from the preparations prior to incubation and following the 2 hour incubation at subsequent 15 minute intervals. Contractions were elicited from the preparations either by indirect stimulation of the preparation through the motor nerve or via direct stimulation of the muscle itself. The ratio of indirect/directly elicited maximum tetanic tension was calculated at each time point and presented in this block diagram. The mean + s.e.m. are presented.
Figure 10

- Indirect: direct tetanic tension

- $10^5$ MACH + 10 Hz + 10 mM Ca$^{2+}$
- $10^5$ MACH + 10 Hz + 10 mM Ca$^{2+}$ + leupeptin

Graph showing indirect: direct tetanic tension over minutes after incubation.
post-contracture, the preparations produced a directly elicited maximum tetanic tension of $17.4 \pm 1.8g$ (± s.e.m., n=9) which is 78% of that initially measured, prior to ACh treatment. At 60 mins. post-contracture this had recovered to $21.0 \pm 1.5g$ (± s.e.m., n=9) which is 95% of that exhibited initially. The results when compared to incubation in normal Krebs solution, for a similarly brief period of time, show no significant difference in direct tetanic tension (Mann-Whitney U-test, at the 5% level of significance). Therefore, it appears that brief exposure to ACh does not affect the contractile capacity of the muscle.

The indirectly elicited tetanic tension of these preparations was also measured and the ratio of indirect/directly elicited maximum tetanic tension calculated. Immediately after the 2 hour incubation the ratio was $0.47 \pm 0.07$ (± s.e.m., n=7) however this subsequently recovered to $0.90 \pm 0.03$ (± s.e.m., n=7) when calculated at 60 minutes post-contracture. This is not significantly different from that observed in control incubation experiments (Mann-Whitney U-test, at the 5% level of significance). Therefore, brief ACh contracture has no significant effects on the functional contacts of the preparations, and the large contracture elicited from the preparations did not cause any mechanical damage to the preparations.

d) Effects of leupeptin, an inhibitor of CANP.

In order to elucidate the route of action of various of the experimental regimes leupeptin, an inhibitor of CANP, was added to some of the incubation media.

As already established, incubation of hemi-diaphragm preparations in normal Krebs containing 0.2mg/ml leupeptin, for 2 hours, has no detrimental effects on the muscle. The indirectly elicited tetanic
tension was then investigated. Immediately after the 2 hour incubation stimulating the phrenic nerve elicited 94% of tension produced before incubation and following a further 60 minutes recording period the indirectly elicited tetanic tension was 91% of that recorded initially. We then calculated the ratio of indirect/direct tetanic tension of leupeptin-treated preparations, to investigate any changes in functional contacts of the preparation. These results are summarized in Table 4. Prior to incubation the ratio was 0.99 ± 0.01 (± s.e.m., n=7), and maintained at a similar level throughout the experiment. At 60 minutes post-incubation, the ratio was 0.83 ± 0.03 (± s.e.m., n=7) which although indicating a certain degree of denervation is not significantly different from that observed in preparations incubated in normal Krebs for 2 hours (Mann-Whitney U-test, p=0.28). Therefore, the inclusion of leupeptin in the incubation media has no detrimental effects on the preparations.

II) Immunoblot Results

The combined techniques of SDS-Page electrophoresis and Western Blotting were employed to further investigate the manifestations of the in vitro manipulations on the diaphragm preparations. Any changes occurring in the cytoskeletal proteins, during the 2 hour incubation period were investigated, using the immunoblot technique.

Antibodies recognizing the mammalian neurofilament proteins NF-H, NF-M, and NF-L were employed in this study in an attempt to probe the events occurring in the cytoskeleton of a specific population of neurones.
Using SDS-Page electrophoresis three neurofilament proteins were visualized in samples taken from normal diaphragm nerve-muscle preparations. The proteins were separated on a polyacrylamide gel and then visualized by staining the gel with Coomasie Brilliant Blue. Figure 11 illustrates the neurofilament proteins visualized with Coomasie Brilliant Blue stain. These neurofilament proteins had apparent molecular weights of 200Kd (NF-H), 155Kd (NF-M) and 68kd (NF-L). These values are consistent with the results of various authors (Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978). The Coomasie Brilliant Blue stained bands relating to the neurofilament proteins were identified by plotting the distance travelled down the polyacrylamide gel by standard molecular weight proteins (6-H, Sigma Chemical Co.) (see Appendix II), and from there extrapolating the distances of the neurofilament proteins. The relative mobilities can be seen in Figure 11, lanes 1 and 3.

The neurofilament, cytoskeletal elements of the tissue samples were further examined by immunoblot methods using monoclonal antibodies to the neurofilament proteins, thus visualizing the immunoreactive neurofilament components in the experimental samples. Equal volumes of solubilized samples extracted from diaphragm preparations were loaded into 2 lanes and subjected to electrophoresis and Western blotting. One of the lanes was then subjected to immunoblotting with an antibody reacting against NF-H and NF-M (RT97; donated by Dr.J.Wood, Sandoz Institute) and one lane was reacted against an antibody recognizing NF-L (68Kd; Boehringer Manheim). Neurofilament proteins appear as bands of immunoreactive protein (immunobands) that correspond to NF-H (immunoband 1), NF-M (immunoband 2) and NF-L (immunoband 3), as seen in control
Figure 11: Coomasie Brilliant Blue Stain of Molecular Weight Markers and Neurofilament Proteins Extracted From Normal Hemi-Diaphragm Preparations

Samples of proteins from normal diaphragms were extracted, solubilized and separated on a 10% acrylamide gel by electrophoresis. The resultant gel was stained with Coomasie Brilliant Blue (CBB) stain to visualize the separated protein bands within it. Lane 1 is an example of CBB stained molecular weight markers (6H, Sigma Chemical Co.) which correspond to proteins with molecular weights of 205Kd (myosin), 116Kd (β-galactosidase), 97.4Kd (phosphorylase), 66Kd (albumin), 45Kd (albumin), 29Kd (carbonic anhydrase) (see Appendix II). Lane 2 is an example of a CBB stained normal diaphragm protein sample. The CBB stained bands which are marked relate to the neurofilament subunit proteins - NF-H (200Kd), NF-M (155Kd) and NF-L (68Kd). Lanes 3 is an example of CBB stained molecular weight markers (Sigma Chemical Co.) which correspond to proteins with molecular weights of 66Kd (albumin, bovine), 45Kd (albumin, egg), 36Kd (glyceraldehyde-3-phosphate dehydrogenase, from rabbit muscle), 29Kd (carbonic anhydrase), 24Kd (trypsinogen) and 20Kd (trypsin inhibitor, soya bean).
Figure 11

Lanes

1
2
3

M.W. markers

M.W. markers

Lane 1:
- 210>
- 116>
- 97>
- 66>
- 45>
- 29>

Lane 2:
- 200>
- 155>
- 68>

Lane 3:
- <66
- <45
- <36
- <29
- <24
- <20
samples of normal diaphragm (Figure 12, lanes 1 and 2). Control samples of chick optic nerve were run on the same gel with the diaphragm samples and demonstrate a major immunoband (1) which corresponds to 61Kd chick neurofilament protein (Figure 12, lane 5).

Hemi-diaphragm preparations which were incubated in solutions containing $10^{-2} M$ ACh and raised Ca$^{2+}$ concentration (10mM), whilst having their motor nerves stimulated, exhibited an extensive reduction in the ratio of indirect/directly elicited maximum tetanic tension as a result of incubation. This decrease in the ratio of indirect/direct tetanic tension may have been due to a reduction in the number of nerve-muscle contacts. To investigate this hypothesis the endplate regions from these preparations were prepared for SDS-Page electrophoresis and then analysed using immunoblot techniques. The immunoblot profiles from these preparations demonstrated a subsequent change in the neurofilament proteins. Figure 12, lanes 3 and 4 illustrates the immunoblot results of the experimental hemi-diaphragm samples. NF-L is no longer present in a recognizable form in the samples (lane 4; immunoband 3 is absent, compared to lane 2; immunoband 3, from a normal diaphragm). The total loss of NF-L is accompanied by a reduction in the intensity of immunobands 1 and 2 (lane 3 compared to lane 1) correlating to the NF-H and NF-M neurofilament subunit proteins.

The immunoblot profiles illustrated in Figure 12 demonstrate that the incubation of the diaphragm preparations in solutions containing $10^{-2} M$ ACh, 10mM Ca$^{2+}$, in addition to motor nerve stimulation, results in a breakdown of the cytoskeletal elements within a population of nerve terminals. These immunoblotting results are consistent with the results from the in vitro physiological recordings, reported in this Chapter,
Figure 12: **Immunoblot Profiles of Neurofilament Proteins From Diaphragm Preparations**

Diaphragm preparations were incubated for 2 hours in solutions containing $10^{-2}$M ACh, 10mM Ca$^{2+}$, in addition to motor nerve stimulation. Control diaphragm preparations were incubated for 2 hours in normal Krebs solution. The diaphragm preparations were then prepared for SDS-PAGE. Samples of the solubilized proteins were separated on a 10% polyacrylamide gel by electrophoresis. The proteins were then electro-transfered from the gel to nitrocellulose paper and the nitrocellulose paper subjected to the immunoblotting technique and reacted with antibodies against the 3 neurofilament subunit proteins. Lanes 1 and 2 were loaded with samples from a normal diaphragm preparations. Lane 1 was then reacted with an antibody against NF-H and NF-M. Lane 2 was reacted with an antibody against NF-L. Immunoband 1 correlates with NF-H, immunoband 2 (NF-M) and immunoband 3 (NF-L). Lanes 3 and 4 were loaded with samples from diaphragm preparations incubated in solutions containing $10^{-2}$M ACh, 10mM Ca$^{2+}$, in addition to motor nerve stimulation. Lane 3 was reacted with an antibody against NF-H and NF-M. Lane 4 was reacted with an antibody against NF-L. Again immunoband 1 correlates to NF-H, immunoband 2 (NF-M) and immunoband 3 (NF-L). Lane 4 demonstrates that following incubation of diaphragm preparations in solutions containing ACh and high Ca$^{2+}$ the NF-L neurofilament subunit protein was absent. Lane 3 demonstrates that NF-H and NF-M are relatively reduced compared to controls (c.f. lane 1). Lane 5 was loaded with a control sample of neurofilaments from embryonic chick (donated by Prof. Warner) and reacted with an antibody against embryonic chick neurofilament. Immunoband 1 (lane 5) correlates to a 61Kd chick neurofilament.
Figure 12
which illustrate that incubation of hemi-diaphragm preparations for 2 hours in these conditions results in a reduced tension ratio, indicative of a decreased number of functional contacts.
DISCUSSION

The establishment of the mature adult pattern of innervation occurring during the normal developmental programme is known to be dependent upon activity, and more specifically, upon the activity of the postsynaptic membrane. During this period of reorganization there is a coincident increase in neuromuscular activity (Navarrete & Vrbova, 1983). The molecular mechanisms which control and determine the elimination of excess synaptic contacts are, however, not fully understood. In an attempt to further elucidate this problem the experiments presented in this Chapter endeavour to mimic the events which may be occuring at the neuromuscular junction during this period of reorganization period.

The results from the in vitro experiments performed in this Chapter confirm the results of O'Brien and colleagues (1980; 1984) and demonstrate that incubation of hemi-diaphragm nerve-muscle preparations for 2 hours in solutions containing ACh, in addition to motor nerve stimulation over this period, has an adverse effect on the functional innervation of the preparations. That is, the ratio of indirect/directly elicited tetanic tension of the 9-11 day old hemi-diaphragm preparations is reduced. Hemi-diaphragm preparations incubated in solutions containing ACh and raised external Ca$^{2+}$ concentration (10mM), in addition to motor nerve stimulation, demonstrated a further decrease in the ratio of indirect/directly elicited tetanic tension, indicative of an alteration in the innervation of the preparations. Inclusion of Ca$^{2+}$ in the experimental media resulted in greater reductions in the calculated tension ratio but not to a significant extent. The ratio of
indirect/direct tetanic tension reflects the functional innervation of the preparation and gives an indication of the extent of denervation of the preparations, as a consequence of incubation. The results presented in this Chapter demonstrate that a loss of contacts between nerve and muscle can be achieved in the diaphragm muscles by a combination of ACh and stimulation. These results demonstrate that the ACh-induced disruption of nerve-muscle contacts is a universal phenomenon, and occurs in the predominantly fast diaphragm muscle in a similar manner to that reported to occur in the slow soleus muscle (O'Brien et al., 1982; 1984).

Furthermore, the experimentally-induced alterations in the functional innervation of the hemi-diaphragm preparations could be prevented by the addition of leupeptin, an inhibitor of the CANP enzymes, to the incubation media.

A further extension of these results demonstrated that samples taken from hemi-diaphragm preparations incubated in experimental conditions of ACh and raised Ca\(^{2+}\), plus motor nerve stimulation, when subjected to SDS-Page electrophoresis showed immunoblotting profiles of neurofilament proteins (NFP's) which had been specifically affected. Immunoblot profiles from hemi-diaphragm preparations demonstrated an altered state of the NFP's - that is, an absence of one of the neurofilament subunit proteins. The immunoband relating to the lower molecular weight neurofilament subunit protein (68Kd) was either absent or altered to such an extent that it was no longer recognized by the antibody.

The large reduction in the innervation ratio of the hemi-diaphragm preparations following incubation in ACh, plus motor nerve stimulation
is probably due to a massive loss of nerve-muscle contacts within the preparations. O'Brien and colleagues (1980) exposed neonatal soleus muscles to ACh for 2 hours and reported dramatic ultrastructural changes in the pre- and postsynaptic elements of the neuromuscular junction. The damage they observed was enhanced by raising the Ca\(^{2+}\) concentration in the external media. Exposure of hemi-diaphragm preparations to ACh, in addition to motor nerve stimulation, may have damaging effects on the neuromuscular junction of the hemi-diaphragm preparations in a similar manner, resulting in functional denervation of the preparations. A hypothesis which may explain these results is that the conditions of ACh, plus motor nerve stimulation, culminate in a Ca\(^{2+}\) influx into nerve terminals which in turn activates a neuronal calcium-activated neutral protease (CANP). It is proposed that the activated CANP then proceeds to disassemble the cytoskeletal elements of axons within the preparations. Under these experimental conditions, the results presented in this Chapter show that the action of CANP is primarily one of degrading the NF-L subunit protein of the cytoskeletal structure of a specific population of axons.

Incubation of hemi-diaphragms in solutions containing ACh only had no adverse effects on the ratio of indirect/directly elicited tetanic tension. It seems likely that ultrastructural changes may have taken place, but not to the extent where any fibres became denervated. The full extent to which the overall innervation of the preparations was affected by ACh incubation is not directly obvious using the technique of measuring the total directly elicited tetanic tension of the preparations. It seems likely that, in addition to the observed denervation of muscle fibres, the levels of polyneuronal innervation
within the preparations are also affected by the incubation. Indeed, in an ultrastructural study O'Brien et al. (1980) demonstrated that incubation of neonatal rat soleus muscles in ACh for 2 hours significantly reduced the number of axon profiles per endplate. It seems likely therefore, that incubation of hemi-diaphragm preparations in ACh for 2 hours did affect the levels of innervation of the preparations, but not to the extent where a significant number of muscle fibres became denervated. Exposure to ACh and additional motor nerve stimulation greatly affected the innervation ratio of the hemi-diaphragm preparations, probably through an extensive reduction in the number of functional nerve-muscle contacts. Raising the external Ca$^{2+}$ concentration around the hemi-diaphragm preparations further affected the calculated tension ratio, although, not to a significantly greater extent. O'Brien et al. (1980) reported that raised external Ca$^{2+}$ concentration resulted in enhanced ultrastructural damage to the pre- and postsynaptic elements of the neuromuscular junction. In the experiments reported in this Chapter it seems likely that further widespread damage occurred in the hemi-diaphragm preparations as a result of the additional raised levels of external Ca$^{2+}$, which did not result in a significantly greater denervation of the preparations. More extended damage may have occurred to other nerve-muscle contacts within the preparations which are not be reflected in the ratio of indirect/direct tetanic contractions. The levels of polyneuronal innervation of the preparations are likely to be significantly affected. It can be said that raising the external calcium concentration around the hemi-diaphragm preparations resulted in a greater disruption of contacts and subsequent denervation of muscle fibres within the
preparations. In addition to denervated fibres other nerve-muscle contacts within the preparations are likely to have been disrupted by a similar action.

ACh is known to facilitate the entry of Ca\(^{2+}\) into the endplate region (Jenkinson & Nicholls, 1961; Evans, 1974; Bregestovski et al., 1979) and causes a contraction of underlying sarcomeres. Katz & Miledi (1969) demonstrated that depolarization of nerve cells results in a Ca\(^{2+}\) influx. Incubation of hemi-diaphragm preparations for 2 hours in solutions containing ACh in addition to motor nerve stimulation probably results in an influx of Ca\(^{2+}\) into specific nerve terminals of the preparations. Degenerative axonal changes in transected neurites have been shown to be calcium-dependent in tissue cultures of dorsal root ganglia (Schlaepfer & Bunge, 1973) and in excised and isolated nerve fibres of rat peripheral nerve (Schlaepfer, 1974). The observed degeneration of the axoplasm is initiated by granular disintegration of neurofilaments; these changes in neurofilaments were not observed in similar samples incubated in media lacking Ca\(^{2+}\), or where Ca\(^{2+}\) had been chelated. Disintegration of the neurofilamentous structure of transected nerves is accompanied by a disappearance of neurofilament proteins from the electrophoretic profile of the nerve homogenates (Schlaepfer & Micko, 1978; Bignami et al., 1981; Kamakura et al., 1983). This loss of neurofilament proteins has also been demonstrated in samples of excised nerves subjected to a Ca\(^{2+}\) influx (Schlaepfer & Micko, 1979). It seems possible that the experiments presented here, ACh is facilitating the influx of Ca\(^{2+}\) into the nerve terminals of the hemi-diaphragm preparations. The subsequent raised internal levels of Ca\(^{2+}\) are probably involved in the breakdown of the cytoskeletal structure of
terminals, thus altering the innervation ratio of the preparations.

An alternative explanation for the decreased ratio of indirect/directly elicited tetanic tension demonstrated in preparations incubated for substantial periods of time in solutions containing ACh plus motor nerve stimulation, may not be a reflection of functional innervation of the preparations, but may instead be the consequence of persistent depolarization of the nerve, as a result of long term exposure to ACh (Katz & Thesleff, 1957). ACh-induced desensitization of the preparations would result in a failure to recruit fibres upon stimulation of the motor nerve, and would be manifest as a reduction in the calculated tension ratio. O'Brien et al. (1980) in their study on neonatal rat soleus muscles observed a similar initial desensitization response as a result of incubation in ACh for prolonged periods. The results reported by these authors agree with those presented in this Chapter, in that the initial desensitization was transient and quickly disappeared. Recovery from ACh-induced desensitization was complete within 15 minutes in all of the preparations studied. The ratio of apparent functional innervation thus quickly recovered. Indeed, incubation in ACh alone demonstrated no significant effects on the calculated innervation ratio, therefore suggesting that the reduction in the ratio of indirect/directly elicited tetanic tension is not in fact due to desensitization of the preparations but may be the consequence of a reduced number of functional nerve-muscle contacts. The level of ACh employed in this series of experiments, though high, is within the range calculated to be present during normal neuromuscular activity (Matthews-Bellinger & Salpeter, 1978).

The extensive reduction in the ratio of indirect/direct tetanic
tension observed in these experiments was prevented by the inclusion of leupeptin, a powerful inhibitor of the CANP enzymes (Suzuki et al., 1981) in the incubation media. The protective nature of leupeptin suggests the involvement of a CANP in the breakdown of functional nerve-muscle contacts.

Guroff (1964) was the first to identify a CANP in rat brain. Subsequently a CANP which degrades neurofilaments was isolated from brain and spinal cord of rat (Zimmerman & Schlaepfer, 1982; 1984), and from bovine brain (Malik et al., 1983). A CANP has been identified in peripheral nerve (Kamakura et al., 1983) and is associated with, and bound to, the cytoskeleton (Tashiro & Ishizaki, 1982) in the CNS and PNS. CANP has been demonstrated to be localized at the neuromuscular junction of monkey skeletal muscle, associated with the basal lamina, axolemma and in association with neurofilaments of myelinated and unmyelinated axons (Badalmente et al., 1987). In light of this work it is possible that the addition of leupeptin to the incubation media may be preserving a whole population of terminals from enzymatic calcium-activated proteolytic degradation.

The first evidence to suggest that the disintegration of neurofilaments in axons is not directly affected by calcium but a calcium-activated thiol protease came from the work of Schlaepfer & Freeman (1980), working on intact neurofilaments from rat spinal cord. The extracted neurofilament samples were shown to be chemically and structurally degraded the presence of calcium, by a factor which they could readily solubilize from the spinal cord tissue. Morphological studies of frozen sections of rat sciatic nerve provide evidence that neurofilament breakdown is mediated by a calcium activated protease
Incubation of frozen sections in media with Ca\textsuperscript{2+} resulted in a widespread disintegration of the neurofilaments. Schlaepfer & Zimmerman (1981) demonstrated that the same Ca\textsuperscript{2+}-dependent disintegration of neurofilaments is universal in neural tissues. The same pattern of disintegration occurs in myelinated and unmyelinated fibres of peripheral nerve, optic tract and spinal cord. In 1982, Zimmerman & Schlaepfer purified a soluble factor from rat brain and spinal cord which degrades neurofilament proteins.

Two calcium activated neutral proteases have been identified in mammals and have subsequently been named \(\mu\text{CANP}\) and mCANP depending on their requirements of Ca\textsuperscript{2+} for activation (Suzuki \textit{et al.}, 1981)(also known as calpain I and II). \(\mu\text{CANP}\) requires 10 \(\mu\text{M}\) Ca\textsuperscript{2+} for activation (Kishimoto \textit{et al.}, 1981), which is much lower than the 0.5mM Ca\textsuperscript{2+} required to activate mCANP, purified from rat brain (Zimmerman & Schlaepfer, 1984). The CANP's have identical effects on myofibrils (Dayton \textit{et al.}, 1981) and on the degradation of neurofilament proteins (Zimmerman & Schlaepfer, 1984). The CANP's, calpain I and calpain II, and their endogenous inhibitor calpastatin, are found widely distributed in most avian and mammalian cells (reviewed by Murachi, 1983 and Suzuki, 1987). Calpain I & II have been indicated to be present in all parts of the neurone, including the nerve terminal, and are mainly associated with the cytoskeleton (Badalamente \textit{et al.}, 1987; Perlumutter \textit{et al.}, 1988).

The identification of \(\mu\text{CANP}\), which requires relatively low, physiological levels of Ca\textsuperscript{2+} for activation, lead to the suggestion that \(\mu\text{CANP}\) may have a physiological role in the turnover of cytoskeletal elements. Homeostasis in the mature non-growing nerve requires that the
synthesis and assembly of cytoskeletal proteins is balanced by a concomittant breakdown and turnover of elements. \( \text{Ca}^{2+} \)-mediated proteolysis of neurofilaments in nerve terminals is postulated as a regulating mechanism of neurofilament turnover in active, nongrowing axons (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976). Lasek & Black (1977) indicated that radiolabelled cytoskeletal proteins are rapidly turned over on reaching axon terminals, and concluded that synaptic endings are the active sites of cytoskeletal degradation. As a result of previous experimental data and their findings, Lasek & Black speculated that the breakdown of cytoskeletal elements is regulated by activation of proteases as a result of local increases of calcium concentration. Schlaepfer et al. (1985) in a study comparing the breakdown of neurofilament proteins \textit{in situ}, with that occurring as a result of the activity of a purified CANP demonstrated a similar pattern of degradation, and suggested that the normal turnover of neurofilaments is a result of the actions of CANP. Thus experimental evidence tends to the support a role for CANP activity in the normal regulation and turnover of cytoskeletal proteins.

Exposure of hemi-diaphragm preparations to conditions of ACh and motor nerve stimulation, in an attempt to mimic neuromuscular activity, may cause a \( \text{Ca}^{2+} \) influx into the nerve terminal. Depolarization of nerve cells is known to cause a \( \text{Ca}^{2+} \) influx (Katz & Miledi, 1969). The raised internal levels of \( \text{Ca}^{2+} \) then activate a neuronal CANP attached to the cytoskeleton (Tashiro & Ishizaki, 1982; Ishizaki \textit{et al.}, 1985). The activated neuronal CANP brings about a disassembly of the cytoskeletal structure of a susceptible population of terminals. In the experimental conditions performed in this Chapter, leupeptin, which is able to enter
axon terminals (Roots, 1983), may inhibit neuronal CANP and so protect the terminals from this proteolytic degradation. Indeed, Roots (1983) injected leupeptin into goldfish optic tectum and demonstrated a reduced breakdown and concomitant accumulation of neurofilament proteins.

Alternatively, CANP may be involved in the metabolism and stabilization of AChR's at the neuromuscular junction. Tissue culture experiments with nerve-muscle preparations demonstrate that nerve-induced dispersal of pre-existing AChR clusters is markedly affected by protease inhibitors including leupeptin (Kuromi & Kidikoro, 1984). Leupeptin has been demonstrated to slow the turnover rate of AChR in cultured muscle cells (Romstedt et al., 1983). Leupeptin can decrease the degradation rate of AChR's in tissue culture by 7-10 fold (Romstedt et al., 1983). In the experiments in this Chapter, the activated CANP may be involved in the destabilization of AChR's and the addition of leupeptin to the experimental media may be having its effect by protecting the AChR's from degradative attack. Destabilization of the AChR's could possibly be due to the actions of CANP on the cytoskeletal network which surrounds the receptors. This cytoskeletal network is thought to either anchor the receptors to the postsynaptic membrane or afford the receptor protection from degradation (Salpeter & Loring, 1985). CANP has been shown to be involved in the modelling of the extracellular matrix; a CANP has been demonstrated to be involved in the binding of glutamate to rat hippocampal membrane receptors in the presence of Ca²⁺ and binding is markedly increased due to the actions of CANP on the intermediate filament, fodrin (Baudry & Lynch, 1980; Siman et al., 1985). Siman and colleagues (1987) reported experiments in which they performed intraventricular infusion of high concentrations of
leupeptin. As a consequence of the leupeptin inhibition of CANP activity, they observed an accumulation of neurofilaments and another intermediate filament, brain spectrin, in subsequent cytoplasmic fractions. It is possible therefore, that the leupeptin-induced preservation of nerve-muscle contacts observed in the experiments in this Chapter, may be due to the inhibition of membrane-affiliated CANP's as opposed to neuronal CANP's. Interestingly, during the elimination of multiple innervation occurring after experimentally-induced reinnervation, terminals which are eliminated are preceded by an elimination of the overlying AChR (Rich & Lichtman, 1989). The authors suggest that the elimination of the postsynaptic AChR may initiate the removal of the overlying nerve terminals.

The in vitro experimental observations reported in this Chapter suggest a proteolytic degradation of the cytoskeletal structure of specific axon terminals in the hemi-diaphragm nerve-muscle preparations. Additional evidence supporting this hypothesis is derived from immunoblotting experiments. Incubation of hemi-diaphragm preparations in solutions containing ACh, Ca$^{2+}$ and motor nerve stimulation had previously been shown to affect the ratio of functional innervation. Immunoblot profiles of samples of neurofilament proteins, from hemi-diaphragm preparations, demonstrated changes in the neurofilament proteins as a consequence of the experimental incubation. The NF-L neurofilament subunit protein was preferentially affected by the experimental manipulation. Calcium-dependent proteolysis of neurofilaments can be demonstrated in a wide variety of tissues, although, the susceptibility of individual neurofilament proteins to this proteolysis is not uniform. Schlaepfer & Freeman (1980) demonstrated
that isolated neurofilaments from rat spinal cord were disrupted by a 
Ca^{2+}-dependent tissue-derived factor. NF-L and NF-M protein subunits 
were preferentially degraded by this tissue factor. The prefered 
degradation of the lower molecular weight neurofilament proteins has 
also been observed during the early phases of Wallerian degeneration 
(Schlaepfer & Micko, 1978). The NF-L neurofilament subunit protein is 
thought to form the backbone to a neurofilament (Willard & Simon, 1981). 
Degradation of this subunit protein would result in a collapse of the 
neurofilament and would subsequently result in the loss of functional 
contacts at the endplate. These results are consistant with the 
preferential proteolyis of the NF-L neurofilament protein demonstrated 
in the experiments performed in this Chapter where conditions of ACh, 
Ca^{2+} and motor nerve stimulation result in a massive reduction in the 
number of functional contacts within the preparations, in addition to 
the loss of NF-L in immunoblot profiles.

An additional interesting observation from the results presented 
in this Chapter was a significant general increase in tension output 
from hemi-diaphragm preparations which had been incubated for 2 hours in 
Krebs containing 10^{-2}M ACh, 10mM Ca^{2+} and 0.2mg/ml leupeptin, in 
addition to motor nerve stimulation. Direct stimulation of these 
preparations, at the end of the experimental period, following the 2 
hour incubation period, produced a tension output which was 45% greater 
than that produced by the same group of preparations prior to the 
icubation. Hemi-diaphragm preparations which were incubated in similar 
conditions, with the exclusion of leupeptin demonstrated no such 
dramatic increase in tension ouput. The inclusion of leupeptin to the 
icubation media may be affecting the force output of the preparations
by inhibiting the CANPs within the muscle fibres. The simple polypeptide, leupeptin, may be directly exerting an influence on the muscle fibres since it has been demonstrated that leupeptin can cross muscle fibre membranes (Libby & Goldberg, 1978). Once within the muscle fibres leupeptin may be in a position to inhibit muscle CANP's. Muscle specific CANP has been localized in the Z-lines of myofibrils (Ishiura et al., 1980; Dayton et al., 1981) and is enriched near the muscle sarcolemma (Barth & Elce, 1981; Dayton et al., 1981). This proteolytic enzyme is known to influence the release of α-actinin and troponin C from myofibrils, concomitant with the disappearance of the Z-line (Reddy et al., 1975). The enzymatic action of CANP subsequently results in the fragmentation of the myofibril. Significantly, CANP has been shown to be involved in the turnover of myofibrillar proteins in muscle cells (Busch et al., 1972; Reddy et al., 1975; Dayton et al., 1976a; 1976b; 1981). Experimental manipulations of dystrophic chick muscles treated with E64c (an inhibitor of CANP) completely inhibited the normal degradative action of CANP on the muscle structural proteins (Sugita et al., 1983). Other experimental work employing intramuscular injections of inhibitors of CANP (leupeptin and pepstatin) delayed the normal process of degeneration and atrophy of dystrophic muscles (Stracher et al., 1977; Sher et al., 1981). The role of CANP in the normal turnover of muscle proteins has, however, been disputed (Gerard & Schneider, 1980). Nevertheless, in the present experimental conditions it is possible that leupeptin, in conditions of raised external Ca$^{2+}$ may be exerting its influence by inhibiting muscle specific CANP enzymes, therefore limiting the normal breakdown of muscle proteins and in so doing, providing more contractile material to be utilized for force...
production by the preparation.

In conclusion, the results presented in this Chapter suggest that functional nerve-muscle contacts in developing hemi-diaphragm muscles can be disrupted by experimental conditions which mimic neuromuscular activity. Disruption of nerve-muscle contacts occurs via a degradation of a specific neurofilament protein - the subunit protein which forms the backbone of the neurofilamentous structure within specific axons and terminals. The results show that this activity-dependent degradation of contacts occurs via a CANP since leupeptin, an inhibitor of the protease, can protect terminals from degradation.
CHAPTER THREE

The Effects of Reducing Target Activity on the Development of Muscle Properties and Pattern of Innervation of the Soleus Muscle
INTRODUCTION

During the development of the mammalian neuromuscular system the differentiation and development of both the peripheral nerves and the muscle cells is initially an independent process. As development proceeds, the motoneurone and its target become dependent upon continued functional interactions for their further development.

The development of muscle fibres and their specific properties is dependent upon motor nerve innervation. Differentiation of mesenchymal cells to the point of primary myotube establishment occurs in the absence of innervation, however, any further development and maturation of the primary muscle mass is critically dependent upon innervation (Carlson, 1973; Harris, 1981b). Early in development, after fusion of myoblasts into mononucleated myotubes, the early muscle cells contain a specific myosin heavy chain isoform (Buller et al., 1982; Crow & Stockdale, 1986), known as embryonic myosin (Trayer et al., 1968; Whalen et al., 1981; Harris et al., 1989; Condon et al., 1990a), which is expressed by primary myotubes prior to their expression of adult myosin isoforms (Whalen et al., 1981). Once secondary myotubes have formed, the absolute necessity for functional innervation gradually diminishes and denervation of the muscle does not produce profound destruction of the muscle mass. In the absence of innervation a retardation in the production of new muscle fibres and subsequent growth of the whole muscle does however occur (Zelená, 1962; Carlson, 1973).

Early in the development of the neuromuscular system each muscle fibre is innervated by several axons converging at a single endplate. Under the influence of this polyneuronal innervation the muscle fibres
are uncommitted to a single motoneurone and possess immature characteristics. At birth the enzyme levels in the oxidative and glycolytic pathways are low in all fibres of rat EDL and soleus muscles, although the different muscles can be distinguished in terms of the relative levels of the metabolic enzymes (Németh et al., 1989). In addition, neonatal muscles are relatively resistant to fatigue (Hammarberg & Kellerth, 1975). As polyneuronal innervation is eliminated, muscle fibre properties become more specialized and can be distinguished into distinct fibre types.

Prior to differentiation into adult types all muscle fibres are slow contracting and relaxing. Subsequently, in neonatal rats, the contractile speeds of both the fast and slow fibres increases with age until they differentiate into the adult types, and the contraction speeds differ markedly (Close, 1964). In kittens, initially all fibres become faster contracting. The slow muscle fibres then achieve their slow rate of contraction whilst the fast fibres increase their speed of contraction to that characteristic of an adult fast twitch muscle (Buller et al., 1960; Brown, 1973). Maturation of the speeds of contraction of fast and slow muscles is dependent upon underlying developmental changes in the time course of the release and uptake of $\text{Ca}^{2+}$ ions from the sarcoplasmic reticulum (SR) (Martonosi, 1982). During development there is an increase in the surface area of the SR and a subsequent increase in the capacity to pump $\text{Ca}^{2+}$ (Luff & Atwood, 1971; Kelly, 1980). The rate of uptake of $\text{Ca}^{2+}$ accelerates after birth (Drachman & Johnston, 1973) and is correlated with an increase in speed of contraction and relaxation of the muscle during development.

As normal postnatal development of the rat skeletal muscle proceeds
the immature muscle fibres are transformed into highly specific adult muscle fibres. This transformation of muscle fibre types is a result of increasing neuromuscular activity of the animal (Navarrete & Vrbova, 1983), and correlates with the emergence of functional specializations of the muscle groups. Between postnatal days 10-21, concomitant with an increased locomotor activity, the levels of oxidative enzymes within each fibre type changes and the specialization of fast fibres into oxidative and glycolytic phenotypes occurs (Németh et al., 1989) until the adult patterns of activity are achieved. Fatigue resistance properties of the developing muscle fibres change over postnatal development, and the ability to resist fatigue decreases in the fast twitch muscle fibres (Hammarberg & Kellerth, 1975).

Muscle fibres comprising an adult rat motor unit are homogenous. Kugelberg and colleagues (1968; 1979) were the first to determine the universal homogenity of muscle fibres in a single motor unit, using the glycogen depletion technique. The authors depleted specific muscle fibres in a single motor unit of glycogen; upon histochemical examination, the muscle fibres belonging to a single motor unit have the same histochemical appearance as each other. Enzyme activites of random muscle fibres demonstrate variable levels of activity, whereas those in one motor unit possess nearly identical contents of enzymes of the oxidative pathway (Németh et al., 1981). Within an adult motor unit muscle fibres also contain identical isomyosins (Gauthier et al., 1983). The homogenuity of muscle fibres within an adult motor unit suggests that the neural input to muscle fibres determines their phenotypic expression.

Classical cross-reinnervation experiments provide compelling
evidence of the essential role of innervation in determining the properties of skeletal muscles (Buller et al., 1960). In these original experiments the authors found that innervating the slow soleus muscle with the nerve of the fast flexor digitorum longus (FDL) muscle transformed the properties of the soleus muscle, so that it became faster contracting and relaxing. A transformation in properties could also be achieved by re-innervating the FDL muscle with the slow soleus nerve. Many characteristic properties of muscle fibres are transformed by cross- innervation. Cross-reinnervated muscles express transformed isomyosins (Buller et al., 1969; Samatha et al., 1970; Srihari et al., 1981; Sreter, 1980) as well as the regulatory proteins of the myofibrills (Amphlett et al., 1975; Dhoot et al., 1981; Dhoot & Perry, 1983). The SR is transformed following cross-innervation (Mommaerts et al., 1969), as are enzyme activites and isozymes of the enzymes of energy metabolism (Romanul & van der Muellen, 1967; Karpati et al., 1975). The phenotypic expression of muscle fibre properties can be permanently modified by the specific activity of the innervating motoneurone. When the nerve supply to a muscle is kept intact, but the activity of the motoneurone innervating the muscle altered by interfering with the afferent inputs, the contractile properties of the innervated muscle are altered in a similar manner to that observed in cross-reinnervation experiments. Thus, the essential nature of the interaction between the nerve and muscle in the normal development of muscle fibre properties is highlighted. Indeed, motoneurone activity appears to determine the physiological and biochemical properties of the muscle fibres within each motor unit.

In neonates the size of an individual motor unit is large, with
motor axons innervating up to five times as many muscle fibres as the same axons in the adult (Brown et al., 1976; Thompson & Jansen, 1977; O'Brien et al., 1978). This large size of a neonatal motor unit is a consequence of extensive polyneuronal innervation, which is eliminated during the first 2 to 3 weeks of postnatal life, as a result of which the size of individual motor units decreases, and the adult pattern of innervation is achieved (Brown et al., 1976; Lowrie et al., 1985). Excess synapses are eliminated at each endplate until, in the adult, each muscle fibre is innervated by a single axon.

An interesting question that arises from the study of the elimination of polyneuronal innervation and the development of the normal adult pattern of innervation at the neuromuscular junction, is whether this reorganization process follows a preprogrammed pattern or if the organization of the adult pattern of innervation is more plastic and can be modified.

The loss of excess neuromuscular contacts and the subsequent reduction in the territory of individual motor units is not thought to be a pre-programmed event. This is illustrated in experiments where partial denervation was performed prior to the normal reduction of neonatal motor unit size; subsequent motor unit sizes remained large and the remaining axons maintained their neonatal territories (Betz et al., 1980; Lowrie et al., 1985; Fisher et al., 1989; Vrbová & Fisher, 1990; Connold & Vrbová, 1990; Connold & Vrbová, 1991). If the patterns and distribution of innervation were predetermined, then motoneurones would be unable to alter their peripheral field to changing demands placed upon them by normal development and by an experimentally-altered target.

Partial denervation experiments in neonates reported by other
research groups demonstrated a delayed decrease in motor unit size. Brown et al. (1976), Thompson & Jansen (1977), and Fladby & Jansen (1987) partially denervated neonatal muscles in rats and mice at birth. As a consequence the authors reported that the normal decline in motor unit size was delayed and the average size of the remaining motor units was larger than normal, when examined after synapse elimination was complete. However, they concluded that the neonatal motor unit size was not maintained, and reported the presence of denervated fibres. This is in contrast to a recent study by Fisher et al. (1989) where competition was removed in the rat soleus muscle at postnatal day 5 by partial denervation. The authors demonstrated that the remaining motor units maintained their large neonatal peripheral field and the normal reduction in territory failed to occur.

It is possible to explain these contradictory results. The results of Brown et al. (1976) are preliminary results and are based on a study of 3 animals which were denervated at different times (4, 7, 7 days) postnatally. The conclusions are based on two of these results since one animal, which showed no reduction in the neonatal motor unit size, was excluded on the grounds that the animal was in a transitional state. The authors recognize the difficulty in drawing any conclusive results from this data due to the large scatter in the data, as a result of the small number of animals investigated. The study of Thompson & Jansen (1977) was performed on the A/O strain of rat, partial denervation being performed between 1-5 days postnatally. The first five days of postnatal life are critical in the development of the neuromuscular junction and operations at PN1 may have very different consequences to an operation at PN5. The authors examined their experimental animals 2-20 weeks after
the initial operation and found that the motor units of partially denervated muscles were greatly reduced in size, leaving a large number of muscle fibres completely denervated. The motor units were however, larger than those seen in normal mature animals. In some cases the motor units had expanded their peripheral fields up to 4 times that seen in normal animals; in other cases they had expanded until they innervated all of the denervated fibres. Since no indication was reported of the precise time at which the partial denervation occurred, or when the animals were examined after the operation, it is difficult to deal with these ranging results. Fladby & Jansen (1987) partially denervated mouse soleus muscles, just after birth, and demonstrated that in animals which had more than 7 motor units remaining innervating the muscle, the muscle was fully innervated; in cases where less than 7 motor units remained the number of innervated fibres was less than normal and the motor unit sizes were reduced.

The experimental results from partial denervation experiments (Brown et al., 1976; Thompson & Jansen, 1977; Betz et al., 1980; Lowrie et al., 1985; Fisher et al., 1989; Vrbová & Fisher, 1989; Connold & Vrbová, 1990; Connold & Vrbová, 1991) suggest that motoneurones of neonates and those of slightly older animals react differently to an altered peripheral field. It may be that early neonatal motoneurones have not reached a point in their maturation where they are capable of metabolically supporting an enlarged peripheral field, and there is an upper limit above which the motor units cannot further expand. Partial denervation carried out slightly later in the developmental programme (e.g. 5 days postnatally, Fisher et al., 1989) may occur at a point when the motoneurone has achieved a certain level of maturity and is capable
of maintaining a larger than normal peripheral field.

The plasticity of the patterns and distribution of innervation demonstrated in neonates, with the establishment of polyneuronal innervation and its subsequent elimination, is also retained in the adult. Adult motor unit territory is not rigidly determined and can be altered by modifying the innervation of the target tissue. After partial denervation in adult skeletal muscle the remaining motor units enlarge their peripheral fields and can expand up to 4/5 times their previous size (Brown & Ironton, 1978; Thompson & Jansen, 1977; Gorio et al., 1983; Fisher et al., 1989; Connold & Vrbová, 1991). If the majority of axons to a muscle are removed, the axons which remain in contact with the target immediately sprout to occupy some or all of the muscle fibres deficient of a nerve supply (Weiss & Edds, 1946; Hoffman, 1950; Edds & Small, 1951; Edds, 1953; Brown et al., 1981). Connold & Vrbová (1991) demonstrated that postsynaptic activity is important for the sprouting process. They temporarily paralysed muscles following partial denervation, and demonstrated that as a result of the target inactivity the expansion of muscle unit territory normally seen following partial denervation did not occur.

Synaptic activity of the target organ is an important factor in the regulation of the rate of synapse elimination, and in the subsequent differentiation and maturation of the neuromuscular junction. Decreasing the level of neuromuscular activity reduces the rate of elimination of polyneuronal innervation. Blocking muscle activity presynaptically by BoTx or TTX (Thompson, 1979; Brown et al., 1981) or postsynaptically with α-BTX (Duxson, 1982; Callaway & Van Essen, 1989) dramatically slows, or even halts, synapse elimination. Conversely, increasing the
activity of the nerve or muscle, by implanted stimulating electrodes, increases the rate of synapse elimination (O'Brien et al., 1978; Thompson, 1983). It appears therefore that the activity of the target is essential for the normal development of the neuromuscular junction and the elimination of excess synaptic inputs. The experiments laid out in Chapter 2 demonstrate that neuromuscular activity is important in the breakdown of functional contacts via a proteolytic degradation of the cytoskeletal structure of axon terminals. It is proposed that this mechanism is involved in the elimination of polyneuronal innervation, since Connold et al. (1986) reported that inhibitors of a protease prevent the elimination of polyneuronal innervation in neonatal rat soleus muscles.

The importance of the interaction between the nerve and muscle for their normal differentiation and maturation is known to be age-dependent. $\alpha$-bungarotoxin induced paralysis of neonatal rat soleus muscles (Duxson, 1982; Callaway & van Essen, 1989) delays the normal pattern of differentiation, and the elimination of polyneuronal innervation. Greensmith and colleagues (1988; 1991) paralysed rat soleus muscles at birth with $\alpha$-bungarotoxin and found that instead of simply delaying the maturation of the neuromuscular junction, contacts were disrupted. There appears to be a critical time period in the development of the neuromuscular junction when normal development is highly dependent upon continued and functional interaction between the nerve and the muscle fibres.

The soleus muscle of the rat is innervated by axons from two lumbar spinal roots, L4 and L5, which are found in the corresponding rami. In adults, axons from the L5 ventral ramus make the greater contribution to
the innervation of the soleus muscle (Brown et al., 1976; Lowrie et al., 1985; Miyata & Yoshioka, 1980; Thompson, 1983). Overall, in neonates the axons from the L4 and L5 ventral roots overlap to a great extent. During the elimination of polyneuronal innervation the motor units of the axons in the L4 ventral ramus loose more territory than those emanating from the L5 v.r. (Lowrie et al., 1985; Miyata & Yoshioka, 1980; Thompson, 1983), although the elimination of polyneuronal innervation proceeds at the same rate for the axons in both ventral roots (Miyata & Yoshioka, 1980). Thompson (1983) suggested that the difference in the overall loss of territories of the two ventral roots is due to the relative number of axons from the two ventral roots, L4 axons loosing proportionally more muscle fibre contacts than L5 axons.

Investigating the properties of the L4 and L5 axons, whose territories overlap, Lowrie and colleagues (1985) found that, during early postnatal life, the quantal content of terminals on soleus muscle fibres was less from the L4 terminals than that from the L5 terminals. As the size of the L4 motor units decreases the transmitter output of these axons increases so that by 18 days, when the overlap of territory is only 2%, the quantal content of the L4 terminals equalled that of the L5 terminals. The differential loss of L4 axons during the postnatal elimination of excess synapses is proposed to be a consequence of the relative "weakness" of the L4 axons (Lowrie et al., 1985; Miyata & Yoshioka, 1980).

Interestingly, work by Herrera & Grinnell (1980) on the frog sartorius muscles demonstrated a correlation between the size of the peripheral field of a motoneurone and the release of transmitter at its terminals. They surgically reduced the size of the sartorius muscle and
consequently the size of the adult motor units decreased. The outcome of this reduced motor unit size was a reciprocal increase in the transmitter output at the frog neuromuscular junction (Herrera & Grinnell, 1980; 1985; Pockett & Slack, 1982). These results seem to suggest that the motoneurone in the adult was not able to monitor the reduced demands of its peripheral connections and therefore was unable to alter its output according to those needs. This experimental approach was extended over a longer period of time and it has been demonstrated that given sufficient time the motoneurone can alter its supply of raw materials to meet the reduced demands of the periphery (Pockett & Slack, 1983; Herrera & Grinell, 1985). Lowrie et al. (1985) performed the converse of the Herrera & Grinnell experiments. Their approach was to reduce the number of axons innervating the same muscle mass, by rhizotomy at different times after birth, and then investigate the relationship between the quantal content and motor unit size. Partial denervation in neonates prevented the normal reduction in the territory of the remaining axons; and those axons with an enlarged peripheral field possessed normal levels of quantal content at their terminals. Lowrie and colleagues (1985) also partially denervated soleus muscles at a later stage in development (young adults); the remaining axons expanded their peripheral field but the quantal content of the remaining terminals was less than normal. As a consequence the authors concluded that before synaptic reorganization within the muscle is complete, motoneurones are able to adapt their function according to increased peripheral demands as a consequence of partial denervation, whereas at later stages of postnatal development, the motoneurone is less plastic and less able to adapt to altered peripheral demands.
The reorganization of innervation occurring during the elimination of polyneuronal innervation is concomitant with changes in the peripheral demands placed on the motoneurone. Once the adult pattern of innervation has been achieved, a functional maturation of the neurone and its terminals proceeds. Both transmitter synthesis and the output from the functional nerve terminals increases (Diamond & Miledi, 1962; Kelly, 1978; O'Brien & Vrbova, 1978). Those terminals which survive the elimination process continue to develop and stabilize, receiving a greater supply of essential materials from the maturing motoneurone cell body.

Chapter 2 demonstrates that activity is important in the regulation of contacts at the neuromuscular junction during early postnatal development and proposes a mechanism by which excess synaptic contacts may be eliminated. Greensmith & Vrbova (1991) have demonstrated the importance of active communication between the motoneurone and the target muscle during early stages in development. This Chapter will attempt to investigate the importance of postsynaptic activity in the development of the adult soleus muscle and its pattern of innervation. Neuromuscular transmission was temporarily interrupted by blocking the response of the postsynaptic membrane of the soleus muscle in the early stages of postnatal development, at a time when the elimination of excess synapses had not occurred and when synaptic contacts are both newly established and relatively weak. The development of the pattern of innervation of the soleus muscle and the subsequent development of the muscle fibre properties was investigated.
MATERIALS AND METHODS

I) Animals

All operations were performed on neonatal Wistar albino rats, of both sexes. The operations were performed on the day of birth, between 6 and 12 hours after birth (this day is known as postnatal (PN) day 0). Animals were supplied by Joint Animal House, University College London, or by Charles Rivers, Animal Suppliers, U.K., and fed ad libitum food and water with a constant light/dark cycle.

II) Muscle Paralysis

The soleus muscles of experimental animals were paralysed at birth, by employing the snake toxin α-Bungarotoxin (α-BTX), obtained from Sigma Chemical Company. α-BTX binds irreversibly to the post-synaptic nicotinic acetylcholine receptor (AChR) and so interrupts the response of the post-synaptic membrane. Duxson (1982) developed a suitable method for applying the toxin to neonatal rats. The toxin is incorporated into inert silicone rubber, which forms a matrix around the toxin allowing a steady release of the toxin into the animal. This method was employed to apply α-BTX to neonatal rats with slight modifications in the size of the implant and the amount of α-BTX according to age.

Effective, long term paralysis was achieved by implanting the highest concentration of toxin compatible with the survival and comfort of the animals. Animals which showed signs of respiratory distress as a consequence of the toxin treatment, were killed with an overdose of...
anaesthetic. 10-20% of animals died after implantation indicating that a sub-lethal dose of α-BTX was employed in these experiments.

III) Preparation of Toxin for Implantation

A small known quantity of α-bungarotoxin was mixed and dry diluted in a sterile petri dish. A calculated volume of silicone rubber solution (Dow Corning, 3140 RTV, non toxic) was added and the three compounds thoroughly mixed in a fume cupboard. This mixture was allowed to set overnight at room temperature and was subsequently stored at 4°C.

When dry, small strips were cut from this rubber matrix with dimensions of approximately 3mm x 1mm x 0.1mm, weighing 1mg. These strips were further subdivided into three (since accurate weighing of 0.3mg strips, to acquire the required weight and size proved too difficult). The resultant strips for implantation at birth contained approximately 7 μg of α-BTX and 120 μg NaCl.

In one series of experiments animals were reoperated 3 days after the initial operation. Silicone matrices with dimensions of 3mm x 2mm x 1mm, weighing 2/3mg were implanted. The strips for implantation at birth contained 14 μg α-BTX and 240 μg NaCl.

Silicone rubber containing NaCl only, for control implantations, was prepared using the above method. The same size and weight strips were cut from this matrix so that the resultant strip for implantation also contained 120 μg NaCl. Second implantations occurred in some experiments where NaCl only strips were implanted 3 days after the first operation. These strips contained 240 μg of NaCl.

Sterile precautions were employed each time the toxin and control NaCl "plugs" were handled.
IV) Application of Toxin

Newborn rats were operated within 6-12 hours of birth, using ether anaesthesia and sterile precautions. A small, longitudinal incision was made in the skin on the lateral side of the right hindlimb. A smaller incision was then made in the underlying fascia to reveal the hindlimb muscles. A silicone rubber strip containing either α-BTX or NaCl only was implanted alongside the soleus muscle. The implant was placed between the soleus muscle and flexor hallucis longus muscles at the midpoint of the soleus, away from the point of nerve entry. The overlying skin was sutured with 0.4 silk thread (Ethicon) and the animals returned to their mother when fully recovered from the anaesthesia.

In some animals a second operation was performed 3 days after the first operation, on PN 3. The animals were reanaesthetized with ether and an incision was made as explained above. The first silicone rubber strip was identified, its position checked, and then removed from the hindlimb. The second implant was then inserted. The overlying skin was sutured with 0.7 silk thread (Ethicon). The animals were returned to their mother when fully recovered from anaesthesia.

The application of toxin in small rubber strips does not cause any mechanical damage to the muscle fibres. Fibres do not become denervated and the only permanent mechanical effects of the silicone implant is a small quantity of connective tissue found in the area of the implant, as demonstrated in Figure 1.
Figure 1: Transverse Section of Adult Rat Soleus Muscle After α-Bungarotoxin Treatment at Birth

Photomicrograph of a transverse section (12 μm) of an adult rat soleus muscle stained with Haemotoxylin-van Gieson's stain. Soleus muscles of newborn rats were paralysed at birth with α-BTX. 2-10 months later the soleus muscles were removed from the experimental animals, frozen, sectioned and subjected to histological examination. The combined Haemotoxylin-van Gieson stain stains muscle nuclei grey, cytoplasm yellow, and connective tissue red. The figure demonstrates that treatment at birth with α-BTX does not permanently damage the muscle fibres; a small amount of connective tissue can be seen at the site of the implant. Scale bar = 50 μm.
Figure 1
V) Release of Toxin

Previous experiments have shown that various compounds are released from silicone rubber strips, and that this method of applying substances to muscles is both easy and effective. No studies have specifically dealt with the release of \( \alpha \)-BTX.

\(^3\)H Quin2 (a radioactive analogue of the calcium chelator BAPTA (1,2-bis(2 amino-phenoxy)ethane-NNN'N'tetra aceitic acid)) has been shown to be released from a silicone rubber matrix for approximately 3 days, 35% of the total \(^3\)H Quin2 being released from the matrix during the first 24 hours (Connold et al., 1984).

Connold et al. (1986) have also demonstrated the release of leupeptin (N-acetyl-L-leu-L-leu-arginal, an inhibitor of calcium activated neutral protease) from rubber implants; 70% of the total leupeptin content of a strip being released over the first 24 hours, further release of the compound continues for the following 2 days. Connold et al (1986) demonstrated that release follows an exponential pattern and therefore can be explained in terms of simple diffusion.

It is reasonable to assume that \( \alpha \)-BTX is also released from the silicone matrix in a simple manner. However, the release of the toxin from the matrix is not the only factor involved in functional paralysis. In neonates the turnover of AChR's is rapid and new AChR's are constantly synthesized and incorporated into the postsynaptic membrane. It was therefore important to study the extent and efficacy of the neuromuscular block.

VI) Efficacy of Neuromuscular Paralysis

In order to assess the extent of neuromuscular block and muscle
paralysis *in vitro* (18-24 hours of age) or *in vivo* (9 days of age) isometric tension recordings were made of experimentally paralysed muscles.

Between 18 and 24 hours after treatment, animals were anaethesized with ether and the operated muscles, plus intact motor nerve, were dissected free. The nerve-muscle preparations were placed in an organ bath and perfused with aerated Krebs solution, at room temperature. The proximal end of the muscle was fixed in the organ bath to the silicone base with a pin. The distal tendon of the preparations was attached to a strain guage (Dyanometer UFI) with 0.1 silk thread (Ethicon).

At older ages, *in vivo* tension recordings were performed. The treated soleus muscle was dissected free from the overlying musculature, and the distal tendon attached to a strain guage (Dynamometer UFI) with 0.1 silk thread. The sciatic nerve was dissected and severed.

In both *in vitro* and *in vivo* recordings of muscle tension the muscle length was adjusted to obtain maximum twitch tension at supramaximal stimulus strength. Isometric muscle contractions were elicited by stimulating the cut end of the soleus nerve with bipolar silver electrodes, using a pulse width of 20 µs, or by directly stimulating the muscle via silver electrodes placed either side of the muscle, using a pulse width of 2ms. Maximal tetanic tensions were elicited at optimal frequencies, which varied with age, and ranged between 20-40Hz. Contractions were visualized on an oscilloscope screen (Tetronix R5113), photographed and the tension measured. The ratio of indirectly/directly elicited tetanic tension was calculated, indicating the level of functional innervation of the preparation. As a result of these experiments the level and time course of muscle paralysis due to
α-BTX treatment could be assessed.

VII) **Isometric Tension Recordings of Muscle Contraction**

Two to ten months after the initial operation had been performed the experimental rats were anaesthetized with chloral hydrate (4.5% aqueous solution, 1ml/100g body weight, intra-peritoneal) and were prepared for *in vivo* physiological recordings of muscle tension.

The soleus muscles of each hindlimb of the experimental animal was dissected free from overlying musculature and the leg rigidly secured to a fixed table. The position of the plug was noted. The distal tendon was attached to a strain gauge (Dynamometer UFI) by a silk thread, 0.7-2.0. The muscle length was adjusted to obtain the maximal twitch tension at supramaximal stimulus strength.

In some experiments the L4 and L5 ventral rami (v.r.) innervating the operated leg were dissected free along with the soleus muscles of both hindlimbs. An incision was made in the skin of the animal overlying the region of the sciatic nerve. The sciatic nerve was then isolated from surrounding musculature and followed proximally to the point where the ventral rami, L4 and L5, join to form the nerve. The v.r. were then dissected as far proximally as possible, sectioned and a ligature tied to each ramus separately. The soleus muscles of both legs were prepared for tension recordings. All other muscles in the hindlimb were denervated by either crush or section of their motor nerve supply, and their distal tendons severed. The distal tendons of the soleus muscles were tied with 0.7-2.0 silk thread and then sectioned. The legs of the experimental animal were then fixed with pins through the ankle and knee.
joints to a rigid table and the distal tendons of the freed soleus muscles were connected to strain gauges (Dyanomo-meter UFI) appropriate to the expected force development of the muscle, by 0.7-2.0 silk thread. Muscle length was adjusted to obtain maximum twitch tension at supramaximal stimulus strength.

Care was taken at all stages in the dissection not to damage the nerve and blood supply to the soleus muscles.

Isometric contractions from the soleus muscles were elicited by stimulating the cut end of either the L4 or L5 v.r., the sciatic nerve or the motor nerve to the soleus muscle, via a pair of bipolar silver electrodes, using square wave pulses with a width of 0.02-0.05msecs, for 500-700 msecs. Twitch and tetanic tensions (20-80 Hz; 0.6msecs) were measured. The resulting contractions were amplified and displayed on an oscilloscope screen (Tetronix R5113), and photographs were taken of the traces.

All exposed tissue was kept moist throughout the experiment with oxygenated Krebs-Henseleit solution. Experiments were carried out at room temperature (23°C).

VIII) Changes in Contractile Properties

In order to observe any changes in contractile properties in the muscles as a result of paralysis at birth, the time-to-peak (TTP) and 1/2 relaxation times (1/2RT) were measured from the oscilloscope traces. TTP is the time taken by the muscle to contract and produce a maximum twitch contraction, measured in msecs. 1/2RT is the time taken in msecs. for the muscle to relax to 1/2 of the maximum twitch tension.
IX) Motor Unit Number & Size

The number of motor units within the whole muscle or within each ventral root was estimated. To estimate the number of motor units in both operated and contralateral control muscles the nerve to soleus was stimulated by single pulses every 4 seconds. Similarly, to estimate the number of motor units in each v.r. the cut end of each ramus was stimulated by a single pulse every 4 seconds. The stimulus intensity applied to either the soleus motor nerve or a specific ventral ramus was gradually increased. With increasing stimulus intensity, axons with different thresholds were activated which resulted in a successive recruitment of individual motor units. This was manifest in stepwise increments in twitch tension which were recorded on an oscilloscope screen. The stimulus intensity was gradually increased until a plateau was reached when all of the motor units had been recruited and the tension would not increase any further. A composite picture of the tension recorded from each individual motor unit was achieved. This was validated by repeating the procedure. Increments of twitch tensions were measured to estimate the number of motor units so as to avoid fatigue of the muscle with repeated tetanic contractions.

This method for measuring the number of motor units in a desired muscle has previously been employed by several research groups (Thompson & Jansen, 1977; Brown & Ironton, 1978; Gorio et al, 1983; Fisher et al, 1989; Vrbová & Fisher, 1990).

The average motor unit size for the operated muscle was found by dividing the maximum tetanic tension by the total number of motor units identified in the muscle. This value was then expressed as a percentage
of the mean size of a motor unit from the contralateral control muscle, obtained by the same method.

X) Glycogen Depletion

In some experiments where the maximum tetanic tension of fibres innervated by specific v.r. had been investigated a technique of glycogen depletion was employed to identify the muscle fibres in one of the v.r., innervating the operated soleus muscle. Glycogen in the motor units was depleted by repetitive stimulation of the muscle at 40Hz for 250msecs. Stimulation usually took 45-60 minutes or until the tetanic contractions obtained in response to stimulation had been reduced to zero.

XI) Histology and Immunohistochemistry

Following the physiological measurements of muscle tension the operated and contralateral soleus muscles of the experimental animal were removed, weighed and prepared for histology.

The operated and contralateral muscles were mounted side by side against a pin embedded in a cork block. The muscles were stretched to resting length, covered in tissue fixing compound (OCT compound, BDH), quickly frozen in melting isopentane, and cooled with liquid nitrogen. Muscles were wrapped in aluminium foil and stored at -70°C until required for sectioning. Control and operated muscles were mounted side by side on the cork so that they could be treated together. Muscle sections of both the operated and control muscles were consequently of equal thickness and subjected to identical histological regimes, thus
facilitating unbiased treatment of operated and control muscles.

Transverse sections from the middle third of each muscle block were cut within two weeks of freezing. 12 μm transverse sections were cut on a cryostat at -22°C and picked up on either pre-gelatinized or pre-polylysined glass slides. Sections were air dried for 30 minutes and slides wrapped and stored at -20°C, until processed for muscle histology.

i) Haematoxylin-van Gieson stain

Muscles were routinely stained for Haematoxylin-van Gieson (H/V) using the method of Culling (1963) to check for damage of muscle fibres as a result of α-BTX treatment or mechanical damage of the silicone rubber strips. H/V stains showed that there was no degeneration or swelling of muscle fibres. As a result of toxin treatment or the control experimental procedure there was a small amount of connective tissue present around the area of the muscle which had been in contact with the silicone implant (see Figure 1).

Haematoxylin stains muscle nuclei grey and cytoplasm yellow, whilst the fuchsin in the van Gieson's stains connective tissue red.

ii) Periodic-acid Schiff stain

The Periodic acid-Schiff (PAS) stain (Pearse, 1960) was used to demonstrate the presence of glycogen in muscle fibres. The PAS stain depends on the oxidation of 1:2 glycol groups (CHOH-CHOH) by periodic acid resulting in the formation of dialdehydes. The dialdehydes then react with the Schiffs (fuchsin sulphuric acid) to produce a pink-purple stain. Glycogen, as well as other polysaccarides, are stained a pink-purple colour.
iii) Succinic-Dehydrogenase stain

The succinic-dehydrogenase stain (SDH) was used to indicate the oxidative capacity of muscle fibres. Succinic-dehydrogenase is a substrate-specific oxidative enzyme which is found on inner mitochondrondial membranes.

The SDH stain employs a colourless soluble tetrazolium salt (Nitroblue tetrazolium) which is reduced to a deeply coloured formazan product (blue-purple colour) deposited at the site of the enzyme activity. Slow fibres stain more darkly than other fibres as they have more mitochondria.

The method of Nachlas et al (1957) was employed.

iv) Myosin ATPase Stain

According to the method of Round et al. (1980). The ATPase enzyme present within muscle fibres is especially dependent upon the influence of pH. Therefore, the pH effect has been traditionally been used to demonstrate different fibre types by preincubating the tissue section at various pH values. Alkaline pre-incubation was employed in these experiments; as a result slow fibres are unstained and the other muscle fibre types are recognized by a dark reaction product.

v) Slow Myosin Antibody Stain

A slow myosin antibody (a gift from Dr. Dhoot) recognizing a slow myosin heavy chain was used to identify specific myosin types of operated muscle fibres. See Appendix III for detailed methods.

XII) Measurements of Muscle Fibre Number & Size

In several animals, the total number of muscle fibres in each
soleus muscle was counted, using a camera lucida device attached to a microscope. In those muscles in which the muscle fibres innervated by one specific ventral ramus had been depleted of glycogen, cryostat cut sections were stained for glycogen using the PAS method (Pearse, 1960). The number of depleted muscle fibres innervated by one specific ventral ramus were white, those which were undepleted were pink. The number of muscle fibres innervated by each ventral ramus and the total number of muscle fibres in each muscle was then counted using the camera lucida device (Figure 2).

In some cases the cross sectional area of a random sample of muscle fibres in specific muscles was measured. Random sampling was achieved by dividing the muscle, by means of a grid, into a number of small squares in columns and rows. Rows of fibres were selected across the muscle section at various intervals and in each muscle the area of 300-400 muscle fibres (approximately 10-15% of the total number of fibres) was calculated by tracing around the fibre perimeter, using a camera lucida device attached to a microscope. The areas from these drawings were transferred to a graphics tablet attached to an Apple II computer. The mean fibre area, and the size distribution was then calculated for each of the muscles.
Photomicrograph of a transverse section (12 μm) of an adult rat soleus muscle. The soleus muscles of newborn rats were treated at birth with α-BTX, 2-10 months later the animals were subjected to isometric tension recordings. The L4 and L5 ventral rami were stimulated and the contractions recorded on an oscilloscope screen. In some experiments the muscle fibres innervated by one of the ventral rami were depleted of their glycogen stores by repetitive stimulation through one of the ventral rami. The muscles were then removed, frozen, sectioned and subjected to histological examination. The periodic-acid Schiff's stain stains polysaccharides (including glycogen) pink. The number of muscle fibres innervated by each of the ventral rami could then be distinguished, depending on their staining with the PAS stain, and counted using a camera lucida device attached to a light microscope. Scale bar = 100 μm.
Figure 2
RESULTS

The experiments performed in this Chapter attempt to look at the importance of early interaction between the nerve and muscle in the normal development and maturation of the soleus muscle. In these experiments neuromuscular activity to the soleus muscle was reduced either i) temporarily, or ii) for a more prolonged period of time, immediately after birth when the muscle and nerve are still developing and the contacts between the two are relatively immature and weak.

The investigations covered in this Chapter followed two paths, i) the effects of paralysis at birth on the development and maturation of the muscle, and ii) the effects of paralysis at birth on the pattern of innervation of the adult soleus muscle.

I) Initial Effects of α-BTX Paralysis

1) Efficacy of Neuromuscular Paralysis

Initially, a behavioural study was undertaken, using visual criteria, to assess the extent of paralysis of the soleus muscle, caused by the application of α-BTX at birth. Paralysis of the soleus muscle resulted in disuse of the hindlimb by the experimental animal and was manifest in exaggerated dorsiflexion of the ankle. In addition, the spontaneous movements of the hindlimb were observed. When the operated animal was held in the air by the tail, limited or no, toe-spreading reflex was elicited from the α-BTX treated leg when compared to the unoperated control leg and also compared to normal unoperated
littermates. Control animals treated with NaCl containing implants showed no impairment of spontaneous movement or use of the hindlimb.

In any cases where the α-BTX treated animal was able to use the operated leg in the normal manner, incomplete paralysis was assumed and the animal killed, with an overdose of anaesthetic. Occasionally, systemic spread of the toxin resulted in breathing difficulties of the experimental animals (20% of cases). Any animal showing signs of respiratory distress was killed. Similarly any animal which did not gain weight, relative to littermates, in the days following the operation was killed with an overdose of anaesthetic.

To quantify the extent of paralysis following α-BTX treatment, the operated soleus muscles were removed from the animals, at various time intervals after implantation, and *in vitro* physiological tension recordings performed. Twitch and tetanic contractions were elicited from the preparations either indirectly, by stimulating the cut end of the motor nerve, or by direct stimulation of the muscle via silver electrodes placed either side of the muscle. The ratio of indirectly/directly elicited maximum tetanic tension was established as an indication of the extent of paralysis of the preparations. The recovery from paralysis is demonstrated in Figure 3.

Soleus muscles examined 18 hours after the implantation of the α-BTX were 100% paralysed. The preparations exhibited a ratio of indirectly/directly elicited tetanic tension of 0.00 ± 0.02 (± s.e.m., n=6). The treated muscles subsequently recovered from the paralysis. Twenty four hours after implantation the preparations exhibited a ratio of indirect/direct tetanic tension of 0.19 ± 0.09 (± s.e.m., n=3); so that 24 hours after treatment with α-BTX, 81% of the muscle fibres were
Figure 3: Recovery of the Soleus Muscle of Newborn Rats From $\alpha$-Bungarotoxin Induced Paralysis at Birth

Muscle tension was measured *in vitro* at early ages after paralysis, and *in vivo* at later stages. Isometric muscle contractions were elicited indirectly by stimulating the cut end of the sciatic nerve with bipolar silver electrodes, using a pulse width of 20 $\mu$s. Alternatively the muscle was stimulated directly via silver electrodes placed either side of the muscle, using a pulse width of 2 ms. The amount of tension developed (in grammes) was then calculated and the ratio of indirectly/directly elicited tetanic tension calculated. This bar chart summarizes the ratio of indirect/direct tetanic tension of paralysed muscles immediately after treatment with $\alpha$-BTX and follows the time course of the recovery from paralysis. The mean $\pm$ s.e.m. are represented in this diagram. The n values are: 18h (n=6), 24h (n=3), 48h and 5d (Greensmith, L; PhD thesis) and 9d (n=4).
Figure 3

Ratio of indirect / direct tetanic tension

Time after treatment

still paralysed. By 2 days, post-treatment, 52% of the muscle fibres had recovered from the paralysis (Greensmith & Vrbova, 1991).

α-BTX binds specifically and irreversibly to the AChR (Jones & Salpeter, 1983) resulting in a failure of neuromuscular transmission. Recovery from paralysis, as observed in the soleus muscles, is likely to be a consequence of new AChR's being incorporated into the developing endplate region of the neonatal soleus muscle. As a result, the extent of paralysis of the treated muscle is reduced. By postnatal day 9 the treated muscles had fully recovered from the paralysis induced at birth. The ratio of indirect/directly elicited tetanic tension in 9 day old treated muscles was $1.00 \pm 0.11$ (± s.e.m., n=4). Figure 4 is an example of traces of 9 day old α-BTX treated soleus muscles. Noticeably muscles treated shortly after birth with α-BTX were unable to maintain tetanic tension at frequencies above 40 Hz. Figure 4 shows an example of this inability to maintain tetanus.

In normal age-matched muscles, and NaCl-treated muscles the ratio of indirect/direct tetanic tension was always above 0.95. Contralateral controls were not used to compare the effects of paralysis on the operated leg as it has previously been found that systemic effects of the toxin affect the contralateral leg in an unpredictable manner (Greensmith, L; PhD thesis, 1989).

As to be expected, treatment at birth with α-BTX also affects the maximum tetanic tension produced by the muscles, as a result of direct stimulation. Directly elicited maximum tetanic tensions produced by treated muscles was significantly less than those elicited from normal muscles. Greensmith (1989) demonstrated that even at 5 days of age, the effects of paralysis were still apparent, with less tension developed
Figure 4: Isometric Tension Recordings from 9 day old Rat Soleus Muscles Following Paralysis at Birth

Typical examples of traces of isometric muscle contractions from 9 day old soleus muscles. Soleus muscles of newborn animals were paralysed 6-12 hours after birth. Subsequently, in vitro and in vivo physiological recordings of muscle tension were made as an indication of the extent of paralysis of the treated muscles. Trace a) is an example of a directly elicited maximum tetanic contraction from a 9 day old soleus muscle, following α-BTX treatment at birth. Direct tetanic tension was elicited by placing bipolar silver electrodes either side of the muscle, and applying a stimulus with a pulse width of 2ms. Trace b) is an example of an indirectly elicited maximum tetanic contraction from the same 9 day old soleus muscle. Indirect tetanic tension was elicited by stimulating the cut end of the sciatic nerve with bipolar silver electrodes, using a pulse width of 20 μs. Traces a) and b) demonstrate that by 9 days of age the soleus muscles had fully recovered from α-BTX-induced paralysis. However, at 9 days of age the muscle was still unable to maintain contractions at higher frequencies, as a result of indirect stimulation.
Figure 4

a)

2g

b)

2g

200 msec
by the treated muscles. Treated muscles were capable of producing only 54% of that produced by normal age-matched muscles. However, by 9 days of age the maximum tetanic tension developed by treated muscles was $4.38 \pm 0.88g$ ($\pm$ s.e.m., $n=4$) compared to $5.22 \pm 0.24g$ ($\pm$ s.e.m., $n=21$) developed by normal soleus muscles of the same age, which is not significantly different (Mann-Whitney U-test, $p=0.37$). This initial decreased force output is due to the delayed development and growth of the paralysed muscles.

ii) Effects of Paralysis Revealed By Light Microscopy

Staining of transverse sections from operated muscles with Haemotoxylin-van Gieson stain at 7, 14, 21 and 28 days of age following $\alpha$-BTX treatment, demonstrated that implantation of the rubber strip containing $\alpha$-BTX or NaCl causes no damage to the developing muscle fibres. Muscle nuclei were always placed peripherally and the only visible effects of the implant on the soleus muscle was the presence of red stained connective tissue. Figure 5 is an example of an $\alpha$-BTX treated soleus muscle stained with Haemotoxylin-van Gieson's stain at 14 days of age.

Transverse sections of $\alpha$-BTX treated muscles and unoperated age-matched soleus muscles (from littermates) were reacted for SDH activity at 7, 14, 21 and 28 days of age. The SDH stain gives an indication of the oxidative enzyme activity of the muscle fibres. These enzymes are considered to be related to muscle activity (Kugelberg & Lindegren, 1979). Transverse sections of soleus muscles, up to 14 days of age, consistently showed less intense staining compared to normal unoperated control muscles. Some preparations demonstrated this reduced level of
Figure 5: Transverse Section of 14 Day Old Soleus Muscle Treated at Birth With α-BTX, Stained With Haemotoxylin-van Gieson’s Stain

Soleus muscles of newborn rats were treated at birth with α-BTX. At various time intervals following α-BTX-induced paralysis the muscles were removed, frozen and sectioned. 12 μm transverse sections of the operated soleus muscles were stained with Haemotoxylin-van Gieson’s stain (H/G). This figure is a microphotograph of 14 day old soleus muscle treated at birth with α-BTX and stained with H/G at 14 days. It demonstrates that no permanent damage has occurred to the muscles fibres as a result of α-BTX treatment at birth. Muscle nuclei are stained grey and can be seen to be placed peripherally. Connective tissue found in the area of the implant is stained red. Scale bar = 10 μm.
Figure 5
oxidative activity in the area of the implantation up to 28 days of age. These results are shown in Figure 6.

II) The Effects of Paralysis at Birth on the Development of Soleus Muscles

i) Muscle Tensions and Weights

In one group of animals soleus muscles of newborn rats were paralysed at birth with α-BTX, using a single implant. In another group of animals paralysis was prolonged by further treatment with α-BTX 3 days later. Control animals were subjected to treatment with NaCl.

Two to ten months later, when paralysis had long worn off the animals were reanaestheized. Indirectly elicited twitch and tetanic tensions were recorded from operated and contralateral control muscles by stimulation of their motor nerves. Figure 7 shows examples of isometric tension recordings from the control and operated muscles 2-10 months after the operation. For each individual experiment the maximum tetanic tension of the operated muscle was expressed as a percentage of that produced by the contralateral control soleus muscle. The results are summarized in Table 1. From Figure 7 it can be seen that soleus muscles paralysed at birth, and examined in adulthood develop less tension than NaCl-treated muscles; a more prolonged paralysis results in an even greater reduction in the tension output of the adult soleus muscles. Soleus muscles treated with NaCl at birth produce 116 ± 7% (± s.e.m., n=7) of tension when compared to their contralateral control muscles. Though the tension produced by NaCl-treated soleus muscles appears to be greater than that produced by normal control soleus...
Soleus muscles were paralysed at birth with α-BTX. Muscles were removed at various time points after paralysis, frozen, sectioned and reacted for SDH activities using the method of Nachlas et al (1957). Photomicrograph a) is taken from a 7 day old control soleus muscle, and b) is from a littermate treated with α-BTX at birth. Photomicrograph c) is taken from a 14 day old control soleus soleus muscle, whilst d) is from an age-matched, littermate treated with α-BTX at birth. Scale bar = 20 μm.

Photomicrograph e) is taken from a 28 day old control soleus muscle, and f) is from a littermate treated with α-BTX at birth. This Figure demonstrates that at 14 days of age, after α-BTX treatment at birth the operated muscle had less SDH activity compared to control muscles. Even at 28 days of age, muscles treated with α-BTX showed less SDH activity compared to controls. Scale bar = 50 μm.
Figure 7: Isometric Tension Recordings of Adult Soleus Muscles after \( \alpha \)-Bungarotoxin Treatment at Birth

Examples of records of single twitch and tetanic contractions (20, 40, 80Hz) from the soleus muscles of adult experimental rats. Contractions were elicited by stimulation of the soleus motor nerve with bipolar silver electrodes, with a pulse width of 0.02msec. Trace a) is a contralateral control of b) which is from an animal that had a NaCl implant alongside the soleus muscle at birth. Trace c) is a contralateral control of d) which is from an animal where \( \alpha \)-BTX was implanted alongside the soleus muscle at birth. Trace e) is a contralateral control of f) which is from an animal where an \( \alpha \)-BTX implant at birth was followed 3 days later by a further \( \alpha \)-BTX implant.
Figure 7

a) 50g

b) 50g

c) 50g

d) 50g

e) 100g

f) 50 msec

50msec
Table 1: **Maximum Tetanic Tension and WetWeights of Adult Soleus Muscles After Paralysis at Birth**

The soleus muscle of newborn rats were treated at birth with α-BTX or NaCl. Two to ten months later the animals were prepared for isometric tension recordings. The maximum tetanic tension was measured from the control and operated soleus muscles by stimulating the cut end of the sciatic nerve with bipolar silver electrodes, using a pulse width of 0.02msecs. Following the tension recording experiments the muscles were removed and weighed (grammes). This table summarizes the mean ± s.e.m. of the maximum tetanic tension (grammes) and the wet muscle weight (grammes) from the experimental animals. In addition, the tetanic tension and weight of the operated muscles were expressed as a percentages of the contralateral control muscles for each animal.
### Table 1

#### MAXIMUM TETANIC TENSION (grammes)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALATERAL</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX</td>
<td>13</td>
<td>137.8±12.4</td>
<td>99.3±11.2</td>
<td>74%</td>
</tr>
<tr>
<td>2BTX</td>
<td>4</td>
<td>206.7±13.5</td>
<td>88.7±6.8</td>
<td>43%</td>
</tr>
<tr>
<td>NaCl</td>
<td>7</td>
<td>122.3±9.6</td>
<td>142.1±10.5</td>
<td>117%</td>
</tr>
</tbody>
</table>

#### MUSCLE WEIGHT (wet weight in grammes)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALATERAL</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX</td>
<td>13</td>
<td>0.169±0.01</td>
<td>0.150±0.01</td>
<td>88%</td>
</tr>
<tr>
<td>2BTX</td>
<td>6</td>
<td>0.250±0.02</td>
<td>0.198±0.04</td>
<td>64%</td>
</tr>
<tr>
<td>NaCl</td>
<td>9</td>
<td>0.163±0.01</td>
<td>0.161±0.01</td>
<td>101%</td>
</tr>
</tbody>
</table>
muscles the apparent increase in tension output is not significant (Mann-Whitney U-test). Muscles treated with α-BTX at birth produced less tension, 74 ± 4.5% (± s.e.m., n=13) than unoperated, contralateral control muscles. This reduction in tension output, as a result of temporary paralysis at birth, is significantly different from normal muscles (Mann-Whitney U-test, p<0.01). More prolonged treatment with α-BTX (2BTX) has a more pronounced effect on the tension output of the operated muscles. The tension developed by muscles subjected to prolonged paralysis (2BTX) was considerably smaller, 43 ± 1.3% (± s.e.m., n=5), and significantly different from normal untreated muscles (Mann-Whitney U-test, p<0.01). Noticeably muscles subjected to prolonged paralysis (2BTX) also produced significantly less tension than those paralysed for a short period of time (BTX) (Mann-Whitney U-test, p<0.01). It is apparent from Figures 7 and 8 that BTX and 2BTX muscles are weaker than unoperated contralateral muscles.

The contralateral control muscles of animals subjected at birth to more prolonged paralysis (2BTX), appear to develop more tension than the contralateral control muscles of temporarily paralysed muscles (BTX) and NaCl-treated muscles (see Table 1). The larger tension output of 2BTX muscles, both operated and control, is probably due to the fact that the animals in that group were all males and relatively large. As a result, the average tension output of 2BTX muscles was larger than that from soleus muscles of animals in other experimental groups.

At the end of the physiological tension recording experiments the operated and control soleus muscles were removed from the animals and weighed. The effects of paralysis at birth, already demonstrated in
Figure 8: Maximum Tetanic Tension and Muscle Weights of Adult Rat Soleus Muscles After Paralysis at Birth

Block diagram a) is of the effects of paralysis at birth on the maximum tetanic tension of adult soleus muscles. The maximum tetanic tension of each treated soleus muscle obtained in response to stimulation of the soleus nerve is expressed as a percentage of its contralateral control muscle tension. Block diagram b) is of wet muscle weight from adult soleus muscles treated at birth with NaCl or with α-BTX. All animals were examined 2-10 months after treatment, and then their muscles removed and weighed. All error bars are + s.e.m.
Figure 8

a) Max. Tetanic Tension

b) Wet Muscle Weight
terms of tetanic tension, were paralleled in the weights of these operated muscles. Muscle weights were expressed as a percentage of the contralateral control muscles and are presented in Table 1. Figure 8 illustrates the changes in muscle weight and demonstrates that reductions in muscle weights and tetanic tensions paralleled one another in the α-BTX treated muscles, though the change was less than that seen in tension output. The muscles of animals temporarily paralysed at birth with α-BTX (BTX) when examined in adulthood weighed 88 ± 3.7% (+ s.e.m., n=13) of contralateral controls and those subjected to more prolonged paralysis weighed significantly less, 64 ± 3.7% (+ s.e.m., n=6). Both of these groups of treated muscles weigh significantly less than NaCl-treated muscles (Mann-Whitney U-test, p< 0.05 and p< 0.001, respectively). Control muscles treated with NaCl at birth showed no significant difference between operated and control 101.2 ± 4.4% (+ s.e.m., n=9) (Mann-Whitney U-test). Thus, these results are consistent with the findings obtained by tension recordings.

ii) Contractile Properties

The TTP and 1/2RT were measured in animals in all experimental groups. The operated values were expressed as a percentage of the control muscles and are summarized in Table 2. Table 2 shows that α-BTX treatment at birth, whether temporary (BTX) or for a more prolonged period of time (2BTX), or treatment with NaCl at birth, has no significant effects on the time-to-peak measurements (Mann-Whitney U-test). However, the 1/2 relaxation measurements are affected following treatment at birth for a prolonged time period. 2BTX treated soleus muscles relax significantly slower than controls (Mann-Whitney U-test,
Table 2: Contractile Properties of Adult Rat Soleus Muscles Following Treatment at Birth With \( \alpha \)-Bungarotoxin

Soleus muscles of newborn rats were treated at birth with \( \alpha \)-BTX or NaCl. An additional group of animals was treated at birth with \( \alpha \)-BTX and then again 3 days later. Two to ten months after the initial operations the muscles were examined. This table presents the time-to-peak (TTP) and half relaxation time (1/2RT) values measured from single twitch contractions. In addition to the real values, the operated values were expressed as a percentage of the value obtained from the contralateral control muscles. All values are mean \( \pm \) s.e.m.
### Table 2

#### CONTRACTILE PROPERTIES

**TIME TO PEAK /msecs.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRAL.</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX</td>
<td>7</td>
<td>106.4 ± 8.4</td>
<td>98.8 ± 6.3</td>
<td>97 ± 11.3%</td>
</tr>
<tr>
<td>2BTX</td>
<td>4</td>
<td>76.0 ± 6.3</td>
<td>86.3 ± 8.5</td>
<td>118 ± 22.3%</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
<td>80.0 ± 11</td>
<td>75.0 ± 10</td>
<td>94 ± 3.3%</td>
</tr>
</tbody>
</table>

**HALF RELAXATION TIME /msecs.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRAL.</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX</td>
<td>7</td>
<td>96.0 ± 5.1</td>
<td>126 ± 20</td>
<td>123 ± 13.3%</td>
</tr>
<tr>
<td>2BTX</td>
<td>4</td>
<td>67.5 ± 4.7</td>
<td>110 ± 19</td>
<td>161 ± 18.9%</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
<td>111.0 ± 11.2</td>
<td>99 ± 13</td>
<td>91 ± 5.8%</td>
</tr>
</tbody>
</table>
iii) Motor Unit Numbers and Sizes

The lower tension output of the operated muscles demonstrated in the previous section may have been a consequence of a lower number of motor units in the operated muscles when compared to NaCl-treated controls. In order to assess if the lower tension output and weight of the transiently paralysed muscles is due to a lower number of motor axons innervating the operated muscles, estimates of the total number of motor units in the soleus muscles were made. Estimates were made by recording and counting the increments of twitch tension elicited by stimulation of the soleus muscle motor nerve by stimuli of increasing intensity. Figure 9 shows examples from such an experiment. In normal, control experiments estimates of the number of motor units in the soleus muscle ranged between 24-29 units, with a mean number of 26.9 ± 0.6 (± s.e.m., n=9). This value is in agreement with those reported by other workers (Gutmann & Hanzlikova, 1966; Close, 1967; Gorio, et al, 1983; Fisher, et al, 1989; Vrbová & Fisher, 1989). The mean number of motor units in BTX treated muscles was 27.5 ± 1.6 (± s.e.m., n=4), with values varying between 22-30. These estimates of motor unit numbers in unoperated and temporarily paralysed muscles are not significantly different from one another (Mann-Whitney U-test, p= 0.35). However, in soleus muscles subjected to prolonged paralysis (2BTX) at birth the number of motor units was much smaller and varied between 13-16, with a mean of 14.3 ± 0.6 (± s.e.m., n=4). The number of motor units in 2BTX treated muscles is significantly different from that in normal control muscles (Mann-Whitney U-test, p= 0.005).
Figure 9: Examples of Oscilloscope Traces used to Make Estimates of Motor Unit Numbers in Experimental Muscles

The number of motor units within muscles paralysed with α-BTX at birth was estimated. Estimates of the number of motor units were made by stimulating the soleus motor nerve with single pulses every 4 seconds. The stimulus intensity was gradually increased so that axons with different thresholds were successively activated and motor units successively recruited. The stepwise increments in twitch tension were recorded on an oscilloscope screen and subsequently photographed. Trace a) is from an animal treated at birth with NaCl, and has 29 motor units. Trace b) is from an animal treated at birth with α-BTX, and has 25 motor units. Trace c) is from an animal which was treated at birth and 3 days later with α-BTX, and has 13 motor units. All animals were examined 2-10 months after the initial treatment.
Figure 9

a. 50g

b. 50g

c. 25g

100 msec
To obtain more information about the motor units, the mean motor unit size was estimated. To determine changes in motor unit size in the operated muscles, a value for the average tetanic tension per motor unit was obtained for both operated and control muscles by dividing the maximum tetanic tension of each muscle by the number of motor units identified in the same muscle. The value obtained from the operated muscle was expressed as a percentage of the value of the contralateral muscle. Table 3 summarizes the results obtained from all the experimental groups. In normal animals this value is 100%.

In animals subjected to temporary paralysis (BTX) at birth, the motor unit size of the operated muscles was 69.4 ± 4.9% (± s.e.m., n=5), which is smaller than that of the control, unoperated muscle. The motor unit size in operated animals was significantly different from the sizes expected in normal animals (Mann-Whitney U-test, p<0.008). Animals subjected to more prolonged paralysis (2BTX) at birth, the motor unit size of the operated muscles was 84.3 ± 2.1% (± s.e.m., n=4) of control muscles. This is significantly different from that expected in normal animals (Mann-Whitney U-test, p<0.014). Indeed, the motor unit size of muscles treated at birth with α-BTX for more prolonged periods (2BTX) is significantly different from that treated temporarily (BTX) (Mann-Whitney U-test, p>0.01).

iv) Muscle Fibre Numbers and Sizes

Tetanic tension and muscle weight results show that soleus muscles paralysed at birth produce less tension in adulthood than unoperated, control muscles. The relatively reduced tension output of α-BTX treated muscles may be due to effects of α-BTX on the development and growth of
Table 3: The Mean Motor Unit Size of Adult Soleus Muscles Following Paralysis at Birth

Following treatment at birth with α-BTX, the number of motor units in the adult soleus muscles was estimated. The average motor unit size was then calculated by dividing the maximum tetanic tension by the total number of motor units identified in that muscle. This value was then expressed as a percentage of the mean size of a motor unit in the contralateral control muscle. The expected value for normal muscles is 100%. The values are collated in this Table. All values are mean ± s.e.m.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>RANGE</th>
<th>MEAN</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTX</td>
<td>6</td>
<td>22-30</td>
<td>27.5 ± 1.6</td>
<td>69 ± 5%</td>
</tr>
<tr>
<td>2BTX</td>
<td>4</td>
<td>13-16</td>
<td>14.3 ± 0.6</td>
<td>84 ± 2%</td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>24-29</td>
<td>26.9 ± 0.6</td>
<td>100%</td>
</tr>
</tbody>
</table>
muscle fibres. To establish whether the reduced tension output of α-BTX treated muscles was due to a loss in muscle fibres or was as a result of extensive muscle fibre atrophy, the muscles were subjected to quantitative histological examination.

The total number of muscle fibres was counted in several contralateral control and operated muscles of 2-10 month old rats. Table 4 summarizes the results obtained from muscles examined in all experimental groups. The total number of muscle fibres, 2500-2900, counted in normal soleus muscles and in unoperated contralateral control muscles, is similar to that reported by other authors (Zelená & Hník, 1963; Frank et al., 1975; Kugelberg, 1976; McArdle & Sansone, 1977; Lowrie & Vrbova, 1984). In addition, the number of muscle fibres in the operated muscle was also expressed as a percentage of that in the contralateral control muscles. Figure 10 illustrates the results of muscle fibre counts, and it can be seen that permanent loss of muscle fibres occurred after temporary α-BTX treatment at birth. 19% of fibres is lost after temporary α-BTX treatment. The number of muscle fibres is significantly different from the number of muscle fibres in contralateral control muscles (Mann-Whitney U-test, p=0.036). With more prolonged paralysis at birth (2BTX) 47% of fibres in the operated muscles is lost. The total number of muscle fibres in 2BTX operated muscles is significantly different from the total number of muscle fibres in the contralateral control muscles (Mann-Whitney U-test, p=0.025). Muscle fibre numbers in NaCl treated muscles were not significantly different from normal untreated muscles, or from contralateral control muscles (Mann-Whitney U-test). In animals treated for a prolonged period (2BTX) the relative loss of muscle fibres (%con)
Table 4: The Number of Muscle Fibres in Adult Soleus Muscles Following Treatment at Birth with α-Bungarotoxin

The soleus muscles in the right leg of newborn rats were treated 6-12 hours after birth with α-BTX or NaCl. A second group of animals, treated at birth with α-BTX, were treated a second time with α-BTX, 3 days later (2BTX). 2-10 months after the initial operation the muscles were removed and frozen in freezing isopentane. 12 μm sections were cut on a cryostat and subsequently stained for glycogen using the PAS method. The total number of muscle fibres in the operated and control muscles was counted, using a camera lucida device attached to a light microscope. The values in the table are mean values ± s.e.m.
Table 4

TOTAL NUMBER OF MUSCLE FIBRES

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALATERAL (left)</th>
<th>OPERATED (right)</th>
<th>% OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>2750 ± 105</td>
<td>2735 ± 141</td>
<td>99.6%</td>
</tr>
<tr>
<td>BTX</td>
<td>8</td>
<td>2821 ± 234</td>
<td>2278 ± 200</td>
<td>81.1%</td>
</tr>
<tr>
<td>2BTX</td>
<td>5</td>
<td>2529 ± 174</td>
<td>1662 ± 225</td>
<td>56.8%</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
<td>2899 ± 239</td>
<td>2731 ± 194</td>
<td>94.6%</td>
</tr>
</tbody>
</table>
Figure 10: Number of Muscle Fibres in Adult Rat Soleus Muscles after α-Bungarotoxin Induced Paralysis at Birth

Muscles were paralysed at birth with α-BTX and the animals left to mature. Two to ten months later the muscles were removed, frozen and sectioned. 12 µm transverse sections were stained with Haemotoxylin-van Gieson stain and the number of muscle fibres in the operated and control muscle counted using a camera lucida device attached to a light microscope. The number of fibres in each operated muscles was expressed as a percentage of the number of muscle fibres in the contralateral control muscle. The block diagram summarizes the results, the mean of each group is shown and the error bar is ± s.e.m. The number of rats in each group, from left to right, is n = 5, 8, 5, 5.
Figure 10

Total number of Muscle fibres (% OP/CON)

- Normal
- BTX
- 2BTX
- NaCl

Treatment at birth
is significantly different from that seen in muscles treated temporarily with $\alpha$-BTX (BTX) (Mann-Whitney U-test, p=0.003). These values of muscle fibre counts are in close agreement with the values of innervation assessed physiologically. It therefore appears that the reduction in tension developed by operated muscles is caused by a smaller than normal number of muscle fibres innervated by the motoneurones to the soleus.

To detect any changes in muscle fibre area and size distribution as a result of early postnatal muscle paralysis, the mean cross-sectional area of the adult control and operated muscles was calculated. Table 5 illustrates the mean muscle fibre area of operated and contralateral control muscles in each experimental group. Although there is no obvious difference in the mean muscle fibre area of the 3 experimental groups, there is a change in the size distribution of the muscle fibre areas. This is highlighted in Figures 11 and 12. In Figure 11 transverse sections of operated and contralateral control muscles, stained with Haemotoxylin-van Giesen, illustrate the differences in the cross-sectional areas of the adult muscles. Figure 12 shows typical frequency distributions of muscle fibre cross-sectional areas from animals in each experimental group. For each animal the distribution of control and operated muscles are superimposed upon one another. There is little difference between the distribution of operated and control muscles treated with NaCl (Figure 12c). Muscles paralysed at birth with $\alpha$-BTX display a wider spread in the distribution of muscle fibres (Figure 12a); larger and smaller fibres are present in the operated muscle compared to the contralateral control muscle. Muscles subjected to more prolonged paralysis at birth (2BTX) display an even greater spread in the distribution of muscle fibre area (Figure 12b).
Table 5: Muscle Fibre Areas of Rat Soleus Muscles Following Paralysis at Birth

Soleus muscles of newborn rats were paralysed at birth with α-BTX. Two to ten months later, the muscles were removed, frozen, sectioned and stained with Haemotoxylin-van Gieson's stain. The perimeter of a 10% random sample of muscle fibres (approximately 300 muscle fibres) was traced using a camera lucida device attached to a light microscope. Samples were taken from operated and contralateral control muscles. This data was then transferred to an Apple II computer via a graphics tablet, and the cross-sectional area calculated. Values of muscle fibre area (μm²) are presented in this table. In addition, the area of muscle fibres on the operated leg are expressed as a percentage of that measured from the contralateral control muscle.

<table>
<thead>
<tr>
<th>Time (months)</th>
<th>Mean Fibre Area (μm²)</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.34</td>
<td>78.5%</td>
</tr>
<tr>
<td>10</td>
<td>1.98</td>
<td>75.2%</td>
</tr>
<tr>
<td>TREATMENT</td>
<td>N</td>
<td>CONTRALATERAL</td>
</tr>
<tr>
<td>-----------</td>
<td>---</td>
<td>---------------</td>
</tr>
<tr>
<td>BTX</td>
<td>2</td>
<td>1410.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4931.6</td>
</tr>
<tr>
<td>2BTX</td>
<td>4</td>
<td>5214.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6526.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3555.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3070.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>2</td>
<td>2662.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3287.6</td>
</tr>
</tbody>
</table>
Figure 11: **Transverse Sections Taken from Adult Soleus Muscles Following Paralysis at Birth Stained with Haemotoxylin-van Gieson’s stain**

Soleus muscles were treated at birth with either $\alpha$-BTX or NaCl. A second group of animals, initially treated with $\alpha$-BTX at birth, were treated 3 days later with $\alpha$-BTX. Two to ten months after the treatment the muscles were examined. Transverse sections (12 µm) were stained with Haemotoxylin-van Gieson’s stain. Using a *camera lucida* device attached to a light microscope the perimeter of 10% of the total number of muscle fibres was traced. This information was transferred, using a graphics tablet, to an Apple II computer which calculated the area of the random sample of muscle fibres.

The photomicrographs presented in this Figure demonstrate the changes in fibre areas seen in operated muscles. Photo. a) is taken from an adult soleus muscle paralysed at birth with $\alpha$-BTX, b) is the contralateral control muscle. Photo. c) is taken from a soleus muscle paralysed at birth with $\alpha$-BTX and then subjected again to $\alpha$-BTX 3 days later; photo. d) is the contralateral control muscle. Photo. e) is taken from an adult soleus muscle treated at birth with NaCl, whilst f) is from the contralateral control muscle. Scale bar = 100 µm.
Figure 11

a

b

c
d
e
f
Soleus muscles were paralysed at birth with α-BTX. Two to ten months later, the muscles were removed, frozen, sectioned and stained with Haemotoxylin-van Gieson's stain. The perimeter of a 10% random sample (approx. 300 fibres) was traced using a camera lucida device and transferred to an Apple II computer via a graphics tablet. The areas (μm²) of these muscle fibres was calculated and plotted in a frequency histogram. The frequency histograms of the operated and contralateral control muscles for a single animal were superimposed upon each other. In all cases the hatched area on each histogram is from the contralateral control muscle and the unhatched area from the operated muscle. Histogram a) is an example of the distribution of muscle fibre areas from an animal treated at birth with α-BTX. Histogram b) is an example of the distribution of fibre areas from an animal treated with α-BTX (2BTX) for a more prolonged period of time. Histogram c) is an example of a NaCl treated muscle.
Figure 12

Muscle fibre area $\mu m^2$
v) Histology and Immunohistochemistry

The effects of paralysis at birth on muscle fibre type in the adult soleus muscle was investigated. 12 μm transverse muscle sections were stained for succinic dehydrogenase (SDH) activity. In contralateral control soleus muscles all the fibres stained for SDH activity. The muscles were composed mainly of large fibres of intermediate staining intensity, interspersed with fewer small, darker stained fibres (Figure 13a). After temporary β-BTX induced paralysis there were fewer small darkly stained fibres and the level of staining for SDH appeared to be less in the operated muscle compared to the control muscles (Figure 13c). More prolonged paralysis with α-BTX (2BTX) at birth resulted in a more marked difference in the SDH activity. 2BTX treated muscles demonstrated a paler staining for SDH activity in the remaining large fibres (Figure 13e) compared to normal unoperated control muscles and BTX treated muscles.

The alkaline pre-incubated fast myosin ATPase stain was performed on operated and contralateral control muscles. Normal unoperated muscles and NaCl treated muscles show a mixture of darkly stained and unstained fibres (Figure 13b). In muscles temporarily paralysed at birth with α-BTX most of the darkly stained fibres were lost. One or two darkly stained fibres were regularly seen in the operated muscles (Figure 13d). After more prolonged paralysis (2BTX), in adulthood none of the fibres were stained. Only rarely were such small darkly stained fibres seen. Figure 13f illustrates an example of these darkly stained fibres, and demonstrates the very small size of these fibres compared to the unstained fibres surrounding it.

10 μm transverse sections of operated and contralateral control
Figure 13: **Transverse sections of Adult Rat Soleus Muscles Following Treatment at Birth with α-Bungarotoxin or NaCl. Stained For SDH and ATPase Activities**

12 μm transverse sections of adult rat soleus muscles were stained for succinic dehydrogenase (SDH) and ATPase activities. Photomicrographs a), c) and e) are taken of sections stained for SDH activity. Photomicrograph a) is from a control muscles, c) is from a muscle paralysed at birth with α-BTX and e) from a muscle paralysed for a more prolonged period of time. Photomicrograph b), d) and f) are taken from sections stained for ATPase activity (alkaline pre-incubation). Photomicrograph b) is from a control muscle, d) from a muscle paralysed at birth with α-BTX and f) from a muscle paralysed for a more prolonged period of time. Scale bar= 50 μm.
muscles were stained with an antibody (96J, donated by Dr. Dhoot) against one of the slow myosin heavy chains (HC). Contralateral control muscle sections stained with the antibody demonstrated that approximately 20% of the total number of muscle fibres were antibody-negative. This is in contrast to sections from operated muscles. Soleus muscles paralysed at birth either temporarily, or for a more prolonged period of time, in adulthood were composed wholly of antibody-positive stained fibres. Figure 14 illustrates the different staining patterns of the slow myosin heavy chain seen in operated muscles compared to controls.

III) Rearrangement of the Contribution of Axons From L4 and L5

Ventral Rami After Paralysis at Birth

Since paralysis at birth reduces the tension output and the total number of muscle fibres in the adult soleus muscle, the next series of experiments were designed to investigate whether this loss is evenly distributed between the axons innervating the muscle (L4 or L5 axons).

Two to ten months after the initial operation the animals were reanaesthetised and prepared for in vivo physiological tension recordings, as before. The preparation of the animal was extended to include the dissection of the L4 and L5 ventral rami (v.r.). Tension experiments were performed stimulating the cut ends of the two ventral rami to elicit the proportions of the previously operated soleus muscles, which were innervated by axons emanating from each v.r.
Figure 14: Transverse Sections of Adult Rat Soleus Muscles Stained with a Slow Myosin Antibody, Following Paralysis at Birth

Soleus muscles were treated at birth with α-BTX either temporarily or for more prolonged periods. Two to ten months later, the adult muscles were removed from the animal and frozen. 12 μm transverse sections of adult rat soleus muscle were stained with an antibody against a slow myosin heavy chain (96J). Antibody-positive muscle fibres are darkly stained, relative to the unstained antibody-negative fibres. Photomicrograph a) is from a control muscle, b) from a muscle paralysed at birth with α-BTX and c) from a muscle subjected to more prolonged paralysis. Scale bar = 100 μm
i) Tetanic Tensions of Temporarily Paralysed Muscles Elicited Through the L4 or L5 V.R.

Table 6 summarizes the amounts of tension produced on stimulation of each v.r. in operated muscles in each experimental groups. In normal adult animals examined at 2-10 months of age, stimulation of the L5 v.r. elicited 70% of the maximal tetanic tension of the normal soleus muscle; stimulation of the L4 v.r. elicited 30% of the maximal tetanic tension of the soleus muscle. This result is similar to values reported by other authors. Lowrie et al. (1985) reported the proportion of tension produced by L4 of 20% and that of L5 of 80%. In adult muscles, unlike the situation in the neonatal muscles, there is virtually no overlap of territory of the two ventral rami (L4 and L5) innervating the soleus muscle. Figure 15 shows examples of isometric tension recordings from normal soleus muscle demonstrating the proportions of tension elicited by stimulating the L4 and L5 v.r.

In each experiment the tension produced by stimulating each ventral ramus was expressed as a percentage of the total tension produced by the operated muscle. Soleus muscles temporarily paralysed at birth (BTX) produced less tension in response to stimulation of the motor nerve than contralateral control muscles. When the proportion of tension produced on stimulation of the L4 and L5 v.r. was investigated, it became apparent that the contribution of each v.r. to the innervation of the operated soleus was also altered as a result of the paralysis at birth. After temporary paralysis, 44% of the operated muscle was innervated by axons emanating from the L4 v.r. and 66% from the L5 v.r. Figure 16 illustrates the results from these experiments and the changes in the proportion of the operated muscles innervated by axons emanating from
Table 6: Maximum Tetanic Tension Produced by Stimulating L4 and L5 Ventral Roots in Operated and Control Animals

The soleus muscles of newborn rats were paralysed 6-12 hours after birth with α-BTX or with NaCl. In some animals treated at birth with α-BTX the treatment was repeated 3 days later. Two to ten months after the initial operation, the animals were prepared for physiological recordings of muscle tension. Isometric contractions from the operated soleus muscle were elicited by stimulating the cut end of either the L4 or L5 ventral rami, or the sciatic nerve. Isometric contractions were elicited from contralateral control muscles by stimulating the cut end of the sciatic nerve. Bipolar silver electrodes were used, with 0.02-0.05msecs. width square wave pulses, with durations of 500-700 msecs. The mean ± s.e.m. of the maximum tetanic tension for each group, and via each route, are presented in this Table.
### Table 6

**MAXIMUM TETANIC TENSION / grammes**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALAT. (left)</th>
<th>OPERATED (right)</th>
<th>L4 (right)</th>
<th>L5 (right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>107.3±9.2</td>
<td>98.4±10.3</td>
<td>28.9±3.3</td>
<td>69.5±7.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>4</td>
<td>116.5±7.5</td>
<td>146.5±15.7</td>
<td>28.3±11.8</td>
<td>115.9±14.3</td>
</tr>
<tr>
<td>BTX</td>
<td>8</td>
<td>132.4±14.8</td>
<td>102.1±11.1</td>
<td>39.6±5.9</td>
<td>62.5±8.0</td>
</tr>
<tr>
<td>2BTX</td>
<td>2</td>
<td>233.3</td>
<td>98.0</td>
<td>67.6</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>242.4</td>
<td>103.5</td>
<td>76.8</td>
<td>26.8</td>
</tr>
</tbody>
</table>
Figure 15: **Isometric Tension Recordings From Adult Rat Soleus Muscles, Obtained By Stimulating the L4 and L5 Ventral Rami Separately**

Examples of typical traces of isometric muscle contractions from adult rat soleus muscles. Soleus muscles were paralysed 6-12 hours after birth with α-BTX and subsequently *in vivo* physiological recordings of muscle tension were made. Maximum tetanic contractions were elicited by stimulating either the cut end of the L4 or L5 ventral rami, via bipolar silver electrodes, using a pulse width of 0.02msecs. Contractions elicited by stimulating the experimental muscle through the L4 ventral ramus, L5 ventral ramus, or sciatic nerve, at 40Hz, were superimposed upon one another on an oscilloscope screen. This composite image was photographed and the amount of tension (grammes) produced via each route was calculated. Trace a) is from a normal animal, b) from an animal temporarily paralysed at birth with α-bungarotoxin and c) from an animal subjected to more prolonged paralysis (2BTX). The maximum tetanic tension elicited from each ventral ramus is marked on each trace. The upper trace is always that obtained from the operated muscle by stimulating the sciatic nerve.
Figure 15

a)

L5
L4

50g

b)

L5
L4

50g

c)

L4
L5

25g

100msec
Figure 16: **Percentage of Operated Adult Rat Soleus Muscle Innervated by Axons Emanating From the L4 and L5 Ventral Rami**

Block diagram representing the percentage of the operated soleus muscle innervated by axons emanating from the L4 and L5 ventral rami. Muscles were treated at birth with α-BTX (as previously described) then examined 2-10 months later. Isometric tension recordings were made by stimulating the soleus muscles through the L4 and L5 ventral rami, using bipolar silver electrodes, with a pulse width of 0.02msecs. The maximum tetanic tension elicited by stimulating a specific ventral ramus was expressed as a percentage of the total tension of the operated muscle, elicited by stimulating the sciatic nerve, in a similar manner. The mean ± s.e.m. are represented in this Figure and the n values are 7, 4, 8 and 2 respectively.
Figure 16

Maximum Tetanic Tension (% op/con)

Treatment at birth

Normal | NaCl | BTX | 2BTX

L4 | L5 | L4 | L5 | L4 | L5 | L4 | L5
the L4 and L5 v.r. In 2 animals subjected to more prolonged paralysis (2BTX) at birth, there is an even greater shift in the proportion of the operated muscle innervated by the 2 ventral rami. In the 2 animals studied, the L4 v.r innervated 69% and 74% of the operated muscles and the L5 v.r. innervated 29% and 26%, respectively, of the operated muscles.

These results however, do not take into account the fact, previously demonstrated, that the operated muscles produce less tension than unoperated contralateral control muscles and loose muscle fibres. The next step was to take into account the reduced tension output of the operated muscle compared to the contralateral muscle when calculating the proportion of the remaining muscle fibres innervated by the 2 ventral rami. This was achieved by expressing the maximum tetanic tension of the operated muscle as a percentage of the total force of the control muscle, and then calculating the proportion of this reduced tetanic tension innervated by axons emanating from one specific ventral ramus. These results are collated in Table 7, and Figure 17 illustrates the changes in distribution of innervation as a result of the paralysis at birth. In BTX treated muscles, stimulation of the L4 v.r. elicited 28.4 ± 3.5% (+ s.e.m., n=8) of the tension produced by the remaining muscle fibres; stimulation of the L5 v.r. elicited 46.1 ± 3.9% (+ s.e.m., n=8) of the tension produced by the reduced muscle mass. As previously demonstrated stimulation of the L4 v.r. in normal animals elicites 30% of the tension produced by the whole muscle and stimulation of the L5 v.r. elicits 70% of the total tension. It therefore appears that the reduction in tetanic tension of the operated muscle as a consequence of paralysis at birth occurs at the expense of the
Table 7: Proportion of Maximum Tetanic Tension of Operated Soleus Muscle Innervated by Axons from the L4 and L5 Ventral Rami

Table collating the proportion of the operated muscle innervated by axons emanating from the L4 and L5 ventral rami. Muscles were paralysed at birth and examined 2-10 months later. The maximum tetanic tension elicited by stimulating a specific ventral ramus was expressed as a proportion of the operated muscle and expressed as a percentage of the maximum tetanic tension produced by the contralateral control muscle. All values are mean ± s.e.m.
Table 7

PROPORTION OF MTT OF OPERATED MUSCLE AS % OF CONTRALATERAL CONTROL MUSCLE

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>L4 VENTRAL RAMUS</th>
<th>L5 VENTRAL RAMUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>30.3 ± 2.4</td>
<td>69.0 ± 5.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>4</td>
<td>27.4 ± 11.0</td>
<td>95.6 ± 8.1</td>
</tr>
<tr>
<td>BTX</td>
<td>7</td>
<td>28.4 ± 3.5</td>
<td>46.1 ± 3.9</td>
</tr>
<tr>
<td>2BTX</td>
<td>2</td>
<td>31.8</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.1</td>
<td>12.0</td>
</tr>
</tbody>
</table>
Figure 17: Proportion of the Maximum Tetanic Tension of the Operated Soleus Muscle Innervated by Axons From the L4 and L5 Ventral Rami as a Percentage of the Contralateral Control Muscle

Block diagram representing the proportion of the operated muscle innervated by axons emanating from the L4 and L5 ventral rami. Muscles were paralysed at birth and examined 2-10 months later (as previously described). The maximum tetanic tension elicited by stimulating a specific ventral ramus was expressed as a proportion of the operated muscle and then calculated as a percentage of the total tension produced by the contralateral control muscle. The mean ± s.e.m. are represented in this figure and the n values are 7, 4, 8 and 2 respectively.
Figure 17

Proportion of tetanic tension (% CON)

<table>
<thead>
<tr>
<th>Treatment at birth</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>140</td>
</tr>
<tr>
<td>NaCl</td>
<td>120</td>
</tr>
<tr>
<td>BTX</td>
<td>100</td>
</tr>
<tr>
<td>2BTX</td>
<td>80</td>
</tr>
</tbody>
</table>

L4
L5
muscle fibres innervated by axons emanating from the L5 ventral ramus (see also Table 7).

In 2 animals subjected to more prolonged paralysis at birth (2BTX) the results illustrates this point even further. In these 2 animals stimulation of axons emanating from the L4 v.r. elicited 27% and 31.8% of the reduced muscle tension; stimulation of the L5 v.r. elicited 13.1% and 11.2% respectively. Figure 17 illustrates these changes in the pattern of innervation of the operated muscles and demonstrates that the reduction in maximum tetanic tension as a consequence of paralysis at birth occurs solely at the expense of the fibres innervated by L5 motor axons.

ii) Motor Unit Numbers

Using the method previously demonstrated, the number of motor units in each ventral ramus was investigated. The results are summarized in Table 8. In normal and NaCl treated animals the L4 v.r. contains 8-12 motor units with an average of $9.3 \pm 0.7$ (± s.e.m., n=6); the L5 v.r. contains between 16-20 motor units, with an average of $17.2 \pm 0.7$ (± s.e.m., n=6). In muscles paralysed at birth with $\alpha$-BTX, estimates of the number of motor units in the L4 ventral ramus were between 6-13, with a mean of $10.5 \pm 1.0$ (± s.e.m., n=6). Investigation of the L5 ventral ramus gave estimates of 14-20, with a mean of $17 \pm 0.9$ (± s.e.m., n=6). These values for motor unit numbers are not significantly different from those made for normal and NaCl treated animals (Mann-Whitney U-test, at the 5% level). In the 2 animals subjected to more prolonged paralysis at birth estimates for the number of motor units in the L4 v.r. were 10 and 12, whereas estimates of the number of
Soleus muscles of newborn rats were paralysed with α-BTX. Two to ten months later estimates of the number of motor units within each ventral ramus innervating the adult soleus muscle were made. The cut end of each ventral ramus was stimulated by a single pulse every 4 seconds. By gradually increasing the stimulus intensity individual motor units were successively recruited, until all motor units had been recruited. A composite picture of the tensions recorded from each motor unit was obtained and the number of motor units counted.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>L4</th>
<th>L5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>9.3±0.7</td>
<td>17.2±0.7</td>
</tr>
<tr>
<td>BTX</td>
<td>6</td>
<td>10.5±1.0</td>
<td>17.0±0.9</td>
</tr>
<tr>
<td>2BTX</td>
<td>2</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>3</td>
</tr>
</tbody>
</table>
motor units in the L5 ventral ramus were 4 and 3 respectively. The number of motor units in the L4 v.r. was significantly different from that estimated for normal animals (Mann-Whitney U-test), and the estimate of the number of motor units in the L5 v.r. was significantly different from that estimated for normal animals (Mann-Whitney U-test, p = 0.03).

iii) Muscle Fibre Numbers

The number of muscle fibres innervated by each ventral ramus was investigated using the technique of glycogen depletion pioneered by Kugelberg (1976). The fibres innervated by one of the rami were depleted of their glycogen stores by repetitive stimulation of the ramus. Transverse sections were taken from frozen operated and contralateral control muscles and stained with the Periodic acid-Schiffs stain (see Figure 1). The number of stained and unstained fibres were counted. Table 9 summarizes these results. Figure 18 illustrates the proportions of muscle fibres innervated by each of the ventral rami in the operated muscle as a percentage of the number of muscle fibres in the contralateral control muscle. The results from muscle fibre counts indicate that when soleus muscles are paralysed at birth with α-BTX, then in adulthood the total number of muscle fibres is less than that in controls. This loss of muscle fibres appears to be at the expense of those muscle fibres innervated by L5 terminals (see Figure 18). This is consistent with the results obtained from tension experiments, observing maximum tetanic tensions elicited through each ventral ramus.

The results presented in this Chapter demonstrate that during the early postnatal period continued interaction between the nerve and
Table 9: Number of Muscle Fibres in the Adult Rat Soleus Muscle, Innervated with Axons From the L4 and L5 Ventral Rami, Following Paralysis at Birth

Soleus muscle of newborn rats were treated with α-BTX as previously described. Two to ten months later, the muscle fibres innervated by one of the ventral rami were depleted of their glycogen stores by repetitive tetanic stimulation. The muscles were then removed from the animal, frozen and sectioned. 12 μm transverse sections were cut on a cryostat and subsequently stained for glycogen using the PAS method. The total number of muscle fibres in the operated muscle was counted using a camera lucida device. The number of muscle fibres innervated by each ventral ramus was counted, differentiation based on the presence or absence of staining for glycogen. The values in the Table are mean ± s.e.m.
Table 9

<table>
<thead>
<tr>
<th>OPERATION</th>
<th>N</th>
<th>CONTRAL. (left)</th>
<th>OPERATED (right)</th>
<th>L4 (right)</th>
<th>L5 (right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>2749±189</td>
<td>2828±208</td>
<td>793±175</td>
<td>1896±172</td>
</tr>
<tr>
<td>NaCl</td>
<td>3</td>
<td>2887±35</td>
<td>2793±103</td>
<td>786±149</td>
<td>2007±76</td>
</tr>
<tr>
<td>BTX</td>
<td>5</td>
<td>2648±351</td>
<td>2327±283</td>
<td>753±102</td>
<td>1555±317</td>
</tr>
</tbody>
</table>
Figure 18: Proportion of the Total Number of Muscle Fibres in the Operated Soleus Muscle Innervated by Axons Emanating From the L4 and L5 Ventral Rami, as a Percentage of the Total Number of Muscle Fibres in the Contralateral Control Muscle

Block diagram representing the proportion of the total number of muscle fibres innervated by axons emanating from the L4 and L5 ventral rami. Muscles were paralysed at birth and examined 2-10 months later. The muscle fibres innervated by one of the ventral rami was depleted of their glycogen stores by repetitive stimulation via the specific ventral ramus. The muscles were then removed from the animal, frozen and sectioned. 12 µm transverse sections were cut on a cryostat and subsequently stained for glycogen using the PAS method. The number of muscle fibres innervated by axons from one of the ventral rami was expressed as a proportion of the number of fibres in the operated muscle, and then calculated as a percentage of the total number of muscle fibres in the contralateral control muscle. The mean ± s.e.m. are represented in this Figure and the n values are 4, 3, 5 and 2 respectively.
Figure 18

Proportion of total number of muscle fibres (% CON)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>NaCl</th>
<th>BTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Treatment at birth

100-1
80-
60-
40-
20-
0 -

Normal NaCl BTX
muscle are necessary for the normal development of both the muscle properties and its pattern of innervation. Temporary disconnection from the target results in the loss of muscle fibres and consequently the tension output of the muscle. More prolonged disconnection from the target results in more serious impairment of development. In addition, the results in this Chapter demonstrate that the loss of muscle fibres seen following temporary disconnection between the nerve and muscle in the early postnatal period occurs predominantly at the expense of muscle fibres innervated by terminals from the L5 spinal ventral root.
DISCUSSION

In the experiments presented in this Chapter target activity was temporarily prevented in newborn rat soleus muscles by applying α-bungarotoxin. The present results show that short term interruption between the neonatal nerve and muscle causes long term impairment in the adult muscle, principally due to a loss of muscle fibres. Neonatal soleus muscles in which nerve-muscle activity was prevented for a more protracted period were more seriously impaired in adulthood; these adult muscles presented a more extensive loss of tension and muscle fibres. The contractile properties of the adult soleus muscle were also affected as a consequence of a short term disconnection from its motoneurones in the early postnatal period. In addition, adult muscles treated at birth with α-BTX possess lower oxidative capacity than controls and are composed completely of slow myosin heavy chain-positive fibres.

The characteristic properties of specific muscle fibres develop under the influence of their innervation. Motor nerves are able to determine the properties of undifferentiated muscle fibres. If myotubes do not become innervated, their differentiation is arrested and they atrophy and become replaced by fat (Hamburger, 1928; Drachman et al., 1968). In rat soleus muscles primary myotubes become slow fibres and secondary myotubes give rise to fast fibres (Rubinstein & Kelly, 1981). Soleus muscles paralysed at birth with α-BTX develop into adult muscles which are composed wholly of slow myosin positive fibres. Removal of the activity of neonatal soleus muscle by α-BTX treatment may be arresting the development of any secondary myotubes present in the soleus muscle immediately following birth. However, it seems likely that the cause of
different contractile and enzymic properties observed in the soleus muscles following $\alpha$-BTX treatment is a consequence of an altered pattern of innervation.

The results presented in this Chapter demonstrate that active communication between the neonatal nerve and muscle is necessary for the establishment of the normal adult pattern and distribution of innervation of the soleus muscle of the rat. Temporary paralysis of the rat soleus muscle at birth results in a change in the distribution of innervation in the adult muscle compared to that seen in a normal animal. The loss of muscle fibres seen after temporary disconnection does not appear to be the result of a random loss of fibres, instead the loss of fibres is biased towards those innervated by terminals from the L5 v.r. If disconnection from the target is prolonged during the early postnatal period, then a greater number of fibres is lost; again this appears to be at the expense of those fibres innervated by terminals of axons emanating from the L5 v.r., since the proportion of tension developed by stimulating the L5 v.r. is greatly reduced compared to controls.

A possible explanation of the loss of muscle fibres innervated by terminals from the L5 spinal root may be the consequence of the proximity of the muscle fibres to the implant. Muscle fibres in the newborn soleus muscle in closest proximity to the $\alpha$-BTX containing implant will be paralysed for the longest period of time. Indeed, staining for succinic dehydrogenase (an enzyme of oxidative metabolism and thought to be an indicator of the level of muscle fibre activity (Kugelberg & Lindgren, 1979)) demonstrates reduced levels of staining in the soleus muscle up to 14 days following $\alpha$-BTX treatment. Generally,
in muscles paralysed at birth, the area of the developing muscle closest to the implant always demonstrated lower levels of staining of SDH activity; that is, muscle activity in the area of the implant is reduced more extensively than that in other areas of the muscle. Those muscle fibres closest to the implant are therefore paralysed for longer periods of time and subsequently may die and be replaced by connective tissue (Hamburger, 1928; Drachman, 1968). It could be argued that these muscle fibres are innervated specifically by terminals from the L5 ventral root. Indeed, the α-BTX containing implant is placed in the same position in the hindlimb for each operation, between the soleus and flexor hallucis longus muscles. It could be possible, therefore, to explain the preferential loss of muscle fibres innervated by terminals from the L5 ventral ramus on the basis of their proximity to the α-BTX containing implant. However, in a study of the mouse soleus muscle there is no suggestion of regional preference of innervation from the L4 and L5 terminals (Fladby, 1987). Camera lucida drawings of adult soleus muscles demonstrate an even distribution of innervation of terminals from L4 and L5 motoneurones within the soleus muscle, and a lack of regional grouping. This is illustrated in Figure 19a. The even distribution of muscle fibres innervated by axons from L4 and L5 motoneurones is also apparent in adult soleus muscles paralysed at birth with α-BTX (see Figure 19b).

Segmentotopic innervation has, however, been reported in other mammalian skeletal muscles. In rat gluteus maximus muscles the L4 and L5 ventral rami dominate the innervation of the rostral and caudal part of the muscle, respectively. However, even in this muscle segregation of innervation in the newborn rat is much less pronounced (Brown & Booth,
Figure 19: *Camera Lucida* Mapping of Muscle Fibres in Adult Rat Soleus Muscles Innervated By L5 Nerve Terminals

Adult rat soleus muscles were depleted of their glycogen stores by repetitive stimulation of the muscle, through the axons emanating through the L5 ventral ramus. The muscle was then removed from the animal, frozen, sectioned and stained for polysaccharides (including glycogen) using the periodic-acid Schiff stain (Pearse, 1960). The orientation of muscle fibres innervated by L5 terminals (fibres depleted of glycogen) was then recorded using a *camera lucida* device. Figure a) represents the orientation of muscle fibres innervated by L5 terminals in a normal adult rat soleus muscle and Figure b) represents the orientation of muscle fibres innervated by L5 terminals in an adult rat soleus muscle following α-BTX induced paralysis at birth.
1983), and the authors report that in neonates both roots are able to activate the entire muscle independently. The lateral gastrocnemius (LG) muscle of the rat has specific regional innervation by axons from L4 and L5 terminals. However, in the newborn animal the LG muscle is evenly supplied by terminals from axons from the L4 and L5 spinal roots (Bennett & Lavidis, 1984). In view of these results, and the camera lucida drawings presented in Figure 19, it seems unlikely that the preferential loss of muscle fibres innervated by terminals from the L5 spinal root is due to their positioning in the neonatal muscles and their proximity to the α-BTX implant.

The loss of nerve-muscle contacts following paralysis at birth may not be the result of temporary disconnection between the nerve and muscle. An alternative explanation could be the result of direct effects of α-BTX on the pre-synaptic terminal. Direct effects of α-BTX on the pre-synaptic terminal would be dependent upon the presence of pre-synaptic AChR's. Electron microscope level, autoradiographic studies, using 125I-labelled α-BTX, designed specifically to detect low levels of α-BTX binding, failed to demonstrate significant levels of pre-synaptic binding at the neuromuscular junction (Fertuck & Salpeter, 1976; Porter & Barnard, 1978; Jones & Salpeter, 1983). The possibility of transient presynaptic AChR in neonates cannot be ruled out, however their presence has not been reported in the literature. Indeed, Greensmith & Vrbová (1991) paralysed soleus muscles at 5 days with α-BTX and demonstrated no significant change in the rate of synapse elimination. Any potential presynaptic AChR's therefore can be assumed to be eliminated in the 5 day old soleus since α-BTX had no effect on the neonatal innervation pattern.
It could be argued that the $\alpha$-BTX used in these experiments (Sigma Chemical Company) which is 95% pure, may contain either $\beta$-BTX or another toxic impurity which may be damaging the neonatal nerve terminals. However, recent studies carried out in these laboratories using a purified source of $\alpha$-BTX demonstrate that the results of experiments using the purified $\alpha$-BTX and commercially obtained $\alpha$-BTX are not significantly different (Connold & Vrbová, 1990; Connold & Vrbová, 1991; Greensmith & Vrbová, 1991). Indeed, nerve terminal damage by $\beta$-BTX impurity seems an unlikely explanation since it has been suggested that $\beta$-BTX, which inhibits ACh release in adult rats, has little effect on neonates (Gunderson, 1981). In addition, $\alpha$-BTX treatment of neonatal rat soleus muscles has no effect on the quantal content of intact nerve terminals compared to controls (Greensmith et al., 1988). $\beta$-BTX involvement would be expected to decrease the quantal content of terminals in treated muscles. A light microscopical study of nerve terminals chronically treated with $\alpha$-BTX found no pathological change in the nerve terminal as a result of treatment (Drachman et al., 1982). These results are consistent with the works of Duxson (1982) who demonstrated that $\alpha$-BTX paralysis of young muscles affected the development of post-synaptic specializations, whilst the pre-synaptic terminals were unaltered. The presence of an impurity in the commercial $\alpha$-BTX and its direct effect on the presynaptic terminal can not be ruled out, however the impurity would need to be extremely toxic at very low concentrations. It seems therefore, unlikely that $\alpha$-BTX is having direct damaging effects on the pre-synaptic terminals.

The altered pattern of innervation observed in adult soleus muscles following temporary paralysis of the newborn muscle are unusual. The
reason for this altered distribution of innervation may be due to a
delayed development and maturation of the muscle fibres and their
postsynaptic membrane as a consequence of early postnatal disruption in
target activity. The development and maturation of the neuromuscular
junction is known to be dependent upon muscle activity. The subneural
apparatus (post-synaptic membrane and associated junctional AChE)
develops early in the development of the neuromuscular junction,
developing rapidly after the motor nerve has contracted the muscle
fibres (Kupfer & Koelle, 1951; Hirano, 1967). If the postsynaptic AChR's
are blocked in chick embryos with α-BTX then the normal maturation of
the nerve and muscle fails to take place (Giacobini et al., 1973; Perry
et al., 1974). As a result of this delayed development the content of
acetylcholinesterase (AChE) in the paralysed chick muscles was reduced.
The accumulation of AChE at the developing neuromuscular junction is
known to be dependent upon the activity of immature muscle fibres
(Gordon et al., 1974; Cangiano et al., 1980; Ljømo & Slater, 1980b).

Greensmith & Vrbová (1991) paralysed rat soleus muscles at birth
and observed delayed development of the neuromuscular junction.
Specifically, paralysis of newborn rats with α-BTX resulted in a
prolonged response of the target in 5-7 day old rats; that is, the
treated muscles demonstrated prolonged endplate potentials (EPP's)
similar to that of immature endplates. The authors suggest this is due
to the persistence or incorporation of immature AChR's into the
postsynaptic membrane. Immature AChR's have longer channel open times
than mature AChR's (Salpeter & Loring, 1985; Brenner & Sakmann, 1983;
Scheutze & Role, 1987). The development of AChR's and the establishment
of their mature characteristics is known to be regulated by activity
(Brenner et al., 1987; Brenner & Rudin, 1989). The accumulation of AChE and the transition of the AChR seen in developing animals is dependent upon activity. Temporarily removing target activity with α,-BTX may be delaying the development of the neuromuscular junction.

Greensmith & Vrbova (1991) demonstrated that following α,-BTX paralysis of the soleus muscle at birth, there were fewer nerve-muscle contacts shortly after transmission was resumed. The prolonged response of the target reported in these experiments appears to be affecting the immature nerve terminals on the rat soleus muscle. Indeed, increased activity has been shown in Chapter 2 to be involved in the disruption of functional contacts at the developing neuromuscular junction. An increased EPP on the temporarily paralysed muscle may cause a prolonged response of the target. Potassium ions are known to be released from muscle fibres during depolarization of the muscle fibre membrane (Hodgkin & Huxley, 1952) and neuromuscular activity is known to result in the accumulation of K⁺ in the synaptic cleft (Hohlfield et al., 1981). Raised potassium ions within the synaptic cleft are thought to cause an influx of Ca²⁺ into the nerve terminal through voltage-gated calcium channels. As demonstrated in Chapter 2, Ca²⁺ influx into the nerve terminal activates a neuronal calcium-activated neutral protease (CANP) which degrades the neurofilament proteins within the terminals and axons innervating the neonatal muscles. It is postulated that the degradative action of CANP is the cause of the loss of nerve-muscle contacts seen after α,-BTX induced paralysis. In a study of paralysis of the soleus muscle at later stages in development, Duxson (1982) reported that nerve-muscle contacts remained intact. Paralysis at later developmental stages is unlikely to greatly affect the maturation of the
neuromuscular junction to such an extent as that seen in newborns, since at later stages in development a substantial amount of AChE has already been developed. As a consequence paralysis at later stages in development will have less marked effects on the deposition of AChE.

The results presented here demonstrate that soleus muscles recover from paralysis over the first 9 days after birth and new nerve-muscle contacts are formed on the soleus muscle by axons from the L4 and L5 ventral ramus after recovery of transmission. It is possible that at this stage there is a mismatch between the re-established innervation and the relatively immature muscle fibres, as a result of which some of the newly formed contacts are lost. The results presented in this Chapter suggest that either, the L5 terminals are unable to make new contacts on the soleus muscle fibres, or that these contacts once established cannot be maintained and withdraw as activity returns. Presumably, over the following days the remaining terminals mature and are then capable of maintaining contact with the relatively immature muscle fibres. A question which arises is whether there is any difference between the terminals of the L4 and L5 axons which makes the L5 terminals more likely to be eliminated from α-BTX treated muscles?

A study by Lowrie and colleagues (1985) demonstrated that in neonatal rat soleus muscles the L5 terminals have a higher quantal content than L4 terminals. Indeed, the quantal content of terminals from the L4 ventral ramus is less than half that of the terminals from the L5 ventral ramus. In addition, there is some evidence to suggest that the quantal content of nerve terminals to fast muscles is higher than that to slow muscles (Tonge, 1974). Soleus muscles of newborn rats, treated with α-BTX at birth initially loose nerve-muscle contacts (Greensmith &
New contacts are reformed on the soleus muscle on recovery of transmission (Greensmith & Vrbová, 1991). The terminals from L5 v.r. reinnervating the treated soleus muscle will be releasing more ACh than the L4 terminals, into the developmentally-arrested neuromuscular junction. It therefore seems likely that the L5 terminals which are releasing more ACh will be less able to persist at the immature endplate. The release of large amounts of ACh by the L5 terminals will result in long periods of depolarization of the associated postsynaptic membrane compared to that seen by L4 terminals. The concomitant increased muscle activity of the muscle fibres innervated by L5 terminals is likely to result in the elimination of the L5 terminals from the endplate region. Chapter 2 demonstrated that activity is involved in the elimination of functional contacts in an in vitro preparation via a calcium-activated proteolytic degradation of the cytoskeletal structure of terminals.

The results presented in this Chapter are unusual since during the elimination of polyneuronal innervation at the developing neuromuscular junction Lowrie et al. (1985) and Miyata & Yoshioka (1980) demonstrated a preferential loss of L4 terminals in the rat soleus muscle. This they associate with the relative "weakness" of the L4 terminals. Work by other authors (Thompson, 1983; Gordon & Van Essen, 1983) has failed to demonstrate a preferential elimination of terminals during the elimination of polyneuronal innervation. However, Duxson (1982) and O'Brien et al. (1984) have demonstrated that during the elimination of polyneuronal innervation the small terminals are those which are preferentially eliminated. Axon diameter in myelinated nerve fibres is determined by the presence of neurofilaments (Friede & Samerajski, 1970;
Weiss & Mayr, 1971; Berthold, 1978) which are thought to account for the radial growth of axons and the stability of axonal processes (Hoffman et al., 1985). It may be that the susceptibility of one terminal over another for elimination at an endplate may be dependent upon the state of "maturity" of the neurofilaments within the terminal.

"Mature" neurofilaments are composed of 3 neurofilament subunit proteins (NF-H, NF-M and NF-L) which have different developmental patterns of expression (Czonak et al., 1980; Shneidman et al., 1989). During the development of the rabbit retinal ganglion cells NF-M and NF-L are present at birth, whereas NF-H does not appear until later (Levine et al., 1982; Willard & Simon, 1983), so giving rise to "immature" neurofilaments. The NF-H is expressed relatively late in development (Shaw & Weber, 1982; 1983; Willard & Simon, 1983; Pachter & Liem, 1984; Harry et al., 1985; Nova et al., 1985; Carden et al., 1987; Schlaepfer & Bruce, 1990). The first appearance of NF-H occurs at E15 (Carden et al., 1987), and increases slowly during development, remaining below the levels of NF-M and NF-L for several weeks postnatally. The addition of NF-H to the "immature" neurofilament confers a mature, more stable state onto the cytoskeletal neurofilament, presumably due to the cross-linking role of NF-H.

Motoneurones situated more cranially may differentiate and mature faster than those in a caudal position (cranio-caudal direction: Jacobson, 1970). Therefore, it may be that the L4 terminals mature more quickly than L5 terminals in the postnatal period, and acquire a more stable cytoskeleton (composed of "mature" neurofilaments and microtubules) earlier in development. The relatively mature L4 terminals may therefore be more able to cope with the immature conditions present
in paralysed muscles. The prolonged EPP's (Greensmith & Vrbová, 1991) observed in soleus muscles paralysed at birth with α-BTX, would therefore be more detrimental to L5 nerve terminals, since the cytoskeleton of the L5 terminals may be more susceptible to proteolytic attack.

In conclusion, the result presented in this Chapter demonstrate that continued, active communication between the nerve and muscle during the early postnatal period is essential for the establishment of the normal pattern of innervation. Temporary paralysis at birth alters the pattern of innervation of the adult soleus muscles and subsequently the characteristic contractile and enzymic properties of the soleus muscle.
CHAPTER FOUR

The Role of Activity in the Development of the Fast Hindlimb

Muscles of the Rat
INTRODUCTION

The normal development of skeletal muscles is dependent upon the interaction between the motor nerve and muscle fibres. Adult skeletal muscle is dependent upon its motor nerve and the functional activity imparted upon it. Notwithstanding that, adult skeletal muscles can survive temporary denervation as long as it is allowed, eventually, to be reinnervated. If reinnervation is prevented and the skeletal muscle is deprived of its innervation, then the muscle atrophies and finally degenerates (Tower, 1939).

Initially during embryogenesis, both the developing muscles and nerves are independent of each other. This independence however, is a transitory state and once the muscle and nerve have made initial contact with each other and developed the pre-requisite characteristics for interaction i.e. the production of, and recognition of ACh; then the two separate components become a functional unit. Having made initial contact with each other, the muscle fibres and motoneurone become dependent upon one another for their continued development and subsequent survival. If muscles are disconnected from their motoneurone during embryonic development they continue to develop for a limited period of time, but ultimately disintegrate and die (Eastlick & Wortham, 1947; Drachman, 1968; Gordon et al., 1974; Srihari & Vrbová, 1978). As development proceeds the properties of the skeletal muscles and motoneurones composing a functional unit become highly specialized and a marrying of properties occurs.

The precise matching of the properties of the muscle fibres and the motoneurone within a single motor unit raises the question of whether
the developing axons explore, find and innervate myotubes with which they can form a functional unit and whose potential characteristics will match those of the motoneurone. Various experiments investigating axonal guidance indicate that innervation of limb muscles occurs in an orderly and stereotyped manner. Motor axons leave the embryonic spinal cord through specific segmental ventral roots and select a specific and characteristic pathway through the lumbar plexus. Axons travel along peripheral stumps and leave at specific branch points in order to reach appropriate muscles. In experiments on embryonic chicks Lance-Jones & Landmesser (1980) surgically reversed short sections of embryonic chick lumbar spinal cord, prior to axonal outgrowth. In the experimentally manipulated chicks, axons grew out of the spinal cord and once they reached the plexus they recrossed so that the experimental dislocation was corrected; the axons subsequently went on to innervate the appropriate target muscle. In experimental situations in chick embryos where limb buds were made devoid of muscle cells by X-irradiation the motor nerves were still able to grow out to the periphery even in the absence of a muscle target (Lewis et al., 1981). It appears that axonal outgrowth and guidance occurs along roughly defined routes, though guidance appears to occur independently of specific target muscles. A passive guidance of motor nerves appears sufficient to explain axon outgrowth to the periphery, but is insufficient to explain specific innervation of muscle fibres. Indeed, motor nerves can grow into and innervate foreign tissue in the case of ectopically grafted limb buds (Hollyday et al., 1977). In X-irradiated muscles motor nerves grow out into the periphery even in the absence of a target, although the muscle nerve branches consistently fail to develop (Lewis et al., 1981).
Muscle fibres are not initially innervated by a specific motoneurone but instead become hyperinnervated by axons from many different motoneurones (Redfern, 1970; Brown et al., 1976; O'Brien et al., 1978). Research by Thompson and colleagues (1984) investigating the innervation of neonatal rat soleus muscles, using the glycogen depletion technique, demonstrated that at PN8, when soleus muscles are innervated by more than one motor axon, individual motor units are composed predominantly by either fast or slow muscle fibres. These results indicate that a matching of muscle fibre properties and motor innervation has occurred prior to the elimination of polyneuronal innervation and suggests that initial contacts are specific and not the result of random innervation. These results however were not confirmed in a study by Jones et al. (1987a) on rat lumbrical muscles. Jones et al. (1987a) demonstrated that 3-5 day old lumbrical muscles are essentially a random mixture of fibre types. They also demonstrated that the number of slow muscle fibres in the rat lumbrical muscles are constant after birth (Jones et al., 1987b), and so they argued that the elimination of polyneuronal innervation is the important factor for the establishment of mature homogenous motor units and matching of the properties of the muscle fibres and the motoneurones, and that initial innervation is a non selective process. Fladby & Jansen (1988) confirmed the earlier work of Thompson et al. (1984) and demonstrated that 5 day old soleus muscles possess motor units which are predominantly composed of either fast or slow muscle fibres. The authors conclude that synapse elimination is not a major factor in the establishment of the selective innervation of muscle fibres. In a recent review, Fladby & Jansen (1990) suggest the early selective innervation of fast and slow muscles.
is a major factor matching of properties of muscle fibres and motoneurone and suggest further sharpening of patterns is due to a transfer of terminals between inappropriately innervated muscle fibres at later stages in development. Duxson et al., (1986) demonstrated that secondary myotubes receive their innervation by the transfer of terminals from hyperinnervated primary myotubes. Once hyperinnervation has been eliminated and muscle fibres are supplied by a single motoneurone the matching of properties between the muscle fibres and motoneurone is firmly established.

During the development of muscle fibre properties in the embryo primary myotubes have been demonstrated to express embryonic isoforms of myosin heavy chain (MHC) as well as a slow MHC isoform (Dhoot, 1986; Narusawa et al., 1987; Harris et al., 1989; Condon et al., 1990a). Muscle fibre differentiation occurs to some extent in the absence of innervation. As differentiation and development proceeds a population of primary myotubes cease to express the slow MHC isoform, whilst continuing to express the embryonic MHC isoform (Harris et al., 1989). These myotubes proceed to develop into muscle fibres possessing fast myosin; the remaining myotubes develop into slow muscle fibres (Harris et al., 1989). As secondary myotubes develop along the scaffold made by the primary myotubes they have been demonstrated to express embryonic MHC but not slow MHC (Harris et al., 1989). These myotubes later develop into fast muscle fibres, but can be converted at even later stages in development into slow muscle fibres (Harris et al., 1989).

Immature, hyperinnervated muscle fibres contract and relax slowly, possibly as a consequence of immature isoforms of MHC (Trayer et al., 1968; Whalen et al., 1981; Dhoot, 1986; Narusawa et al., 1987; Harris et
Immature muscle have uniformly slow rates of \( \text{Ca}^{2+} \) release and re-uptake from the sarcoplasmic reticulum (Martonosi, 1982).

Once muscle fibres belong to a specific motor unit and are functionally innervated by a single motoneurone then the characteristic properties develop under the influence of the innervating motor nerve. Characteristic myosin isoforms develop and the activities of the enzymes of oxidative metabolism differentiate until they reach the different levels characteristic of adult skeletal muscles. The contractile speeds of the developing muscles alter, so that initially slow and fast muscles become faster contracting. The slow muscle fibres gradually achieve their slow rate of tension development whilst fast muscles continue to increase their contractile speeds (Buller et al., 1960; Close, 1964; Brown, 1973). If disconnected from neural input, the development of fast muscles is arrested and the increase in speed after birth is much reduced (Brown, 1973).

Activity is an important factor in the development of matching properties between muscle fibres and those of the motoneurones that innervate them. The importance of the neurone in the determination of muscle fibre properties was initially demonstrated in the cross-innervation experiments of Buller et al. (1960a). The authors demonstrated the plastic nature of muscle fibre properties, and the fact that muscle fibres can be modified by the pattern of innervation they received. By cross-innervating fast muscles with a nerve from a slow muscle, and vice-versa, the authors demonstrated that the phenotype of the cross-innervated muscle was completely transformed. They attributed this to the effects of a trophic factor released from the nerve.
However, experimental manipulations imposing different patterns of activity upon muscle by chronic electrical stimulation, has been demonstrated to bring about altered phenotypic expression of muscle fibre properties (Salmons & Vrbová, 1969; Salmons & Sreter, 1976; Pette & Vrbová, 1985).

Muscles which are disconnected from their motoneurone at birth by nerve crush injury continue to grow for a short period of time (Vrbová, 1952; Zelená, 1962). However, they cannot be maintained in the absence of innervation and atrophy. If reinnervation is allowed, the muscles recover, though this recovery is poor (Romanes, 1946; Bueker & Meyers, 1951; Zelená & Hník, 1963; McArdle & Sansone, 1977). Even a short period of disconnection has permanent effects on the tension development of the affected muscles. Sciatic nerve crush at birth has been demonstrated to result in a reduced number of muscle fibres in the reinnervated slow soleus muscle (Zelená & Hník, 1963). The authors report that the remaining muscle fibres are however markedly larger than normal (Zelená & Hník, 1963).

With increasing age and maturity disconnection of the muscle from its motor innervation becomes less devastating to the muscle fibres. Consequently, denervation of skeletal muscle at slightly later developmental stages has less pronounced effects on the skeletal muscles. The relatively slow immature soleus muscle almost completely recovers from sciatic nerve crush injury at 5 days, whereas the fast EDL and TA muscles are permanently impaired (Brown, 1973; Lowrie et al., 1982). Lowrie and colleagues demonstrated that this is not due to a misguided reinnervation attempt by the disconnected axons, since upon reinnervation all of the axons reach the muscles and are accurately
guided back to their original target (Lowrie et al., 1982). Interestingly, Lowrie & Vrbová (1984) demonstrated that the impairment in muscle fibres properties is due to a substantial loss of muscle fibres, which are lost upon reinnervation of the muscle 10 days after the original injury. A prolonged period of disconnection from the motoneurone, by repeated nerve injury, has recently been demonstrated to have more marked effect on the developing muscles and leads to even poorer recovery of muscles (Lowrie et al., 1990).

The susceptibility of muscle fibres to temporary disconnection from their specific motoneurones is no longer apparent in adult skeletal muscles. Muscle fibres subjected to peripheral axonal injury in adulthood almost completely recover from temporary denervation, provided that reinnervation proceeds unhindered (Gutmann & Young, 1944; Beranek et al., 1957; Miledi, 1960a; 1960b; McArdle & Alberquerque, 1973; Lowrie et al., 1982).

The age-dependent pattern of the vulnerability of muscle fibres to axonal injury is mirrored in the motoneurone. Similarly, if nerve-muscle interactions are interrupted during the early postnatal life then motoneurones are adversely affected. Sciatic nerve crush injury at birth results in extensive degeneration and death of motoneurones — up to 70% of the immature motoneurones subjected to axonal peripheral injury have been reported to die (Romanes, 1946; Buerker & Meyers, 1951; Lowrie et al., 1987). The vulnerability of neonatal immature motoneurones decreases with age. If axonal injury occurs slightly later in development, the motoneurone cell death is reduced and not as extensive (Romanes, 1946; Lowrie et al., 1982; Lowrie & Vrbová, 1984; Lowrie et al., 1987; Crews & Wigston, 1990). However, if the reinnervation of
target muscle by its motoneurones is delayed or prevented then the motoneurones are less likely to survive the peripheral assault (Kashihara et al., 1987; Lowrie et al., 1990). Following neonatal nerve injury motoneurones survive for two weeks disconnected from the target (Kashihara et al., 1987). In parallel with muscle fibre survival, peripheral axonal section in the adult (4 weeks postnatally) has no adverse effects on motoneurone survival (Schmallbruch, 1984) and the susceptibility of motoneurones to peripheral injury is no longer discernable.

An interesting question to arise from the work demonstrating the age-dependence of the effects of peripheral axonal injury on the development and survival of muscle fibres is to question what is conferring the capacity to survive injury onto the developing muscle fibres. Following nerve crush injury at birth motoneurones die (Romanes, 1946; Buerker & Meyers, 1951; Lowrie et al., 1987). Muscle fibre loss following injury at birth can therefore be explained in terms of this substantial loss of innervation. In adults no motoneurone death occurs following nerve injury and subsequently there is a complete recovery in the muscle fibres following this peripheral injury, assuming that reinnervation occurs (Lowrie et al., 1982; Schmallbruch, 1984). However, between the two extremes there is the point in the early postnatal period when nerve crush injury at 5 days results in no motoneurone cell death (Lowrie et al., 1982) but does however result in the functional and structural impairment of the reinnervated muscle fibres (Lowrie et al., 1982). Lowrie & Vrbová (1984) demonstrated that nerve crush injury at 5 days results in a reduced number of muscle fibres in the reinnervated fast muscles. This loss of muscle fibres seems to occur
upon reinnervation and coincides in time with the reinnervation of the temporarily denervated muscles. Lowrie & Vrbová (1984) postulated that the poor recovery of the immature muscles after nerve crush injury may depend upon the ability of the denervated muscle fibres to respond to the ingrowing nerve and accept innervation. The inactivity following denervation may have prevented muscle development and so may have caused a mismatch between the developmental state of the muscle and its innervating motoneurone.

The experiments performed in this Chapter attempt to examine the importance of target activity and subsequent arrest in muscle development in the first postnatal days on the response of immature muscles to temporary deprivation of motor innervation. The experiments were designed to investigate the importance of target activity in the changing response of the muscle to denervation injury. Whether target activity is involved in the developmental process by which the muscle fibres become less susceptible to temporary denervation is addressed. Target activity was reduced in the fast EDL and TA muscles of the rat hindlimb by applying α-bungarotoxin to the muscle immediately after birth. Five days later the animals were subjected to neonatal sciatic nerve crush injury. The effects of this interruption of nerve-muscle interactions in the early postnatal period and its effect on the response of the muscle to temporary denervation were investigated in the adult animal. In addition, the effects of temporary disruption in nerve-muscle interactions either temporarily or for a more prolonged period on the development of normal muscle fibre properties in the adult fast muscle were investigated.
MATERIALS AND METHODS

I) Animals

All operations were performed on neonatal Wistar albino rats, of both sexes. Animals were supplied by Joint Animal House, University College London, and fed *ad libitum* food and water and kept in a constant light/dark cycle.

II) Surgery

In one experimental group, newborn rats were operated on between 6 and 12 hours after birth (known as postnatal (PN) day 0). The animals were anaesthetized with ether, and sterile precautions were observed throughout the procedure. A small, longitudinal incision was made in the skin on the lateral side of the right hindlimb. A smaller incision was made in the underlying fascia to reveal the hindlimb muscles. A silicone rubber strip containing α-BTX (for preparation, see Chapter 2, Materials and Methods, section III) weighing 0.4mg was implanted between the *tibialis anterior* muscle (TA) and the *extensor digitorum longus* (EDL) muscle. The silicone rubber implant was a long sheet-like implant with a large surface area, with approximate dimensions of 3mm x 2mm x 0.05mm. The implant was placed between the two muscles away from the nerve, taking care not to cause any damage to muscle, nerve or blood supply. The overlying skin was sutured with 0.4 silk thread (Ethicon) and the animals returned to their mother when fully recovered from the anaesthesia. The strips containing α-BTX contained approximately 9 µg α-BTX and 160 µg NaCl.
A further group of control animals underwent the same surgical procedure and were implanted with silicone rubber strips, of equal shape and size, containing NaCl only. Control implants contained 160 µg of NaCl.

Some animals which had been operated at birth and implanted with α-BTX were reoperated 3 days later. At this time point recovery from paralysis was becoming apparent - the operated animals were able to make use of the hindlimb in a manner similar to that of age-matched littermates. The animals were re-operated under ether anaesthesia and sterile conditions as previously described, to reveal the hindlimb muscles. The original silicone implant was removed and a second implant was inserted into the same position in the hindlimb. The second implant weighed 0.75mg and again was sheet-like in nature (4mm x 2.5mm x 0.05mm) and contained 17 µg α-BTX and 300 µg NaCl. Control implants contained 300 µg of NaCl.

In another experimental group, the EDL and TA muscles of neonatal animals were treated 6-12 hours after birth with α-BTX as previously described. Five days later (PN5) the animals were subjected to further surgery. The 5 day old rats were reanaesthetized with ether, and under sterile conditions a small incision was made in the skin overlying the right thigh. The hamstrings were then separated to expose the sciatic nerve. The sciatic nerve was crushed between watchmakers forceps, under a light microscope (Zeiss), at a site proximal to the branching point of the common sciatic into the lateral and medial popliteal nerves. Sciatic nerve crush was performed in each animal with the same pair of watchmakers forceps and for 10 seconds each time, in order to standardize the procedure. After crushing, the sciatic nerve was checked
for continuity of the epineurium (a crush injury leaves the epineurium intact, whereas, axotomy injury to nerves results in a severed epineurium). The overlying skin was then sutured with 0.4 silk thread (Ethicon) and the animals allowed to recover from the anaesthesia and then returned to their mother.

Control animals which had been previously been treated at birth with silicone implants containing NaCl alone were also subjected to an identical sciatic nerve crush injury at 5 days of age, using ether anaesthesia and sterile precautions.

All operations were performed on the right hindlimb so that the effects of treatment in the early postnatal period could be assessed in comparison to the contralateral control hindlimb.

III) Efficacy of Muscle Paralysis

The extent of the paralysis of the EDL and TA muscles following treatment with α-BTX birth, and the effects of nerve crush injury was assessed by observing the use of the hindlimb, particularly the movements of the ankle joint. In addition to spontaneous movements, movements of the ankle joint and the toe-spread reflex were elicited and assessed. The movements and usage of the limb was compared to unoperated littermates.

IV) Isometric Tension Recordings of Muscle Contraction

10 weeks to 10 months after the experimental manipulations (α-BTX induced paralysis and nerve crush) had been performed the experimental Wistar rats were reanaesthetised with chloral hydrate (4.5% aqueous
solution, 1ml/100g body weight, intra-peritoneal) and were prepared for in vivo physiological recordings of muscle tension.

The extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of each hindlimb of the experimental animal was dissected free from the overlying skin and surrounding musculature. The distal tendons of the operated and control EDL and TA muscles were freed from the retinaculum and the tendons ligatured firmly with 2.0 silk thread (the ligatures were then secured with "superglue"). An incision was made in the skin of the experimental animal overlying the region of the sciatic nerve. The sciatic nerve was freed and sectioned. Care was taken at all points in the dissection not to damage the nerve or blood supply to the muscles. The legs of the experimental animal were then rigidly secured to a fixed table.

The distal tendons of the operated and contralateral control EDL or TA muscles were attached to strain guages (Transducer Techniques, Precision Measurements Systems) with 2.0 silk thread and the muscle length was adjusted to obtain maximum twitch tension at supramaximal stimulus strength.

Isometric contractions from the specific muscle were elicited by stimulating the popliteal branch of the sciatic nerve via a pair of bipolar silver electrodes using square wave pulses with a width of 0.02-0.05msecs, for 500-700 msecs. The resulting contractions were recorded through an analogue-digital converter (Loredon Inc.) and then passed into an IBM computer, which analysed the information (Scope, Loredon Inc.). Maximum twitch and tetanic contractions (40-100Hz) and the contractile speeds of the isometric muscle contractions were analyzed.

All exposed tissue was kept moist throughout the experiment with
oxygenated Krebs-Henseleit solution, and all experiments were carried out at room temperature (23°C).

V) Changes in Contractile Properties

In order to observe any changes in the contractile properties of the fast muscles studied, the time-to-peak (TTP) and 1/2 relaxation time (1/2RT) were studied. The computer programme calculated these values from the trace of a single twitch muscle contraction. TTP is the time taken by the muscle to contract and produce a maximal twitch contraction. 1/2RT is the time taken in milliseconds for the muscle to relax to 1/2 of the maximal twitch tension.

VI) Motor Unit Number and Size

The number of motor units within the experimental and contralateral control EDL muscles was estimated. To estimate the number of motor units in the adult EDL muscles the sciatic nerve was stimulated every 4 seconds, achieving a twitch contraction from the muscle. The stimulus intensity applied to the sciatic nerve was gradually increased. As the intensity of the impulse was increased axons with different thresholds were successively activated resulting in the successive recruitment of individual motor units. This was manifest as stepwise increments in twitch tension which were amplified and displayed on an oscilloscope screen (Tetronix R5113), and photographs were taken of the traces.

The average motor unit size for the operated muscle was found by dividing the maximum tetanic tension of the operated muscle by the total number of motor units identified in that muscle. This value was
expressed as a percentage of the mean size of a motor unit from the contralateral control muscle, obtained by using the same method.

VII) Fatigue Index

The fatigue resistance of the experimental and control muscles was tested by stimulating the muscles continuously at 40Hz, for 250mseconds every second, for a period of 3 minutes. The tetanic contractions were displayed on a penrecorder (Electromed, Multitrace 2). The decrease in tension after 3 minutes of such stimulation was measured and the fatigue index calculated.

\[
\text{Fatigue Index} = \frac{\text{initial tension} - \text{tension after 3 minutes stimulation}}{\text{initial tension}}
\]

A fatigue index of 0 indicates a completely fatigue resistant muscle, and a fatigue index of 10 indicates at the end of the experiment no tension was produced by the muscle; that is, the muscle was completely fatigued.

VIII) Histology and Immunohistochemistry

At the end of the physiological tension recording experiment the experimental and contralateral control muscles were removed from the animal, weighed and prepared for histology.

The operated and control muscles were mounted side by side against a pin embedded in a cork block. The muscles were stretched to a resting length, covered in a tissue fixing compound (OCT compound, BDH), and quickly frozen in melting isopentane, cooled with liquid nitrogen. Muscles were wrapped in aluminium foil and stored at -70°C, until
required for sectioning. Control and operated muscles were mounted side by side so that the muscles were treated uniformly and therefore could be readily compared.

Transverse sections from the middle of each muscle block were cut within two weeks of freezing. 12 µm transverse sections were prepared on a cryostat, at -22°C, and picked up on pre-polylysined glass slides. Sections were air dried for 30 minutes and slides wrapped and stored at -20°C, until required for histological and immunohistochemical analysis.

The staining techniques described in Chapter 3, "Materials and Methods" were performed routinely on the fast muscles studied in this Chapter. The transverse sections taken from the fast EDL and TA muscles were processed for SDH activity and with an antibody raised against a slow myosin heavy chain (HC). The method employed when processing with the slow myosin antibody can be found in Appendix IV.
RESULTS

The experiments performed in this Chapter attempt to look at the importance of early interaction between the nerve and muscle in the normal development and maturation of the fast twitch extensor digitorum longus (EDL) and tibialis anterior (TA) muscles. In these experiments neuromuscular activity to the EDL and TA muscles was reduced both temporarily and for a more prolonged period with α-bungarotoxin. In another group of animals the muscles were disconnected from their motoneurones by nerve crush injury at 5 days. In some cases target activity was reduced in the EDL and TA muscles at birth by application of α-BTX and the nerve crush injury was subsequently inflicted at 5 days of age.

The investigations in this Chapter look at the effects of disruption of nerve-muscle interactions in the neonate on the development of adult fast muscles.

I) Initial Effects of Treatment at Birth

i) Behavioural Recovery From Treatment

Immediately following surgery the animals were assessed to determine the extent of functional impairment and subsequent recovery. A behavioural study was employed observing movement of the ankle-joint and the toe-spreading reflex (Gutmann, 1943).

Paralysis of the EDL and TA muscles of the rat hindlimb with α-BTX resulted in a misuse of the hindlimb by the experimental animal. This
was manifest in an exaggerated plantar-flexion of the ankle. All comparisons were made with contralateral control legs and unoperated littermate animals. The animals treated at birth with α-BTX seemed to have impaired function of their ankle movement for 4-5 days following treatment, after which normal movements and usage of the hindlimb returned.

The EDL and TA muscles of a second group of animals were paralysed at birth with α-BTX. 3 days later the animals were reoperated and the EDL and TA muscles treated again with α-BTX. Recovery of function was followed. The animals seemed to have additional impaired function of the operated hindlimb for 4-5 days after the second implant, and had recovered normal functional use of the hindlimb by postnatal day 7-8.

In cases where the α-BTX treated animals were able to use the operated leg in the normal manner, the paralysis was assumed to be incomplete and the animal was not included in the experiment. These animals were killed with an overdose of anaesthetic. Systemic spread of the toxin occasionally caused breathing difficulties in the experimental animals (20% of cases) in which case the animals were killed with an overdose of anaesthetic. The percentage of animals affected by systemic spread of the toxin is similar to the seen by other authors (Greensmith, 1989; Greensmith & Vrbová, 1991) and similar to that reported in Chapter 3. Those animals which did not gain weight, relative to unoperated littermates, in the days following the operation were killed with an overdose of anaesthetic.

Animals treated at birth with NaCl demonstrated normal use of the hindlimb. The surgical operation and implantation of a silastic plug between the EDL and TA muscles resulted in no apparent functional
In one group of animals the sciatic nerve was crushed in the early perinatal period. Animals were treated with a NaCl-containing implant at birth, 5 days later the sciatic nerve was crushed in the right hindlimb. Sciatic nerve crush injury results in paralysis of the hindlimb muscles involved in movement of the ankle joint and the toes. Immediately following sciatic nerve crush the results of the injury were manifest as no abduction of the toes or dorsiflexion of the ankle. Functional recovery of the hindlimb muscles was seen after 9-10 days, when the toe-spaying reflex returned along with dorsi-flexion of the ankle of the operated leg. This time course of recovery is in agreement with that reported by other authors who similarly crushed the sciatic nerve in 3-6 day old Wistar rats (Lowrie et al., 1982; Lowrie & Vrbová, 1984; Lowrie et al., 1990).

In another group of animals, sciatic nerve crush injury at 5 days was preceded by α-BTX treatment at birth. The EDL and TA muscles recovered from α-BTX paralysis at PN day 4-5. On PN day 5 the sciatic nerve was crushed. Observations of functional recovery and use of the hindlimb indicated that recovery took place 9-10 days after the sciatic nerve injury (PN 14-15).

Any animals capable of using their operated right limb in a relatively normal manner, following the sciatic nerve crush were killed with an overdose of anaesthetic, since the crush injury was assumed to be incomplete.

In adulthood, those animals subjected to nerve crush injury in early neonatal life continued to have impaired use of the hindlimb. Although the experimental animals were capable of initiating
contractions of the muscles in the operated leg, the functional use of the leg was very different from that observed in the unoperated, contralateral control leg. When the adult animal was held off the ground by the tail the operated leg appeared weak and demonstrated exaggerated plantar-flexion - the operated leg assuming an extended position. This impaired function persisted in all of the animals following the initial nerve crush injury. Examination of the adult animal highlighted this fact; the hindlimb muscles of the operated leg appeared to be extensively wasted compared to the contralateral control leg.

II) The Effects of Disruption of Nerve-Muscle Contacts at Birth on the Development of the Adult Fast Muscles

i) Muscle Tensions and Weights

The fast twitch muscles, EDL and TA, of the neonatal rat hindlimb were treated at birth with a single $\alpha$-BTX or NaCl implant. Some animals were treated again 3 days later with a second $\alpha$-BTX or NaCl, implant. Another group of animals was subjected to sciatic nerve crush injury at 5 days of age following $\alpha$-BTX induced paralysis at birth. Control animals were treated with NaCl prior to sciatic nerve injury at 5 days of age. The animals were left to recover.

Ten weeks to 10 months later, by which time $\alpha$-BTX paralysis had long worn off, and those muscles subjected to nerve crush injury had been reinnervated, the experimental animals were reanaesthetised with chloral hydrate anaesthesia, and prepared for physiological examination of the EDL and TA muscles. Indirectly elicited twitch and tetanic contractions were recorded from both operated and contralateral control
muscles by stimulating their motor nerves. The results from these experiments are collated in Table 1. For each experiment the maximum tetanic tension developed by the operated muscle was expressed as a percentage of that produced by the contralateral control muscle. Table 1 demonstrates that fast muscles paralysed at birth with a single treatment of \(\alpha\)-BTX, when examined at 10 weeks to 10 months, develop relatively less tension than that produced by control muscles treated with NaCl only. More prolonged paralysis (2BTX) results in a greater reduction in tension output of the adult EDL and TA muscles, compared to NaCl treated controls.

EDL muscles treated with NaCl at birth and again 3 days later produced 107 \(\pm\) 7\% (\(\pm\) s.e.m., n=6) of the tension, and TA muscles subjected to identical treatment produced 100 \(\pm\) 6\% (\(\pm\) s.e.m., n=9) of the tension produced by their contralateral control muscles. These results are not significantly different (Mann-Whitney U-test). EDL muscles treated at birth with \(\alpha\)-BTX however produced significantly less tension, 86 \(\pm\) 5\% (\(\pm\) s.e.m., n=9) than contralateral control muscles (Mann-Whitney U-test, \(p=0.01\)). The reduced tension output of \(\alpha\)-BTX treated muscles was also significantly less than that produced by NaCl treated muscles (Mann-Whitney U-test, \(p=0.018\)). TA muscles treated at birth with \(\alpha\)-BTX exhibited a similar reduction in tension output. \(\alpha\)-BTX treated TA muscles produced 81 \(\pm\) 4\% (\(\pm\) s.e.m., n=8) of the tension of contralateral control muscles. The reduction in tension output of the adult TA muscles following \(\alpha\)-BTX induced paralysis at birth is significantly different from that produced by NaCl treated controls (Mann-Whitney U-test, \(p<0.001\)).

More prolonged treatment with \(\alpha\)-BTX (2BTX) immediately after birth
Table 1: **Maximum Tetanic Tensions of Adult EDL and TA Muscles Following Treatment at Birth**

The fast EDL and TA muscles were paralysed at birth with α-BTX or subjected to neonatal nerve crush injury (as previously described). Ten weeks - 10 months later the animals were prepared for isometric tension recordings. The **maximum tetanic tension** was measured from the contralateral control and operated EDL and TA muscles by stimulating the cut end of the sciatic nerve with bipolar silver electrodes, using a pulse width of 0.02msecs. The Table summarizes the mean ± s.e.m. of the maximum tetanic tension (grammes) of the adult EDL and TA muscles from the experimental animals. In addition, the tetanic tension of the operated muscle was expressed as a percentage of that of the contralateral control muscle for each animal.
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<th>CONTRALATERAL</th>
<th>OPERATED</th>
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<td>BTX</td>
<td>9</td>
<td>261.3 ± 8.1</td>
<td>224.3 ± 11.1</td>
<td>86.1 ± 4.5</td>
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<td>2BTX</td>
<td>12</td>
<td>313.5 ± 20.8</td>
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<tr>
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<td>273.0 ± 34.0</td>
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**TIBIALIS ANTERIOR**

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<td>378.3 ± 34.1</td>
<td>37.4 ± 2.9</td>
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<td>1081.9 ± 82.6</td>
<td>575.6 ± 53.9</td>
<td>53.4 ± 4.1</td>
</tr>
<tr>
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<td>9</td>
<td>1141.0 ± 119</td>
<td>1142.6 ± 119</td>
<td>100.4 ± 5.5</td>
</tr>
</tbody>
</table>
has a slightly greater effect on the tension output of the adult EDL and TA muscles. The muscles produced 73 ± 4% and 72 ± 3% of the tension of contralateral controls (± s.e.m., n=12 and n=13 respectively) and are significantly different from that produced by NaCl treated control muscles (Mann-Whitney U-test, p<0.001 and Mann-Whitney U-test, p<0.001 respectively).

After sciatic nerve crush at 5 days (preceded by NaCl treatment) the amount of force produced by the adult reinnervated EDL muscles was greatly reduced; reinnervated EDL muscles produced significantly less, 59 ± 4% (± s.e.m., n=7) tension than contralateral controls (Mann-Whitney U-test, p=0.009). Similarly, reinnervated TA muscles produced significantly less tension, 53 ± 4% (± s.e.m., n=7) than contralateral controls (Mann-Whitney U-test, p<0.0001). The relative tension (% con) produced by reinnervated fast muscles is similar to that previously reported by others authors (Lowrie et al., 1982, 1990).

In another group of animals the EDL and TA muscles were paralysed with α-BTX at birth, prior to sciatic nerve crush at 5 days. The tension produced by the reinnervated EDL muscles was 54 ± 4% (± s.e.m., n=12) of that produced by contralateral controls and is significantly different (Mann-Whitney U-test, p<0.001). The tension produced by animals subjected to nerve crush injury preceded by NaCl treatment is not significantly different from that produced by muscles subjected to nerve crush injury at 5 days following a period of paralysis at birth (Mann-Whitney U-test). Interestingly, the reinnervated TA muscles were much more severely affected by paralysis at birth followed by nerve crush injury. The reinnervated TA muscles produced 37 ± 3% (± s.e.m., n=12) of tension of the contralateral control muscles, which is
significantly different from that produced by TA muscles treated with NaCl only prior to nerve crush injury (Mann-Whitney U-test, p<0.001). Preceeding nerve crush injury at 5 days with a period of muscle paralysis significantly affected the tension output of the adult reinnervated TA muscles (Mann-Whitney U-test, p<0.01). Examples of these experiments illustrating the reduced tension output of the adult muscles can be seen in Figure 1.

These results demonstrate that temporary paralysis of fast EDL and TA muscles at birth affects the tension development of the adult muscles. More prolonged paralysis at birth has greater effects on the adult fast muscles. Sciatic nerve crush injury at 5 days has been demonstrated to greatly impair the tension development of the adult fast muscles as they develop approximately 50% of the tension of controls. Interestingly, if 5 day nerve crush injury is preceeded by a temporary period of muscle paralysis at birth, a more pronounced effect on the tension development of the adult reinnervated TA muscles is observed. However, preceeding nerve crush injury with α-BTX treatment had no further significant effects on the tension development of the adult EDL muscles.

At the end of the physiological tension recording experiments the operated and control muscles were removed from the experimental animal and weighed. The operated muscle weight was expressed as a percentage of the weight of the contralateral control muscle and the values are collated in Table 2. The effects of paralysis and nerve crush injury in early neonatal life were paralleled in the weights of the operated muscles. The fast EDL and TA muscles which had been briefly paralysed with α-BTX were less well developed than their contralateral control
Figure 1: Isometric Tension Recordings of Adult EDL and TA Muscles Following Neonatal Nerve Crush Injury, Preceeded By \(\alpha\)-BTX or NaCl Treatment at Birth

Examples of records of maximum tetanic contractions (100 Hz) elicited from adult EDL and TA muscles. Contractions were elicited by stimulating the sciatic nerve with bipolar silver electrodes, with a pulse width of 0.02msecs. In all of the traces presented the upper tetanic contraction was elicited from the contralateral control muscle and the lower contraction was elicited from the operated muscle from the same animal. The two traces in a) are from an adult EDL muscle, following nerve crush injury at 5 days preceded by NaCl treatment at birth. Trace b) presents traces from an adult TA muscle subjected to nerve crush injury at 5 days preceded by NaCl treatment at birth. The traces presented in c) are examples of tetanic contractions from an adult EDL muscle subjected to nerve crush injury at 5 days preceded by \(\alpha\)-BTX treatment at birth and the traces presented in d) are examples of tetanic contractions from an adult TA muscle subjected to nerve crush injury at 5 days preceded by \(\alpha\)-BTX treatment at birth.
Table 2: *Wet Weights of Adult Fast EDL and TA Muscles Following Treatment at Birth*

The EDL and TA muscles of newborn rats were treated with α-BTX at birth; some of these animals were treated again 3 days later with α-BTX. Other animals were treated at birth with α-BTX or with NaCl, and were then subjected to nerve crush injury at 5 days. Control animals were treated at birth with NaCl and then again with NaCl 3 days later. Ten weeks - 10 months later the operated and contralateral control muscles were removed from the experimental animals and weighed (grammes). This Table summarizes the mean ± s.e.m. of the wet weight of the EDL and TA muscles from the experimental animals. In addition, the wet weight of the operated muscle was expressed as a percentage that of the contralateral control muscle in each animal.
Table 2

MUSCLE WEIGHT/wet weight in grammes

EXTENSOR DIGITORUM LONGUS

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALATERAL</th>
<th>OPERATED</th>
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<td>BTX</td>
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<td>0.197 ± 0.03</td>
<td>0.190 ± 0.03</td>
<td>92.8 ± 1.0</td>
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<td>16</td>
<td>0.200 ± 0.02</td>
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<td>0.077 ± 0.01</td>
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<td>2NaCl</td>
<td>10</td>
<td>0.187 ± 0.02</td>
<td>0.180 ± 0.02</td>
<td>96.8 ± 3.6</td>
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<td>0.362 ± 0.06</td>
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<td>0.670 ± 0.04</td>
<td>0.652 ± 0.04</td>
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muscles. α-BTX treated EDL and TA muscles weighed 90-95% of their contralateral control muscles. The relative wet muscle weights (%con) of both the treated EDL and TA muscles are significantly different from the relative weights of NaCl-treated muscles (Mann-Whitney U-test, 5% level). More prolonged paralysis (2BTX) of the fast muscles at birth affected the muscle weights of the adult muscles to a greater extent, so that the weight of the operated EDL and TA muscles in adulthood was 80-85% of the contralateral controls. The relative wet weights of 2BTX muscles are significantly different from NaCl-treated muscles (EDL: Mann-Whitney U-test, p<0.025; TA: Mann-Whitney U-test, p<0.002).

In one group of animals where sciatic nerve crush injury at 5 days was preceded by NaCl treatment at birth, the adult muscles demonstrated extensive atrophy compared to contralateral control muscles and weighed significantly less. Reinnervated EDL muscles weighed 54 ± 3% (± s.e.m., n=8) of contralateral control muscles and reinnervated TA muscles 49 ± 2% (± s.e.m., n=8) of contralateral control muscles. The muscle weights of the adult reinnervated EDL and TA muscles are significantly different from the weights of their contralateral controls (Mann-Whitney U-test, p<0.003, and p<0.004 respectively).

In this experimental series the greatest impairment in muscle weight was seen when sciatic nerve crush injury at 5 days was preceded by a period of α-BTX induced paralysis. The development of the muscles was substantially effected. EDL muscles paralysed at birth then subjected to nerve crush injury at 5 days weighed 47 ± 2% (± s.e.m., n=14) of that of contralateral controls (Mann-Whitney U-test, p<0.001). The reinnervated TA muscles were even more extensively effected by the treatment. The TA muscles weighed only 39 ± 2% (± s.e.m., n=13) of
contralateral control muscles (Mann-Whitney U-test, p<0.001). Interestingly, EDL muscles paralysed at birth with α-BTX prior to nerve crush injury at 5 days, weighed significantly less than those muscles subjected to nerve crush injury, with NaCl pre-treatment (Mann-Whitney U-test, p<0.025). This significant difference reflected that seen in similarly treated TA muscles (Mann-Whitney U-test, p<0.01).

In conclusion, the extent of muscle fibre atrophy demonstrated in the experimental animals paralleled the reduced tension output of the muscles. This is illustrated for the EDL muscles in Figure 2 and for the TA muscles in Figure 3.

iii) Motor Unit Numbers and Sizes

In order to assess if the lower tension output and weight of the operated EDL muscles is due to a lower number of motor axons innervating the operated muscles, estimates of the total number of motor units in the EDL muscles were made. Estimates were made by recording and counting the increments of twitch tension from the EDL muscles, elicited by stimulation of the sciatic nerve by stimuli of increasing intensity. Examples of such an experiment are shown in Figure 4. The number of motor units in the operated and contralateral control EDL muscles was estimated and collated in Table 3. In addition, the estimate of the number of motor units in the operated muscle was expressed as a percentage of that estimated for the number of motor units in the contralateral control muscle, for each individual animal. Estimates for the number of motor units within the TA muscles was not attempted since the method employed could not accurately estimate the high number of motor units expected in adult TA muscles. Edstrom & Kugelberg (1968)
Figure 2: Maximum Tetanic Tension and Muscle Weights of Adult Rat EDL Muscles Following Interruption of Nerve-Muscle Interactions at Birth

Block diagram a) is of the effects on the maximum tetanic tension of adult EDL muscles, of interrupting nerve-muscle interactions at birth (as previously described). The maximum tetanic tension of each treated EDL muscle obtained in response to stimulation of the sciatic nerve is expressed as a percentage of its contralateral control muscle tension. Block diagram b) is of the wet weight of adult EDL muscles following treatment at birth. The wet weight of the operated muscle was expressed as a percentage of that of the contralateral control muscle. All animals were examined 10 weeks - 10 months after the initial treatment. The mean + s.e.m. are shown for each group.
Figure 2

a) Maximum Tetanic Tension

b) Muscle Weight

BTX 2BTX BTX +NC NaCl +NC 2NaCl

(%) op/(con)
Block diagram a) is of the effects on the maximum tetanic tension of adult TA muscles, of interrupting nerve-muscle interactions at birth (as previously described). The maximum tetanic tension, elicited by stimulating the muscles through the sciatic nerve, of the operated muscle was expressed as a percentage of the maximum tetanic tension of the contralateral control muscle. Block diagram b) illustrates the wet weight of the operated TA muscle expressed as a percentage of the wet weight of the contralateral control muscle. All animals were examined 10 weeks - 10 months after the treatment at birth. The mean ± s.e.m. are shown for each group.
Figure 3

(a) Maximum Tetanic Tension

(b) Muscle Weight

BTX 2BTX BTX NaCl 2NaCl +NC +NC 242
The number of motor units within adult EDL muscles treated at birth was estimated by stimulating the sciatic nerve with single pulses every 4 seconds. The stimulus intensity was gradually increased so that axons with different thresholds were successively activated and motor units were successively recruited. The stepwise increments in twitch tension were recorded on an oscilloscope screen and subsequently photographed. Trace a) is from a contralateral control EDL muscles, which has 40 motor units. Trace b) is from an EDL muscle treated at birth with α-BTX and then treated again with α-BTX 3 days later, and has 25 motor units. Trace c) is from an adult EDL muscle treated at birth with NaCl and then subjected to nerve crush injury at 5 days, and has 21 motor units. Trace d) is taken from an adult EDL muscle treated at birth with α-BTX and then subjected to nerve crush injury at 5 days, and has 14 motor units.
Table 3: The Mean Motor Unit Numbers of Adult EDL Muscles Following Treatment at Birth

Following treatment at birth with either α-BTX and/or neonatal nerve crush injury (as previously described) the number of motor units in the adult EDL muscles was estimated. The sciatic nerve was stimulated by single pulses every 4 seconds. With increasing stimulus intensity, axons with different thresholds were activated, resulting in a successive recruitment of individual motor units. This was manifest in stepwise increments in twitch tension which were recorded on an oscilloscope screen and could be counted. The estimates of motor unit numbers in the operated and contralateral control muscles are presented in this Table. In addition, the estimate of the number of motor units in the operated muscle was expressed as a percentage of the estimate of the number of motor units in the contralateral control muscle, in each animal. The values presented in this Table are mean ± s.e.m.
Table 3

ESTIMATES OF MOTOR UNIT NUMBERS

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRAL.</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>2BTX</td>
<td>4</td>
<td>40.3 ± 5.1</td>
<td>23.4 ± 4.5</td>
<td>64 ± 2%</td>
</tr>
<tr>
<td>BTX + crush</td>
<td>7</td>
<td>34.3 ± 1.5</td>
<td>13.9 ± 1.2</td>
<td>41 ± 4%</td>
</tr>
<tr>
<td>NaCl + crush</td>
<td>3</td>
<td>31.7 ± 3.0</td>
<td>19.5 ± 2.5</td>
<td>65 ± 17%</td>
</tr>
<tr>
<td>2NaCl</td>
<td>1</td>
<td>46</td>
<td>43</td>
<td>93%</td>
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</table>
estimated that TA muscles contain 77 or 73 motor units, according to calculations based on tension recordings or histochemical mapping.

The number of motor units in the contralateral control EDL muscles from all of the experimental groups ranged between 27-50, with a mean of 36.9 $\pm$ 1.7 (± s.e.m., n=15) (see Figure 4a). Close (1967) and Balice-Gordon & Thompson (1988) reported similar estimates (40) of motor unit numbers in adult EDL muscles. The number of motor units within adult EDL muscles treated at birth with $\alpha$-BTX was not calculated since the amount of tension produced by the adult operated muscle was not very different from that produced by the contralateral control muscle, and it was assumed that any difference in motor unit numbers between the operated and contralateral control muscles would be a small difference and therefore difficult to accurately assess. In addition, Burls et al. (1991) demonstrated that treatment of EDL and TA muscles shortly after birth has no appreciable effects on motoneurone numbers. However, when adult EDL muscles treated at birth for a prolonged period of time with $\alpha$-BTX (2BTX) were examined they demonstrated a lower number of motor units in the operated muscles compared to estimates of the number of motor units within the contralateral control muscles (see figure 4b). The number of motor units in adult EDL muscles paralysed at birth for a prolonged period ranged between 12-31, with a mean of 23.4 $\pm$ 4.5 (± s.e.m., n=4). This estimate of the mean motor unit number is significantly different from that estimated in contralateral control muscles (Mann-Whitney U-test, p=0.029).

EDL muscles subjected to nerve crush injury at 5 days, preceded by NaCl treatment at birth, demonstrated a greatly reduced number of motor units in the reinnervated adult muscle (see Figure 4c). The estimated
number of motor units in the adult reinnervated EDL ranged between 13-25, with a mean of 19.5 ± 2.5 (± s.e.m., n=3). This estimate is significantly different from that estimated for contralateral control muscles (Mann-Whitney U-test, p=0.028). Interestingly, preceding nerve crush injury with a temporary period of α-BTX induced muscle paralysis had a more pronounced effect on the number of motor units within the reinnervated adult EDL muscle (see Figure 4d). Estimates of the number of motor units in the reinnervated EDL muscles ranged between 10-20 with a mean of 13.9 ± 1.2 (± s.e.m., n=7). This value is significantly different from that estimated for contralateral control muscles (Mann-Whitney U-test, p<0.0001). Noticeably, the estimates made of motor unit numbers in the EDL muscles paralysed at birth with α-BTX prior to nerve crush injury is significantly different from that estimated for EDL muscles treated with NaCl at birth prior to nerve crush injury (Mann-Whitney U-test, p=0.028).

The results presented in this section indicate that a number of motoneurones is lost in adult EDL muscles following prolonged treatment at birth with α-BTX. The number of motor units within EDL muscles is reduced following nerve crush injury at 5 days (NaCl pre-treatment). The number of motor units estimated for EDL muscles subjected to nerve crush injury preceded by a short period of muscle paralysis was greatly reduced, indicating the loss of a larger number of motoneurones.

To determine any changes in the size of individual motor units within the reinnervated EDL muscles, the mean motor unit size was then calculated by dividing the maximum tetanic tension produced by the operated muscle by the number of motor units within that muscle. The mean motor unit size of the operated muscles was then expressed as a percentage of that
calculated for the contralateral control muscle. For normal muscles this value would be 100%. The results are summarized in Table 4.

Table 4 demonstrates that within adult EDL muscles paralysed at birth with $\alpha$-BTX and then subjected to $\alpha$-BTX treatment again 3 days later, the average tension of individual motor units is $115 \pm 13\%$ (s.e.m., n=4) of control motor units. EDL muscle subjected to nerve crush injury at 5 days have an average tension of individual motor units of $98.7 \pm 1.7\%$ of controls (s.e.m., n=3). This is not the case for EDL muscles paralysed at birth with $\alpha$-BTX and then subjected to nerve crush injury at 5 days. In these muscles the mean motor unit tension was $134.4 \pm 7.4\%$ (s.e.m., n=7) of controls. This mean motor unit size is significantly different from that seen in EDL muscles subjected to nerve crush injury alone (NaCl pre-treatment) (Mann-Whitney U-test, 5% level). These results show that the motor units of $\alpha$-BTX and nerve crush treated adult EDL muscles have a larger territory than NaCl and nerve crush injury treated muscles.

iii) Physiological Properties of Treated Muscles

The contractile characteristics and fatiguability of the operated fast twitch muscles were assessed to ascertain any changes in these properties induced by treatment at birth. Alterations in the TTP and 1/2RT measurements, and in the fatigue indices, tend to reflect altered motoneurone activites (see Lowrie et al., 1987). The TTP and 1/2RT measurements of the operated and contralateral control muscles are presented in Table 5. Table 5 demonstrates that no significant effects on the contractile characteristics (TTP and 1/2RT) were seen in any of the experimental groups. These results also confirm those of other
Table 4: Estimates of Mean Motor Unit Sizes Within Adult EDL Muscles Following Treatment at Birth

Following treatment at birth with either α-BTX or/and neonatal nerve crush injury (as previously described), the average motor unit size was estimated in the adult EDL muscles. The average motor unit tension was calculated by dividing the maximum tetanic tension of the operated muscle by the total number of motor units identified in that muscle. The average motor unit tension was also estimated for the contralateral control muscle. The value of mean motor unit tension of the operated muscle was then expressed as a percentage of the mean motor unit tension of the contralateral control muscle. The expected value for normal muscles is 100%. The values presented in this Table are mean ± s.e.m.
### Table 4

<table>
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<tr>
<th>Treatment</th>
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<th>Control</th>
<th>Operated</th>
<th>% Op/Con</th>
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<tr>
<td>2BTX</td>
<td>4</td>
<td>8.8 ± 1.0</td>
<td>10.2 ± 0.9</td>
<td>115 ± 13%</td>
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<tr>
<td>BTX + Crush</td>
<td>7</td>
<td>7.2 ± 0.4</td>
<td>9.8 ± 1.1</td>
<td>134 ± 7%</td>
</tr>
<tr>
<td>NaCl + Crush</td>
<td>3</td>
<td>9.0 ± 1.1</td>
<td>8.6 ± 0.8</td>
<td>99 ± 17%</td>
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<tr>
<td>2NaCl</td>
<td>1</td>
<td>9.2</td>
<td>9.8</td>
<td>106%</td>
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Table 5: Contractile Properties of Adult EDL and TA Muscles Following Treatment at Birth

The fast EDL and TA muscles were treated at birth with an α-BTX implant; some animals were treated again 3 days later with α-BTX. Other animals were treated at birth with α-BTX or NaCl, and then subjected to sciatic nerve crush injury at 5 days. Control animals were treated at birth with NaCl and then treated again 3 days later with NaCl. Ten weeks - 10 months later the muscles were prepared for isometric tension recording. This Table collates the time-to-peak measurements (TTP) and half relaxation time (1/2RT) measurements from single twitch contractions from the operated and contralateral control muscles. All values are mean ± s.e.m. Following all of the experimental treatments at birth, the TTP and 1/2RT values of the adult EDL and TA muscles are not significantly different (Mann-Whitney U-test).
Table 5

**CONTRACTILE PROPERTIES**

**EXTENSOR DIGITORUM LONGUS**

<table>
<thead>
<tr>
<th>TREATMENT</th>
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<th>TIME TO PEAK</th>
<th>1/2 RELAXATION TIME</th>
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</thead>
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<tr>
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<td></td>
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</tr>
<tr>
<td></td>
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<td>OP</td>
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<td><strong>TIME TO PEAK</strong></td>
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<td></td>
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<tr>
<td><strong>1/2 RELAXATION TIME</strong></td>
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</tr>
<tr>
<td><strong>TREATMENT</strong></td>
<td><strong>N</strong></td>
<td><strong>CON</strong></td>
<td><strong>OP</strong></td>
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<td>BTX</td>
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<td>NaCl + crush</td>
<td>7</td>
<td>24±2</td>
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<tr>
<td>2NaCl</td>
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<td>23±2</td>
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**TIBIALIS ANTERIOR**

<table>
<thead>
<tr>
<th>TREATMENT</th>
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<th>1/2 RELAXATION TIME</th>
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<tbody>
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<td><strong>TREATMENT</strong></td>
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<td><strong>CON</strong></td>
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authors that nerve crush injury at 5 days does not affect the TTP (Lowrie et al., 1982; 1987; 1990).

The fatigue indices (FI) were calculated for the experimental EDL and TA muscles within each group. An index of 0 indicates absolute resistance to fatigue and an index of 1 indicates complete fatiguability. The fatigue index calculated for the operated muscle was expressed as a percentage of the contralateral control muscle. The values of the FI for both the operated and contralateral control muscles and the % op/con are collated in Table 6. Figure 5 presents examples of such experiments and shows traces of the response of operated and control muscles to the intermittent stimulation used to fatigue the adult fast twitch muscles.

Subjecting normal fast EDL and TA muscles to intermittent stimulation at 40Hz for 3 minutes, reduces the tension produced by approximately three quarters. The fatigue index of normal EDL muscles was $0.74 \pm 0.02$ (± s.e.m., n=20) - this is the mean of the fatigue indices from all of the contralateral control muscles in this experimental series. The FI of normal TA muscles (again calculated from all of the contralateral control muscles) is $0.72 \pm 0.02$ (± s.e.m., n=16). This is similar to that reported by other authors (FI = 0.66; Lowrie et al., 1982)

Fast muscles temporarily paralysed at birth (BTX) demonstrate a slightly lower resistance to fatigue in adulthood when compared to contralateral control muscles (see Table 5). This difference is not, however, significant for either the EDL or TA muscles (Mann-Whitney U-test). More prolonged paralysis (2BTX) of the EDL and TA muscles again
Table 6: Fatigue Indices of Adult EDL and TA Muscles Following Treatment at Birth

Fast EDL and TA muscles of newborn animals were treated at birth with α-BTX; some of these animals were treated again 3 days later with α-BTX. Other animals were treated at birth with α-BTX or NaCl, and then subjected to sciatic nerve crush injury at 5 days. Control animals were treated at birth with NaCl and were then treated again 3 days later with NaCl. Ten weeks - 10 months later the fatigue resistance of the experimental and control muscles was tested by stimulating the muscles continuously at 40Hz, for 250 milliseconds every second, for a period of 3 minutes. The fatigue indices of the operated and contralateral control muscles was calculated as previously described. In addition, the calculated fatigue index of the operated muscle was expressed as a percentage of that calculated for the contralateral control muscle for each animal. All values are mean ± s.e.m.
### Table 6

**FATIGUE INDEX**

#### EXTENSOR DIGITORUM LONGUS

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>N</th>
<th>CONTRALATERAL</th>
<th>OPERATED</th>
<th>%OP/CON</th>
</tr>
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<tr>
<td>BTX</td>
<td>6</td>
<td>0.74 ± 0.05</td>
<td>0.60 ± 0.09</td>
<td>78.8 ± 7.7</td>
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<td>2BTX</td>
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<td>0.79 ± 0.09</td>
<td>0.60 ± 0.11</td>
<td>75.7 ± 5.8</td>
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<td>BTX + crush</td>
<td>6</td>
<td>0.74 ± 0.05</td>
<td>0.22 ± 0.04</td>
<td>29.8 ± 5.5</td>
</tr>
<tr>
<td>NaCl + crush</td>
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<td>0.70</td>
<td>0.38</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.73</td>
<td>0.48</td>
<td>66</td>
</tr>
<tr>
<td>2NaCl</td>
<td>3</td>
<td>0.75 ± 0.02</td>
<td>0.78 ± 0.06</td>
<td>108 ± 5.9</td>
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#### TIBIALIS ANTERIOR

<table>
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<tr>
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<th>CONTRALATERAL</th>
<th>OPERATED</th>
<th>%OP/CON</th>
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<tr>
<td>BTX</td>
<td>3</td>
<td>0.70 ± 0.01</td>
<td>0.64 ± 0.06</td>
<td>92.7 ± 8.5</td>
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<tr>
<td>2BTX</td>
<td>4</td>
<td>0.77 ± 0.04</td>
<td>0.60 ± 0.06</td>
<td>78.0 ± 9.3</td>
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<tr>
<td>BTX + crush</td>
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<td>0.64 ± 0.03</td>
<td>0.21 ± 0.11</td>
<td>32.0 ± 14.4</td>
</tr>
<tr>
<td>NaCl + crush</td>
<td>2</td>
<td>0.76</td>
<td>0.36</td>
<td>47</td>
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<tr>
<td></td>
<td></td>
<td>0.60</td>
<td>0.39</td>
<td>65</td>
</tr>
<tr>
<td>2NaCl</td>
<td>3</td>
<td>0.76 ± 0.00</td>
<td>0.82 ± 0.07</td>
<td>108.7 ± 8.2</td>
</tr>
</tbody>
</table>
d. 1000g

e. 500g

f. 250g

1 min
results in a slightly lower resistance to fatigue in both fast muscles compared to their contralateral controls. Again this difference seen in operated muscles compared to contralateral controls is not significantly different (Mann-Whitney U-test).

Those muscles which had been subjected to nerve crush injury at 5 days, preceded by NaCl treatment, were in adulthood much more resistant to fatigue than contralateral control muscles (see Figure 5). The reinnervated EDL muscles had 54% and 66% of the fatigue indices of the contralateral control muscles, whilst reinnervated TA muscles demonstrated 47% and 65% respectively. The values of FI for reinnervated EDL muscles is comparable to that reported (0.36) by Lowrie et al. (1982) following 5 day nerve crush injury in Wistar rats.

Most strikingly, the fatiguability of the fast muscles which were temporarily paralysed with α-BTX prior to a 5 day nerve crush injury was greatly reduced (see Figure 5). The FI of reinnervated EDL muscles was 30 ± 6% (± s.e.m., n=6) of that of contralateral control muscles and is significantly different (Mann-Whitney U-test, p<0.001), whilst the FI of reinnervated TA muscles was 32 ± 14% (± s.e.m., n=3) of that of contralateral controls and which is significantly different (Mann-Whitney U-test, p<0.05).

Control EDL and TA muscles treated at birth and 3 at days with NaCl, demonstrated no significant changes in the fatigue characteristics of the adult muscles. The altered fatigue properties are illustrated in Figure 6.

iv) **Histology and Immunohistochemistry**

The changes observed in the fatigue properties of the experimental
Fatigue Index (% of control)

Figure 6

EDL

TA

BTX  2BTX  BTX +NC  NaCl +NC  2NaCl

Fatigue Index

100

80

60

40

20

0

20  40  60  80  100

BTX  2BTX  BTX +NC  NaCl +NC  2NaCl
muscles were accompanied by changes in the capacity of the muscles for oxidative metabolism. This was investigated by staining transverse frozen sections of the operated and contralateral control muscles for succinic dehydrogenase activity (SDH). The fast EDL and TA muscles were treated at birth as previously described and examined in adulthood. Contralateral, unoperated muscles and NaCl-treated adult EDL and TA muscles demonstrated a mosaic pattern of staining for SDH activities with a mixture of fibres which have either a low (large, palely staining) or high (small, darkly staining) oxidative capacity. In normal fast muscles large glycolytic fibres (palely staining) comprise approximately 30-40% of the total number of muscle fibres. This is illustrated in Figure 7.

Staining for oxidative enzymes in adult EDL and TA muscles, which had been temporarily paralysed at birth with α-BTX, demonstrated no apparent difference in the overall staining intensity of the muscle fibres in the operated muscles compared to the contralateral controls. More prolonged paralysis of the fast muscles at birth (2BTX) similarly resulted in no marked difference in the overall staining intensity of the muscles.

Sciatic nerve crush injury at 5 days, preceeded by NaCl treatment at birth, resulted in adult EDL and TA muscles which, when stained for SDH activity, revealed reinnervated muscles with greatly altered patterns of SDH activities. This altered pattern of staining can be seen in Figure 7. Reinnervated EDL and TA muscles possessed a predominance of muscle fibres with a high content of oxidative enzymes (small, darkly stained) relative to contralateral controls. Most noticeably, in the reinnervated muscles the normal mosaic pattern of staining was absent
EDL and TA muscles of newborn animals were treated with either α-BTX or NaCl within 6-12 hours of birth. Five days later the neonatal animals were subjected to sciatic nerve crush injury. The animals were allowed to recover. 10 weeks - 10 months later the muscles were removed from the experimental animals, frozen, sectioned and stained for SDH activities using the method of Nachlas et al. (1957). Photomicrographs a) - d) are taken from transverse sections of adult reinnervated EDL muscles. Photo. a) is the contralateral control of b) which is taken from an EDL muscle treated at birth with NaCl and then subjected to nerve crush injury at 5 days. Photo c) is of the contralateral control of d) which is taken from a reinnervated EDL muscle treated at birth with α-BTX and then subjected to nerve crush injury at 5 days.

Photomicrographs e) - h) are taken from transverse sections of adult reinnervated TA muscles. Photo e) is taken from the contralateral control of f) which is taken from a TA muscle treated at birth with NaCl and then subjected to nerve crush injury at 5 days. Photo g) is the contralateral control of h) which is taken from an adult reinnervated TA muscle treated at birth with α-BTX and then subjected to nerve crush injury. Scale bar = 150 µm.
and fibres of similar staining intensities (i.e. oxidative capacities) tended to group together. These observations are similar to those reported by other authors (Lowrie et al., 1982; Lowrie et al., 1990).

Preceeding nerve crush injury with α-BTX induced paralysis had a more marked effect on the staining pattern of SDH activity within reinnervated adult muscles. This altered pattern is illustrated in Figure 7. Reinnervated adult EDL and TA muscles were composed almost totally of muscle fibres with high levels of oxidative enzymes compared to contralateral controls. All of the muscle fibres were small, darkly stained fibres (high oxidative capacity) and paley stained fibres were very rarely seen. Indeed, in some of the reinnervated adult TA and EDL muscles all of the muscle fibres stained darkly for SDH activity.

Transverse sections of EDL and TA muscles from all experimental groups were stained with an antibody against a slow myosin heavy chain (SMHC) (NOQ7.54D, kindly donated by Dr. Robin Fitzsimmons) to study any changes which may have occured in the number and distribution of slow fibres in the fast muscles following treatment at birth. Camera lucida drawings of whole sections of EDL and TA muscles stained with the SMHC antibody are presented in Figure 8, and illustrate the patterns of distribution of the positively-stained fibres. Immunocytochemical staining of NaCl treated and contralateral control muscles stained for the SMHC demonstrated a small number of positively stained fibres within the adult fast muscles. In adult EDL muscles the SMHC-positively stained fibres were evenly distributed throughout the adult muscle though they tended to be more prevalent in the deeper parts of the muscle. In adult TA muscles the SMHC-positively stained fibres were again evenly
distributed, in a mosaic pattern, however they were localized within the
depth portion of the muscle. Photomicrographs of SMHC stained fibres in
adult contralateral control EDL and TA muscles can be seen in Figure 9.

Fast EDL and TA muscles paralysed either temporarily (BTX) or for
more prolonged periods (2BTX) with α-BTX demonstrated no marked
difference in the SMHC-positive staining pattern between operated and
contralateral control muscles.

Fast muscles which had been treated at birth with NaCl and then
subjected to sciatic nerve crush injury at 5 days demonstrated marked
changes in the pattern of staining to the slow myosin antibody in the
adult reinnervated muscles. Reinnervated adult EDL and TA muscles

demonstrated a clustering of SMHC-positive fibres which was not
observed in the contralateral control muscles. The clustering of
positively stained fibres in adult EDL and TA muscles is graphically
illustrated in Figures 8 and 9. In the reinnervated EDL muscles the slow
myosin HC positive fibres are irregularly distributed throughout the
muscle, compared to the contralateral control muscle and appear in parts
of the muscle where positively stained fibres are not expected.
Similarly, adult reinnervated TA muscles demonstrate irregular grouping
of the positively stained fibres compared to the contralateral control
muscles (see Figure 8).

In addition to the irregular clustering of positively stained
fibres, both reinnervated EDL and TA muscles possessed more positively
stained fibres compared to contralateral control muscles. As an
indication of this increased staining the number of SMHC positively
stained muscle fibres was counted. A reinnervated EDL muscle had 255
positively stained fibres, compared to 100 positively stained fibres in
EDL and TA muscles were treated at birth with α-BTX and NaCl and then subjected to sciatic nerve crush injury at 5 days. Ten weeks - 10 months later the muscles were removed from the experimental animals, frozen, sectioned and stained against a slow myosin heavy chain (MHC) antibody. Photomicrograph a) is taken from the contralateral control of b) which is of an adult reinnervated EDL muscle treated at birth with NaCl and then subjected to sciatic nerve crush injury at 5 days - examined at 9 months. Photo. c) is taken from the contralateral control of d) which is is of a reinnervated adult EDL muscle following treatment at birth with α-BTX, followed by sciatic nerve crush injury at 5 days - examined at 4 months. Photomicrograph e) is taken of the contralateral control of f) which is of a reinnervated adult TA muscle following treatment at birth with NaCl followed by sciatic nerve crush injury at 5 days - examined at 9 months. Photo. g) is taken from the contralateral control of h) which is taken is of an adult reinnervated TA muscle following treatment at birth with α-BTX and a subsequent nerve crush injury at 5 days - examined at 4 months. Scale bar = 150 μm.
Figure 9
the contralateral control muscle (255% of the control). A reinnervated TA muscle had 262 positively stained fibres compared to 66 positively stained fibres in the contralateral control muscles (397% of the control). The reinnervated muscles were also small compared to their contralateral control muscles, and have been previously shown to contain about half of the normal number of muscle fibres (Lowrie et al., 1982).

Preceeding nerve crush injury with temporary α-BTX-induced paralysis at birth, had marked effects on the distribution of SMHC-positively stained fibres, in adult reinnervated muscles. The results are illustrated in the camera lucida drawings presented in Figure 8. Adult reinnervated EDL and TA muscles demonstrated an irregular pattern and distribution of positively stained fibres compared to controls. The normal pattern of mosaic staining of adult fast muscles was no longer present. Instead, an irregular grouping of positively stained fibres was observed, spread throughout the whole reinnervated muscles. The clustering of the positively stained fibres is illustrated in Figure 9.

In addition, the number of positively stained muscles fibres in temporarily-paralysed reinnervated EDL and TA muscles was counted. The number of positively-stained fibres in the operated EDL muscles was 328 compared to 182 positively stained fibres in the contralateral control muscle (180% of the control). A temporarily paralysed-reinnervated TA muscle had 627 positively stained fibres in the operated muscle compared to 101 in the contralateral control muscle (621% of the control). The large number of positively stained fibres in the reinnervated TA muscle compared to contralateral control is unexpected. Lowrie et al. (1988) demonstrated that following nerve crush injury at 5 days the number of slow fibres in the TA muscles of 2 month old adults was equal to the
number present in contralateral control muscles. Before 2 months of age the number of SMHC positive fibres in the reinnervated muscle was greater than that in the control muscle; the number of positive fibres gradually decreasing until at 2 months the number of positively-stained fibres equalled that of controls. The temporarily-paralysed reinnervated EDL and TA muscles counted for slow myosin positive fibres presented here were 3 months of age. The high relative number (%con) of SMHC positively stained fibres may be indicative of a delayed development in the temporarily paralysed-reinnervated muscles. The number of positively stained fibres may decrease in older animals to equal that of controls - in a similar, but delayed, manner to that seen in reinnervated muscles (Lowrie et al., 1988). Alternatively, the greater number of SMHC positively stained fibres in the reinnervated muscles may be an indication of an altered population of slow fibres in the temporarily-paralysed reinnervated TA muscles. This remains to be investigated - examination of muscles and counts of SMHC positively stained fibres, at varying time points following reinnervation would be interesting.
DISCUSSION

The experiments in this study endeavoured to look at the importance of muscle activity in the early postnatal period, on the development of the fast hindlimb muscles of the rat. The results presented in this study demonstrate that by reducing the activity of the target during the first few days of life, the normal development of the fast hindlimb muscles of the rat is impaired. By prolonging the period of time during which the activity of the immature muscle fibres is reduced, the more severe the impairment in the adult muscles.

In addition, the experiments assessed the role of target activity in the changing response of the muscle fibres to neonatal nerve crush injury. Peripheral nerve injury at birth results in poor reinnervation and recovery of function of the fast hindlimb muscles, whereas the same injury imposed on an adult results in complete recovery of innervation and recovery of the muscle fibres (Beuker & Meyer, 1951; Zelená & Hník, 1963; McArdle & Sansone, 1977; Lowrie et al., 1982). In parallel, immature motoneurones die more readily after axonal injury than mature motoneurones (Romanes, 1946; Lowrie et al., 1982). The results presented in this Chapter confirm earlier findings that sciatic nerve crush injury inflicted on 5 day old rats, permanently impairs the development of the fast EDL and TA muscles, and leads to marked changes in the physiological properties of the reinnervated muscles. In addition, the results presented in this Chapter demonstrate that reducing the activity of the target prior to the nerve crush, results in an even greater impairment in the function of the fast reinnervated muscles. These findings suggest an important role for the activity of the target
in the establishment of a mature state in the neuromuscular system, from which the muscle fibres and motoneurones can survive a temporary period of disconnection.

The data presented in this Chapter, in addition to that presented in Chapter 3, demonstrate the importance of continued interaction between the muscle and the motor nerve for the normal development of the mammalian hindlimb muscles; the longer the disruption of nerve-muscle interactions the greater the impairment. EDL and TA muscles paralysed at birth with α-BTX were reduced in size and produced less tension in adulthood, than controls. However, the extent of the impairment observed in the fast muscles following paralysis at birth was not as great as that manifest in similarly-treated slow soleus muscles (data presented in Chapter 3) - (see Figure 10).

The different magnitude of impairment seen in the fast muscles following paralysis at birth, when compared to the slow soleus muscle is unusual. Especially since previous experimental work, in which the sciatic nerve of 5 day old animals was crushed, shows a greater selective impairment of the weight and tension development of the fast EDL and TA muscles, compared to the soleus muscle (Lowrie et al., 1982; Lowrie & Vrbová, 1984; Lowrie et al., 1987). A similar insult performed at birth however, demonstrates that following injury at a very early postnatal stage, there is little difference in the recovery of the reinnervated fast and slow muscles (Lowrie et al., 1987).

The reasons for the greater susceptibility of the slow soleus muscle to reduced postsynaptic activity in the early postnatal period, when compared with the EDL and TA muscles, are unclear. It may be possible to explain the differences in susceptibility of the hindlimb
Figure 10

Maximum Tetanic Tension (% con)

EDL  TA  SOLEUS

BTX  2BTX  BTX  2BTX  BTX  2BTX
muscles in terms of their relative maturity at the time of the imposed paralysis. In an extensive study on the formation and development of the hindlimb muscle groups in embryonic rats, Condon and colleagues (1990a) demonstrated that the muscles within the anterior muscle group, including the EDL and TA muscles, are formed as distinct individual muscles before those in the superficial posterior muscle group, including the soleus muscle, thus, indicating a differential in maturity. In addition to the difference in times of origin, the muscles within the anterior muscle group (EDL and TA) show different patterns in the expression of a slow myosin heavy chain (MHC) compared to the superficial posterior group of muscles. By E17, in the rat hindlimb, all primary myotubes, in all muscle groups, express slow MHC (Condon et al., 1990a). Earlier in development, at E15, embryonic EDL and TA muscle masses express slow MHC in all of the primary myotubes. However, in the soleus muscle mass only 50% of the primary myotubes have reached a stage in development where they are capable of expressing this protein (Condon et al., 1990a). The authors conclude that there is a general anterio-posterior gradient in the timing of slow myosin expression which they suggest is a reflection of a corresponding gradient in the timing of myogenesis. These results of Condon and colleagues indicate that the anterior muscles, EDL and TA, are more mature at birth, than the superficial posterior muscles, including soleus. The relative levels of maturity of the EDL and TA muscles may explain the differences in response to paralysis at birth on the development of the adult muscles; as seen in this thesis. It is possible that the neonatal EDL and TA muscles are more capable of withstanding a temporary period of disconnection from their motor nerve than the relatively immature soleus.
muscle. Even with this proposed advantage, the fast EDL and TA muscles temporarily paralysed at birth do not fully recover from a period of disconnection, and the adult muscles are impaired in both size and ability to produce force when compared to controls. The results presented demonstrate that for normal development to proceed, constant interactions between the target and the motor nerve are necessary during the early postnatal stages.

The possibility that there are maturity related differences in the neuromuscular junctions of the postnatal EDL and TA muscles compared to those of the soleus muscles, could possibly explain the marked inability of the slow soleus muscles to cope with a period of postsynaptic inactivity. There is some evidence to suggest that differences in the neuromuscular junctions of these two types of muscle do exist. During postnatal development, the endplates on fast muscle fibres appear to be morphologically stable from the third postnatal week, whereas the endplates on slow fibres are more inclined to change their detailed morphology (Wigston, 1987).

It cannot be ruled out that the technique employed for paralysing the immature muscles, that of applying α-BTX in the form of a silastic implant, could not be easily transferred from the soleus preparation to the EDL and TA preparation. The better recovery of the adult fast EDL and TA muscles from paralysis at birth, could be the consequence of a lower initial level of paralysis. The different parameters involved in paralysing EDL and TA muscles, such as the size and shape of the muscles, were taken into account when approaching the experiments. An increased quantity of α-BTX was applied to the two fast muscles, compared to that used to paralyse the soleus. Consistent with the
experiments performed in Chapter 3, a sublethal dose of the toxin was applied to the hindlimb muscles, which in 20% of cases resulted in the death of the experimental rats. This sublethal dose thus ensured optimal conditions for extensive paralysis of the muscles in the hindlimb of the rats. In addition, the dimensions of the $\alpha$-bungarotoxin containing implant were altered to take into account the differences in the surface areas of the EDL and TA muscles, compared to the soleus muscles. However, the combined mass of the EDL and TA muscles is greater than that of the soleus muscles and the amount of toxin applied to the muscles may have been insufficient to cause temporary but complete paralysis. It is also possible that the deeply placed muscle fibres within the TA muscle may be more difficult to reach by diffusion of the toxin from the implant and therefore, it is a possibility that the more deeply placed fibres were unaffected by the $\alpha$-BTX and remained active throughout the early postnatal period. Incomplete paralysis seems, however, an unlikely explanation for the different responses of the fast and slow muscles to the period of reduced postsynaptic activity, especially since the larger TA muscles were consistently more impaired in adulthood, as a consequence of the $\alpha$-BTX treatment, than the relatively thin, and small EDL muscles.

In the present Chapter the results presented show that nerve crush injury during the early postnatal period prevents the normal development of the adult fast muscle, leading to changes in the physiological properties of the reinnervated muscles. These results, demonstrating the incomplete recovery of the fast muscles to nerve crush injury at 5 days confirm the findings of other authors (Lowrie et al., 1982; Lowrie & Vrbová, 1984; Lowrie et al., 1987; Burls et al., 1991).
The fast reinnervated EDL and TA muscles developed half of the tension and weight of controls. In addition, to the altered bulk and force output of the muscles, the adult reinnervated EDL and TA muscles demonstrated a large-scale reduction in the number of large, glycolytic muscle fibres present (assessed by lack of SDH activities). Those large glycolytic fibres which remained in the reinnervated adult muscles were distributed in an abnormal manner and tended to be clustered in small groups. The normal mosaic distribution of glycolytic fibres was no longer apparent. The increase in the oxidative capacity of the fast muscles following reinnervation (more darkly, stained small fibres) was manifest as an increased resistance of the EDL and TA muscles to fatigue.

The most striking finding of these experiments is the large impairment of the reinnervated EDL and TA muscles following nerve crush injury at 5 days, preceded by a period of temporary muscle paralysis at birth. Interestingly, the EDL muscles were consistently less affected by the temporary period of paralysis and the insult to the motor nerve, than the TA muscles (this phenomenon has been reported by other authors: Lowrie et al., 1987). TA muscles subjected to paralysis at birth and subsequent nerve crush injury produced 37% tension and 39% weight of controls. EDL muscles however, were noticeably less impaired and produced 47% tension and 54% weight of controls. This difference is unusual and may again be related to the relative level of maturity of the two muscles or, as was suggested by Lowrie & Vrbová (1984), the large size of the TA muscle may make it more difficult to reinnervate or reinnervation may occur over a longer time period. The longer the time needed for successful reinnervation, the longer the period of time
during which the motoneurone and muscles fibres are out of contact with each other. Thus, the prolonged period of separation may affect the ultimate recovery of the muscles from the insult. Lowrie et al. (1990) reported that the extent of the impairment of the developing fast muscles, after nerve injury, depends upon the period of denervation. Indeed, the number of motoneurones surviving neonatal nerve section is significantly reduced if reinnervation is delayed (Kashiara et al., 1987).

The reduced tension and weights of these muscles cannot be explained in terms of inhibited development and growth of new muscle fibres during the early stages of postnatal development, as a result of α-BTX paralysis. TA is relatively mature at birth and 3 days later has its full complement of muscles fibres and contains only a small number of myotubes. At birth, TA muscles have effectively completed their maturation (Zelená, 1962). Similarly, in a study investigating the relative maturity of the EDL muscles at birth, the author concluded that EDL muscles contain the full complement of muscle fibres at birth, and that no new fibres are added postnatally (Ontell, 1979). It seems fair to suggest that the effects of neonatal nerve crush injury are not due to the suppression of new muscle fibre formation. Indeed, Lowrie & Vrbová (1984) in a study of rats subjected to sciatic nerve crush injury at 5 days demonstrated that the substantial reduction in tension seen in the fast muscles following the insult, was due to a rapid loss of muscle fibres after reinnervation had occurred. Prior to reinnervation, the number of muscle fibres in the denervated muscles were similar to controls.

Preceeding sciatic nerve crush injury with a period of muscle
paralysis had very marked effects on other properties of the reinnervated muscles. Reinnervated EDL and TA muscles, when stained for SDH activities, were composed solely of small, darkly staining oxidative muscle fibres. The large, glycolytic muscle fibres which normally make up 30-40% of the total of muscle fibres in rat EDL muscles (Kelly, 1978), were absent from the reinnervated EDL and TA muscles. All of the muscle fibres in the reinnervated muscles were highly oxidative, and demonstrated consistently high intensity staining. The very marked SDH activity staining patterns of the temporarily paralysed-reinnervated muscle were reflected in the fatigue properties of these muscles. The EDL and TA muscles treated with α-BTX prior to nerve crush injury were much more resistant to fatigue than those subjected to nerve crush injury only, at 5 days. Following nerve crush injury at 5 days, adult EDL and TA muscles had 54% & 66%, and 47% & 65% of the fatigue indices of controls, respectively. Preceding nerve crush injury at 5 days with a period of paralysis at birth, resulted in reinnervated adult muscles which were much more resistant to fatigue - EDL muscles had fatigue indices which were 30% of controls and TA, 32% of controls.

It has been previously suggested that the extensive lack of recovery of fast muscles following neonatal nerve injury is due to a mismatch between the characteristics of the reinnervating axons and the denervated muscle fibres (see Lowrie et al., 1982; Lowrie & Vrbová, 1984; Lowrie et al., 1987; Burls et al., 1991). Nerve crush injury isolates the muscle from its motor nerve and so deprives the muscle fibres of the influence which brings about its maturation and development (Buller et al., 1960; Brown, 1973; Close, 1967). Muscle fibre development is retarded upon denervation (Zelená & Hník, 1963;
Engel & Karparti, 1968). By removing the activity of the target during the early postnatal period, many aspects of the normal development of muscle fibres and the neuromuscular junction are retarded. Positional and metabolic stability of ACh receptors (AChR), stabilization of AChR's at the neuromuscular junction (Stanley & Drachman, 1983a; Avila et al., 1989) and the conversion of slow neonatal AChR channel opening times, to the fast opening times characteristic of mature AChR's are all dependent upon activity (Sakmann & Brenner, 1978). Denervation of soleus muscles at birth prevents the transformation of AChR channel properties (Schuetze & Vicini, 1984). Chronic electrical stimulation of the denervated muscles permits the development of the mature shortened opening times (Brenner et al., 1987). Target activity also brings about the deposition of junctional AChE. Paralysis of embryonic muscles prevents the accumulation of AChE (Gordon et al., 1974) and Lømo & Slater (1980a) reported that AChE is not deposited in denervated endplates.

Meanwhile, the motoneurones continue to undergo their normal development and maturation, achieving their adult characteristics, whilst continuing to mature with the circuitry of the CNS. Neonatal, immature motoneurones are very different from adult motoneurones in terms of their synaptic connectivity and their intrinsic electrophysiological properties. Immature motoneurones have high input resistances and low maximal firing rates compared to adults (Fulton & Walton, 1986). The smaller, immature motoneurones are also more easily excited by afferent stimulation. During early development there are no apparent differences between "fast" and "slow" motoneurones. Over the first 3 weeks of postnatal development, motoneurones undergo many
developmental changes which bring about the characteristic properties of mature motoneurones. At 8 to 10 days of age motoneurones have already undergone marked developmental changes and the after-hyperpolarization and depolarization properties of these cells are different from those of 3 day old motoneurones (Navarrete & Walton, 1988). Mature motoneurones innervating slow muscle fibres, fire for long periods of time at low frequencies, whilst those motoneurones innervating fast muscle fibres fire phasically, but with high frequency (Denny-Brown, 1929; Navarette & Vrbová, 1983). Nerve crush injury at 5 days therefore isolates the muscle fibres from the motoneurone during a period of extensive motoneurone development (Navarrete & Vrbová, 1983), which occurs over the first few weeks of postnatal life.

The experiments performed in this Chapter demonstrate that by pre-treating the muscle with α-BTX at birth, followed by nerve crush injury at 5 days, the reinnervation of the fast muscle fibres was not as successful as that seen following nerve crush injury at 5 days only. In adulthood, the temporarily paralysed-reinnervated EDL and TA muscles were extensively impaired in both size and force production. In addition, the reinnervated muscles were completely oxidative in nature, and very resistant to fatigue. Greensmith & Vrbová (1991) treated soleus muscles at birth with α-BTX and examined them 5 days later. They found that upon recovery from paralysis, the soleus muscles have very prolonged endplate potentials, characteristic of immature muscles fibres. α-BTX treatment at birth, therefore retards the development of muscle fibres and the neuromuscular junction. If this treatment is followed by peripheral nerve crush injury at 5 days, the immature muscle fibres are isolated from the influence of the motoneurones for an even
greater period, and so the delayed maturation of the muscle fibres, already established through α-BTX treatment at birth, is accentuated. Upon reinnervation the mismatch between the immature muscle fibres and the reinnervating motoneurones will be great, and even more marked than that after nerve crush only. Once reinnervation has been established the immature muscle fibres are rapidly exposed to mature high frequency activity patterns from the reinnervating motor axons. During normal development, the EDL and TA muscles would gradually be exposed to higher activity patterns with increasing frequency as the motoneurone develops. However, in this experimental situation, the sudden imposition upon reinnervation of high frequency firing rates characteristic of fast motoneurones, is detrimental to the immature muscle fibres. The high frequency firing of mature motoneurones activate the immature muscle fibres, which results in prolonged depolarization. Prolonged depolarization is well known to cause disruption of functional contacts between nerve terminals and muscle fibres (O'Brien et al., 1980; O'Brien et al., 1984; Duxson & Vrbová, 1985; Chapter 2, this thesis). Following crush injury at birth motoneurones fire for longer periods and the aggregate EMG activity is 2/3 times greater than normal. The high frequency firing rates of the mature motoneurones innervating the EDL and TA muscles are possibly disrupting the newly established contacts between motor nerve and muscle fibres, resulting in a loss of muscle fibres, which is known to occur after reinnervation (Lowrie & Vrbová, 1984).

The mechanism of fibre loss is probably that discussed in Chapter 2, whereby the high frequency firing of the mature motoneurones activates the immature muscle fibres causing them to release K⁺.
Muscle fibres release K\(^+\) ions during depolarization (Hodgkin & Huxley, 1952) which accumulate in the synaptic cleft (Hohlfield et al., 1981). The prolonged endplate potential of paralysed muscles is probably accentuating this release. It is postulated that the K\(^+\) ions released from the muscle fibres open voltage-gated Ca\(^{2+}\) channels in the nerve terminals, which results in an influx of Ca\(^{2+}\) into the nerve terminals. Raised internal levels of Ca\(^{2+}\) may then activate neuronal CANP's attached to the axonal cytoskeleton (Tashiro & Ishizaki, 1982; Ishizaki et al., 1985), which, as demonstrated in Chapter 2, bring about the degradation and collapse of the neurofilamentous cytoskeleton of nerve terminals. The collapse of the cytoskeleton, thus brings about a large reduction in the number of nerve-muscle contacts. A substantial number of muscle fibres would therefore be lost as a consequence of denervation. α-BTX induced paralysis of young muscles has previously been demonstrated to cause the loss of contacts (Greensmith & Vrbová, 1991). After partial denervation of rat soleus muscles the motor units innervating the soleus expand their motor unit territory to innervate denervated muscles fibres. α-BTX treatment, after partial denervation, causes a reduction in the number of existing contacts, and prevents the maintenance of the enlarged motor unit territory (Connold & Vrbová, 1990).

In the experiments presented here, the number of motor units in the adult reinnervated EDL muscles was estimated. In the sample studied, the results indicate that following nerve crush injury at 5 days, the number of motor units is reduced (65 ± 17% of controls). Even considering the small sample size and the large standard error of the mean, the results indicate that a number of motor units is lost following nerve crush.
injury at 5 days preceded by NaCl treatment. This therefore suggests that the number of motoneurones innervating the EDL muscles was reduced. This is in contrast to previous reports that nerve crush injury at 5 days results in no significant loss of motoneurones (Lowrie et al., 1982; Subramaniam Krishnan et al., 1985). A recent study however demonstrated that some loss of motoneurones may occur; in that 87% and 85% of the number of control motoneurones are present in the ventral horn of rat spinal cord, following nerve crush injury, preceded by NaCl pretreatment (Burls et al., 1991). The discrepancy between the results presented in this Chapter and those of other authors may be based on the different techniques employed. In the reinnervated muscles studied a large number of muscle fibres are lost (Lowrie & Vrbová, 1984), resulting in smaller motor unit sizes. It is possible that the method of counting increments in twitch tension, to estimate motor unit numbers, may be less accurate with smaller motor units, so that it may be difficult to record each increment in force. In addition, the backlabelling technique used by Lowrie et al. (1982), Subramaniam Krishnan et al. (1985) and Burls et al. (1991), employing horseradish peroxidase (HRP) as a tracer, labels the cell bodies of γ-motoneurones as well as α-motoneurones. It is possible that the lower number of motoneurones innervating the adult EDL muscles following nerve crush injury at 5 days, may be the result of a misdirection of the reinnervating axons, so that a larger than normal proportion of the regenerating axons innervated the denervated TA muscles. This would result in a lower estimate of the number of motor units in the EDL muscles and would also take into account the lack of motoneurone loss observed by other authors (Lowrie et al., 1982; Subramaniam Krishnan et al., 1985). This in itself
is unexpected, since nerve crush injury allows for accurate reinnervation of muscles (see Burls et al., 1991). The discrepancy in the estimates of motor unit number in adult reinnervated EDL muscles following nerve crush injury at 5 days, with those of motoneurone counts made by other authors (Lowrie et al., 1982; Subramaniam Krishnan et al., 1985; Burls et al., 1991) are difficult to explain, especially considering the small sample size. However, the small increments in force produced by recruiting successive motor units, and the fact that many reinnervated muscle fibres have more than one input - some muscle fibres have more than one endplate, and other muscle fibres are innervated at a single endplate by more than one axon branch (Subramaniam Krishnan et al., 1985) - are the most likely reasons for the discrepancy in the results.

EDL muscles temporarily paralysed at birth and then subjected to sciatic nerve crush injury at 5 days demonstrated a pronounced reduction in the number of motor units in the reinnervated adult EDL muscle - estimates of the number of motor units in the adult reinnervated muscles was 41% of controls. These results parallel those of Burls et al. (1991), who showed that rats subjected to similar interruptions in nerve-muscle interactions in the early postnatal period, had much fewer motoneurones innervating the reinnervated adult EDL and TA muscles. The authors reported a loss of motoneurones similar to that seen following nerve crush injury at birth (Burls et al., 1991).

The experiments presented in this Chapter suggest that constant interactions between the nerve and its active target are necessary to bring about the change in response to peripheral injury. It is possible that the activity of the target brings about changes in the motoneurone
which makes it less susceptible to nerve injury. By blocking target activity in the early postnatal stage, the influence of the target is removed and therefore the motoneurone may not develop fully. Upon reinnervation, the immature motoneurone may be unable to maintain contact with all of the muscle fibres. Alternatively, the altered response to injury may be dependent upon the level of maturity of the target itself, and the ability of the regenerating axons to maintain contact with the immature muscle fibres. It appears that the motoneurone and muscle need to be interacting with one another and developing under the influence of one another in order to establish a system which is capable of withstanding a later period of separation. If temporary separation occurs during the early stages of development the incomplete recovery from this injury is most likely to be due to a mismatch of the characteristics of the muscle fibres and the motoneurone within a motor unit upon reinnervation, as a consequence of the short period of independent development.

An additional interesting observation, demonstrated in the results presented in this Chapter, concerning reinnervation of the fast EDL and TA muscles following nerve crush injury at 5 days, is that reinnervation is not a selective process, and instead occurs randomly. This is illustrated by the slow myosin heavy chain (MHC) staining patterns seen in the fast reinnervated muscles. Normal adult EDL and TA muscles demonstrate slow MHC staining in a mosaic pattern in the axial regions of the muscles. Following nerve crush injury however this pattern is no longer apparent; the slow MHC positive fibres can be seen clustered in groups, and are no longer confined to the axial regions of the muscles. These findings are in agreement with the work presented by Lowrie et
al. (1988), and demonstrate the random nature of reinnervation. Counts of the number of slow MHC positive fibres in the reinnervated adult muscles indicate that a greater number of slow fibres are present in the reinnervated muscles compared to controls. It has been previously demonstrated that nerve crush injury at 5 days impairs the adult muscles, and the reinnervated fast muscles lose 50% or more of their muscle fibres (Lowrie et al., 1982). Since the number of slow MHC positive fibres actually increases in the reinnervated muscles then it suggests that the reduction in tension output and size of the reinnervated adult EDL and TA muscles is not due to an even and random loss of muscle fibres but instead, it appears that the fast muscle fibres are preferentially eliminated. This preferential elimination of fast fibres appears to be even more marked in muscles treated at birth with α-BTX and then subjected to nerve crush injury at 5 days. The temporarily-paralysed reinnervated adult muscles, especially TA, appear to be composed of a greater number of slow MHC positive fibres, than controls. The weight and force production of these muscles is even less than that produced by the muscles subjected to nerve crush injury only, and in addition, the number of motor units within the reinnervated EDL muscles was markedly reduced. These results seem to suggest that the impairment of the reinnervated muscles fibres is due to a preferential loss of the fast motor units within the reinnervated muscles. Since, in the case of EDL, the size of the remaining motor units is increased, and the number of slow fibres is increased it would appear that the slow motoneurones are increasing their peripheral fields by sprouting. The previously fast fibres are then undergoing changes in the expression of myosin isoforms, expressing slow MHC.
The preferential loss of fast fibres and/or preservation of slow fibres has previously been reported in Chapter 3, in investigations on the soleus muscle in which the minority of fast fibres is lost. This work also goes along with work which demonstrates that fast muscles are more susceptible to neonatal nerve injury than slow muscles (Lowrie et al., 1987). In addition, some other reports on adults have suggested that slow motor units have a selective advantage over fast motor units during reinnervation (Lewis et al., 1982; Kuno et al., 1974; Foehring et al., 1986).

The results presented in this Chapter suggest that the increasing ability to withstand peripheral nerve injury demonstrated with increasing age, is due to coincident and matched development of the motor nerve and muscle fibres. Once this interaction is interrupted, then the intimate matching of the development and properties becomes unsynchronized. It appears that "fast" motoneurones are less able to maintain contact with the immature muscle fibres they re-encounter upon reinnervation than "slow" motoneurones. This is exacerbated by making the muscle fibres even more immature by arresting their development with α-BTX treatment. In conclusion, during early postnatal development when muscle fibres and the motoneurones are undergoing extensive developmental changes, constant interaction with the target emerges as an important factor in the survival of muscle fibres and their motoneurones.
CHAPTER FIVE

General Discussion: The Influence of Nerve-Muscle Interactions on the Maturation of the Neuromuscular System
Within the developing neuromuscular system, motoneurones and muscle fibres initially develop independently of one another. Primary myotubes appear in embryonic muscles in normal numbers and distributions in the absence of innervation (Butler et al., 1982; Phillips & Bennett, 1984). As development proceeds, both the muscle fibres and the motoneurones acquire the ability to interact with one another; the motoneurones change from growing structures into secreting structures, whilst the target cells develop the capacity to receive transmitter and to form functional units with the nerve cells. Further differentiation and development of both the nerve and muscle cells is highly dependent upon a constant interaction between the two.

Axons growing into the pre-muscle masses make many synaptic contacts with the primary myotubes and the muscle cells become hyperinnervated. Shortly after the incoming axons reach and innervate their targets and neurogenic locomotor activity of the embryo begins, then a period of naturally occurring motoneurone cell death occurs (Hamburger & Oppenheim, 1982), where up to 70% of a population of a motoneurones may die (Oppenheim, 1981). Embryonic motoneurones are dependent upon their target cells for survival, and naturally occurring motoneurone cell death can be increased if populations of motoneurones are deprived of their target (Hamburger, 1958; Oppenheim et al., 1978). During the period of cell death, the motoneurones exclusively contact primary myotubes, and the widescale death of motoneurones takes place before the onset of secondary myotube formation (Ross et al., 1987a). Secondary myotubes develop in a second wave of myogenesis and form along
the template of the primary myotubes. This second generation of muscle cells are innervated by transfer of synaptic contacts from the hyperinnervated primary myotubes (Duxson et al., 1982). For a period in embryonic and postnatal development, all muscle fibres are hyperinnervated and have an excess of synaptic contacts. The achievement of the adult pattern of innervation and the establishment of the motor unit sizes of adult muscles, is brought about by a reduction in the peripheral field of the motoneurone and the elimination of excess synaptic inputs on individual muscle fibres. The number of synapses innervating each muscle fibre is subsequently reduced until the adult situation of a single nerve terminal innervating a single muscle fibre is achieved. This process of synaptic reorganization generally occurs when the overall locomotor activity of the animal is rapidly increasing, and the animal is acquiring greater mobility.

1) The Role of Muscle Activity in the Regulation of Synaptic Inputs

Postsynaptic activity is a major contributory factor in the withdrawal of excess terminals at an endplate during the elimination of polyneuronal innervation (Srihari & Vrbová, 1978; Sohal & Holt, 1980; Duxson, 1982; Duxson & Vrbová, 1985; Callaway & Van Essen, 1989; Greensmith & Vrbová, 1991). The experiments performed in Chapter 2 attempted to elucidate the mechanism by which target activity influences the maintenance or disruption of functional synaptic contacts.

Previously, it has been demonstrated that by reducing postsynaptic activity during the period of most rapid loss of synapses, the rate of elimination of excess synaptic inputs is delayed (Duxson, 1982; Greensmith & Vrbová, 1991). In an in vitro preparation of the slow
soleus muscle, increasing the activity of the postsynaptic membrane by prolonging acetylcholine-induced depolarization, increases the rate of elimination of polyneuronal innervation (O'Brien et al., 1980). The experiments performed in Chapter 2, employing an *in vitro* preparation of rat diaphragm muscle, demonstrate that, prolonging the depolarization of the postsynaptic membrane by ACh treatment and electrical stimulation of the motor nerve results in a widespread disruption of functional nerve contacts. The activity-induced disruption of terminals culminates in a large number of muscle fibres becoming denervated. The results presented in Chapter 2 demonstrate that ACh-induced disruption of functional contacts in neonatal muscles is common to fast muscles as well as slow muscles (the diaphragm preparation is predominantly composed of fast fibres), and is probably a universal phenomenon.

The process of synaptic remodelling which occurs during early postnatal development is known to be dependent upon calcium ions. O'Brien and colleagues (1980; 1984) demonstrated that the rapid disruption of nerve-muscle contacts evoked by exposure to ACh could be enhanced by the additional exposure of *in vitro* soleus preparations to high external levels of Ca$^{2+}$. *In vivo* experiments further examined the role for Ca$^{2+}$ in synaptic remodelling during early postnatal development. By treating neonatal rat soleus muscles with Ca$^{2+}$-chelators, Connold et al. (1986) were able to demonstrate that lower external Ca$^{2+}$ concentrations, which were insufficient to reduce the levels of muscle activity, were however, sufficient to prevent the normally occurring reduction in polyneuronal innervation. *In vitro* manipulations of the number of functional contacts within a nerve-muscle preparation can be prevented by the addition of an inhibitor of a
proteolytic enzyme, calcium-activated neutral protease, to the incubation media (O'Brien et al., 1984). Chapter 2 demonstrates that the number of functional contacts in in vitro diaphragm preparation are similarly susceptible to exposure to high external levels of Ca$^{2+}$, in combination with depolarizing effects of ACh and motor nerve stimulation. A large number of contacts were disrupted by the depolarizing conditions and high levels of external Ca$^{2+}$, to the extent that many muscle fibres lost all of their contacts and became denervated. In addition, experimental evidence is presented which demonstrates that most of the contacts disrupted by ACh-induced depolarization and conditions of raised external Ca$^{2+}$ can be protected from degradation by the action of a protease inhibitor, leupeptin. It is postulated that a neuronal CANP is degrading the cytoskeleton of axons within the diaphragm preparations. By adding leupeptin to the incubation media, the proteolytic action of CANP is inhibited and the nerve-muscle contacts are protected from disruption. The in vitro experiments presented in Chapter 2 illustrate the essential role of activity and CANP-proteolytic action in the regulation and withdrawal of synaptic contacts. Moreover, the experiments demonstrate that the withdrawal of terminals as a consequence of depolarization and raised external Ca$^{2+}$ levels is brought about by a breakdown of the neurofilamentous structure of the axons and terminals that are in the process of being withdrawn. Chapter 2 demonstrates that the proteolytic breakdown of neurofilament proteins occurs principally because the neurofilament subunit protein (NF-L), which is thought to form the backbone of the neurofilament structure, is preferentially degraded as a consequence of the experimental conditions. It is assumed that the preferential degradation
of the NF-L subunit protein results in the collapse of the cytoskeletal structure of the terminals and subsequently results in the retraction of the terminals from the endplate region.

A mechanism has previously been proposed which links muscle activity with the elimination of terminals at an endplate. Vrbová and colleagues (1988) proposed that K⁺ ions released from muscle during neuromuscular activity (Hodgkin & Huxley, 1952) link the activity of postsynaptic membrane with the elimination of excess synapses. Electrophysiological recordings of polyneuronal innervation demonstrate that presynaptic terminals of neonatal rat soleus muscles exposed to high external K⁺ solutions are effected by the exposure, and the number of synaptic contacts per endplate was reduced by the treatment. Skeletal muscles release K⁺ ions upon depolarization, which accumulate in the synaptic cleft (Hohlfield et al., 1981). It is probable that the K⁺ ions in the synaptic cleft rise to high levels, since stimulation of the optic nerve of neonatal rats results in K⁺ ions accumulating to levels as high as 21 mM (Connors et al., 1982). Vrbová et al. (1988) proposed that during the period of increased neuromuscular activity, when locomotor activity of animal is rapidly increasing, K⁺ ions are released from the muscle and accumulate in the synaptic cleft. The subsequent raised levels of K⁺ ions cause the opening of voltage-gated Ca²⁺ channels within the nerve terminals. These channels, once opened, close slowly and allow for the influx of high levels of Ca²⁺ into the nerve terminals. Once internal Ca²⁺ has reached critical levels, cellular CANP's are activated. Calcium activated neutral proteases (CANP's) are found in muscle and nervous tissue and neuronal CANP's are strongly associated with the cytoskeleton (Schlaepfer & Hasler, 1979;
Kamakura et al., 1983; Ishizaki et al., 1985). Chapter 2 demonstrates that under depolarizing conditions, an activated CANP degrades the neurofilament subunit proteins. The proteolytic degradation of neurofilament proteins, specifically the "backbone" subunit, results in the collapse of the axonal cytoskeleton and the subsequent withdrawal of excess terminals from the endplate region. This mechanism encompasses the experimental evidence that muscle activity and prolonged depolarization cause a disruption of neuromuscular contacts and in addition, the involvement of Ca\textsuperscript{2+} and CANP in the elimination of functional contacts. K\textsuperscript{+} ions potentially fit the role of an activity-dependent, muscle derived trophic factor which is thought to be released from muscles, thereby influencing the survival or elimination of excess terminals at a single endplate (Brown et al., 1981).

The preferential elimination of one terminal over another at an endplate is probably based on the size differentials between the converging nerve terminals. On a hyperinnervated muscle fibre, many axon profiles of varying sizes have been observed at a single endplate (Duxson, 1982; O'Brien et al., 1984). During the elimination of polyneuronal innervation there is an overall decrease in the small and medium sized axon terminals innervating the soleus muscle (Duxson, 1982), whilst the number of large axon terminals increases. The establishment of the mature pattern of innervation, via the elimination of superfluous synapses at an endplate occurs, therefore, at the expense of the smallest terminals. The largest terminals which remain, will subsequently mature and increase in size and geometric complexity (Tuffery, 1971). In experimentally manipulated systems, AChE injected into neonatal skeletal muscles in vivo results in a rapid reduction in
the number of contacts on polyneuronally innervated muscles, the small and medium sized terminals are eliminated whilst the large terminals remain (Duxson & Vrbová, 1985). Vrbová et al. (1988) suggested that smaller terminals are eliminated on account of their surface area to volume ratio. The smaller terminals would be subjected to a relatively greater influx of Ca\(^{2+}\) ions than the larger terminals at the same endplate. The smaller terminals would therefore suffer a greater extent of proteolytic degradation of their neurofilamentous structure, and would consequently be preferentially eliminated from a polyneuronally-innervated muscle fibre.

2) Permanent Effects of Target Inactivity in the Early Postnatal Period

The importance of target activity in the reorganization of synapses has been demonstrated in Chapter 2, and the mechanism involving CANP degradation of the neurofilamentous structure of developing axons has been discussed. During the early postnatal period the adult pattern of innervation of skeletal muscles is achieved by the elimination of excess synaptic inputs from individual muscle fibres and by a pruning of the peripheral fields of motoneurones. The rates of elimination of polyneuronal innervation can be altered by experimental manipulations of neuromuscular activity. By decreasing general neuromuscular activity in hindlimb muscles, the rate of elimination of excess synaptic input is delayed (Benoit & Changeaux, 1975; Riley, 1978; Zelená et al., 1979; Miyata & Yoshioka, 1980). Conversely, experimentally increasing neuromuscular activity results in a speeding up of the rate of elimination of polyneuronal innervation (O’Brien et al., 1978; Zelená...
et al., 1979; Thompson, 1983). The involvement of the target in the elimination of polyneuronal innervation is demonstrated in experiments in which the activity of the postsynaptic membrane is reduced or eliminated in early postnatal development, which results in a reduction in the rate of elimination (Srihari & Vrbová, 1978; Duxson, 1982; Callaway & Van Essen, 1989; Greensmith & Vrbová, 1991).

The role of target activity in the establishment of the adult pattern of innervation was addressed in Chapter 3. Postsynaptic activity of the newborn soleus muscle was temporarily removed, whilst leaving the activity of the motoneurone intact. By temporarily removing target activity in the early postnatal period, the development of the soleus muscles was permanently impaired. The adult muscles never fully recovered from the period of paralysis - they were smaller, produced less force and were composed completely of slow myosin heavy chain positive fibres. The reduced force output of the muscles was shown to be due to a loss of muscle fibres. The most interesting observation from the experiments performed in Chapter 3, is that following temporary paralysis of the soleus muscle in the first days of life, the subsequent pattern of innervation of the adult muscle was altered, and there was a change in the characteristic distribution of innervation of the muscle by nerve terminals of axons emanating from either L4 or L5 ventral ramus (v.r.). Adult soleus muscles are normally innervated by axons from the L4 and L5 ventral rami; 70% of the muscle fibres are innervated by terminals from the L5 v.r. and the remaining 30% are innervated by L4 nerve terminals. Following temporary paralysis at birth, the distribution of innervation of the adult soleus muscles was altered, so that 65% of the remaining muscle fibres were innervated by L5 nerve
terminals and 45% by L4 nerve terminals. Prolonging temporary paralysis, in early development, further affected the distribution of innervation so that L5 nerve terminals innervated 30% of the remaining muscle fibres and L4 nerve terminals innervated 70% of the remaining muscle fibres. The results presented in Chapter 3 demonstrate that the poor recovery of the adult muscles was due to a loss of muscle fibres. The arrested development of the soleus muscles was not however as a consequence of random loss of muscle fibres, instead there appeared to be a preferential loss of those muscle fibres innervated by axons emanating through the L5 v.r. The results illustrate the critical role of the target in the establishment of the normal pattern of innervation of the soleus muscle and demonstrate that a reduction in postsynaptic activity during the early stages of postnatal development, affects not only the rate of establishment of the adult pattern of innervation (Duxson, 1982; Greensmith & Vrbová, 1991) but also the pattern and distribution of innervation itself.

The altered pattern of innervation of the adult soleus muscles following temporary paralysis at birth, can be explained by the model of synapse elimination discussed in the previous section. Paralysis of the soleus muscles at birth arrests the development of the muscle fibres. The results presented in Chapter 3 illustrate this arrested development - soleus muscles express low levels of the enzymes of oxidative metabolism, produce less force and weigh less than controls. Greensmith & Vrbová (1991) paralysed soleus muscles at birth and observed them 5 days later. Many nerve-muscle contacts were lost and the contacts which remained presented prolonged endplate potentials. On recovery of soleus muscles from paralysis, a mismatch in the properties of the relatively
mature nerve terminals and the immature muscle fibres would occur. In a study investigating the transmitter release from nerve terminals on soleus muscles fibres Lowrie et al. (1985) demonstrated that L5 nerve terminals release almost twice as much transmitter as L4 nerve terminals at 5 days of age. Therefore, the developmentally-arrested soleus muscle fibres are innervated by L4 and L5 nerve terminals, of which the L5 terminals are releasing most transmitter. Chapter 2 demonstrates that high levels of activity bring about the disruption of functional contacts between nerve terminals and muscle fibres. It is suggested therefore, that the reduction in the number of adult muscle fibres innervated by terminals from the L5 v.r., is because the neonatal, more active L5 nerve terminals are unable to maintain contacts on the immature muscle fibres. The L5 nerve terminals are eliminated at an endplate in preference to L4 nerve terminals on account of their higher quantal content. The less active L4 nerve terminals are more suited to the immature muscle fibres and are therefore able to maintain more functional contacts.

3) The Role of the Target in the Age-Dependent Response to Peripheral Nerve Injury

In the early stages of development, when contacts have been made between the developing motoneurone and muscle fibres, the survival of both the nerve and muscle cells is dependent upon a constant interaction between the two. This interdependence is essential through early postnatal life, but the consequences of temporary disruption, due to peripheral nerve injury, become less devastating as development proceeds.
and the neuromuscular system becomes more mature.

Muscles which are disconnected from their motoneurones at birth, by nerve crush injury, continue to grow for a short period of time (Vrbová, 1952; Zelená, 1962). However, in the absence of innervation over the long term, they die. If reinnervation of the denervated target occurs, then the muscles recover, although this recovery is poor (Romanes, 1946; Beuker & Meyers, 1951; Zelená & Hník, 1963; McArdle & Sansone, 1977; Lowrie et al., 1987). Concomitant with poor muscle recovery following sciatic nerve crush injury at birth, the motoneurones are also greatly affected, so that up to 70% of the immature motoneurones die (Romanes, 1946; Beuker & Meyers, 1951; Lowrie et al., 1987).

In adult animals this susceptibility of muscle fibres and motoneurones to disconnection is no longer apparent. Muscle fibres virtually recover completely from peripheral nerve injury, provided that reinnervation is allowed to occur (Gutmann & Young, 1944; Beranek et al., 1957; Miledi, 1960a; 1960b; McArdle & Alberquerque, 1973; Lowrie et al., 1982). Similarly, motoneurones do not die if they are temporarily disconnected from their muscle fibres in later stages of maturation (Schmallbruch, 1984).

The transition in the response of the nerve and muscle cells to peripheral nerve injury occurs, during the first few days of postnatal life, and the injury becomes progressively less harmful with age (Lowrie et al., 1987). If nerve crush injury is inflicted at 5 days of age, the response of the muscle fibres to temporary disconnection from their motoneurone is dependent upon the type of muscle studied. Reinnervated slow muscles recover almost completely from 5 day nerve crush injury (Lowrie et al., 1982), whereas recovery of the fast muscles is not as
successful, and they develop only 50% of the tension of controls (Lowrie et al., 1982; Chapter 4 of this thesis). Following 5 day peripheral nerve crush injury there is no significant motoneurone cell death and all of the motoneurones appear to reinnervate the muscles (Lowrie et al., 1982; however, see Chapter 4 of this thesis). Nevertheless, these motoneurones are not unscathed by the peripheral nerve injury and the mean motoneurone size is smaller and the size distribution is altered compared to normal. In the EDL and TA motoneurone pool, the largest motoneurones which normally innervate fast-fatiguable muscle fibres in EDL muscles, are permanently reduced in size (Lowrie et al., 1987). Moreover, motoneurones subjected to peripheral nerve injury at 5 days are, after reinnervation, more active and excitable compared to normal (Navarette & Vrbová, 1984). Thus, it appears that during the first few days of postnatal life the motoneurone acquires the ability to survive in the absence of target influence. Muscle fibres similarly develop a capacity to withstand temporary disconnection from motor innervation over the first few days of life. This capacity appears to develop in fast twitch muscles at a slower rate than in slow twitch muscles (see Lowrie & Vrbová, 1984). If reinnervation of target muscles is prevented or delayed following the nerve injury then motoneurones are less likely to survive (Kashihara et al., 1987). Muscle fibre recovery from a prolonged insult is equally as unsuccessful (Lowrie et al., 1990). These results suggest the importance of a constant interaction between the motoneurone and the muscle fibres during early development - the longer they are separated the less likely they are to successfully recover.

The importance of target activity in the changing response of fast muscle fibres to peripheral nerve injury was investigated in Chapter 4.
By removing or reducing postsynaptic activity of the fast muscles in the first few days of postnatal life, the effect of sciatic nerve injury at 5 days was exacerbated. Fast muscles developed less tension and were smaller in size than those subjected to nerve crush injury at 5 days only. In addition to the reduced bulk of the muscles, other properties of the reinnervated muscles were greatly affected. The reinnervated muscles were more resistant to fatigue and the enzymes of oxidative metabolism were correspondingly greatly increased. The temporarily-paralysed, reinnervated muscles possessed muscle fibre properties similar to those expressed by fast muscles subjected to nerve crush injury at birth (see Lowrie et al., 1987).

The results presented in Chapter 4 demonstrate the importance of target activity in the increasing independence of muscle fibres from the motoneurone. The importance of target activity on the ability of the motoneurone to survive peripheral nerve injury has recently been demonstrated (Burls et al., 1991). These experiments highlight the critical role of a constant interaction between the muscle fibres and the motoneurones during early postnatal development. Constant interaction between the nerve terminals and the muscle fibres results in a coincident development between the two, and the formation of a functional, highly specialized unit. In the absence of a constant interaction, there is a mismatch in the properties of the muscle fibres and motoneurones, an upon reconnection the muscle fibres and motoneurones are no longer compatible, and death ensues. The effects of interruption of nerve-muscle interactions are most apparent at birth (Lowrie et al., 1987), when most marked changes in the characteristic properties of muscle fibres and motoneurones are occurring.
During the first two to three weeks of life a functional differentiation of fast and slow motoneurones takes place (Navarrete & Vrbová, 1983; Fulton & Walton, 1986). "Slow" motoneurones increase their duration of firing whilst the firing frequency remains low, whereas "fast" motoneurones increase the rates of firing, but fire for shorter periods than neonatal motoneurones (Navarrete & Vrbová, 1983). "Fast" motoneurones also become more excitable with increasing age as their input resistance decreases (Navarrete et al., 1989).

The developmental changes occurring in muscle fibre properties in early postnatal development are dependent upon motor innervation and activity. The stabilization of AChR's at the neuromuscular junction is dependent upon motor nerve innervation (Avila et al., 1989), and the conversion of AChR-ion channel opening times from the slowly opening neonatal channels, to the more rapidly opening adult channels, is dependent upon the motoneurone (Brenner et al., 1987). Muscle activity has also been demonstrated to play an important role in the deposition of acetylcholinesterase (AChE) at the neuromuscular junction. Chick embryos paralysed with curare, fail to accumulate AChE (Gordon et al., 1974), whereas chronic electrical stimulation of curarized muscles causes the development and accumulation of AChE at the neuromuscular junctions (Rudin et al., 1980).

Nerve crush injury isolates the muscle fibres from the motoneurone during a period of most rapid developmental changes. The development of denervated muscle fibres is retarded (Zelená & Hník, 1963; Engel & Karparti, 1963). Meanwhile, the central connections to the motoneurones continue to develop unhindered (Stelzner, 1982). Upon reinnervation there is a mismatch between the state of development of the motoneurones
and the muscle fibres, and the motoneurones are unable to establish or maintain contacts on the immature muscle fibres. Following nerve crush at 5 days, fast muscles become impaired. Lowrie & Vrbová (1984) demonstrated that impairment was a consequence of a loss of muscle fibres upon reinnervation. Similarly, Kashihara et al. (1987) demonstrated that motoneurones separated from their target muscle survive for the period of disconnection but die upon reinnervation of the target muscle.

Disconnection of muscle fibres from the influence of the motor nerve retards the development of the muscle fibres. Immature muscle fibres are likely to have developed and accumulated only low levels of AChE, and the AChR-associated ion channels are likely to be open for long periods of time. Upon reinnervation the immature muscle fibres are exposed to large amounts of high frequency activity from the relatively mature motoneurones. The immature muscle fibres when activated, will present a prolonged depolarization, which, as demonstrated in Chapter 2, will bring about the disruption of neuromuscular contacts. Those motoneurones which are most active will be mismatched with the muscle fibres and therefore unable to maintain contacts.

In Chapter 4, by reducing target activity in the first days of postnatal life, the development of the muscle fibres was arrested prior to a period of disconnection from the motoneurone by nerve crush injury. Upon reinnervation, the extent of mismatch in the properties of the muscle fibres and motoneurones was extensive. Thus, the motoneurones were unable to maintain effective contacts on the immature muscle fibres, which resulted in permanent impairment of the fast muscles.
In conclusion, the experiments performed in this thesis have demonstrated the critical role of muscle activity in the development of the adult neuromuscular system. During postnatal development the activity of the muscle plays an important role in the maintenance and disruption of functional synaptic contacts. Elimination of functional contacts on muscle fibres involves the activity of the muscle fibres and proteolytic CANP degradation of neurofilament proteins in axon terminals. By interrupting the interaction between muscle fibres and motoneurones in early postnatal development the essential role of constant interactions between the two is highlighted.
APPENDIX I

Cholinesterase Stain for Young Mammalian Endplates

1) Muscles were fixed in a slightly stretched position in buffered 4% formaldehyde at 4°C for 30 minutes.

   Fixative: Paraformaldehyde (EM grade) 4g
   distilled water 40ml
   heated to 60°C and then 4 drops of 4% NaOH were added to dissolve the paraformaldehyde. Cooled.
   0.5% CaCl₂ 1ml
   sucrose 5g
   DMSO 1ml
   0.2M phosphate buffer 50ml
   made up to 100ml with distilled water

2) Tissue transferred to washing solution for 30 minutes at 4°C.

   Washing solution: 0.2M phosphate buffer 50ml
   sucrose 5g
   0.5% CaCl₂ 1ml
   DMSO 1ml
   Made up to 100ml with distilled water

3) Sections were incubated, on ice, for 1 hour, in:-

   Incubation medium: Acetylthiocholine iodide 10mg
   0.1M Sodium hydrogen maleate (pH 6.0) 13ml
   100mM tri-sodium citrate 1ml
   30mM copper sulphate 2ml
   glass distilled water 2ml
5mM potassium ferricyanide 2ml
sucrose 3g

Solutions were added together in the order in which they appear and shaken fully between each addition.

4) The tissue was then transferred to distilled water for 10 minutes, at 4°C.
5) The tissue was immersed in potassium ferricyanide for 10 minutes at room temperature (0.25g, made up to 100ml with distilled water).
6) The tissue was washed in distilled water, two washes of 5 minutes each.
7) The tissue was mounted on a gelatine coated slide and allowed to dry overnight.
8) The mounted tissue was then cleared with histoclear (2 x 5 minutes) and mounted in DPX.
The molecular weight of given proteins can be determined by comparing their electrophoretic mobilities with those of known protein markers. A linear relationship is obtained when the logarithms of the molecular weights of standard polypeptide chains are plotted against their electrophoretic mobilities in polyacrylamide gels.

A mixture of six proteins (SDS-6H, Sigma, U.S.A.) was used in these experiments in order to establish the molecular weights of the proteins identified by specific monoclonal antibodies, following an immunoblot procedure.

**SDS-6H:**

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>MOL. WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic Anhydrase (Bovine Erythorocytes)</td>
<td>29,000</td>
</tr>
<tr>
<td>Albumin (Egg)</td>
<td>45,000</td>
</tr>
<tr>
<td>Albumin (Bovine Plasma)</td>
<td>66,000</td>
</tr>
<tr>
<td>Phosphorylase B (Rabbit Muscle)</td>
<td>97,400</td>
</tr>
<tr>
<td>β-Galactosidase (E. Coli)</td>
<td>116,000</td>
</tr>
<tr>
<td>Myosin (Rabbit Muscle)</td>
<td>205,000</td>
</tr>
</tbody>
</table>
APPENDIX III

Slow myosin heavy chain antibody staining 1

1) Sections dried for 30 minutes at room temperature (r.t.).

2) Fixed in absolute alcohol for 10 minutes at r.t..
   Washed thoroughly in phosphate buffered saline (PBS) + 0.1% BSA (bovine serum albumin).

3) The endogenous peroxidase activity was blocked by incubating sections in 0.3% H₂O₂ in PBS for 30 mins.

4) Sections rinsed in PBS for 3 x 5 mins.

5) Non-specific binding sites were blocked by incubating sections in Normal Rabbit Serum (1:30 dilution, in PBS) for 30 mins.

6) Excess serum was drawn off and sections washed in 3 x 5 minutes in PBS.

7) Sections were then incubated in the primary antibody, recognizing rat slow myosin heavy chain. The primary antibody was 96J (kindly donated by Dr.T.Dhoot). 96J was used at a working dilution of 1 in 100 (diluted in PBS).
   Sections were incubated in primary antibody for 1 hour in a humid chamber.

8) Rinsed in PBS for 3 x 5 mins.

9) Sections were next incubated in secondary layer antibody, Rabbit anti-mouse IgG conjugated with Horse Radish Peroxidase (Nordic) for 1 hour, in a humid chamber. The secondary layer antibody was diluted 1 in 50 in PBS.

10) Rinsed in PBS for 3 x 5 mins.

11) The antigen-antibody complex was then visualized by incubating the
sections in a 0.05% Diamino benzadine (DAB) solution (in PBS), containing 2% CoCl₂. Sections were incubated in the DAB solution for 5 mins, then 1% H₂O₂ was added to the DAB solution and the sections incubated for a further 5-10 mins.

12) The reaction was stopped by washing the sections in tap water.

13) Sections were then dehydrated in successive washes of 70%, 90%, 100%, 100% alcohol of 3 minutes each.

14) Finally, sections were cleared in 2 x 3 mins. Histoclear and slides mounted in DPX (mounting medium).
APPENDIX IV

Slow Myosin Heavy Chain Antibody Stain II

1) Sections dried at room temperature for 30 minutes.

2) Fixed in absolute alcohol for 10 minutes at r.t.  
   Rinsed in phosphate buffered saline (PBS) + 0.1% BSA.

3) Endogenous peroxidase activity blocked by incubating sections in  
   $0.3\% \text{H}_2\text{O}_2$ in PBS for 30 minutes.  
   Rinsed thoroughly in PBS.

4) Non-specific binding sites were blocked by incubating sections in Normal  
   Horse Serum diluted in PBS (1:30), for 30 minutes. Incubation was  
   carried out in an humid chamber.  
   The excess serum was shaken off the sections, and then sections  
   were rinsed in PBS.

5) Sections incubated in the primary antibody NOQ7.54D (donated by Dr.  
   R. Fitzsimmons) for 1 hour. Ascites were used undiluted and  
   the incubation was carried out in an humid chamber.  
   Primary antibody was shaken off sections and sections were then  
   rinsed in 3 x 10 minutes washes of PBS.

6) Sections were incubated in secondary antibody, biotinylated anti-  
   mouse IgG, made in horse (Vector Labs.) diluted in PBS (1:200), for  
   2 hours. Incubation was carried out in an humid chamber.  
   Secondary antibody was shaken off sections and then sections washed  
   3 x 10 minutes in PBS.

7) Sections incubated for 1 hour in a standard biotin-avidin complex  
   (Standard Vecta stain Elite ABC kit).
Sections were washed in PBS for 5 minutes.

Sections then washed for 2 x 5 minutes in Tris, pH 7.6.

8) Sections incubated in 0.05% DAB solution (diamino benzadine), made in Tris, pH 7.6, for 10 minutes.

9) 1% H$_2$O$_2$ added to DAB solution, and sections incubated for a further 5-10 minutes.

DAB reaction stopped by washing sections in tap water.

10) Sections counterstained with toluidine blue solution for 30 seconds.

11) Sections dehydrated in successive washes of 70%, 90%, 100%, 100% alcohol for 3 minutes each.

12) Sections cleared in Histoclear for 2 x 5 minutes and mounted in DPX mountant.
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