Studies on the mechanosensory innervation of muscle using organotypic culture, reinnervation and immunohistochemistry

El-Tarhouni, Amal Ibrahium

Studies on the Mechanosensory Innervation of Muscle Using Organotypic Culture, Reinnervation and Immunohistochemistry

A thesis presented in candidature for the degree

of

Doctor of Philosophy

by

Amal Ibrahium El-Tarhouni, M.Sc. (Benghazi / Libya)

Department of Biological Sciences, University of Durham

Durham, 1996

THIS WORK IS DEDICATED WITH GRATITUDE

TO

MY FAMILY FOR LIFE, SACRIFICE, SUPPORT

AND PATIENCE.

Acknowledgements

I would like first of all to express my gratitude to Allah, who enabled me to carry out this work, then to the people who helped me during the course of this study, above all my supervisor Dr. R. W. Banks. I am thankful to him for his co-operation, constant advice, encouragement and guidance throughout the experimental phase of this study and the writing-up stage. I would also to thank Dr. Colin Jahoda and his wife Mandy for their advice and help, particularly with the tissue culture work.

My sincere thanks to Mrs. A. C. Richardson for introducing me to the use of the electron microscope and other histological techniques. My gratitude goes to Mr. D. Hutchinson for his skilled photographic assistance and to Sue Raven and Judith for their help with the English of the manuscript.

Special gratitude and personal thanks are due to my friends Dr. Mirela Cuculescu and to Dr Lydia Wong for their help, encouragement and support during my studies.

I would like to express my warmest thanks and gratitude to all my friends and colleagues from the biology department and all my friends from Durham with whom I spent many pleasant hours throughout the time I spent in Durham.

Last but not least, I am especially indebted to my mother for her encouragement, patience and sacrifices, and to members of my family for their co-operation, understanding and support throughout my studies.

Memorandum

This work reported in this thesis was carried out at the Biology Department, University of Durham between May 1992 and March 1996. This has not been submitted for any other degree and is the original work of the author except where acknowledged by means of appropriate references.

Statement of copyright

Financial support

Foundation based on a scholarship from Garyounis University Benghazi (Libya). I am gratefully acknowledged for the financial support for the work described herein.

Some of the material contained in this thesis has been published

El-Tarhouni, A. & Banks, R. W. (1995). The distribution of Calretinin in muscle receptors of the cat. J. Physiol. (Lond) 487, P77.

The structural and functional relationships in the sensory innervation of muscle spindles revealed by abnormalities following reinnervation. (represented as a poster in Wellcome Trust workshop meeting).

Contents

Chapter 1

General Introduction

Chapter 2

An *in vitro* Study of the Development of Sensory Neuromuscular Connections in the Rat.

Chapter 3

Structural and Functional Relationships in the Sensory Innervation of the Muscle Spindles Revealed by Abnormalities Following Reinnervation

Chapter 4

The Distribution of Calretinin in Muscle Receptors of the Cat.

List of Tables:

Abstract

Studies on the Mechanosensory Innervation of Muscle Using Organotypic Culture, Reinnervation and Immunohistochemistry

This thesis studies sensory innervation in mammals using an organotypic co-culture of spinal cord-dorsal root ganglion and skeletal muscle of embryonic rat, the histological changes of reinnervated muscle spindles after nerve section and the localisation of the calcium-binding protein calretinin in cat mechanoreceptor organs. The immediate importance of this project concerns the better understanding of how the normal process of development differs from reinnervation following nerve lesion or section. A range of classical and well defined materials and methods as been used in the work described.

The thesis is divided into five chapters:

Chapter 1 reviews aspects of the mechanosensory organs which have been studied experimentally in relation to their sensory innervation, including proprioceptive muscle spindle development, reinnervation, and finally, the presence of the calcium-binding protein, calretinin in the mechanoreceptor organs. This provides an introduction and background to the work.

Chapter 2 describes the organotypic organisation of spinal-cord, dorsal-root ganglia and skeletal muscle co-culture *in vitro*. Results show that slices of the spinal-cord, dorsal-root ganglia survive well under experimental conditions and can live for several weeks with feeding every 1-3 days. Sensory neurons can develop and grow in a medium without any additional promoting factor. The presence of structurally identifiable synapses indicates that other neurons are also maintained in culture and have functional connections. In the

organotypic culture new muscle fibres can form either from the original explant or from the additional explant.

In chapter 3 I describe two abnormal endings present in spindles of the tenuissimus of the cat that had been reinnervated following section of the nerve more than one year previously. The reconstruction of the endings of these two spindles supports the hypothesis of modulation of the primary-ending response by the mechanical properties of the intrafusal muscle fibres, rather than by intrinsic properties of the Ia afferent itself. They further indicate that, in the absence of a Ia afferent, intrafusal-fibre differentiation can be maintained by a group II afferent.

Chapter 4 concerns the localisation of the calcium-binding protein calretinin, which was studied immunohistochemically in the abductor digiti quinti medius muscle of the cat hind limb. The calretinin immunoreactivity was found in some intrafusal fibres, the primary endings and the capsule of the muscle spindles and the sensory terminals of tendon organs and Paciniform corpuscles. The present findings contradict a recent hypothesis that calretinin is associated with rapid adaptation, but suggest that calretinin has a specific function in muscle proprioceptors.

Finally, Chapter 5 outlines the conclusions of this study and gives some suggestions for continuation of the work in the future.

Chapter 1

General Introduction

1.1. Definition of Muscle Spindles

Muscle spindles are complex mechanoreceptor organs distributed in differing numbers throughout the skeletal muscles of vertebrates. They are also called neuromuscular spindles Ruffini, (1893) or stretch receptors and are sensitive to muscle length (Barker, 1974; Boyd, 1985 and Hunt, 1990). They are essential for our awareness of the position of limbs as they are responsible for the fine control of movement. Muscle spindles were first noticed and described by Weismann, (1861) in frog muscle.

It is generally thought that the first mechanoreceptor appeared in early tetrapods in antigravity muscles associated with posture and locomotion. The physiology and histology of muscle spindles has been more extensively studied in mammals than in other vertebrates and more is known about cat spindles than those of any other species. Cat spindles are good models for human spindles.

1.1.1. Structure of Muscle Spindles

Each muscle spindle contains several slender muscle fibres (intrafusal) the ends of which are attached to connective tissue, tendon or extrafusal endomysium. For most of their length they are enclosed by a fusiform cellular multilamellated capsule containing a fluid. This encloses the sensory innervation and extends as a sleeve on each side to enclose part of each pole. In the equatorial region of the muscle spindle the bundle of intrafusal fibres is separated from the capsule by a prominent periaxial space. The equatorial length of the periaxial space and the length of capsule vary according to the number of sensory endings present. Two types of innervation are supplied to the intrafusal bundle:

A. Primary and secondary sensory endings occur in the equatorial region and respond to active and passive changes in muscle length.

B. Motor endings occur in the polar regions, where the intrafusal fibres are contractile.

1.1.1a. Intrafusal Fibres

The intrafusal fibre bundle is the largest part of each muscle spindle. The bundle is composed of bag_1 fibres, bag_2 fibres and chain fibres. In cat spindles the maximum number of fibres per spindle is 9-12 and the minimum number is 3-4. Each fibre is subdivided into an equatorial, two juxtaequatorial and two polar regions which are clearly distinguishable. According to a widely accepted definition, zone A is the sensory region which includes the equatorial and both juxtaequatorial zones, whereas zone B is intracapsular and zone C the extracapsular part of the polar region (Boyd, 1985; Barker & Banks, 1994).

The three intrafusal fibre types, bag_1, bag_2 and chain, can be distinguished from one another in all three spindle regions. At the equator of cat spindles, the bag_2 fibre has the largest diameter of up to 25 μm, bag_1 is thinner, about 20 μm in diameter, and the chain fibres are 10 to 12 μm in diameter. The juxtaequatorial regions flank both sides of the equator; here the intrafusal fibres have single axial nuclei and the myofibrillar content increases. The sensory A region of cat spindles, including both the equator and juxtaequatorial zones, is several hundred micrometers long, depending on the limit of the innervation, usually provided by one Ia and none or up to several spindle II axons (Barker, 1974 and Barker & Banks, 1994).

Along the A zone, the axial bundle of intrafusal fibres is surrounded by the periaxial space and capsule; at the two ends of the A zone the spindle diameter decreases and the capsule forms a narrow capsular sleeve around the polar regions of the fibre bundle. Bag fibres extend beyond the capsule, the extracapsular portions of the bag_2 fibre being as a rule longer than that of the bag_1 fibre. The chain fibres are intracapsular,

except for the long chain and the intermediate chain fibres (Kucera, 1980). In polar regions the intrafusal fibres are completely filled with myofibrils and possess peripheral nuclei. The bag fibres display a characteristic arrangement of their myofibrils, they are large and confluent, the sarcoplasmic reticulum is poorly developed and mitochondria are small and rare.

According to Ovalle, (1971) the ultrastructure of the intrafusal fibres shows a conspicuous M-line in each sarcomere in the polar region of chain fibres of cat lumbrical muscle, similar to the M-line in sarcomeres of the extrafusal muscle fibre. In contrast, the M-line of bag fibres was said to be ill-defined, and in the form of two faint parallel lines. Ovalle, (1972) found the structure of the two forms of M-line in the equatorial regions of both intrafusal fibre types. Landon, (1966) revealed the presence of an M-line in the sarcomere of chain fibres, and also said it was ill-defined in bag fibres. However, it was subsequently found that a prominent M-line is present throughout the length of the chain fibres but only in the polar region of the bag_2 fibres. The bag_1 fibres have a M-line consisting of two faint parallel lines throughout most of the length of the fibre (Banks *et al.* 1975, 1977 and Barker *et al.* 1976).

The bag fibres are surrounded by many elastic fibrils which are absent from chain fibres (Gladden, 1976). In cat spindles, this elastic outer layer is more prominent around the bag_2 than the bag_1 fibre.

1.1.1b. Capsule

The spindle capsule is a modified extension of the perineurium and endoneurium connective cellular layers that enclose the spindle nerve (Shantha *et al.* 1968). Barker & Banks, (1994) have agreed with this interpretation. For most of its length the intrafusal bundle is surrounded by a fusiform capsule, which at the equatorial region consists of about 6-9 layers of very thin, flat cells. The number of layers gradually decreases towards the polar regions and ends in a single layer, usually with the end of the chain

fibres. Capillaries adjacent to the outer layer of the capsule, and those enclosed in its layers, accomplish an important role in providing the spindle with oxygen (Kennedy & Yoon, 1979). They hardly ever enter into the periaxial space (Barker, 1974). A group of myelinated axons of different diameters enters the capsule, passing through the periaxial space to innervate the intrafusal muscle fibres. Unmyelinated axons were also detected to pass through together with myelinated axons inside capsular lamellar layers (Merrillees, 1960).

1.1.1c. Sensory Innervation

The intrafusal fibres are innervated by two types of sensory axons: the large primary and smaller secondary afferent. According to Stacey, (1969), most of the axons supplying a muscle are sensory. The myelinated sensory fibres are divided into 3 groups according to their diameter: Group I axons range in diameter from 12 to 20 μm and have conduction velocities from 72-120 m/sec. This group contains axons which terminate in muscle spindles as primary afferents (Ia afferent) as well as in the tendon organs (Ib afferent). The group II afferents range in diameter from 4-12 μm and have conduction velocities from 24-72 m/sec. This group includes the afferents from the secondary endings of the intrafusal fibres of the muscle spindles, and afferents from other mechanoreceptors such as paciniform corpuscles, pacinian corpuscles and free endings. The diameters of group III afferents range from 1 to 7 μm and their conduction velocities from 12-30 m/sec. They terminate almost always as free endings in muscle.

Most of the sensory afferents are non-myelinated ; according to Stacey, (1969) all of the non-myelinated, and some of the myelinated sensory axons, terminate as free endings on blood vessels and in fat and connective tissue, whereas most of the remaining myelinated sensory axons are supplied to muscle spindles and tendon organs.

i. The Primary Endings and Axons

Most studies have been carried out on cat and rabbit muscles using silver techniques and reconstruction technique (Barker, 1974; Banks *et al.* 1982 and Banks, 1986).

Generally each muscle spindle is supplied by one thick sensory fibre (afferent), known as a group Ia, which enters the spindle near the equator of the capsule. Within the periaxial space the axon divides into several branches to form the terminal branches (sensory endings) on the densely nucleated equatorial regions of the three types of intrafusal muscle fibres (bag_1, bag_2 and chains). These terminals collectively form the primary ending.

Sometimes two axons enter the same spindle to terminate around bag fibres. Banks, (1973) found that some spindles contained two separately innervated primary endings, the endings lying side by side or end to end. Banks *et al.* (1982) found some cat spindles innervated by two Ia axons, referring to the endings as double primaries.

The terminals appear to consist of spirals, half rings and a few complete rings. According to Boyd, (1962), large spirals are distributed to the bag fibres and small spirals to the chain fibres. They are arranged closely together around the middle of each nuclear bag, but more widely spaced to either side, where they may be disposed as irregular forms or run diagonally (Barker, 1948). Spirals are more common and more extensive around chain fibres than around bag fibres. The total number of spiral turns per unit length is greater in the middle of the bag_1 than the bag_2 primary terminals and the bag_1 possess more extensive irregular portions than the bag_2 (Barker & Banks, 1994). Banks *et al.* (1982), studied the sensory innervation of the cat hindlimb muscle spindles by using reconstruction, they showed there is a close relationship between nucleation and innervation. The bag_1 fibre usually receives more preterminal branches than the bag_2 and chain fibres.

The terminals in the bag fibres are set closely and arranged largely as regular bands while at each end they are disposed loosely and irregularly. The terminals on the chain fibres are widely separated. Sometimes a terminal wraps two or three adjacent fibres to form an interlocking "sensory cross-terminal" (Adal, 1969). The sensory terminals usually occur between chain fibres, but may also occur between a chain and a bag fibre (Barker, 1974; Banks *et al.* 1982) and between a bag_1 and bag_2 fibre (Walro & Kucera, 1987).

Banks *et al.* (1982), used transverse sections and graphic reconstruction to illustrate all the important details of the terminals. Banks, (1986), used a reconstruction technique to describe the form and distribution of the primary terminals of cat tenuissimus muscle spindle. He found that all ring features on the intrafusal muscle fibres are open and all the terminals remain separate and do not fuse, most of them are located on the bag_1 fibre and the position of each terminal determines its form.

ii. The Secondary Endings and Axon

The secondary afferent terminals are supplied by afferents thinner than group Ia axons, and these are known as group II axons. They usually enter the spindle together with the primary afferent. They also have lower conduction velocities, typically ranging from 24 to 72 m/sec (Matthews, 1972). The terminals are few in number and about one third or one half shorter than the primaries (Barker & Banks, 1994). Secondary endings are located on one or both sides of the primary ending. In cat and rabbit usually only one secondary ending is present adjacent to the primary, but there may be up to five on one side and up to eight on both sides (Banks & Stacey, 1988). The afferent may branch to supply two endings in one spindle located on each side of the primary or located in different spindles (Barker, 1974). Almost all of the secondary endings in cat spindles lie on the static intrafusal fibres: 75% on chain fibres, 17% on the static bag_2 fibre and only the remaining 8% on the dynamic bag_1 fibres (Barker & Banks, 1994).

1.1.1d. The Motor Innervation

The mammalian muscle spindle is supplied with motor innervation at its poles. Two kinds of motor system are involved in spindle motor innervation: a fusimotor (γ) system, which is entirely intrafusal and a skeletofusimotor (β) system, in which intrafusal and extrafusal muscle fibres shared innervation, (Barker & Banks, 1994).

1.2. Tandem Spindle

The term tandem muscle spindle was introduced by Cooper and Daniel, (1956) to describe those spindles that have two or more periaxial spaces each with its own intrafusal bundle but linked end to end usually by a single bag_2 fibre.

1.3. Paciniform corpuscles

Paciniform corpuscles, mechanoreceptor organs that are sensitive to vibrations, are found in all muscles of any size (Barker, 1962). They are supplied by a medullated fibre which loses its myelin and runs inside a lamellated sheath.

1.4. Tendon Organs

The tendon organs were first described in detail more than a hundred years ago by Golgi, (1880) in birds and in mammals, and hence are often called Golgi tendon organs. The structure and function of the tendon organs has been reviewed by Barker, (1974) and Jami, (1992). They are spindle-shaped mechanoreceptors protected by a multilayered capsule. Their body consists of branching and fusing collagen bundles innervated by axon terminals of a Ib myelinated axon. At the muscular end, the receptor body is attached through individual tendons to a group of extrafusal muscle fibres.

1.5. Reinnervation and Recovery of Muscle Spindles after Nerve Injury

Following nerve crush, regenerating axons grow through their original endoneurial tubes along the peripheral nerve stump into the muscles and reinnervate the muscle receptors after a relatively short duration of denervation. After nerve section, reinnervation is slower, more random and incomplete (Brown & Butler, 1976 ; Banks & Barker, 1989).

Morphological observations showed that muscle spindles can be reinnervated after peripheral nerve lesions. The regenerated axons of the afferent or efferent neurons appear to end in their appropriate sites of the spindle, both primary and secondary endings are restored to their normal sites (Ip *et al.* 1977 and Barker *et al.* 1985 & 1986). Barker & Milburn, (1984), describe the appearance of the reinnervated spindles as abnormal, the regenerated primary endings are shorter with few transverse bands, while the secondary endings showed signs of growth through the primary region. Banks *et al.* (1985) and Banks & Barker, (1989) studied by light microscopy the morphological complexity of the innervation pattern of the spindles that occurs after cutting and either reuniting the same nerve, or cross-uniting different nerves.

1.6. Innervation Determines the Differentiation of the Intrafusal Fibre Types

The muscle spindles begin to develop at an early stage of histogenesis when skeletal muscle consists completely of primary myotubes (Milburn, 1973 & Kucera *et al.* 1988a, b & 1989). The sensory axons that grow into a muscle primordium find contact with a certain number of primary myotubes. The sensory innervation induces the differentiation of the primary myotubes into specific myofibre phenotypes, which are distinct from those of extrafusal muscle fibres.

Each primary myotube that receives the sensory innervation differentiates into an intrafusal fibre, of the bag_2 type. The secondary myotubes differentiate under the effect of sensory terminals into a bag_1 and one or more chain fibres. The Ia afferents are the first to contact the myotubes, then the secondary sensory axons follow later. The secondary axons can be identified only when their innervation separates from the Ia axons. The motor endings were observed in rat soleus muscle 2-days before birth (Kucera *et al.* 1988a). Milburn, (1984) also observed that the motor endings contact nascent spindles soon after the arrival of afferents or possibly at the same time in the cat.

Intrafusal myotubes originally develop in one bundle enclosed by a common basal lamina. The sensory terminals which surround the bag_2 myotubes later envelop, beneath the common basal lamina, the outer surface round the bundle including the myoblasts and assembled myotubes. During their later development, bag_1 and chain fibres separate sequentially from the common bundle, during this process the intrafusal fibres get their own basal lamina, sensory and fusimotor terminals.

The intrafusal fibres progressively differentiate into three distinct fibre types: the largest and longest being the bag_2 fibre, a thinner bag_1 fibre and thin and short chain fibres, each with a single row of axial nuclei in the region innervated by sensory endings. The spindle differentiation is completed by the formation of a periaxial space and of an adult innervation pattern. The Ia terminals are fundamental for the differentiation of the intrafusal fibre phenotypes whereas the fusimotor innervation to the morphogenesis of muscle spindles is less clear.

The development of muscle spindles or intrafusal fibres has not been reported to occur *in vitro*. Elliot & Harriman, (1974) have made an attempt to grow intrafusal fibres *in vitro* from muscle spindles but the intrafusal fibres did not successfully differentiate *in vitro* even when myoblasts were dissociated from embryonic chick muscles and cultivated with spinal ganglia cells (Tolar *et al.*1974). All muscle fibres

were found to be completely cross-striated throughout the culture period (4-5) weeks, and there were no signs of intrafusal fibres.

1.7. Peripheral Projections of Calretinin Immunoreactive Neurons

Calcium ions (Ca^{2+}) are second messengers that control a wide variety of cellular functions. Their actions include the regulation of metabolic pathways; the synthesis and release of hormones and neurotransmitters; muscle and non-muscle motility, lipid and carbohydrate metabolism and mitosis (Carafoli, 1987). However Ca^{2+} do not act alone, many cells contain a variety of cytosolic Calcium-binding proteins (CaBPs) which either modulate or mediate the actions of these ions. Among the many CaBPs in the nervous system, parvalbumin, calbindin-D_{28k} and calretinin are particularly striking in their abundance and in the specificity of their distribution.

Calretinin is a CaBP expressed mainly in the nerve cells, it is most homologous to the avian intestinal CaBP calbindin-D_{28k}. Both calretinin and calbindin were probably neuronal proteins in origin and they are expressed in largely separate sets of neurons both in birds and in mammals (Résibois & Rogers, 1992). Antibodies against CaBPs are used for neuroanatomical studies of vertebrate nervous system. They give excellent cytoarchitectonic staining (Résibois & Rogers, 1992 and Van Brederode *et al.* 1991).

On the basis of calretinin immunoreactivity among a variety of cutaneous and muscle receptors in rat, Duc *et al.* (1994) have suggested that calretinin-expressing neurons innervate particular mechanoreceptors that display physiological characteristics of rapid adaptation to stimuli.

1.8. The objectives of this work

The initial impetus of this study derives directly from recent work in this laboratory which concentrated on afferent reinnervation of muscle proprioceptors following nerve or muscle injury, and on comparative abundance of muscle spindles and their sensory innervation.

The common theme to this research is the interaction between the dorsal root ganglion cells that supply muscle spindles and the intrafusal muscle fibres on which the sensory terminals of the neurons lie. The interaction can have several components. Predominant among these are the long-term effects that are thought to be responsible for differentiation and maintenance of the differentiated state, and short-term effects that may be important in mechanosensory transduction. For this purpose, the first part of this thesis was designed to study neuromuscular development *in vitro*, by culturing slice preparations of foetal rat spinal cord and dorsal root ganglia together with a piece of muscle from newborn rat. This represented an *in vitro* system for the investigation of the developmental interaction of nerve and muscle, particularly in the formation of sensory receptors of muscle by using the standard techniques of light and electron microscopy.

The second aim was to the study the structural and functional relationships involved in the sensory innervation of muscle spindles, revealed by abnormalities following reinnervation. The third and final part was inspired from an histological observation made by Duc *et al.* (1993) that found calretinin in a subpopulation of large neurons, whose myelinated axons selectively innervate all muscle spindles. In 1994 the same workers suggested that the presence of calretinin in primary afferents correlated with the electrophysiological properties of rapid adaptation.

Chapter 2

An *in Vitro* Study of the Development of Sensory Neuromuscular Connections in the Rat

2.1. Introduction

Nervous tissue cultures offer the possibility of carrying out physiological and morphological investigations under well controlled experimental conditions. Furthermore cultures are well suited for pharmacological studies where the nerve cell can be observed either in the living state or after fixation and staining. With the development of appropriate fluorescent staining techniques individual cells can be labelled by intracellular injection. A combination of these different techniques has yielded valuable information about the function and relative location of specific types of neurons within explanted central nervous system.

Organotypic tissue culture techniques have been available since the beginning of this century. The term organotypic indicates that the tissue under study retains at least some essential features characteristic of the tissue *in vivo*.

Organotypic cultures of nervous tissue provide experimental access to individual neurons similar to that provided by dissociated cell cultures. Organotypic cultures offer the advantage that the original cytoarchitecture of the explanted tissue is well preserved and cell interactions could occur similarly as *in vivo*.

Many different techniques have been used to maintain explants of nervous tissue in culture. Of these, the most promising one is the roller-tube technique which was first

described by Hogue, (1947), and then improved by Costero & Pomerat, (1951) who introduced the technique of culturing the tissues on a coverslip placed in a roller-tube.

The cultivation of tissue on coverslips was found to be adequate for tissue of nervous origin by Willmer, (1965). This cultivation method has been modified by many authors and finally characterised in great detail by Gähwiler, (1981 & 1988). Using this method, slices of many different parts of the central nervous system have been kept in culture for several weeks.

Among the most important characteristics of the cultures obtained using the method described by Gähwiler, (1981), are the organotypic organisation of the explants and the appearance of the monolayers which develops over a period of 2-3 weeks and makes it possible to visualise individual cells with phase-contrast microscopy.

The basic principle of the roller-tube technique consists of attaching slices of the explant to a glass coverslip by means of a clot prepared from plasma and incubating them in rotating tissue culture tubes. These contain adequate medium and are placed in a roller drum, which rotates at a specific speed at a suitable angle, and incubated for several weeks. The rotation results in a periodic alteration of the gas / liquid interface to which the cultures are exposed; that is the cultures are sometimes immersed but sometimes only covered by a film of medium.

This process not only facilitates gas and medium exchanges, but also allows identified cells to be observed in living unstained tissue after about 2 weeks *in vitro*. Many studies carried out using this procedure have shown that the neurons grown in culture for 2-3 weeks exhibit morphological as well as electrophysiological properties which are similar to those found in slices of adult animal (Gähwiler, 1984 and Zimmer & Gähwiler, 1984).

Braschler *et al.* (1989) were the first to use the roller-tube technique, with modifications to the original description protocol by Gähwiler, (1981), to study the organotypic organisation of spinal cord-dorsal root ganglia-skeletal muscle co-cultures on the same coverslip.

Dribin & Barrett, (1980) concluded that neuritic outgrowth enhancement occurred on collagen and polylysine collagen-substrates media conditioned by rat, mouse or chick muscles or fibroblast. Also they noted that the rat and mouse conditioned media always produced more enhancement than chick condition media.

Casey *et al.* (1989), in their studies pointed out that the organotypic slice cultures provide an excellent system for the analysis of axonal connectivity and development. However, the study of the molecular mechanisms of this development necessitated the use of a chemically defined culture medium which allowed a high degree of cytoarchitectural maintenance while promoting neural metabolism and process outgrowth.

Peterson & Crain, (1972) have shown that when using a collagen substrate and long strips of teased adult skeletal muscle cultured with foetal rodent spinal cord, the fibres which are oriented towards the ventral root nerve fibres of the foetal spinal cord explant will regenerate rapidly as the neural outgrowth makes contact with the muscle.

The nerve cells which differentiate during the first week in the cerebellar cortex culture are the Purkinje cells (Gähwiler, 1981), which retain many important physiological and pharmacological properties in organotypic cultures. These cells morphologically grow multipolar dendritic trees with perisomatic dendrites emerging from the cell bodies, while in hippocampus culture, the pyramidal cells are capable of growing dendritic trees reminiscent of those *in vivo*.

The first probable demonstration of the formation of neuromuscular junctions *in vitro* can be traced back to the studies of Harrison, (1907), in which he noted that cultures of frog embryonic tissue exhibited evidence for enhanced spontaneous muscle contraction which could be correlated with the presence of spinal cord tissue in the explants. Szepsenwol, (1946 & 1947), showed that it was possible to form neuromuscular junctions in combined cultures of spinal cord and somite from chick embryos.

The development of neuromuscular relationships has been recently studied in several types of tissue culture systems. Bornstein *et al.* (1968) correlated histochemical and electron microscopic observations showing the development of organotypic neuromuscular junctions in mouse embryo myotome cultures.

In these preparations the neural and muscle tissues were explanted together, prior to synaptogenesis, but without disruption of the early topographic relationships already found in the embryo. A more flexible experimental approach involving pairing *in vitro* of completely separate spinal cord and skeletal muscle tissues has been used by several groups of investigators (Spenger *et al.* 1989a, b and 1991).

All of these studies showed evidence of the formation of neuromuscular synapses in cultured chick embryo tissues. James & Tresman, (1968) and Nakai, (1969) used spatially separated explants of spinal cord and skeletal muscle. Veneroni & Murray, (1969), presented completely dissociated muscle fibres to chick spinal cord, and Shimoda *et al.* (1969a & b), dissociated both the skeletal muscle and ventral spinal cord cells prior to culture. In all these cases of coupling between separated nerve and muscle cells in culture, the cytologic features of the resulting neuromuscular junctions appear immature.

Abundant ultrastructural evidence for functional interaction between cultured explants of mammalian neural tissue has been found (Crain, 1966, Crain, 1968 and Bung

et al. 1967). Synaptic junctions have been demonstrated morphologically in cultures of dissociated cells from chick spinal cord.

Hirano, (1967) observed that the first sign of neuromuscular junction formation was the thickening of the postjunctional membrane, whereas in the rat, the appearance of synaptic vesicles in the nerve terminals was reported to be the first event in junction formation *in vivo* (Kelly & Zacks, 1969).

The purpose of this part of my work was to investigate the mechanisms whereby the system of large-diameter muscle afferents important in motor control attain their highly organised pattern characteristic of different muscles. *In vivo* the muscle-fibre component of the skeletal muscles is formed by a series of generation of myotubes. Primary myotubes arise in the absence of innervation, whereas the nerve-dependent secondary myotubes are associated with older primaries (Kelly & Zacks, 1969).

The muscle spindle is a mechanosensory receptor organ that contains an intrafusal bundle of modified muscle fibres whose early development is morphologically indistinguishable from the surrounding incipient skeletomotor fibres. Its sensory nature is due to the presence, on the intrafusal muscle fibres, of the peripheral axon terminals of a group of dorsal root ganglia cells (Barker & Milburn,1984).

The differentiation of the intrafusal fibres commences at about the time of arrival of the sensory innervation and is dependent on its continued presence. It is not known whether there is a population of intrafusally determined primary myotubes that must be located by the incoming afferents or whether the myotubes are multipotent and become intrafusally specified after sensory contact. Kucera *et al.* (1989), studied the early differentiation of muscle spindles in rat muscles, on days 16-17 of gestation. They observed that both motor and sensory axons initially form primitive contacts with primary myotubes. During the initial period, sensory and motor axons or different

sensory axons may coinnervate one myotube. Transient contact with myotubes trigger the development of muscle spindle. Therefore this study focuses primarily on two questions :

1. Is the earliest generation of intrafusal muscle fibres specified before or after the initial sensory neuromuscular contacts?
2. What are the structural characteristics of these neuromuscular contacts?

The principal techniques will be light and electron microscopy of the spinal cord-dorsal root ganglia from rat foetuses, and skeletal muscle in co-cultures (Spenger *et al.* 1991). Since it is known that, *in vitro*, dorsal-root ganglion cells can establish contact with myotubes in organotypic co-culture with spinal cord slices, such a preparation offers the possibility to determine whether dorsal-root ganglion cells can initiate intrafusal fibre differentiation by co-culturing with myoblasts derived from a muscle known to contain few or no spindles in the adult for example digastic muscle which was studied by Saed, (1990) should answer the question about intrafusal-fibre specification. Successful establishment of a differentiable co-culture system would also facilitate a more fundamental approach to intrafusal development for example gene expression than currently seems likely *in vivo*.

2.2. Materials and Methods

2.2.1. General Techniques

i. Cleanliness

The slice culture technique is designed to optimise the conditions for growth and development of nervous tissue. Because explanted nervous tissue is very sensitive to oil, detergents, precipitated protein and other substances, great care was taken to ensure that all foreign substances were cleaned from any surface that came in contact with the cultures.

ii. Sterile Technique

Conditions optimal for growth and development of nervous tissue are also optimal for growth of some bacteria, fungi and viruses, therefore precautions were taken to avoid contaminating the cultures with unwanted organisms. A sterile laminar flow hood was used and continual observations were done in sterile conditions to keep the cultures uncontaminated.

2.2.2. Culture Medium

2.2.2.1. Choice of Culture Medium

The culture medium provides essential nutrients to the growing cultures and thus the preparation and composition of the culture medium are of critical importance to the production of high quality cultures. Two basic types of culture medium were used.

2.2.2.1a. Serum-Free Medium

In initial trials, serum-free medium was used, but none of the cultures survived.

2.2.2.1b. Serum-Based Medium

The serum-based medium is in widespread use today and became popular because it works very well. Essentially it consists of a nutrient solution such as Eagle's basal medium, a balanced salt solution and horse or calf serum. The serum offers the following advantages for the culture technique :

1. An effective buffer for the medium thus allowing the culturing process to take place without the need to regulate the concentration of CO_2 in the incubator.

2. Contains a variety of growth factors, hormones and nutrients that promote the health of cultured tissue.

2.2.2.2. Preparation of Culture Medium

The culture medium was usually prepared the same day or the day before the actual culturing was undertaken, because it is better for the growth of the cultures to use freshly prepared medium. Since the medium was used to feed the cultures for several weeks, sterile techniques were used to ensure that the medium was uncontaminated. Prepared medium was kept in the refrigerator at 4°C.

2.2.3. General Procedures for Obtaining Slices

2.2.3a Sterilisation

All surgical tools and instruments that came in contact with the tissue were sterilised either by heat or by cleaning with alcohol.

2.2.4. Choice Of Animals

Pregnant rats (14-16 days) belonging to the wistar CFHB strain were used throughout the study.

i. Dissections

It was necessary that the dissections proceeded quickly to reduce the length of time between dissecting and the time when the spinal cord and dorsal root ganglia were separated and exposed to Gey's balanced salt solution (BSS). Any prolonged delay caused deterioration in the tissue and a consequent reduction in the quality of the cultures.

ii. Preparation of Spinal Cord Slices with Attached DRGs

1. The 14-days pregnant rats were killed by dislocation of the neck. In order to avoid contamination of the abdominal wall with fur, the ventral surface was swabbed with 70% alcohol, the skin was cut along the transversal median line just above the diaphragm, and then the sides were grasped and pulled apart to expose the untouched ventral surface of the abdomen.

2. The abdominal wall was cut longitudinally along the median line with sterile scissors revealing the viscera. At this stage the uteri filled with embryos could be clearly seen and were dissected and put into a petri dish containing BSS. All the dissection steps were rapidly carried out under a laminar flow hood to maintain sterility.

3. The intact amniotic sacs were freed from the wall of the uterus and each foetus was removed to a sterile petri dish where it was decapitated. Immediately the trunk was transferred to another petri dish containing sterile BSS where the tail and legs were cut off and the viscera were carefully removed under a dissecting microscope.

4. The back was fixed on a block of agarose for support then cut transversely into 350μm thickness slices by means of a 752M vibroslice. Slices were transferred to a petri dish containing BSS.

5. The spinal cord slices with attached dorsal root ganglia were punched out of these sections using a hypodermic needle that had been sawn off transversely and sharpened. The needle was positioned in such way as to remove as much extramedullary tissue as possible.

iii. Preparation of Skeletal Muscle Tissue

New-born rats were sacrificed by decapitation. After disinfection of the trunk, the skin and fasciae of the back were cut open widely and a piece of paravertebral muscle was dissected out. This long muscle strip was cut into 3-4 pieces and chopped with a razor blade into long thin strips under a dissecting microscope. The muscle pieces were transferred into a petri dish containing (BSS) and kept at room temperature.

2.2.5. Protocol for Preparing Organotypic Slice Cultures

2.2.5a General Techniques

Four different procedures were attempted in order to discover optimal conditions for maintaining explant co-cultures of spinal cord-dorsal root ganglia and skeletal muscle.

A. Method 1

The first method used was an explant membrane technique (Stoppini *et al.* 1991). Tissue was cultivated on a sterile, transparent, porous and low protein-binding membrane (Millicell-CM, Millipore) at the interface between culture medium and air, and kept in petri dishes in an incubator in a stationary state. The culture medium passively diffused through the membrane to the tissue. The tissue surface was constantly covered by a thin film of culture medium. No improvement in the quality of tissue cultures was detectable in any of the 40 cultures prepared using this technique because of the outgrowth of non-neuronal cells.

B. Method 2

The second method (Mandys *et al.* 1991) involved cultivation of the tissue in a semi-solid medium consisting of two layers of agar. For the preparation of the lower layer, 1.5 ml of culture medium was added to 100 ml of 0.5% agar in distilled water and aliquots of this mixture were pipetted into 35 mm petri dishes to create a continuous layer, approximately 2-3 mm thick. After the lower layer became solid, the slices of the spinal cord-dorsal root ganglia and the muscle were placed on top of the agar. For the upper layer, 0.5 ml of medium was added to 100 ml of 0.5% agar and small portions of this solution were very carefully pipetted between the tissue slices, such as not to cover the upper surface of the slices, but to allow exposure to the humidified atmosphere of the incubator which was kept at 36.5°C. Twelve cultures were prepared without changing the medium. Unfortunately, the tissue slices gradually became immersed in the upper layer and zero growth rate was obtained.

C. Method 3

The third technique which I tried was a modification of the method of Caterina *et al.* (1993). The entire spinal cord (the spinal cord together with dorsal root ganglia) was split longitudinally in two halves along the posterior median groove, the ventral sides facing each other, and a muscle strip was placed in the middle, facing the two ventral sides. The tissues were mounted on a coverslip using the plasma clot technique, which will be described in detail in relation to the fourth method. Ten such preparations were made and put in test tubes with 1.5 ml of medium and incubated at 36.5°C and 5.8% CO_2 with continuous rotation. This technique did not yield any results because the pieces of the tissue came off the coverslip soon after the roller drum started to rotate.

D. Method 4

The fourth technique I tried was an improvement of the third one, where only sections of spinal cord were used instead of the whole tissue. This technique was successful and will be described in more detail. It was initially introduced by Hogue, (1947), then modified by many authors and finally characterised in great detail by Gähwiler, (1981 & 1988), and referred to as the roller-tube technique. This technique is the most commonly used method for maintaining differentiating central nervous system and dorsal root ganglia organ culture. The roller-tube technique has the advantage of being cheap and can be used for preparing large numbers of cultures that may be maintained for several weeks in culture. However, the longevity of the cultures can be achieved only by ensuring that all work for preparing and maintaining the cultures is done in a sterile environment and that all tools for preparing the cultures are clean and sterile.

2.2.6. Mounting and Embedding the Slices

The embedding procedure described by Gähwiler, (1981) was used. Slices of tissue were mounted on glass coverslips. The coverslips used were small enough to fit into the culture tube but large enough to hold the cultures. All the coverslips were cleaned in alcohol, washed thoroughly in distilled water and sterilised in glass petri dishes at 100^{o}C for two hours.

2.2.6a. Plasma Clot

The method of attachment of the tissue slices to the coverslips is very important. The means of attachment needed to be strong enough to stand the rolling action and should also be a substrate that is conducive to growth of the cultures. Using a pair of sterile forceps, the coverslips were placed in a sterile petri dish and a drop of rabbit plasma (Sera-Lab) was placed in the centre of each coverslip and spread over the surface to coat the slide in order to keep the explants in place. Coagulation of the plasma was begun by adding one or two drops of thrombin (Sigma), then under the dissecting

microscope one spinal cord slice was placed in the centre of each drop using a fine spatula. Before coagulation was complete a piece of paravertebral muscle from a new born rat was quickly positioned at a distance of 1-2 mm from the ventral side of the spinal cord explant in order to place the target for the sensory neurons as well as the motoneurons at a well defined site. The coverslips with cultures were kept in a petri dish for several minutes so the plasma coagulated well before placing the coverslip in the culture tube.

2.2.7. Growth Medium

The growth medium consisted of a mixture of two volumes of basal medium (Dulbecco's modified Eagle's medium glutamine free), one volume of Hanks basal salt solution and one volume of heat-inactivated horse serum (all purchased from GIBCO). 1ml of a 50% glucose solution and 0.5 ml of a 200Mm L-glutamine solution (GIBCO) were added to each 100 ml of freshly prepared medium, through a sterilising filter.

The basal medium and the Hanks basal salt solution were stored at 4°C. The basal medium was purchased glutamine free in order to prepare specific, accurately controlled concentrations of glutamine.

The serum had been screened for mycoplasma to reduce the chances of contamination of the cultures. Serum was stored at -20°C and thawed at room temperature. Complement was inactivated by heating the serum in a water bath at 56°C for 30 minutes before using.

2.2.7a. Medium and Medium Change

The coverslips bearing the cultures were inserted into plastic test tubes. About 1.5 ml of the culture medium was added in the first week, then in the second week the culture medium was increased to 2 ml. This volume allowed the cultures to be immersed in medium during half a rotation and to be out of the medium during half a rotation. The

cultures were observed by phase contrast inverted microscopy every three to four days, after which they were washed and fed with fresh growth medium. The first spontaneous contractions of the muscles started on day 7 *in vitro*. Due to enhanced spontaneous muscle activity, an acid shift of the culture medium occurred. The medium volume was then increased from 1.5 to 2 ml in each culture tube to increase the buffering capacity of the medium. The interval of medium change was decreased from three to four days during the first week to two days at the beginning of the second week.

2.2.8. Incubation

Culture tubes were placed in a roller drum in an incubator at 36.5°C and 5.8% CO_2. Incubation took place in a dry atmosphere, and the roller drum was set to 120 revolutions per hour. An original modification to the method described by Gähwiler, (1981 & 1988) was that the culture tubes were placed at an angle of 55° instead of 5°.

2.2.9. Care and Maintenance

Healthy cultures metabolised the culture medium and needed to be fed with new medium two times a week. As the medium gets metabolised by a healthy culture it becomes more acidic and turns from a pinkish red to a salmon-pink colour. Feeding of the cultures was always done under a laminar flow hood using sterile precautions. Only a few cultures were usually removed from the incubator at any time. The technique used was to pour out the old medium into a beaker, wash the culture with BSS, then fresh medium was added. The cultures kept up to 4-weeks *in vitro*.

2.2.10. Microscopic Observations

Light microscopy observations were made continually throughout all the experiments and photographic recordings of the cultured tissues were made at regular intervals on a phase contrast inverted microscope.

i. Histology Preparation

For histological preparation the cultures were taken and fixed at intervals in Karnovsky, (1965) fixative, following the routine procedure described below.

Solution A

Paraformaldehyde	2 g.
Distilled water	40 ml.
1N Na OH	2-6 drops.

The paraformaldehyde and water were heated in a beaker on a hot plate. Drops of NaOH were slowly added while the mixture was shaken, until a clear solution was obtained.

Solution B

25% Glutaraldehyde	10 ml.
0.2 M Sodium cacodylate buffer pH 7.3	40 ml.

The two solutions (A & B) were stored separately at 4°C and mixed just before use.

1. Fixation in Karnovsky's fixative for 1-1.5 hours at 4°C.
2. Overnight storage in buffered solution (0.2 M Sodium cacodylate).
3. Post- fixation for 0.5- 1 hours at 4°C in 1% buffered Osmium tetroxide by mixing 2% osmium tetroxide 1 : 1 with 0.2 M sodium cacodylate to give 1% OsO4 in 0.1M buffer.

4. Dehydration at room temperature in a graded series of alcohol.
   70% alcohol for 15 minutes (3 changes)
   95% alcohol for 15 minutes (3 changes).
   Absolute alcohol 30 minutes (3 changes).

5. Infiltration
   Absolute alcohol / propylene oxide 1 : 1 30 minutes (3 changes).
   propylene oxide 30 minutes (3 changes).

Infiltrate in 1 : 1 propylene oxide / Araldite in bottle for 30 minutes at 45°C with lids off.

Infiltrate in absolute Araldite in a bottle at 45°C for 30 minutes.

Embed in Araldite 45°C for 12 hours, transfer to 60°C for further 2 days.

ii. Removal of Glass Coverslips from Cultures Embedded in Araldite using Hydrofluoric Acid

Using this technique (Moore, 1975) the glass coverslip from a culture could be removed easily leaving the surface of the araldite quite smooth and suitable for subsequent thin or ultra thin sectioning for electron microscopy. The following procedure was carried out in a fume cupboard.

1. Hydrofluoric acid (40% or 60%) was poured into a polyethylene beaker.
2. Two or three of the araldite blocks with attached coverslips were immersed for 1-3 hours depending on the concentration of hydrofluoric acid.
3. The blocks were removed from the acid by means of polyethylene forceps and washed

in a beaker containing sodium hydrogen carbonate then washed under tap water.

iii. Preparation of Semi-Thin Sections (1 μm)

The sections (1μm thickness) of the small piece of the culture that contained the spinal cord-dorsal root ganglia and muscle were cut parallel to the coverslips on which they were grown by using glass knives mounted in a Reichert OMU3 microtome.

Staining (Toluidine blue)

1 μm thick sections were gathered up with a glass rod and placed on a slide in a drop of water. The slide was then dried on a hot plate to spread and fix the sections on the slide. Drops of 1% toluidine blue solution were placed directly on the sections and the slide was left for about 1 minute on a hot plate. It was then rinsed gently with distilled water to remove excess stain, dried on the hot plate and mounted in D.P.X. All

the sections were examined by light microscopy and were photographed using a microflex UFX camera on a Nikon Optiphot microscope with a suitable filter.

iv. Preparation of Ultrathin Sections

Ultrathin sections were automatically cut by the microtome, however it was common for the block to need trimming first. Section thickness was estimated by interference colour ; and sections appearing silver or gold were selected. They were spread using chloroform vapour, collected on a 150-200 mesh copper grid coated with formvar, then stained with uranyl acetate and lead citrate and examined with a Philips 400 T electron microscope.

2.2.11. Acetylcholinesterase- Staining

Initially in this investigation I used the AchE-histochemical staining technique and the Dil fluorescent dye technique separately for morphological characterisation of the development of the cultures, and to label individual axons which innervated the muscle, then the cultures were fixed and the detailed histological was carried out. Acetylcholinesterase (AchE) activity was demonstrated in fixed cultures using the histochemical method of Karnovsky & Roots, (1964). After one to several weeks *in vitro* the spinal cord-dorsal root ganglia-muscle co-cultures (SC-DRG-M) were fixed and stained for AchE to investigate the persistence of AchE in the innervated regions.

1. Growth medium was decanted from the cultures and the cultures were rinsed 3 times with BSS.
2. The cultures were fixed in 1% glutaraldehyde in 0.135 M phosphate buffer (pH 7.2) at 4°C for 5 minutes.
3. Cultures were washed for 30 minutes with several changes of an acetate-buffered saline solution.
4. The cultures were then incubated with Karnovsky-Roots medium.

2.2.11a. Incubation Medium

5 mg acetylthiocholine iodide were added to 6.5 ml acetate buffer (pH 6.0), with thorough shaking. The following were then added with stirring.

0.5 ml 0.1 M sodium citrate.

1.0 ml 30 mM copper sulphate.

1.0 ml distilled water.

1.0 ml 5 mM potassium ferricyanide.

The incubation took place for 15-60 minutes at room temperature. 0.01%Triton X-100 was added to the medium to increase penetration of the substrate.

5. The medium was then replaced by distilled water and the cultures were washed for 15 minutes.

6. The cultures were dehydrated with ethanol and mounted under a coverslip with a drop of glycerol.

7. The sites of AchE activity appeared as black grains.

2.2.12. DiI Labelling of Explant Cultures

An attempt was made to label the sensory neurons with DiI (1,1'-dioctadecyl - 3,3,3',3'-tetramethylindocarbo-cyanin perchlorate) (Honing & Hume, 1989). A solution of 0.1% DiI dissolved in absolute ethanol was injected from glass micro pipettes by air pressure into the dorsal root ganglia after the fixation of the cultures and allowed to diffuse in darkness at 4°C for a few weeks. The cultures were observed under a fluorescence microscope with a blue filter in order to locate labelled neurons.

2.3. Results

Observations

2.3.1. Morphology of the Culture System in Living State.

On the day of explantation, the dorsal root ganglia (Fig 3) were located very close to the spinal cord to which they were connected by very short dorsal roots (Fig 2). The tissue slices were too thick for visualisation of individual cells.

The spinal cord kept its organotypic configuration in vitro, the dorsal and ventral part could be distinguished and also the location of dorsal root ganglia. Out of 197 cultures, 50 died and 43 were washed out during rotation. Therefore the following description is based on observations of 104 cultures, through a phase-contrast microscope when the nutrient medium was changed. The co-cultures were maintained for 21-27 days, by which time the explanted muscles had regenerated to form cross-striated contracting fibres. The spontaneous muscle contractions observed in the cultures could be due either to innervation or electrical coupling between neighbouring fibres. After examining all the material I found that the results were best described as follows.

i. The Initial Stage Co-culture

During the initial two or three days of culture the outgrowth of nerve fibres radiated from the ventral surface and dorsal root ganglia cells. The neurite outgrowth and the neuronal precursor cells were able to grow in the absence of nerve growth factor, and after 24-hours other cells showed a radial outgrowth from the co-cultures. These cells are myoblasts (Fig 5A), they are spindle shaped, short, thick and granular, and the myoblasts nuclei are typically oval in shape with one or two round nucleoli in the clear karyoplasm.

ii. The Intermediate Stage Co-culture

At the intermediate stage of development (3-10 days *in vitro*), the neurites emerged from the explants in thick bundles, and as they extended further from the cultures they gradually separated (Fig 4A & B). The spinal cord-dorsal root ganglia explants flattened little by little at their margins due to cell migration, while their central area remained thick. The flattening of the cultures is a most desirable feature, because it is the basis for the visualisation of individual neurons with phase-contrast microscopy. The flattening of the margin of the explants and the formation of the glia network indicated a healthy culture.

After 8-days the dorsal root ganglia spread ventro-laterally around the spinal cord tissue from their original dorsal location forming a single sheet of cells leading to an epithelial-like structure and the neurons could be clearly distinguished from other cell types. Each had a dark cytoplasm with round or oval centrally located nucleus (Fig 6B).

In successful cultures three cell types were distinguished, these were:

A. Non-neuronal background cells.

B. Dorsal root ganglia cells (Fig 6B).

C. Spinal cord neurons (Fig 6A).

The non-neuronal population consisted of pale grey, flat cells of various sizes and without long processes. Neurons were easily distinguished from these background cells by their processes of a relatively larger size, greater thickness, prominent nuclei and nucleolus.

The dorsal root ganglia cells and the spinal cord cells were differentiated from one another by the following criteria.

1. The spinal cord cells had a large number of thicker, more tapering and more highly branched processes than did the dorsal root ganglia cells.

2. The spinal cord cell bodies were often elliptical or stellate.

3. The dorsal root ganglia cells were characteristically round and generally more plump than spinal cord cells and the cytoplasm of the dorsal root ganglia cells often had more prominent granules than did the spinal cord cells cytoplasm.
4. The nucleus of the dorsal root cells was generally centrally located and the phase dark nucleolus was situated near the centre of the nucleus whereas in the spinal cord cells both the nucleus and the nucleolus were more often found eccentrically placed.

The dorsal root ganglia cells, which at the end of first week in culture clearly rested on top of the background cells, often appeared flattened. The spinal cord cells also stayed on top of the glia cells.

The muscle fibres differentiated either from proliferation of the muscle pieces from the newborn rats when co-cultured or from myoblasts of the perispinal myotomes in cultures without coexplanted muscle.

iii. Late Stage Co-culture

The thickness of the explant itself made it difficult to examine culture constituents other than large neurons. Therefore, stained semi-thin sections of the explant were prepared.

A. Light Microscopy

The sections showed three zones (data not present). The superficial zone or the top surface which was exposed to the feeding medium and was relatively free of cell bodies, the middle zone which contained the majority of cells and the deep zone or the necrosis area, where nutrition was apparently inadequate. In the necrosis area large spaces and debris containing processes gave the zone an alveolar appearance. So the best organisation of neurons was generally seen in semi-thin sections.

The dorsal root ganglia cells spread ventrolaterally around the spinal cord tissue. They spread out in a monolayer with a typical epithelial-like form, nerve fibres and Schwann cells were seen among them. Two type of neurons were seen, large and small neurons. The small neurons tended to be grouped together more than the large neurons. Cytoplasm organisation varied as seen in (Fig 7A & B), the small neurons (n1) contained fairly dense and homogeneous cytoplasm while the large neurons (n2) had lighter and less homogeneous cytoplasm. Mitosis was seen among migrant cells.

The spinal cord *in vitro* consisted of both developed and less mature neuronal perikarya (Fig 8), and myelination occurred around the axons after three weeks. The neuroblasts always had a multipolar character, they were spindle-shaped or stellate with radially arranged processes.

In semi-thin sections new myotubes were seen to have formed alongside the parent fibres which had degenerated and been cleared away by macrophages, leaving a bundle of regenerated myotubes. The myotubes developed by 6-10 days *in vitro*, and by 8-13 days the cross-striated muscle fibres appeared (Fig 5B). Striated fibres increased in number after two weeks, and contractile activity developed by 7-18 days. One often saw slender muscle fibres which had central nuclei and no striations (Fig 5C). The 21-day old cultured fibres under electron microscopy appeared mature with cross-striated myofibrils distributed throughout the cytoplasm, with distinct A, I, Z, and M bands present (Fig 5D). Soon after this period *in vitro* the muscle began to atrophy, cross-striations disappeared completely, fat droplets formed and swelling developed in the long muscle fibres.

The muscle differentiation proceeded normally only where it was oriented towards the ventral edge or surface of the cord explant. In 3 cultures I tried changing the orientation of the muscle co-culture to the dorsal edge of the spinal cord-dorsal root ganglia. The cultures were examined after two days and the medium was changed. One

culture was found to have died, while the other two appeared alive and neurites had emerged from their ventral surface, the muscle remained as at the moment of the establishment of the cultures; no axonal outgrowth could be observed to reach the muscle, after 4-days the spinal cord-dorsal root ganglia died and the muscle started to atrophy, and after 6-days the muscle had degenerated completely, and the spinal cord tissue had started to disintegrate into small pieces of tissue.

B. Electron Microscopy

One of the best indicators of healthy cultures was their fine structure, and electron microscopical studies demonstrated that these cultures displayed cellular and subcellular features of healthy neurons even after long periods *in vitro*. Within the investigated time period, few degenerating profiles and little cell debris were evident in the neuropil.

Most of the neurons were identified by their size and shape, characteristic nissl substance and nuclear morphology. Although neurons were found in groups, intervening layers of glial processes prevented their direct contact. The neuronal somata varied in shape and size, contained large nuclei and the chromatin was quite uniformly dispersed. The nuclei of small neurons were usually centrally placed, the nucleoli were prominent and the cytoplasm contained the usual organelles, mitochondria which were round or ovoid or slender rods, Golgi apparatus, endoplasmic reticulum and lysosomes which were scattered throughout the perinuclear cytoplasm. Nissl substance filled the narrowing part in the periphery of the perikaryon (Fig 9A & C).

A type of neuron has been found with large folded nuclei which were eccentrically located, and the fold contained clustered ribosomes, mitochondria and vacuoles (Fig 9B). The nuclear pores were visible in another neuron, the outer membranes of the envelope were more ruffled than the inner one, and ribosomes were associated with the outer membrane.

2.3.2. Neuronal Processes and Synapses

The dendritic cytoplasm resembles perikaryal cytoplasm in its complement of organelles and tubules, which were more concentrated along the length of the dendrite. The mitochondria were oriented parallel to the length of the process as usual. In thin dendrites the tubules were the most prominent organelles, scattered among them were neurofilaments, clusters of ribosomes and mitochondria.

Axons contained mitochondria, neurofilaments and tubules. Some processes in which tubules were found were identified as axons because of the presence of myelin or because they were continuous with terminals that contained the characteristic synaptic vesicles.

Synapses that appeared morphologically normal developed between neurons and nerve processes *in vitro*. On the presynapic side typical vesicles gathered that occurred singly or aggregated in clusters on the synaptic membrane. The presynaptic and postsynaptic membranes were more dense and sometimes appeared thickened. The large axon terminals contained mitochondria but smaller ones did not.

Neurofilaments and tubules were absent from the presynaptic terminals and the extracellular material was usually visible in the synaptic cleft. Eight-axodendritic synapses were observed in this study. In axodendritic synapses, the synaptic cleft was enlarged and filled with particularly dense extracellular substance, and the additional dense material just inside the postsynaptic membrane made the membrane appear thicker (Fig 10A).

Dendro-dendritic synapses, axo-axonic and axo-somatic synapses were also observed. In axo-somatic synapses, the presynaptic component contained mitochondria and synaptic vesicles, the cleft was slightly narrower than in axodendritic synapses, and there was conspicuous curvature of the synaptolemma (Fig 10B).

2.3.3. Nerve Fibres and Myelination

The nerve fibres typically contained neurofilaments, various tubular components, mitochondria and vesicles (Fig 11A). Myelin sheaths displayed a typical lamellated structure, they did not appear uniform over the extent of a whole internodal segment, but varying numbers of interruptions were present. In some myelination cultures schmidt-lanterman clefts were seen in longitudinal thin sections, a cleft is a series of splits in myelin lamellae at the major dense line with portions of Schwann cell cytoplasm enclosed. Nodes of Ranvier were covered by Schwann cell processes within a continuous basement membrane (Fig 11B) and the paranodal myelin terminated in loops, the loop nearest the node arising from the outermost myelin unit. In addition to tubules, many dense particles were found within the terminating loops.

2.3.4. Neuromuscular Junctions

i. Analysis of (AChE) Stain and Fluorescent Dye

An attempt was made to demonstrate the enzyme AchE in the region of nerve endings by the histochemical method of Karnovsky & Roots (1964). In the early stages of development *in vitro* the AchE persisted as a diffuse or disorganised patch on the surface of muscle fibre, but at late stages of development a negative reaction for AchE was obtained.

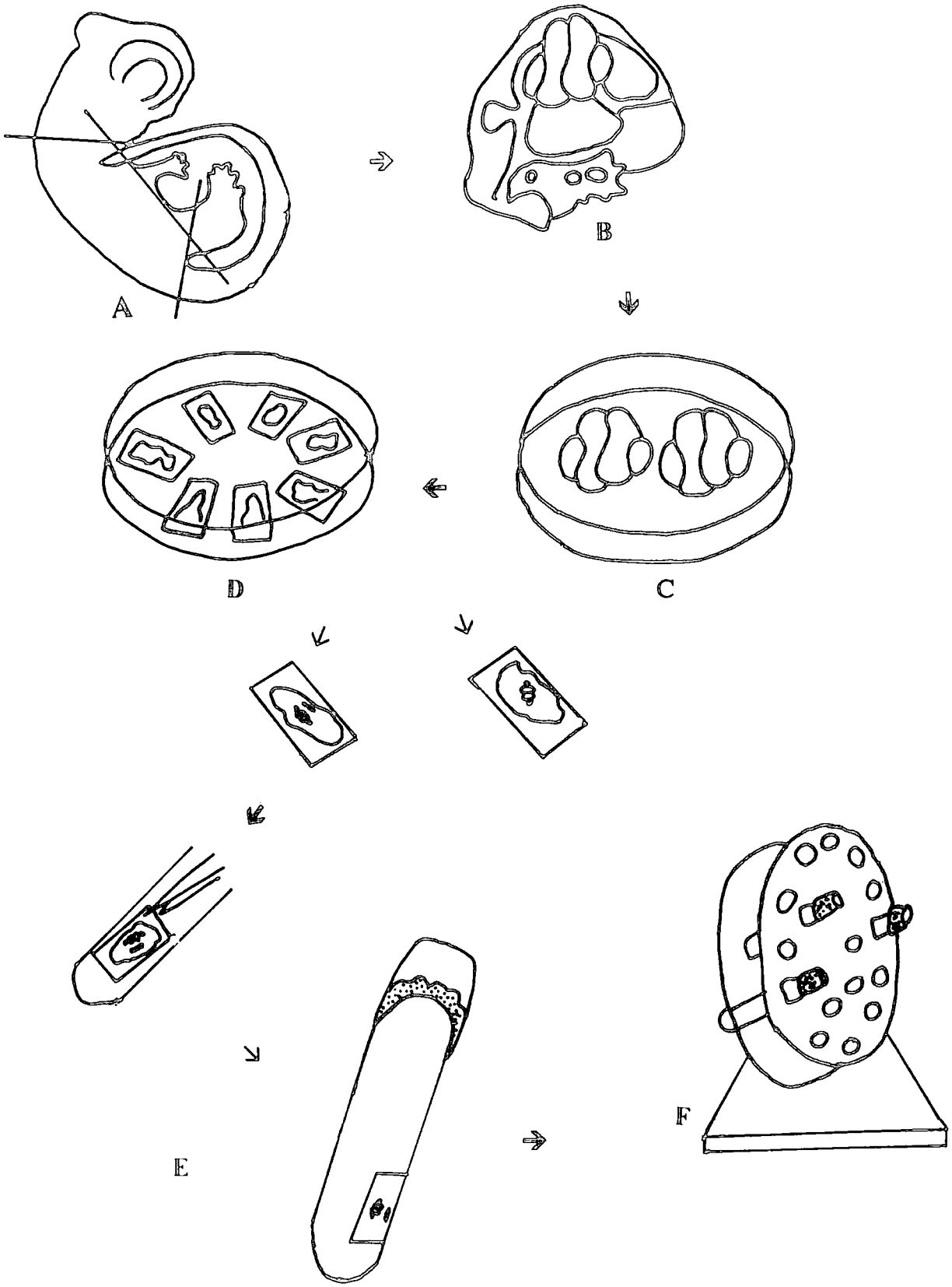
The numbers of patches present in each culture was not what was expected based on the number of muscle fibres placed into the cultures originally, so the actual distributions of AchE have so far proved to be negative. With the highly fluorescent dye Dil, (Honig & Hume 1989), individual nerve cells could be visualised in the early days in cultures (Fig 13).

ii. Development of neuromuscular junctions

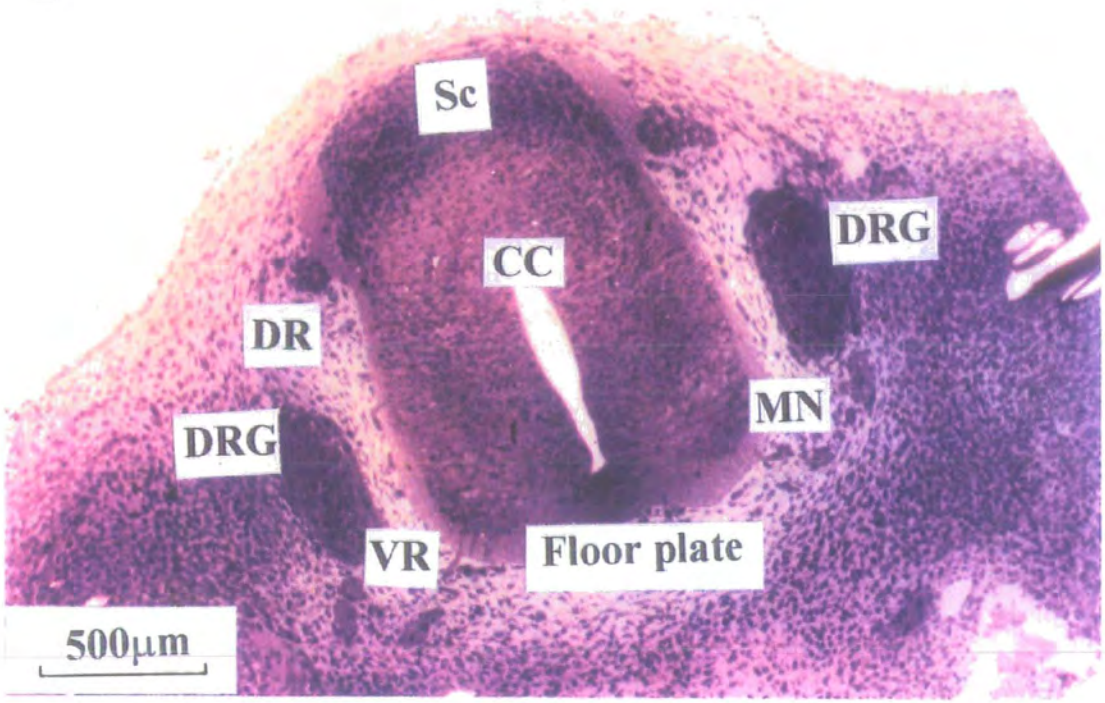
After 24-hours in culture, neurons began to send out processes, neuromuscular contacts started to form, and many neuromuscular contacts were observed in the living

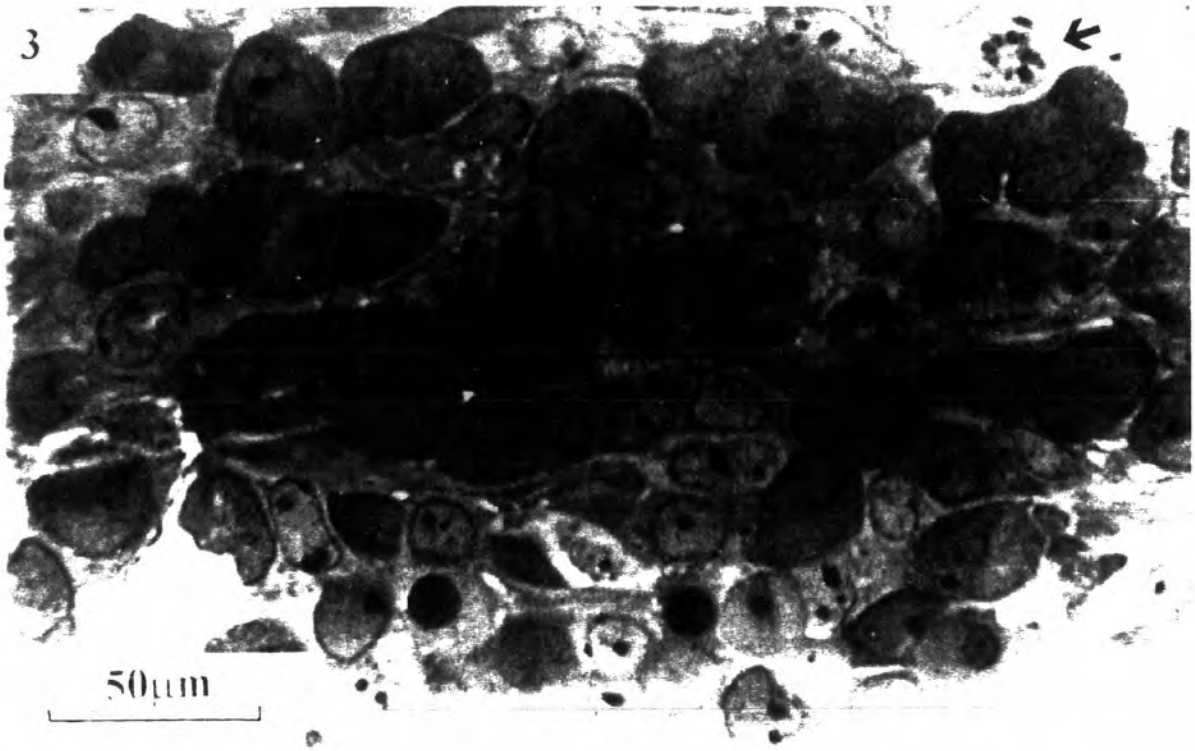
state by phase-contrast microscopy. A well differentiated motoneuron forming a junction with a regenerated muscle fibre was observed after 8-days culture (Fig 12). This was confirmed under the electron microscope (The material was lost). In this, the nerve process had made junctional contacts with the myotube, and a number of vesicles structurally very similar to synaptic vesicles in the mature neuromuscular junctions that have acetylcholine as the transmitter, were observed in the nerve ending. Most of these vesicles were round, but some had an irregular shape. The vesicles were often located close to the prejunctional membrane. In addition to synaptic vesicles mitochondria were present and microfilaments. The width of the junctional cleft was variable, in some places it was large and in other places narrow; the cleft had dense material.

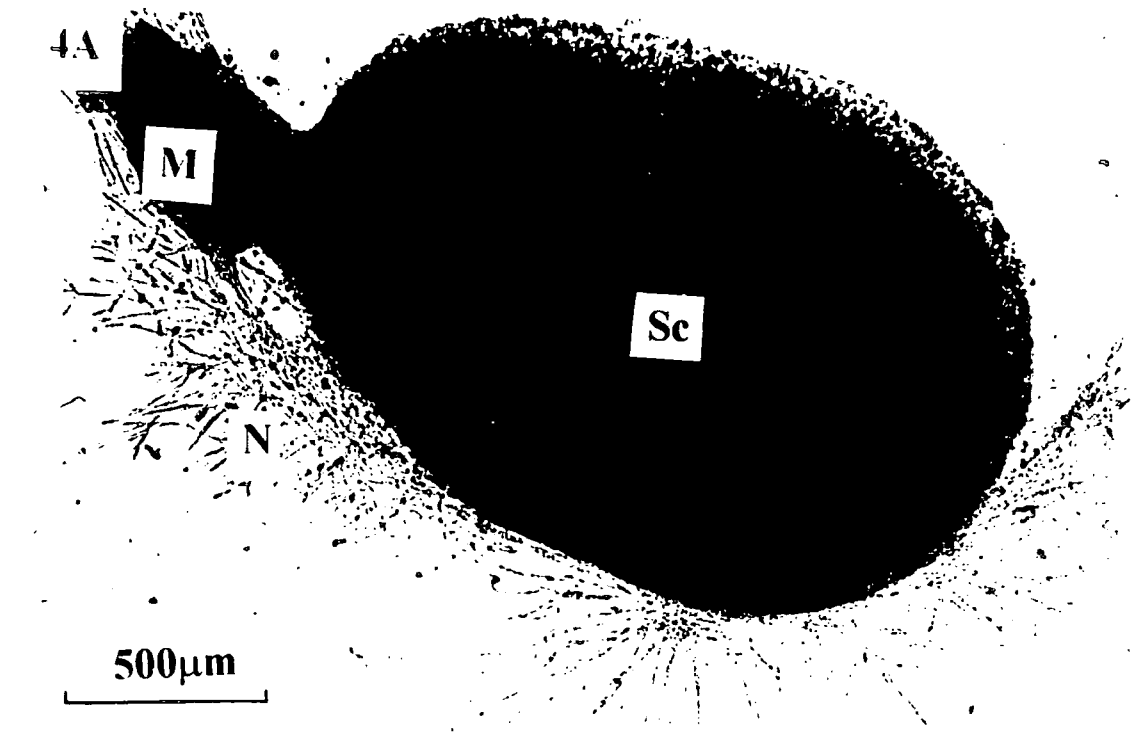
1

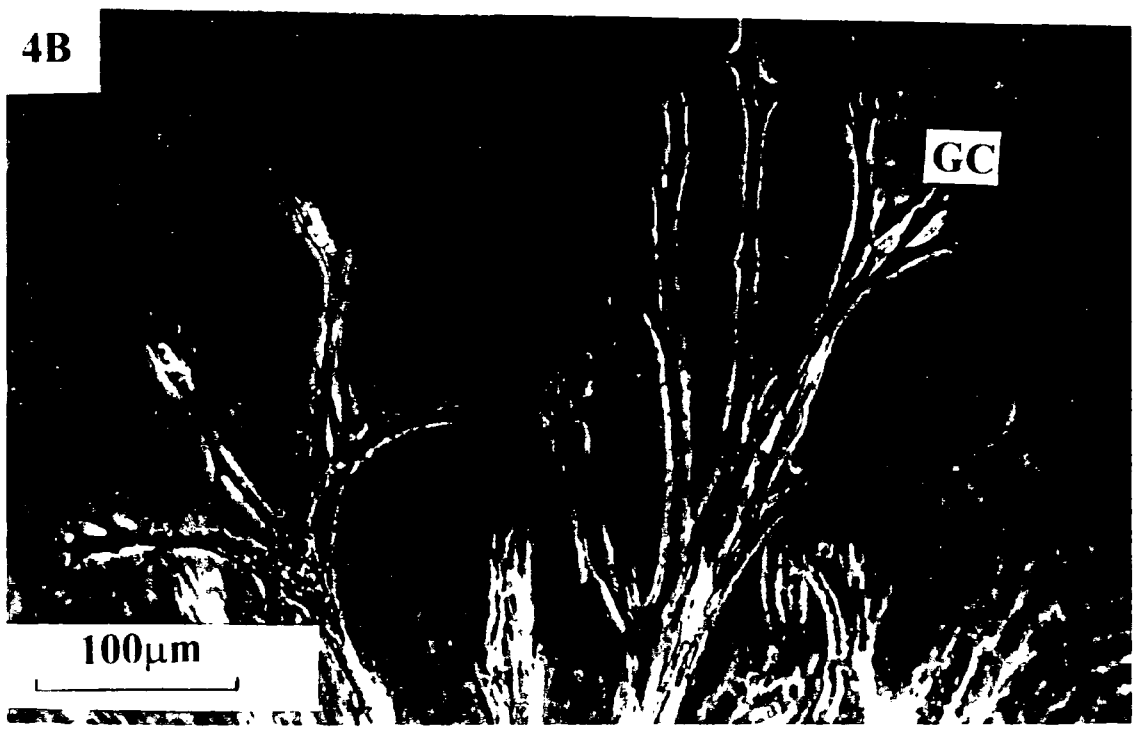


2

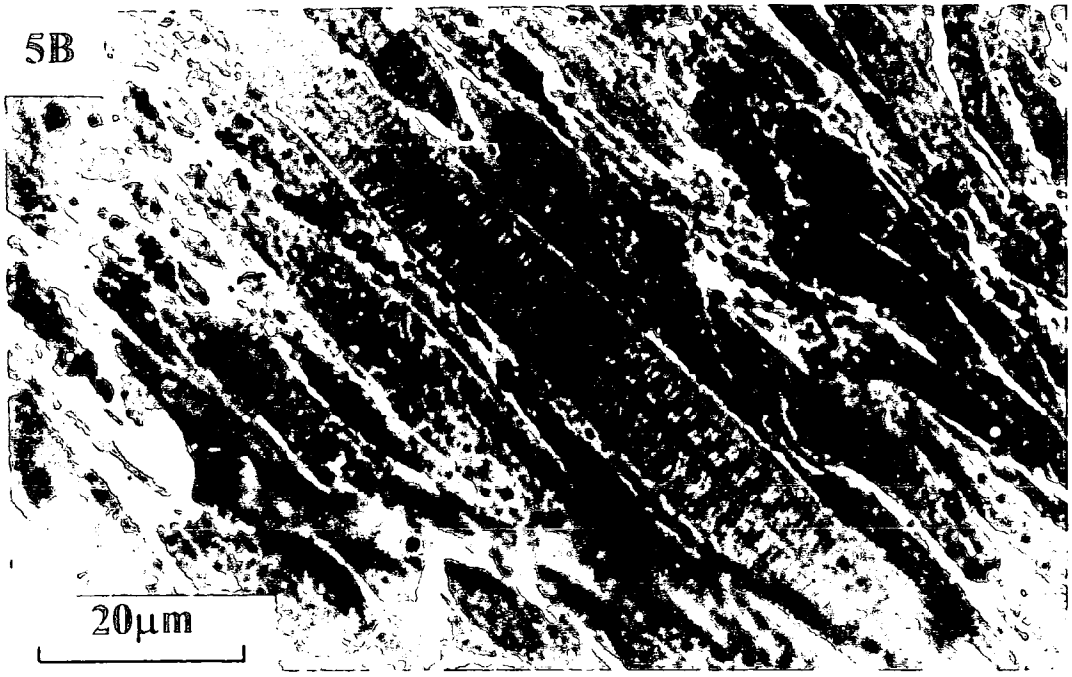


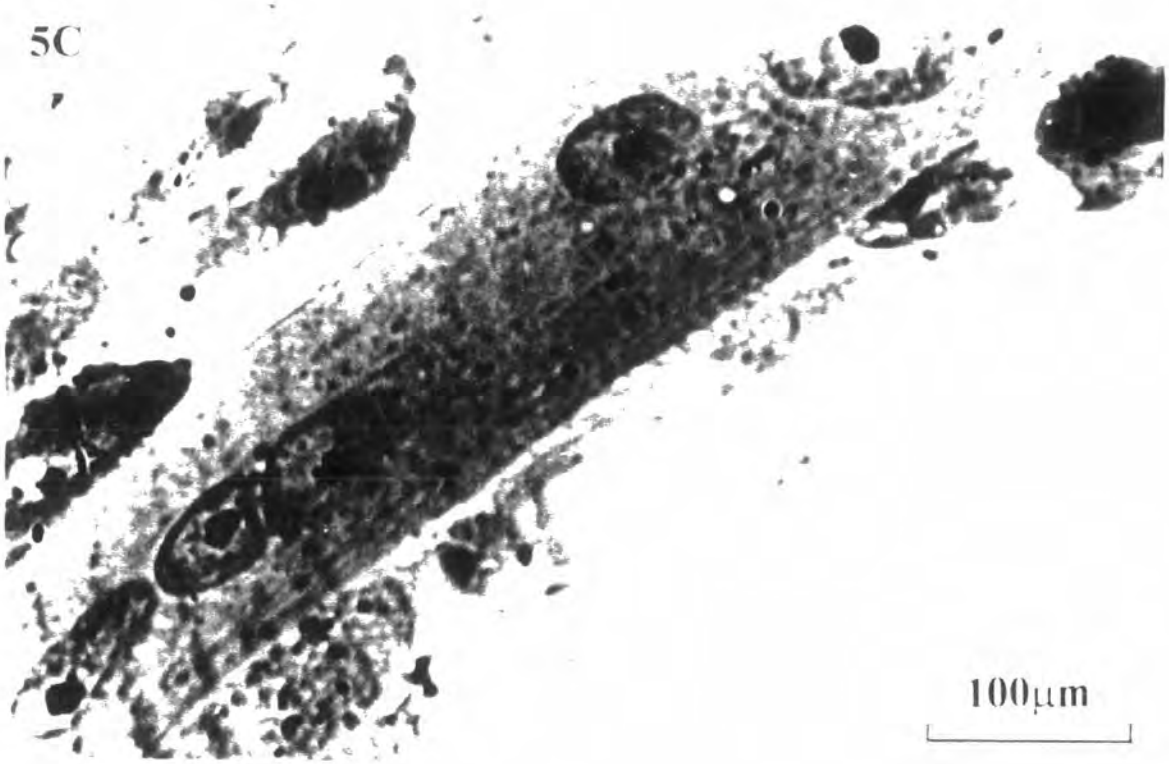

3
50μm


4A
M
Sc
N
500µm

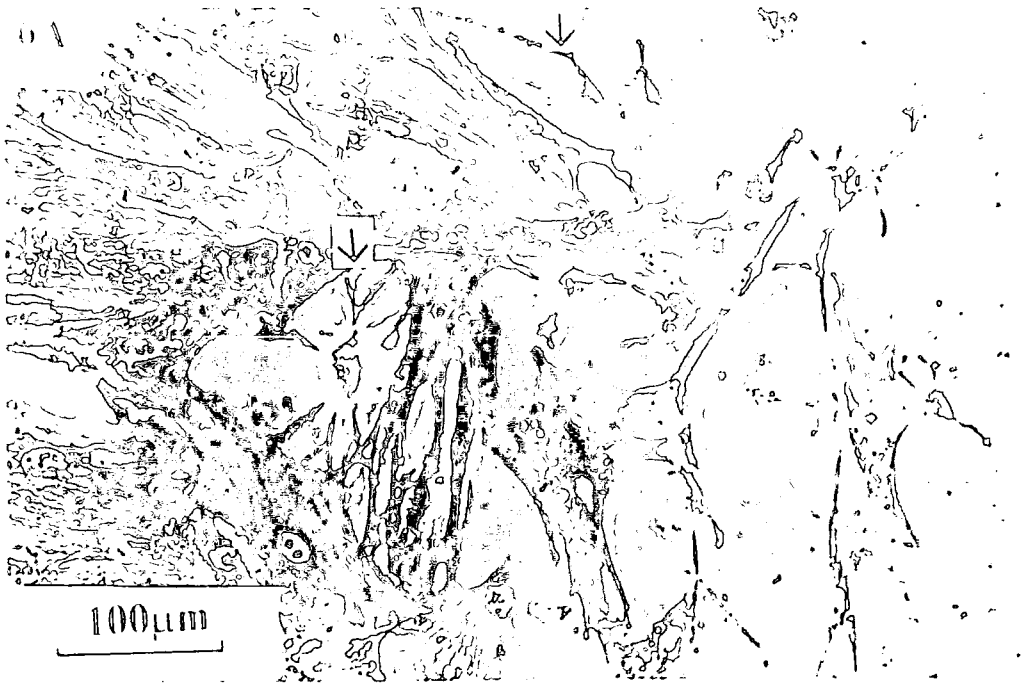

4B
GC
100µm


5A
100μm

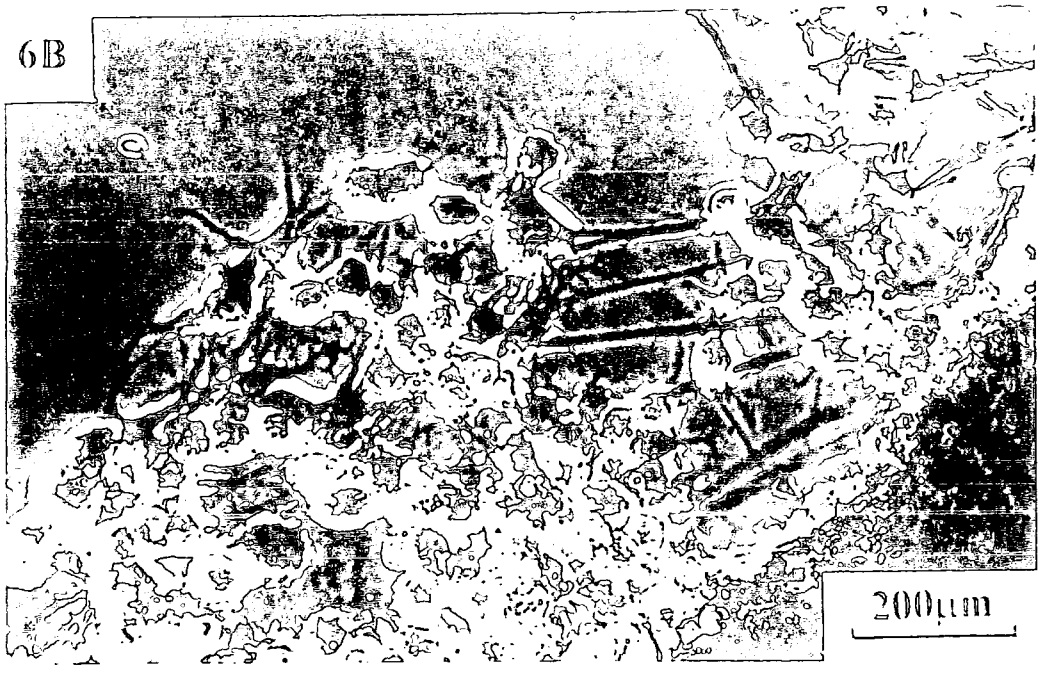

5B
20μm

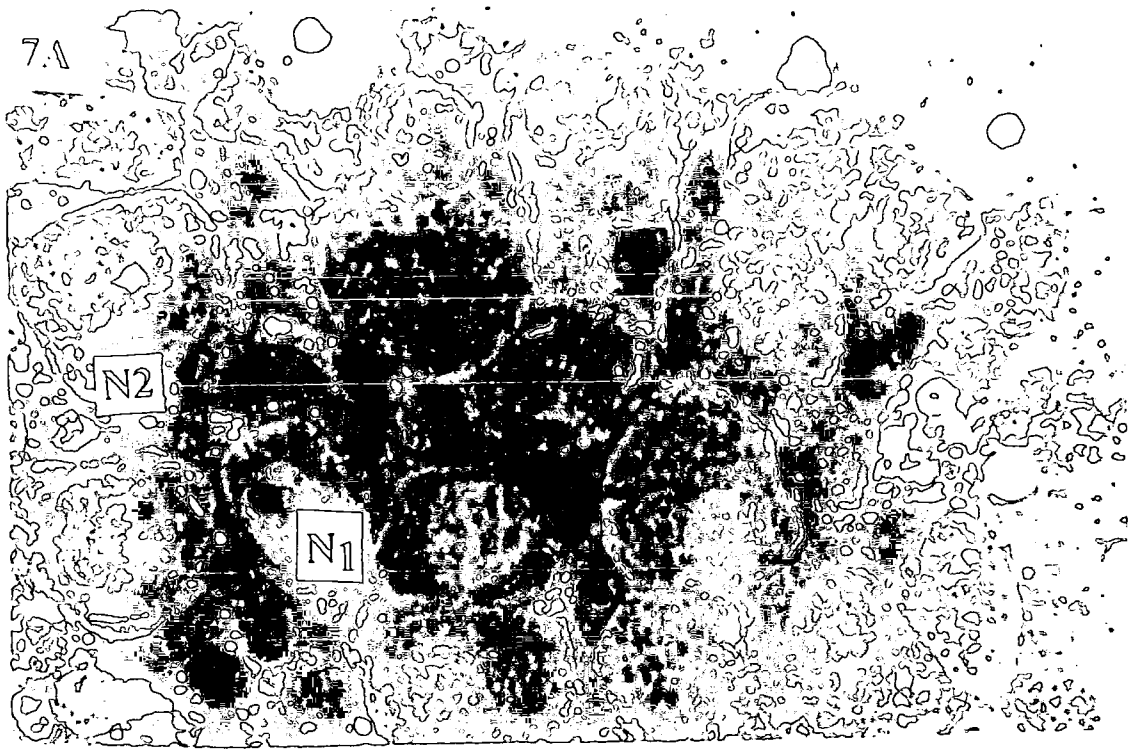

5C
100μm

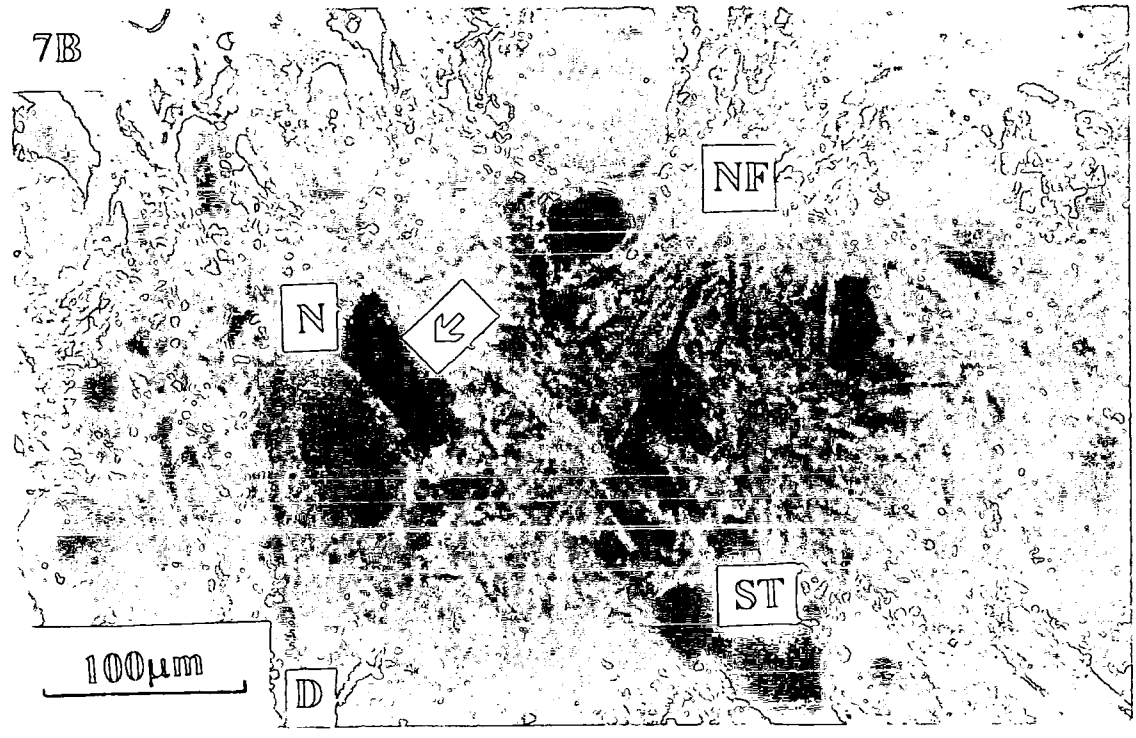

5D
Z
I
A
1µm

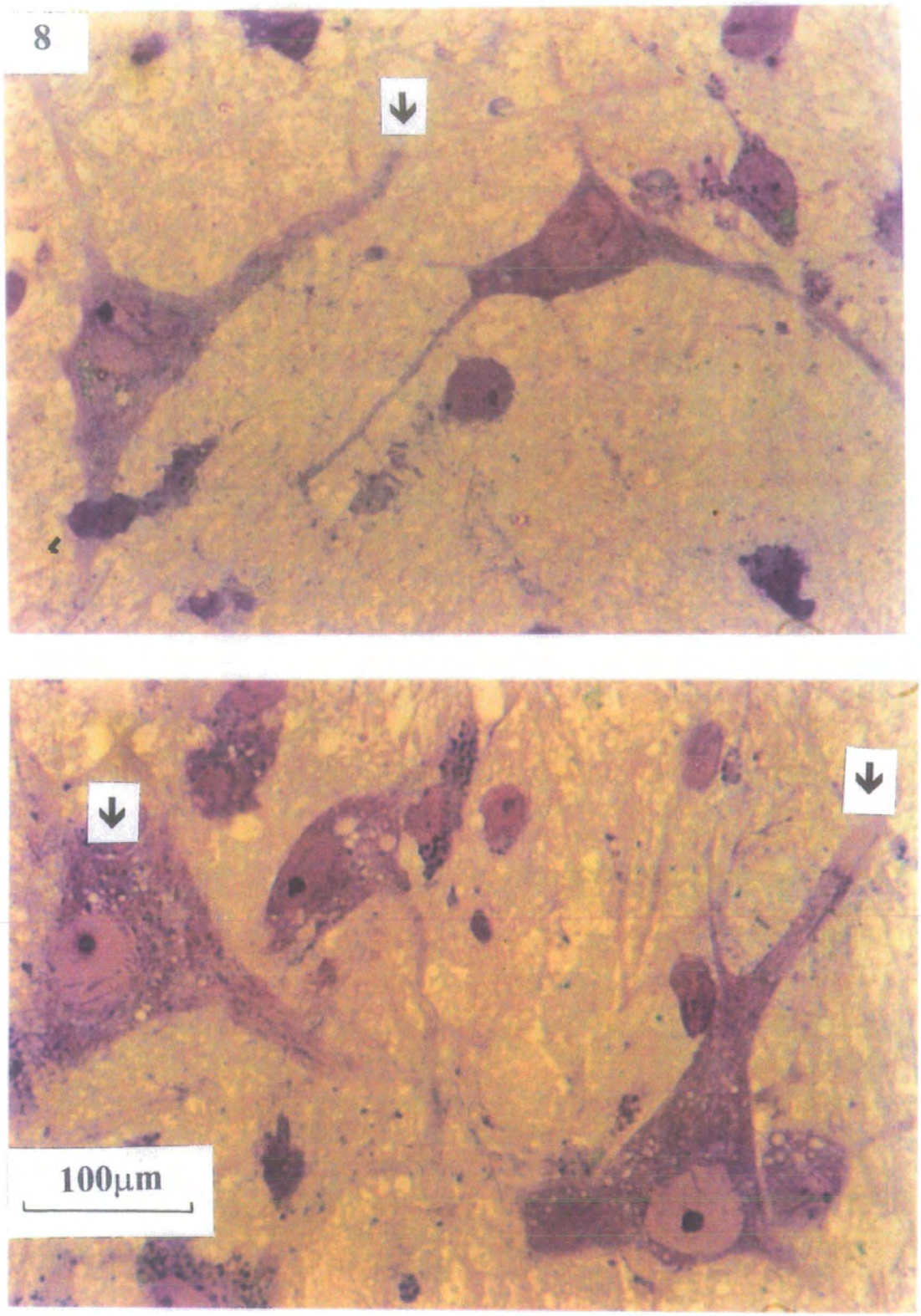


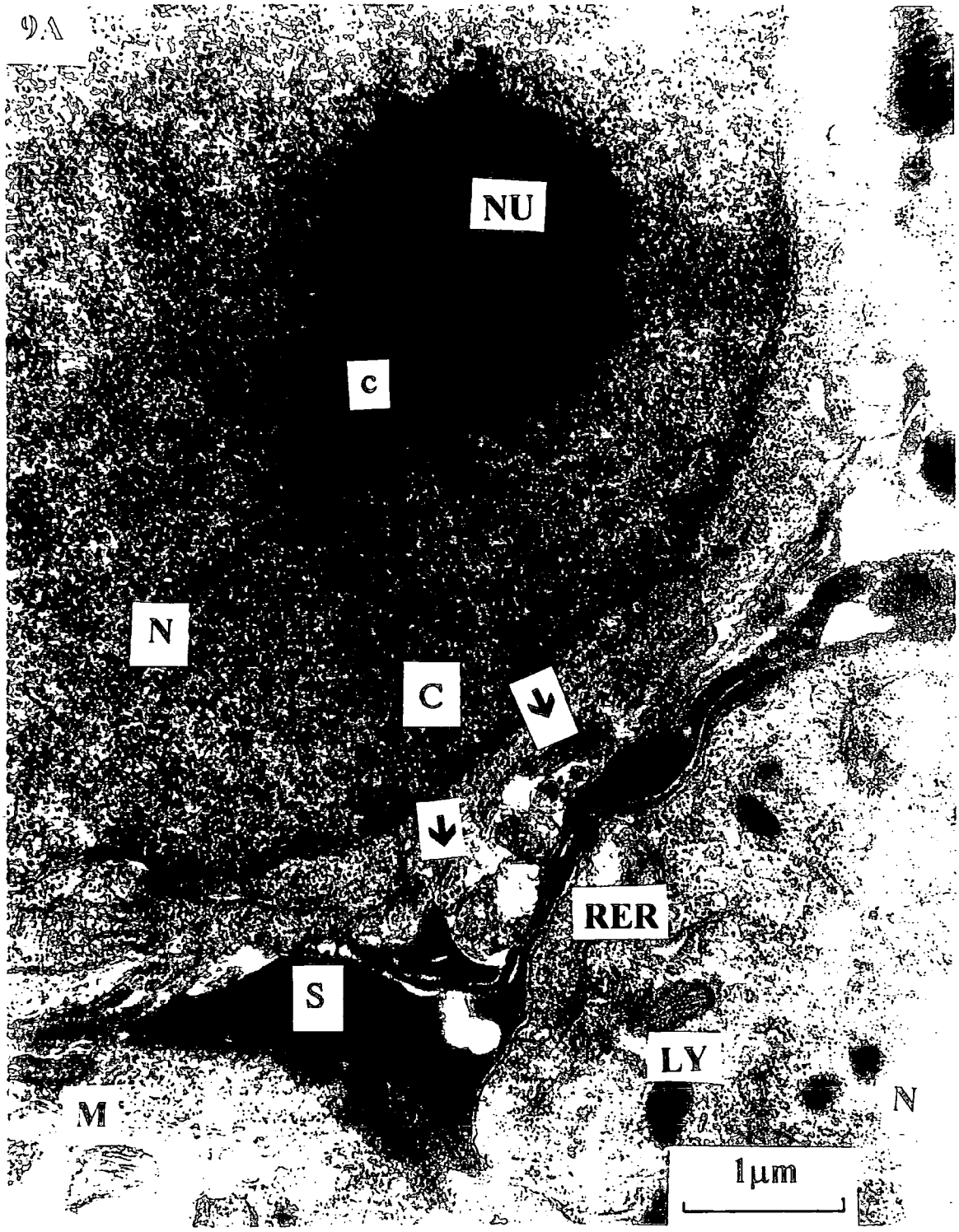
6B

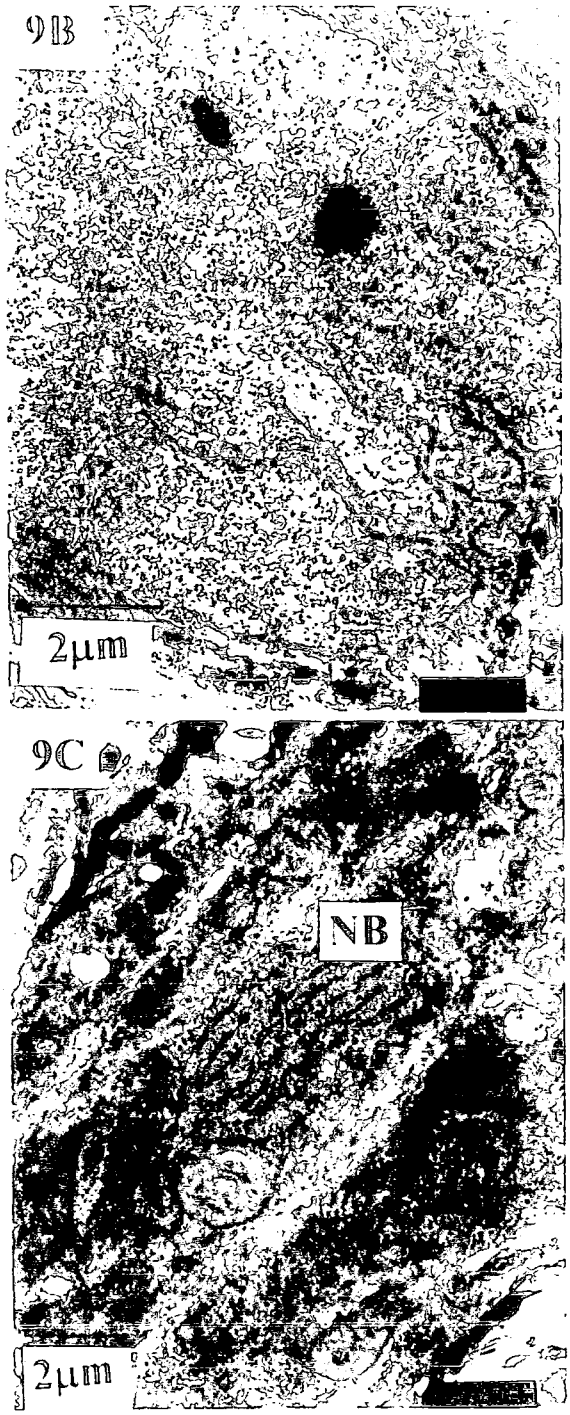


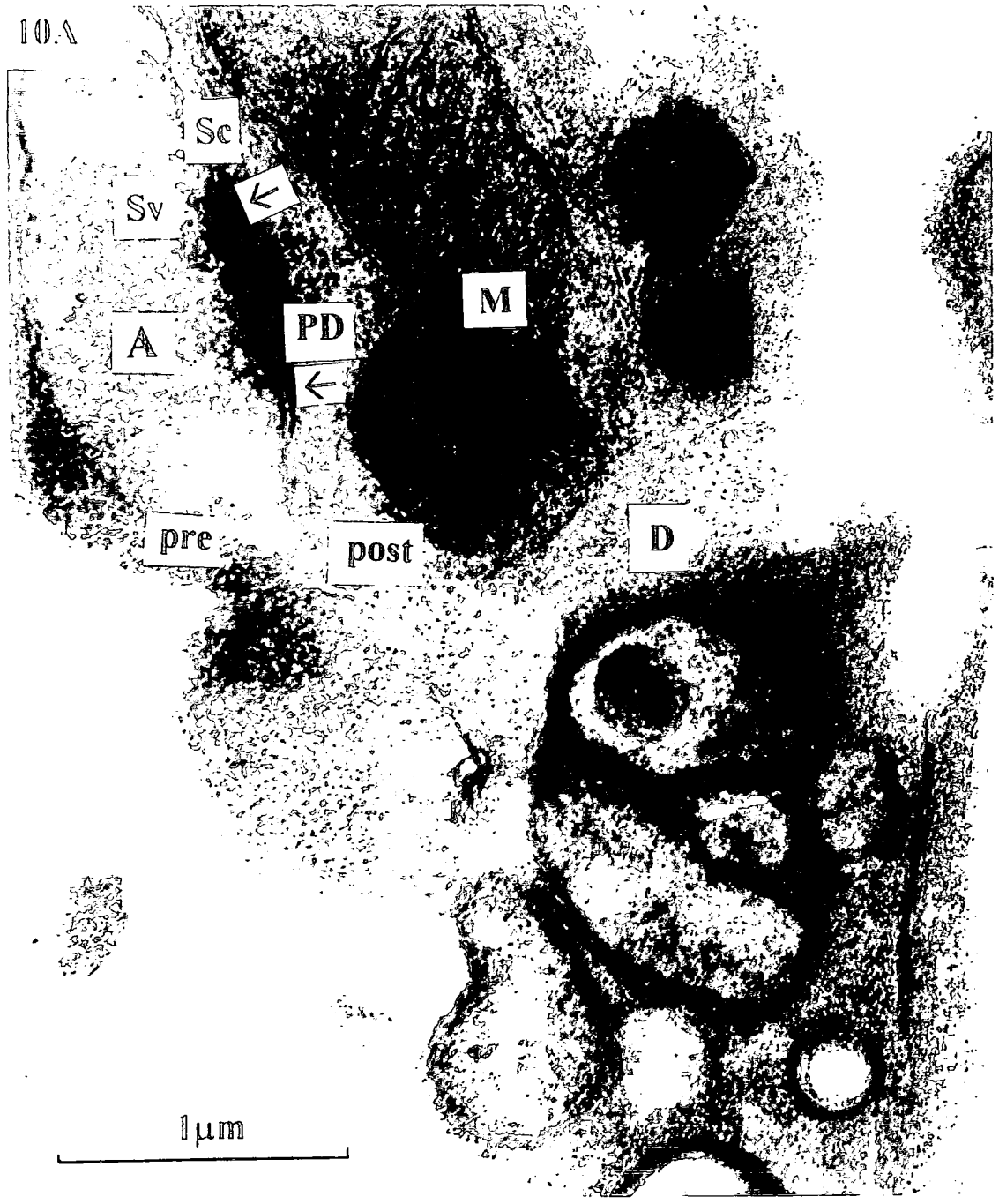

7.1
N2
N1

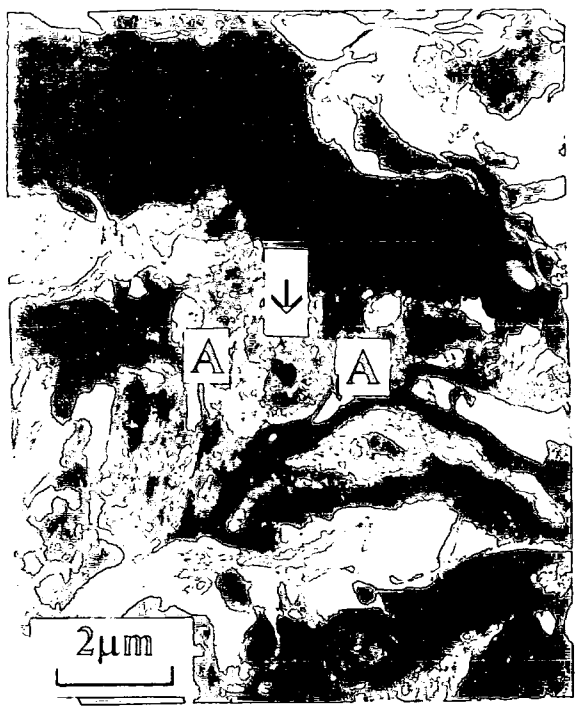

7B
NF
N
ST
100µm
D

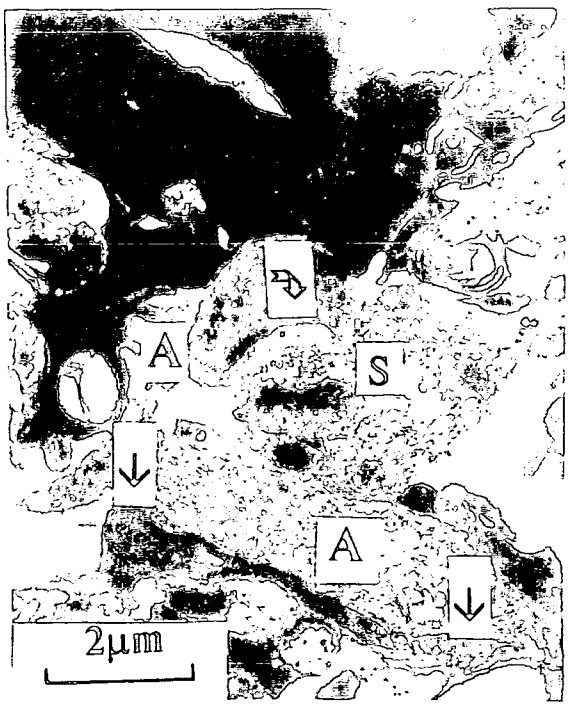

8
100μm


NU
c
N
C
RER
S
LY
M
N
1μm


9B
2μm
9C
NB
2μm


10.1
Sc
Sv
A
PD
M
pre
post
D
1µm

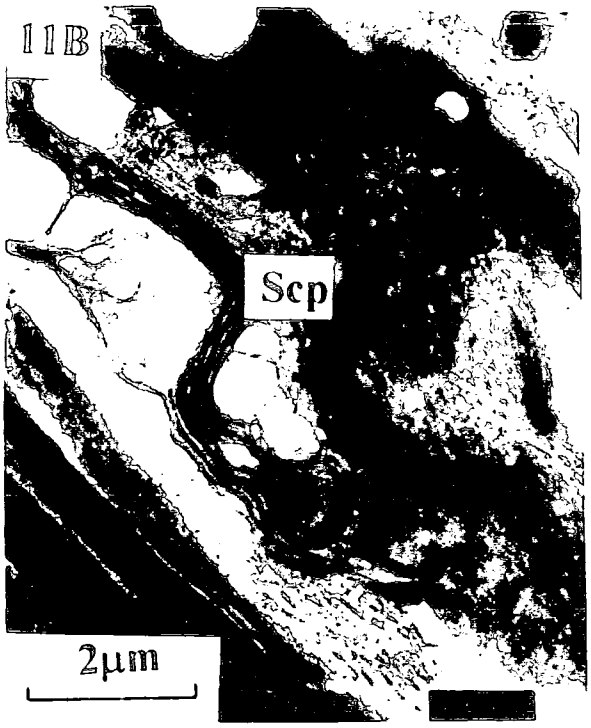

A
A
2μm

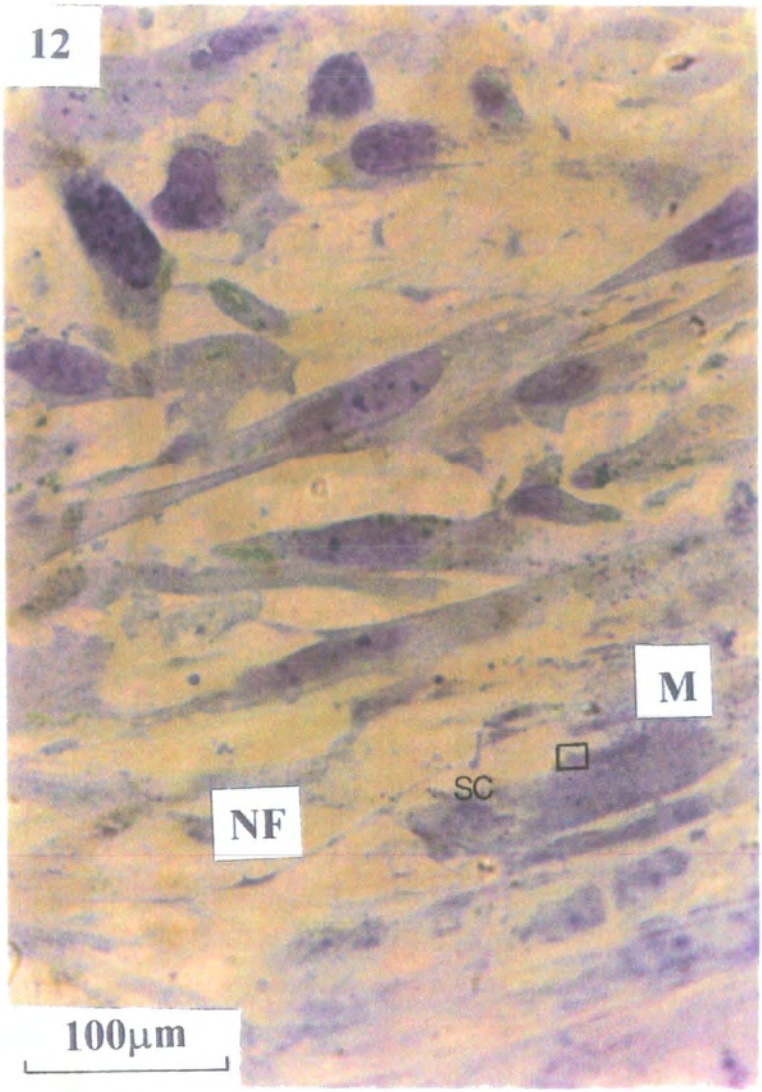

A
S
A
2μm

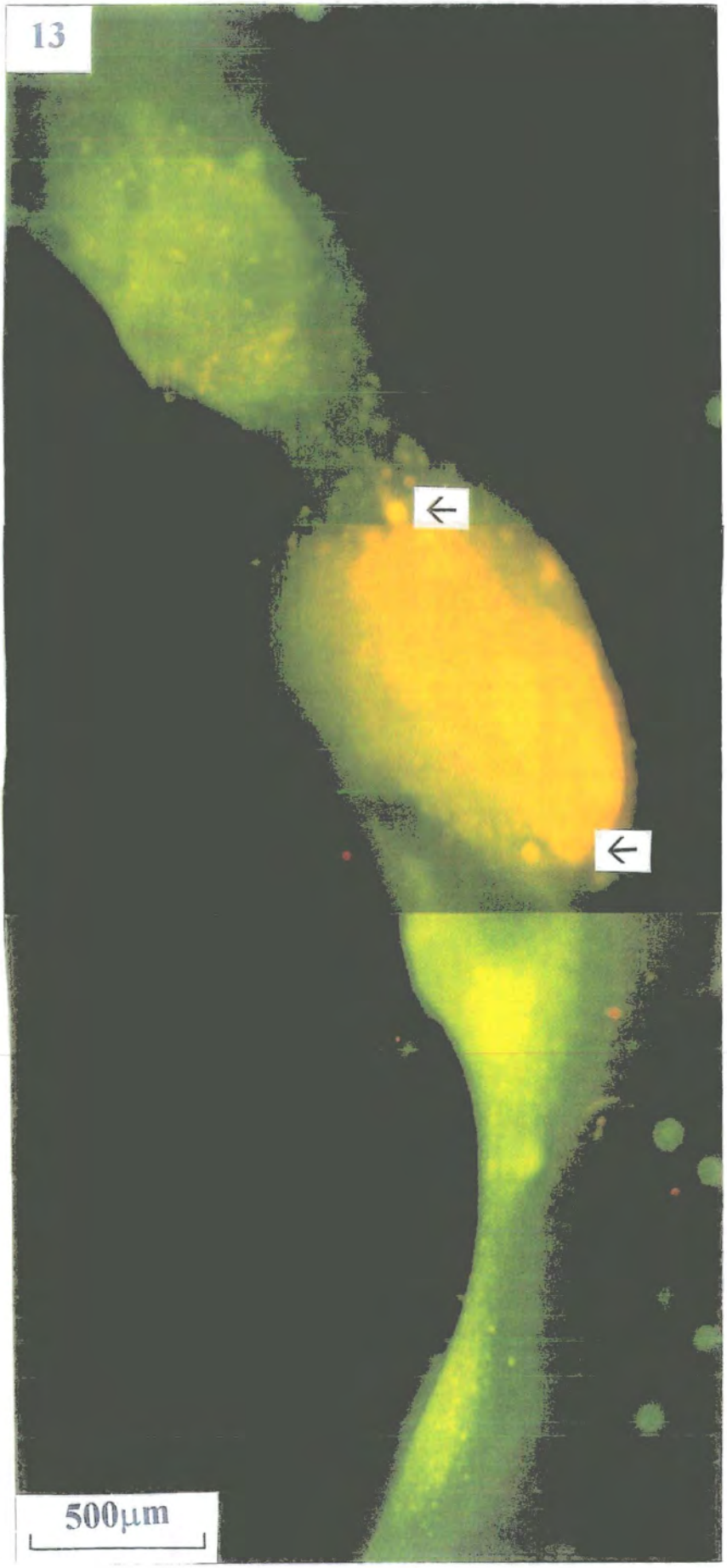

A
D
2μm


Sv
2μm


11A
MY
N
1μm


11B
Scp
2μm


12
M
SC
NF
100μm


13
500μm

2.4. Discussion

The culturing nervous tissue is widely used as a model system for studying a variety of neurobiological problems. In organotypic cultures, neurons mature and differentiate morphologically and intricate functional synaptic networks are formed in vitro.

The roller-tube technique described has been used to culture tissue derived from different parts of the nervous tissue such as cerebellum, hippocampus, hypothalamus, spinal cord, cerebral cortex, brain stem and midbrain as well as co-culture of spinal cord-dorsal root ganglia and skeletal muscle (Braschler *et al.* 1989 and Spenger *et al.* 1991).

This method yields thin organotypic cultures which have advantages over other techniques. Thus the organotypic organisation of the tissue facilitates cell identification which appears more difficult to achieve in cultures of dissociated cells. The roller-tube cultures can be viewed with phase-contrast or fluorescence microscopy.

The roller-tube technique (Gähwiler, 1981) described in this study provides an excellent environment for spinal cord-dorsal root ganglia slices. The coverslips provided good substrate for neurites, as they enabled long survival of neurons and their neurites without the use of any the growth promoting substances or growth factors commonly used for the improvement of neuronal cell growth or survival *in vitro*, (Tanaka *et al.* 1982 ; Laerum *et al.* 1985 ; Martinou *et al.* 1989 and Ebendal, 1989).

Axonal outgrowth was observed even after two weeks of cultivation in some of the cultures. The outgrowth of neurites from the cultured tissue into co-cultured tissues had been described for a variety of co-cultured systems, (Braschler *et al.* 1989 and

Spenger *et al.* 1991). Based on light and electron microscopy results, most of these processes originated from the dendrites of neurons.

The best results were obtained when the spinal cord slices together with the skeletal muscles were embedded in plasma and the muscle was positioned on the ventral surface of the spinal cord slices. There was no axonal outgrowth, nor any processes growing out from slices when the muscles were placed near the dorsal side.

The flattening of the spinal cord never reached the condition of a monolayer which is achieved with most other brain explants (Gähwiler, 1981), while the dorsal root ganglia always flattened into a true monolayer leading to an epithelial-like structure (Spenger *et al.* 1991).

In the spinal cord-dorsal root ganglia and muscle co-culture there was a large variety of different cell types which could be distinguished by morphological criteria. The morphologic differentiation of the nerve cell could be demonstrated up to 4 weeks in cultures, both the spinal cord and dorsal root ganglia cells were different from the underlying non-neuronal layer of cells which formed the dominant component of the cultures.

These background cells were fibroblast and glia cells, flat grey cells whose nuclei were not prominent, and with few or no processes. The neurons by contrast were thick cells with prominent nuclei and frequently prominent nucleoli. According to morphological characters, the neuronal population was divided into two subgroups:

Dorsal root ganglia cells were characterised by a small number of thin processes, a prominent nucleus with a distinct central nucleolus and phase dark cytoplasmic granules. Spinal cord cells on the other hand possessed a much more elaborate set of processes, and a less prominent nucleolus (Peacock *et al.* 1973).

Muscle seemed to keep growing for up to 3-weeks if the culture conditions were favourable. Mature muscle fibres coexisted with dividing myoblasts after the period of incubation. Although these cultures were stopped after 4-weeks, Capers, (1960) maintained chick embryonic muscle fibres for four months.

The neuron at the time of explantation was small with few processes and it seems certain that the many processes and well organised nissl substance of the cultured neurons indicated differentiation in culture. Myelin sheaths could not be seen by light microscopy at the time of explantation, but it is certain that myelin forms *in vitro*, (Bunge, *et al.* 1965), and because synapses were found in the middle zone it seemed very likely that synapses formed in vitro.

The synapses found in the explant resembled those present in central nervous tissue *in vivo*. They displayed the characteristic accumulation of synaptic vesicles, increased density and thickening of apposing membranes, and in the large terminals, mitochondria, (Palay, 1956 & 1958). In axodendritic synapses the clefts were widened and contained dense material, and in the postsynaptic process there was an additional substance associated with the membrane type 1 synapse, (Gray, 1962).

The electron microscopy study revealed a variety of synaptic types occurring among nerve processes in deeper regions of the middle zone. Evidence for formation of synaptic connection *in vitro* was also reported in cultures of thalamo-cortical system, (Yamamoto *et al.* 1992) and between basal forebrain afferents fibres and cerebral cortex neurons (Distler & Robertson, 1993).

It is difficult in work of this kind to distinguish between casual association and significant morphological union between neural and muscular elements. A further difficulty lies in the fact that Schwann cells have not been seen at neural terminals.

Furthermore it has been impossible to follow a particular neurite from its origin to the terminal, after it has joined in a fasciculation.

Similar difficulties are reflected in the work of Peterson & Murray, (1965), Peterson & Crain, (1968), and also Bornstein, (1966) who presented a beautiful picture of well differentiated muscle fibres coexisting with myelinated nerve fibres found in cultures, although he did not show terminal structures nor did he identify the nerve fibres as motor or sensory. Recent *in vitro* studies suggest that group Ia afferents do not survive well in culture, (Spenger *et al.* 1991).

Using electron microscopy to study an 8-day old culture, neuritic contacts to muscle were seen in cultures of rat newborn embryo skeletal muscle coupled with spinal cord dorsal root ganglia. The electron micrographs of motor nerve endings on regenerated muscle fibre showed structures characteristic of simple synapses, including vesicles, an appropriate gap separating adjoining membranes containing electron-dense material, and thickening of the postsynaptic membrane. The ultrastructure of muscle fibres revealed a membrane specialisation resembling the gap junction, which may mediate the electrical coupling between muscle fibres.

From another point of view, the possibility had to be considered that the dorsal root ganglia cells send dendrite to the muscle to form a receptor of a muscle spindle. At present, however, this study has provided no information about the mechanism of muscle spindle formation and the possibility that muscle spindles may develop *in vitro*. Nor do I know whether processes from motor or sensory neurons can associate equally well with muscle cell.

The cholinesterase positive loci have not been detected in the late stage in these cultures in contrast to the intense staining produced by Karnovsky & Roots, (1964) method. This could be due to lack of innervation, resulting in the late degeneration of

the fibre. The DiI and AChE techiques yielded remarkably similar data in the late stage, however I saw no evidence of a population of outgrowth fibres labelled by DiI that was not labelled by AChE.

Chapter 3

Structural and Functional Relationships in the Sensory Innervation of the Muscle Spindles Revealed by Abnormalities Following Reinnervation

3.1. Introduction

The intrafusal fibre bundle is an intrinsic part of each muscle spindle. The bundle is composed of nuclear-bag fibres and nuclear-chain fibres. The nuclear-bag fibres are further subdivided into bag_1 and bag_2 fibres (Banks *et al.* 1975 & 1977). Each fibre is subdivided into an equatorial, two juxtaequatorial and two polar regions which are distinct in their structure. According to a widely accepted definition, zone A is the sensory region which includes the equatorial and both juxtaequatorial zones, whereas zone B is the intracapsular and zone C the extracapsular part of the polar region (Banks *et al.* 1977).

The intrafusal-fibre types are identified or distinguished from one another by their diameters, lengths and equatorial nucleation. The intrafusal fibres receive their sensory innervation from two groups of neurons. In each spindle this is usually provided by one Ia afferent, which at the entry into the spindle divides into myelinated branches that separately supply the bag_1, bag_2 and chain fibres, and either none or up to several spindle II axons (Barker. 1974 ; Barker & Banks. 1994). Both Ia and II groups are myelinated axons whose size and conduction velocities place them in the two largest and fastest groups of peripheral nerve fibres.

The two types of sensory ending exhibit characteristic differences in their responses to muscle stretch. It is unclear whether these differences are due to intrinsic properties of the endings or to the mechanical properties of the three types of intrafusal fibres.

Ia myelinated fibres in the cat have diameters ranging from 12-20 μm at the point of entry to muscle spindles and have conduction velocities in the range of 72 to 120 m/sec (Matthews, 1972). Every spindle is innervated by a Ia axon, which forms a primary ending and is thought to initiate and maintain the differentiation of the intrafusal muscle fibres. Group II axons terminate as secondary endings, which lie adjacent to the primaries. The secondary afferent fibres have smaller diameters than the primaries, they also have lower conduction velocities, ranging from 24 to 72 m/sec (Matthews, 1972) and also less extensive terminals. They appear to terminate in irregular spirals or rings. They are not invariably present in every spindle, but when present they seem to have rather little effect on the differentiation of the intrafusal fibres.

Ia axons lose their myelin sheaths close to the intrafusal fibres and terminate in annulospiral endings which encircle each fibre at the equator with terminals having the appearance of a spiral. The distribution of primary endings to intrafusal fibres in cat muscle is not uniform, more than one-third of their total contact area in a spindle lying on the dynamic bag_1 fibre (Barker & Banks, 1994): the same fibre receives, however only 8% of the total of the secondary endings.

Most secondary endings in cat spindles lie on the static intrafusal fibres: 75% on chain fibres, 17% on the bag_2 fibre and only the remaining 8% on the dynamic bag_1 fibre (Barker & Banks, 1994).

Both bag fibres are surrounded by numerous elastic fibrils which are absent from chain fibres, in cat spindles this elastic layer is more prominent around the bag_2 than bag_1 fibres (Gladden, 1976). The sarcomeres of bag_1 fibres are longer than those of bag_2 fibres, chain fibres and extrafusal fibres (Banks *et al.* 1977). The M lines are either absent or appear as two faint lines. Bag_1 fibres have thicker Z lines than bag_2 fibres. In the bag_1 fibres, M lines reappear in the extreme polar and extracapsular zones, while in

the bag_2 fibres they are already apparent in the B zone. In chain fibres myofibrils are well defined and they have thin Z lines and prominent M lines.

Banks *et al.* (1982) and Banks, (1986), described the form and distribution of the primary terminals of cat tenuissimus muscle spindle by using a reconstruction technique. They found that all the ring features on the intrafusal muscle fibres are open, all the terminals remain separate and do not fuse, most of them are located on the bag_1 and the position of each terminal determines its form. They also note that there is a close association between nucleation and innervation.

Muscle spindles can be successfully reinnervated after nerve injury or crush. Light microscopy observations testify that regenerated axons of efferent or afferent neurons appear to end in the appropriate regions of the spindle (Ip *et al.* 1977, Barker *et al.* 1985 & 1986). Following crushing, the regenerating axons grow through their original endoneurial tubes along the peripheral nerve into the muscles and reinnervate the muscle receptors after a relatively short period of denervation.

After sectioning of the nerve however, reinnervation is slower, more random and incomplete (Brown & Butler, 1976; Banks & Barker, 1989). The axons often grow back to inappropriate sites where they may, however, form functional endings. Successful reinnervation of muscle spindles can be attained by alien mechanosensitive axons (Banks and Barker, 1989). These experiments indicated that cross-connected Ib afferents are able to terminate in sites of Ia or spindle II endings and to respond to stretch like normal Ia or spindle II afferents. Conversely, spindle II afferents also appeared to be able to terminate in tendon organs after nerve section and self-reinnervation. Banks & Barker, (1989) conclude that Ia, Ib and spindle II afferents retain dual specificity and can thus make functional reconnections with either spindle or tendon organs.

Banks *et al.* (1985) and Banks & Barker, (1989) studied the morphological complexity of innervation patterns that can occur in reinnervated spindles after sectioning and re-uniting or cross-uniting nerves. Their results were based on light microscopy and defined the limit of what is likely to result under optimal conditions for reinnervation. They pointed out the need to identify nerve terminals ultrastructurally.

Recent work in this laboratory has included the afferent reinnervation of muscle proprioceptors following nerve sectioning, and the comparative abundance of muscle spindles and their sensory innervation. The results have highlighted the need for a better understanding of the normal pattern of muscle afferents. This work forms part of a large study in muscle receptor reinnervation following nerve section (Banks & Barker,1989).

Here I will examine the distribution of the sensory terminals, and the structural properties of the associated intrafusal fibres, of two abnormal endings present in spindles of the tenuissimus muscle of the cat that had been reinnervated following sectioning of its nerve more than one year earlier.

In the first case a group Ia axon identified by its conduction velocity (61 m/s) had reinnervated an extensive site probably occupied originally by a primary and a secondary ending. Normally such an axon would supply a primary ending alone, which might be the only ending present. Some features of the response were similar to those of a normal secondary ending.

In the second case a group II afferent identified by its coduction velocity (26 m/s) has reinnervated a site previously occupied by a muscle spindle primary ending. Its response to muscle stretch, particularly to the phase when the muscle is changing length, is more primary than secondary-like.

3.2. Materials and Methods

3.2.1. Initial preparation and physiology

The surgery and physiological recording had been done by Dr Banks. The experiment was carried out on two adult cats, the left tenuissimus muscles and their nerves were exposed and nerves sectioned. After recovery and one year later the responses of reinnervated spindles to ramp and hold stretches were recorded, their locations were marked by epimysial stitches, then the muscles were dissected from the cats which had been killed by sodium pentobarbitone overdose.

3.2.2. Histological preparation

The histological preparation was carried out by Dr Adal. The muscles were immersed in a 1% veronal acetate-buffered osmium tetroxide solution for about 30 minutes at 4ºC, and were then teased in the Karnovsky, (1965) fixative at room temperature under a binocular microscope. After partial separation of the extrafusal muscle fibres, the muscle spindles were recognised easily by their dark stained myelinated nerve supply and also by their capsule. The spindles were teased away from the rest of the muscle, and photographed before embedding. The spindles were then dehydrated through a series of alcohols and embedded in epoxy resin.

3.2.3. Sectioning

The light microscopy work was carried out by myself. Serial 1μm thick transverse sections of the spindles were cut on a Reichert OMU3 ultramicrotome and collected on strips of coverslip in ribbons of tens, maintained in sequential order and stained for light microscopy with 1% toluidine blue.

i. Photography

Every section containing part of the nerve terminal or sensory ending was photographed on 35mm film with a Microflex UFX camera on a Nikon Optiphot microscope using a 100X oil immersion objective.

ii. Reconstructions

The muscle fibre types were identified on the basis of diameter and position (Barker *et al.* 1976). Isometric reconstructions were made by tracing the relevant features of each photograph on to A4 paper, then every ten sections were traced on to graph paper with the use of coloured pens to distinguish between the various components.

Adjacent tracings were placed along an oblique line to yield the needed amount of spatial analysis. The centre of the bag_1 fibre was always fixed on this line, and the bag_2 fibre was oriented so that its centre was horizontally to the right. The bag_1 fibre was thus artificially straightened, while the position of the bag_2 fibre was confined to a plane that passed through the bag_1 fibre's centre, and the chain fibres were positioned relative to that plane.

The reconstructions were completed using the same colours to draw the envelopes of every group of ten tracings, and then by making tracings of the envelopes in Indian ink. A number of line thickenings and shadings were used in the final drawing. The standard line was used to indicate the sarcolemma of intrafusal muscle fibres, nerve terminals, nodes of ranvier and unmyelinated axons. The myonuclei were drawn with fine outlines and nucleoli were shown as dots.

iii. Ultrastructural Characteristics

In order to know more details about the structure of the reinnervated spindles, ultrathin sections were cut transversely and longitudinally on a Reichert OMU3 ultramicrotome. The block was trimmed and sections giving a silver to light gold interference pattern were cut. They were picked up on 150 mesh, formvar-coated copper grids and were double-stained with uranyl acetate and lead citrate for 10 minutes. Each section was examined with a Philips 400T electron microscope, the range of magnification being between 6,000X and 13,000X.

A normal spindle from the tenuissimus muscle of an unoperated animal was used as a control to study the normal innervation pattern in tenuissmus muscle spindle. This spindle was derived from the series of histophysiological experiments of Banks *et al.* (1995b). It was serially sectioned throughout the sensory region in 1μm thick longitudinal sections whose outlines were traced directly using a drawing tube attachment on a Nikon Optiphot using a 100X oil immersion objective.

3.3. Results

The distribution of the sensory axons to the three types of intrafusal muscle fibre of reinnervated tenuissimus spindles and the control spindle has been determined using reconstuctions from serial sections which were cut transversely or longitudinally (section thickness 1μm), and stained with toluidine blue. The morphological features of the intrafusal muscle fibres of the muscle spindles removed from reinnervated tenuissimus muscle of the cat that had been reinnervated following section of its nerve more than one year earlier allowed them to be recognised as distinct types: of bag_1, bag_2 and chain fibres.

3.3.1. Histological analysis of the normal muscle spindle

The spindle in the tenuissimus muscles from unoperated cat was studied as a control to study the normal innervation pattern of muscle spindle. The spindle contained one bag_1 fibre, one bag_2 fibre and four chain fibres. In this spindle one chain fibre, referred to as chain fibre 1, occupied an unusual position to one side of the bag_1 fibre, while the other three chain fibres, lay on the other side of the bag_1, between it and the bag_2 fibre. The Ia afferent first divided dichotomously, with one branch supplying the bag_2 and the neighbouring 3 chain fibres as is quite normal. The distribution of the second branch was, however, highly unusual as it supplied not only the bag_1 fibre but also produced extensive terminals on chain 1 fibre.

The terminals consisted of spirals and many half rings. Those on the bag fibres covered a large portion of the fibres and were arranged as regular transverse bands around the middle of each nuclear-bag and more loosely in an irregular form to either side. A few terminals on bag_1 fibre were distributed to the chain 1 fibre by cross-terminals, also cross-terminals occurred between chain fibres and between chain fibres and the bag_2 fibre.

3.3.2. Histological analysis of the abnormal muscle spindles

3.3.2a. Ia afferent with secondary-like response

In this case an abnormal ending was produced by a group Ia axon conduction velocity, (61 m/s) that had reinnervated an extensive site probably occupied originally by a primary and a secondary ending (Fig 2A). Some features of the response were similar to those of a normal secondary ending (Fig 2B); correlated with these was the absence of sensory terminals on a particular intrafusal fibre, the bag_1 fibre (see discussion), which was modified in ways indicating dedifferentiation due to the absence of a connection with the sensory ending.

The reconstruction of the ending of this afferent, which was located predominantly in the old primary and secondary site, revealed the presence of sensory terminals on the bag_2 fibre and the chain fibres, and confirmed the absence of sensory terminals from the bag_1 fibres (Fig 2C). All the intrafusal fibres contained central nuclei (Fig 2D). The bag_1 fibre was clearly visible when the spindle was embedded in epoxy resin, due to its unusually large size and segregation from other intrafusal fibres. The bag_1 fibre abnormally was striated throughout the primary ending region, and this region contained a single row of nuclei in place of the normal nuclear bag.

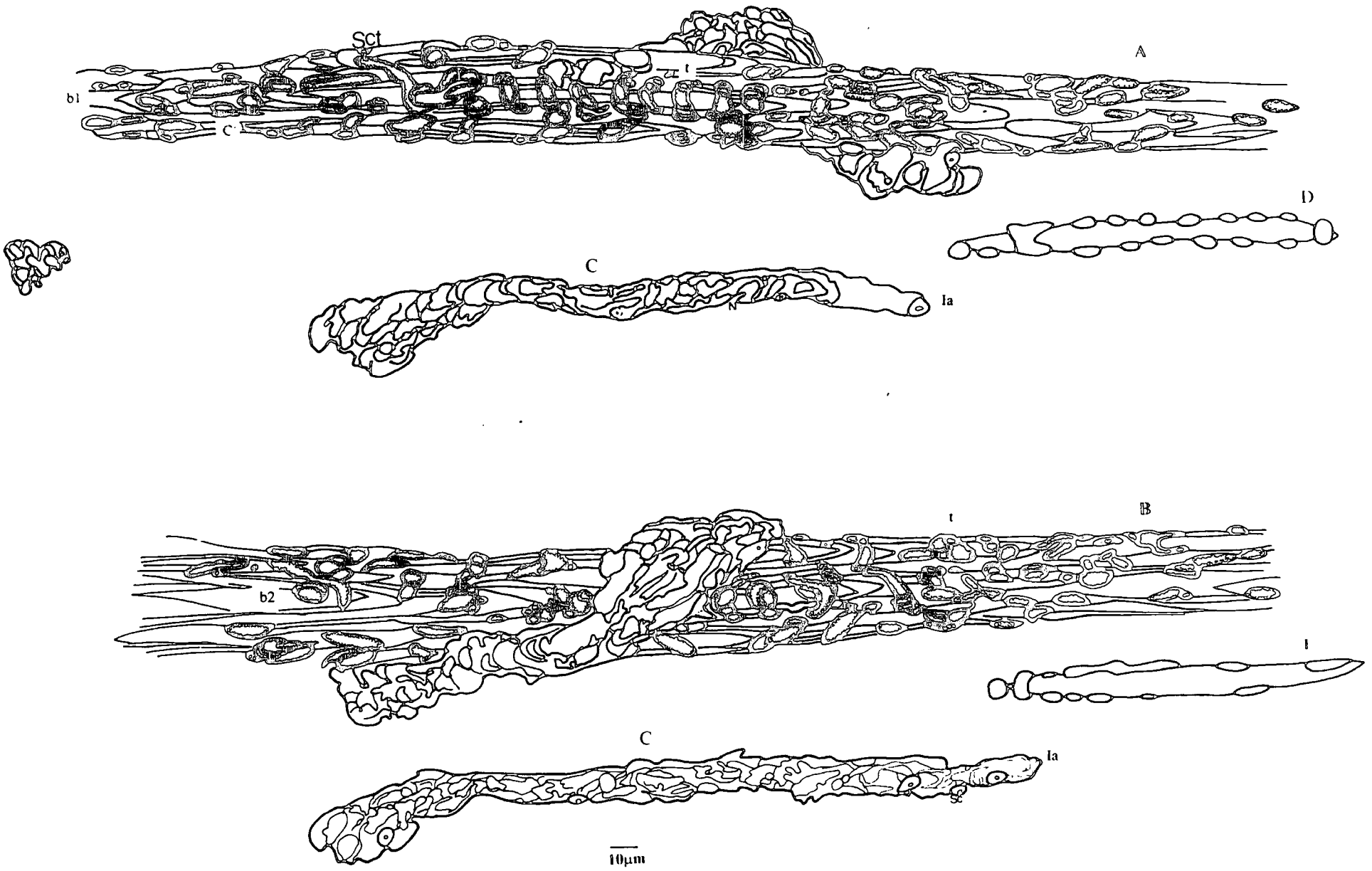
The ultrastructural characteristics of the bag_1 fibre from the sensory region of the spindle were studied, in both transverse and longitudinal sections. Examination of thin transverse sections from the sensory region of the spindle revealed the presence of an M-line in the A-band of the bag_1 fibre which appeared as a band of cross-bridges between the thick filaments. The myofibrils had a constant hexagonal arrangement with one thick filament in the centre.(Fig 2E). In longitudinal sections it was difficult to observe the M-line because the bag_1 fibre was folded beneath the grid.

3.3.2b. II spindle afferent with a primary-like response.

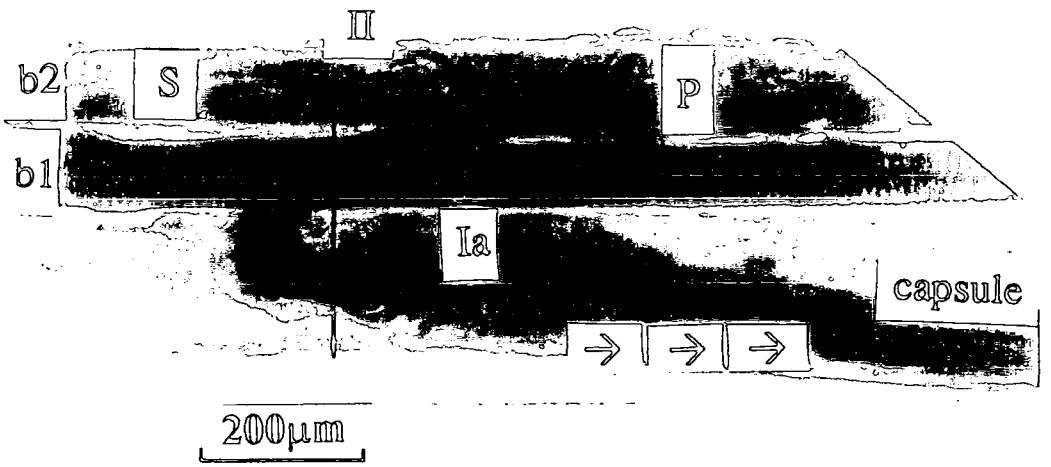
In this case a group II afferent identifiable by its conduction velocity, (26 m/s), reinnervated a site previously occupied by a muscle spindle primary ending (Fig 3A). The afferent appeared with an unusually small ending that was restricted to the bag_1 and bag_2 fibres with two small branches of the II afferent which became unmyelinated and appeared to end on the bag fibres. The larger ending looped around outside the limit of the reconstruction and returned alongside the bag_1. The chain fibres lacked sensory terminals (Fig 3D).

Its response to muscle stretch, particularly to the phase when the muscle was changing length was more primary-than secondary-like (Fig 3B). The intrafusal fibres were structurally differentiated, showing that a group II axon was able to substitute for a group Ia axon in the maintenance of the differentiation.

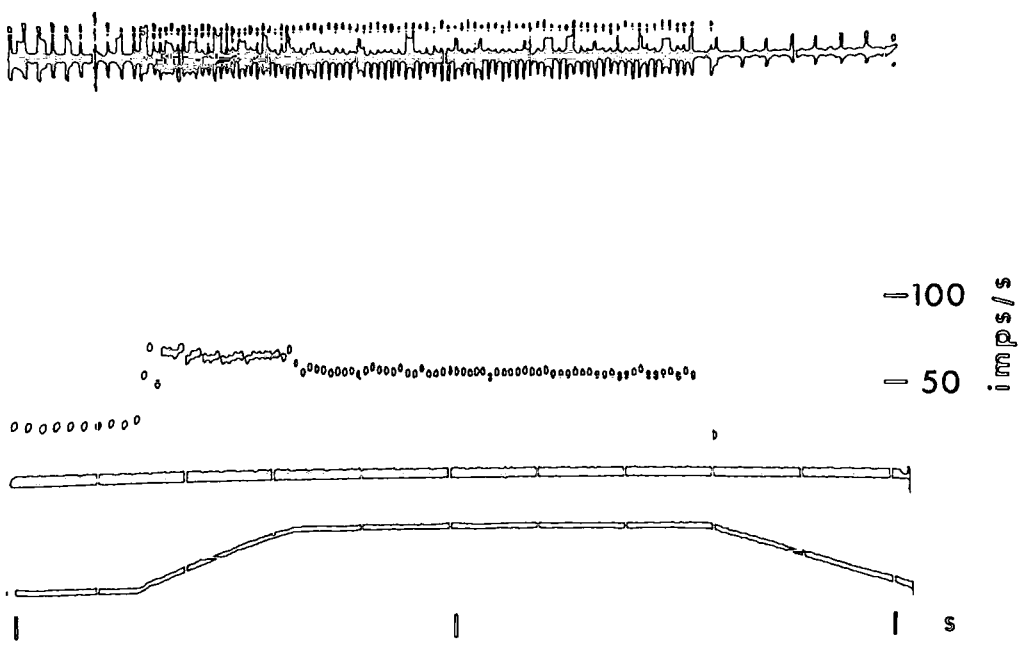
The intrafusal fibres were enclosed in a spindle-shaped capsule, with a distinct periaxial space (Fig 3C). Bag_1 and bag_2 fibres were about the same length but bag_2 fibres were greater in diameter than bag_1 fibres, while the chain fibres were smaller in diameter. Satellite cells were found in close contact with intrafusal muscle fibres in the sensory region. The nuclei of the bag fibres were clustered in the equatorial area beneath the sensory nerve endings. The chain fibres which did not receive sensory terminals were striated throughout and contained a single row of rounded nuclei, which lay centrally along the equatorial region of the muscle fibres.


Sct
A
t
b1
c
D
C
la
B
t
b2
1
C
la
10µm

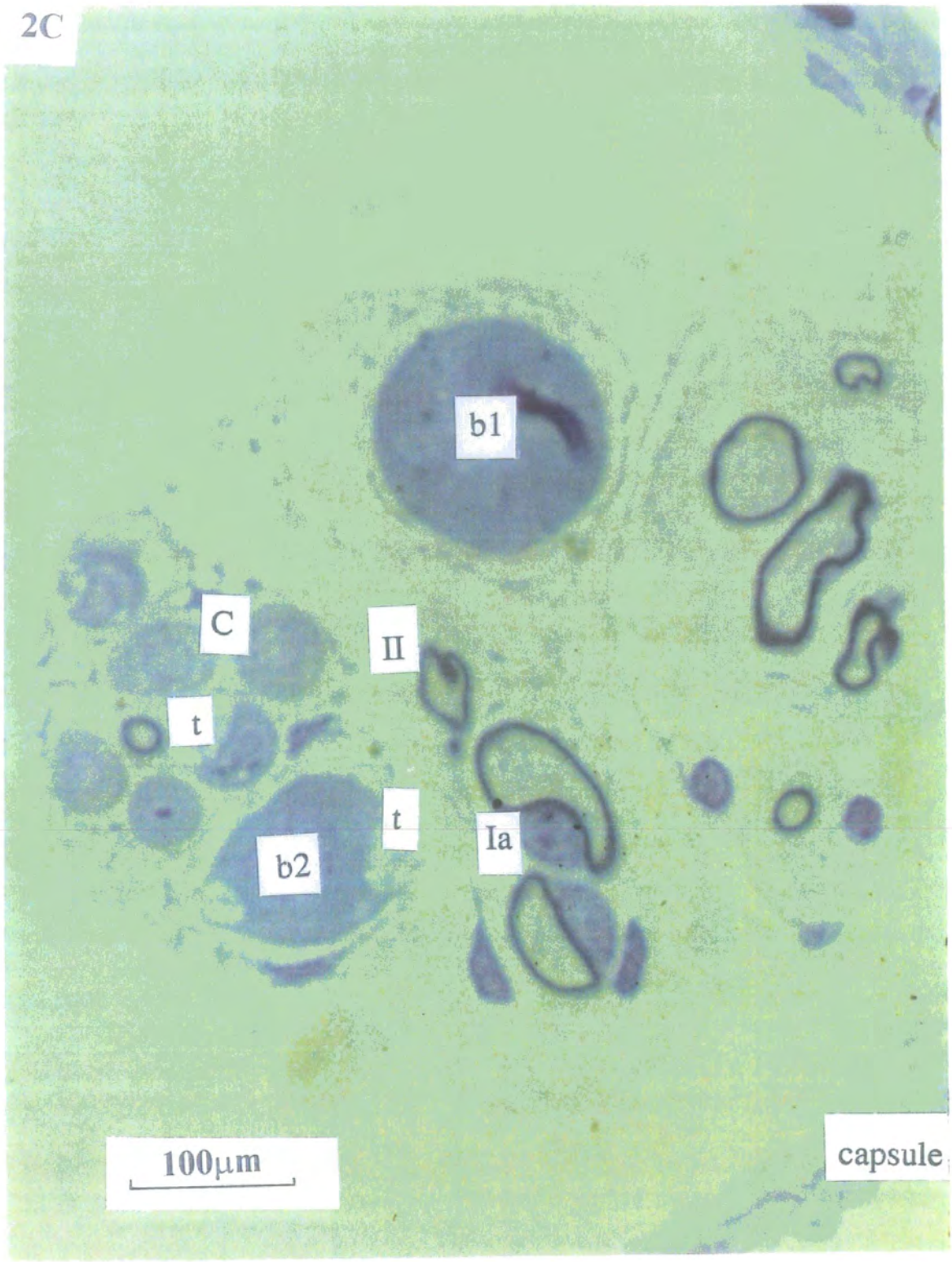
2A

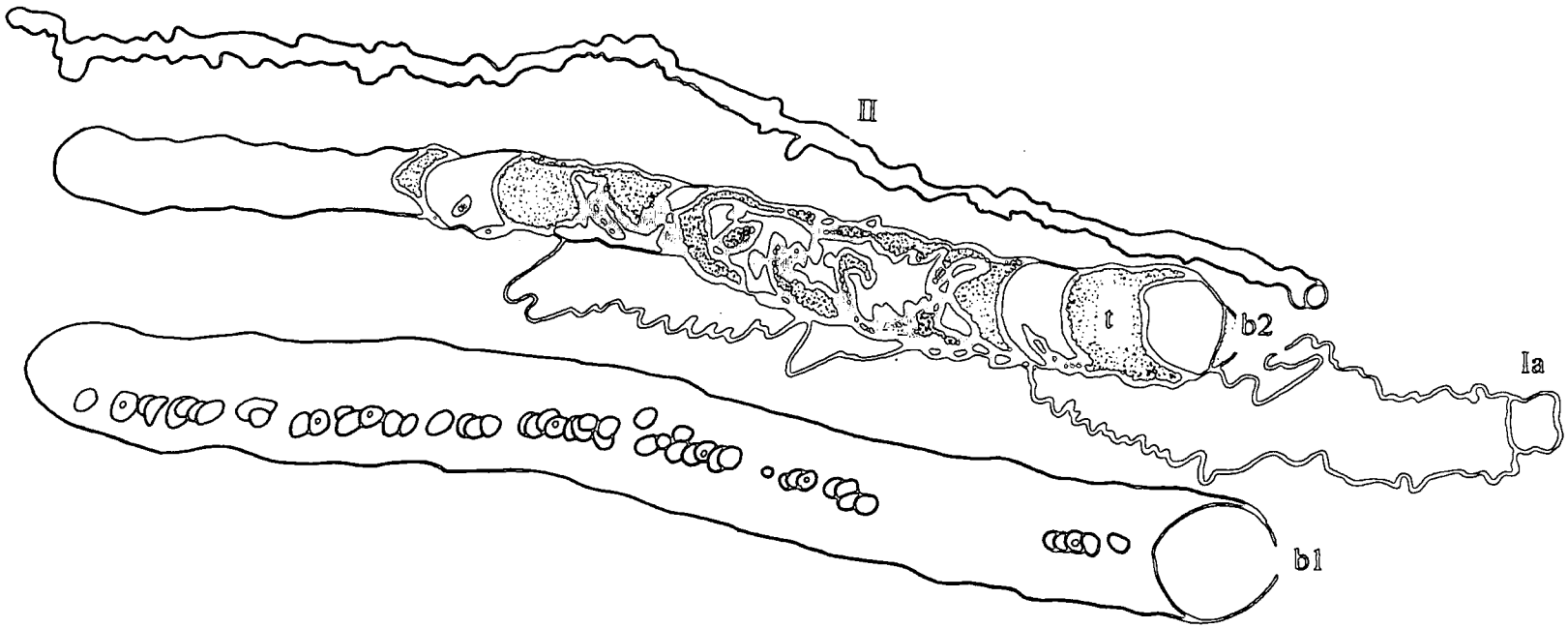


2B



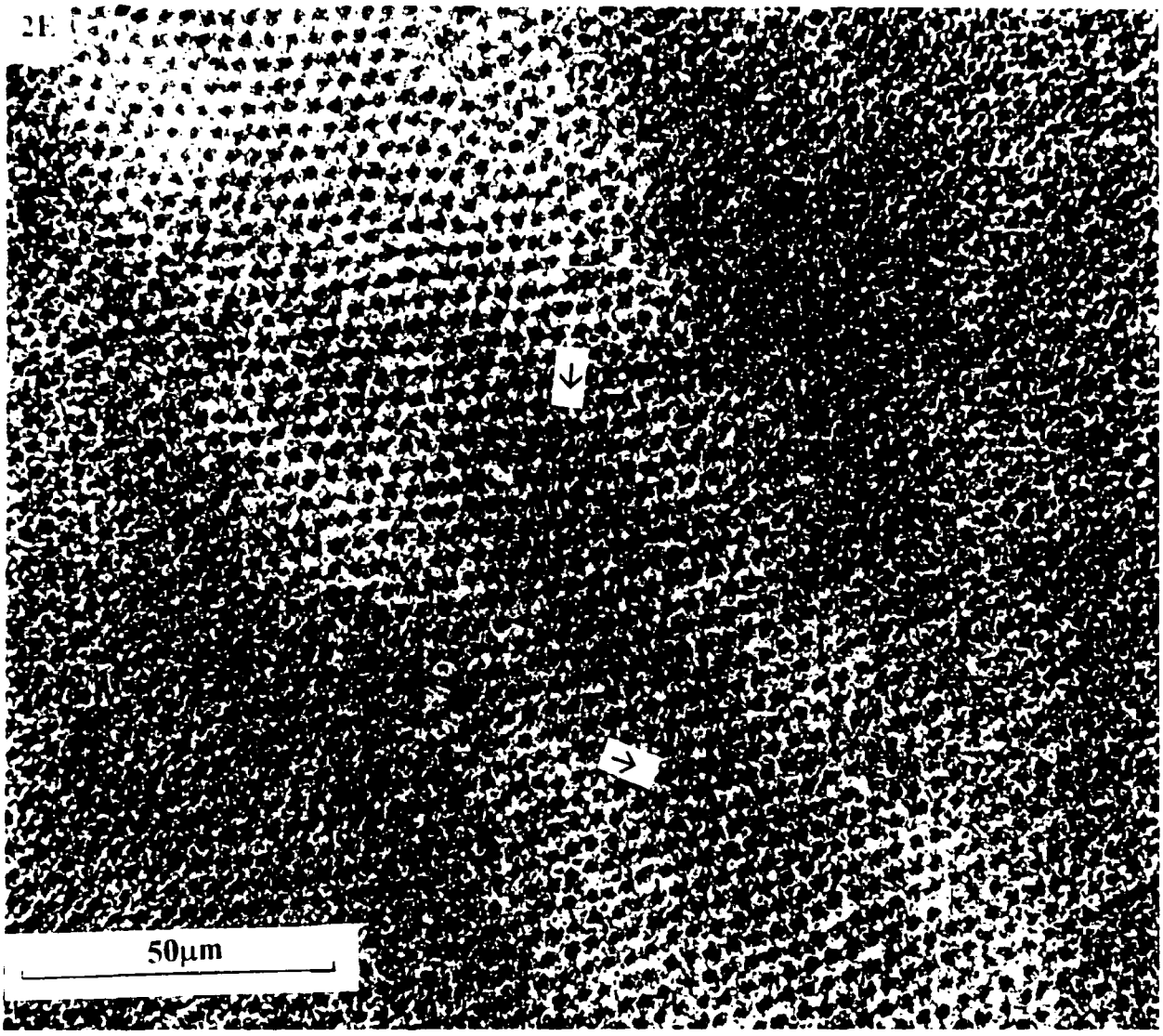
2C



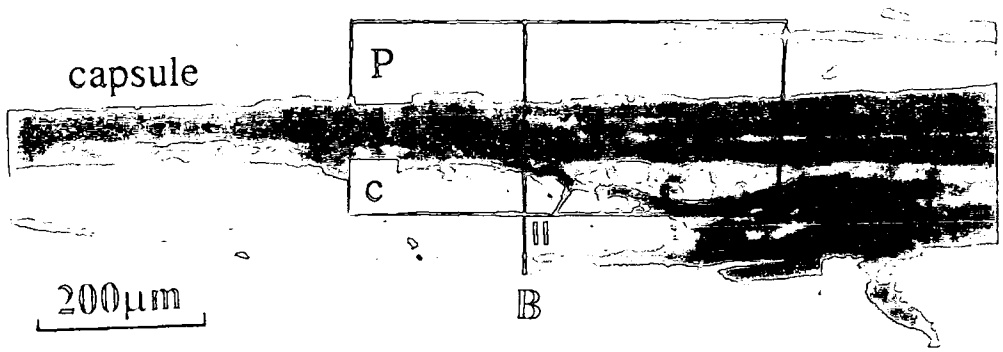

II
t
b2
Ia
b1


2D
C

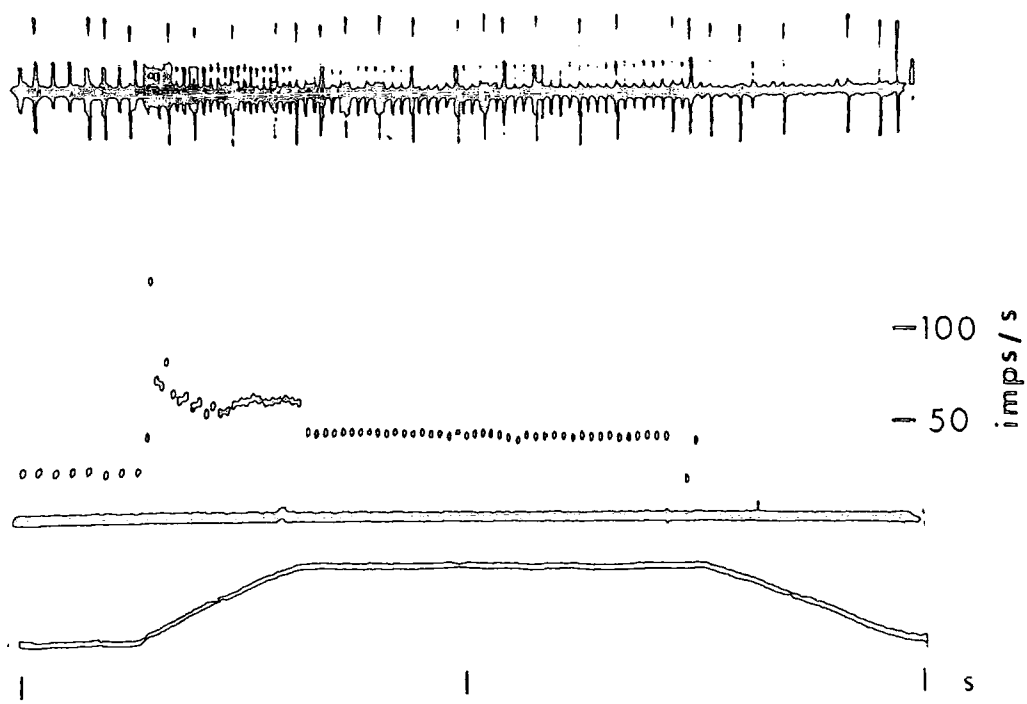
2E

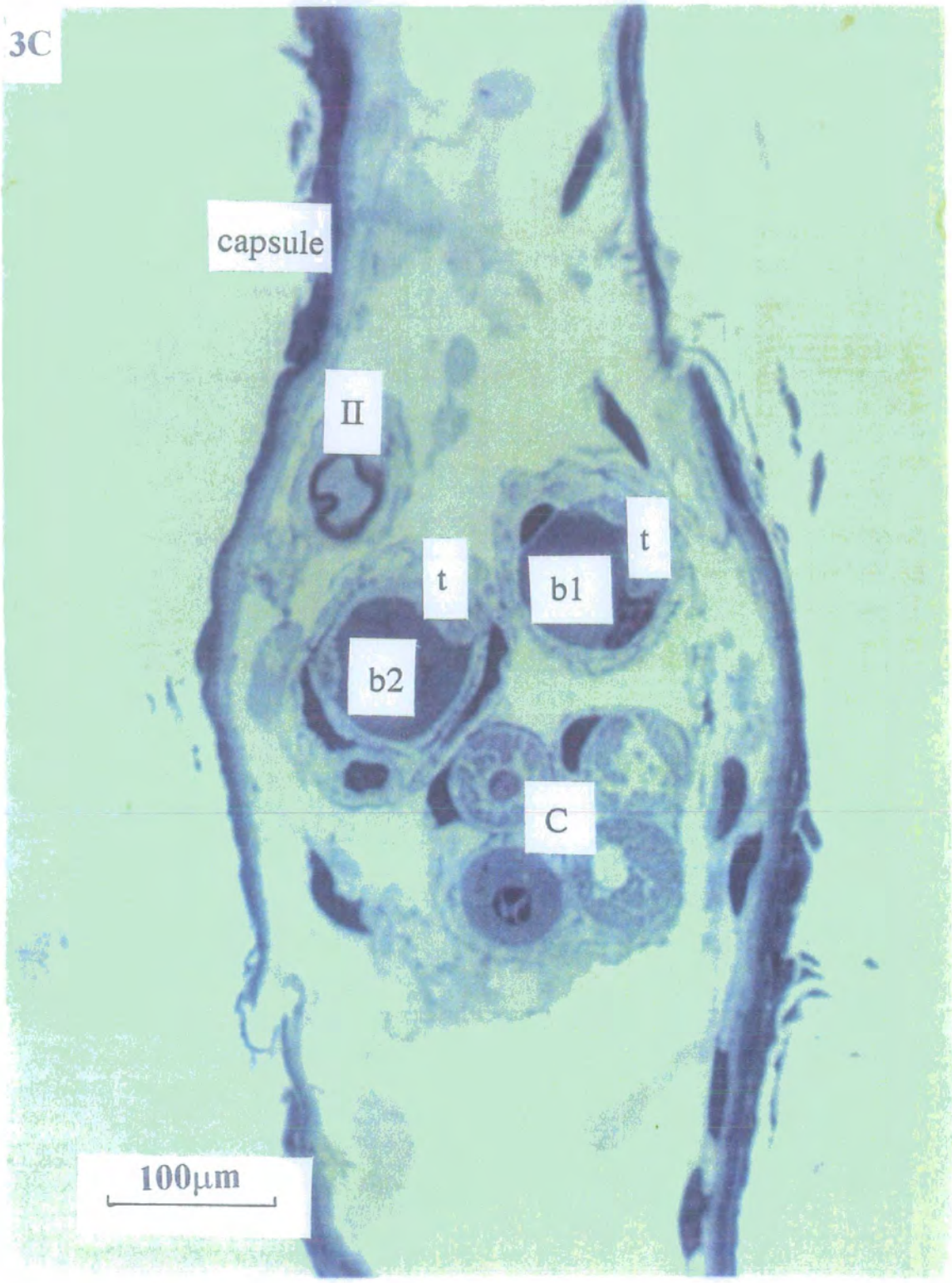


3A



3B




3C
capsule
II
t
t
b1
b2
C
100μm

3D

3.4. Discussion

Reinnervation of muscle receptors after nerve injury is known to occur in the adult, but often results in various abnormalities such as afferents returning to inappropriate sites or intrafusal fibres remaining denervated (Banks & Barker, 1989). These abnormalities are potentially informative about the long-term effects that are thought to be responsible for differentiation and maintenance of the differentiated state, and short-term effects that may be important in the mechanosensory aspect of the interaction between dorsal-root ganglion cells and intrafusal muscle fibres.

The pattern of branching of the sensory afferents of normal and regenerated primary and secondary afferents and the distribution of their terminals to the three types of intrafusal-fibres was determined by reconstruction of the spindles from serial 1μm thick transverse and longitudinal sections of cat tenuissimus muscle spindles.

3.4.1. Innervation pattern in control spindle

The development of intrafusal fibres, involving three generations of myotubes is very similar to the pattern of extrafusal development (Milburn, 1973). The intrafusal fibres develop sequentially in the order bag_2, bag_1 and chain fibres (Milburn, 1973). Bag_2 fibre formation is initiated when the primary sensory axon contacts a first generation myotube; these myotubes seem to be similar to myotubes destined to become extrafusal fibres. Zelená, (1957) confirmed that bag_1 and chain fibres each develop from second and third generation myotubes respectively, in association with the older bag_2 fibres. They separate from the bag_2 fibres during maturation (Barker & Milburn, 1984).

In cat spindles the chain fibres are typically found close to the bag_2 fibres, whereas bag_1 fibres tend to lie somewhat apart from the rest of the intrafusal bundle (Barker *et al.* 1976). In the present study, the reconstruction of the normal spindle,

which was treated as a control, showed one of the chain fibres, referred to as chain one, in an unusual position to one side of bag_1. The other three chain fibres lay on the other side of bag_1, between bag_1 and bag_2 fibres in typically close association with the bag_2. Similar observations have been reported in rat muscle spindle by Kucera, (1982), he suggested that the chain fibre had formed from myotubes positioned alongside the bag_1 rather than the bag_2 fibre.

The consistent feature of the primary afferent of bag_1, bag_2 and chain spindle units in the cat is a first order branch which terminates solely on the bag_1 fibre, and whose terminals are arranged as regular transverse bands in the middle portion of the bag fibre, and disposed irregularly at each end (Banks *et al.* 1982). Moreover, the dynamic bag_1 fibres do not usually share terminals of the primary afferent with static bag_2 or chain fibres in the spindle of the cat (Banks *et al.* 1982). However in the control spindle of the present study a few terminals on the dynamic bag_1 fibre were distributed directly to the chain one fibre by cross-terminals. Moreover, the remaining terminals of the chain one fibre were also derived from the first-order branch of the Ia afferent that otherwise supplied only the bag_1 fibre. While the existence of such a close relationship between bag_1 and chain fibres is very rare in cat spindles, it has been found more commonly in the spindles of rat extensor digitorum longus muscle by Walro & Kucera, (1987), who suggested that the organisation of the dynamic and static systems of muscle spindles differed among different groups of mammals.

3.4.2. The abnormal endings in relation to intrafusal-fibre differentiation

Detailed reconstruction of two abnormal endings, having known response properties, provide evidence consistent with the hypothesis that continued contact of individual intrafusal fibres with a sensory neuron is necessary for the maintenance of their differentiated state, but that the sensory neuron need not be a Ia afferent (Zelená, 1957).

3.4.2a. Ia afferent with secondary-like response

The Ia afferent, the response of which appeared similar to the response of a normal secondary ending, showed signs of growth through the primary region which was probably occupied originally by primary and secondary endings. The Ia afferents are essential for the differentiation of the intrafusal fibres. The nuclei in the mid regions of intrafusal myotubes are induced by the sensory terminals (Zelená, 1957; Milburn, 1973 & 1984; and Kucera & Walro, 1991), and the myonuclei begin to accumulate in the region of contact with the sensory nerve endings. The reconstruction of the reinnervated Ia afferent showed this ending to be incomplete, in that terminals were not supplied to all three types of intrafusal muscle fibres (bag_1, bag_2 and chain fibre). In this case, one of the two types of bag fibre lacked terminals, the bag_1 fibre that did not receive sensory terminals was striated throughout the primary ending site. Banks *et al.* (1982), by using a reconstruction technique of normal tenuissimus spindle, found a close association between nucleation and innervation. This association begins when spindle development is initiated by the contact of a Ia axon with a developing myotube that would (Zelená, 1957; Milburn, 1973 and Barker & Milburn, 1984) otherwise have matured into an extrafusal muscle fibre. Therefore the Ia afferent is the critical factor modifying the pre-existing program of gene expression in the precursor myotubes, and mediates the differentiation of the myotubes into intrafusal rather than extrafusal fibres (Kucera & Walro, 1990). After deafferentation, the equatorial nuclei disappear and are replaced by myofibrils, and the periaxial space is reduced (Tower, 1932; Boyd, 1962 and Kucera, 1980). Reconstruction of this abnormal ending showed that the bag_1 fibre which lacked sensory terminals had a single row of well-separated nuclei, which lie centrally along the equatorial region of the fibre. This modification indicated dedifferentiation due to the lack of a connection with the sensory ending.

i. Identification of the M-line in the bag_1 fibre

The two types of ultrastructure usually present in the intrafusal-fibres have been described by many workers (Barker, 1974). The nuclear-bag fibres have usually been correlated with dM condition, which appear as a double faint parallel lines and nuclear-chain fibres with M condition. Banks, (1977) found that the bag_1 fibres, unlike the bag_2 and chain fibres, were characterised throughout by the poor development of the M-line. This line occurs in the middle of the sarcomere and, in studies of a range of muscles, has been shown to increase in prominence with increasing speed of muscle contraction and shortening (Matthews, 1972). In the central equatorial region of the bag_1 fibre the M-line is usually totally absent, but a weakly developed one may appear towards the poles. With the aid of the electron microscope, I noticed in the present study that the bag_1 fibre which lacked sensory terminals had an M condition. The M-line appeared as a dark line in the middle of the H zone, which lies at the centre of the sarcomere. In the H zone no thin filaments were present therefore it is clear that these cross-bridges are a permanent part of the structure of the thick filaments.

3.4.2b. II spindle afferent with a primary-like response

In this case, corresponding to the response, the afferent returned to the site formerly occupied by the primary ending. The reconstruction of this ending showed that the intrafusal fibres were structurally differentiated, and clearly indicated that a group II afferent is able to substitute for a group Ia afferent in the maintenance of the differentiation, the development and maintenance of spindles being normally dependent on the presence of the Ia afferent (Milburn, 1984). This study clearly indicated that, in the absence of a Ia afferent, intrafusal-fibre differentiation can be maintained by a group II afferent alone.

3.4.3. Mechanosensory transduction

The present reconstruction also provided evidence consistent with the hypothesis that the major features of the mechanosensory responses are dependent on the mechanical properties of the different types of intrafusal-fibre rather than the intrinsic properties of the Ia afferent itself (Poppele & Quick, 1985). Corresponding to their responses, the first ending appeared to prefer a site formally occupied by a secondary ending, where the ending produced terminals closely similar to those of a normal secondary ending. The ending was large with terminals restricted to bag_2 and chain fibres, the equatorial nucleation of the intrafusal fibres present except in the bag_1 fibre which lacked innervation and was striated throughout the primary region. The second ending of a small diameter afferent was located predominantly in an old primary site, was abnormally short, had few terminal bands around the nuclear bags and the chain fibres lacked innervation. Nevertheless the ending was able to respond in an manner resembling a normal primary ending (Barker *et al.* 1986).

Chapter 4

The Distribution of Calretinin in Muscle Receptors of the Cat

4.1. Introduction

As one of the important intracellular signalling molecules, calcium ions (Ca^{2+}) are involved in fundamental physiological processes, such as signal transduction. It has become clear that the effects of Ca^{2+} are closely related to a superfamily of the structurally related calcium-binding proteins (CBPs), which bind calcium with high affinity.

The prototype CBP is calmodulin (CM). CM was identified in 1967 and has been intensively studied since then. The activity of more than 20 enzymes are regulated by Ca^{2+}-CM complexes. The central nervous system contains a large variety of calcium-binding proteins, (Heizmann & Celio, 1987) most of which belong to the troponin C superfamily and share a common structural motif described as the "EF-hand" domain (Kretsinger, 1980). The "EF-hand" structure, a consensus amino acid sequence, is involved in calcium-binding activity for over 160 CBPs (Persechini *et al.* 1989).

One of these CBPs is calbindin D_{28K}, first identified in the duodenum as the main molecular response to vitamin-D derived hormones. Wasserman & Taylor (1966), later described it in other locations, including the kidney, the pancreatic islets and the brain (Jande *et al.* 1981). Parvalbumin (PV) and calretinin (CR) have been also found widely distributed in the central nervous system. Parvalbumin (PV) was first found in muscular tissue (Lehky *et al*, 1974), and also in the brain (Celio & Heizmann, 1981).

Calretinin (CR) is a calcium-binding protein which was first identified by gene cloning cDNA of chick retina (Rogers, 1987), and is present in various types of neurons in chick central and peripheral nervous system (Rogers, 1991 & Winsky *et al.* 1989). It includes five putative calcium-binding sites of the "EF-hand" type, and is closely related to calbindin D_{28K}, 60% of their amino acids sequences being identical, (Rogers, 1987 and Rogers *et al.* 1990). It was once thought to be a neuronal specific CBP (Parmentier & Lafort, 1991), but has since been found in colonic cancer cells (Gotzos *et al.* 1992).

Calretinin (CR), when compared to CB-D_{28K} and PV is present largely in different sets of neurons, although the three CBPs do overlap to some extent (Rogers & Résibois, 1992). Calmodulin (CM) has been found in glial cells in the cerebellum, (Caceres *et al.* 1983) and in large motoneurons in the spinal cord, whereas CR-like immunoreactivity has been found in dorsal horn neurons rather than motoneurons and glial cells (Ren *et al.* 1993).

CR has been identified mainly in the central nervous system, particularly in the retina in neurons belonging to sensory pathways, and its functions have not been elucidated. It is believed to be important in the intracellular transport of Ca^{2+} and also to act as a calcium buffer (Rogers, 1987).

Taylor, (1974), Jande *et al.* (1981) and Rogers, (1987 & 1991) and Rogers *et al.* (1990), observed that although several different calcium-binding proteins are known to be present in the brain, including parvalbumin, calbindin D_{28K} and calretinin, it is not known whether they simply act as calcium buffers, in which role they could passively modulate several aspects of neuronal activity, or whether they play a more active role in calcium-mediated signal transduction. Rogers & Résibois, (1992) described again the distribution of calretinin and calbindin in rat brain and their results agree with an independent survey by Arai *et al.* (1991).

The differential distribution of CBPs in certain regions of the central nervous system suggests that each CBP may perform distinct functions. For instance, CB-D_{28K} and PV are located in distinct compartments in the thalamus where they may be involved in relaying sensory information of different modalities, (Rausell & Jones, 1991).

All these proteins are present in neuronal cells, although CB-like immunoreactivity is also present in ependymal cells of all the ventricles. Garacia-Segura *et al.* (1984), said it is already clear that each protein is present in different subsets of neurons, but the overlap between them has not been defined. Later on Nitsch *et al.* (1993), analysed the organisation of the calretinin containing system of the monkey hippocampus and their results suggested that there are two separate calretinin-containing systems in the primate hippocampus, an intrinsic and an extrinsic excitatory system. Gotzos *et al.* (1992), found that calretinin and calmodulin play important roles in mediating the Ca^{2+} signal during division in colonic cancer cell line WiDr cells.

The significance of the binding of Ca^{2+} by CBPs may be related to either the triggering of a physiological process or modulation of intracellular Ca^{2+} concentrations. The precise function of most CBPs however, is still largely unknown (Baimbridge *et al.* 1992). Using primarily the immunohistochemical technique, interesting distribution patterns of CBPs have been described in the central nervous system. CB-D_{28K} and PV are present largely in different groups of neurons in the cerebral cortex (Van Brederode *et al.* 1991), in hippocampus (Sloviter, 1989), in superior colliculus, (Mize *et al.* 1992) and spinal cord (Ren *et al.* 1993 and Antal *et al.* 1990).

Duc *et al.* (1993) studied the calretinin immunoreactivity in chicken dorsal root ganglia and they found CR is expressed by a subpopulation of large A-neurons, the axons of these neurons selectively innervate all muscle spindles and most of the herbst corpuscles associated the feathers in the limb. They suggested that the presence of

calretinin in primary afferents may be correlated with the electrophysiological properties of rapidly adapting mechanoreceptors.

Recently the same group (Duc *et al*, 1994), answered this question and confirmed that on the basis of calretinin distribution among a variety of cutaneous and muscle receptors in the rat, calretinin correlated with the rapid adaptation of the mechanoreceptors.

The objective of this study is to obtain evidence bearing on the hypothesis of Duc *et al.* (1994), which says calretinin is correlated with, and presumably involved in, rapid adaptation of the mechanoreceptors. They reported that calretinin is present in primary endings of muscle spindles and in the axon terminals of Paciniform corpuscles, said to be rapidly adapting, whereas it was absent from secondary endings of the muscle spindles and from tendon organs, said to be slowly adapting. This hypothesis could not possibly be correct for many reasons.

1. The responses of muscle mechanosensory receptors are not readily or easily classifiable as either rapidly or slowly adapting, since under suitable conditions of stimulation they will exhibit both type of behaviour (Matthews, 1972).

2. Whereas Paciniform corpuscles may be classified as rapidly adapting sense organs, this adaptation is partly determined by the mechanical properties of the capsule lamellae as well as by the properties of the nerve terminals itself. After removal of the capsular lamellae the nerve terminal shows a slow adaptation, thus proving that the capsule modifies the response properties of Paciniform corpuscles (Loewenstein & Skalak, 1966).

Studies on reinnervated spindles and tendon organs carried out in this laboratory by Banks & Barker, (1989), indicate that the intrinsic mechanism of mechanosensory

transduction is similar not only in the primary and secondary endings of muscle spindles but also in the sensory ending of the tendon organs, and that the characteristic responses of these endings are largely shaped by the mechanical properties of the accessory structures with which they are in contact. For example:

3. Successful reinnervation of muscle spindles by cross-innervation (Banks & Barker, 1989), in which foreign afferents including Ib were given the opportunity of reinnervating spindles in the absence of their native Ia, or spindle II afferents. This study revealed the ability of regenerating Ib afferents to terminate in spindles in sites originally occupied by primary Ia or secondary II endings and to respond appropriately to their site of reinnervation.

4. Further evidence that the accessory structures largely determined the shape of the response of muscle receptors came from reinnervated tenuissimus spindles (Banks & Barker, 1989). In this case a group II afferent, identified by its conduction velocity (26m/s) has reinnervated a site previously occupied by a muscle spindle primary ending, following section of the nerve supplying the muscle a year earlier. Its response to muscle stretch, particularly to the phase when the muscle is changing length is more primary than secondary-like (see chapter 3).

The working hypothesis of this part of my project was based on the reinnervation studies of Banks & Barker, (1989) and on my own observations on reinnervated spindles (see chapter 3) which together indicate that as in paciniform corpuscles the adaptation properties of muscle receptors also depend on the non-neuronal components such as intrafusal muscle fibres or tendon bundles with which the sensory endings are in contact. Here I shall re-examine the distribution of calretinin in the abductor digiti quinti medius (adqm) muscle of the cat. This is an intrinsic muscle of the hind foot. It originates from the fifth metatarsal, and from the ventral surface of the calcaneus, and it connects by a thin tendon into the lateral side of the base of the proximal phalanx of the fifth digit. It

was chosen for this work because it is small in size, and contains a highly predictable distribution of a variety of mechanoreceptors (Banks *et al*, 1995a). Also its muscle spindles regularly contain several secondary endings; this is not the case in most rat muscle whose spindles quite frequently lack secondary endings altogether. Another aspect studied on the same experimental model was the sensory innervation of the abductor digiti quinti medius muscle.

4.2. Materials and Methods

The abductor digiti quinti medius (adqm) muscle of the cat hind limb was chosen because it meets the following technical requirements: it is small in size (about 30 mg) and because of its convenient size and shape and the fact that the direction of its fibres is such as to permit easy orientation for longitudinal sectioning. It is also easy to tease. It has three receptors, muscle spindles, tendon organs and paciniform corpuscles, therefore it provides a practical model to re-examine calretinin-immunoreactivity.

4.2.1. Technique for Muscle Removal

Five cats were killed with an overdose of sodium pentobarbitone (sagatal). Immediately after the death of the cat the abductor digiti quinti medius muscles were carefully dissected out from their attachments, each muscle was oriented on a piece of card and fixed for 2 hours with paraformaldehyde fixative: 1 volume of 0.075 M L-lysine, 3 volumes of phosphate buffered saline, 4% paraformaldehyde and 0.23% periodate, (Duc *et al.* 1994).

The fixed muscles were incubated in 30% sucrose for 24 hours, frozen in liquid nitrogen, then transferred to a cryostat and left for a few hours. If the muscles were not immediately required, they were stored in sealed frozen bags either in the freezer or the cryostat for a few days.

4.2.2. Preparation of the Tissue

i. The muscles were prepared first as described by Duc *et al.* (1994).

Two muscles were defrosted and gently teased into small bundles of fibres and placed in Tris-buffer (pH 7.3) in Petri dishes and stained according to Duc *et al.* (1994). A weak reaction was obtained in all spindles and tendon organs except one tendon organ which was more heavily stained because part of the capsule was torn during the teasing.

Then the procedure was repeated, tearing the capsule using fine forceps to facilitate the penetration of the antiserum. When this was successful, a clear stain was obtained.

ii. Cryostat sectioning

Four muscles were used for this technique including the control. The frozen muscles were handled with pre-cooled forceps and were cemented to cryostat chucks with glue (Cryomatrix, Agar). A small quantity of Cryomatrix was put on the platform of a pre-cooled chunk in the refrigerated chamber. As the glue began to solidify the muscle was oriented in the matrix on the chuck, more glue was added around the muscle to provide further support during sectioning. The chuck with the frozen muscle was transferred to the microtome and maintained at -20°C. Serial longitudinal sections were cut at 20-25 μm thickness and six consecutive sections were placed on each microscope slide and the slides numbered in series. The slides were then stored in trays in the cryostat at -20°C until required for staining. This technique did not work as the sections came off the slide. After the treatment with H_2O_2, an attempt was made to modify the immuno-staining procedure described by Duc *et al.* (1994). I found the staining had to be carried out on free-floating teased fibres with tearing on the capsule to facilitate the penetration of the antiserum and on free floating sections of 50 μm thickness in order to get through the capsule and to get the whole spindle. For thicknesses of less than 50 μm it was necessary to reconstruct the whole spindle.

4.2.3. Muscle Fibre Immunohistochemistry

The calretinin (CR) immunoreactivity was detected in the muscles using the peroxidase-antiperoxidase method (Dako universal PAP kit). The rabbit antiserum used (SWant 7686) was reconstituted with 200 μL double distilled water and stored frozen in micro centrifuge tubes (1.7 ml volume) at -80°C. For continuous use the antiserum was kept at 4°C to avoid repeated freezing and thawing.

i. Procedure for the Immunolabelling of the Teased and Sectioned Muscles with Rabbit Antiserum

The following immuno-staining procedure is a modification of those described by Duc *et al.* (1994) and the Dako universal PAP.

The PAP method is the most commonly used immunoperoxidase staining method not only because of its high sensitivity, but also because reliable reagents are available commercially.

The structure of the PAP complex contains three peroxidase and two immunoglobulin molecules. Thus three peroxidase molecules can be bound to the antigen sites by the action of one bridge (secondary) antibody. Therefore the staining intensity of the immunoperoxidase reaction is a function of peroxidase activity.

ii. The Steps of the Immuno-Staining Procedure Were:

1. The free-floating tissue sections and teased fibres were treated for 20 min with H_2O_2 to inhibit endogenous peroxidases.
2. Rinsed in Tris-buffer 3 x 10 min.
3. Normal rabbit serum (blocking serum) applied for 30 min.
4. Incubated for 2-3 days with the rabbit antiserum at a dilution of 1 / 1000 to 1 / 2000 in Tris-buffer pH 7.3 with 10% horse serum and 0.4% Triton X 100 at 4°C.
5. Rinsed in Tris-buffer 3 x 10 min.
6. Transferred into immunoglobulin anti-rabbit serum (Link antibody) and incubated at room temperature for 4 hours.
7. Rinsed in Tris-buffer 3 x 10 min.
8. The peroxidase complex applied for 2 hours at room temperature.
9. Rinsed in Tris-buffer 3 x 10 min.
10. Peroxidase activity revealed by incubation with reagent 3,3' diaminobenzidine for 30 min. (one DAB tablet and one urea hydrogen peroxide tablet dissolved in 5 ml of distilled water) which gives a brown reaction product.

11. The free-floating sections and teased tissues were mounted on slides, with glycerol and covered with coverslips.

All the specimens were examined by light microscopy and were photographed using a Microflex UFX camera mounted on a Nikon Optiphot microscope with a blue filter.

In control sections the rabbit antiserum was omitted and the same staining procedure was followed.

4.2.4. Silver Impregnation Method

To study spindle innervation the abductor digiti quinti medius (adqm) muscles were stained with silver impregnation and teased according to the method of Barker & Ip, (1963). The procedure was as follows :

1. The muscles were fixed for 4-6 days in a mixture of freshly prepared chloral hydrate, 1g; 95% alcohol, 45ml; distilled water, 50ml; 70% concentrated nitric acid, 2ml.
2. Washed for 24 hours in running tap water.
3. Placed for 24-48 hours in 95% alcohol, 25 ml; ammonia, 1 drop.
4. Blotted surplus fluid, and incubated for 5 days in 1.5 % silver nitrate at 37°C.
5. Reduced for 2 days in freshly prepared hydroquinone, 2 g ; 25% formic acid, 100 ml.
6. Rinsed in distilled water; cleared and stored in glycerine before teasing.

To search for sensory innervation the muscles were teased under a stereomicroscope using transmitted and reflected light. The teased specimens were mounted in glycerol on microscope slides and the cover-slips were ringed with pitch.

4.2.5. Measurement of Sensory Terminal Contact Areas

The photographs of the longitudinal sectioned spindles were taken at two focal levels of a single primary ending used to estimate the radii of the outer (free) together with the length of the imaginary chord cutting the outline in the line of the intrafusal fibre, which was identified by the degree of prominence of the terminals (Banks, 1986), using a set of standard circles. The terminal outlines on each muscle fibre were selected in the sections that contained the greatest equatorial diameter of each fibre and usually appear sharply focused.

4.3. Results

4.3.1. Analysis of Calretinin Distributions

Localisation of the calcium-binding protein calretinin (CR) in the muscle spindle, tendon organs and Paciniform corpuscles of the abductor digiti quinti medius muscles was immunohistochemically investigated using the peroxidase-antiperoxidase method. In the initial trial using the same procedure for preparing the tissue, the result was negative except for one tendon organ in which part of the capsule fortuitously turned back during the teasing process, and that part of the sensory terminals which was exposed to the antibody was heavily stained (Fig 5A & B). The results presented in this work are based on two teased muscles and four that were longitudinally sectioned, including the control. The calretinin immunoreactivity was expressed by all the preterminal branches of the Ia afferent and also by all the terminals of the sensory ending of muscle spindles (Fig 2A & B) which are wrapped around the specialised muscle fibres that form an intrafusal bundle. It was immediately obvious that unlike the surrounding extrafusal fibres many of the intrafusal fibres showed calretinin immunoreactivity and that the intensity of the immunoreactivity varied according to intrafusal fibre type (Table 2 & Fig 2A & B). The chain fibres were intensely positive, bag_1 and extrafusal fibres were unlabelled (negative) and bag_2 fibres intermediate. Blood capillaries were seen to be positively stained.

In fourteen sectioned muscle spindles (Table 1) the calretinin immunoreactivity was detected throughout the length of labelled intrafusal fibres and most of the annulospiral nerve endings were visualised with (CR) antiserum whereas in teased spindles a weak reaction was obtained first because the capsule was intensely positive to the calretinin immunoreactivity. The capsule is typically formed from the perineurial epithelium of its nerve supply and since it acts as a highly active diffusion barrier Shantha *et al.* (1968) it presumably prevents the diffusion of the antiserum to the intrafusal fibres. An attempt was therefore made to tear the capsule using fine forceps to facilitate the

penetration of the antiserum. When this was successful, then good results were obtained similar to those for the sectioned spindles.

In the primary endings immunoreactivity occurred in the sensory terminals, especially those on the bag fibres, thus facilitating identification of fibre types (Fig 2B). Although the secondary endings, which are extensively developed only on chain fibres, were negative (Fig 3A & B), the sensory terminals of the tendon organs were positively stained (Fig 6A & B). In Paciniform corpuscles calretinin immunoreactivity was intensely expressed by all axon terminals of the corpuscles but not by lamellar cells (Fig 7A & B). The bag fibres were distinguished by the degree of prominence of the terminals, those on the bag_1 fibre being more prominent than those on the bag_2 (Banks, 1986).

4.3.2. Control

In control sections in which the first antibody was omitted no significant immunostaining could be detected.

4.3.3. Structure and Arrangement of Muscle Spindles

The spindles were examined in longitudinal sections at 50μm thickness. The number of intrafusal fibres ranged from five to nine.

According to the morphological features of the intrafusal muscle fibres, and details of the primary endings usually each spindle possessed zero or two bag_1 fibres, one to two bag_2 fibres and the chain fibres varied in number from one to seven (Table 4 & Fig 9). There are usually more chain fibres in small spindles than bag fibres. Two spindles appeared tandemly linked to tendon organs. In each case the spindle and tendon organ capsules were continuous, so that only intrafusal fibres inserted into the tendon organ. A unique blood capillary was seen only in one spindle to pass through the capsule

into the periaxial space and travel close to the intrafusal muscle fibres in the equatorial region.

4.3.4. Innervation of Muscle Spindles

i. Nerve Supply

A single spindle received one large afferent nerve fibre to the primary ending on all the intrafusal fibres and usually one to several smaller afferents to the secondary endings principally on the chain fibres.

ii. Sensory Endings

Sensory endings often occupied small channels in the surfaces of the intrafusal muscle fibres in the form of spirals that overlaid the nuclear bags and the middle regions of the nuclear chains. Terminations lying parallel to the axis of the intrafusal fibre occurred on either side of the spiral, especially on the bag_1 fibre, (Fig 2B) as described by Banks *et al.* (1982), in several hind limb muscles.

A. Primary Endings

The primary sensory nerve endings (Fig 2A) are derived from a myelinated nerve fibre of large diameter which goes to the group of intrafusal muscle fibres and occurs as annulo-spirals wound around the equatorial regions of the intrafusal muscle fibres. There were differences in the form and disposition of the terminals on the two bag fibres: in bag_1 fibres transversely orientated, regularly arranged terminals wrapped closely together around the bag fibres and were arranged on either side by an unsymmetrical arrangement of the terminals, many of which were set parallel with the fibre axis; by contrast the terminals on the bag_2 fibre were more widely spaced and nearly all were transversely orientated with minimal irregularity at each end.

B. Secondary Endings

The secondary sensory nerve endings are extensively distributed to the chain fibres, most spindles containing one or more secondary afferent endings. These endings lie on one or both sides of the primary ending, and occur largely in the form of loose widely spaced spirals (Fig 3A & B).

iii. Terminal Indentation

The terminal outlines as seen in optical section were bordered on their outer unattached (free) surface by curves resembling segments of circles. Following Banks, (1986), mean values of the radii of curvature, O, together with the half-length, C of the chord spanning the ends of the curves and the ratio are given in Table 3. The surface of the muscle fibres of the intrafusal fibres were indented by the terminals, most deeply in chain fibres and slightly in bag_1 fibres, but these surfaces were not usually sufficiently clearly resolved to measure their curvature.

iv. Motor Endings

The motor endings in the intrafusal muscle fibres were not detected immunohistochemically by using the calcium-binding protein calretinin, because of the difficulty in obtaining good preparations comparable to the silver impregnation staining technique. This could be due to the staining artifact, or probably to incomplete penetration of the antiserum to the sites of the motor endings, or due to the intensive staining of the chain fibres. The calretinin immunoreactivity was detected only on the extrafusal motor endplates (Fig 8) while the intrafusal motor endings were poorly developed.

4.3.5. Analysis of Silver Preparations

4.3.5a. Number of Spindles and Tendon Organs

Five spindles and two tendon organs were teased from one muscle. For the analysis of sensory innervation only two spindles were chosen because of superior staining, good teasing quality and unbroken afferents.

The spindles were not uniformly distributed throughout the muscle, the majority were confined to a circumscribed region of the muscle periphery. All spindles were situated in parallel with extrafusal fibre bundles. The sensory region of $spindle_1$ contained three endings (S_1 P S_1) and $spindle_2$ contained two endings (P S) (Fig 1).

4.3.5b. The Form of the Terminals

The terminals of the primary endings were annulospiral on both bag and chain fibres. They were thicker and wider on the bag fibres; in the mid-equatorial regions of bag fibres the turns were set more closely than on the adjacent regions, nuclei of intrafusal fibres were seen. The number of bands appeared to be more in bag_1 than in bag_2 fibres. The secondary endings were distributed to chain fibres usually in the form of fine sprays, though some coils and spirals were present, particularly close to primary endings.

4.3.6. Paciniform Corpuscles

Each Paciniform corpuscle was recognised in longitudinal sections by its envelope of delicate unstained lamellae which wrapped the nerve fibre. Their incidence varied from 2-3 in each muscle. Paciniform corpuscles are pressure receptors often closely associated with the tendon organs. They are laminated, cylindrical structures which consist of a capsule formed by a series of connective tissue sheaths organised in an "onion skin" arrangement around a central cavity known as the inner core. Each Paciniform corpuscle is supplied by a single myelinated nerve fibre. In eight examined

Paciniform corpuscles (Table 1), calretinin (CR) immunoreactivity was intensively expressed by all axon terminals of the corpuscles but not by lamellar cells (Fig 6A & B).

4.3.7. Tendon Organs

The total number of tendon organs is shown in (Table 1). They were distributed throughout the muscle wherever tendon and muscle fibres united. Most were distributed as single receptors, but a few were linked with the muscle spindle.

The tendon organ (also called Golgi tendon organs) lies at the musculo-tendinous junction, connected in series with a group of extrafusal muscle fibres. Their function is complementary to that of the muscle spindle. They are spindle-shaped, surrounded by a capsule, and supplied by a single medullated nerve fibre large in diameter (Ib afferent) which breaks up into a series of sensory terminals which are close to the surface of the tendon fascicles (Fig 4). Those sensory terminals were positively stained (Fig 5A & B).

Methods	Spindles	Tendon organs	Paciniform corpuscles
Teased	3	2	-
Sectioned	14	3	8
Total	17	5	8

Table 1

showing the number of spindles, tendon organs and paciniform corpuscles observed using the two preparation techniques prior to the calretinin immunoreaction.

Receptor	Structure	Staining density
Muscle spindle	Capsule	++
	Bag_1	-
	Bag2	++
	Chains	+++
	Primary ending	+++
	Secondary ending	-
Tendon organ	Capsule	++
	Sensory terminals	+++
Paciniform corpuscle	Sensory terminals	+++
	Lamellae	-
Extrafusal muscle	Fibres	-
	Motor ending	++
Blood capillaries		++

Table 2

Shows comparison of the distribution of calretinin immunoreactivity in the Muscle spindle, Tendon organ, Paciniform corpuscles and other stained structures in the abductor digiti quinti medius muscle.

+++ high density, ++ moderate density and - not found.

	O	C	O/C
Bag_1 fibres			
spindle 1	10	0.525	19
spindle 2	9.2	0.475	19.4
Bag_2 fibres			
spindle 1	20	0.925	21.6
spindle 2	18.25	0.67	27
Chain fibres			
spindle 1	14	0.6	23.3
spindle 2	11	0.5	23.2

Table 3

Showing the radii of curvature of the outer borders of terminal outlines on the three types of intrafusal muscle fibre, the half length of the corresponding chord and ratio.

O. Radius of the outer boundary.

C. Half-length of chord.

The values represent the means of six observations and are recorded in μm.

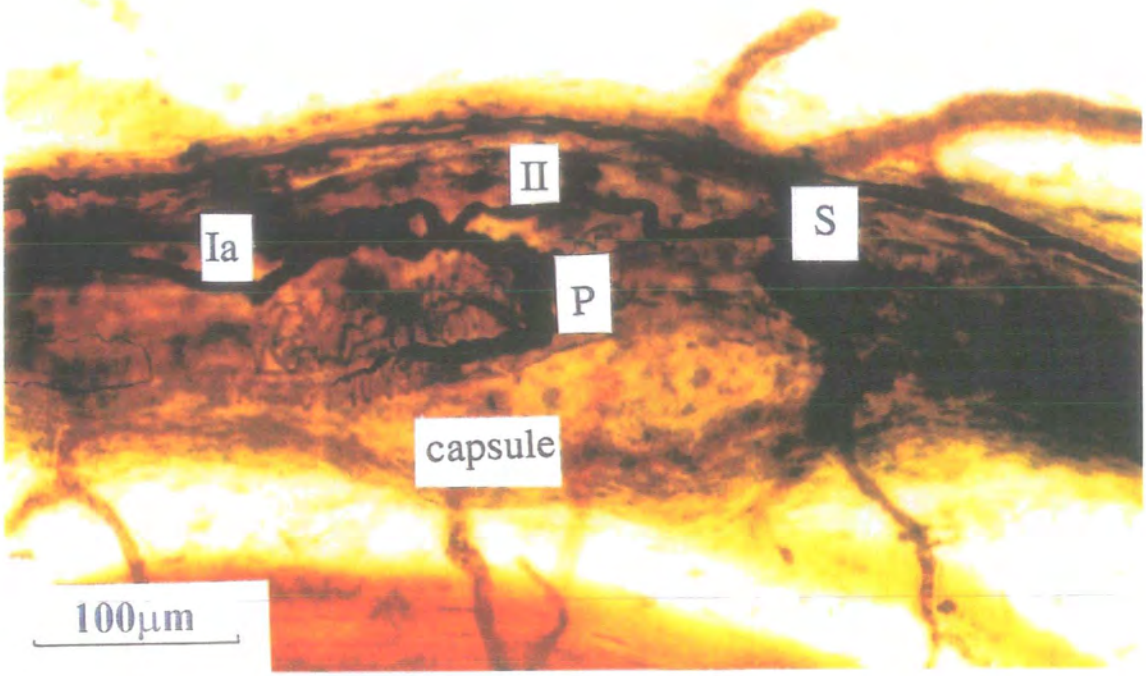
Total no of bag fibres	No of chain fibres								
	1	2	3	4	5	6	7	Total	% of Total
1(b_2)	-	-	-	-	-	-	1	1	7
2(b_1/b_2)	-	-	-	1	3	2	-	6	43
3($b_1/2b_2$)	-	-	1	2	-	-	-	3	21
4($2b_1/2b_2$)	-	-	2	-	2	-	-	4	28
Total	-	-	3	3	5	2	1	14	

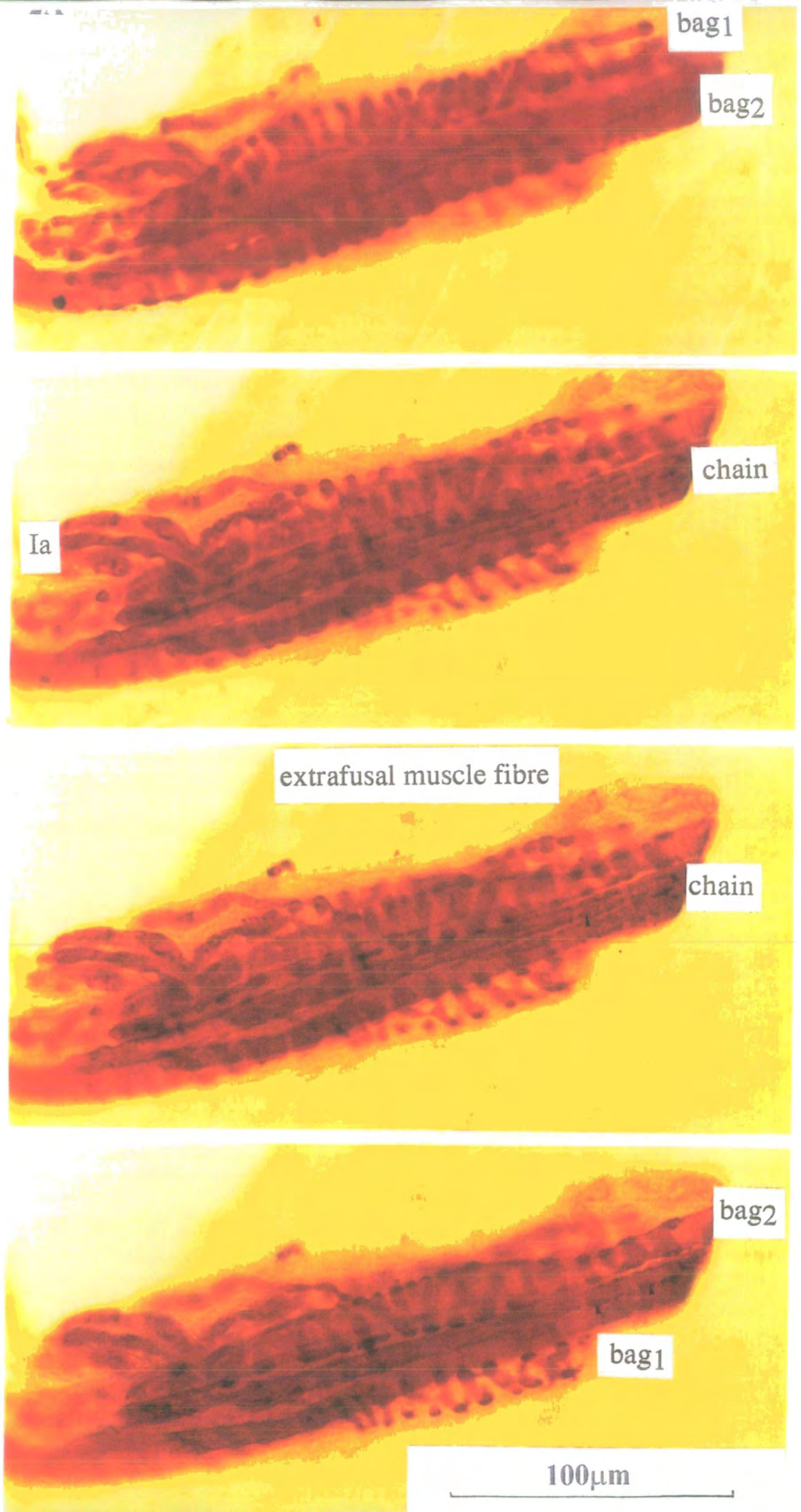
Table 4

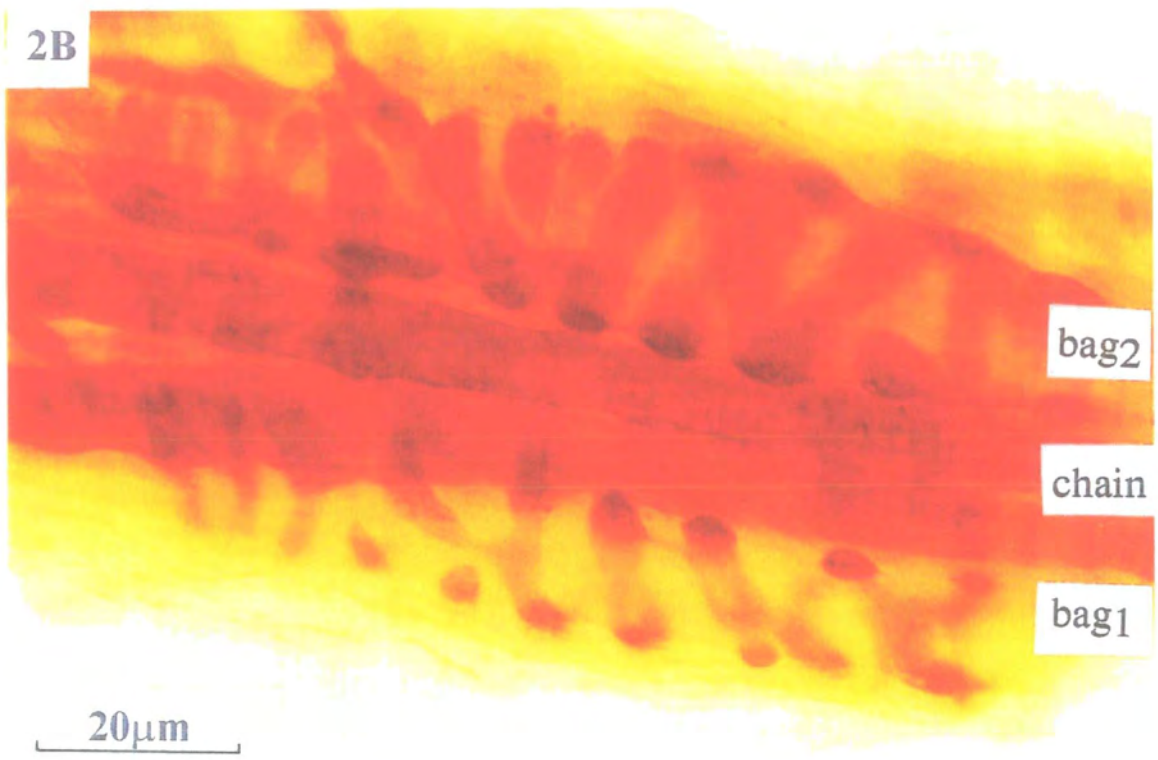
Intrafusal fibre contents of muscle spindles in abductor digiti quinti medius muscle.

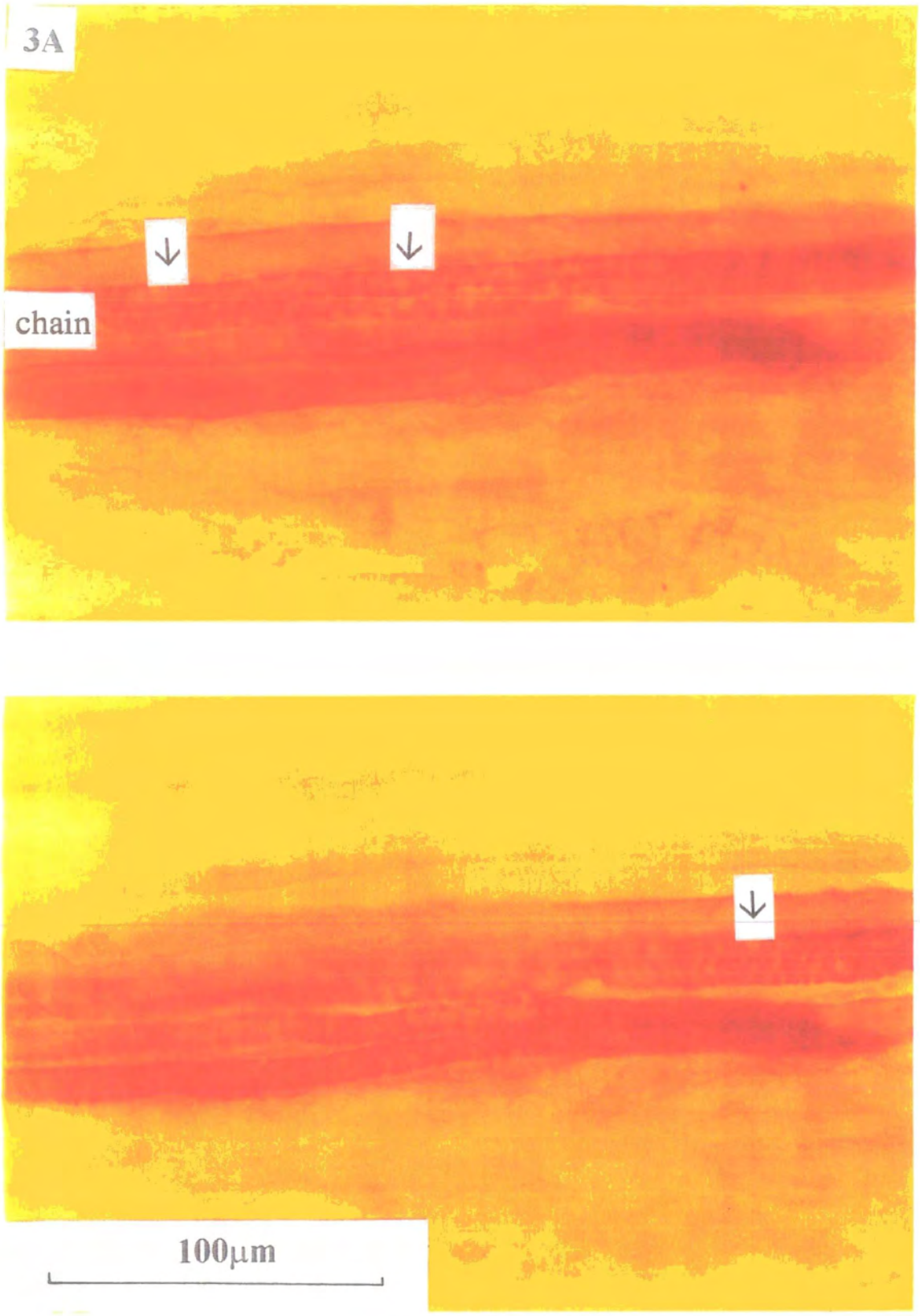
The data represent counts from 14 spindles in three adqm muscles. Each column in of the table indicates the number of spindles containing a specific nuclear-bag fibre content (left) and chain-fibre content (top). Note that most muscle spindles contained two to four nuclear bag and three to seven nuclear chain fibres.

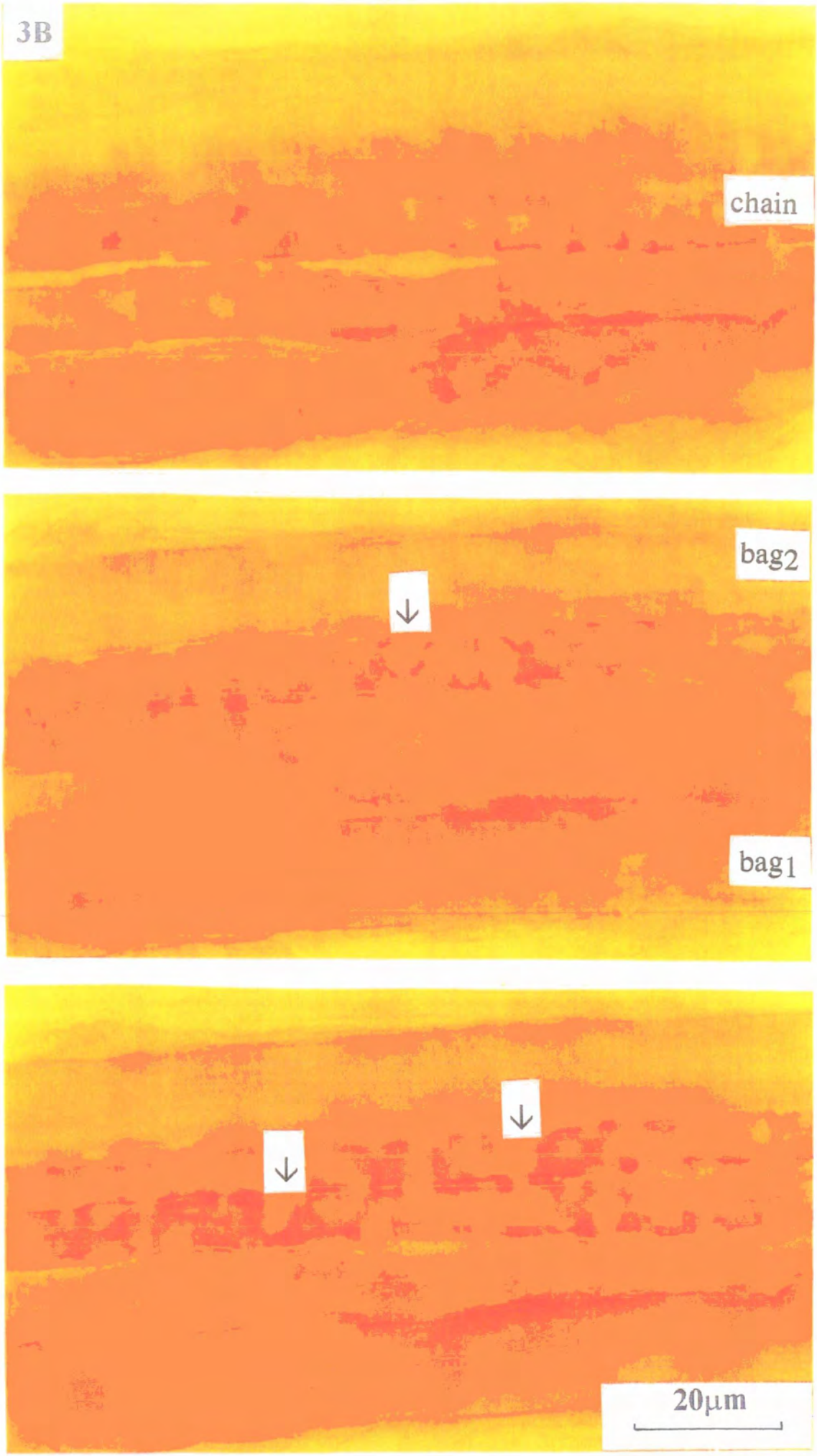
1

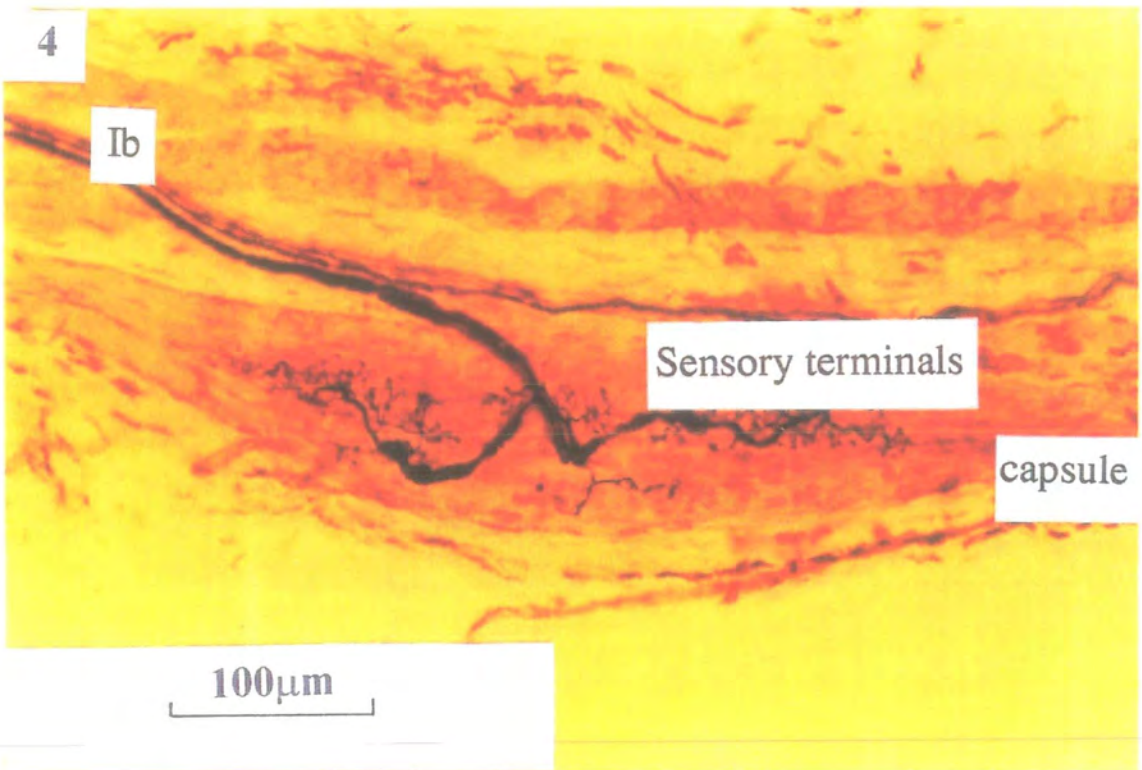


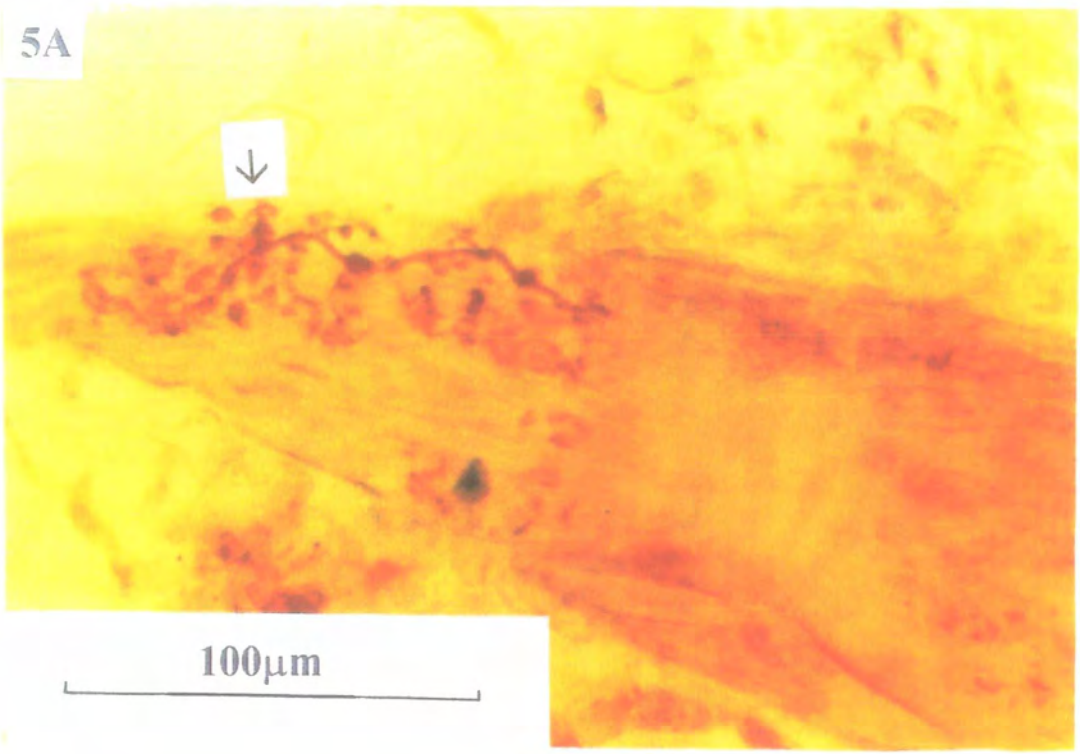

bag1
bag2
chain
Ia
extrafusal muscle fibre
chain
bag2
bag1
100μm

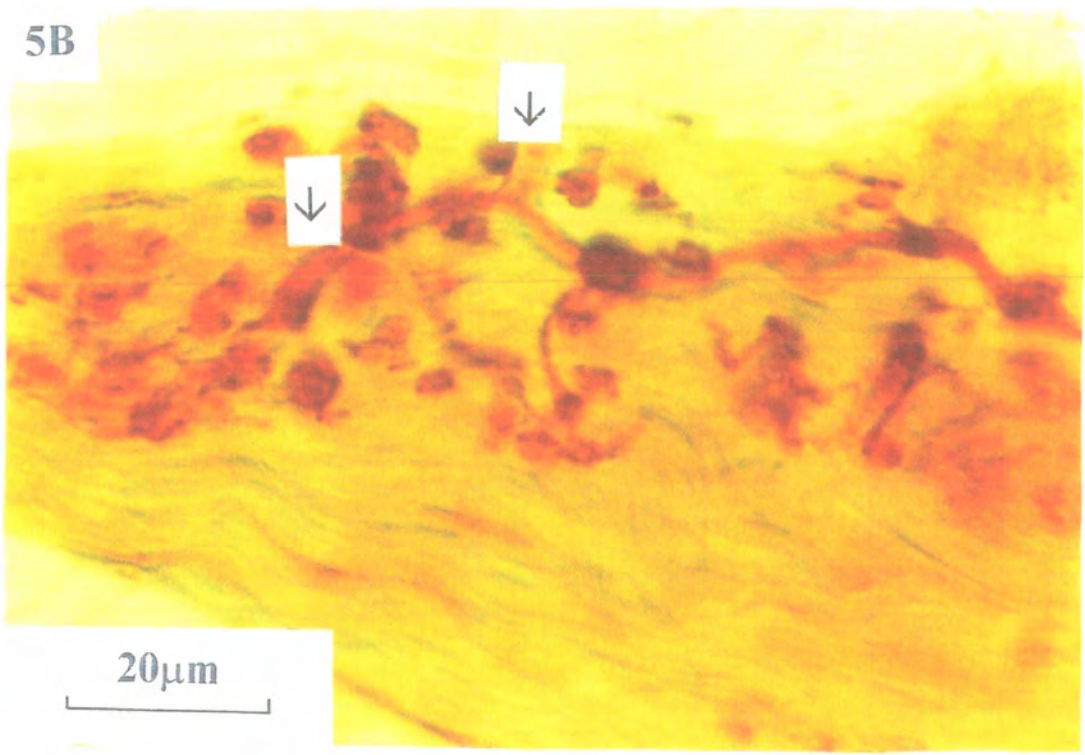

2B
bag2
chain
bag1
20μm


3A
chain
100μm

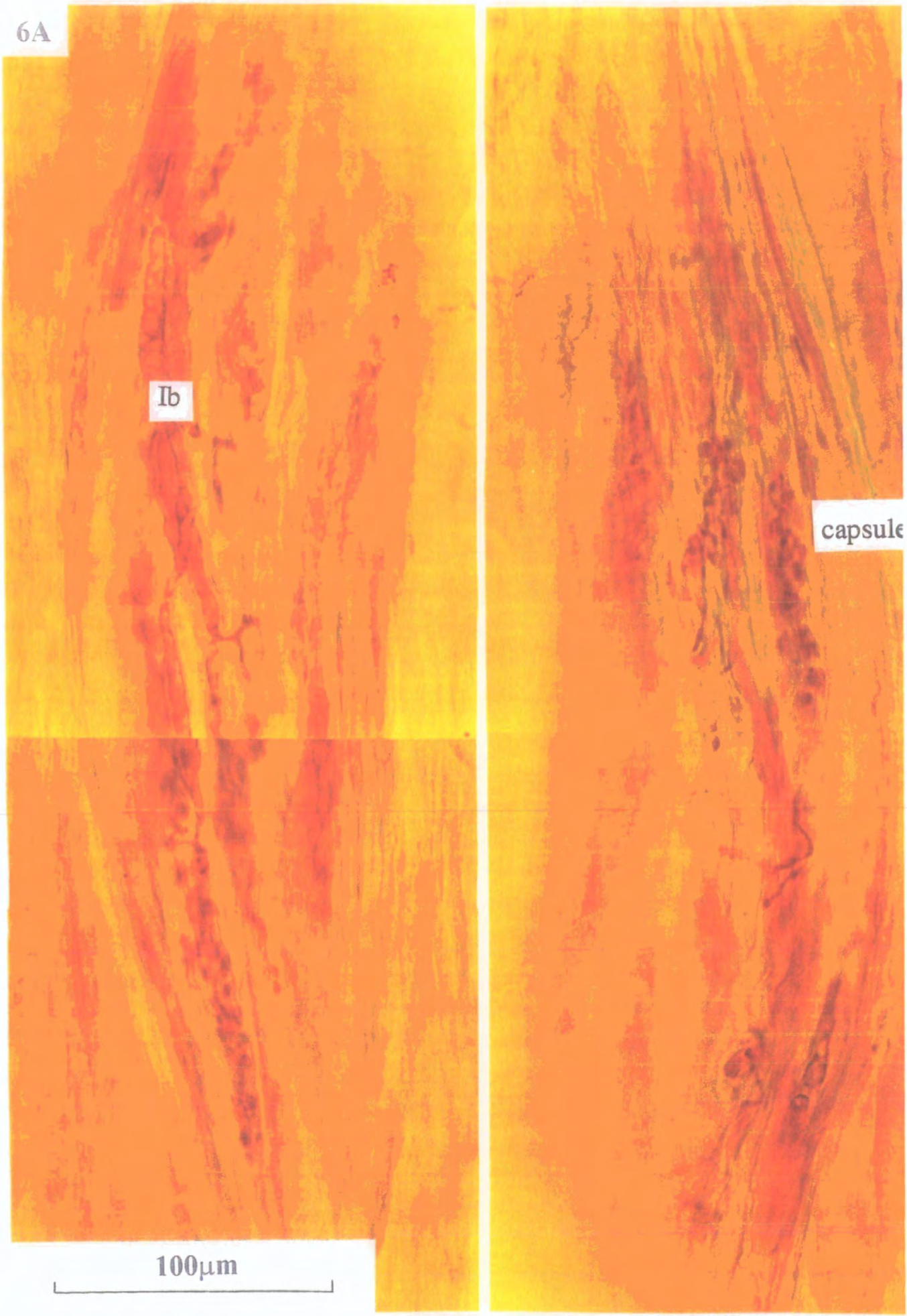

3B
chain
bag2
bag1
20μm

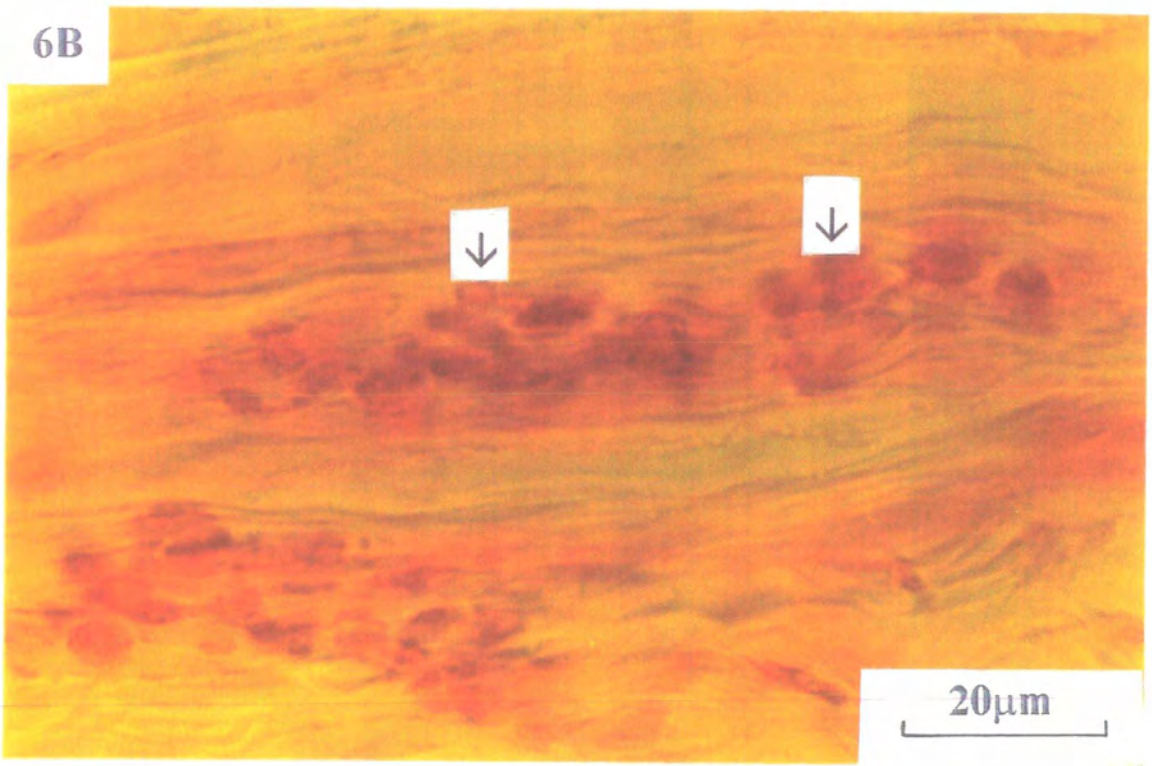

4
Ib
Sensory terminals
capsule
100μm

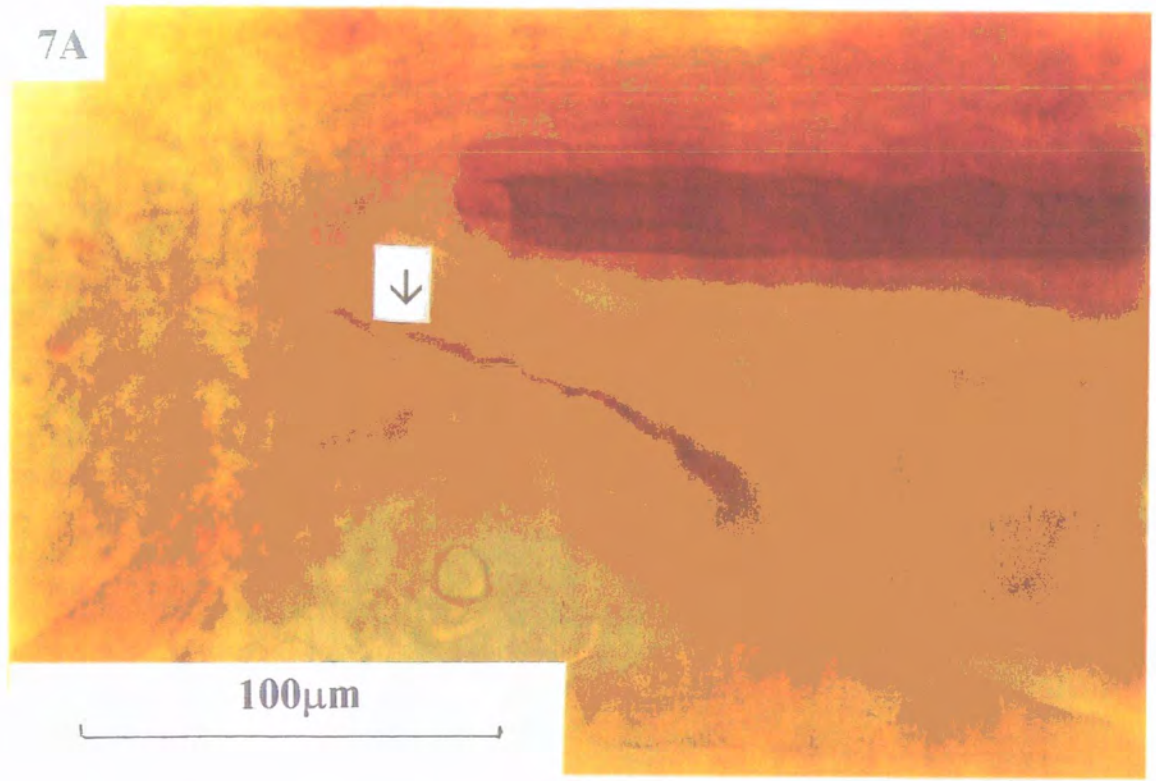

5A
100μm

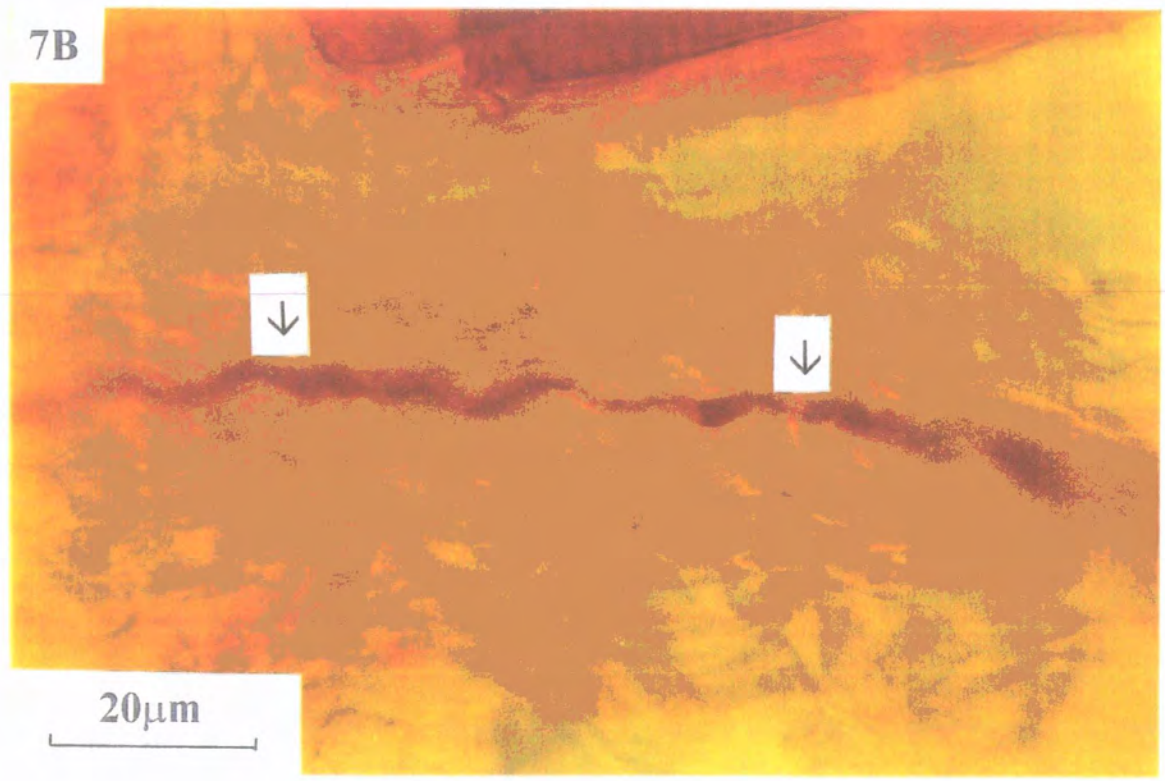

5B
20μm

6A




6B
20μm


7A
100μm


7B
20μm

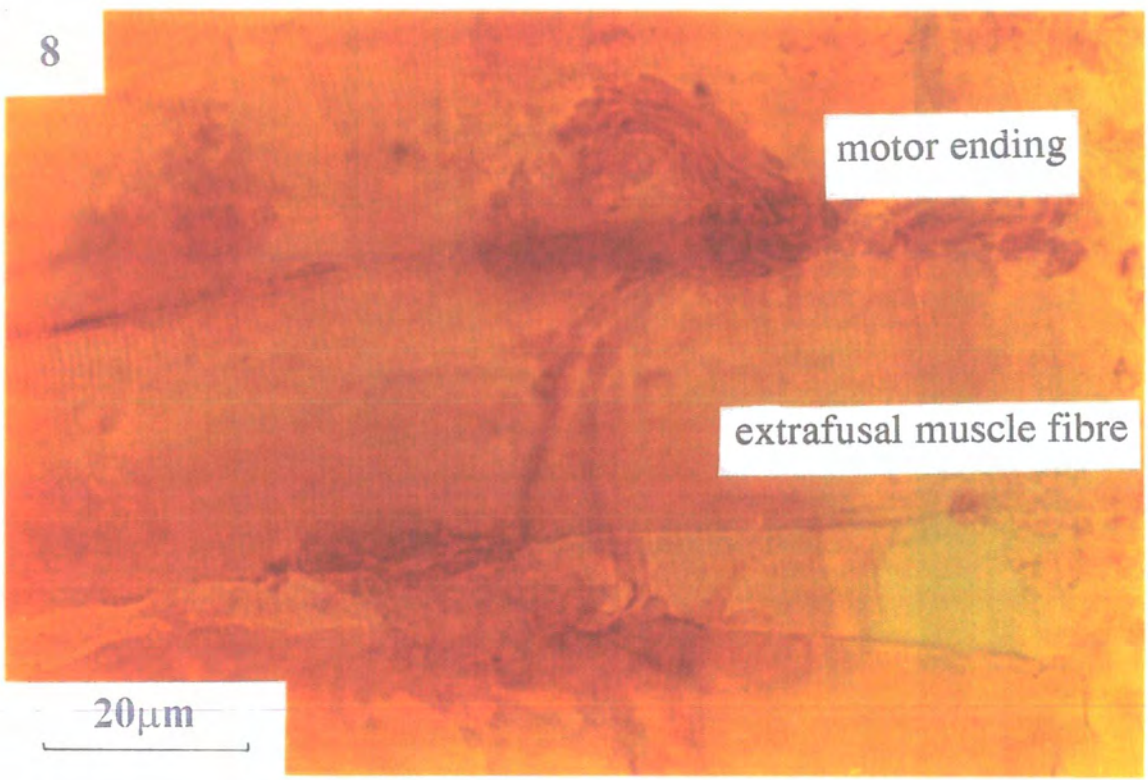
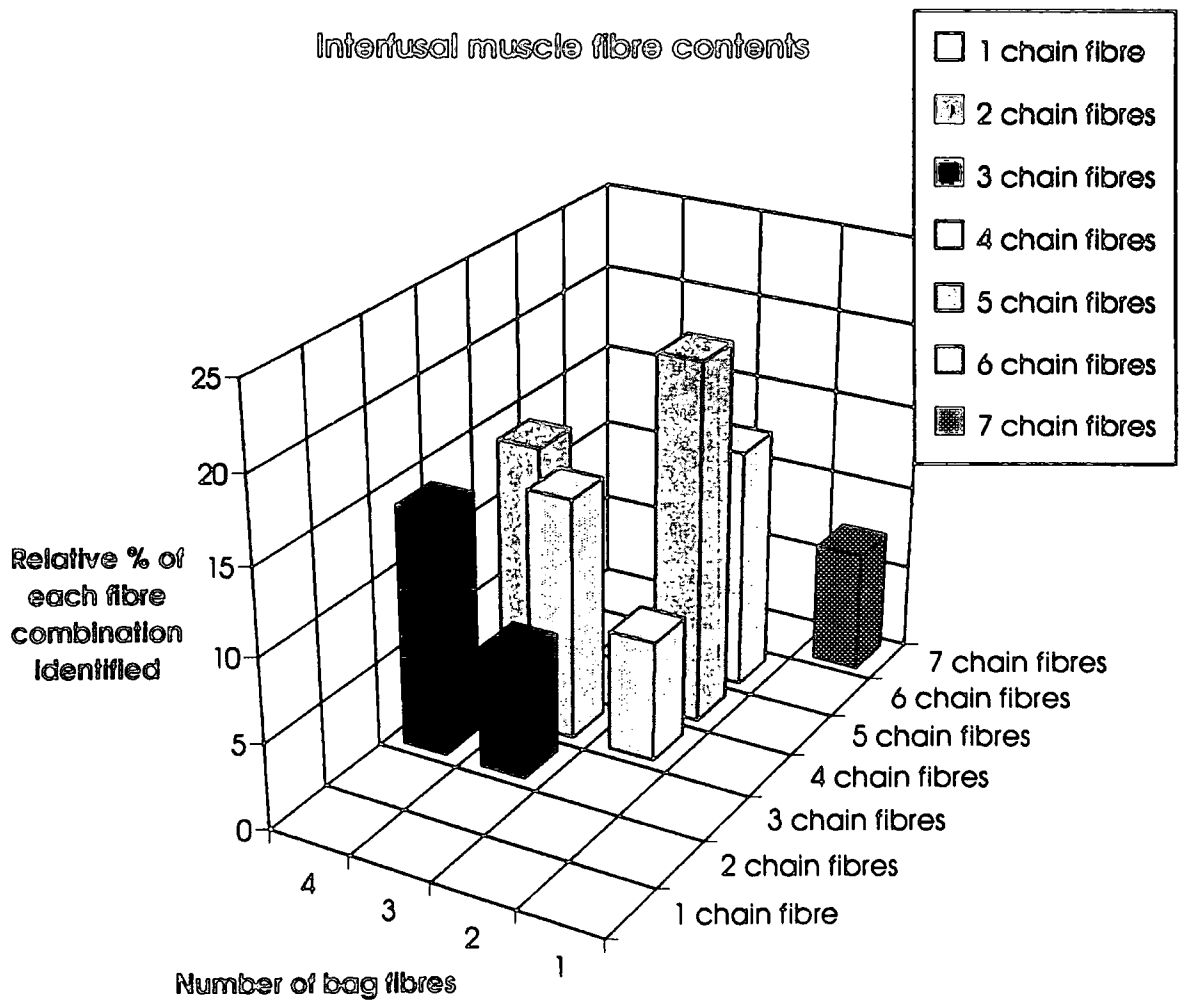

8
motor ending
extrafusal muscle fibre
20μm

Figure 9. Histogram shows the percentage numbers of spindles containing a given number of intrafusal muscle fibres in sets of longitudinal sections taken from three muscles.



4.4. Discussion

4.4.1. Methodology

In this study I localised calretinin immunoreactivity in the abductor digiti quinti medius (adqm) muscle from cats using rabbit antiserum (SWant 7686). Résibois *et al.* (1990) suggested the possibility that anti-calretinin antisera may cross-react with calbindin D_{28K} because of the high degree of homology between calretinin and calbindin D_{28K}. In the case of the anti-calretinin antiserum used in this study, however, this possibility is excluded. The anti-calretinin antiserum detected only calretinin but not calbindin D_{28K} on a protein blot of the muscle receptors (Schwaller *et al.* 1993). Localisation of calretinin was previously examined immunohistochemically in rat muscle using the same antibody (Duc *et al.* 1994). Results presented here show significant differences.

4.4.2. Comparison of the Results with the Description of Duc *et al.* 1994.

Calretinin is a highly conserved calcium-binding protein (Parmentier, 1990). Although it is expressed in specific subsets of neurons its role therein is unknown.

In the present study my results clearly show a different picture to that described by Duc *et al.* (1994). According to those authors calretinin immunoreactivity could not be detected in the subsets of the nuclear bag or nuclear chain fibres, while in this study there are clear differences in the distribution of calretinin immunoreactivity according to the type of the intrafusal muscle fibres: chain fibres were intensely positive, bag_1 fibres and the extrafusal fibres were virtually negative and bag_2 fibres were intermediate. So this observation is in disagreement with Duc *et al.* (1994).

In the primary endings immunoreactivity was especially clear in the sensory terminals on the bag fibres. In the chain fibres immunoreactivity appeared to be present

in some sensory terminals and not in others. The difficulty of observing immunoreactivity in chain-fibre terminals could be partly due to the intense staining of the fibres themselves, but it was clear in some cases that chain-fibre terminals were relatively poorly stained (Fig 2A). The secondary endings, which are extensively developed only on chain fibres, appeared negative, though this again could be due in part to intense staining of the chain fibres. The difference between primary and secondary endings is in agreement with Duc *et al.* (1994), however, in complete contrast to their results immunoreactivity for this CaBP was seen in the tendon organs. The sensory terminals of tendon organs were positively stained, whereas in Duc *et al.* study tendon organs were negatively stained, so this is the second disagreement with Duc *et al.* (1994).

My results on the pattern of the differences in distribution of calretinin immunoreactivity between the different muscle receptors therefore show significant differences from those of Duc *et al.* (1994) and do not support the suggestion that calretinin plays a role in rapid adaptation. In particular, in the tendon organs which Duc *et al.* (1994) stated do not exhibit calretinin immunoreactivity because of their function they claim that the tendon organs and the secondary endings are slowly adapting while the primary endings and Paciniform corpuscles are rapidly adapting but the present study shows beautifully positive stained tendon organs, therefore the presence of calretinin in the primary endings, Paciniform corpuscles and of tendon organs and the absence of the calretinin immunoreactivity from the secondary endings does not correlate with the electrophysiological properties of rapid adaptation.

4.4.3. Functional Consideration

No direct evidence has been obtained to link this calcium-binding protein to specific electrophysiological characteristics. Nevertheless the highly specific distribution, especially that within the spindle primary endings, clearly indicated a particular physiological role that has yet to be elucidated.

The physiological role of calretinin in neurons remains speculative. Calretinin has a similar Ca^{2+} binding site structure (EF-hand) as other Calcium-binding proteins including calmodulin, calbindin D_{28K} and parvalbumin (Kretsinger *et al.* 1988).

Calretinin is believed to be important in the intracellular transport of Ca^{2+}, it is not known whether it simply acts as a calcium buffer (Braun, 1990), in which role it could passively modulate several aspects of neuronal activity, or whether it plays a more active role in calcium-mediated signal transduction also thought to participate in phosphorylation. The precise function of most CBPs is still largely unknown.

Li *et al.* (1995), revealed that calbindin D_{28K} acts as an endogenous Ca^{2+} buffer which plays an important role in determining the firing patterns of hypothalamic neuroendocrine neurons. Oxytocin-releasing cells are normally tonically active whereas vapropressin-releasing cells fire in phasic bursts due to the balance between Ca^{2+}-dependent depolarizing and hypopolarizing processes. Introduction of anti-calbindin antibody into oxytocin cells caused them to fire phasically, whereas introduction of calbindin into vapropressin cells caused them to fire continuously. From studies which show that calretinin is closely related to calbindin D_{28K}, 60% of their amino acids being identical, and from the evidence presented here on the distribution of calretinin in muscle receptors, it seems likely that calretinin is involved in modulating the responses of the muscle receptors.

The available information requires extensive investigation and a comparison study of the cells expressing Calretinin in different species may lead to the identification of functional specialisations which might help to elucidate the role of this highly conserved protein.

4.4.4. Receptor Distribution

The majority of spindles and tendon organs in the abductor digiti quinti medius (adqm) muscle appear to form a receptor matrix with a consistent anatomical localisation in the muscle periphery. This part of the muscle contains a greater proportion of sensory organs than other muscle regions.

4.4.4a. Receptor Morphology

Muscle spindles and tendon organs were found to be morphologically similar to those observed in other cat limb muscles. The ratio of tendon organs in adqm appeared related to the spindle content, with a mean ratio of spindles to tendon organs 2 : 1. The same ratio has been observed in flexor carpi radialis (Richmond & Stuart, 1985). In other limb, tail and neck muscles the muscle spindles also outnumber tendon organs but by a ratio of 3 : 1 (Barker, 1974; Bakker & Richmond, 1982 and Goldfinger & Fukami, 1982).

i. Types of Intrafusal Muscle Fibres

Several authors have described two morphological types of intrafusal fibres known as bag and chain fibres (Barker & Hunt, 1964, and Bridgman *et al.* 1969). This classification has been extended to three type of fibres, now named bag_1, bag_2 and chain fibres (Barker & Banks, 1994). The number of intrafusal fibres in abductor digiti quinti medius muscle varied from five to nine. Based on the different sizes of the intrafusal fibres and on details of the primary sensory ending I was able confidently to identify bag_1 and bag_2 fibres and chain fibres in the immunohistochemically stained and silver-impregnated materials. The spindles contained up to two bag_1, two bag_2 fibres and seven chain fibres. The presence of multiple bag fibres is a feature that has been noted in the superficial lumbrical muscle (Decorte *et al.* 1990). The extra bag_2 could occur if a primary sensory axon made the initial morphogenetic contact with more than one first generation myotubes, each of which then developed into a bag_2 fibre within the capsule. The occurrence of an extra bag_1 fibre might be due to the associatiation of two second

generation myotubes with the bag_2 fibre during spindle development, both of which then develop into bag_1 fibres (Kucera, 1983).

ii. Distribution of Terminals

In terms of the distribution of the terminals in the bag and chain fibres my results are similar to Boyd, (1962) ; Banks *et al.*(1982) and Banks, (1986). Most of the terminals of the secondary endings were distributed to chain fibres largely in the form of loose widely spaced spirals while the primary endings differed in form on the two bag fibres: in bag_1 fibres the terminals were regularly arranged, wrapped closely together around the nuclear bag and the terminals on the bag_2 fibres were more widely spaced with minimal irregularity at each end. Analysis of the primary endings in the teased spindles revealed that the bag_1, bag_2 and chain fibres were often innervated separately.

iii. Capsule

The spindle capsule is formed from the perineurial epithelium of its nerve supply (Shantha *et al.* 1968), and acted as a barrier preventing the diffusion of the antiserum in the first attempt at staining.

iv. Blood Capillaries

A large blood vessel was seen coursing for a long distance through the capsule into the periaxial space and travelling close to the intrafusal muscle bundle in the equatorial region. The present observation indicates that abductor digiti quinti medius muscle spindles are highly vascular, similar findings have been described in rabbit spindles (Banks & James, 1973). The presence of capillaries within the spindles may be related to the myoglobin content of intrafusal fibres, it is well known that myoglobin either acts as an oxygen store or accelerates the diffusion of oxygen in regions where demand would otherwise exceed supply (James, 1971a).

4.4.4b. Paciniform Corpuscles

The presence of Paciniform corpuscles in muscles has been recognised for nearly 100 years (Ruffini, 1897). Barker, (1962) concluded from counts in teased muscle preparations that Paciniform corpuscles occurred frequently in numbers of 2-12 receptors in each muscle. Richmond & Stuart, (1985) found between 3-20 Paciniform corpuscles in each flexor carpi radialis muscle. In the present study 2-3 Paciniform corpuscles were found in each muscle. Paciniform corpuscles in the abductor digiti quinti medius muscle were usually found in the periphery of the muscle close to the spindle, which differ greatly from the observations of Stacey, (1969), Barker, (1974) and Richmond & Stuart, (1985).

Chapter 5

Conclusions and proposal for future work

The work described in chapter 2 in this thesis was designed to illustrate the early differentiation of intrafusal muscle fibres after sensory neuromuscular contact. Organotypic cultures consisting of spinal-cord and dorsal root ganglia from foetuses together with a piece of muscle from new born rats, were used as a model system to test the hypothesis that intrafusal and extrafusal muscle fibres are both derived from the same population of myotube. As discussed in chapter 2 the cultures were studied in 4 states.

A. In the living state.

B. In the fixed state (light and electron microscopy sections).

C. In stained whole mounts for cholinesterase activity and

D. Labelling with fluorescent DiI dye (intracellular injection).

Three distinguishable cell types occurred in the successful cultures: non-neuronal cells which were pale grey flat cells of various sizes and with no long processes; dorsal-root ganglia cells; and spinal-cord cells, that were differentiated from each other by their shape, DRG cells being rounder and plumper than SC cells, and by the more prominent granules of the cytoplasm of the DRG cells.

The main finding in this part of the study is that the sensory neurons in the embryonic rat dorsal-root ganglia flattened into a single sheet of cells leading to an epithelial-like structure, the cells being clearly distinguishable from other cell types. The muscle fibres differentiated either by proliferation of the muscle pieces from the new born rats, only where they were oriented towards the ventral surface of the cord explant, or from myoblasts of the perispinal myotomes in cultures without co-explanted muscle.

The electron microscopy of the cultures revealed a well-preserved ultrastructure characteristic of dorsal-root ganglia cells. Synapses appeared morphologically normal between neurons and nerve processes and myelination was seen to occur *in vitro*. An attempt was made to demonstrate the enzyme AchE in the sensory neuromuscular junctions that form *in vitro* by the histochemical method of Karnovsky & Roots (1964), or labelling with fluorescent DiI dye. Both methods clearly showed similar results, which were positive in the early stage and negative in the late stage. This study established that in organotypic cultures, sensory neurons mature and differentiate morphologically in a medium without addition of any growth promoting substance or growth factors and the presence of structurally identifiable synapses indicates that other neurons are also maintained in culture and have functional connections.

Based on results presented in chapter 3 a group Ia axon had reinnervated an extensive site probably occupied originally by a primary and a secondary ending, normally such an axon would supply a primary ending alone, which might be the only ending present. Some features of the response were similar to those of a normal secondary ending: correlated with these was the absence of sensory terminals on a particular intrafusal muscle fibre, the bag_1, thought to confer much of the dynamic sensitivity on the normal primary ending. The bag_1 fibre was modified in ways indicating dedifferentiation presumably due to the lack of a connection with the sensory ending. In particular the fibre was of unusually large diameter and the ultrastructural characteristics of the bag_1 fibre from the sensory region of the spindle showed a well developed M-line visible as a band of cross-bridges between thick filaments. In another muscle a group II axon that would normally supply a secondary ending alongside a primary, but here had reinnervated a former primary-endings site. The ending, which was usually small, nevertheless included the bag_1 fibre. The response of the ending showed typically primary-like properties. The intrafusal fibres were structurally differentiated, showing that a group II axon is able to substitute for a group Ia axon in the maintenance of the differentiated state.

The histological analysis of the normal spindle, which was used as a control, showed a close relationship between the primary-ending terminals of the bag_1 and those of a chain fibre, which is very rare in cat spindles. The particular chain fibre was separated from the remaining 3 chain fibres by the bag_1 fibre. The bag_1 and associated chain fibre were innervated by the same first-order branch of the Ia axon. A few terminals on bag_1 fibre were distributed to the chain 1 fibre by cross terminals.

As discussed in chapter 4, my results on the localisation of calretinin in muscle receptors show significant differences from those described by Duc *et al.* (1994), and do not support the suggestion that calretinin plays a role in rapid adaptation. According to those authors calretinin immunoreactivity could not be detected in the subsets of the nuclear bag or nuclear chain fibres, while this study showed different degrees of calretinin immunoreactivity according to the type: the chain fibres were intensely positive, bag_1 and extrafusal fibres were negative and bag_2 fibres intermediate. The sensory terminals of the sensory endings of muscle spindles are wrapped around specialised muscle fibres that form an intrafusal bundle, and it was immediately obvious that unlike the surrounding extrafusal fibres.

In the primary endings, immunoreactivity occurred in the sensory terminals especially those on the bag fibres. Although the secondary endings which are extensively developed only on chain fibres were negative, the sensory terminals of the tendon organs were positively stained, whereas in the study by Duc *et al* tendon organs were said to be negatively stained. In Paciniform corpuscles calretinin immunoreactivity was intensely expressed by all axon terminals of the corpuscles but not by the lamellar cells.

It is clear that there are many aspects of the work described in this thesis which could be expanded in future studies. In particular, investigation of the developmental interaction of nerve and muscle, particularly in the formation of the sensory receptors of muscle. As this technique was new to the department the methods were not producing

sufficient new observations relevant to the initial problem of the sensory innervation of the muscle spindle, because the prospective Ia sensory neurons failed to express their inductive influence. Therefore more work is required in this area.

It has recently been claimed by Duc *et al.* (1994), that calretinin is specifically associated with rapidly adapting mechanoreceptors, among which, the authors of the claim, include the spindle primary ending. It is further claimed that calretinin is somehow involved in the production of rapid adaptation. If so, this could be considered as contrary evidence to the hypothesis of the importance of the intrafusal mechanical propertied in sensory transduction. For this reason, together with the difficulties of describing muscle receptors as either rapidly or slowly adapting, it was necessary to carry out a new study on the distribution of calretinin in muscle.

References

ADAL, M. N. (1969). The fine structure of the sensory region of cat muscle spindle. J. Ultrastruct. Res. 26, 332-354.

ANTAL, M., FREUND, T. F. & POLGÁR, E. (1990). Calcium-binding proteins, parvalbumin and calbindin D_{28K}- immunoreactive neurons in the rat spinal cord and dorsal root ganglia: a light and electron microscopy study. J. Comp. Neurol. 295, 467-484.

ARAI, R., WISKY, L., ARAI, M. & JACOBOWITZ, D. M. (1991) Immunohistochemical localization of calretinin in the rat hindbrain. J. Comp. Neurol. 310, 21-44.

BAIMBRIDGE, K. G., CELIO, M. R. & ROGERS, J. H. (1992). Calcium-binding proteins in the nervous system. Trends. Neurosci. 15, 303-308.

BAKKER, D. A. & RICHMOND, F. J. R. (1982). Muscle spindle complexes in muscles around upper cervical vertebrae in the cat. J. Neurophysiol. 48, 62-74.

BANKS, R. W. (1973). Morphological and histochemical studies on muscle spindles. Ph. D. Thesis: University of Sheffield.

BANKS, R. W. (1986). Observations on the primary sensory ending of tenuissimus muscle spindles in the cat. Cell. Tissue. Res. **246**, 309-319.

BANKS, R. W. & BARKER, D. (1989). Specificities of afferents reinnervating cat muscle spindles after nerve section. J. Physiol. (Lond). **408**, 345-347.

BANKS, R. W., BARKER, D. & BROWN, H. G. (1985). Sensory reinnervation of muscle following nerve section and suture in cats. J. Hand Surg. **10**, 340-344.

BANKS, R. W., BARKER, D. & STACEY, M. J. (1982). Form and distribution of sensory terminals in cat hindlimb muscle spindles. Phil. Trans. Roy. Soc. **B299**, 329-364.

BANKS, R. W., HARKER, D. W. & STACEY, M. J. (1975). Correlation between ultrastructure and histochemistry of mammalian intrafusal muscle fibres. J. physiol. (Lond). 252, 16-17.

BANKS, R. W., HARKER, D. W. & STACEY, M. J. (1977). A study of mammalian intrafusal muscle fibres using a combined histochemical and ultrastructural technique. J. Anat. **123**, 783-796.

BANKS, R. W., HULLIGER, M., SCHEEPSTRA, K. A. & OTTEN, E. (1995a). Pacemaker competition and the role of preterminal-branch tree architecture: a combined morphological, physiological and modelling study. In Alpha and

Gamma motor system. (Eds. A. Taylor ; M. H. Gladden & R. Durbaba). Plenum press: New York 255-260.

BANKS, R. W., GLADDEN, M. H., SUTHERLAND, F. & YOSHIMURA, A. (1995b). The sensory and motor innervation of the abductor digiti quinti medius muscle of the cat. In Alpha and Gamma motor system. (Eds. A. N. Taylor ; M. H. Gladden & R. Durbaba). Plenum press: New York 213-215.

BANKS, R. W. & JAMES, N. T. (1973). The blood supply of rabbit muscle spindles. J. Anat. 114, 7.

BANKS, R. W. & STACEY, M. (1988). Quantitative studies on mammalian muscle spindles and their innervation, In Mechanoreceptors Development, structure and function (Eds P. Hník, T. Soukup, R. Vejsada and J. Zelená), New York: Plenum press, 263-269.

BARKER, D. (1948). The innervation of muscle spindle. Quart. J. Micr. Sci. 89, 143-186.

BARKER, D. (1962). The structure and distribution of muscle receptors. In D. Barker (Ed) : Symposium on muscle receptors. Hong Kong: Hong Kong university press, 227-240.

BARKER, D. (1974). The morphology of muscle receptors In D. Barker, C.C. Hunt, and A. K. McIntyre (Ed): Hand book of sensory physiology III/2, Muscle receptors New York: Springer-verlag, 1-190.

BARKER, D. & BANKS, R. W. (1994). The muscle spindle. In Myology Vol. 1 2nd edition (Eds. A. G. Engel & C. F. Armstrong).Mcgraw hill: New York, 333-360.

BARKER, D., BANKS, R. W., HARKER, D. W., MILBURN, A. & STACEY, M. J. (1976). Studies of the histochemistry, ultrastructure, motor innervation, and regeneration of mammalian intrafusal muscle fibres. In Understanding the stretch reflex (Eds. Homma), prog. Brain Res. 44, 67-88. Amsterdam, Excerpta medica.

BARKER, D. & HUNT, J. P. (1964). Mammalian intrafusal muscle fibres. Nature (Lond). 303, 1193.

BARKER, D. & MILBURN, A. (1984). Development and regeneration of mammalian muscle spindles. Sci. Prog. 69, 45-64.

BARKER, D. & IP, M. C. (1963). A silver method for demonstrating the innervation of mammalian muscle in teased preparations. J. Physiol. (Lond). 169, 73-74.

BARKER, D., SCOTT, J. J. A & STACEY, M. J. (1985). Sensory reinnervation of cat brevis muscle spindles after nerve crush. Brain Res. 29, 131-138.

BARKER, D., SCOTT, J. J. A & STACEY, M. J. (1986). Reinnervation and recovery of cat muscle receptors after long-term denervation. J. Exp. Neurol. 94, 184-202.

BORNSTEIN, M. B. (1966). Observations on mouse nerve tissue in culture. Anat Rec. 148, 362.

BORNSTEIN, M. B., IWANAMI, H., LEHRER, G. M. & BREITBART, L. (1968). Observations on the appearance of neuromuscular relationships in cultured mouse tissue. Z. Zellforsch. 92, 197-206.

BOYD, I. A. (1962). The structure and innervation of the nuclear bag muscle fibre system and the nuclear chain muscle fibre system in mammalian muscle spindles. Phil. Trans. Roy. Soc. B245, 81-136.

BOYD, I. A. (1985). Autonomic control of intrafusal muscle fibres. In The muscle spindle (Eds. I. A. Boyd & M. H. Gladden) Macmillan: London. 145-146.

BRASCHLER, U. F., IANNONE, A., SPENGER, C., STREIT. J. & LÜSCHER, H. R. (1989). A modified roller tube technique for organotypic co-cultures of embryonic rat spinal cord, sensory ganglia and skeletal muscle. J. Neurosci. Methods 29, 121-129.

BRAUN, K. (1990). Calcium-binding proteins in avian and mammalian central nervous system : Localisation, development and possible functions. Progr. Histochem. Cytochem. 21, 1-64.

BRIDGMAN, C. F., SHUMPERT, E. E. & ELDRED, E. (1969). Insertion of intrafusal fibres in muscle spindles of the cat and other mammals. Anat. Rec. 164, 391-402.

BROWN, M. C, BUTLER. R. G. (1976). Regeneration of afferent and efferent fibres to muscle spindles after nerve injury in adult cats. J. Physiol. 260, 25-39.

BUNGE, M. B., BUNGE, R. P., PETERSON, E. R. & MURRAY, M. R. (1967). A light and electron microscope study of long-term organised cultures of rat dorsal root ganglia. J. Cell Biol. 32, 439-466.

BUNGE, R. P., BUNGE, M. B. & PETERSON, E. R. (1965). An electron microscope study of cultured rat spinal cord. J. Cell Biol. 24, 163-191.

CACERES, A., BENDER, P., SNAVELY, L., REBHUN, L. I. & STEWARD,O. (1983). Distribution of the calcium-binding proteins parvalbumin and calbindin-D_{28K} in adult and developing brain tissue. Neuroscience. 10, 449-461.

CAPERS, C. R. (1960). Multinucleation of skeletal muscle in vitro. J. Biophys. Biochem. Cytol. 7, 559.

CASEY, M., HOHN, E. & RICHARD, T. R. (1990). A chemically defined medium for organotypic slice cultures. J. Neurosci. Methods. 32, 63-70.

CARAFOLI, E. (1987). Intracellular calcium homeostasis. J. Annu Rev Biochem 56, 395-433.

CATERINA, M., VALERIE, A. & ENGEL, W. K. (1993). New organotypic model to culture the entire fetal rat spinal cord. J. Neurosci. Methods. 48, 157-167.

CELIO, M. R. & HEIZMANN, C. W. (1981). Calcium-binding protein parvalbumin as a neuronal marker. Nature. 293, 300-302.

COOPER, S. & DANIEL, P. M. (1956). Human muscle spindle. J. Physiol. (Lond) 133, 1-3.

COSTERO, I. & POMERAT, C. M. (1951). Cultivation of neurons from the adult human cerebral and cerebellar cortex. Amer. J. Anat. **89**, 405-467.

CRAIN, S. M. (1966). Development of "organotypic" bioelectric activities in central nervous tissues during maturation in culture. J. Int. Rev. Neurobiol. 9, 1-43.

CRAIN, S. M. (1968). Development of functional neuromuscular connections between separate explants of fetal mammalian tissues after maturation in culture. Anat. Rec. **160**, 466.

DECORTE, L., EMONET-DÉNAND, F. HARKER, D. W. & LAPORTE, Y. (1990). Individual differences in multiple-bag spindles of cat superficial lumbrical muscles. J. Anat. **169**, 1-12.

DISTLER, P. G. & ROBERTSON, R. T. (1993). Formation of synapses between basal forebrain afferents and cerebral cortex neurons: An electron microscopic study in organotypic slice culture. J. Neurocytology. **22**, 627-643.

DRIBIN, L. B. & BARRETT, J. N. (1980). Conditioned medium enhances neuritic outgrowth from rat spinal cord explants. J. Dev. Biol. **74**, 184-195.

DUC, C., WALTER, I. B. & DROZ, B. (1993). Peripheral projections of calretinin-immunoreactive primary sensory neurons in chick hindlimb. Brain. Res. **622**, 321-324.

DUC, C., WALTER, I. B. & DROZ, B. (1994). Innervation of putative rapidly adapting mechanoreceptors by Calbindin and calretinin-immunoreactive primary sensory neurons in the rat. Eur. J. Neuroscience. **6**, 264-271.

EDENDAL, T. (1989). Use of collagen gels to bioassay nerve growth factor activity. In R. A. Rush (Ed), Nerve Growth Factors, John Wiley & Sons Ltd., New York, 81-93.

ELLIOT, B. J. & HARRIMAN, D. G. F. (1974). Growth of human muscle spindles in vitro. Nature. 251, 622-624.

GÄHWILER, B. H. (1981). Organotypic monolayer cultures of nervous tissue. J. Neuroscience. Methods. 4, 329-342.

GÄHWILER, B. H. (1984). Development of the hippocampus in vitro: cell types, synapses and receptors. J. Neuroscience. 11, 751-760.

GÄHWILER, B. H. (1988). Organotypic cultures of neural tissue. Trends Neurol. Sci. 11, 484-490.

GARCIA-SEGURA, L. M., BACTENS, D., ROTH, J., NORMAN, A. W.& ORCI, L. (1984). Immunohistochemical mapping of calcium-binding protein immunoreactivity in the rat central nervous system. Brain Res. **296**, 75-86.

GLADDEN, M. H. (1976). Structural features relative to the function of intrafusal fibres in the cat. Prog. Brain Res. **44**, 51-59.

GOLDFINGER, M. D. & FUKAMI, Y. (1982). Distribution, density and size of muscle receptors in cat tail dorso-lateral muscles. J. Anat. **135**, 371-384.

GOLGI, C. (1880). Sui neri de tendini dell'uomo e di altri vertebratie diun nuovo organo nervaso terminate musculo-tendineo. Mem. Accad. Sci. Torino. **171**, 1-198.

GOTZOS, V., SCHWALLER, B., HETZEL, N., BUSTOS-CASTILLO, M. & CELIO, M. (1992). Expression of the calcium-binding protein Calretinin in WiDr cells and its correlation to their cell cycle. Exp. Cell Res. **202**, 292-302.

GRAY, E. G. (1962). Electron microscopy of synaptic organelles of the central nervous system. In Iv International congress of neuropathology, Vol.II, (Eds. H. Jacob. Stuttgart) Thieme.

HARRISON, R. G. (1907). Observations on living developing nerve fibre. Proc. Soc. Expt. Biol. Med. **4**, 140-143.

HEIZMANN, C. W. & CELIO, M. R. (1987). Immunolocalization of parvalbumin. In Methods of enzymology ,Vol. 39, 552-570. Academic press, New York.

HEIZMANN, C. W. & HUNIZER, W. (1991). Intracellular calcium-binding proteins: more site than insights. Trends Biol. Sci. **16**, 98-103.

HIRANO, H. (1967). A histochemical study of the cholinesterase activity in the neuromuscular junction in developing chick skeletal muscles. J. Arch. Histol. **28**, 89-101.

HOGUE, M. J. (1947). Human fetal brain cells in tissue culture: their identification and motility. J. Exp. Zool. **106**, 85-107.

HONING, M. G. & HUME, R. I. (1989). DiL and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci 12(9), 333-341.

HUNT, C. C. (1990). Mammalian muscle spindle: peripheral mechanism. Physiol. Rev. 70, 643-663.

IP, M. C., VRBOVÁ, G. & WESTBURY, D R. (1977). The sensory reinnervation of hindlimb muscles of the cat following denervation and de-efferentation. Neuroscience. 2, 423-434.

JAMES, N. T. (1971). The histochemical demonstration of myoglobin and succinic dehydrogenase activity in the tibialis anterior muscle of the rabbit. Histochemie. 26, 327-332.

JAMES, D. W. & TRESMAN, R. L. (1968). De novo formation of neuromuscular junctions in tissue culture. Nature. Lond. 220, 384-385.

JAMI, L. (1992). Golgi tendon organs in mammalian skeletal muscle: Functional properties and central actions. Physiol. Rev. 72, 623-666.

JANDE, S. S., MALER, L. & LAWSON, D. E. M. (1981). Immunohistochemical mapping of vitamin D-dependent calcium-binding protein in brain. Nature. 294, 766-767.

KARNOVSKY, M. J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27, 137A.

KARNOVSKY, M. J. & ROOTS, L. (1964). A " direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219-221.

KELLY, A. M. & ZACKS, S. I. (1969). The fine structure of motor endplate morphogenesis. J. Cell Biol. 42, 154-169.

KENNEDY, W. R. & YOON, K. S. (1979). Permeability of muscle spindle capillaries and capsule. Nerve Muscle. 2, 101-108.

KRETSINGER, R. H. (1980). Structure and evolution of calcium-modulated proteins. CRC. CRIT. REV. Biochem. 8, 119-174.

KRETSINGER, R. H., MONCRIEF, N. D., GOODMAN, M. & CZELUSNIAK, J. (1988). Homology of calcium-modulated proteins : Their evolutionary and functional relationships. In M. Morad, W. Nayler, S. Kazda, and M. Schramm (Eds): The calcium channel Structure, Function and Implications. Berlin: Springer- verlag, 16

KUCERA, J. (1980). Myofibrillar ATPase activity of intrafusal fibres in chronically de-afferented rat muscle spindles. Histochemistry. 66, 221-228.

KUCERA, J. (1982). The topography of long nuclear chain intrafusal fibres in the cat muscle spindle. Histochemistry. 74, 183-197.

KUCERA, J. (1983). Multiple-bag-fibre muscle spindles in tenuissimus muscles of the cat. Histochemistry. 79, 457-476.

KUCERA, J., DOROVINI-ZIS, K. & ENGEL, W. K. (1978). Histochemistry of rat intrafusal muscle fibers and their motor innervation. J. Histochem. Cytochem. 26, 973-988.

KUCERA, J. & WALRO, J. M. (1990). Origin of intrafusal fibres in the rat. Histochemistry, 93, 567-580.

KUCERA, J. & WALRO, J. M. (1991). Aggregation of myonuclei and the spread of slow-tonic myosin in developing muscle spindles. Histochemistry. 96, 381-390.

KUCERA, J., WALRO, J. M & REICHLER, J. (1988a). Motor and sensory innervation of muscle spindles in the neonatal rat. Anat. Embryol. 177, 427-436.

KUCERA, J., WALRO, J. M & REICHLER, J. (1988b). Innervation of developing intrafusal muscle fibres in the rat. Am. J. Anat. 183, 344-358.

KUCERA, J., WALRO, J. M. & REICHLER, J. (1989). Role of nerve and muscle factors in the development of rat muscle spindles. Am. J. Anat. 186, 144-160.

KUCERA, J., WALRO, J. M. & REICHLER, J. (1991). Neural organisation of spindles in three hindlimb muscles of the rat muscle spindles.Am. J. Anat. 186, 144-160.

LAERUM, O. D., STEINSVAG, S. & BJERKVIG, R. (1985). Cell and tissue culture of the central nervous system: Recent developments and current applications. J. Acta. Neurol. Scand. 72, 529-549.

LANDON, D. N. (1966). Electron microscopy of muscle spindles. In control and innervation of skeletal muscle (Ed. B. L. Androw), 96-111.

LANDON, D. N. (1972). The fine structure of the equatorial region of developing muscle spindles in the rat. J. Neurocytol 1, 189.

LEHKY, P., BLUM, H. E., STEIN, E. A. & FISHER, E. H. (1974). Isolation and characterisation of parvalbumin from skeletal muscle of higher vertebrates. J. Biol Chem. 249, 4332-4334.

LI, Z., DECAVEL, C. & HATTON, G. I. (1995). Calbindin D_{28K}: role in determining intrinsically generated firing patterns in rat supraoptic neurons. J. Physiology. 488, 3, 601-608.

LOEWENSTEIN, W. R. & SKALAK, R. (1966). Mechanical transmission in a Pacinian corpuscle an analysis and a theory. J. Physiol. 182, 378-386.

MANDYS, V., VAN DER NEUT, R., BÄR, P. R. & GISPEN, W. H. (1991). Cultivation of rat fetal spinal cord slices in a semi-solid medium: a new approach to studying axonal outgrowth and regeneration. J. Neuroscience Methods. 38, 63-69.

MARTINOU, J. C., VAN THAI, A., CASSAR, G., ROUBINET, F. & WEBER, M. J. (1989). Characterisation of two factors enhancing choline acetyltransferase activity in cultures of purified rat motoneurones. J. Neurosci. 9(10): 3645-3656.

MATTHEWS, P. B. C. (1972). Mammalian muscle receptors and their central action. Arnold, Lond

MERRILLEES, N. C. R. (1960). The fine structure of muscle spindles in the Lumbrical muscles of the rat. J. Biophys. Biochem. Cytol. 7, 725-742.

MILBURN, A. (1973). The early development of muscle spindles in the rat. J. Cell Sci. 12, 175-195.

MILBURN, A. (1984). Stages in the development of cat muscle spindles. J. Embryol. Exp. Morphol. 82, 177-216.

MIZE, R. R., LUO, Q., BUTLER, G., JEON, C. J. & NABORS, B. (1992). The calcium-binding proteins parvalbumin and Calbindin-D_{28K} from complementary patterns in the cat superior colliculus. J. Comp. Neurol. 320, 243-256.

MOORE, M. J. (1975). Removal of glass coverslips from culture flat embedded in epoxy resins using hydrofluoric acid. J. Microscopy. 104, 205-207.

NAKAI, J. (1969). The development of neuromuscular junctions in cultures of chick embryo tissues. J. Exp. Zool. 170, 85-106.

NITSCH, R., SERESS, L. & LERANTH, C. (1993). Calretinin-immunoreactivity in the monkey hippocampal formation I . light and electron microscopic characteristics and co-localisation with other calcium-binding proteins. J Neuroscience. 55, 775-796.

OVALLE, W. K. (1971). Fine structure of rat intrafusal muscle fibres. The polar region. J. Cell Biol. 51, 83-103.

OVALLE, W. K. (1972). Fine structure of rat intrafusal muscle fibres. The equatorial region. J. Cell Biol. 52, 382-396.

PALAY, S. L. (1956). Synapses of the central nervous system. J. Biophysic. and Biochem. Cytol. 2, 4 suppl. 193.

PALAY, S. L. (1958). The morphology of synapses of the central nervous system. Exp. Cell Research Suppl. 5, 275.

PARMENTIER, M. (1990). Structure of the human cDNAs and genes coding for Calbindin D_{28K} and Calretinin in R. Pochet, D.E. M. Lawson and C. W. Heizmann (Eds), Calcium-binding proteins in Normal and Transformed cells. New York: Plenum press, 27-34.

PARMENTIER, M. & LAFORT, A. (1991). Structure of the human brain calcium-binding protein Calretinin and its expression in bacteria. Eur. J. Biochem. 196, 79-85.

PEACOCK, J., NELSON, P. G. & GOLDSTONE, M. W. (1973). Electrophysiologic study of cultured neurons dissociated from spinal cords and dorsal root ganglia of fetal mice. J. Develop. Biol. 30, 137-152.

PERSECHINI, A., MONCRIEF, N. D. & KRETSINGER, R. H. (1989). The EF-hand family of calcium modulated proteins. Trend Neurosci. 12, 462-467.

PETERSON, E. R. & CRAIN, S. M. (1968). Reinnervation of denervated mammalian skeletal muscle in vitro. Anat. Rec. 160, 408-409.

PETERSON, E. R. & CRAIN, S. M. (1972). Regeneration and innervation in cultures of adult mammalian skeletal muscle coupled with fetal rodent spinal cord. Exp. Neurol. 36, 136-159.

PETERSON, E. R. & MURRAY, M. R. (1965). Patterns of peripheral demyelination in vitro. J. Ann. N. Y. Acad. Sci. 122, 39.

POPPELE, R. E. & QUICK, D. C. (1985). Effect of intrafusal muscle mechanics on mammalian muscle spindle sensitivity. J. Neuroscience. 5, No.7. 1881-1885

RAUSELL, E. & JONES, E. G. (1991). Chemically distinct compartments of the thamic VPM nucleus in monkeys relay principal and spinal trigeminal pathways to different layers of the somatosensory cortex. J. Neurosci. 11, 226-237.

REN, K., RUDA, M. A. & JACOBOWITZ, D. M. (1993). Immunohistochemical localisation of Calretinin in the dorsal root ganglion and spinal cord of the rat. Brain. Res. Bull. 31, 13-22.

RÉSIBOIS, A., BLACHIER, F., ROGERS, J. H., LAWSON, D. E. M. & POCHET, R. (1990). Comparison between rat brain Calbindin and Calretinin-immunoreactivities. In Calcium binding proteins in normal and transformed cells. (Eds Pochet, R., Lowson, D. E. M. & Heizmann, C. W). New York: Plenum Press, 211-214.

RÉSIBOIS, A. & ROGERS, J. H. (1992). Calretinin in rat brain: An Immunohistochemical study. Neuroscience. 46, 101-134.

RICHMOND, F. H. R. & STUART, D. G. (1985). Distribution of sensory receptors in the flexor carpi radialis muscle of the cat. J. Morphology 183, 1-13.

ROGERS, J. H. (1987). A gene for a novel calcium-binding protein expressed principally in neurons. J. Cell. Biol. 105, 1343-1353.

ROGERS, J. H. (1991). Calretinin: In Novel calcium-binding proteins. (Ed C.W. Heizmann). 251-276. Springer, Berlin.

ROGERS, J. H., KHAN, M. & ELLIS, J. (1990). Calretinin and other CBPs in the nervous system, In R. Pochet, D. E.M. Lawson and C. W. Heizmann (Eds), Calcium binding proteins in Normal and Transformed cells. New York: plenum press, 195-203.

ROGERS, J. H. & RÉSIBOIS, A. (1992) Calretinin and calbindin-D_{28K} in rat brain: patterns of partial co-localisation. J. Neuroscience. 51, 843-865.

RUFFINI, A. (1893). Sur la terminaison nerveuse dans les faisceux musculaires et leur signification physiologique. Archs ital. Biol. 18, 106-114.

RUFFINI, A. (1897). Observations on sensory nerve endings in voluntary muscles. Brain 20 , 368-374.

SAED, H. H. (1990). A study of receptors in jaw muscles of the rat and other vertebrates. Ph.D. Thesis: University of Durham, U. K.

SCHWALLER, B., BUCHWALD, P., BLÜMCKE, I., CELIO, M. R. & HUNZIKER, W. (1993). Characterisation of a polyclonal antiserum against the purified human recombinant calcium binding protein Calretinin. Cell Calcium 14, 639-648.

SHANTHA, T. R., GOLARZ, M. N.& BOURNE, G. H. (1968). Histological observations on the capsule of the muscle spindle in normal and denervated muscle. Acta. Anatomica. 69, 632-646.

SHIMADA, Y., FISCHMAN, D. A. & MOSCONA, A. A. (1969a). Formation of neuromuscular junctions in embryonic cell cultures. Zoology. 62. 715-721.

SHIMADA, Y., FISCHMAN, D. A. & MOSCONA, A. A. (1969b). The development of nerve-muscle junctions in monolayer cultures of embryonic spinal cord and skeletal muscle cells. J. Cell Biol. 43, 382-387.

SLOVITER, R. S. (1989). Calcium-binding protein (Calbindin-D_{28K}) and parvalbumin-immunocytochemistry: Localisation in the rat hippocampus with specific reference to the selective vulnerability of hippocampal neurons to seizure activity. J. Comp. Neurol. 280, 183-196.

SPENGER, C., STREIT, J., BRASCHLER, U. F. & LÜSCHER, H. R. (1989a). Morphological and functional properties of identified motoneurons in

organotypic co-cultures of spinal cord, dorsal root ganglion and skeletal muscle. Eur. J. Neuroscience (suppl. 2), 264.

SPENGER, C., STREIT, J., BRASCJLER, U. F. & LÜSCHER, H. R. (1989b). Morphological and physiological evidence for a functional spinal reflex arc in an organotypic co-culture system. Sco. Neurosci. Abstr. 15. 921.

SPENGER, C., BRASCHLER, U. F., STREIT, J.& LÜSCHER, H. R. (1991). An organotypic spinal cord dorsal root ganglion-skeletal muscle co-culture of embryonic rat. I. The morphological correlates of the spinal reflex arc. Eur. J. Neurosci. 3, 1037-1053.

STACEY, M. J. (1969). Free nerve endings in skeletal muscle of the cat. J. Anat. 105, 231-254.

STOPPINI, L., BUCHS, P. A. & MULLER. D. (1991). A simple method for organotypic cultures of nervous tissue. J. Neuroscience Methods. 37, 173-182.

SZEPSENWOL, J. (1946). A comparison of growth, differentiation, activity and action currents of heart and skeletal muscle in tissue culture. Anat. Rec. 95, 125-146.

SZEPSENWOL, J. (1947). Electrical excitability and spontaneous activity in explants of skeletal and heart muscle of chick embryos. Anat. Rec. 98, 67-85.

TANAKA, H., SAKAI, M. & OBATA, K. (1982). Effects of serum tissue extract, conditioned medium and culture substrata on neurite appearance from spinal cord explants of chick embryo. J. Dev. Brain Res. 9, 390-395.

TAYLOR, A. N. (1974). Chick brain CaBp: Comparison with intestinal vitamin D-induced CaBp. Archs. Biochem. Biophys. 161, 100-108.

TOLAR, M., HANZLIKOVÁ, V. & GUTMANN, E. (1974). The effect of external factors on development of the chick skeletal muscle in vitro. Physiol. Bohemoslov. 23, 182-183.

TOWER, S. S. (1932). Atrophy and degeneration in the muscle spindle. Brain. 55, 77-90.

VAN BREDERODE, J. F. M., HELLIESENEN, M. K. & HENDRICKSON, A. E. (1991). Distribution of the calcium-binding proteins parvalbumin and Calbinidin-D_{28K} in the sensorimotor cortex of the rat. Neuroscience. 44, 157-171.

VENERONI, G. & MURRAY, M. R. (1969). Formation de novo and development of neuromuscular junctions in vitro. J. Embryol. Exp. Morph. 21, 369-382.

WALRO, J. M. & KUCERA, J. (1987). Sharing of sensory terminals between the dynamic bag_1 and static bag_2 fibres in the rat muscle spindle. Brain Res. 425, 311-318.

WASSERMAN, R. H. & TAYLOR, A. N. (1966). Vitamin D3-induced calcium-binding protein in chick intestinal moucosa. Science. 152, 791-793.

WEISMANN, A. (1861). Über das wachsen der quergestreiften. Muskel nach Beobachtungen am Frosh.Z. Rat. Med 10, 263-284.

WILLMER, E. N. (1965). Introduction, In E. N. Willmer (Ed), Cells and tissue in cultures. Methods, Biology and Physiology, vol. 1, Academic Press, London. 1-18.

WINSKY, L., NAKATA, H., MARTIN, B. M. & JACOBOWITZ, D.M. (1989). Isolation, partial amino acid sequence and immunohistochemical localisation of a brain- specific calcium-binding protein. Proc. Natl. Acad. Sci. USA. **86**, 10139-10143.

YAMAMOTO, N., YAMADA, K., KUROTANI, T. & KEISUKE, TOYAMA. (1992). Laminar specificity of extrinsic cortical connections studied in culture preparations. Neuron. 9, 217-228.

ZELENÁ, J. (1957). The morphogenetic influence of innervation on the ontogenetic development of muscle-spindles. J. Embryol. Exp. Morphol. 5, 283-292.

ZIMMER, J. & GÄHWILER, B. H. (1984). Cellular and connective organisation of slice cultures of the rat hippocampus and fascia dentata. J. Comp. Neurol. 228, 432-446.