The development of fusimotor innervation in the cat: an ultrastructural study.

A thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy in the Faculty of Medicine.

by

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This thesis is dedicated to my husband, **Bill** and children,
Catriona, Cameron and **Hugh**.

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Abbreviations.

It is useful to be able to abbreviate some descriptors into initials but this gives problems to the reader so I intend to present this page as a key to all the abbreviations not in common use found in this work.

b1:	bag1 intrafusal fibre
b2:	bag2 intrafusal fibre
ch:	typical chain intrafusal fibre
L.ch:	long chain intrafusal fibre - may be large (L) or thin (T)
caps:	capsule
df:	days foetal
dpn:	days post natal
my:	myelinated
um:	unmyelinated
mep:	motor endplate
a-t:	axon terminal
NMJ:	neuromuscular junction

Summary

1. This study was carried out to investigate if the degree of selectivity of motor innervation to hind limb muscle spindles which occurs in the adult cat is present in the post-natal kitten. Selective axons innervate one type of intrafusal fibre only, while non-selective axons innervate more than one type in the same spindle (Boyd and Gladden 1985).

2. Tenuissimus and peroneal muscle spindle poles from kittens of 50df (n=7, 1dpn (n=14), 12dpn (n=13), 21dpn (n=12) and 28dpn (n=13) were serially sectioned for light and electron microscopy. Ultrathin sections were taken every 2-5μm and semi-thin (1μm) sections collected in-between. If it was suspected that information was lost in the 1μm thick sections these were re-embedded and subsequently ultrathin sectioned. Electron micrographs were taken of the ultrathin sections and from these the spindles were reconstructed so that the lengths of primary and sensory endings, and capsule and intrafusal fibre lengths were estimated. Numbers and degree of myelination of the motor axons were noted. The distribution of motor axons to the intrafusal fibres was determined by following both myelinated and unmyelinated axons through serial sections: this has not been possible previously in such young material. Numbers, lengths, types and distance from the equator of motor endings were calculated.

3. Results of each of the ages studied were compared statistically for each feature studied in order to assess any significant change during this period of development.

4. Bag fibres were distinguished from chain fibres by size and arrangement of central nuclei under the primary sensory endings. The bag fibres were approximately twice the diameter of chain fibres and had their central nuclei

arranged in a group whereas the chain fibre nuclei were arranged in a line. Many intrafusal fibres were found to decrease in cross-sectional area in the equatorial region.

5. Bag1 fibres were distinguished from bag2 fibres by a) the grouping of the bag2 fibre with chain fibres in the equatorial region, and b) the more granular appearance of the bag2 fibre (due to more mitochondria and glycogen inclusions) than the bag1 fibre. Bag fibre cross-sectional area measurements were not consistently different from each other.

6. A formula was constructed in order to determine when any chain fibres should be included as long chain fibres. This was necessary because although the majority of chain fibres were shorter than bag fibres (typical chain fibres), long chain fibres were also found; these can be of a typical chain or bag fibre diameter. Since the total length of spindles increased with age, the absolute length of chain fibres could not be used to discriminate between long and typical chains.

7. Endings were identified as sensory when the muscle basement membrane encompassed the axon terminal. Motor endings were distinguished from sensory endings by the presence of the basement membrane between the axon terminal and muscle surface. Additional features which confirmed the identity were the variety of types of axon terminal vesicle (round, flat, dense cored) found in sensory endings and the position of the endings along the intrafusal fibre. Occasionally the basement membrane was not present between the axon terminals and muscle membrane in motor endings of the 50df spindles.

8. There were 119 motor axons in this study of which 77 were unmyelinated. Of the 59 total spindle poles, 36 were from tenuissimus muscles and 23 from peroneal muscles. Four tandem spindles were present in this material. There were 55 bag1 fibre poles, 66 bag2 fibre poles, 240 typical chain poles and 20 long chain poles of either large or thin diameter. In total there were 586 motor endings.

9. The length of the primary and secondary sensory endings, capsule and intrafusal fibres increased with age.

10. Motor axons.

It was not possible to categorise the motor axons as γ or β.

The numbers of motor axons supplying spindle poles was found to be 2-3 for each age group. Numbers of motor axons supplying individual intrafusal fibre poles were, 1-2 for bag fibres and 0-1 for chain fibres, both typical and long. These figures are in the same range as the numbers of γ axons which supply individual fibres of adult spindles. Polyneuronal innervation of the type where many axons supply a single motor endplate therefore does not occur during the period studied, but does in rat spindles, even after birth (Kucera, Walro and Reichler 1988).

The distribution of motor axons to the intrafusal fibres, even as late in development as 28dpn, was less selective than in adult spindles. Myelination of motor axons was 92% complete by 28dpn. The selectivity did not depend on myelination prior to 21dpn but myelinated axons had a smaller proportion of inappropriate innervation by 28dpn.

All bag2 fibres, 90% of bag1 fibres and 76% of typical chain fibres had some motor innervation by 28dpn. These values were very similar to adult innervation values. The innervation of long chain fibres was more variable in the young but they tended to be innervated when present, whereas only 25% are innervated in the adult.

11. Motor endings.

a) Numbers

Although the number of motor axons remained constant at 2-3 for each whole spindle pole, the mean number of motor endings per spindle pole decreased from 12 (range 3-19) at 12dpn to 8 (range 1-14) at 28dpn.

Non-selective axons tended to have more motor endings for each axon than selective axons. Axons selective to b1: numbers of motor endings increased with age. (mean values from 1 to 3.5). Axons selective to b2: numbers of motor endings decreased with age, (mean values from 4 to 1). Axons selective to typical chain fibres: numbers of motor endings did not change significantly throughout the study, (mean values of 2.5-3). Axons ending on b2+chain fibres: numbers of motor endings peaked at 12dpn, (mean value 8.7). Axons to other combinations of intrafusal fibres: numbers of motor endings were consistently high, (mean values of 5 to 7).

Despite the decrease in total number of motor endings, the distribution also varied with the intrafusal fibre type; numbers of endings on bag2 fibres decreased from an average of 4 at 50df to 2 at 28dpn. Numbers of endings on the bag1 fibre showed no significant change throughout the study, (mean value 3). Typical chain fibres had a peak number of motor endings (1.5) at 12dpn. Long chain motor ending numbers decreased from means of 4 to 2 by 28dpn.

b) Length.

The mean lengths of motor endings of the whole sample increased with age. Lengths of endings peaked on the b1 fibre at 12dpn. Surprisingly, lengths of motor endings of axons selective to bag1 fibres remained constant. Axons selective to b2 and chains had motor endings that increased in length with increased developmental age.

Motor endings at the termination of myelinated axons were not significantly longer than those of unmyelinated axons. The lengths of motor endings of unmyelinated axons reached a peak length at 12dpn.

c) Type.

The endings were arranged in categories from post-synaptic features according to the classification of Arbuthnott et al, 1982, into ma (superficial), mb (indented) and mc (complex protrusions). Intermediary categories of maf, mab,mabf, mbf and mac were introduced to accommodate the many endings of intermediate forms found. The type of motor endings found in this period of mainly post-natal development were predominantly of the simplest or ma-types for motor axons ending on all types of intrafusal fibres. There was a slight move towards two populations of ending type on typical chain fibres: complex mc-type endings and simple ma-type endings by 28dpn.

In adult spindles, type ma endings are found on both bag2 and chain fibres; type mb are found on bag1 fibres and type mc are found on chain fibres. Arbuthnott et al (1982) also found an md type ending on a long chain fibre. No md endings were found in the present study.

In developing spindles it was found that any intrafusal fibre could support several types of motor ending and any one motor axon could have several types of motor ending.

d) Distance from the equator.

The distance of the motor endings from the equator increased as the animals grew and each part of the spindle was expanded. This was unrelated to its type or its length for all ending types on all intrafusal fibres.

12. One interpretation of these data is that the motor innervation of developing spindles has no fibre specificity before 12 postnatal days. However the pattern of innervation of bag1 fibres suggests that it may receive fibre-specific innervation before this stage, possibly the dynamic γ or β innervation, as well as the random innervation received by the other fibres. In either model, fibre-specific connections would emerge to produce an adult pattern of intrafusal fibre motor innervation, but it is clear from this study that the process is still not complete by 28dpn.

Introduction

Many years of research have led to the conclusion that in adult cats the motor innervation to hind limb muscle spindles is in some measure selective to the different intrafusal fibres. Intrafusal fibres already have their adult myosin staining characteristics from 5 days before birth (Rowlerson et al 1985: Rowlerson 1987 a, b) and motor endings can be observed in muscle spindles in 34-38day foetal cats (Milburn 1984). The present study was undertaken to observe with electron microscopy whether the adult pattern of motor innervation is established from the beginning or whether remodelling is necessary.

The structures in skeletal muscle first described by Kolliker (1862) and named muscle spindles by Kuhne (1863) are now known to be sense organs which respond to muscle length and changes in muscle length. However it was not until Sherrington (1894) characterised the structure, and the histologist Ruffini (1898) stained the nerves that the study of the muscle spindle as we know it to-day began. Prior to this, muscle spindles were alternately thought to be concerned with the generation of skeletal muscle fibres, or a focus of inflammation!

The mammalian muscle spindle is composed of a bundle of intrafusal fibres, these being surrounded by a cell and connective tissue structure, the capsule. The capsule surrounds the central region of the intrafusal fibres, about 1mm long, where it is expanded (the equatorial region) and filled with fluid (the fluid space), of a mucopolysaccaride nature. The capsule closely surrounds the intrafusal fibres for about 1mm on either side of the equator (the sleeve region).

Intrafusal fibres fall into two groups, chain and bag fibres, established from histology by Boyd 1962, in cats, and by Cooper and Daniel 1963, in humans. Bag fibres are large in both diameter (16-20μm) and length (up to 10mm in total length) and there are usually two of them in tenuissimus muscle spindles. Chain fibres are small (8-10μm diameter) and between 2 and 10 in number. Although there is a size difference, the distinction refers to the arrangement of the central nuclei, there being a single line or chain of nuclei in the equatorial region of the chain fibres (Boyd 1960) and in the bag fibres the nuclei are in a group or bag (Barker 1948). The majority of chain fibres with a diameter approximately half that of the bag fibres end within the spindle capsule or protrude for only a short distance outside and these are termed `typical' chain fibres. In some instances the chain fibre extends far out beyond the sleeve region and is comparable in length with the bag fibres; hence it is called a long chain fibre. (Barker et al 1976; Kucera 1980a, 1984a and b; Kucera and Hughes 1983). Chain fibres with intermediate lengths have also been observed (Kucera 1980b, 1982a). Some long chain fibres remain as thin as typical chain fibres, while others become comparable in thickness with that of the bag fibres.

Innervation of muscle spindles in adult cats.

1) Sensory innervation.

When Ruffini described his muscle spindles he not only saw the muscle fibres but excitingly he could draw three types of nerve endings. (fig. 1a). In the equatorial region he always saw one large diameter myelinated axon enter the capsule with or without other smaller diameter axons. This is the sensory region and one of these large diameter axons has ramifications which wrap themselves around the equatorial region of all the intrafusal fibres. Ruffini

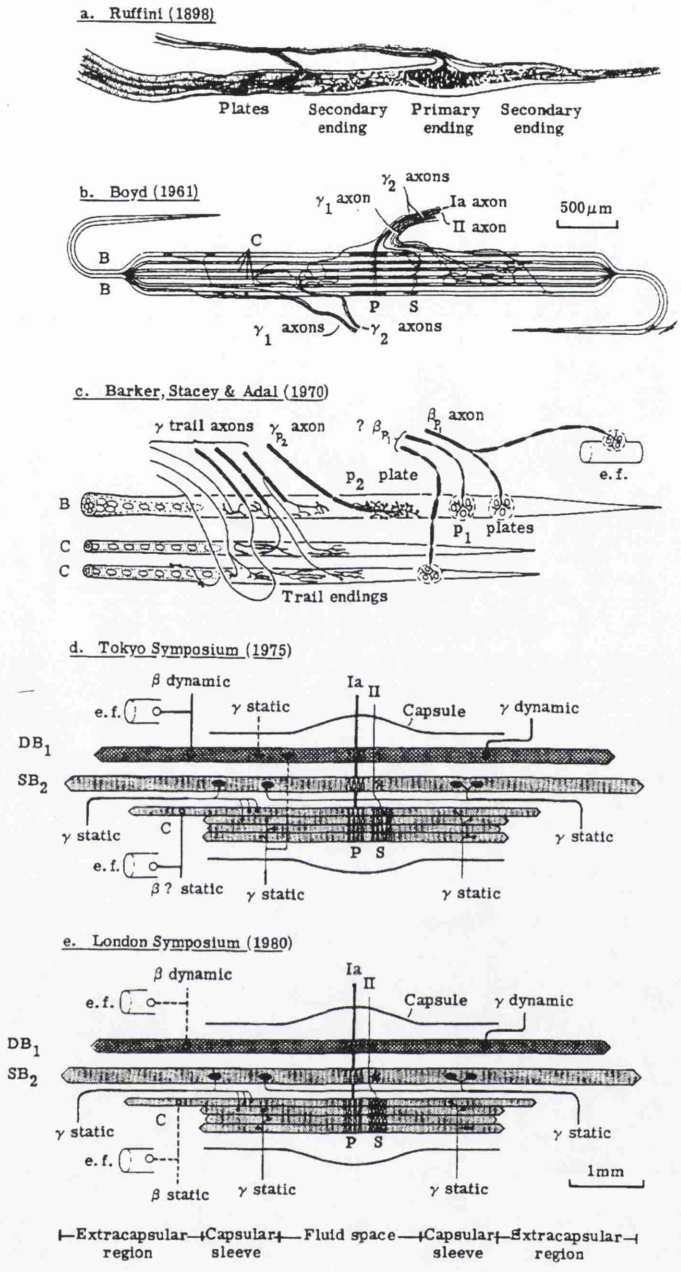


Figure 1

Progress in understanding of the motor innervation of mammalian muscle spindles

a) Ruffini (1898) Primary ending, secondary ending and plate endings on intrafusal muscle fibres.
b) Boyd (1961) Selective motor control of two large nuclear bag intrafusal fibres (B) by γ_1 axons to γ_1 end-plates, and of four or five small nuclear chain fibres (C) by γ_2 axons to γ_2 network small nerve endings.
c) Barker, Stacey and Adal (1970). Non-selective motor control of nuclear bag fibres (B) and nuclear chain fibres(C) by three types of motor nerve ending: p_1 plates, p_2 plates and trail endings.
d) Tokyo Symposium (1975) Motor innervation of two types of nuclear bag fibres (B_1 & B_2) by dynamic and static fusimotor axons. Motor connections shown by continuous lines agreed by Boyd and Barker. Additional static γ connections to bag_1 fibre postulated by Barker shown in broken lines.
e) London Symposium (1980) Agreed that motor innervation of dynamic bag_1 fibre (DB_1) is usually by dynamic γ and β axons only. Agreed that static γ axons innervate static bag_2 fibre only, chain fibres only or both together. Dynamic and static β connections in some spindles only (broken lines).

(from Boyd, 1981, Sci. prog Oxf., 67, p208/9)

called this the primary sensory ending which has a group 1a afferent nerve fibre (Matthews 1972, Barker 1974), which travels unbranched from the spindle to the dorsal root ganglion. There may be two or three secondary endings and these are positioned on either side of the primary ending and are numbered for convenience outward from the primary ending (S1, S2 etc.)(Boyd 1962). Secondary sensory endings lie predominantly on the chain fibres with a more dispersed arrangement called a flower-spray ending. The primary sensory ending is usually described as a spiral as it appears to be wound around all the intrafusal fibres, (Banks et al 1982). There has been discussion recently as to whether this may not be a spiral but a rod with projecting ribs. This idea has been pursued by Patak et al (1992) histologically on teased silver-stained preparations where they did not necessarily encounter a complete spiral around the intrafusal fibres. Gregory and Proske (1987, 1988), found differences in compliance of the central region of the muscle spindles when comparing the immature and adult spindles to responses to succinyl choline and stretch. These physiological differences may be due to structural differences in the connection of the primary sensory ending, especially during development.

The opening of the research on the functional properties of the primary and secondary receptor endings was due to Cooper who in 1961 classified functionally single group 1a or group II nerve fibres on the basis of their conduction velocities. Her work demonstrated that group Ia fibres were more sensitive to the velocity of muscle stretch than the group II fibres. This work has been confirmed by others and reviewed by Matthews 1962. The function of the secondary sensory ending is to signal static lengths (Matthews and Stein, 1969).

2) Motor innervation

a) γ fusimotor innervation

Ruffini also described a third type of ending which lay further away from the sensory endings in the sleeve region of the spindle fibres. These he identified as motor "plates" and they are the terminations of the smallest motor axons with the slowest conduction velocities (15-55 m/s), the γ axons. These axons have terminations (fusimotor endings) only on the muscle spindle intrafusal fibres. It is now known and in fact could be inferred from Ruffini's drawings that branches of axons supplying extrafusal fibres also innervate the muscle spindles, usually quite far out in the polar region. These were named ß axons because they were thought to have intermediate conduction velocities (60-90m/sec).

The function of the 'plate' type of ending described by Ruffini was not known for many years. Leksell (1945) and Hunt and Kuffler (1951) started the elucidation of the fusimotor control of the muscle spindle. These authors showed that selective stimulation of the small γ motor neurones caused excitation of the spindle afferents. Using a powerful technique of recording from single afferent fibres while simultaneously stimulating single efferent fibres, Hunt and Kuffler showed that fusimotion is the exclusive function of the γ motoneurones. Tetanic stimulation of these efferent fibres provoked a striking excitation of spindle afferents without causing any measurable tension in the parent muscle.

Individual γ axons were called either static γ or dynamic γ fibres depending upon whether they decreased or increased the size of the dynamic response of primary afferents to ramp stretches, Matthews, 1962. (The static response is

defined by Jansen and Matthews, 1962, as the frequency of firing 0.5 sec after completion of the dynamic phase of extension. The dynamic response is defined as the difference between the discharge frequency immediately before the end of the dynamic phase of extension and the static response.) This was later confirmed by Crowe and Matthews 1964; Brown et al 1965; Appelberg et al 1966 and Bessou et al 1966.

By 1962 it was becoming clearer that the large numbers of motor endings observed by Ruffini could be reduced to two main categories (Barker 1962, Boyd 1962) but the definitions were not the same in both camps and would not be resolved for more than two decades! The discrete "plate" endings were thought to occur largely (Boyd 1962) but not exclusively (Barker 1962) on the bag intrafusal fibres, while the diffuse ramifications type of motor ending was according to Boyd (1962) restricted to the chain fibres (diffuse γ network). (fig. 1b). Crowe and Matthews 1964 and Matthews 1964 proposed a scheme of independent selective control of the two types of intrafusal fibres, to explain the physiological findings. Dynamic γ axons would selectively innervate the bag fibres having plate endings on those intrafusal fibres. By contrast the static γ axon would innervate the chain fibres having the diffuse or "trail" ending (Barker 1962).

From the beginning this scheme was challenged. Barker and Ip (1965) maintained that the γ axons could terminate on either bag or chain fibres with plate endings. The same was true for the trail endings, and so a major difference arose between those who believed in a selective innervation of the muscle spindle intrafusal fibres and those who did not. Further research into the morphological characteristics of the motor endings showed from light and

electron microscopy that there were two types of motor plate ending; namely p1 and p2 (Barker 1966; Barker et al 1970) as seen in fig 2. Confusion increased in trying to categorise the motor endings when Barker found p1 plates on chain fibres as well as on bag fibres. When a nerve is cut, the nerve endings degenerate at rates proportional to the diameter of the supplying nerve fibre and can be used to distinguish between sources of α and γ fusimotor supply. Experiments indicated that the p1 plates were terminations of ß axons and so leaving p2 plates and trail endings as the terminations of γ axons. This again conflicted with Matthews' scheme, as ß axons were considered, up to that time, to have purely dynamic fusimotor action.

Another problem was to be thrown into the arena when histochemical techniques, used to characterise extrafusal skeletal muscle, were applied to the intrafusal fibres. The nuclear bag fibres were found to show different properties with regard to their content of oxidative enzymes and myofibrillar ATPase (Barker and Stacey 1970; Banks 1971; Ovalle and Smith 1972). Ovalle and Smith named bag fibres with relatively low ATPase activity after acid pre-incubation, bag1 and those with relatively high ATPase activity after acid pre - incubation, bag2.

ATPase activity had been found to be linked to speed of contraction of the muscle (Barany 1967). The mechanical properties of the bag fibres could be divided into two groups, those with a slow contraction velocity (the 'dynamic' bag fibre) and those with relatively fast contraction velocities (the 'static' bag fibre). This work was done in 1976 by Boyd. In 1977 these results were confirmed by Gladden, after which the dye Alcian Blue was injected into the dynamic bag fibre and noting that the slower contracting bag fibre was

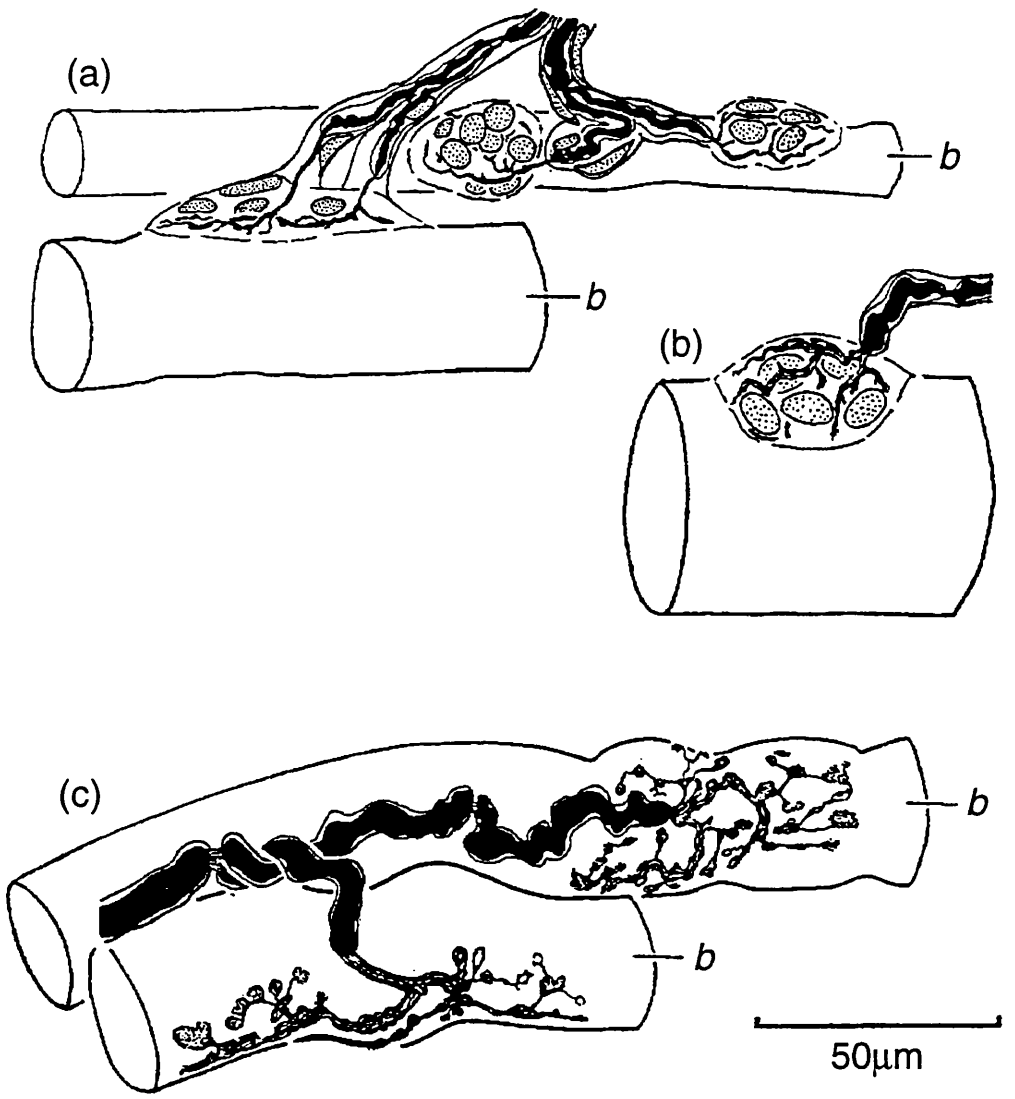


Figure 2

Drawings of plate endings traced from photographs of teased, silver preparations of de-afferentated spindles.

(a) Three p_1 plates supplying a pes interosseous spindle.
(b) An extrafusal motor endplate from the same muscle as in (a) for comparison.
(c) Two p_2 plates supplying a pes interosseous spindle.

b: nuclear bag muscle fibre

Nucleii have been omitted from (c).

(*adapted from Barker, Stacey and Adal, 1970, Phil. Trans. p330.*)

stained. Further observations at the poles of the two longest fibres, showed significantly more elastic fibres surrounding the longest bag fibre (Gladden 1976), which was found to be the faster contracting fibre. This fibre was also the darker stained fibre with the myosin ATPase reaction. This is therefore called the static bag2 fibre shortened nowadays to bag2 fibre. At this stage, Boyd (1976a) was sure that the different responses of the bag fibres to γ axon stimulation were due to the mechanical properties of the two fibre types.

There have been four main lines of work with the aim of clarifying the motor innervation of the muscle spindle;

1. chronic denervation sparing only a single γ static axon (Barker et al 1973) with histological follow-up to determine what fibres that axon supplied;
2. application of the glycogen-depletion technique of Edstrom and Kugelberg 1968 to the muscle spindle; this marked the intrafusal fibres operated by identified γ axons (Brown and Butler 1973);
3. visual observations on isolated spindles of intrafusal fibre movement provoked by stimulation of individual γ fibres (Bessou and Pages 1975); (Boyd et al, 1975, 1977).
4. intracellular recording from muscle fibres with subsequent histological identification (Barker et al 1973; Gladden, 1976)

Even by 1973 the evidence seemed to support the view that static γ axons innervated both bag and chain fibres non-selectivity, whereas dynamic γ axons were specific for bag fibres. Supporters of the glycogen depletion method were the last to be convinced that the motor innervation of the spindle was in any respect selective, as stimulation of the dynamic γ axon always depleted the bag1 fibre but stimulation of the static γ axon could give depletion of the chain fibres as well as a bag fibre, the bag1 being as often depleted as the

bag2. However, against this evidence, cinematographic observations of isolated muscle spindles during stimulation of known γs, γd and βd axons (Bessou and Pages 1975 and Boyd et al 1977) were firmly in favour of specificity. With regard to the intrafusal fibre a single fibre was never observed to be operated by both a static and a dynamic γ axon.

By 1980 selective innervation received further support when several sets of evidence were presented at the Muscle Receptors and Movement Symposium in London (fig. 1e). Gladden confirmed that dynamic γ axons gave rise to local responses in the bag1 fibre only ; she also demonstrated that static γ axons gave rise to local responses in the bag2 fibre or to propagated action potentials in chain fibres; stimulation of static axons never depolarised the bag1 fibre. Barker et al, using serial reconstruction and teased silver stained spindles, reported that they never found the axons traced to the bag2 and chain intrafusal fibres to have connections on the bag1 fibre. They reported one instance of a γ axon (presumed dynamic) which innervated a bag1 fibre along with a chain fibre but otherwise the bag1 fibre had selective motor innervation. Boyd also showed on video in detail the three types of intrafusal fibre with their distinct motor innervation and how the response of the primary and secondary endings depended on the mechanical properties of each of the three types of intrafusal fibre when individual γ axons were stimulated. The expression of the mechanical response clearly depended on selective innervation of each fibre type.

With the weight of the above evidence it was concluded that the glycogen depletion method gave artefacts especially concerning the bag1 fibre results (Decorte et al 1984). Laporte's group has shown that static γ axons could

deplete the glycogen stores of the bag1 fibre even though there were no connections on to that intrafusal fibre! Why this should occur is a puzzle. Perhaps activity in adjacent intrafusal fibres could affect the bag1 fibre mechanically causing depletion of already low levels of glycogen.

b) β fusimotor innervation

Some spindles receive skeleto-fusimotor innervation or ß innervation also. These were first identified physiologically by Bessou et al (1963) and histologically by Adal and Barker (1965). The response of the primary sensory ending to the ß innervation can be dynamic or static. Boyd et al showed (1975 and 1977) that the intrafusal fibre innervated by the dynamic ß axon was the same as that innervated by the dynamic γ axon namely the bag1 fibre. Barker, Laporte and colleagues in 1977 confirmed by glycogen depletion the almost exclusive distribution of dynamic ß axons to the bag1 fibre. Dynamic ß innervation occurs in 30 - 70% of spindles depending upon the muscle. Harker et al, (1977) found some of the fast conducting ß axons innervating the longest chain fibres far out into the pole. Jami and colleagues (1979) observed these ß axons to have a static action on the primary sensory ending and so also established a ß static action in spindles although this is more rare than the ß dynamic innervation.

It was agreed by the early 1980's (Fig. 1e) that the dynamic intrafusal system, comprising the bag1 fibre and its controlling dynamic γ and dynamic ß axons, was largely independent of the static intrafusal system of the bag2 and chain intrafusal fibres controlled by static γ axons either independently or together. Attention will now be focused on the form of the connections between these types of axons and specific categories of intrafusal muscle fibre.

3) Form of the motor endings.

As mentioned previously, Barker and his colleagues (1970) classified the intrafusal motor endings into p1 and p2 plates, and trail endings (fig 2) in teased silver preparations of cat muscle spindles. It was also shown that types of motor endings could be distinguished by differing rates of degeneration after nerve section. The p1 plates degenerated at the same rate as extrafusal endplates, and were therefore presumed to be β-innervated. The same types of motor ending could also be found in other mammals; rat (Gladden 1969), rabbit (Barker and Stacey 1970b;) and man (Kennedy 1970; Swash and Fox 1972). At that time, of course, spindles were still thought to be composed of only two types of intrafusal fibres, bag fibres and chain fibres. Since then Barker and his colleagues have re - examined their work in the light of the three intrafusal fibre classifications and have re-affirmed that p1 plates are the terminations of ß axons and p2 plates and trail endings are the terminations of dynamic and static γ axons respectively.

In identifying the form of the motor endings post-synaptic and pre-synaptic features were considered. In the early work of Barker et al (1970), most emphasis was placed on the pre-synaptic features including the pre-terminal axons. This was in part because only light microscope preparations were available, stained with gold or silver salts in which the pre-synaptic features were more obvious. Also, as previously mentioned, at this time it was thought that the spindle muscle fibres were only of two types; bags and chains. After the work of Ovalle and Smith (1972) in separating bag fibres into bag1 and bag2 Banks et al (1985) re-examined the motor endings of the 1970 paper in the light of the three types of intrafusal muscle fibres. Their conclusions remained essentially unchanged i.e. that p1 plates (ß), were found on bag1,

long or intermediate chains, p2 plates (dynamic γ) were found on bag1 when all ß endings had degenerated following nerve section, and trails (static γ) were found on both bag2 and typical chains. Barker made it clear that his classification was based solely on pre-synaptic features and that there may have been some overlap in the categories.

The trail endings were described as ramifications of the γ axons that 'trail' along the surface of the bag or chain fibre, often for a hundred or more micrometers. Their terminations were simple, on the surface of the muscle fibre or more complex, having brush-like tapers, knobs or hooks. In the more recent work (Banks et al, 1985) the term' trail-plate' was offered as the area occupied by the trail ramifications, which was a discrete entity.

The p2 plates of Barker et al (1970), and Banks et al (1985) were also described as having knobs, rings or less frequently tapers and the ending was closely applied to the surface of the muscle. This made it appear very similar to the trail ending and indeed the authors stated that the p2 plates could be confused with the trail endings especially at the polar end of the trail ramification!

The p1 plate resembled an extrafusal end plate having axon terminals of mainly tapers located on a sole plate with a Doyère eminence (the muscle surface was raised).

The p2 plate is in total smaller than the p1 plate or trail ending and can be considered more discrete. Despite Barker's claim that his criteria were based on only pre-synaptic features, it seems that most of the distinction between p1

and p2 plates is the presence or otherwise of the raised sole plate of the post-synaptic muscle membrane (fig. 2).

The post-synaptic features that Banks, Barker and Stacey, (1985) now consider as important are indentation of the motor terminals into the muscle membrane and the way in which this varies with the distance of the ending from the middle of the sensory region of the spindle. However their conclusion that on all types of fibre pole the more polar the ending, the more indented are the terminals removes its usefulness as a distinguishing criterion between motor ending types.

During the 1970's and 1980's, Kucera used serial sections stained to show cholinesterase activity. He described two types of motor ending on cat intrafusal muscle fibres as simple `rim' type endings and larger more complex ' plate' endings. (Kucera, 1980b; Kucera 1982a; Kucera 1982b). He considered these to be the trail and plate type endings, respectively, described by Barker. Both Kucera (1982b: Kucera and Walro 1986) and Barker (1966) agreed that chain fibres had on average one plate per pole: as the axons supplying these plates often branched profusely, the plate endings of Kucera and the trail endings of Barker could be equated. However Kucera indicated that there were possibly more than two types of plate motor endings in spindles and further subdivided his 'plate' endings according to their staining intensity and length. He proposed that the intrafusal fibre as well as the motoneurone may have a strong influence in determining the form of the motor ending, since he could recognise numerically more ending types than the physiological categories of motor axon known to exist.

The rim endings of Kucera were thought to be autonomic due to their simplicity and absence from some spindles, (Barker et al 1980). Kucera, Dorovini-Zis and Engel (1978) described 'diffuse' endings in rat spindles stained for cholinesterase activity ; these seemed to be homologous with the 'rim' endings in the cat of Kucera and therefore could not be identified as the trail endings of Barker.

Most of this work has been done by light microscope observation and it was not until the mid 1970's that the ultrastructural form of the various motor endings could be studied with the electron microscope which meant that both pre- and the post-synaptic structure could be observed. When considering the synaptic structure the question of whether the motor axon or the intrafusal muscle characteristics have a greater influence on the form of the motor ending generated considerable debate. At first Barker thought that the former was more important (Barker et al 1970) but later work convinced them that muscle fibre type and distance from the equator were the determining factors (Barker et al 1976). Studies by Banks 1981, 1983 and Kucera (1980b, 1981, 1982b) have confirmed these latter findings.

Arbuthnott et al (1982) carried out careful ultrastructural studies on the form of the motor endings in the cat tenuissimus muscle spindles; these spindles were first examined by electrophysiological methods to classify the fusimotor innervation, and identify the intrafusal fibres upon which each γ axon terminates. From the above work a new classification of motor endings emerged. All terminations of motor axons were called 'plates'; ma plates (found on bag2 and chain fibres): mb plates (found on bag1 fibres only): mc plates (found only on chain fibres): md plates (found rarely and only far out in

the pole of chain fibres). Drawings of all the possibilities are found in fig. 3. which shows the total cross section of the muscle fibre and axon terminals. Note that at the time of publication of this figure (1982), the nomenclature of 'dynamic' bag 1 fibre and 'static' bag 2 fibre were still in use. The ending types were also equated with the physiological function of the axons which supplied them. Thus mb endings on bag1 fibres were the endings of dynamic γ axons; if γd axons also supplied chain fibres the ending was an mc type; static γ axons ended in ma or mc plates on chain fibres and ma plates on bag2 fibres; md endings were thought to be the ending of a β static axon.

Classification of motor ending types found.

This classification is described in more detail since the same features are recognised in developing spindles. The definition of each ending type is now described with reference to figs. 3 and 4.

ma endings are seen on both bag2 and chain fibres and are the simplest of all. The axon terminals lie superficially on the surface of the intrafusal muscle fibre which may or not have a few shallow, rudimentary folds beneath the terminals or immediately adjacent to them. These could be equated with the trail endings or plates of Barker.

mb endings are only found on bag1 fibres whether innervated by γ or ß axons they are the most deeply indented into the muscle surface, often with a certain amount of folding of the post-synaptic membrane associated with them. These plates could possibly be equated with the p2 plates of Barker. Sometimes a few of the axon terminals are not fully indented into the muscle surface; these

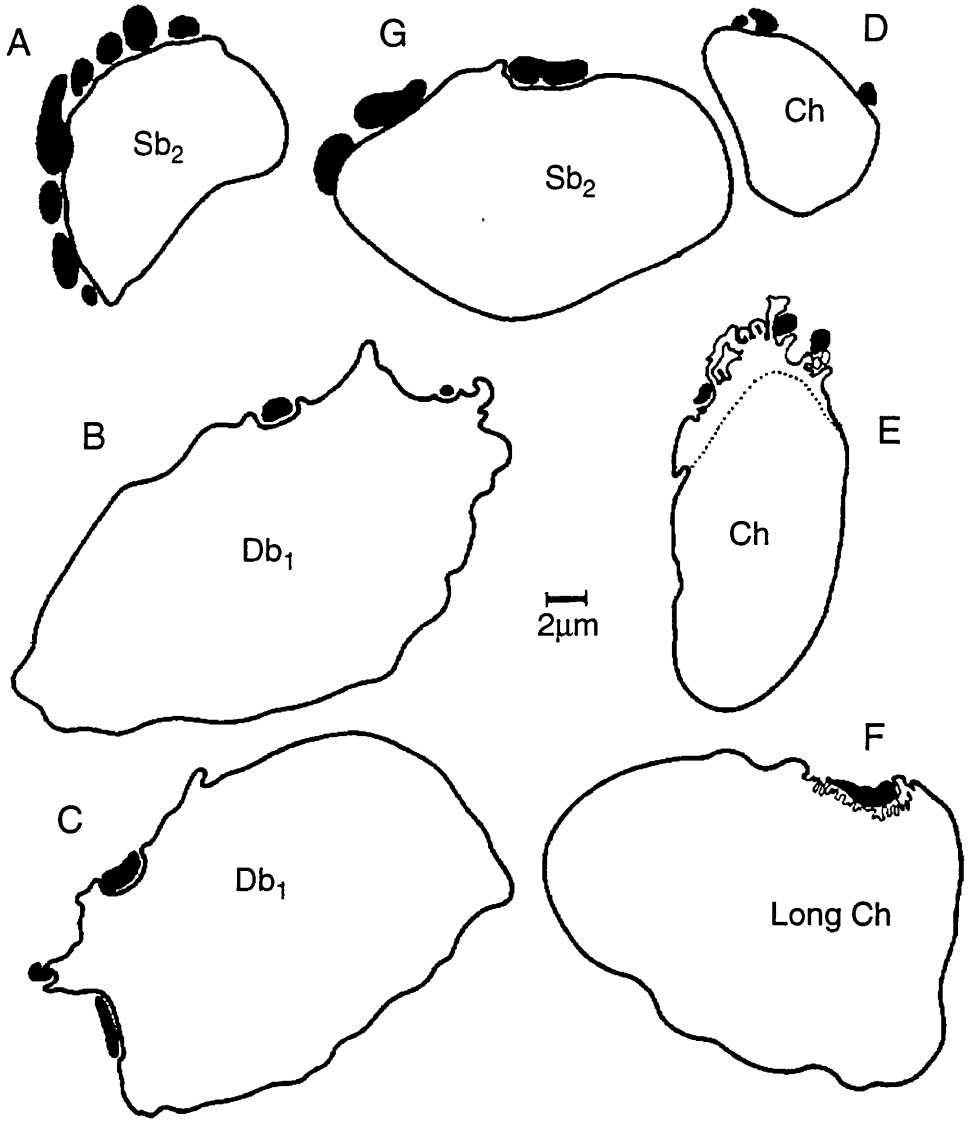


Figure 3

Tracings of low power electron micrographs of entire static bag_2 fibres (Sb_2), dynamic bag fibres (Db_1) or chain fibres (ch) to show the relation of the axon terminals in a single transverse section of a motor plate to the entire intrafusal fibre.

A: m_a plate on static bag_2 fibre (static γ axon).
B: m_b plate on dynamic bag_1 fibre (dynamic γ axon).
C: m_b plate on dynamic bag_1 fibre (dynamic β axon).
D: m_a plate on chain fibre (static γ axon).
E: m_c plate on chain fibre cut obliquely (dynamic γ axon); dotted line shows boundary of myofibrils beneath raised plate.
F: m_d plate on long chain fibre (? static β axon).
G: m_{ab} plate on static bag_2 fibre (presumed static γ axon).

(*adapted from Arbuthnott et al, 1982, J. Physiol. 331, p293.*)

were initially called mab endings but this distinction has been discarded (fig. 4).

mc endings are the most complex as they have the muscle surface thrown up into finger-like folds between and on which the axon terminals are located. They are only found on chain fibres, are rarely found in the same pole as an ma ending (Arbuthnott et al 1992) and are also the shortest.

md endings are very rarely found and closely resemble extrafusal motor endings with their axon terminals deeply indented into the post-synaptic muscle membrane which is heavily folded, the folds often being branched with fused basal lamina. These are thought to be the endings of ß axons and may correspond with the p1 plates.

Because this work (Arbuthnott et al 1982) was correlated with the electrical stimulation of fusimotor axons which resulted in foci of sarcomere convergence on the intrafusal fibres, there is strong evidence linking mb plates with dynamic axons ending on the bag1 fibre selectively, ma plates as the terminations of static axons on both bag2 and chain fibres selectively or non-selectively and mc endings as terminations of other static axons selective to chain fibres only. It was suggested that md endings are the endings of ß axons on long chain fibres, because of their similarity to endings on extrafusal fibres, but there was no direct physiological evidence for this.

Arbuthnott et al (1992) have gone further in their analysis of the morphometry of the static γ innervation. They devised a measurement to compare the length of the periphery of the chain and bag2 intrafusal fibres beneath the motor

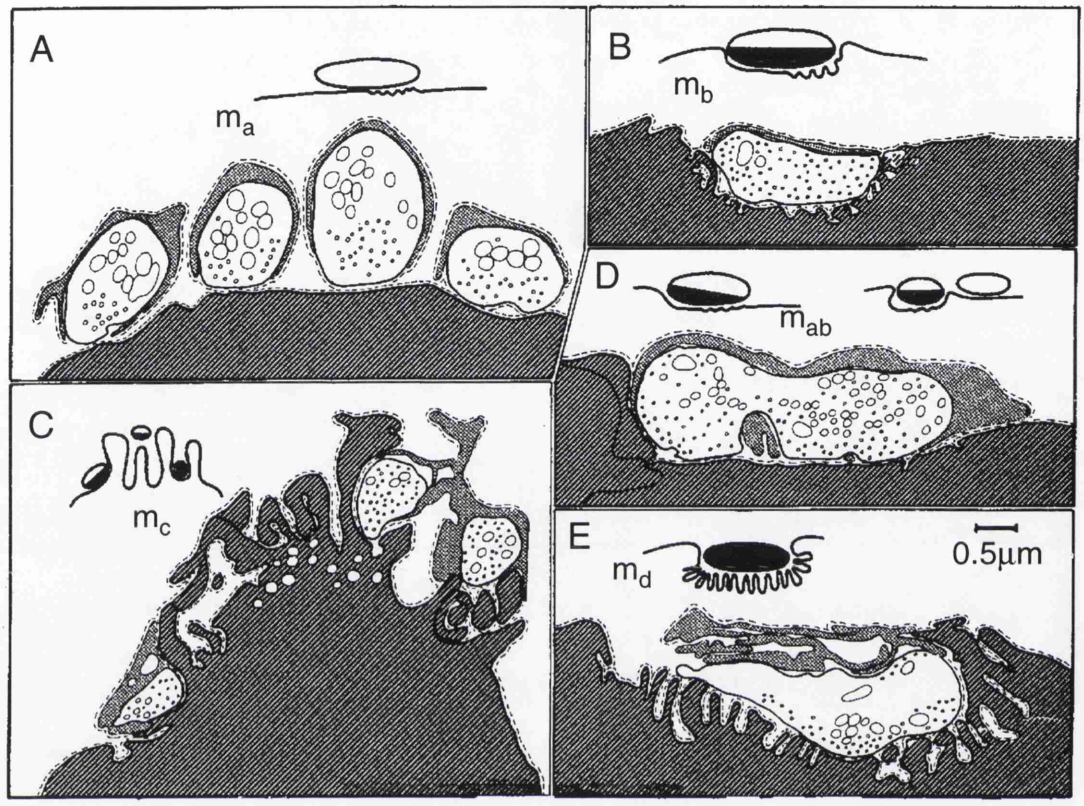


Figure 4

Tracings of high power electron micrographs of axon terminals of each of the five ultrastructural types together with a diagrammatic representation of each type.

Hatched area: Intrafusal fibres
Light stippled area: Schwann cell lid
Black shading: Area of axon terminal indented into the intrafusal fibre

Scale in diagram E applies to all tracings.

(*from Arbuthnott et al, 1982, J. Physiol. 331, p292*)

ending with the length of the periphery without the motor ending. This was called the 'folding index' and the largest folding index was that of the most convoluted post-synaptic membrane, i.e. the mc plate. The data falls into two populations of motor endings;

complex ending: those γs motor axons having terminations on chain fibres alone and having a large folding index. (These would be classified as mc by Arbuthnott et al, 1982).

simple ending: those γs motor axons having terminations on either or both chain or bag2 fibres having a low folding index. (These would be classified as ma by Arbuthnott et al, 1982).

All the motor endings in the above study were of known static γ axons from electrophysiology, the path of the γ axons having been traced through serial electron micrographs.

It is now possible to present a summary of fusimotor innervation of individual spindles in adult cats. The possible combinations of fibres innervated separately or together are;

b1 alone, b1plus long chain fibres innervated by γd axons.

b2 alone, b2plus chain fibres, chains alone innervated by γs axons.

Gladden and Sutherland 1989, suggested that there could be 3 types of static motoneurone as the terminations of all the static axons to chain fibres do not exclusively have mc endings. Even when non-selective static gamma innervation occurs the axon may have a preference for either the bag or the chains, i.e. an axon ending on several chains but also having a foot on the bag2 fibre can be said to be predominantly a chain fibre innervator whereas

an axon ending on the bag2 fibre and also on one or two chain fibres can be said to be a predominantly bag2 fibre innervator.
The selectivity of γd axons extends to other spindles. Boyd (1986), thought that the selectivity of γs axons extended to other spindles where the γs motoneurones were almost selective to the bag2 fibres or γs motoneurones almost selective to chain fibres. He even linked the mc ending of Arbuthnott et al, 1982, to the latter.

Work by Celichowski et al, (1994) in peroneus tertius spindles used three types of primary ending responses to distinguish the active intrafusal fibre when it was stimulated by a single static gamma axon at 30 pulses per second. If only chain fibres were stimulated, their contraction either caused driving of the primary ending discharges or initiated an irregular increase in the ending discharge frequency. When only bag2 fibres were activated, their contraction elicited a sustained and generally regular increase in the discharge frequency. When bag2 and chain fibres were activated simultaneously, impulses were elicited both by unfused contraction of chain fibres and sustained bag2 fibre contraction. Results indicated that the distribution of static gamma axons to spindles did not differ significantly from that expected by chance. This leaves unexplained a) why there are such striking differences in endplate structure of static gamma axons, and b) why Dickson et al (1993) can recruit bag2 fibres and simultaneously inhibit chain fibres by stimulating at single sites in the midbrain.

If innervation of a single spindle is considered, co-innervation of bag1 and bag2 fibres would not be expected for the adult pattern. In the developing cat spindle, this pattern of motor innervation could not be observed even by birth

although the spindle is relatively mature in its composition of intrafusal fibres, sensory endings, capsule and motor axons, (largely non myelinated).

Muscle spindle development.

The development of the muscle spindle was first studied by Sutton (1915) in the extrinsic eye muscles of the pig. Subsequently, Tello (1917, 1922) in man, cat, dog, rabbit and chick, Cuajunco (1927, 1940) in cat and man and Zelena (1957) in rat made detailed observations on developing muscle spindles using the light microscope. This work was done before much was known about the structure of adult muscle spindles, and certainly prior to the discovery of two types of bag fibre, although Cuajunco always maintained that there were three sizes of intrafusal fibres in the adult pig and human spindles.

1. Early prenatal development of muscle spindles.

a) Extrafusal Muscle Development.

Mammalian skeletal muscles begin developing before the appearance of peripheral nerves. As these appear in the muscle primordium, myoblasts fuse to form primary myotubes (Kelly and Zacks 1969) which extend from tendon to tendon (Ontell and Dunn 1978). Secondary myotube development is dependant upon the electrical and contractile activity between the muscle and the incoming innervation (Harris 1981). The myoblasts fuse with each other and are closely opposed to the primary myotube surface, within a common basal lamina forming the 'muscle cluster' of Ontell and Dunn. This multicellular structure acts as a template for the assembly of secondary myotubes which then differentiate and separate from the primary myotube becoming independent muscle fibres within their own basal lamina. The assembly, maturation and separation of the first series of secondary myotubes

takes longer in the cat than the rat, and in the cat the first series secondary myotubes separate between 38 and 41days of foetal development and before subsequent series assemble.

In extrafusal muscle Harris (1981), in rat, has shown that secondary myotubes cannot develop until the primary myotubes have been contacted by branches of the α motor axons; does the same hold true for intrafusal muscle?

b) Intrafusal Muscle Fibre Development.

What determines the contact of sensory or motor axons with particular myotubes? According to Milburn (1984) and Proske (1988) this is a chance occurrence. A primary myotube which is contacted by an α motor axon, goes on to become an extrafusal muscle fibre whereas a myotube contacted by a 1a afferent nerve fibre becomes a muscle spindle. Milburn found in the cat, that 1a axons had arrived in the muscle between the 34 and 38th foetal day (gestation period 63 days). Only those myotubes surrounded by sensory axons will become muscle spindles, (Zelena 1957, 1974; Zelena and Soukup 1974; Milburn 1973,1984; Kucera, 1988,1989, 1990, 1991), the rest becoming dedicated extrafusal muscles. The sensory innervation is also essential for the maintenance of muscle spindles during development. Sensory neurones in the dorsal root ganglion of prenatal rats exhibit spontaneous electrical activity (Fitzgerald 1987) at a time when spindles are forming in hind limb muscles. This may convert the undifferentiated myotubes into intrafusal fibres by the release of neurotrophic substances. Extrafusal fibres only have one motor axon innervating them (ultimately) whereas spindle fibres have both sensory and motor innervation. Early spindle development is thought to be independent of motor innervation. However, the recent work of

Soukup et al (1990) indicates that full maturation of the myosin heavy chain isoforms will not be completed in adult spindle fibres, in the absence of the fusimotor innervation.

2. Late prenatal development of muscle spindles.

Electron microscopy allowed more detail in the developmental process to be described. Landon (1972) and Milburn (1973) used the rat for their studies, finding the rodent useful as in this species, much of the development is postnataly. Rat spindles have two intrafusal fibres at birth (one larger and more nucleated than the other). These are the bag fibres; the full complement of two bag and two chain fibres was thought to occur by each bag fibre splitting to produce the other two (Marchand and Eldred 1969). However it became evident that this was not the mechanism of increased fibre numbers.

The most recent work by Milburn (1984) on developing muscle spindles is in the cat peroneal muscles; she gave an elegant illustration of the process of intrafusal fibre production (fig. 5). The 1a axon that innervated the primary myotube made contact between the 34th and the 38th foetal day of development, stimulated the aggregation of nuclei beneath its terminals and initiated the formation of a thin capsule which is derived from the axonal perineurium and separated the sensory region from neighbouring extrafusal myotubes. The myotube contacted becomes the presumptive bag2 fibre and here in the cat, as in man, (Mavrinskyaya 1967) some simple motor endings were also seen along the intrafusal fibre in the sleeve region.

The secondary myotube is formed by association of satellite myoblasts along the primary myotube surface and by 41 to 44 days of foetal development, the

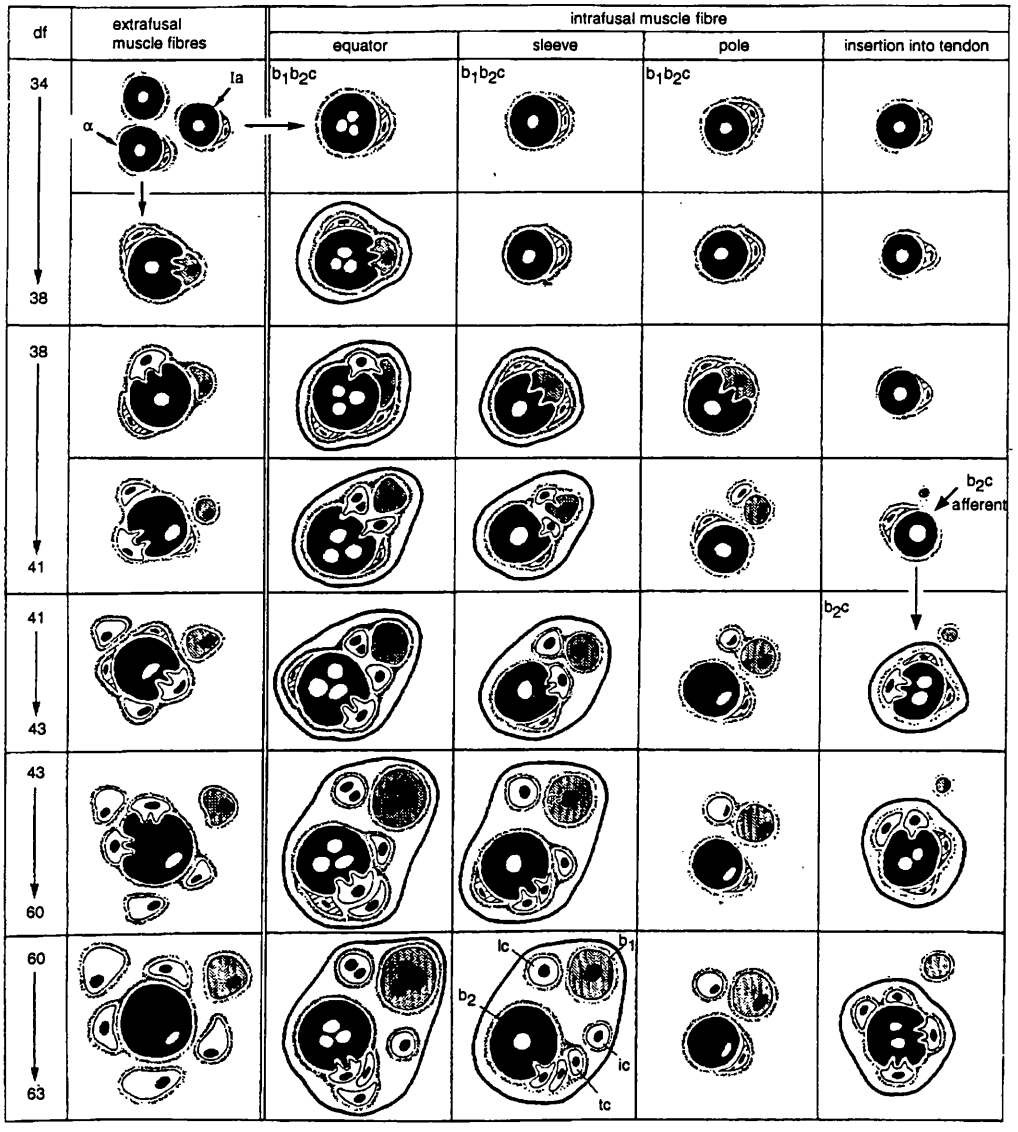


Figure 5

Schematic diagrams of transverse sections of developing extrafusal and intrafusal muscle fibres in cat peroneal muscles.

In the extrafusal fascicle note how the first series secondary myotube (stippled) separates from the primary myotube (black), acquiring its own basal lamina (stippled halo), before the assembly of subsequent series of secondary myotubes (white). The thin fusiform cells (hatched) are myoblasts. The diagrams of intrafusal muscle fibres show how myotube assembly begins at the equator and spreads to the poles, in contrast to their maturation and separation, which begins at the poles and spreads to the equator. The column headed 'insertion into tendon' shows the later arrival of a b_2c afferent axon as compared with a b_1b_2c 1a afferent axon; it is shown innervating the primary bag_2 myotube of a developing spindle and initiating the subsequent development of a b_2c spindle unit of a tandem spindle.

Abbreviations:

α:	alpha motoneurone	df:	days foetal
b_1:	bag_1 fibre	ic:	intermediate chain fibre
b_2:	bag_2 fibre	lc:	long chain fibre
b_1b_2c:	developing b_1b_2c spindle unit	tc:	typical chain fibre
b_2c:	developing b_2c spindle unit	1a:	1a axon

secondary myotube developing in association with the bag2 fibre is ready to separate from it. This is the presumptive bag1 fibre. Separation of myotubes occurs from the pole of the muscle fibres towards the sensory region in contrast to secondary myotube formation which starts beneath the sensory axons at the equator and grows out towards the pole. Presumably the sensory axons wind around the developing fibres holding them closely opposed during formation of the secondary myotubes. Therefore although the myotubes separate the sensory innervation keeps the newly formed intrafusal fibres very closely linked.

The newly formed bag1 fibre may have associated with it another secondary myotube which, according to Milburn is formed from the primary bag2 myotube (although Butler in 1980 thought otherwise) and becomes a long chain fibre. Typical chain fibres are then formed from the initial primary myotube i.e. from the bag2 fibre and not from the bag1 fibre. The sequence of fibre formation, is bag2, bag1, long, intermediate and typical chain fibres. Butler proposed that the grouping of the intrafusal fibres was a result of their development sequence: bag1 fibres forming separately from a different myotube from bag2 and chain fibres but Milburn disagreed indicating that the fibre grouping occurs late in development. In both equatorial and sleeve regions, bag1 fibres are usually in an inner capsular compartment of their own perhaps with one chain which is most often the longest chain fibre in that particular spindle (Barker et al, 1976; Arbuthnott et al 1982, 1992) whereas bag2 and typical chain fibres are compartmentalised together. This led Butler to the conclusion that bag1 and bag2 were developed from different primary myotubes. His study was during late development (55days foetal to 24 days post natal), by which time the grouping had already occurred.

In contrast to Milburn (1973) and Landon's (1972) work, recent work by Pedrosa and Thornell, (1990a) on expression of myosin heavy chain (MHC) isoforms in developing rats, has suggested that all three types of intrafusal fibre originate from three distinct cell lines. This conclusion arises as a result of experiments showing that b2 fibres expressed slow twitch, slow tonic and neonatal MHC whereas b1 fibres expressed neonatal MHC from the beginning and only after a time lag of two days did slow twitch and slow tonic MHC become evident, in spite of the presence of afferent innervation from the start. Also, although myoblasts which would give rise to chain fibres at 20df, had sensory contacts (Kucera et al, 1989) there was no slow twitch or slow tonic MHC present.

Secondary axons probably find their way to the developing spindles along the paths already forged by the primary axons. The first to arrive most probably takes up the position nearest to the primary, the S1 position (Boyd 1962). This occurs between the 41st and 43rd foetal day. Subsequent secondary axons must make connections further into the poles of the intrafusal fibres where separation from the primary myotube has already occurred and therefore in many instances the secondary sensory connections may only be with chain fibres. Also axons arriving later tend to have smaller diameters.

Tandem spindles.

Tandem spindles, are spindles with an intrafusal fibre (often bag2) in common. They occur in varying numbers in different muscles and are thought (Milburn 1984) to occur because of two or more 1a axons innervating a single primary myotube simultaneously. Each sensory region then forms its own population

of secondary myotubes and so produces two spindles with a full complement of intrafusal fibres but with a common bag2 fibre. Tandem spindles without a bag1 fibre are thought to arise due to late arrival of a 1a axon which makes connections with the pole of a bag2 fibre that has already separated from its bag1 fibre and therefore the next sequence of events is the formation of the chain fibres at both sensory regions of this bag2 fibre. This leads to a tandem spindle with a common bag2 and chain fibres, but no bag1 fibre.

The arrival of the motor axons in developing cat spindles is also early (from 34days foetal) and they are thought to be guided by the paths laid out by the 1a axons (Milburn 1984).

Ultrastructural work by Kucera et al (1989) on developing rat spindles indicated brief α motor axon innervation of the putative bag2 fibre prior to encapsulation in contrast to his 1988 paper in which he thought prior alpha innervation unlikely. This means that in rats both sensory and motor contacts are made together on the bag2 myotubes, the α innervation being lost within two days. This initial contact is at 17 days gestation, (full term in rats is 22 days), and by 19 days the second intrafusal fibre has formed, the γ innervation also having arrived at the spindle. Milburn maintains in the cat, that spindle formation from the 34th day of gestation takes place in the presence of both sensory and motor axons and that the motor innervation here is by γ axons. Kucera would argue from rat work that fleeting α innervation occurs immediately prior to sensory innervation which only precedes motor innervation by a day (equating 19 days gestation in rat with 34 days foetal in cat). This fleeting proposed innervation by an α motor axon may leave a residual influence on the ability of the individual intrafusal fibres to capture

and reorganise subsequent motor innervation. The intrafusal fibres may be unable to support both sensory and α motor innervation on the one fibre but this initial trigger allows the spindle fibres to support both sensory and γ motor innervation in adulthood. The larger amount of β innervation found in the new-born kitten (Gregory and Proske, 1985) than in the adult would support the view of an initial influence of non-γ motor innervation.

New-born mouse spindles develop in the presence of α motor innervation (Kozeka and Ontell, 1981) and as discussed above, cat and rat spindles may also, however briefly. The relative importance to continued development of the spindle, of the sensory and motor innervation, was studied in rats which only have two intrafusal fibres at birth. Denervation of rat hind limb muscles at birth arrested spindle formation, the muscles becoming devoid of spindles (Zelena 1957). Nerve crush at 14 days after birth in rats showed no spindle degeneration and normal re-innervation occurred subsequently (Zelena and Hnik 1963). However if only de-efferentation of new born rat spindles was carried out, the normal complement of intrafusal fibres developed (Zelena and Soukup, 1973, 1974), but with some deficiencies in the nuclear bag myosin characteristics (de Kronnie et al, 1982). These experiments showed an initial dependence on the 1a afferent innervation for normal spindle development. This dependence declines with age (Werner 1972, Shiaffino et al 1976). Secondary axons may exert a smaller influence and motor axons have as yet an unknown influence. Recent work by Pedrosa et al, (1990b) on the expression of myosin heavy chain isoforms in developing rat muscle spindles indicated that motor innervation may be necessary for the full maturation of spindle fibre myosin heavy chains to the adult form.

From experiments of cross-innervation and stimulation studies there is abundant evidence that the type characteristics of mammalian skeletal muscle are influenced by the innervating motoneurone (Jolesz and Sreter, 1981). Conversion from the neonatal condition to the adult could be either by motoneurone influences or by selective withdrawal of mismatched connections. As commitment to either fast or slow muscle (extrafusal) or bag2 muscle (intrafusal) occurs early, Jones et al (1987b) believe in selective withdrawal to allow correction of inappropriate contacts. Extrafusal muscle fibres only have motor innervation whereas intrafusal fibres can accommodate both sensory and motor innervation on the same fibre. It would be expected that at some point during development local factors would influence the achievement of the final innervation pattern.

3. Postnatal muscle spindle development.

Development of motor innervation.

a) Polyneuronal innervation in extrafusal fibres.

In new-born rats Redfern (1970) graded the stimulus intensity to the muscle nerve while recording intracellularly the end-plate potentials from single muscle fibres and found that several axons terminated on the one motor end-plate site. This became known as polyneuronal innervation. Similar conclusions were made by Bagust, Lewis and Westerman (1973) in kittens from experiments using motor unit tension overlap measurements.

At some stage between birth and full adulthood some synapses must be eliminated to reach the state of one nerve terminal to one muscle fibre (Redfern, 1970; Bennett and Pettigrew, 1974; Brown et al., 1976; Riley, 1977; O'Brien et al., 1978). From an ultrastructural point of view, synapse

elimination is seen as a reduction in nerve terminal profiles at each neuromuscular junction (NMJ) (Korneliussen and Janssen 1976; Rosenthal & Taraskevich. (1977); Duxson 1982). This reduction process is thought to be by retraction of supernumerary axons rather than by degeneration as no degenerate profiles were observed. This could be thought of as similar to the retraction of pre-synaptic boutons after post-synaptic injury in the central nervous system (Sumner 1975; Sumner and Sutherland 1973). This surely is not a random process as some muscle fibres would reach adulthood uninnervated unless there was a compensating attraction to denervated muscle fibres. Whether the sorting process is driven by the pre or post-synaptic component of the NMJ still is unresolved.

There are initially large numbers of acetylcholine (Ach) receptors which decline in numbers rapidly between 1-2 weeks of post-natal life in the rat (Bambrick and Gordon 1992). During the development of extrafusal muscle, Ach receptors are initially expressed throughout the whole sarcolemma; innervation causes them to become restricted to the neuromuscular junction (Diamond and Miledi 1962; Fambrough and Rash 1971; Bevan and Steinbach 1977) and continued innervation is essential for maintenance of proper discrete connections (Ribchester and Taxt 1983). Continued innervation, therefore must produce some inhibitor to prevent any new sites developing. Any contact, however immature, of motor axons with muscle fibres seems to provide a rudimentary form of transmission (Chow and Poo, 1985). Any contact, even if subsequently axotomised, also seems to leave instructions for receptor formation at extrajunctional sites (Lomo and Slater 1980). The basal lamina between axon and muscle cell and in particular the substance Agrin

within the basal lamina is currently thought to be vital for this recognition process (see Discussion).

Blockage of Ach release by botulinum toxin (Brown et al., 1980, 1981a) and tetrodotoxin (Thompson et al., 1979) during development slows the rate of synapse elimination. Increased nerve activity (O'Brien, 1978; Ribchester and Taxt, 1983) increases the rate of synapse elimination. Blocking the post-synaptic receptor sites with bungarotoxin inhibits the reduction in numbers of axon terminals during development (Duxson 1982)

b) Development of fusimotor innervation

The earliest study of responses from muscle receptors in kittens was that of Skogland (1960) who recorded fusimotor effects from gastrocnemius muscles of 17-20 day old kittens. Fusimotor effects could be recorded earlier in more proximal muscles. Skoglund also recorded a few responses in young animals from axons conducting with velocities in the α range and concluded that the initial motor innervation of the spindles was by α axons, as occurs in extrafusal muscle development, the γ system developing later. No further work was done in this field until 1985 when Gregory and Proske recorded fusimotor responses in kitten soleus muscles as young as 1 day old. They showed that it was possible to distinguish separate static and dynamic responses in 5 day old animals. In all cases there was a clear separation of the γ axon responses and those of the α axons with regard to conduction velocities although the values for the γ axon conduction velocities were in most cases within the range for unmyelinated axons. Gregory and Proske did not accept that the initial motor innervation to the spindles was exclusively α, but did note 4 instances of axons conducting in the α conduction velocity

range with fusimotor effects i.e. β fusimotor axons. From this they concluded that there was a high incidence of ß innervation, higher than found in adult spindles. The α axons may send branches (ß axons) to the spindles which are later retracted or degenerate as do supernumerary contacts of extrafusal muscle fibres.

In summary, adult spindles lie in parallel with the main extrafusal muscle fibres and are involved in the response to stretch of these muscles. Spindles all possess a capsule, usually all three types of intrafusal fibre (b1, b2 and chain fibres, both long and typical), sensory innervation and motor innervation. The motor innervation is mainly by γ motor axons but β axons can also be present. Fusimotor axons supply b1 fibres selectively while b2 and chain fibres may be innervated independently or together. The form of the motor endplates can be simple or have post-synaptic specialisations such as in-folding or protrusion of the muscle surface.

During development, primary myotubes require the contact of α motor axons in order to become dedicated extrafusal fibres. Myotubes which are encircled by the 1a sensory axon mature into muscle spindles. There are two theories of spindle formation. Either, the sensory axon makes contact with a nascent myotube; this becoming the future b2 fibre. Accumulations of myoblasts along this b2 fibre produce firstly the b1 fibre and then chain fibres in descending order of size. Thus all spindle fibres arise from the same primary myotube (Milburn, 1984). Alternately, myotubes from three different cell lines may produce the three separate types of intrafusal fibres (Pedrosa and Thornell, 1990).

The kitten spindle at birth also possesses a capsule, all three types of intrafusal fibre, sensory innervation and motor innervation which already shows some adult features in the form of the terminals such as post-synaptic folding (Milburn 1984). However, from the literature, it was not known whether motor endings on different types of intrafusal fibre in young kittens were supplied by randomly distributed fusimotor axons with subsequent remodelling of the connections, or whether the adult pattern of spindle motor innervation is set early in pre-natal development. Milburn's work (1984) stops prior to birth in kitten spindle development. The present work, also using electron microscopy, investigates the fusimotor innervation of the muscle spindles of tenuissimus and peroneal muscles from 10 days prior to birth to 28 days post-partum. Preliminary findings were reported by Gladden, Spike and Sutherland, 1989.

Methods

Muscle spindles from kitten tenuissimus and peroneal muscles were studied by electron microscopy at 50 day foetal, 1, 14, 21, and 28 days post-natal stages. The muscles were dissected from the hind limbs immediately after death by anaesthetic overdose (sodium pentobarbitone 60 mg/cm^3).

Crown-rump lengths of kittens.

The crown -rump length was determined by measuring kittens from the highest point of the head, along their backs, to the most caudal extent of the buttocks at the base of the tail as described by Evans and Sack 1973.

Tissue preparation

Fixation.

The dissected muscles were placed in 25% gluteraldehyde in a cacodylate buffer for 2-3 hours, after which they were washed in buffer, placed in 1% Osmium Tetroxide for 2 hours, dehydrated through a graded series of alcohols and embedded in Araldite resin. For details of the procedure see appendix 1.

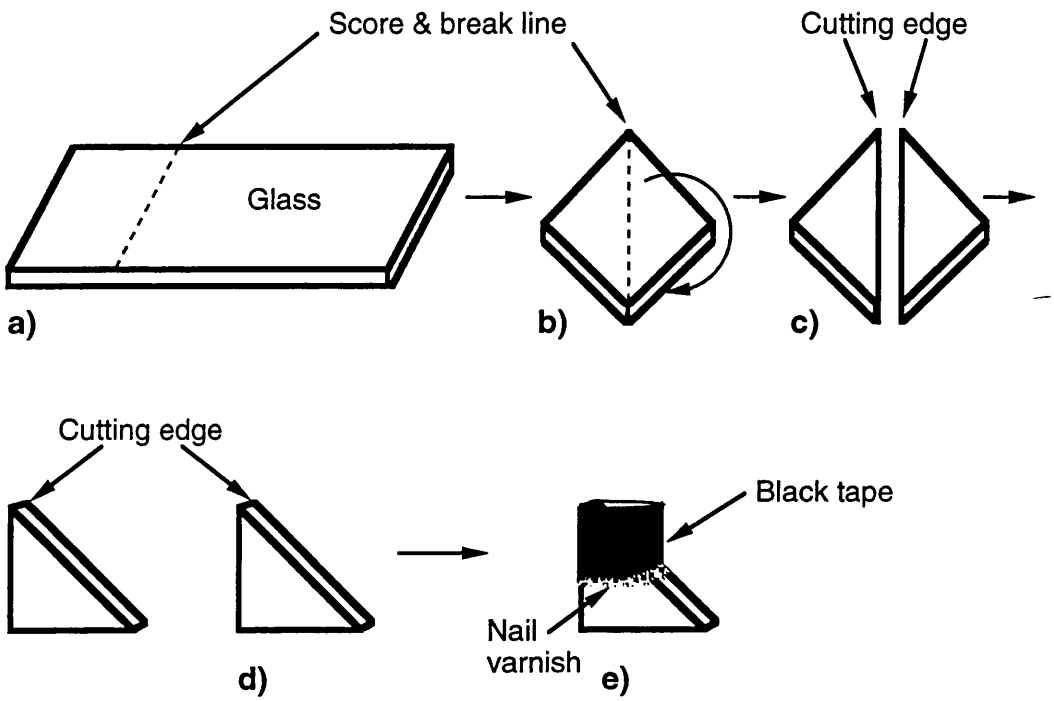
Sectioning.

Transverse ultrathin sections were cut using glass knives made on an L.K.B.7801A knifemaker (fig. 6) and then fitted with a water boat using black tape, sealed with nail varnish.

A ribbon of silver to grey ultrathin sections were cut, approximately 20 sections comprising 1μm of the block on a Reichart UM3 ultramicrotome. These sections could be separated into groups of three or four sections by touching the edge of the ribbon at the section borders with an eyelash mounted on a cocktail stick. The sections were flattened with chloroform vapour.

Figure 6

Diagrams to show the process of making a glass knife for ultramicrotomy



a) The strip of glass is scored and a rectangle broken off.

b) This rectangle is turned over and scored diagonally across.

c) The rectangle is broken to produce 2 rectangular knives (d).

e) Black tape is attached as shown and sealed with nail varnish.

Cross sections of muscle spindles are large relative to the mesh size of any copper grid and therefore grid bars may obscure some features of the sections. Sections therefore require support by a transparent but strong substance such as formvar which is applied across a copper grid with a single hole of 800nm diameter. The following method was devised to overcome the problem.

Groups of sections were lifted (fig. 7) by holding an 800 nm one hole copper grid (without formvar plastic coating) to the surface of the water above the sections to be collected and allowing the sections to be caught by surface tension in a bubble of water below the grid (Arbuthnott 1973). This "collecting grid" was gently placed over a formvar coated grid of the same hole size (fig. 7c). The water was gently withdrawn from between the two grids with a small segment of filter paper and the two grids parted using forceps (fig. 7d,e).

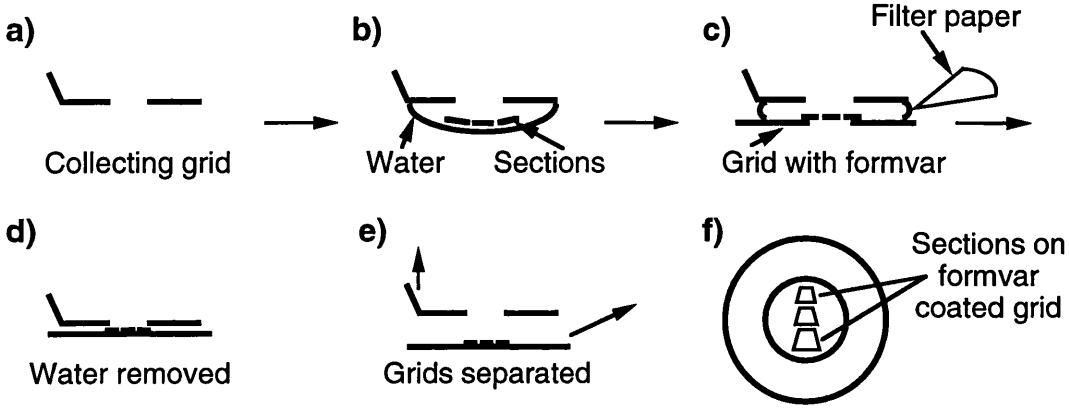
Serial sections were cut throughout the block using a regime of ultrathin sections at 2 or 5μm intervals (depending on the age of the kitten, the younger the kitten the smaller the interval). The intervening 1μm thick sections were lifted in twos with a mounted eyelash and placed in drops of distilled water on glass slides coated with gelatine/chrome alum.

Staining.

Semi- thin 1μm sections were stained with toluidine blue/pyronin Y (appendix 1) (Ito and Winchester 1963) for 1 minute on a hot plate, and gently rinsed with tap water, dried and mounted in immersion oil and covered with a coverslip. Immersion oil was used as a mounting medium so that the sections

Figure 7

Lifting ultrathin sections



a) The collecting grid (without formvar coating) is placed over the sections floating in a ribbon on the water bath.

b) The collecting grid is lifted with its bubble of water containing the sections.

c) The collecting grid is placed over the formvar coated grid.

d) The water is removed with filter paper.

e) The collecting grid is gently lifted away.

f) The sections now on the formvar coated grid are ready for staining.

could be easily removed from the slide, re-embedded and sectioned for electron microscopy if necessary (see later).

Ultrathin section staining.

The ultrathin sections were stained with 2% uranyl acetate, (Stempak and Ward 1964, appendix 1) for four minutes followed by lead citrate (Reynolds 1963, appendix 1) for eight minutes. These stains when made up will last for up to a fortnight if kept in the dark and if the syringe in which they are stored has a filter to catch any precipitate before the solutions pass into the needle. A new needle was used on each day's staining.

In each case drops of stain were arranged in rows on a clean square of 'paraclear' on a clean bench covered with 'bench-coat' paper renewed at regular intervals. These were covered with a watch glass darkened with black tape. In the case of the lead citrate stain, care has to be taken when the drops of stain are being put out as expired carbon dioxide can precipitate lead carbonate from the solutions which will of course deposit on the sections.

The grids were placed section side down on the drops in the dark. After 4 minutes in the uranyl acetate stain the grids were rinsed in a fine stream of distilled water and dried by touching the edge of the grid with filter paper. This was followed by 8 minutes staining in lead citrate, again in the dark, after which the grids were gently rinsed in a weak solution (4 pellets/200ml. water) of sodium hydroxide followed by careful washing in a very gentle stream of distilled water; again the grids were dried by touching the edge of the grid with filter paper.

Photography.

A Zeiss EM109 electron microscope was used to observe and photograph the ultrathin sections. Magnifications of X1296 for low power views of the complete cross section of the spindles and X4680 for features of interest including the motor endings and motor axons were taken. The electron microscope camera used Ilford FP4 film containing 8, 6 x 9 cm. negatives. The film was developed with Ilford PQ universal developer. The micrographs were usually enlarged x 2.5.

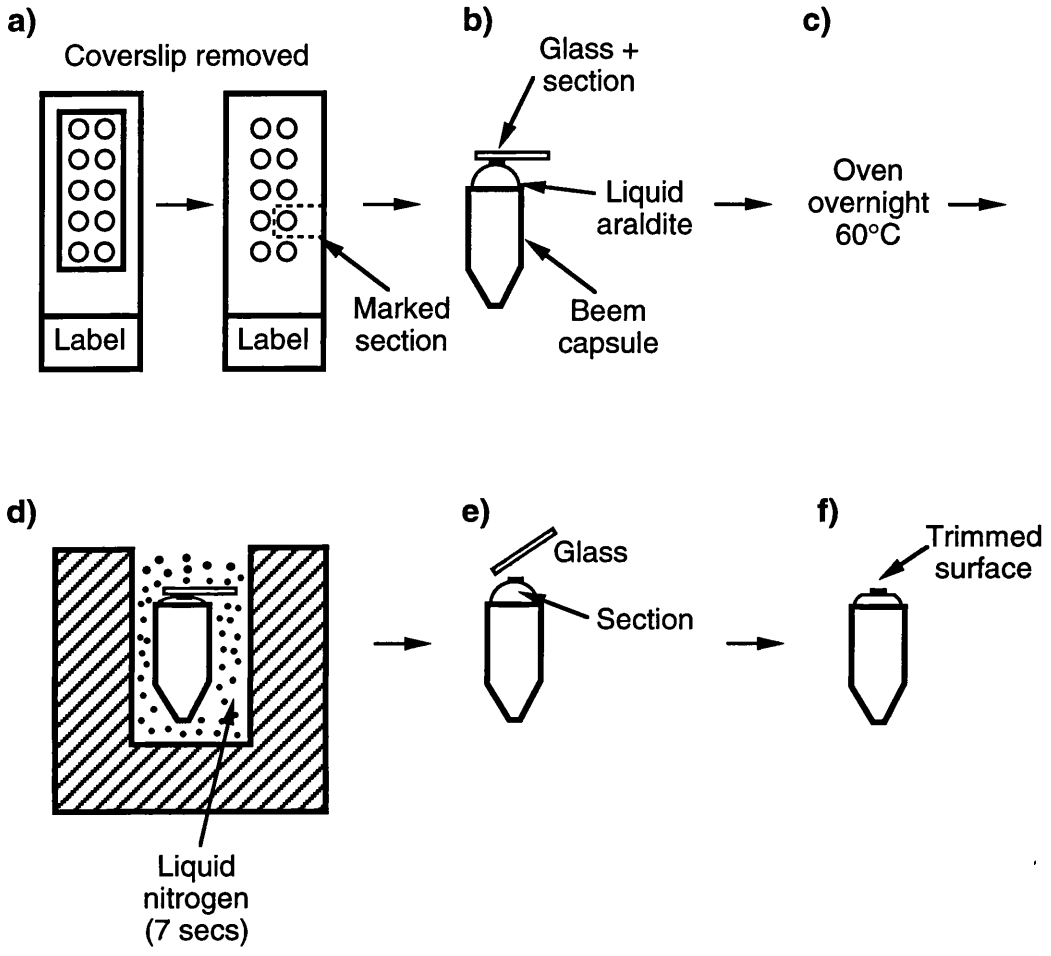
Although all of the tracing of axons was done on the electron micrographs, inspection of the light microscope sections was helpful especially in the case of extremely small motor endings which do not extend between two batches of ultrathin sections! It is possible to see that a motor ending is present, but not what the features are. In these rare cases the 1μm section can be re-embedded.

Re-embedding of 1μm resin embedded sections for ultrathin sectioning.

The section must first be removed from the slide by lifting the coverslip from the slide, cleaning off the immersion oil by gently wiping with a tissue, selecting the section to be re-embedded, using a diamond to score the slide underneath the section and breaking out the relevant piece, (see fig. 8a). The small fragment of the slide was then laid section side down on the top of a BEEM capsule full of liquid Araldite (without bubbles), fig. 8b. The capsule was supported upright in a 60°C oven overnight, fig. 8c. The next day, the capsule was allowed to cool to room temperature before being held in a polystyrene beaker full of liquid nitrogen for 7 seconds, quickly removed and the glass pulled off the top, fig. 8 d,e. The 1μm section was therefore left

Figure 8

Re-embedding 1μm thick sections for ultramicrotomy



a) The coverslip is removed and the slide marked with a diamond around the relevant section.

b) The section is broken out and placed section-side down on liquid araldite in a beem capsule.

c) The capsule is left overnight in a 60°C oven to polymerise.

d) After the araldite is allowed to equilibrate with room temperature it is then plunged into liquid nitrogen for 7 seconds.

e) The glass is quickly removed with forceps leaving the section on top of the araldite.

f) The block can now be trimmed ready for sectioning.

behind on the now solidified Araldite. A face was trimmed round the section (fig. 8f) and the block cut in the normal way, ultrathin sections being cut throughout the 1μm.

Unit of measurement.

Measurements were made on spindle poles. The total length of a pole is the distance from the centre of the primary sensory region to the end of the longest intrafusal fibre. To determine the centre of the primary sensory region the distance between the outer limits of the whole primary sensory region along all the fibres was halved. The centre was therefore taken as at the same point on all intrafusal fibres of any one spindle. If a whole spindle was encountered, it constituted two poles.

Micrograph Analysis.

The micrographs were analysed as follows:

1. By eye for any feature of interest.

Bag fibres were distinguished from the chain fibres by their central bag of nuclei beneath the primary sensory ending, their larger diameter and longer length (where it was possible to section the entire pole). Bag1 fibres were distinguished from bag2 fibres by the separation of the bag1 into its own capsular compartment (Barker et al 1976), less granular appearance (Kucera 1986) and smaller numbers of surrounding elastic fibres at the pole (Gladden 1976).

2. The form of the motor endings.

In adult spindles the sensory terminals are very deeply indented into the muscle surface and deform it considerably. The basement membrane of the muscle totally surrounds both terminals and muscle with no separating basement membrane. In contrast, all forms of the motor terminals of adult muscle spindles, have at least one layer of basement membrane between axon terminals and muscle surface, the basement membrane of nerve and muscle often being fused into one. Sensory terminals have larger numbers of mitochondria and a greater variety of vesicle styles (round, flat, dense-cored, or clear) within one terminal than motor terminals. Distinguishing between sensory and motor endings was quite difficult at the 50df stage as in some cases the basement membrane was not present between the muscle and the motor terminal. If this distinguishing criterion was not present, the position of the terminals along the intrafusal fibre was also used.

An axon terminal is the smallest discrete contact of a terminal branch of any axon. An ending is the whole collection of axon terminals of the final axon branches. In the case of motor endings, an ending includes all the axon terminals of the final branches of the motor axon and the muscle on which the terminals lie.

The classification of Arbuthnott et al 1982 (fig. 4) was used.

ma; simple non-indented, few if any folds;

mb; deeply indented, with some light folding;

mc; complex ending with the post synaptic membrane pushed up into numerous fingers, and the axon terminals lying between and to the side of these structures;

md; deeply indented with many narrow folds.

Some additional categories had to be introduced for the kitten material as there were too many endings with morphology which could not be definitively assigned a specification. (See fig. 9).

maf; the axon terminals lie superficially on the muscle surface but this is very folded although not thrown up into finger-like projections.
mab; the axon terminals are partially indented or some of the terminals are indented. This was also used at first by Arbuthnott(1982) but later simplified into ma or mb;
mabf; as above (mab) but with large numbers of folds;
mbf; the axon terminals are indented into a very folded muscle surface;
mac; the muscle surface is thrown into finger-like projections but the axon terminals lie superficially on the muscle surface, not between the fingers.

As mentioned in Arbuthnott et al 1982, 1992 and Sutherland et al 1985, classification of motor endings takes into account the form of serial sections (hence all axon terminals) of that ending: no decision was made on a single, sample axon terminal. This can be seen in fig. 10 which shows serial sections of an ma and mc ending and it is interesting to note that not all levels of the mc ending showed the pronounced folding. Therefore only looking at one section might give an erroneous classification.

3. The fusimotor innervation.

The fusimotor innervation was traced in the spindles by following the axons from spindle entry (or earlier if possible) through consecutive sections to their

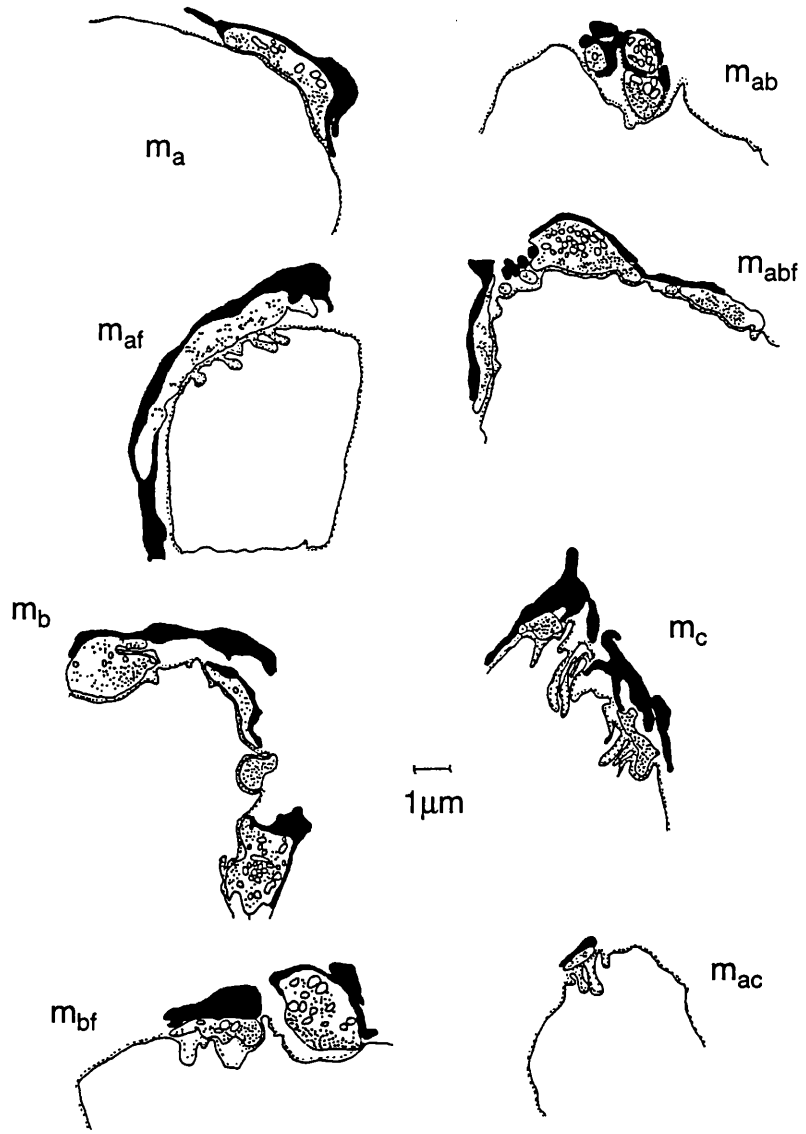


Figure 9

Tracings of motor endings to show the 8 types distinguished in this study.

These are basically four of the five types of Arbuthnott et al, 1982, where
m_a has axon terminals lying superficially on the intrafusal muscle surface;
m_b has terminals indented into the muscle surface;
m_c has terminals superficial and indented into the muscle surface but the muscle surface itself is thrown up into finger-like processes;
m_{ab} has terminals 'half and half' indented into the muscle surface.
In development the superficial axon terminals can be resting on a very folded muscle surface, m_{af}, or 'half and half' indented over a folded muscle membrane, m_{abf}. m_{ac} is similar to m_{af} exept the muscle surface is raised in the m_{ac} type although the axon terminals are all superficial.

Key:
Structure containing stipples (vesicles) is an axon terminal. The open shapes are mitochondria.
Dotted line is basement membrane.
Clear structure is intrafusal muscle.
Black structure is Schwann cell.

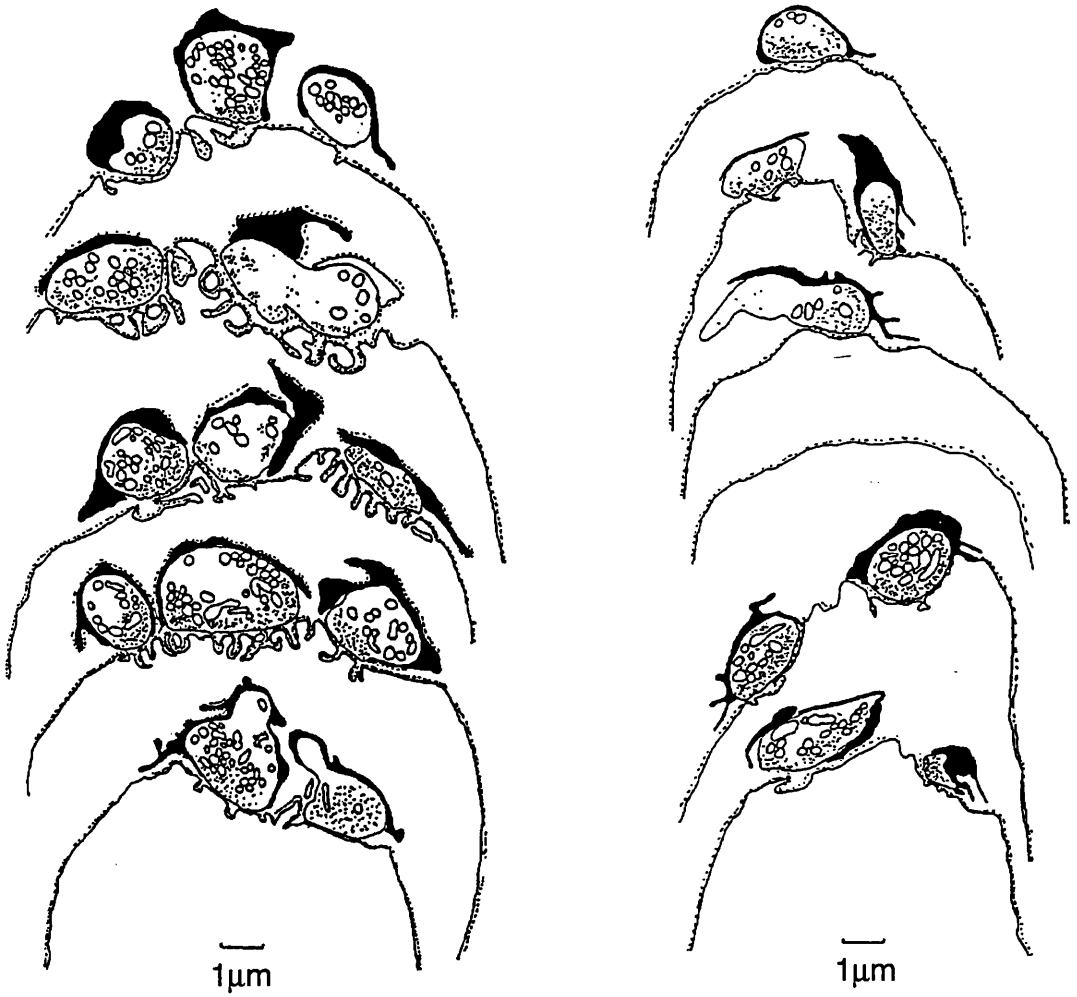


Figure 10

Tracings of sections taken 10μm apart through endplates.

m_c, with very variable folding in successive sections and m_a with more uniform and less intense folding.
Schwann cells are shown in black, the basement membrane is stippled and axon terminals contain hollow outlines (mitochondria) and stipples (vesicles).

(from Arbuthnott, Gladden and Sutherland, 1992, Experimental physiology, 77, p446)

destination as motor terminals on individual intrafusal fibres. These connections were represented on schematic diagrams of spindle poles. An example is given of a 1dpn tenuissimus spindle in fig. 11.

Both myelinated and unmyelinated motor axons were followed in this way, using sequential electron micrographs. In all cases the total unit under consideration was a spindle pole, the centre of the primary sensory ending being the notional mid-point. The selectivity or otherwise of the motor innervation could therefore be recorded.

Database of Morphometric Measurements.

All the following parameters were entered into spreadsheets for each age group, using Microsoft EXCEL from the Kontron IBM computer system, later transferred to Cricket Graph on an IBM PC.

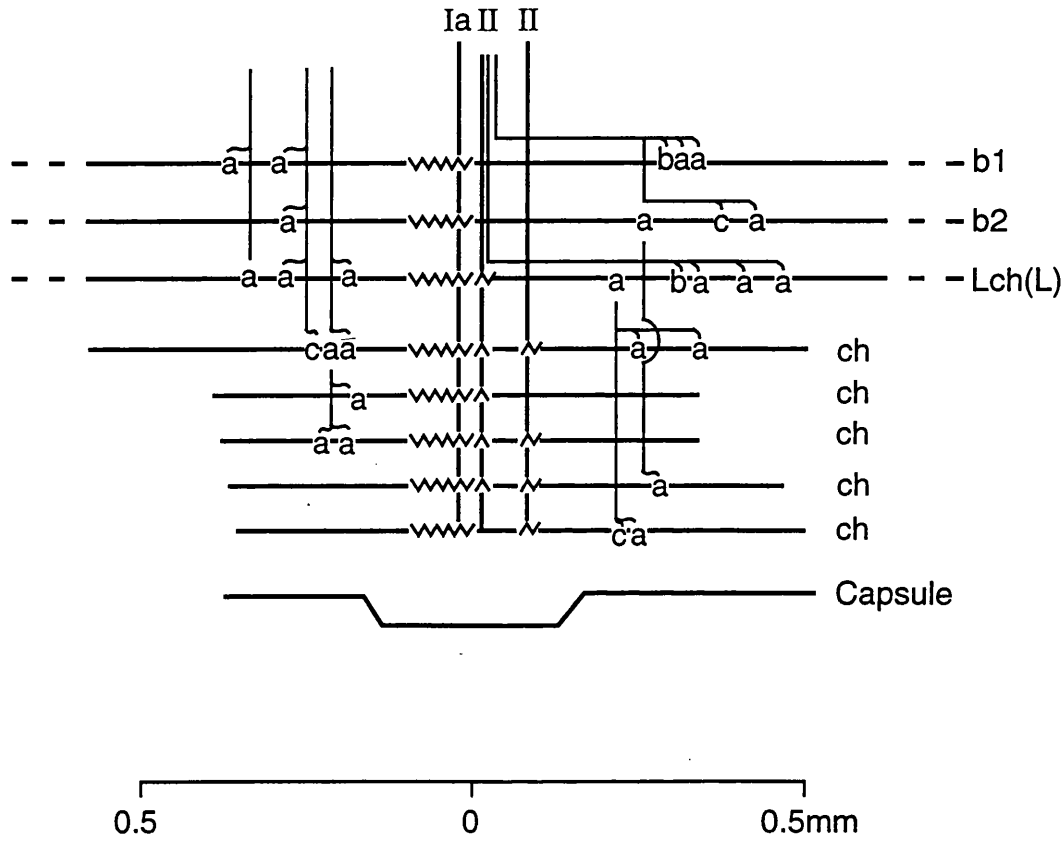
1. Measurement of cross-sectional area of the intrafusal fibres at 50df and 28dpn with Videoplan.

It was the impression that the b2 fibre is always larger in cross section than the b1. To find out whether this is a potential distinguishing feature of the two bag fibres, the transverse areas of the fibres were measured along their length at two age groups, 50df. and 28 dpn..

Area measurements of individual fibres were made using a digitising tablet with attached cursor which outlined the micrograph image of each fibre. Measurements were made every 100μm along the fibre length of 28dpn samples and at 100 and 50μm intervals for 50df samples. Correction for any

Figure 11

Innervation diagram of 1dpn Tenuissimus spindle



This spindle has two bag fibres (b1, b2), a long chain fibre with a large diameter (Lch L), and 5 typical chain fibres. The capsule is also indicated. There is a primary and 2 secondary endings and 5 fusimotor axons with ending types simplified to a, b, c, due to lack of space.

variation in micrograph magnifications were made. Micrographs had a total magnification of either x3240 or x4230.

2. Morphometric measurements of spindle poles.

Lengths of the primary and secondary sensory regions, lengths of the capsule and lengths of the intrafusal fibre poles (where possible) were calculated from the numbers of batches of ultrathin sections within any of these structures multiplied by the interval between batches (2 or 5μm).

Long chain fibres.

In adult cat spindles, it is established that there are chain fibres that are long in length, even as long as bag fibres. These can have either a large or narrow diameter. Where the long chain has a large diameter there is no problem in recognition (the equatorial nuclei are in a line), but if the diameter does not differ from that of typical chains, Barker et al (1976) and Kucera (1982) defined a long chain as one which projected for more than 1000μm beyond the end of the capsule. This is easy to calculate in the adult cat when all the structures are fully grown, but in the kitten, where the spindles are different sizes at different ages it is impossible to give an absolute measurement for designating a long chain fibre. It was decided to express the extracapsular length as a ratio. Values for the adult cat were taken from the work of Kucera (1982) and Arbuthnott et al (1982) of the length of the chain fibre pole that extended beyond the end of the capsule in relation to the total length of the intrafusal fibre pole, in designated long chain fibres of thin diameter of these workers. In other words a ratio was generated thus;

$$R = \frac{\text{length of chain fibre pole outside capsule}}{\text{total chain fibre pole length}}$$

where R is a ratio known as the Long Chain Ratio.
From these calculations, a range was found of between 0.35 and 0.6 for chains with at least 1000μm length beyond the end of the capsule.
In the kitten samples any chain fibre pole with a ratio (R) of greater than 0.35 would be classified as a long chain pole.

3. Morphometry of the motor endings.

The numbers of each type of motor ending per intrafusal fibre and its length and distance from the equator were all recorded. Any length was again determined from counting the batches of ultrathin sections and multiplying by the distance between. No account was taken of any shrinkage in the fixing and embedding process.

In some cases it was not possible to obtain all parameters for all the features studied, and so there are variations in total numbers in any one sample; e.g. there were 82 motor endplates at 21 dpn. but only 81 motor endplates were characterised. Therefore in some calculations such as the numbers of motor endings it would be possible to use 82 as the total. For the numbers of each ending type, only 81 could be used.

4. Computation.

Using the information on these spreadsheets, the results can be expressed as follows:

i) lengths of the sensory endings, capsule and intrafusal fibres with age .

ii) pre synaptic features of the developing fusimotor innervation by:

a) the numbers of motor axons of each spindle pole.

b) the percentage of motor axons that were myelinated.
c) the distribution of the motor axons to the intrafusal fibres.
d) the numbers of motor endings of each motor axon.
e) the lengths of motor endings of each motor axon.

iii) post synaptic features of the developing fusimotor innervation by:
a) the numbers and types of motor endings of each intrafusal fibre.
b) the distance of motor endings from the equator.
c) the lengths of motor endings.

The results are expressed either as raw data or means and standard deviations. The raw data, where possible, has been compiled into a set of bar charts or histograms, scatter diagrams or pie charts in the results and actual figures can be found in tables in appendix 2.

5. Statistics.

Statistical analysis comparing the numbers of motor endings, their length and distance from the equator at different age groups used analysis of variance (ANOVA) to test for a normal distribution of the data. All the data was found to be normally distributed.

Fisher's Least Significant Difference (LSD) test, being a form of Student's t-test for comparison of small sample numbers, was used to compare parameters at the different age groups using an IBM statistics package. The formula involved in the calculations is given below:

$$LSD_{\alpha} = t \sqrt{\{ s^2 [1/ni + 1/nj] \}}$$

where s^2 is the error mean square.

t is the α-level two tail t-value for v degrees of freedom, (df of s^2).

ni is the size of sample i.

nj is the size of sample j.

The results were considered significant at the 95% level.

Results

Tenuissimus and peroneal muscles were removed from kittens of the following ages: four at 50df; two each at 1 and 12dpn and one each at 21 and 28dpn.

The numbers of spindle poles and whole spindles studied for each age, the numbers of intrafusal fibres contained in the spindles and the numbers of motor endings are shown in table 1.

Table 1. The numbers of each parameter used in this work.

	50df	1dpn	12dpn	21dpn	28dpn
Ten. poles	3	14	8	4	7
Per. poles	4	0	5	7	6
whole sp.	3	5	3	4	4
sp. poles	7	14	13	12	13
tandem sp.	0	0	0	1	3
b1 poles	7	14	13	11	10
b2 poles	7	15	17	12	15
Ch. poles	26	59	57	49	49
Lch (T) poles	1	3	2	3	4
Lch (L) poles	0	5	2	0	0
m.e.p. b1	21	37	31	26	24
m.e.p. b2	27	39	49	18	27
m.e.p. on ch	24	63	68	35	45
m.e.p. Lch(T)	0	6	5	2	8
m.e.p. Lch(L)	0	24	6	0	0
Total m.e.p.'s	72	169	159	82	104

The crown-rump lengths of kittens used for this study is shown in table 2. The increase in length with age is expressed as a percentage of the mean length of the previous age.

Table 2. Crown-rump lengths of kittens between 50df and 28dpn.

Age	**50df** n=4	**1dpn** n=2	**12dpn** n=2	**21dpn** n=1	**28dpn** n=1
Mean crown-rump length mm.	106.4±9.2	142.5±2.5	160.0±5.0	185.0±0	212.0±0
% length increase mm.		33.9	12.3	15.6	15.0

Some poles did not have both bag fibres, in which case the most usual one present was the b2 fibre. There were 4 tandem spindles in this study in which two encapsulations shared a b2 fibre.

Analysis.

The results are divided into three main parts:

1) Morphometry of the intrafusal fibres, sensory endings and capsule.

2) Pre synaptic features of developing motor innervation.

Numbers of motor axons, their myelination and distribution; numbers and lengths of motor endings of these axons.

3) Post synaptic features of developing motor innervation.

Distribution of motor endings, their numbers, lengths and distances from the equator along the intrafusal fibres

1. Morphometry of the intrafusal fibres, sensory endings and capsule.

Compilation of the data was used to draw plans of each spindle or pole, with both sensory and motor innervation; an example is given for each age studied (fig. 12.) in which as many features of the innervation encountered as possible are shown. An example of an adult spindle (f) is also shown for comparison. Note (in f) the high degree of selectivity of motor axons to intrafusal fibres; the only case of non-selectivity is one dynamic motor axon which has a termination on to one of the chain fibres in addition to the bag1 fibre. See also fig. 1e.

These schematic diagrams are useful in giving the relative positions of all the muscle spindle features. For instance, it is possible to see that all the motor endings found were within the sleeve region of the capsule; that there were many motor endings present at the early post-natal stages of development; that fewer motor endings were found at 21 and 28 dpn, and whether the axons end on only one or several muscle fibres.

Most of the motor end plates were described on these plans as a,b or c due to lack of space but some may be ab,af,abf,bf.

All the features of muscle spindles were present even at the earliest age 50df development, (fig. 12). There were usually two large intrafusal fibres and between two and nine small fibres. Central nuclei were present and the

Figure 12

Plan of representative spindles and their innervation from each age group compared with a spindle from an adult tenuissimus muscle.

All spindles have a primary axon (Ia) and ending and varying numbers of secondary axons (II) and endings. All spindles have at least 2 bag fibres and several chain fibres. Spindles b) and e) have one or more long chain fibre and spindle c) has a second b2 fibre. Note how the numbers of motor endings/axon becomes less as the age of the animal increases. The scale for the developing spindles a) - e) is the same and half that of the adult f). Again the names of the motor ending types are simplified.
Spindle f) from Sutherland et al 1985, The Muscle Spindle, I.A. Boyd and M.H. Gladden. p52.

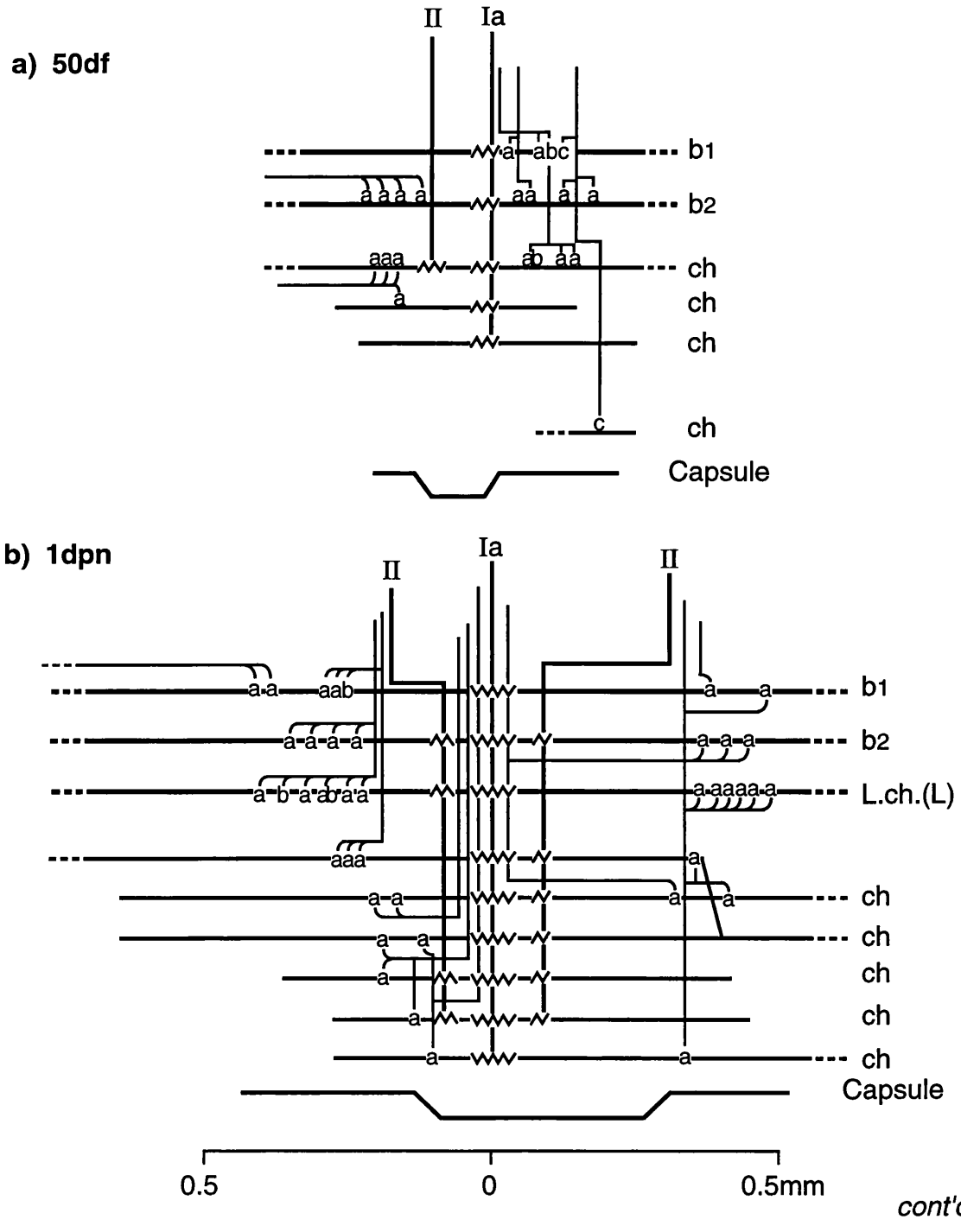


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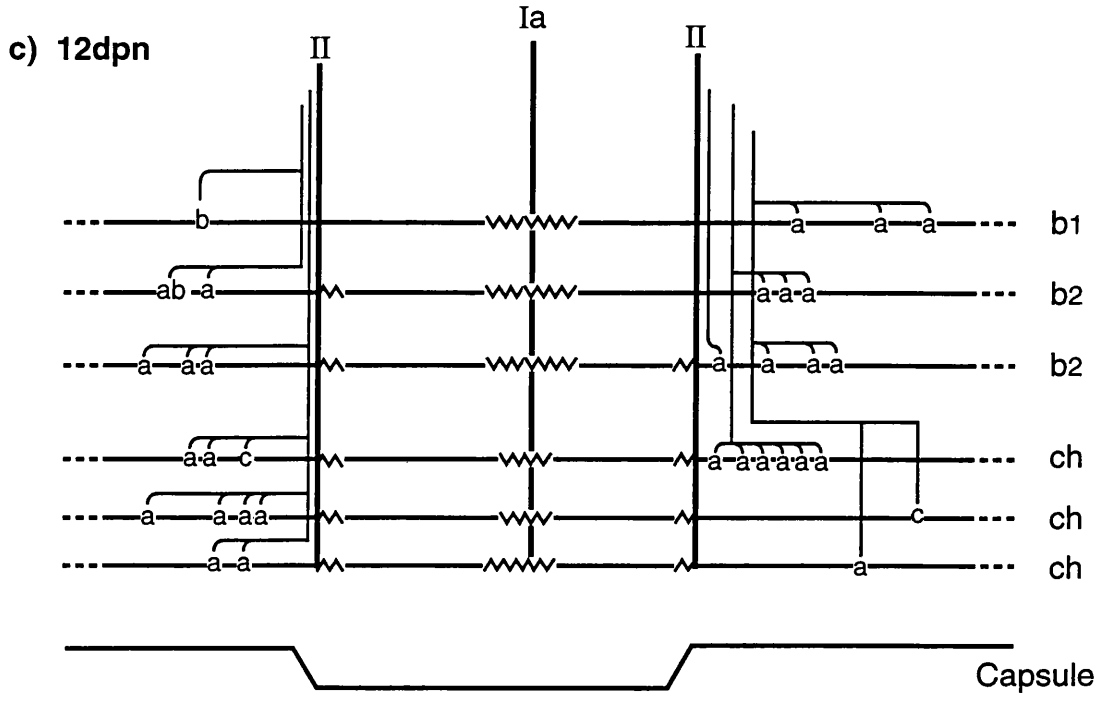
Figure 12 *cont'd*



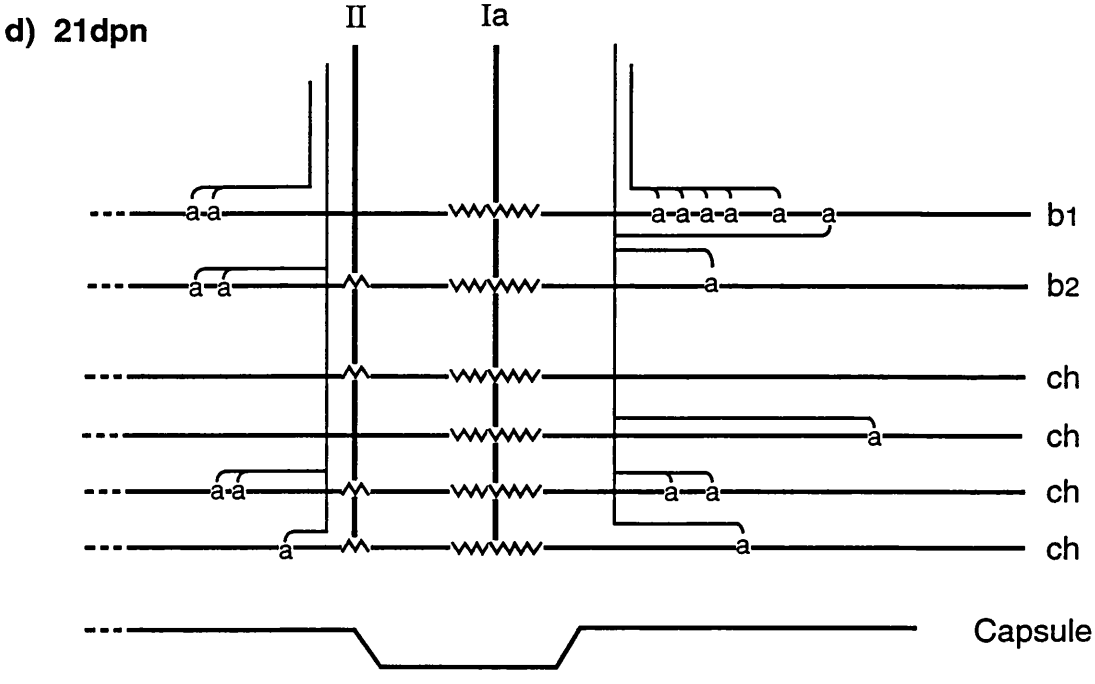


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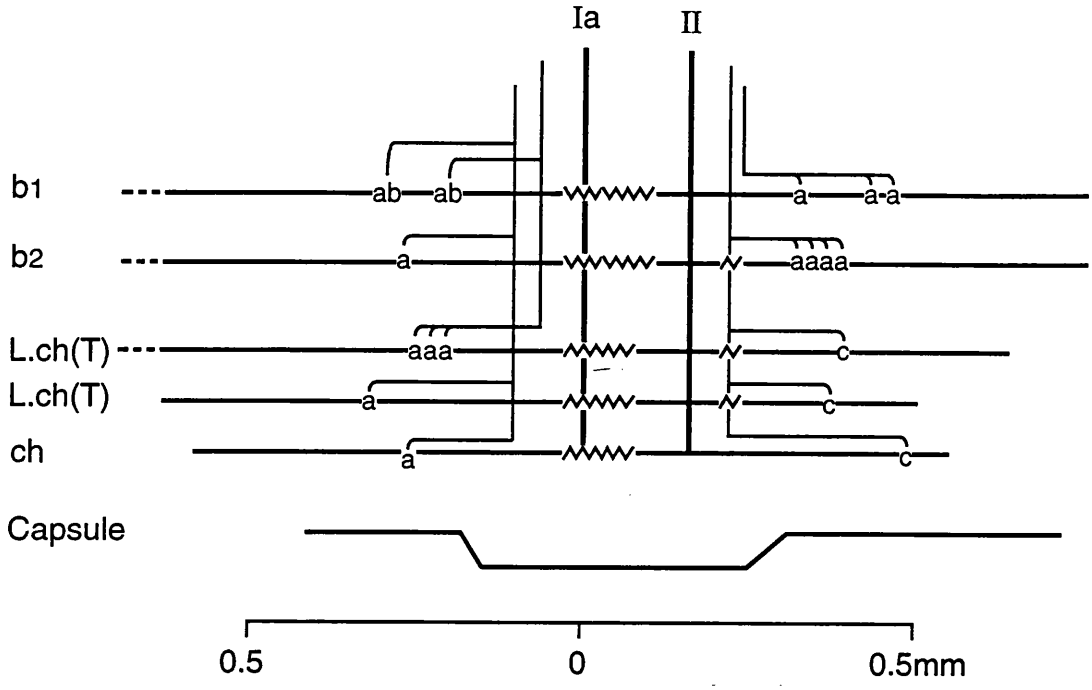
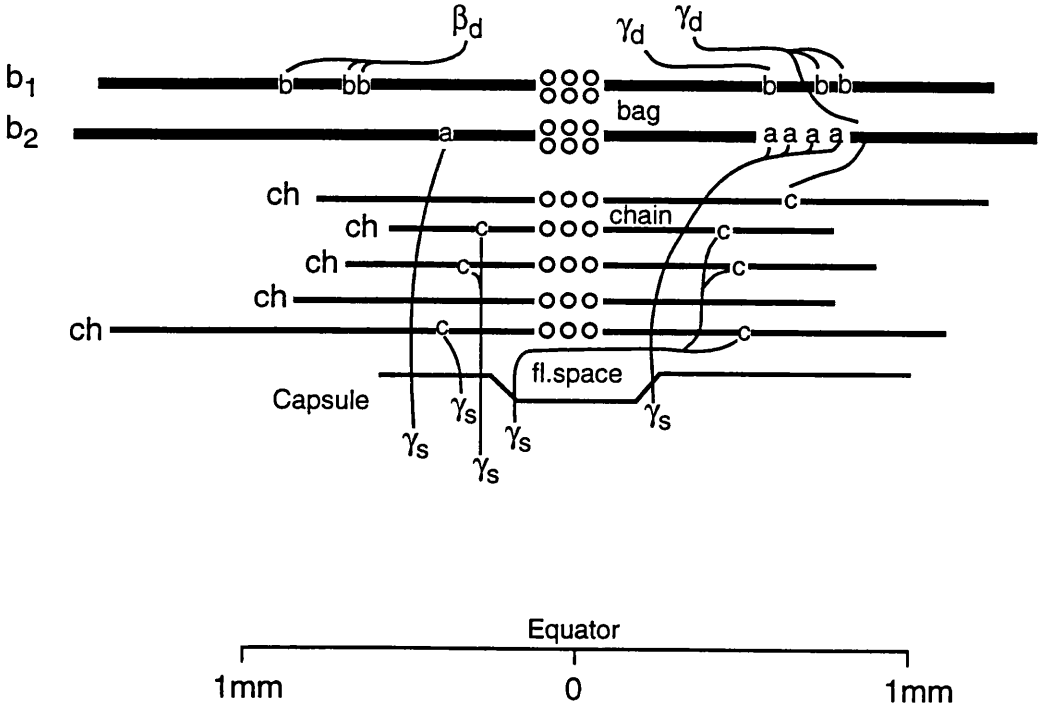
Figure 12 *cont'd*

e) 28dpn



f) Adult



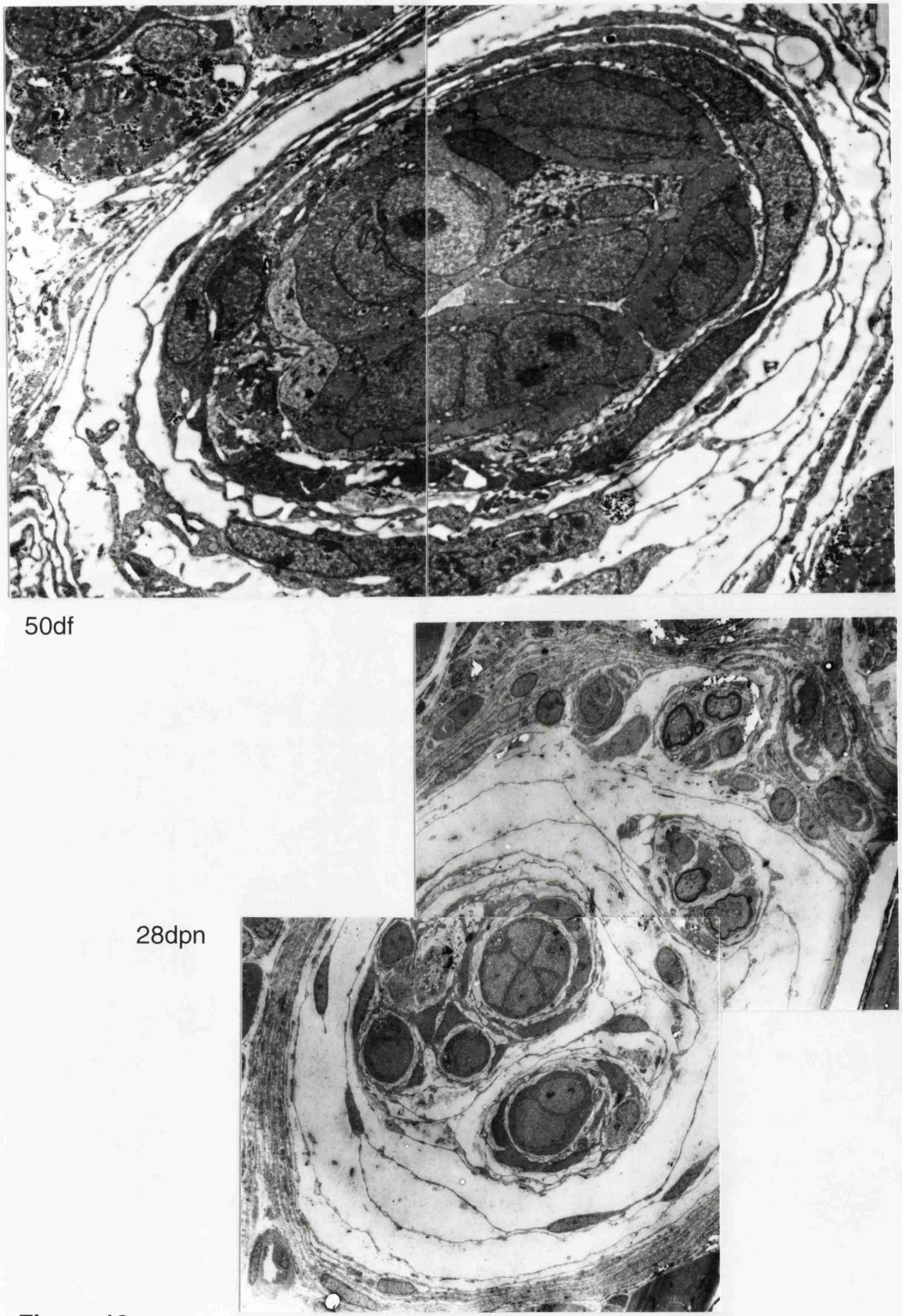


Figure 13

Electron micrographs of cross sections of the equatorial region of two developing spindles; one at 50df and the other at 28dpn.

The intrafusal fibres and capsular layers are all much closer packed at 50df than 28dpn where the fluid space has greatly increased in size, allowing the intrafusal fibres to have room to expand also.

sensory axons, both primary and secondary, were myelinated. Forty four percent of spindle poles possessed at least one secondary ending. There were unmyelinated as well as myelinated motor axons, and these had terminations on the intrafusal muscle fibres. The capsule was present and close to the intrafusal fibres throughout its length at 50df. As can be seen in figure 13, which shows electron micrographs of transverse sections of the equatorial region of a muscle spindle at 50df and 28dpn, as the spindles grew, the intrafusal fibres moved apart from each other and there was more fluid between the outer and inner capsule cell layers, but this was not prominent until 28 days after birth. (Maier and Eldred, 1974; Butler 1980 also observed this).

Cross sectional area of the intrafusal fibres at 50df and 28dpn.

a) Bag Fibre Morphometry.

In adult spindles, the bag1 fibre was usually separated from the other spindle intrafusal fibres by a capsular layer, (fig. 13), but this separation was less prominent in young spindles than in the adult muscle spindles. However, one of the bag fibres was always in closer contact with a group of chain fibres even although there were not so many capsular layers giving separate compartments. The separated fibre was designated the bag1 fibre.

Elastic fibres at the end of the b2 fibre in the adult spindles is another distinguishing feature (Gladden 1976) but in the young material this was not developed and it was not always possible to section the very polar regions of the bag fibres.

Many intrafusal fibres became smaller in the equatorial region where there were very few myofibrils present, the central nuclei taking up the majority of the space. At first it seemed (fig. 14) that the b2 fibre was consistently larger in cross sectional area than the b1 fibre area (fig. 14 a,b,e,f and h,) but there were other instances when the opposite was the case (fig. 14 c,d,g,) and so this was not a reliable distinguishing feature.

In 50df spindles, one pole of the intrafusal bag fibres was consistently larger than the other in cross section (most obvious in fig. 14a, b and d). The larger pole may have been the proximal pole, its growth being further advanced than the distal pole. It is known that there is a proximal/distal gradient of development (Evans and Sack 1973) and this would be expected to occur in spindles as well.

Unfortunately, no note was taken of which direction the muscles were positioned for fixation after dissection and so the sectioning direction for one sample will be different from another. However, where there were two muscle spindles sectioned along the one block, (fig. 14 c,d) the larger pole was oriented in the same direction in each case, suggesting that the size difference may have been related to the proximal/distal gradient. This differential size between the bag fibres of the two spindle poles, was not evident in the 28 dpn samples, (fig. 14 e-h) the growth presumably being more symmetrical by this time.

b) Chain Fibre Morphometry.

The areas of each of the chain fibres was taken in the same manner as the bag fibres. From fig. 15 it can be seen that there was also a reduction in the area

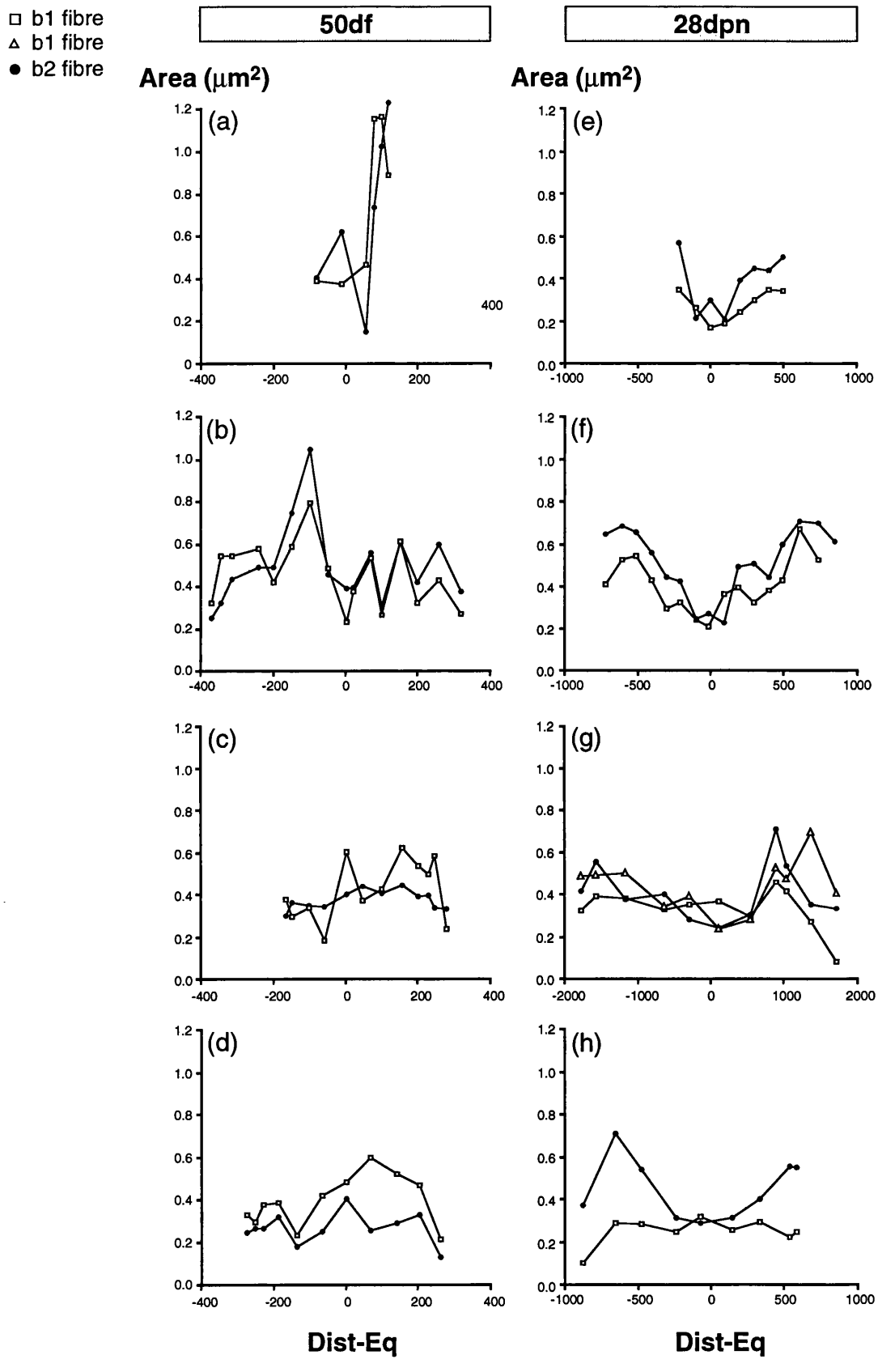


Figure 14

Graphs comparing the cross sectional areas of four bag_1 and bag_2 fibres throughout their whole lengths in spindles at 50df and at 28dpn.

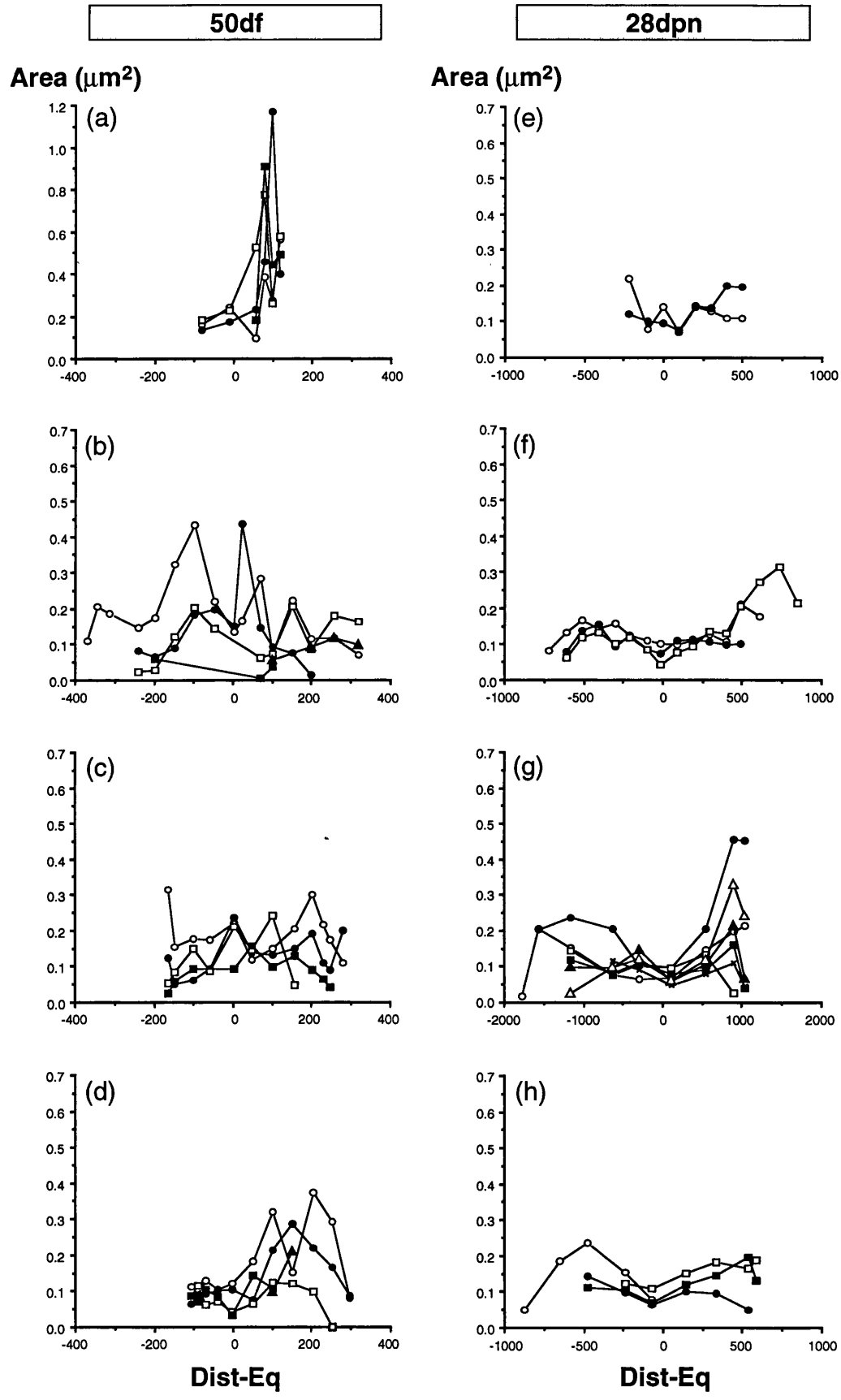


Figure 15

Graphs showing the cross sectional areas of typical chain fibres throughout their lengths in four spindles at 50df and at 28dpn. (Same spindles as Fig. 14)

Each line of joined symbols represents a separate chain fibre.

at the equatorial region although generally smaller than that of the bag fibres. There was less variation in area from one pole to another (exceptions are a and g in fig. 15) and it was certainly not as pronounced as in the 50df bag fibre samples. Variations in intrafusal fibre area throughout its length, may occur due to cutting obliquely and fixation having contracted the myofibrils unevenly. No difference in fibre areas at 50df was found between peroneal and tenuissimus muscles.

Length of primary and secondary sensory endings.

The length of the primary spiral and the secondary ending, where present, was recorded from each spindle. This could only be done on whole spindles, or at least where there was enough length of spindle to see the complete extent of the sensory ending. The mean lengths of primary and secondary sensory endings are shown in table 3.

From the table of mean lengths of the primary and secondary ending lengths with age (Table 3), it comes as no surprise that the length of each structure increases with age as the muscles grow. It is perhaps the variation of lengths from one spindle to another that is more interesting, this being wide even at the 28dpn age group. The secondary sensory endings were particularly variable and at 21dpn the mean length was extremely large in comparison with that at 28dpn as was the range in lengths of primary sensory ending at 28dpn.

Table 3. **Mean lengths ± standard deviation of spindle primary and secondary sensory endings between 50df and 28dpn development.**

Primary ending	**50df** n=5	**1dpn** n=7	**12dpn** n=6	**21dpn** n=3	**28dpn** n=7
Mean length (μm)	**108.2±** 41.2	**163.6±** 58.7	**152.5±** 54.8	**228.2±** 33.3	**227.2±** 150.3
Range (μm)	68-160	70-235	60-230	200-265	150-560
Secondary ending	**50df** n=8	**1dpn** n=9	**12dpn** n=3	**21dpn** n=2	**28dpn** n=4
Mean length (μm)	**42.3±** 19.4	**42.7±** 35.5	**71.7±** 40.7	**320±** 240.4	**97.5±** 122.8
Range (μm)	23-73	10-100	25-100	60-400	20-280

Length of the capsule.

The capsule is traditionally considered in two regions; the A and B regions (Barker 1948). The A region extends from the centre of the spindle measured from the centre of the primary sensory spiral to the end of the fluid space. In the B or sleeve region, the capsule closely envelops the intrafusal bundle from the end of the fluid space to the end of the capsule.

Table. 4. **Mean lengths ± standard deviation of spindle capsule poles; A and B regions and total length between 50df and 28dpn development.**

A region	**50df** n=9	**1dpn** n=12	**12dpn** n=9	**21dpn** n=8	**28dpn** n=12
Mean length (μm)	**90.6±** 50.7	**183.8±** 110.2	**200.6±** 84.5	**177.5±** 24.9	**235.0±** 173.2
Range (μm)	20-155	50-360	95-345	150-220	110-710
B region	n=6	n=9	n=6	n=6	n=9
Mean length (μm)	**128.7±** 66.2	**311.6±** 95.8	**525.0±** 181.6	**312.2±** 120.6	**431.7±** 294.8
Range (μm)	67-243	165-490	320-840	148-440	170-860
Whole pole	n=6	n=10	n=6	n=6	n=9
Mean length (μm)	**211.2±** 47.4	**447.9±** 167.2	**705.8±** 188.0	**478.8±** 122.8	**711.7±** 410.8
Range (μm)	124-263	215-790	525-1050	298-620	320-1570

The mean lengths of capsule in the A and B regions as well as the total capsule length increased with increased age of the animal as expected (Table 4). However, the measurements at 21dpn seem to be smaller than those at 12dpn and 28dpn. This cannot have been due to the kittens of that sample being smaller in size, because from Table 1 it was seen that the kitten at 21dpn was 15.6% larger than that at 12dpn.

Designation of chain fibres as long or typical chains.

For the adult spindle, the descriptor 'long chain fibre' is applied to those projecting 1mm beyond the end of the capsule (Barker et al 1976; Kucera 1982). Since a definition based on absolute length could not apply to developing spindles as described in the methods, the ratio (R) of the length of chain fibre pole projecting beyond the capsule to that of the length of the whole fibre pole was found; any chain fibre pole with a ratio of more than 0.35 was considered to be a long chain pole. A chain fibre pole with a ratio of zero or a negative value was as long as the capsule or ended within it; clearly these are all 'typical' chain fibre poles.

The ratio of 0.35 was based on the relative lengths of chain fibre pole outside and inside the capsule in adult spindles (see Methods). Figure 16 shows that in practice this ratio seemed justified. At 50df, 1 chain fibre pole was re-classified as a long chain pole. At 1 and 12 dpn, no chain fibres became long chain fibres. At 21dpn, there were 2 and at 28dpn 4 chain fibres poles which became long chains with this formula. Any long chain poles with large cross sectional diameters, which may be different physiologically from these narrow long chain poles, were excluded from these calculations.

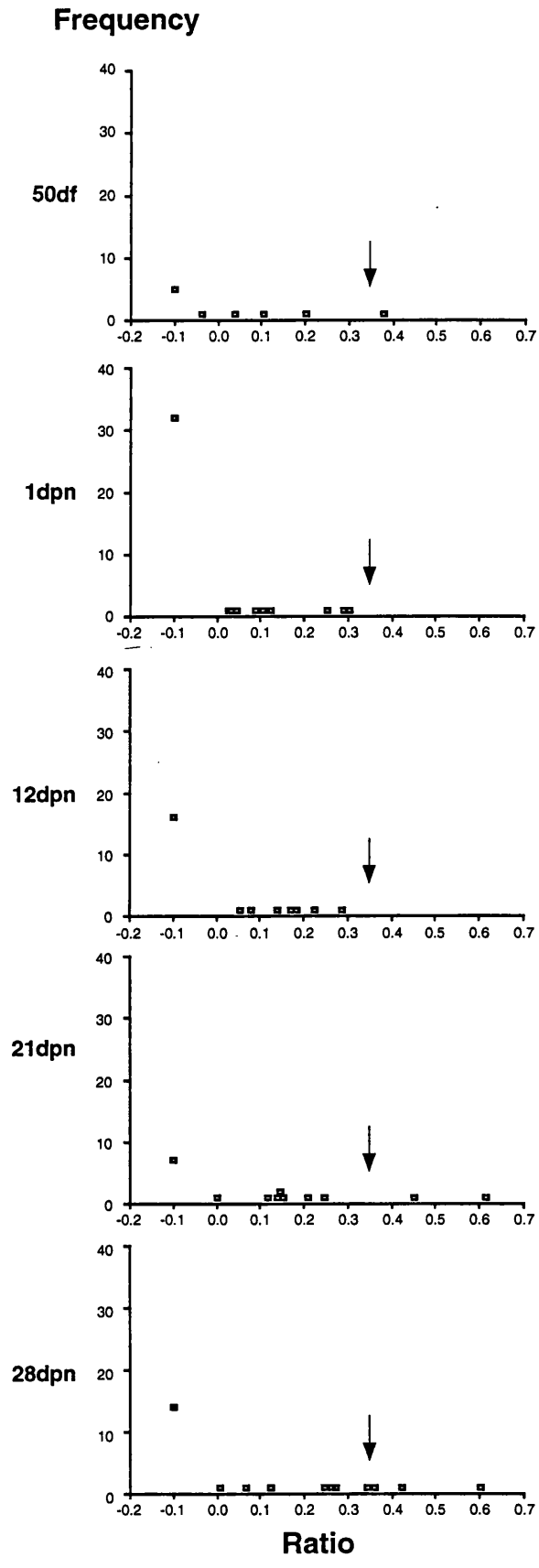


Figure 16

Graphs of the ratio of the length of whole typical chain fibres to the length that projects outside the capsule with the numbers (frequency) of chain fibres measured. The arrow indicates the point (0.35) beyond which any chain fibres should be reclassified as long chain fibres.

Description of motor endings.

The basic classification of Arbuthnott et al 1982, plus the intermediary categories introduced in Methods to account for the many examples that fell between two groups was used.

The following micrographs (fig. 17) show examples from each of the types found except type md as none of these were found in the young animals. These micrographs can be interpreted with reference to fig. 9 where they were drawn in outline.

2. Pre synaptic features of the developing motor innervation.

It is convenient to consider features of the motor axons and their connections separately from the intrafusal fibres and their connections, although the motor endplate is the term for the complete structure of pre- and post-synaptic features where the axon and muscle connect.

a) The numbers of motor axons.

Figs. 18A and 18B show the numbers of motor axons to whole spindles and intrafusal fibre poles for each age groups studied.

When the spindle pole as a whole was considered as in fig. 18.A, the mean number of motor axons to each spindle pole was two to three throughout the study, much the same as in the adult spindles (Kucera 1985; Arbuthnott et al 1982).

However it is interesting that the range of axon numbers was more variable at 50df, 1dpn and 12dpn, one spindle being innervated by 5 motor axons. This

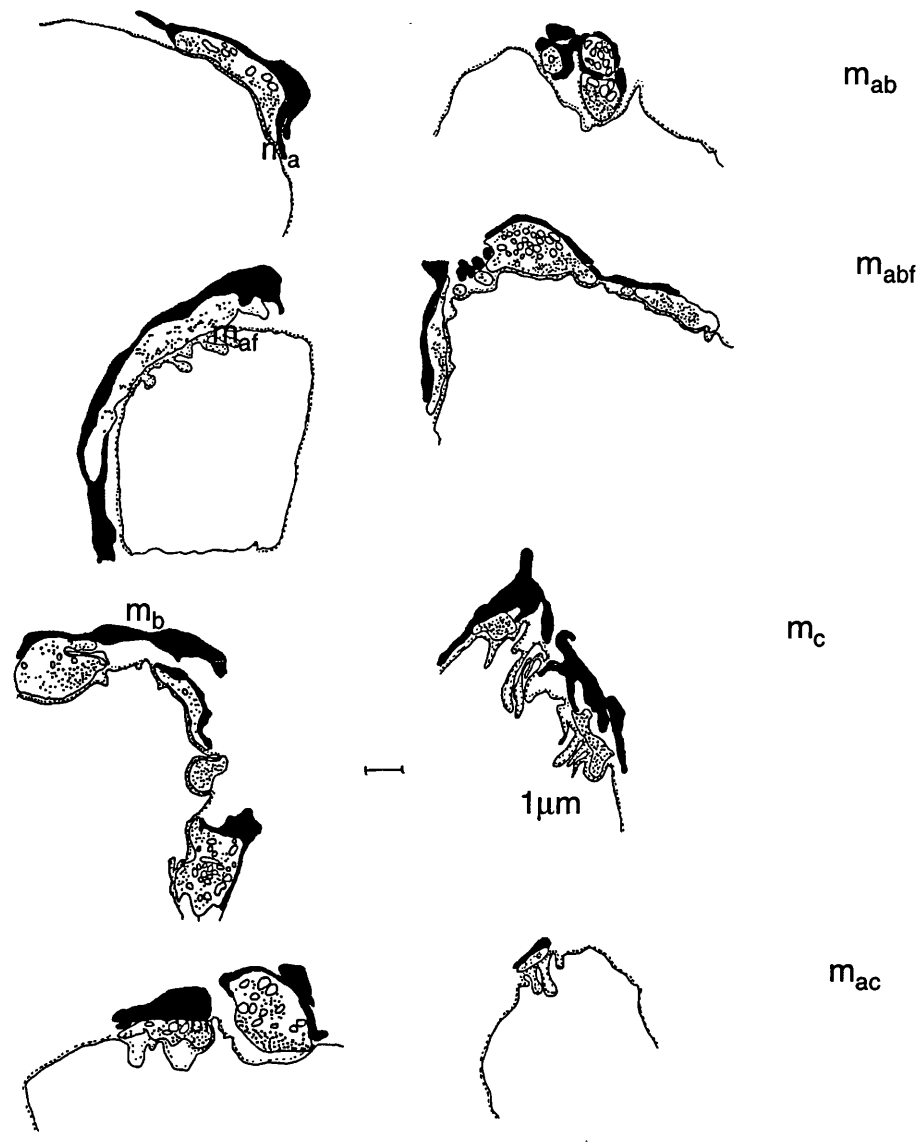


Figure 9

Tracings of motor endings to show the 8 types distinguished in this study.

These are basically four of the five types of Arbuthnott et al, 1982, where
m_a has axon terminals lying superficially on the intrafusal muscle surface;
m_b has terminals indented into the muscle surface;
m_c has terminals superficial and indented into the muscle surface but the muscle surface itself is thrown up into finger-like processes;
m_{ab} has terminals 'half and half' indented into the muscle surface.
In development the superficial axon terminals can be resting on a very folded muscle surface, m_{af}, or 'half and half' indented over a folded muscle membrane, m_{abf}. m_{ac} is similar to m_{af} exept the muscle surface is raised in the m_{ac} type although the axon terminals are all superficial.

Key:
Structure containing stipples (vesicles) is an axon terminal. The open shapes are mitochondria.
Dotted line is basement membrane.
Clear structure is intrafusal muscle.
Black structure is Schwann cell.

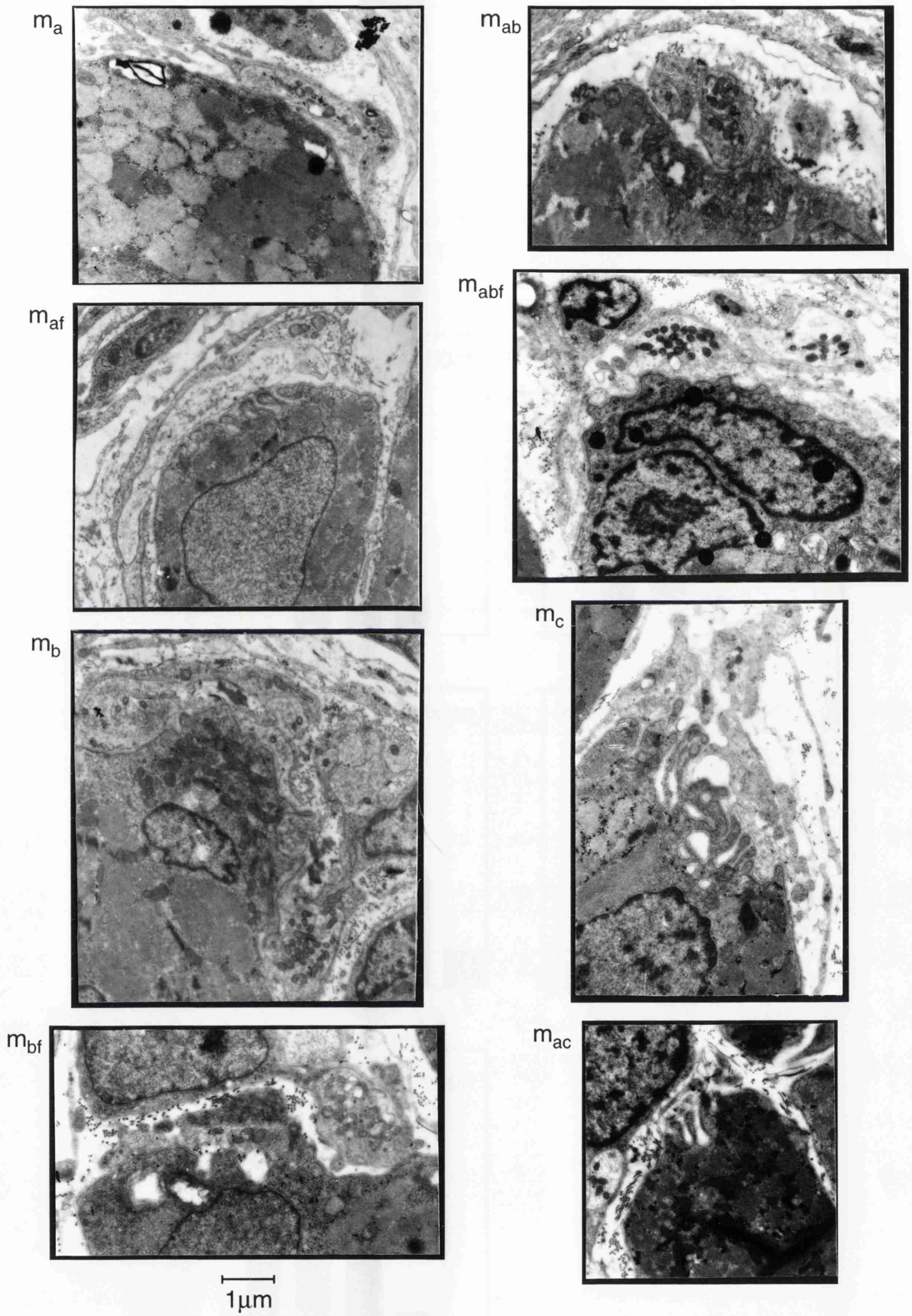


Figure 17

Electron micrographs of the 8 motor ending types studied in this work.

These can be studied with their tracings in fig 9.

Figure 18A

Number of fusimotor axons per whole spindle pole with age.

m = mean.

There was no significant difference of mean numbers of axons per whole spindle pole between any age groups.

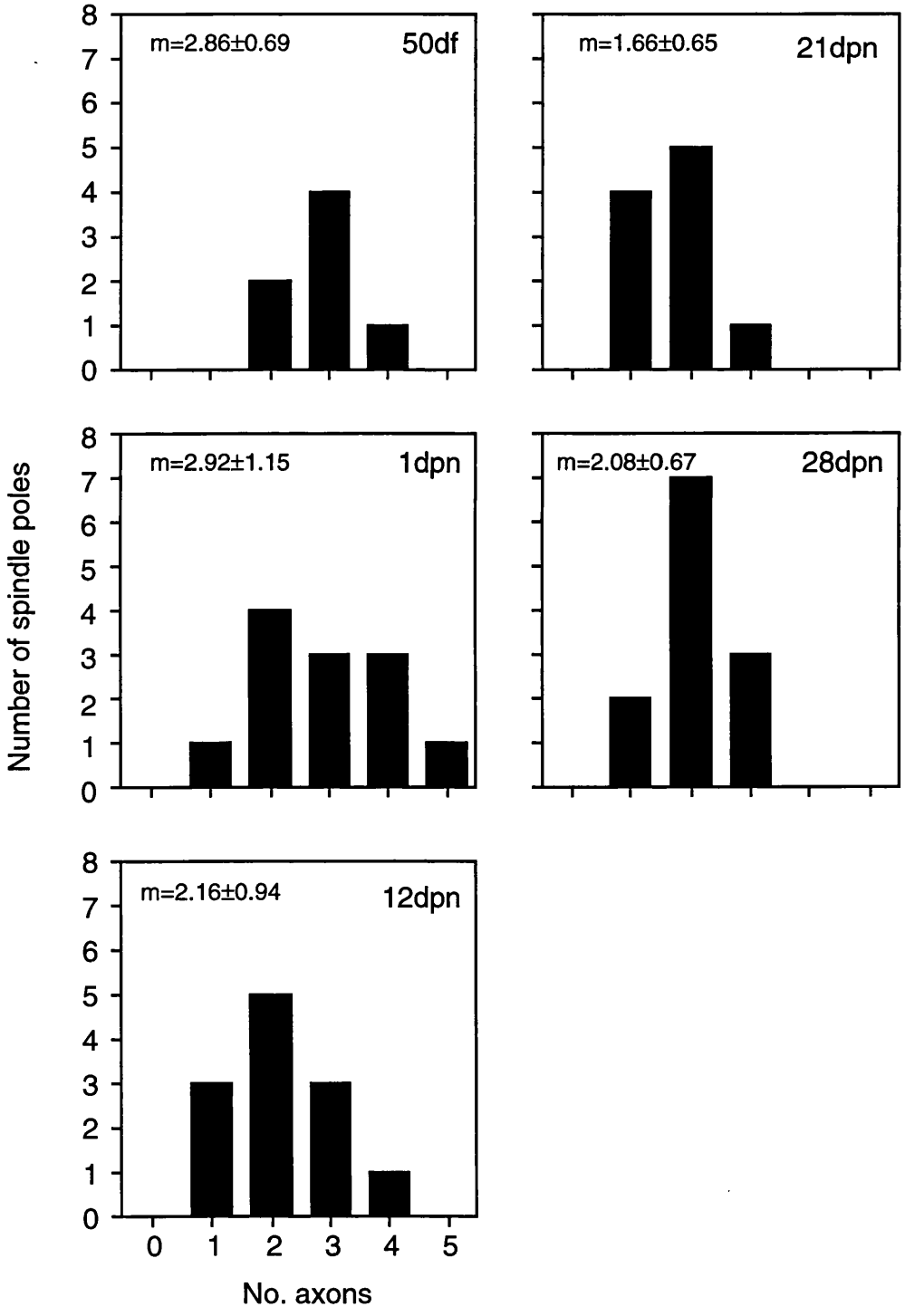
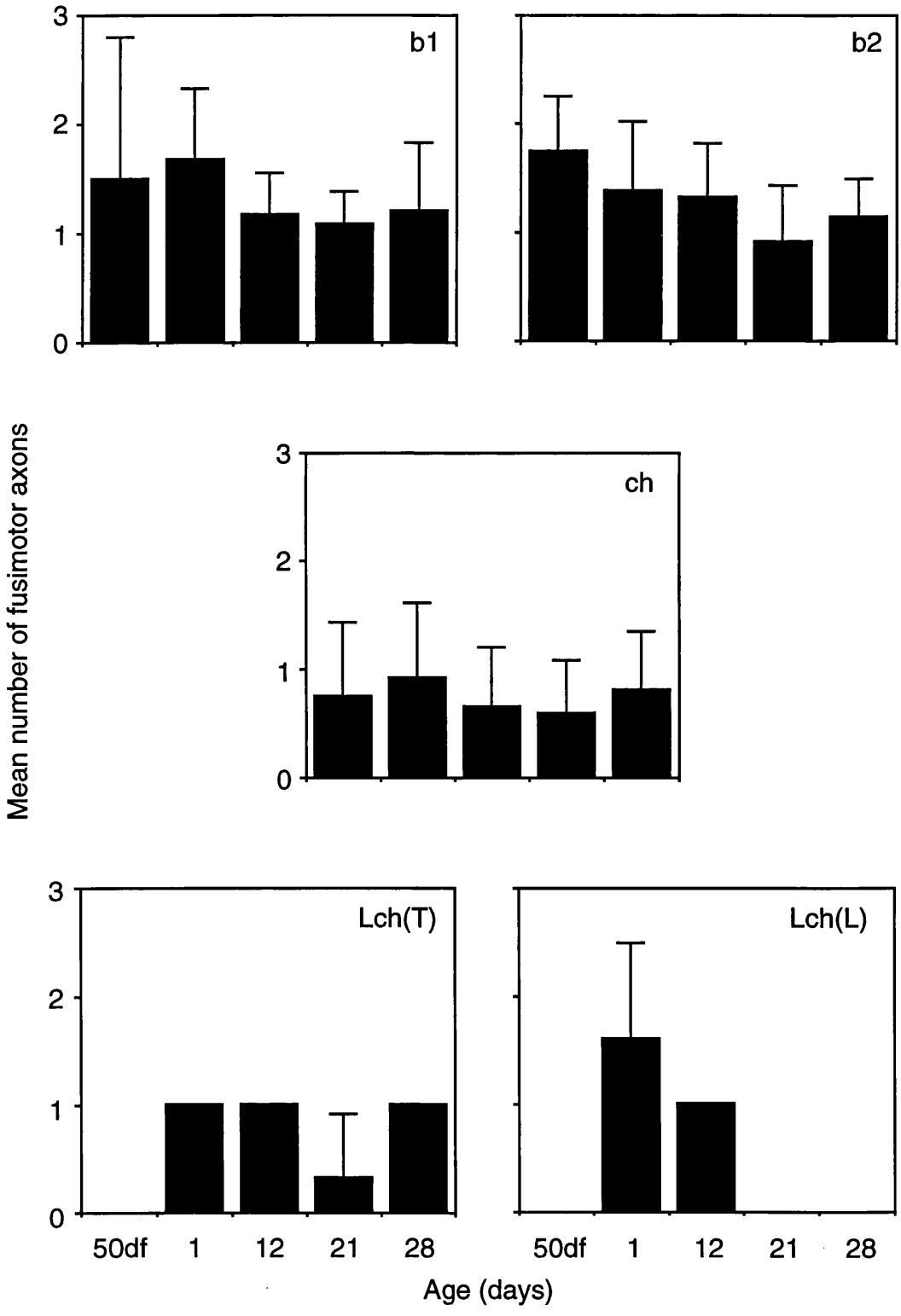


Figure 18B

Mean number of fusimotor axons per individual intrafusal fibre pole.

Lines through the bars are standard deviations.

There was no significant difference of mean numbers of axons per individual intrafusal fibre pole for any fibre type, between age groups.



indicated an excess of motor axons at that time because at older ages the largest number of motor axons in any pole was 3. This concurred with findings in the adult spindles.

In fig. 18B, the number of motor axons to the individual fibre poles was seen. The bag fibres received endings from one axon per fibre pole on average although the b1 at 1dpn. and the b2 at 50df. may have had excess innervation. Chain fibre poles of both typical and long chains, mainly had endings of one axon but also had several non-innervated fibre poles (table 6). This agrees with the numbers found in adult spindles (Kucera 1985).

b) Percentage of motor axons that were myelinated.

Table 5. Percentage of motor axons that were myelinated at the different ages studied.

	50df	1dpn	12dpn	21dpn	28dpn
total nos. axon.	12	36	26	20	25
nos. unmyelinated axon.	12	32	23	8	2
nos. myelinated axon.	0	4	3	12	23
% axons myelinated.	**0**	**11.1**	**11.5**	**60.0**	**92.0**

At 50df all motor axons were unmyelinated. At 1 and 12dpn the percentage of myelination remained constant at 11%; not until 21dpn were more than half of the motor axons myelinated. By 28dpn over 90% of motor axons to spindles were myelinated indicating increased myelination as development proceeded.

c) Distribution of motor axons to intrafusal fibres.

Both unmyelinated and myelinated axons were followed through consecutive electron micrographs to their intrafusal fibre destinations. This has not been possible previously in such young material and was aided by the small intervals (2 and 5μm) between ultrathin sections. It was therefore possible to determine whether a motor axon had terminals on one or several intrafusal fibres and their distributions are shown in fig. 19. Combinations of co-innervated or selective motor innervation are given in the key to the figure. The chart giving information on adult spindles was compiled from Banks (1981), Arbuthnott et al (1982) and Kucera (1985). The numbers of motor axons are tabulated in table 5.

In all cases of young animals, the percentages of b1 fibres selectively innervated never reached the 34% found in the adult. The bag1 innervated along with one chain fibre was a variable finding, even in the adult (mean value of 3% from the pooled data) but an instance of bag1 and more than one chain fibre being innervated together has not been observed in the adult; however, instances were found at the 1, 21 and 28dpn ages.

Selective innervation of bag2 and chain fibres before birth was almost as high as in the adult (taken together, 42% compared with 45%). The proportion reached the lowest at 21dpn (15%), but had increased by 28dpn (24%). On

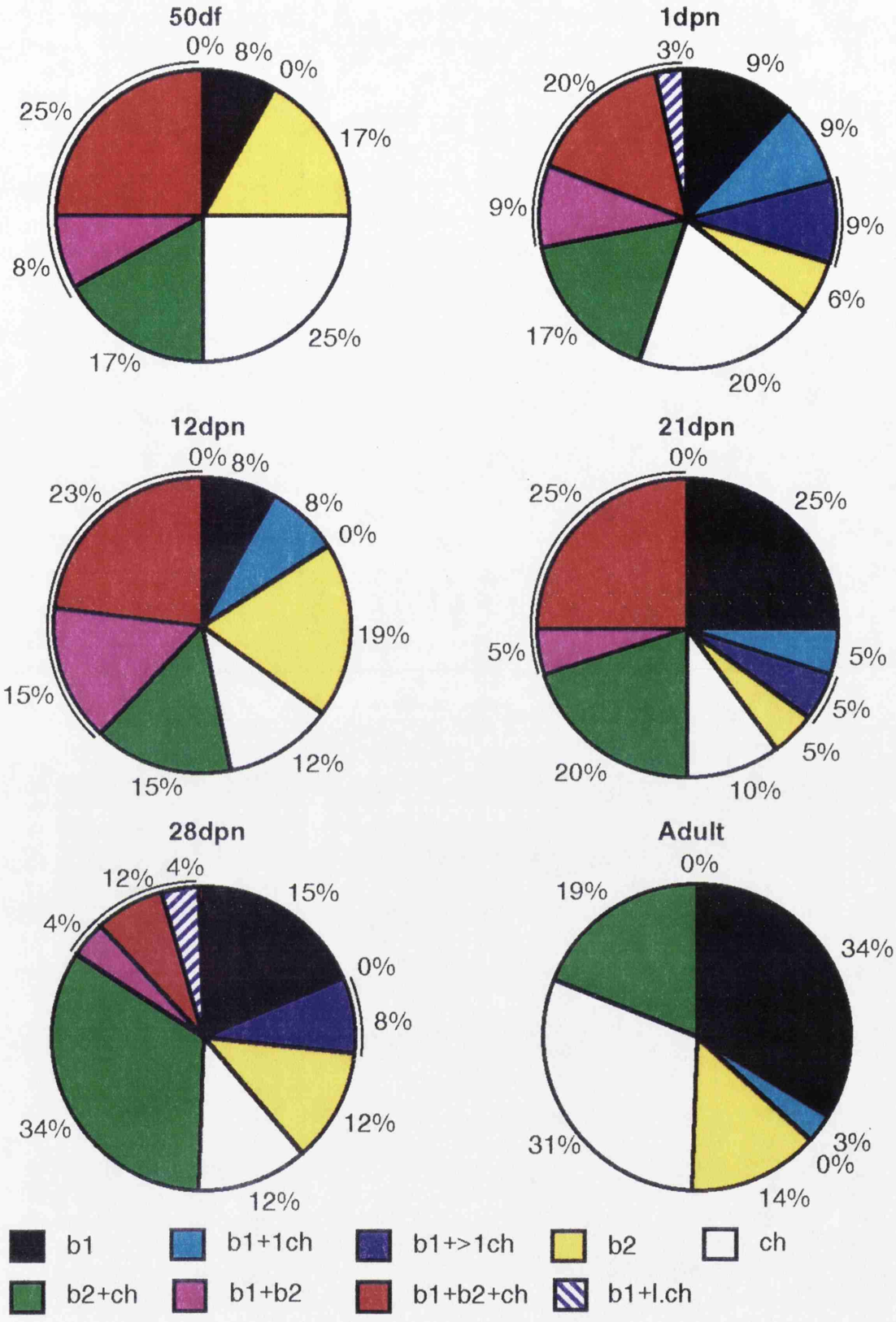


Fig.19

The distribution of motor axons to the intrafusal fibres as a percentage of all axons supplying aspindle pole compared with adult tenuissimus spindle poles.
Categories of innervation are displayed in the charts in the order in which they are listed, starting at the twelve o'clock position and moving clockwise.
0% indicates that one or more categories are absent.
Various combinations of intrafusal fibres innervated are found in the developing spindles (marked by a curved line outside the chart), which are not found in the adult example.

the other hand, the commonest non-selective innervation in adult spindles (b2+ch), remained about the same proportion as in the adult until 28dpn when it was almost doubled.

Co-innervation of a) bag1 and bag2, b) all three intrafusal fibre types and c)bag1 and large diameter long chain fibres occurred only during development. Even at 28dpn these combinations of connections were still present which indicates that there was still a great deal of readjustment of the motor innervation to occur.

There were no axons that exclusively innervated the long chain fibres, either with large or small fibre diameter, in this study.

When the motor axon distributions were separated into unmyelinated and myelinated axons as in fig. 20 A, B respectively it could be seen that at ages up to and including 21dpn, both myelinated and unmyelinated axons made multiple connections with intrafusal fibre types in combinations not found in adult spindles. Between 21 and 28dpn, the percentage of unmyelinated axons making non-adult multiple intrafusal fibre type connections changed from 25 to 100. During the same period, the percentage of myelinated axons making immature multiple fibre type connections decreased from 33 to 20. (At birth, non-adult patterns of multiple intrafusal fibre type innervation occurred with 50% of myelinated axons.)

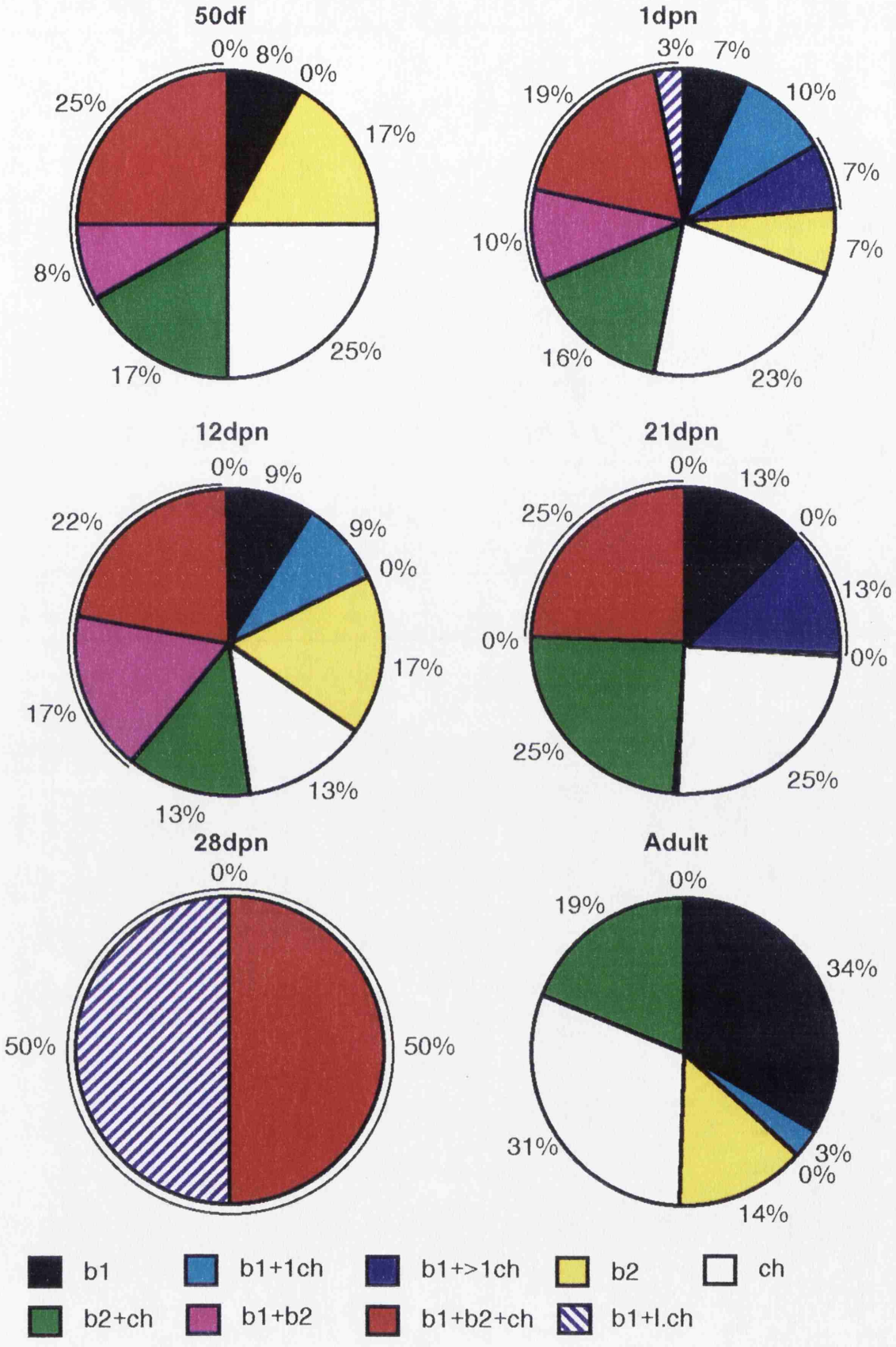


Fig.20A

The distribution of unmyelinated motor axons to the intrafusal fibres compared with adult tenuissimus spindle poles.
Various combinations of intrafusal fibres innervated are found in the developing spindles (marked by a curved line outside the chart), which are not found in the adult example.

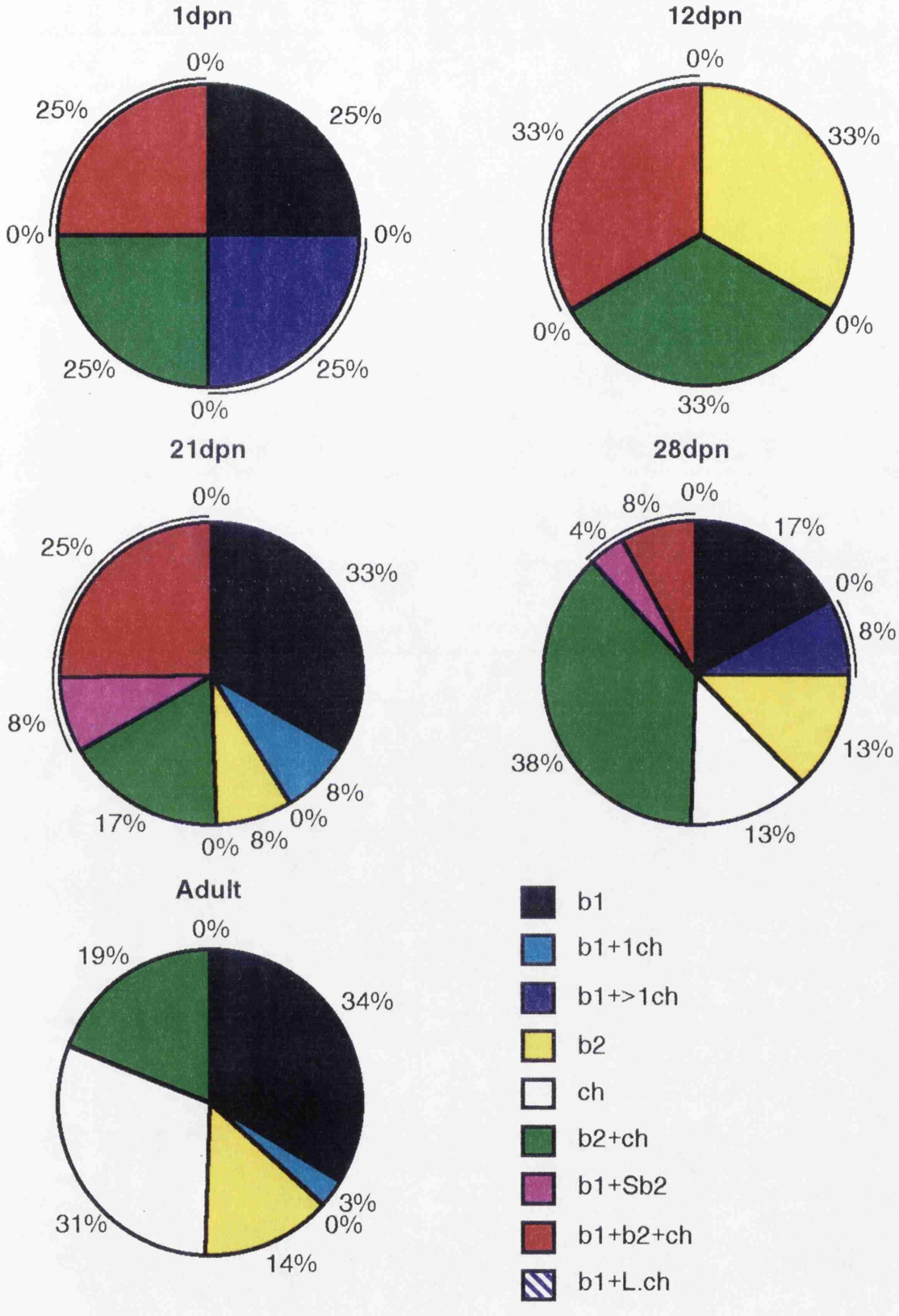


Fig.20B

The distribution of myelinated motor axons to the intrafusal fibres compared with adult tenuissimus spindle poles.

There were no myelinated axons at the 50df stage of development. Various combinations of intrafusal fibres innervated are found in the developing spindles (marked by a curved line outside the chart), which are not found in the adult example.

d) Numbers of endings of each motor axon.

The mean number of motor endings of each motor axon, for all axons together and separating the myelinated from the unmyelinated axons was calculated (Appendix 2, table A).

The mean numbers of motor endings/motor axon were plotted in fig. 21 from figures in table A, appendix 2. The intrafusal fibre on which the axon terminated is given and therefore whether the axon was selective or otherwise to that fibre. Actual numbers of motor endings of each axon can be seen in fig. 22 A, B, C. (For further analysis of this figure see Results section 3.)

The most obvious result of figs. 21 and 22 A, B, C indicated that selective axons had the smallest number of motor endings and that axons non-selectively innervating the intrafusal fibres had the largest number of motor endings. This was irrespective of whether the combination of the fibres innervated was as seen in the adult (b2 + ch) or only in development, ('other' fig. 21). It should be noted that this applied to chain fibres as well as bag fibres; multiple endings on bag fibres means multiple endings on the same fibre, which is not the same for chain fibres for which the axon has the possibility of ending on several chain fibres.

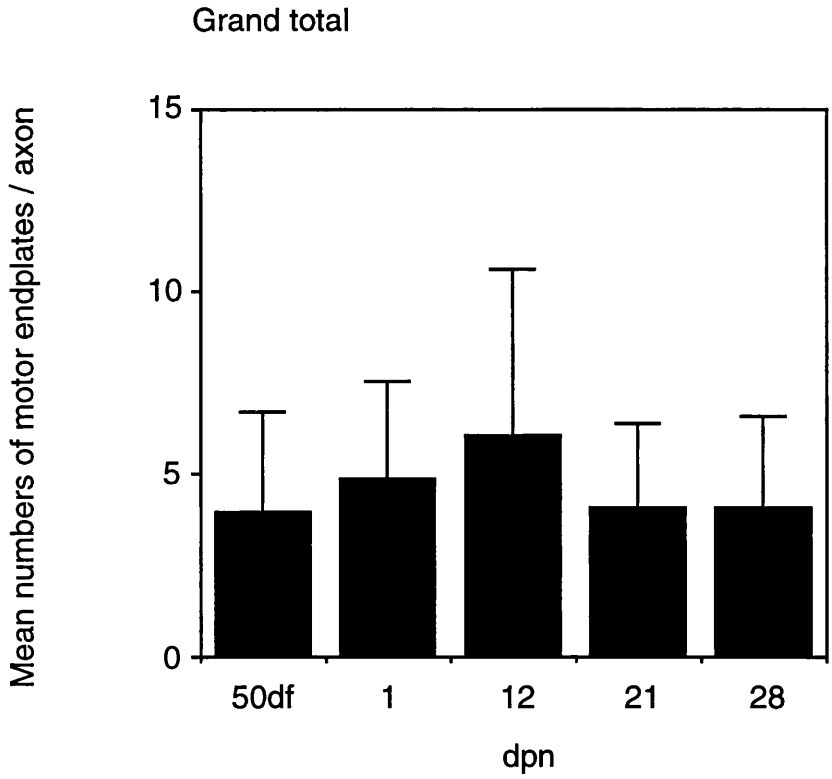
Using Fisher's PLSD test, the mean of the total numbers of motor endings of each motor axon at 12dpn was significantly greater, at the 95% level, from the 50df, 21 and 28dpn means. However, the numbers of motor endings for each axon was not significantly higher at 12dpn if the axons were separated into myelinated and unmyelinated.

Figure 21

Mean numbers of motor endplates for each motor axon.

Total mean numbers of motor end plates / axon of the whole sample (grand total) show a significant peak at 12 dpn.
b1, mean numbers (total) of motor endplates/axons increased with age.
b2, mean numbers (total) of motor endplates/axons decreased with age.
b2ch and n-s, mean numbers (total) of motor endplates/axon showed a significant peak at 12dpn.
(All significances at the 95% level with Fisher PLSD test).

Myelinated (my) as well as unmyelinated (um) axon values are shown separately from the total values.



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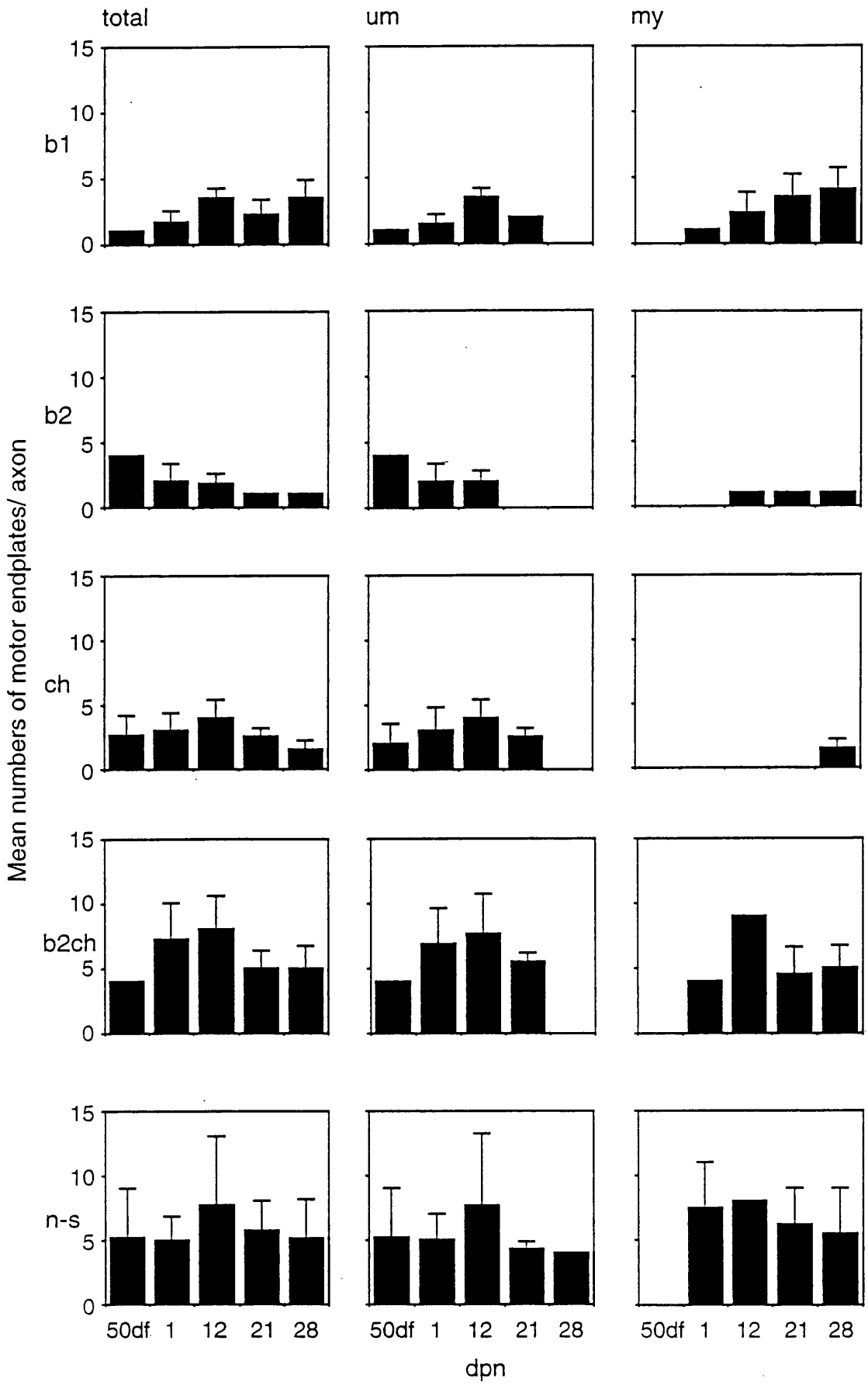


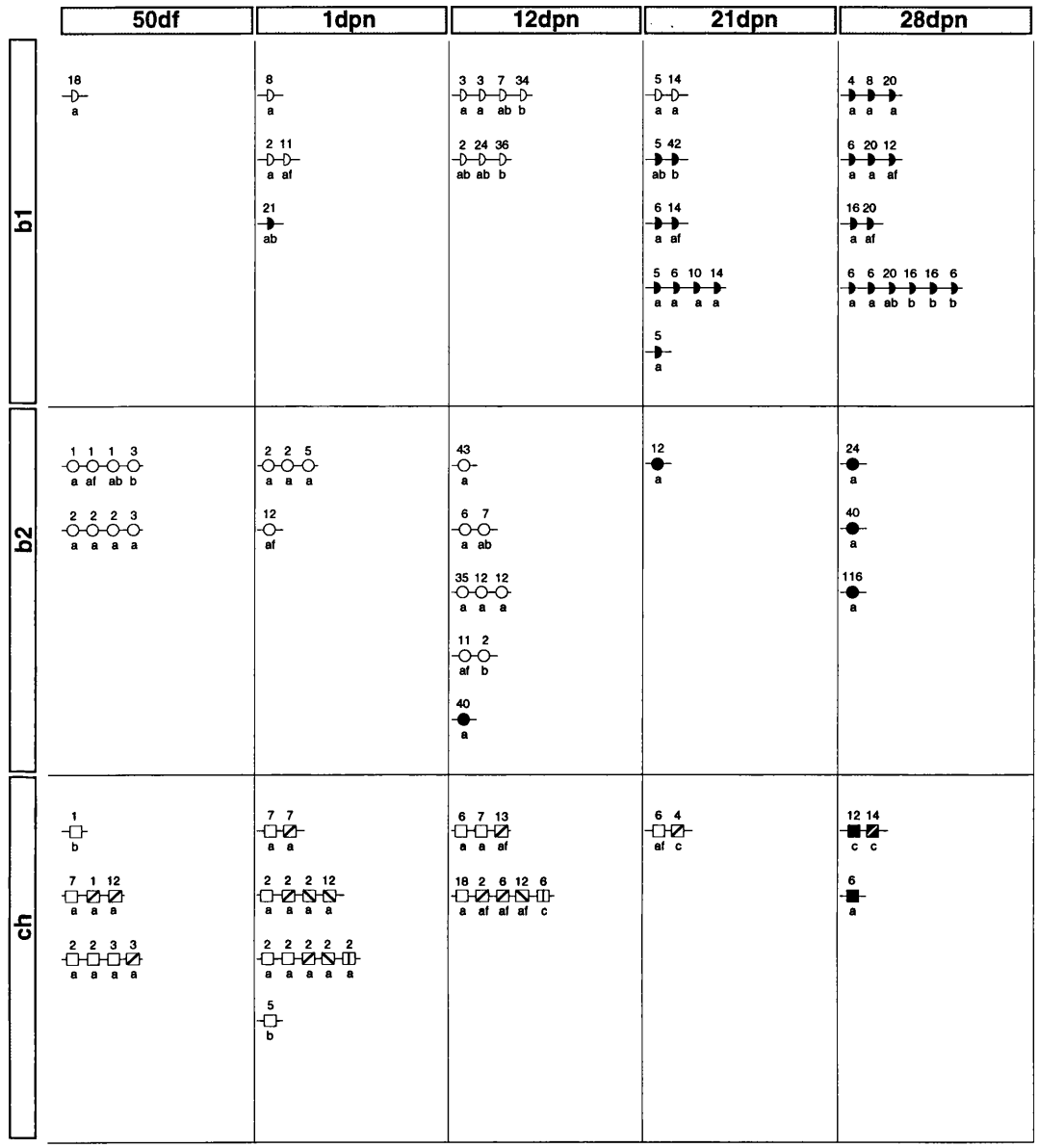


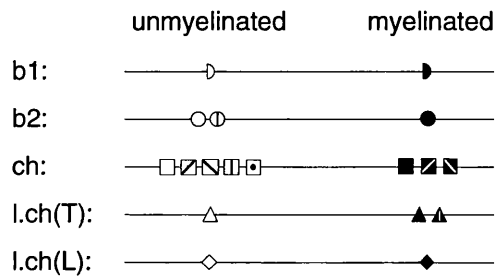
Figure 22a

Motor ending lengths (μm) related to type for motor axons selectively innervating the three intrafusal fibre types, with the age of the kittens.

Filled symbols represent myelinated axons and unfilled symbols represent unmyelinated axons.

Letters beneath the symbols indicate motor ending type.

The key to the shapes of the motor endings indicates which fibre type the motor axon terminates upon.



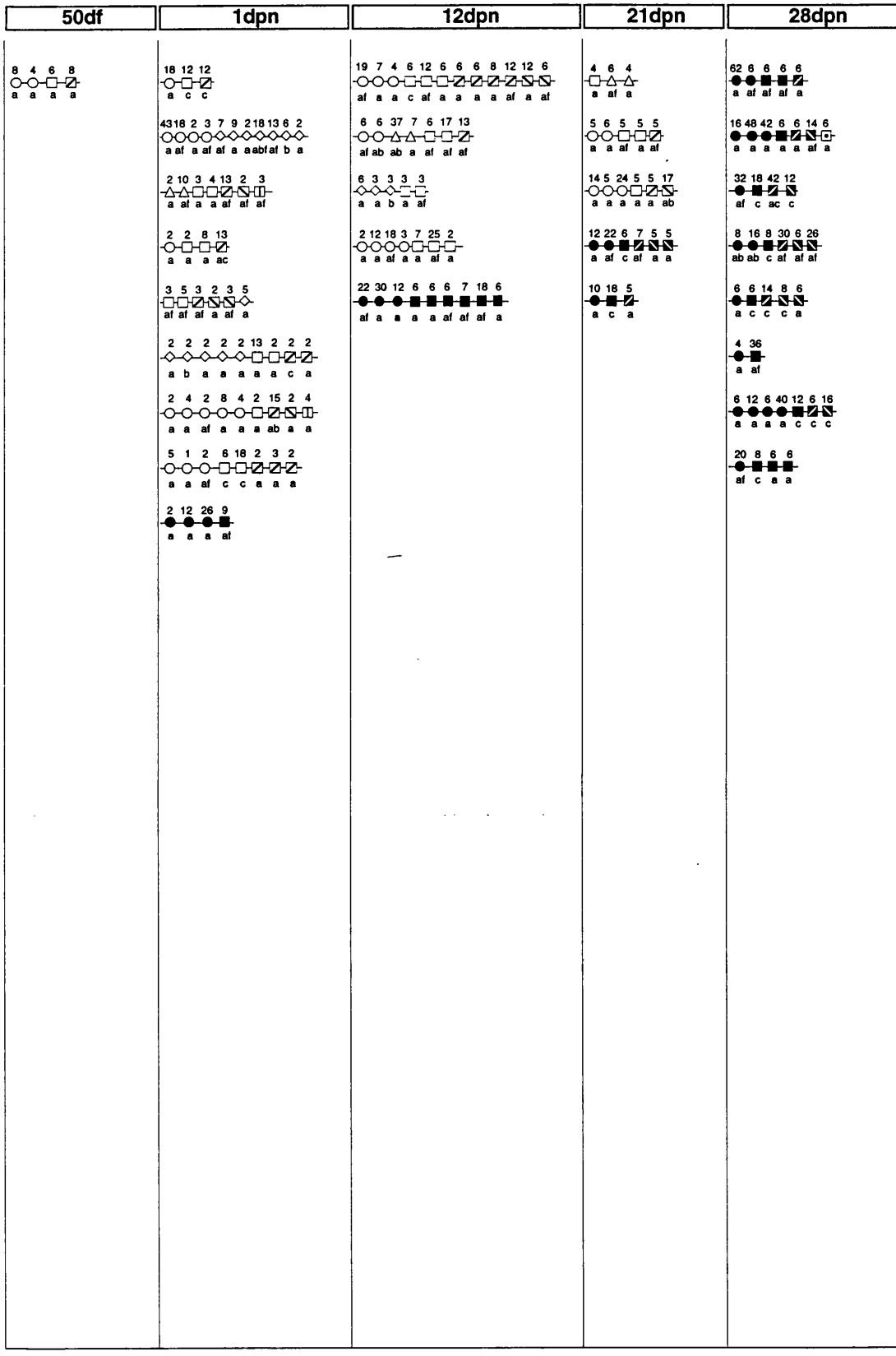


Figure 22b

Motor endplate lengths (µm) related to type for motor axons innervating b2 + ch or ch + L.ch with the age of the kittens.

Symbols as in figure 22a.

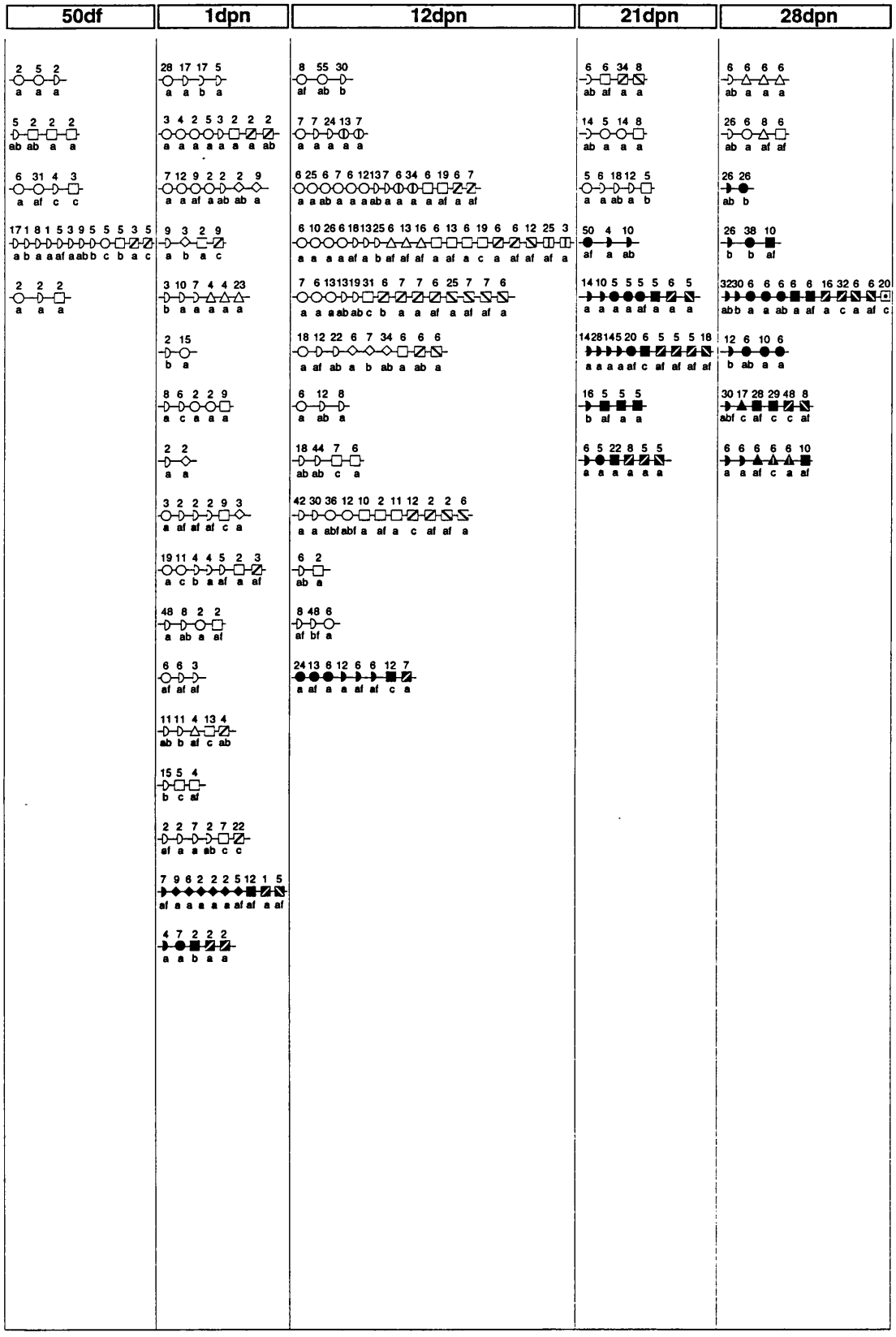


Figure 22c

Motor endplate lengths (μm) related to type for motor axons innervating any combination of intrafusal fibres not already covered in figures 22a and 22b.

Symbols as in figure 22a.

Also from fig. 21 it can be seen that for selective axons with endings on the b2 fibre, mean numbers of motor endings did not follow this trend. Axons ending selectively on the b2 had most mep's at 50df and numbers were significantly reduced at all other ages (Fisher PLSD 95% level). Selective axons ending on b1 fibres had a significant increase in the number of motor endings (Fisher PLSD 95% level) by 28dpn. Selective motor axons to typical chain fibres had no significant difference in numbers of endings of their motor axons at any developmental stage. However, it may be significant that myelinated axons innervated chain fibres selectively only by 28dpn, although these axons had been present since 1dpn..

The numbers of motor endings of non-selective axons to the b2 and chain fibres was of the same order as axons which had connections with a wider variety of intrafusal fibres. Non-selective axons had a significant peak of motor ending numbers at 12dpn which contributed to the peak at 12dpn when the total sample was taken into account .

e) Length of endings of each motor axon.

The mean length of the motor endings was calculated (tables B and E, appendix 2). Fig. 23 shows the data as bar charts; endings at the terminations of myelinated axons were separated from those of unmyelinated axons. The total mean lengths for each age group is also shown.

In the total sample, motor ending lengths increased with increasing age of the kitten, except for motor endplates ending on the b1 at 12dpn where the combined mean lengths of endings of myelinated plus unmyelinated axons

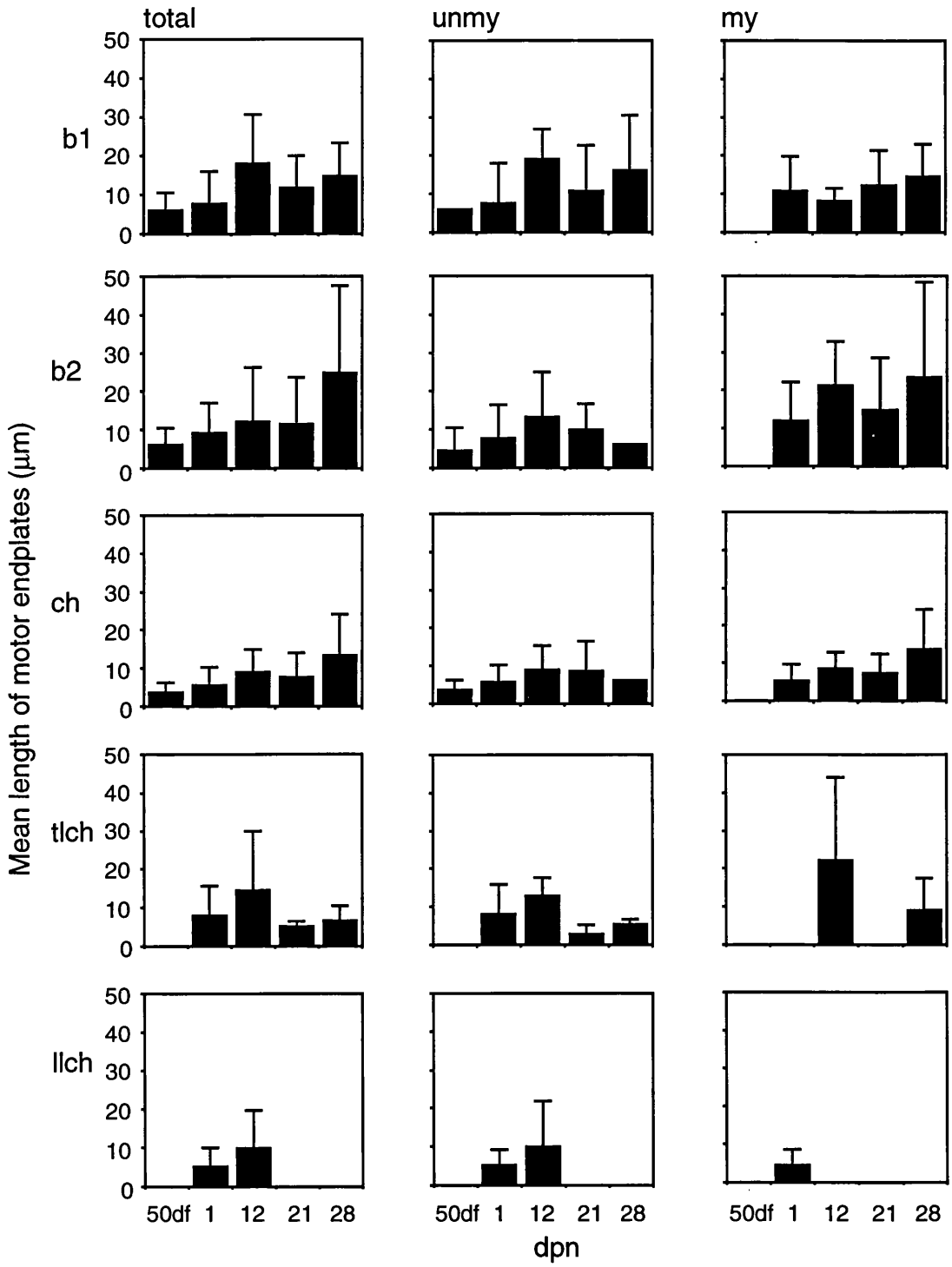


Figure 23

Mean lengths (µm) of motor endings related to the intrafusal fibre on which the axon ends and axon myelination but irrespective of selectivity of axon. Mean lengths of motor endings on b1, + lch + llch showed a significant (95% level) peak at 12dpn.

were greater than for both younger and older motor end plates (Fisher PLSD, 95% level).

From fig. 23 and appendix 2 table B, it can be seen that in all cases (except at 12dpn for endings on the b1 and chain fibres and at 28dpn for endings on the b1 fibre) the mean length of the motor terminals of myelinated axons looks numerically greater than the mean length of unmyelinated motor axon terminals but was not found to be significantly so (Fisher PLSD).

The increase in length at 12dpn for motor endings of unmyelinated axons terminating on b1, b2 and typical chain fibres was significant (Fisher PLSD, 95% level) over that at earlier and later stages.

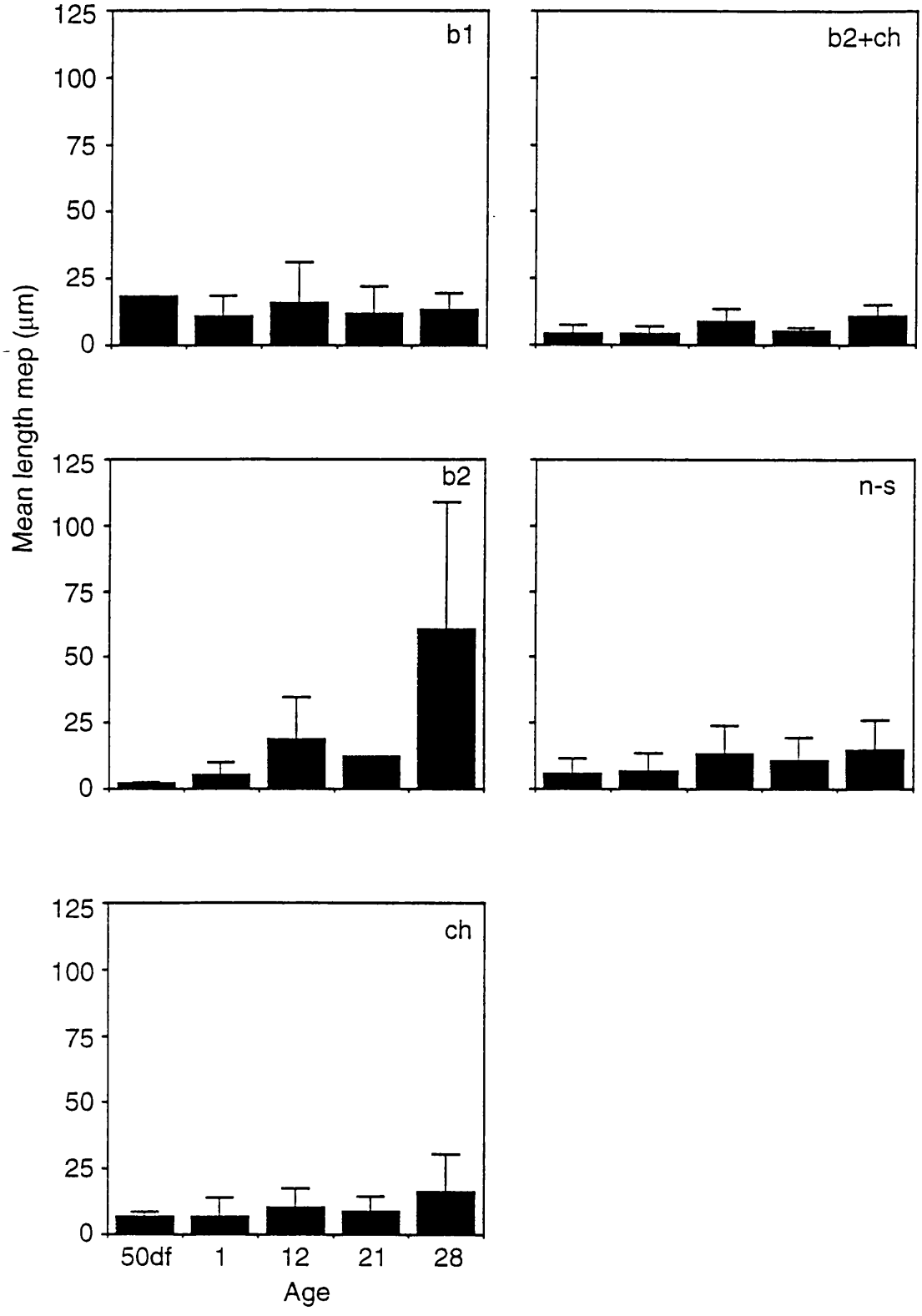
If the mean motor ending lengths are plotted with respect to selectivity of the motor axons to their intrafusal fibres, figure 24, and Fisher's PLSD test carried out, it was found that in all but those motor endplates on the b1 fibre their length increased with increased developmental age. Motor endplate lengths for those axons selective to the b1 fibre did not significantly increase with increased age but remained constant throughout.

3) Post synaptic features of the developing motor innervation.

In adult cat muscle spindles some of the intrafusal fibres lack innervation from motor axons; this is quite normal especially of chain fibres where there are frequently many typical chain fibres in any spindle, often with one or two uninnervated. Table (6) shows that less than full motor innervation also occurred in the developing spindle. It was never found that all chain fibres were innervated at any stage in this study. By 28dpn the percentage of chains

Figure 24

Mean length of motor endplates of axons selective and non-selective (n-s) to the individual intrafusal fibres between 50df and 28dpn development.



not innervated (24) was very similar to the percentage of non-innervated chains found in adult spindles (26). Long chain fibres were innervated when they were present in spindle poles (except at 50df) during development. This was in contrast to the adult figure of 75% of long chain fibres which are non-innervated.

Table 6. Percentage of fibre poles innervated at the different age groups studied.

	50df	1dpn	12dpn	21dpn	28dpn	Adult
b1	71	100	100	100	90	85
b2	100	100	100	82	100	96
ch	52	75	62	60	76	74
Lch(T)	0	100	100	33	100	
					}	25
Lch(L)		100	100			

The b2 fibre (table 6) almost maintained 100% innervation, and being the first fibre formed of the spindle this was not very surprising as it was therefore available for innervation for the same length of time as there were motor axons available to innervate it. The b1 fibre seemed to go through a phase of full

motor innervation (1, 12, 21dpn) to less than 100% innervation which is the adult norm.

a) Distribution of motor endings.

Using the classification of Arbuthnott et al 1982, it is expected that in broad terms, b1 fibres support mb endings, b2 fibres ma endings, chains ma or mc endings and long chains, ma, mc, or md endings.

As previously mentioned in the Methods, these categories had to be expanded to account for types which were found during development but do not fit the adult classification of motor terminals. At each age in this study the intrafusal fibres supported a variety of ending types on the same pole. Despite this, the b2 fibre appeared to have a predominance of ma type endings at all stages of development (figure 25, appendix 2, table C). The b1 fibre also seemed to support a large percentage of ma endings although by 28dpn a slight shift towards the mb type endings was evident. No complex mc endings were present on b1 fibres after 1dpn.

Typical chain fibres perhaps showed the clearest separation of ending types by 28dpn where the motor endings were broadly divided into ma (maf) and mc(mac) types. In only 3 cases were ma and mc endings found on the same intrafusal fibre pole and these may have been the terminations of separate motor axons. On the other 32 innervated chain fibre poles at 28dpn there were either ma or mc motor endings.

Long chain fibres, either thin or large, supported a range of ending types. Large long chains supported ma and mb type endings whereas thin long chains

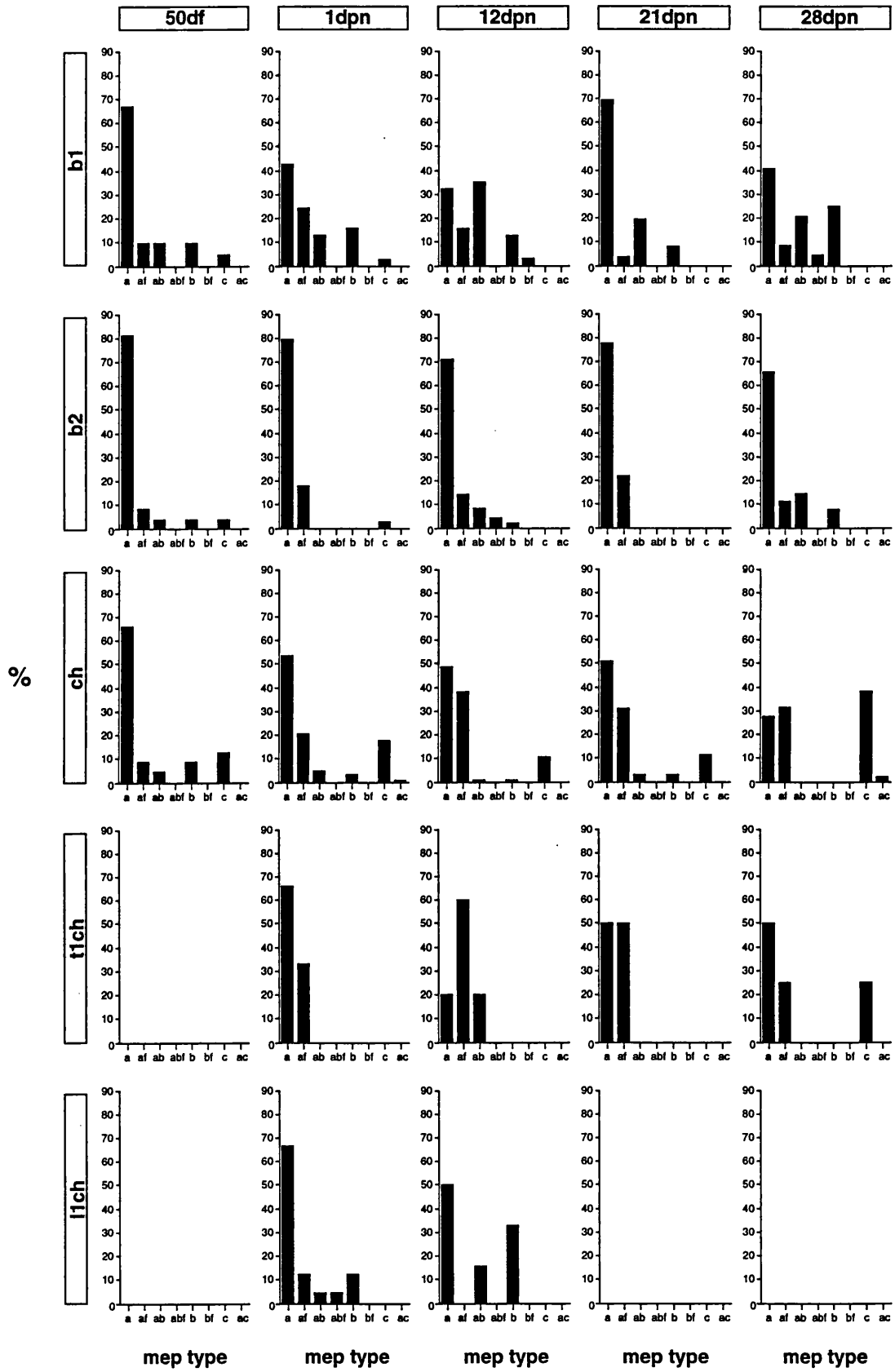


Figure 25

The percentage of each motor endplate type at the different stages of development, (50df - 28dpn) for each type of intrafusal fibre in kitten muscle spindles.

supported predominantly ma type endings. The occurrence of complex mc endings did not occur until 28dpn on thin long chain fibres at which time there were no large long chains in this study.

Motor axons supplying endings to the intrafusal fibres ended in a variety of types of terminal (fig. 22 A,B,C). Even axons which were selective to the three types of intrafusal fibres had several types of terminal on the one axon. Neither could any distinction be drawn between the unmyelinated and myelinated axons with respect to their ending type selectivity.

Some axons had motor endings all of one type (fig. 22A: b2 50df; 1dpn; 12dpn 21dpn; 28dpn and chain fibres 50df; 1dpn; 28dpn. Fig. 22B: 50df. Fig. 22C: 50df; 12dpn; 21dpn). Many more of the axons showed variation in motor ending types. The axons innervating the chains selectively (fig. 22A) at 28dpn showed a complete separation of ending types into the two categories of the adult ma and mc, from the work of Arbuthnott et al (1982). However the sample was very small!

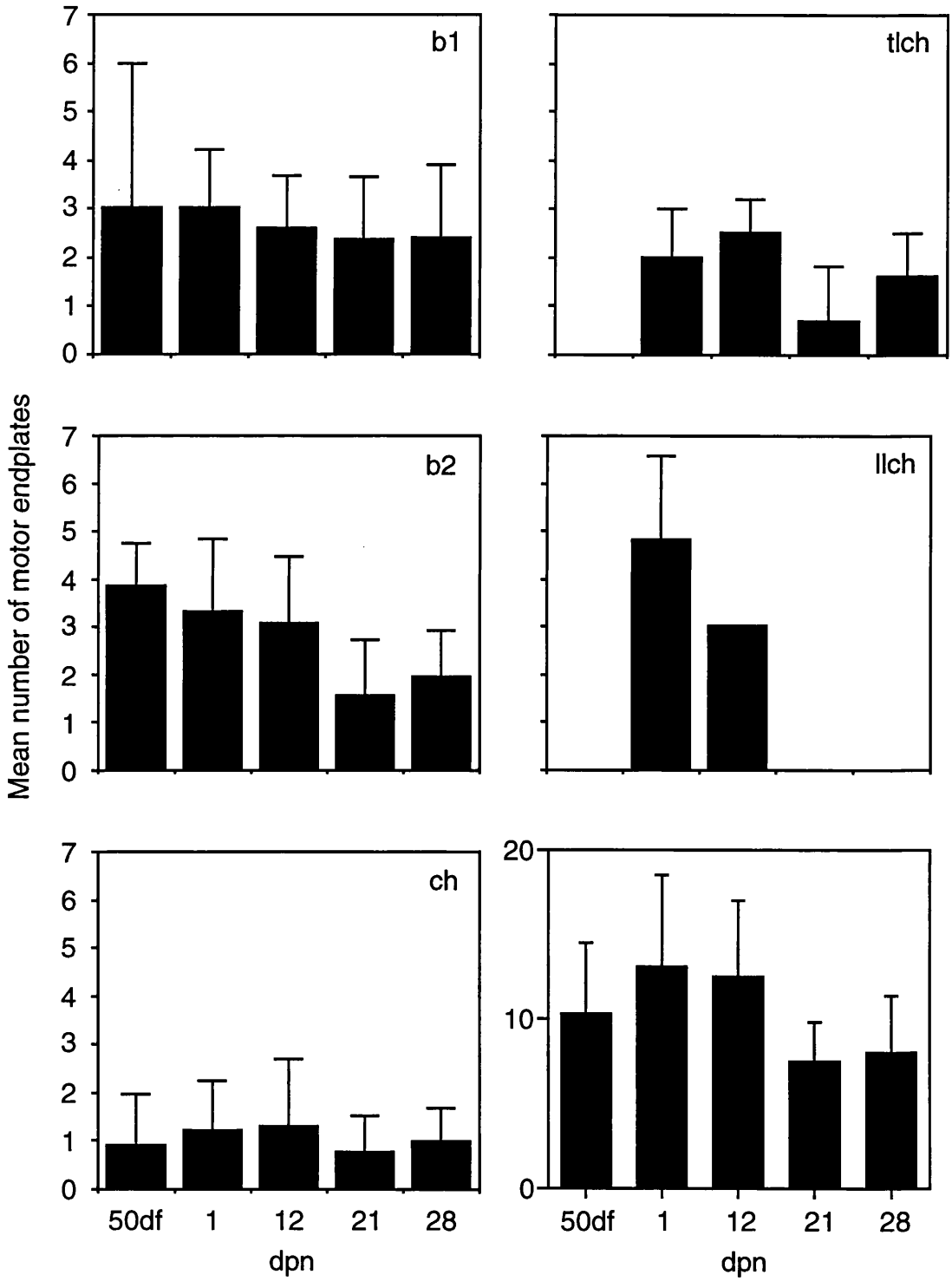
b) Numbers of motor endings on each intrafusal fibre.

The mean of the total number of motor endplates for whole spindle poles was significantly higher at 50df, 1 and 12 dpn than at 21 or 28 dpn at the 95% level as tested with Fisher's PLSD test (fig. 26). This was different from the mean of the total number of motor endplates of each motor axon which showed a peak at 12dpn (fig. 21).

From counts of the mean number of axon contacts on individual types of intrafusal fibre pole (poles without any motor endings were also included) at

Figure 26

Mean numbers of motor endplates for each intrafusal fibre type and for whole spindle poles at the 5 age groups studied.
Mean numbers of motor end plates for whole spindle poles, b2, tlch and llch decreased significantly (95% level) by 28dpn and on typical chain fibres showed a peak at 12dpn.



the different age groups, it was seen (fig. 26) that the b1 fibre had approximately the final number of endings for each fibre pole throughout this development period (fig. 26). No significant change was found in mean numbers of motor endings on each b1 fibre in contrast to the mean numbers of motor endings of each motor axon to the b1 fibre, which showed a rise by 28dpn (fig. 21 and table A appendix 2).

The b2, and long chain fibres started with a higher number of mep for each fibre pole (fig. 26) and settled by 28dpn to a number comparable to that found in the adult i.e. 2-3, and 1 motor endings respectively (Kucera and Walro 1986). Mean numbers of motor endings on b2 fibres were significantly different (Fisher's PLSD test at 95% level) in the same order as the whole sample i.e. a drop in numbers of motor endings on b2 fibres at 21 and 28dpn. This also concurred with the numbers of motor endings of each motor axon on each b2 intrafusal fibre.

For typical chain fibres at one and 12dpn the mean numbers of endings of each fibre were greater than the 21dpn means (fig. 26), in contrast to the constant number of motor endings of each motor axon for each intrafusal fibre throughout. (fig. 21 and table A appendix 2). Only axons selective to typical chain fibres were studied in the previous results. Axons innervating b2 + chain fibres together showed a rise in mean motor ending numbers to a peak at 12dpn. The difference was made by the initial high numbers (4) of motor endings of axons to the b2 fibre.

The numbers of endings on typical chain fibres would be influenced by the numbers of non-innervated fibre poles, and at 21dpn there were more non-

innervated fibre poles than at one or 12dpn (table 6). This initial high number of motor endings on b2 fibres will have contributed to the larger mean numbers of motor endings on whole spindle poles at 50df, one and 12dpn than at 21 and 28dpn.

c) The distance of motor endings from the equator.

The sensory innervation in adult cat spindles is thought to have an inhibitory effect on the indentation of the motor terminals into the muscle surface when motor endings are close to the equator (Banks 1983).

The length between the centre of the primary sensory spiral and the mid-point of any motor ending was measured and recorded as the distance from the spindle equator of that ending. This was a difficult parameter to compare in this study as the spindles were of different final lengths at each age group. As the intrafusal fibres grew there was more length available for axon contacts. Hence an ending that might have appeared near the equator (and therefore under the influence of the primary sensory ending) at 1dpn for instance, may be considered as far away from the equator by 28dpn.

It would have been interesting to observe extracapsular motor endings but no extracapsular endings were found on any intrafusal fibre in this work. Kucera, 1988 also found no extracapsular motor endings in developing spindles of rat, but rat spindles also have fewer extracapsular motor endings in the adult. It might be expected that motor endings placed outside the capsule would be uninfluenced by the sensory innervation.

With these reservations in mind, actual values of distance from the equator of all the motor endings at the different age groups were plotted in fig. 27; the mean values can be found in appendix 2, table D.

There was a shift (although small) of increased distance from the equator of the motor endings as the animal grew except for 21dpn samples which were closer than those at 12dpn. However, there was a wide variation in proximity to the sensory innervation at all age groups.

The ma endings on the b1 were always amongst the closest to the equator; one of the type mb endings on this fibre was a considerable distance from the equator at 28dpn and presumably might be considered as outside the influence of the sensory axons (Kucera and Walro 1986; Banks 1983); is this ending the stable axon contact that will mature into the adult innervation?

The endings on the b2 fibre were at varying distances from the equator, any terminals far out in the pole were only found in the 28dpn samples and those furthest from the sensory region were of both mb and ma types.

Motor endings on the typical chain fibres showed clustering into mainly ma or mc type and not until 28dpn was there any noticeable difference between their positioning on the intrafusal fibre. Like the ma ending on the b2 fibre, these endings were found both close to and at a distance from the equator; the mc endings were in the same range of distance from the equator as the ma group closest to the primary sensory spiral.

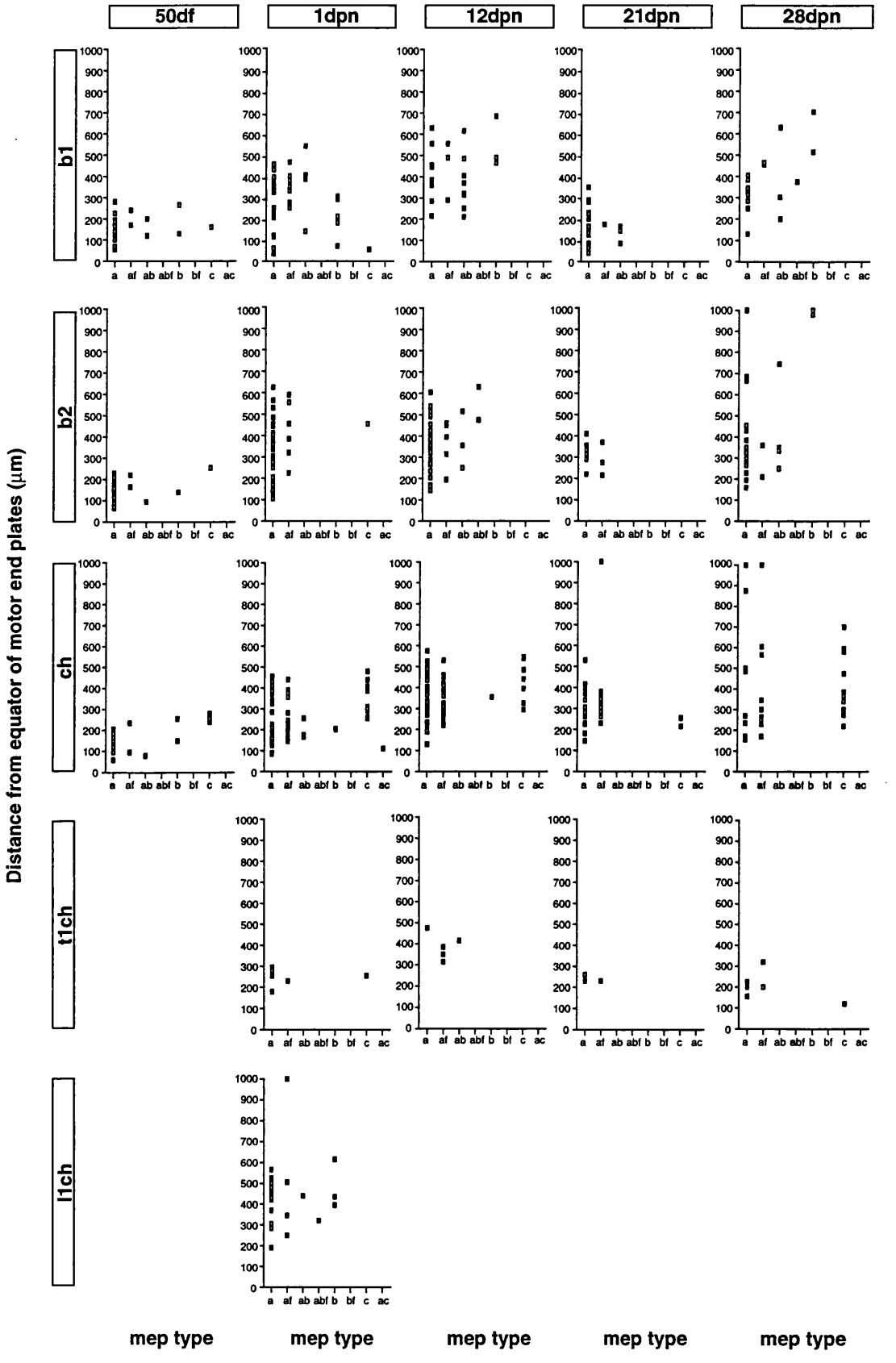


Figure 27

Cluster diagram of the distance of every motor endplate from the equator related to its ending type and intrafusal fibre on which it is sited, at each of the age groups studied.

Both mb and mc motor endings are considered more complex endings than the ma type and yet from this work increased complexity cannot be linked with increased distance from the equator during post natal development.

It is interesting to note that the motor endings on the long chain fibres were very little different in their distance away from the sensory innervation from those on the typical chain fibres.

The distances of the motor endings from the equator are, during development, most likely to be influenced by the numbers (if any) of secondary sensory endings and the length of muscle fibre available for innervation.

d) Lengths of motor endings.

It is to be expected that as the spindles grow, all parameters would increase in size (Table 4) and the length of the motor terminations would be no exception. As was seen in part 2e) in fig. 24 and table B, appendix 2 the length of motor endings when considered with the supplying axons increased with age except for endings of axons selective to b1 fibres which did not change in length throughout the study. When considering the mean length of motor endplates with their fibre type, there was an unexpectedly high value for the length of motor endplates at 12dpn on all intrafusal fibre types (figure 23, totals column and table E appendix 2) but it was only significant for b1 fibres at the 95% level with Fisher's PLSD test. Alternatively this could be interpreted as a reduction in motor ending lengths at 21 dpn. From table 4 it was seen that the kitten increased in length gradually with increased age (including at 21dpn) and so it would have been expected that the lengths of motor endplates would have increased at 21dpn also.

Lengths of each motor ending on individual intrafusal fibres were plotted against ending type category and cluster diagrams obtained, (fig. 28) which showed that a variety of motor ending lengths were possible on all intrafusal fibre types at all age groups.

From fig. 22 A, B, C, showing the motor ending lengths and type in relation to their motor axon, a direct comparison of the length of the motor ending with its classified type could be seen again showing that all types of motor ending were found in a variety of lengths. The axons already selectively innervating the three types of intrafusal fibre (figures 21 and 22A) had often one ending longer than the others. It is tempting to think that this was the most mature motor ending.

In some cases from fig. 22A, the longest ending could correspond to the type for the intrafusal fibre concerned, as expected from the study by Arbuthnott et al (1982); namely mb on b1 fibre at 12 and 21dpn, ma on b2 fibres at 1, 12 and 28dpn, and ma or mc on typical chain fibres throughout the study. It should be pointed out that there was rather a large mean length for the mb type endings on b2 fibres at 28dpn. It might have been anticipated from Arbuthnott et al's (1982) classification of ma type endings on b2 fibres in adult cat spindles, that any non-ma type endings on b2 fibres might be reduced in length by 28dpn.

However, it can be seen that the more complex endings were not necessarily the longest as evidenced by the mb and mc endings being in the same numerical range of lengths as the ma endings at any one age group. Different

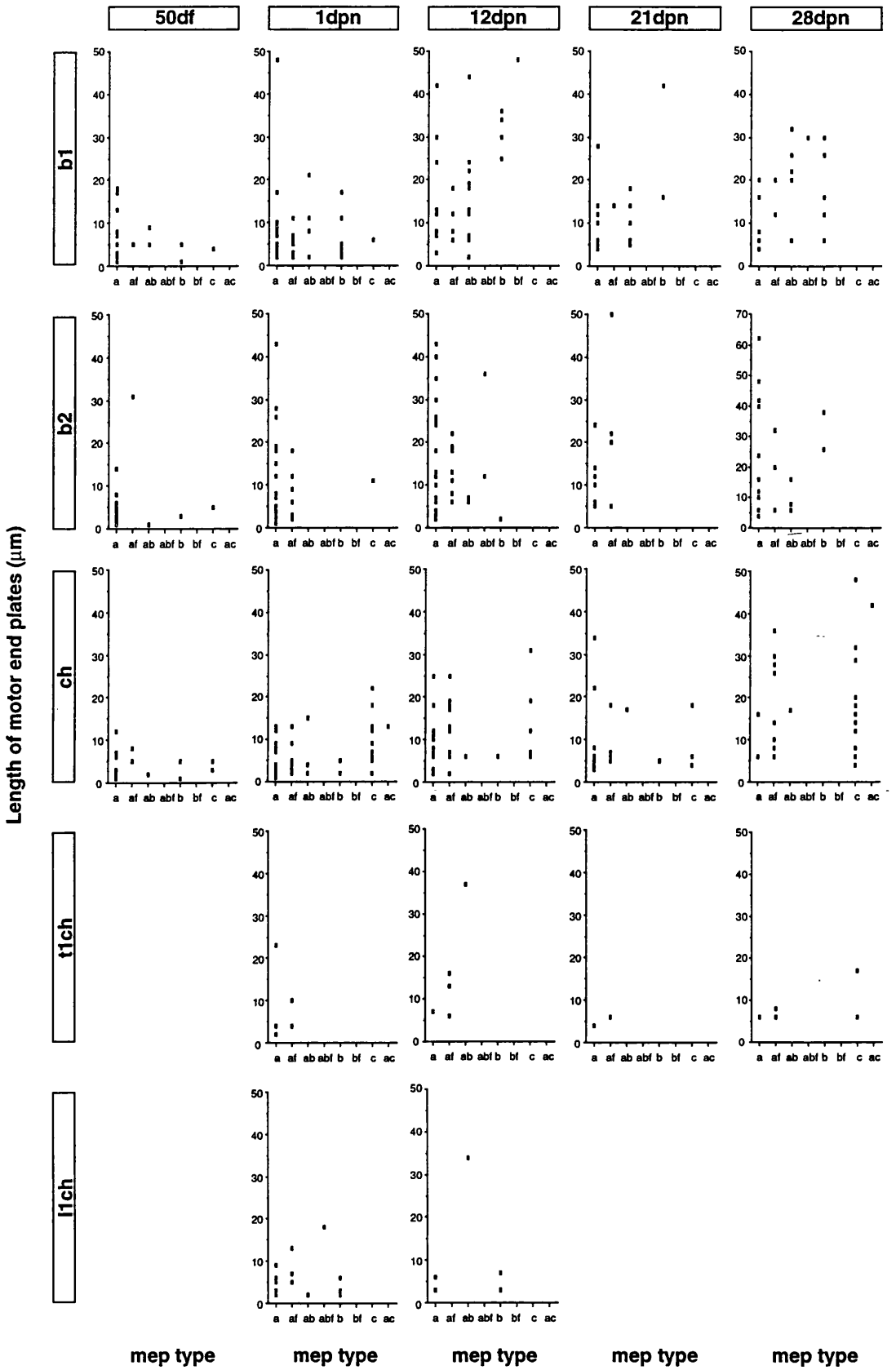


Figure 28

Cluster diagram of the length (µm) of every motor endplate related to its type and intrafusal fibre on which it is sited, at each group studied.

intrafusal fibres may support unequal lengths of the same ending type: a type ma may be 5μm in length on a chain fibre but 10μm on a b2 fibre.

Fig. 28 and table E appendix 2 should be studied in conjunction with table C in appendix 2 as there, the percentage of endings of the various types is given. If maf is considered as a developmental variant of ma-type in character and mab, mabf and mbf as developmental variants of mb-type in character, the majority of the endings fell into the ma-type on all intrafusal fibre types, but only became among the longest on the b2 fibre late in development (appendix 2, table E). The mb-type ending on the b1 fibre as early as 12dpn was larger than the ma-type on the b1 fibre. The mc endings on the typical chain fibres were about the same size as the ma/maf-type endings on these fibres until 28dpn when the mc endings were longer.

Discussion

This Thesis shows that the numbers of motor axons innervating each spindle pole were not significantly different from the adult (2-3), although their distribution was less selective to the intrafusal fibres at all ages. Myelination of these axons increased with age until at 28dpn 92% of motor axons were myelinated. (All sensory axons are myelinated prior to 50df). Motor axons ending selectively on the individual intrafusal fibres had fewer endings than non-selective ones. The total numbers of motor endings reached a peak at 12dpn and thereafter decreased. However the numbers of motor terminals on the individual intrafusal fibres did not necessarily follow this pattern. Intrafusal fibres could accommodate terminations of both selective and non-selective motor axons although there was no evidence of polyaxonal innervation at individual end-plates. Motor end-plate structure was predominantly of the simple ma type at all ages although more complex types (mb and mc) were found even at 50df. There was definite evidence that new contacts are still being made at 50df as there were occasional motor endplates without basement membranes found. It will be argued in the discussion that at least some of the motor innervation is not organised into the adult pattern, even by 28dpn.

At the 50df stage of development, muscle spindles in cat hind limb muscles already possess bag intrafusal fibres, chain fibres, sensory and motor innervation and a capsule. Intrafusal fibre recognition by fusimotor axons and endplate organisation seem to occur at different times. Structures such as sensory innervation and a capsule not found surrounding extrafusal fibres may possibly play a role in the development of the fusimotor innervation.

1) Possible influence of the sensory endings on the developing spindle motor innervation.

a) The distance of motor endings from the equator.

The distance of the motor endings from the equator is a function of the growth of the intrafusal fibres and the number of secondary endings. When there is only a short length of fibre available, the axons will attach where they can and this would include sites close to the equator. Any site close to the sensory innervation may be affected by it and either become more or less stable. An axon attached to this site may become capable of being lost or replaced. As all endings in this study were found to be intracapsular endings, figure 27 shows that even older kittens had motor endings at any distance along the intrafusal fibre within the capsular sleeve, and motor endings were found as close as 100μm to the centre of the sensory ending for all intrafusal fibres at all ages. This indicates that there was no inhibition to axons forming connections in this area of fibre. The sensory endings therefore cannot be preventing the axons making contact with each intrafusal fibre close to the sensory ending during this development period.

Banks (in Press), has indicated that in adult cat spindles the number of motor axons entering a spindle is related to the number of secondary sensory endings. As the mean numbers of motor axons to spindles from 50df development was the same as in the adult, this indicates that there was no initial relationship, but more of the motor axons are likely to survive in spindles with secondary endings.

In adult cat spindles, Banks et al (1985) believe that more indented endings are to be found further away from the equator than the more superficially placed endings. Arbuthnott et al 1982, 1986, 1992 could not positively confirm this

from their data. Motor endings which approach the sensory endings most closely would therefore be more likely to be ma or their variants maf or mab at any age; i.e. the least differentiated. This study showed simple and complex or indented motor endings at varying distances from the equator even in the least mature kittens of 50df (fig. 27). Motor endings on long chain fibres (tlch and llch) might also be expected to be further away from the equator but at 28dpn the endings were as close to the equator as endings on other fibres.

In summary, it could not be shown that the sensory innervation had an influence on either the distance of motor endings from its centre or the type of motor ending exhibited.

b) The fusimotor axons.

Although the motor axons of this study could not definitely be assigned as γ or β, as there were no instances where axons could be traced back to nerve bundles which branch to also innervate extrafusal muscle fibres, it could be assumed that some of each would be present. Axon diameters of γ and β do not significantly differ in adults at the level of the spindle and would not be expected to during development. Endings of β motor axons in adult spindles are often extra capsular but as there were no extra capsular endings found in this study, no clue as to motor axon identity could be gained from that source.

In the sense that polyneuronal innervation means axons from different neurones terminating on the same muscle fibre, this is the normal adult pattern for intrafusal fibres. However, each motor endplate does not have several innervating axons throughout the period of development covered by this study. The numbers of motor axons innervating each whole spindle pole at all age groups was two to three, which was of the same order as in the adult (fig

18A). The numbers of motor axons supplying individual intrafusal fibre poles was also in the same range as in the adult spindles: i.e. 1-2 axons to the bag fibres and 1 to the chain fibres (fig. 18B). Therefore it seemed that the final numbers of motor axons which enter each spindle was determined very early in development, prior to 50df. Even in the adult some spindles have numerous motor axons to one pole, whereas some poles receive only a single motor axon, usually a static motor axon. However, as this study was fixed at a point in time, there was always the possibility that while the numbers of axons were in the same range at any point in time, the actual axons might be different i.e. exchange of one axon for another.

This is a constant dilemma when using any absolute numerical data in dealing with a continuous process. We can only see a snapshot in time of the connections of any axon with any intrafusal fibre. Studies in adult spindles have indicated a fairly stable arrangement of connections but any continual turn-over would not be observed by the present methods. Only those re-connections after axotomy or other injury could be monitored and even in this it would be difficult to know whether the axons returned to the same site of previous connections.

Gregory and Proske (1985) also found no evidence of hyperinnervation in kittens in contrast to Kucera et al (1988), who found polyaxonal innervation of individual motor endings in rat spindles from animals as old as 4 days after birth. Each bag fibre pole in adult spindles can support terminations of more than one axon, so why is it that when one motor ending is present on any intrafusal fibre this does not automatically inhibit another motor axon from making contact? Single site polyneuronal innervation provides opportunities for mutual inhibition not given by multi site polyneuronal innervation. If there

is local polyneuronal innervation which is eliminated, this means that there must be some limited interaction or competition between motor terminals. For chain fibres in adult spindles it is relatively rare to find two end plates on the one fibre pole and even rarer to find the motor endings from two separate motor axons, (Arbuthnott, 1982); thus it is possible that some interaction takes place. However, the fact that the chain fibres are late in developing may mean that their poverty of innervation (relative to bag fibres) is caused by many of them not being present when motor innervation is most vigorous. In other words they may miss a 'critical period' of motor innervation by developing late. For bag fibres, one could argue that the competition between motor endings stays very local, and that each motor ending can control only a short section of intrafusal fibre.

c) Motor axon contacts.

Functional connections are known to decrease the numbers of acetylcholine receptor sites (in extrafusal muscle) available for innervation (Dennis 1981). Work by Burden et al (1979) and McMahan and Slater (1984) on re-innervation of muscle after axotomy of the supplying axon, has indicated that the basal lamina contains the trigger for the accumulation of acetylcholine receptors into clusters. McMahan and Wallace (1989) added basal lamina extracts prepared from the electric organ of the marine ray *Torpedo californica*, (this tissue is derived embryonically from muscle with a dense cholinergic innervation) to cultured myofibres and found that these extracts induced acetylcholine receptor accumulation and post synaptic specialisation. The active component in these extracts, which has been characterised and named Agrin, produces the strongest effect on post synaptic specialisation at the NMJ of regenerating myofibres and regenerating nerve terminals. It is suggested that agrin, synthesised by the motoneuron is transported down the

axons and released to induce differentiation at the developing post-synaptic membrane (McMahan 1990).

In the case of spindles this accumulation of receptors may well be overridden by the sensory ending. The sensory innervation positioned as it is, might have some controlling function over the intrafusal fibre muscle membrane. It could be argued either that the sensory spirals would depress the numbers of receptor sites near to it, or prevent the numbers being reduced when axons made connections with the intrafusal fibres.

The number of motor endings of each motor axon in the whole sample showed a significant increase at 12dpn over that at earlier and later ages (fig. 21). Branching of the axons presumably must have occurred to account for increased motor endings without significant increase in axon numbers. Therefore receptor sites must be still maximally available at 12dpn. This peak number of motor endings at 12dpn could be considered the saturation point of the intrafusal muscle fibre receptors prior to synapse elimination. When considering the numbers of motor endings of motor axons on the individual intrafusal fibres there were varying trends.

For axons selectively ending on the **b1 fibre** the number of endplates of these axons was significantly increased at 28dpn over that at 50df.

The numbers of endings of axons selectively ending on the **b2 fibre** started high at 50df and were reduced by 28dpn.

The numbers of motor endings of axons selective to **typical chain fibres** stayed constant throughout this time of development. The innervation of chains is more complex than that of the bag fibres because chain fibres are still developing until birth. As the numbers of axons seems to be determined early in development (prior to 50df), later formed chains will either be un-innervated

or have branches from existing axons making contact with them. This may explain why Boyd observed that chain fibres seem to work as a group in many spindle poles.

Axons innervating **b2 and typical chains** non-selectively showed a peak number of endings at 12dpn. This and the increasing numbers of endings of each motor axon innervating the b1 and decreasing numbers of endings of each motor axon innervating the b2 will all contribute to the total sample peak in motor ending numbers of individual motor axons at 12dpn.

The numbers of terminations of motor axons which were **totally non-selective** remained consistently high between 50df and 28dpn. These probably constituted the most labile population of motor endings. Axons may stay attached to a non-appropriate intrafusal fibre while they search for another muscle fibre with which to make a suitable connection.

These observations suggest that the sensory innervation may have a facilitating effect on motor ending attachments. This may account for the observations that bag fibres, especially, can support more than one ending on each pole both during development and in adulthood. Motor ending numbers were reduced from a peak number at 12dpn which suggested that the sensory innervation did not inhibit motor endings being removed from intrafusal fibres, at least after 12dpn, during development.

2) **Possible influence of the capsule on the developing spindle motor innervation.**

The capsule may limit the formation of further connections as growth of the intrafusal fibres probably is mainly extracapsular after 12dpn, capsular growth having slowed after 12dpn (table 4). No extracapsular endplates were found in this study as was also the case with the work of Kucera (1988) and Gregory

and Proske (1986). Adult spindle fibres can support both intra- and extracapsular endings on the one pole (Arbuthnott et al 1982) and so perhaps extracapsular γ or ß axons ending on intrafusal fibres are responding to functional requirements of the muscle fibres in later development and adulthood.

3) Possible influence of propagated action potentials on the developing spindle motor innervation.

Multiple intrafusal fibre innervation should also be considered in the light of whether or not the muscle fibre in question responds to nervous stimulation by propagating action potentials. In chain fibres of adult muscle spindles propagated action potentials can occur (Barker et al 1978). The numbers of endings of axons selective to the typical chain fibres remained constant during development (fig. 21), and effective action potentials, could presumably inhibit any further receptor formation in many instances. By 12dpn some of the connections will be secure and so any subsequent attempted connections would be inhibited. The typical chain fibres that do have more than one motor ending per pole may be the oldest of these chain fibres; possibly in these the whole of the muscle surface would have had receptor sites available for innervation prior to any electrical activity.

Barker et al (1978) recorded action potentials from some bag2 fibres but not all, and Gladden (1981) never recorded any action potential from bag2 fibres in adult spindles. The numbers of endings of motor axons selective to the b2 fibre during development was initially high (fig. 21) and as this fibre was available the longest during development it might be expected that many endings would form on this fibre before some would be discarded. As the b2 supports some action potentials, it may be when these do occur this causes

some endings to be disconnected or discouraged. The condition of supporting action potentials in the adults may be residual from development, and the majority of bag2 fibres may loose their capacity to support action potentials. If this were so the stabilisation of the motor ending numbers could all be due to action potential triggering at around 12dpn on the b2 fibre. It should also be remembered that the bag2 fibre is specifically innervated by gamma static axons only, not gamma or beta dynamic or beta static axons.

Both Barker et al (1978) and Gladden (1981) recorded non-propagated junctional potentials in bag1 fibres of adult spindles. What therefore determines the correct complement of endings on bag1 fibres? Presumably there were no propagated action potentials at any time to trigger the loss or inhibit the formation of any unwanted endings. Does the b1 have an early pre-set number of receptor sites so that it can only exchange one motor ending for another, not inhibit or encourage the formation of more or fewer junctions? As was seen in fig. 21, the numbers of endings of each motor axon terminating on the b1 fibre rose significantly by 28dpn indicating branching of the axons already innervating the b1 fibre because the numbers of axons did not also increase.

Motor ending organisation.

a) Numbers of motor endings on each intrafusal fibre pole.

Discreet endings can be counted on the intrafusal fibres at all ages and without reference to the supplying axons (fig. 26). Different conclusions can be drawn from the figures for numbers of endings of motor axons. The total numbers of motor endings reached a peak number at 12dpn, concurring with the total number of endings of each motor axon innervating whole spindle poles.

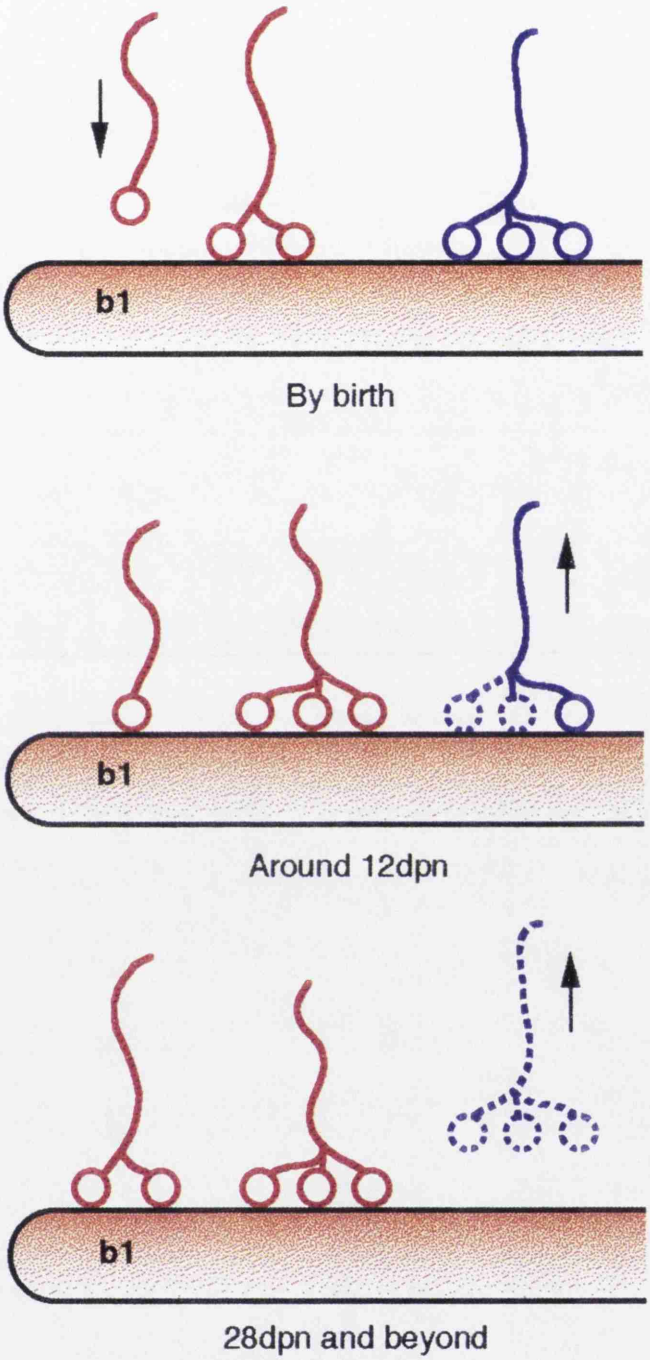
However when the b1 fibre was considered alone, the numbers of endings on each fibre pole stayed constant (Fisher's PLSD test); although there could be as many as 6 motor endings on one bag1 fibre at 50df there were never as many as this after birth. By comparison with innervation of adult spindles, some terminals on bag1 fibres were inappropriate; 28% even at 28dpn (fig. 19). In each individual case, either connections to other fibres must have been removed or connections with bag1 fibres were removed. It seems likely that both processes would operate in a balanced system. Presumably there is some exchange of axons although the numbers stay approximately constant. From figure 29 it is proposed that before and including 12dpn a selective dynamic axon increases its numbers of axon terminals (fig. 21) while another non-selective axon starts to withdraw from contact with the b1 fibre. Between 12 and 28dpn this balance of axon retraction and increase of ending numbers continues, while at the same time, it is possible that another selective axon could arrive to also innervate this intrafusal fibre, thus maintaining axon numbers.

The numbers of endings on the b2 start high at 4-6 which is not outwith the range for adult spindles at any time during development. This decreased with time (fig.26), as with the numbers of motor endings of each axon specific to the bag2 fibre (fig. 21). However the proportion of axons specific to bag2 fibres was very labile (fig. 19); 17, 6, 19, 5, 12% selective b2 innervation from 50df to 28dpn. This suggests that these axons are easily able to innervate other intrafusal fibre types, since the total numbers of axons remains the same.

The combined percentage of specific and non-specific axons innervating chain fibres increased, while the bag1 innervation remained constant although the composition changed. The great increase in proportion of b2+chain

Figure 29

Diagram of bag 1 intrafusal fibre innervation



It has been assumed that there must be some axon replacement of non-selective by selective axons to account for the observations that the total numbers of motor axons per fibre pole does not change significantly between 50df and 28dpn development, yet the number of inappropriate contacts is decreasing from 12dpn onwards.

Red coloured axons are selective and blue coloured axons are non-selective.

innervation from 12 to 28dpn, (15 to 20 to 34%) (fig. 19) may come from axons innervating bag2 fibres since the proportion of axons innervating chains alone is unchanged (between 10-12%). This may explain the fall in the percentage of innervation specific to the bag2 at 21dpn. The increase in selective bag2 innervation between 21 and 28dpn (5 to 12%) is probably derived from retraction of connections with the bag1 fibre since non-specific connections to bag1 fibres were decreasing at that time, (30 to 16%)

Typical chain fibres had a peak number of endings on each pole at 12dpn (fig. 26). These would be the endings of non-selective as well as selective axons which was found by tracing motor axons to their intrafusal fibre. However the mean number of motor axons innervating each fibre pole remained constant at a mean of just below one; this was the same as in the adult and should indicate that there was no superfluous innervation of single chain fibres during development. As 38% of chain fibre poles were not innervated at 12dpn (table 6), the mean numbers of motor axons to each fibre pole will be lowered by non-innervated chain fibre poles. The numbers of motor endings of axons selective to chain fibres was not significantly different at any age group unlike the peak numbers of motor endings of axons innervating chain fibres along with other intrafusal fibres at 12dpn. Excess connections (presumably of non-selective axons) would therefore be being discarded by 28dpn in a continuing process.

Of the 20 long chain fibre poles in this study (table 1), 17 poles were innervated. This is greater than the 25% of long chain fibre poles which are innervated by motor axons in adult spindles (table 6.). All motor innervation in this population of developing long chain fibres was within the capsule whereas there is a mixture of intra- and extracapsular motor innervation of

adult long chain fibres. This would imply that extracapsular endings appear later in development than 28dpn, possibly in response to functional needs.

b) Length of motor endings.

The motor endplates will grow as the kitten grows. Lengths of motor endings, whether measured by reference to the motor axon (figure 23, and table B, appendix 2) or the intrafusal fibre (table E, appendix 2), did increase with increased age of the kitten for those endings on b2 and typical chain fibres. For motor endings on the b1 fibre there was a peak of endplate length at 12dpn when the totals of both selective and non-selective axon terminations were measured (fig. 23). When only those endings of selective motor axons innervating the b1 fibre were measured (fig. 24), the length of motor endings stayed remarkably constant. This suggests that terminations of non-selective motor axons were longer on average than endings of selective axons on this intrafusal fibre.

This suggests that motor endings on b1 fibres at 12dpn could be longer than older endings at 21 and 28dpn, unless the kittens at 12dpn were unusually large. As there were two animals at 12dpn it seems unlikely that both kittens would be exceptionally large. This study showed a linear increase in the crown-rump lengths of the kittens throughout the age groups, which would suggest that any significant difference in values of one age from another would reflect changes that are a consequence of maturity rather than variations in individuals..

Increase in size may not necessarily mean greater maturity as there were variations in ending lengths at all stages during development (fig. 22 A,B,C;

fig. 28). In extrafusal fibres the largest motor ending is usually the most mature (Proske 1992), and the smaller ones are lost during development.

According to Tuffery (1971), growth or elaboration of the neuromuscular junction takes place after myelination of small axon branches of the motor axons as they approach their target muscle. These small branches connect within the same sole-plate length as the original, parent axon in a process known as elaboration. However, in the present work, the percentage of myelinated axons increased with increased age of kittens and myelination was extensive after 12dpn (table 5) when the motor endplates on the b2 and chain fibres were indeed increasing in length but those on the b1 fibre had peaked.

Elaboration of motor endings at 12dpn and then reduction in total NMJ area makes it crucial to know where one motor ending finished and another began and to be able to confidently follow an axon to its ending(s). The work of Balice-Gordon and Lichtmann (1990), on visualisation of growth of extrafusal NMJ's suggests close adhesion of an axon terminal to its target muscle and formation of new motor ending material from within the ending area (intercalation), pre- and post-synaptic material growing in parallel. An alternative suggestion comes from the work of Barker and Ip (1964) who proposed replacement of one motor ending by a new, longer contact as the means of increasing motor ending length. It is thought that the basal lamina has a controlling part to play in the formation and permanence of motor endings as it has been proved (Glicksmann and Sanes, 1983 in developing motor innervation: Sanes, Marshall and McMahan, 1978 in re-innervation) that an axon terminal could be attached to the basement membrane even although the muscle fibre was destroyed. The work of Sanes et al (1978) showing that only the basement membrane is required for formation and continuation of any

motor ending rather favours the intercalation theory of motor ending growth where extra membrane material can be increased from within the motor endplate area.

This process of intercalation could be seen to cause folded basement and muscle membranes if new membrane materials were formed prior to their elongation, suggesting folding may precede increase in measurable longitudinal length of motor endplates. This could explain the constant length of motor endplates of selective axons to the b1 fibre as the 'special' relationship of axon with b1 muscle fibre may lead to increased folding during the time of this study but not increased length of motor terminal. Although the results here indicated no increase in length of motor endplate as measured along the length of intrafusal fibre surface, an increase in 'size' of the motor ending may be by formation of folds into the muscle surface. This process requires the increase in length of both basement membrane and muscle tissue, not measured quantitatively in this work.

c) Spindle neuromuscular junction structure during development.

Intrafusal motor endings are more simple in structure than those on extrafusal fibres. Even the most complex ending, (md ending of Arbuthnott et al, 1982), which is the nearest to an extrafusal neuromuscular junction with its recessed axon terminal into a gutter which has many deep narrow folds is simpler than an extrafusal ending. During the development of extrafusal endings these endings have a simple structure with smooth post-junctional membranes; these are found prior to the electrical activity of the motor axon which is thought to stimulate the formation of folds in the post-synaptic membrane. The conversion from a long acetylcholine receptor channel open time (4ms) to a fast one (1ms) occurred at the same time as development of post-junctional

folding in rat soleus muscle after denervation and re-innervation at ectopic endplates (Brenner et al. 1983). In both developing and mature adult cat spindles, all gradations of complexity are found, through simple endings without folds to very complex ones with deep folds, the muscle membrane thrown up into 'finger-like' processes or depressed into a hollow. Many of these connections are known to be capable of normal characteristic responses to stimulation of the γ motor axons (Arbuthnott et al 1982,1985,1992: Sutherland et al 1985: Boyd and Sutherland 1987 in adult and Gregory and Proske 1985 and 1986 in kittens).

In this study, there were no md endings of Arbuthnott et al, (1982). This is curious as these were thought to be β static endings of long chain fibres extracapsularly. The long chain fibres in this study, as mentioned previously, had totally intracapsularly positioned endings although they encompassed the range of ending types found in this study. Gregory and Proske (1985), from physiological recordings in 5 day old kittens, noted a larger than expected incidence of ß innervation by using conduction velocity measurements as criteria to separate α and γ innervation and noting 4 instances of axons conducting in the α range with fusimotor effects. It was not possible in this study to distinguish between β and γ axons. Gregory and Proske (1985) proposed that spindle motor innervation was initially mainly ß innervation which is subsequently replaced by γ innervation.

Complex mc endings were recognisable at 50df, (fig. 25) which is unexpected from adult spindle motor endings. This would indicate that complexity is not necessarily a sign of 'maturity' and may in fact be a feature of immaturity. As these complex mc endings were found on all fibre types until 1 dpn (fig. 25) this suggests that the immaturity of the intrafusal fibre membrane may support

this type of ending. After 1dpn only the chain fibres support mc endings and it is known from work by Rowlerson et al 1985 that adult spindle chain fibres contain neonatal myosin. As chain fibres are the last to form in development, some of them at least may retain their immature myosin type into adulthood. Simple ma endings were also found on typical chain fibres. By 28dpn motor endplates on chain fibres fell into the two populations of form of motor ending, ma and mc; are the characteristics more influenced by the motor neurone than by the intrafusal muscle fibre, the muscle being the same in each case? Perhaps there are different (histochemically) forms of typical chain fibres. Recent results (Yoshimura et al 1993) indicate that even the typical chain fibres have different histochemical characteristics and even regions of variable histochemistry (Dickson et al 1991). The two motor ending types (ma and mc) were almost never found on the one fibre pole together in the adult cat which supports the idea that the target muscle is involved in the 'fine-tuning' of the motor connections. An alternative approach is that the b2 connection in some other spindle allows chain fibres to support ma type endings because of some local influence as suggested by Arbuthnott et al (1992).

If the proposal by Sanes et al (1978) is correct, that extrafusal neuromuscular contacts are possible when only specialised basement membrane is present which is known to have been formed by release of substances from the motor nerve terminal, then the structure of motor endings ought to be determined early. This initial structure can be thought of as like a scaffold comprised of basement membrane. The work of Balice-Gordon and Lichtman 1990 suggests that the scaffold gets larger from within the first-formed structure in proportion to growth of both muscle and axon terminal.

From work on extrafusal muscle histochemistry (Rowlerson 1980), maturation of the NMJ's of slow muscle types occurs prior to that of fast muscle NMJ's. However physiological work by Jami et al (1988) in kitten spindles, using high amplitudes (0.1-0.5mm) of sinusoidal stretching to test for dynamic responsiveness, indicated that the maturation of dynamic responses lagged behind the static responses in kittens between 5 and 18 days old. Gregory and Proske (1986), using ramp-and-hold stretch of the muscle to distinguish between dynamic and static responses of spindle discharges, stated that static and dynamic responses appeared together at about the same time during development (5dpn). Static motor axons innervate b2 and chain fibres which are of mixed (fast and slow) and fast histochemical types respectively. The b2 fibre is however the first intrafusal fibre formed in development and if there is a timed sequence of axons arriving and subsequent maturation it would be expected that static innervation would be in advance of dynamic. Motor endings in spindles are visible ultrastructurally (Milburn 1984) by 40df and so far no-one has tried to record from kitten spindles prior to birth. Whether this means that the functional maturity does actually lag behind the morphological maturity has yet to be established.

Development of appropriate contacts of fusimotor axons with intrafusal fibres.

a) Myelination of the fusimotor axons.

Unmyelinated axons are very difficult to trace through serial electron micrographs even in adult spindles due to their small (<1μm) diameter. As all features of developing spindles were smaller than in adult spindles the interval between sections becomes important. As the interval between ultrathin sections was 2μm for 50df and 1dpn spindles and 5μm for the remainder it was possible to follow these motor axons to their intrafusal fibres. None of

the unmyelinated axons in this study showed any varicosities or large numbers of dense cored vesicles typical of autonomic axons and so unmyelinated axons were confidently identified as motor axons.

As can be seen by fig. 20 A,B, up to and including 21dpn, both myelinated and unmyelinated motor axons made connections with intrafusal fibres in combinations not found in adult spindles. This suggests that myelination is not necessarily influencing the refinement of motor innervation during this period. Between 21 and 28dpn it was observed that while the percentage of myelinated axons making immature connections declined from 33 to 25%, connections of unmyelinated axons to combinations of intrafusal fibres not encountered in the adult, increased dramatically from 25% to 100%. This could suggest that myelination of motor axons influences the maturity of connections **after** 21dpn or that both processes are happening at the same time.

b) Distribution of motor axons to intrafusal fibres.

The percentage innervation of intrafusal fibres by motor axons is at its maximum during 1dpn (all), 12dpn (bag fibres) and 21dpn (b1) (table 6). Presumably the motor axons are contacting as many of the spindle muscle fibres as possible at birth; later some of the connections will be lost and this can lead to some completely non-innervated intrafusal fibre poles, as is also observed in the adult. There are up to 25% of typical chain fibres which lack innervation in adult spindles (table 6) which seems too much redundant muscle just as a safety insurance against damage to the working chain fibres. Some of this must occur due to chains developing after the main phase of motor axon growth and innervation. The bag fibres also exhibit the occasional non-innervated pole, especially b1. This may be due to early axon connections

being mis-matches. If only one axon has made a connection to the intrafusal fibre, this may be subsequently withdrawn due to being an unsuitable connection. This will leave the intrafusal fibre uninnervated.

Are selective motor axons different from non-selective ones? From this study, selective motor axons certainly had fewer terminals (figs. 21, 22A,B,C) and did not end on such a variety of intrafusal fibres. This suggests that selective axons must be less branched. It would be interesting to know whether the wider distribution of axons to different intrafusal fibres in the adult was also accompanied by a larger number of terminals of each axon.

The distribution of motor axons to the intrafusal fibres and their selectivity of innervation can be seen in fig. 19. Motor axons will terminate on any of the intrafusal fibres. There does not seem to be any particular plan at first and any axon seems to be able to make contact with any and all the intrafusal fibres at once. The situation in adult spindles is somewhat more selective; beta innervation is apparently totally selective (beta dynamic axons innervate bag1 fibres; beta static axons innervate long chain fibres). Gamma dynamic innervation is almost selective to the bag1 fibre. Only gamma static innervation can be non-selective between bag2 and typical chain fibres (Arbuthnott et al 1982). Throughout the present study there were motor axons innervating varying combinations of intrafusal fibres and even by 28dpn, there were still 28% of motor axons innervating combinations of intrafusal fibres not found in adult spindles (fig. 19).

Selective innervation of bag1 fibres had doubled to 15% by 28dpn although this was less than half that of adult spindle selective bag1 innervation. In the adult, dynamic γ motoneurones must differ from static motoneurones because

their axons innervate b1 in all spindles they innervate. In this study it was not possible to observe the intrafusal fibres innervated in several spindles by one motor axon and so there was no evidence that axons innervating b1 in the sectioned spindles will innervate b1 in other spindles. It seems reasonable to suppose that a proportion of the axons innervating these sectioned spindles were dynamic since the total number of motor axons was unchanged and was on average the same as in the adult. These may be broadly selective from the start and specifically target their respective intrafusal fibre. As there was a rise in the total numbers of motor endplates at 12 dpn (fig.21) with a fairly constant axon number (fig. 18A), these results suggest that there could be another type of motoneurone which is not specific and able to modify its connections in response to the activity of the intrafusal fibre to which it is connected. This type of motoneurone may produce an axon which has a greater potential for branching than the others. As the b1 fibre supports a constant number of motor endings throughout development (fig. 26) it suggests an ability of this fibre to be much less receptive to many motor axons ending on it, or a strong capacity to reject other potential axon connections.

Whereas selective innervation to bag2 fibres was not consistent between 50df and 28dpn, the proportion of axons innervating chains alone remained unchanged (10-12%). Combined motor innervation of bag2 and chain fibres accounted for 58% of the motor axons by 28dpn (fig.19). This value was close to 64% of motor axons which innervated bag2 and typical chain fibres in adult spindles. The difference was in the proportion; combined bag2 and chain innervation was a larger part (34% of 58%) of this innervation at 28dpn than in the adult (19% of 64%).

Proposed mechanisms for the development of fusimotor innervation

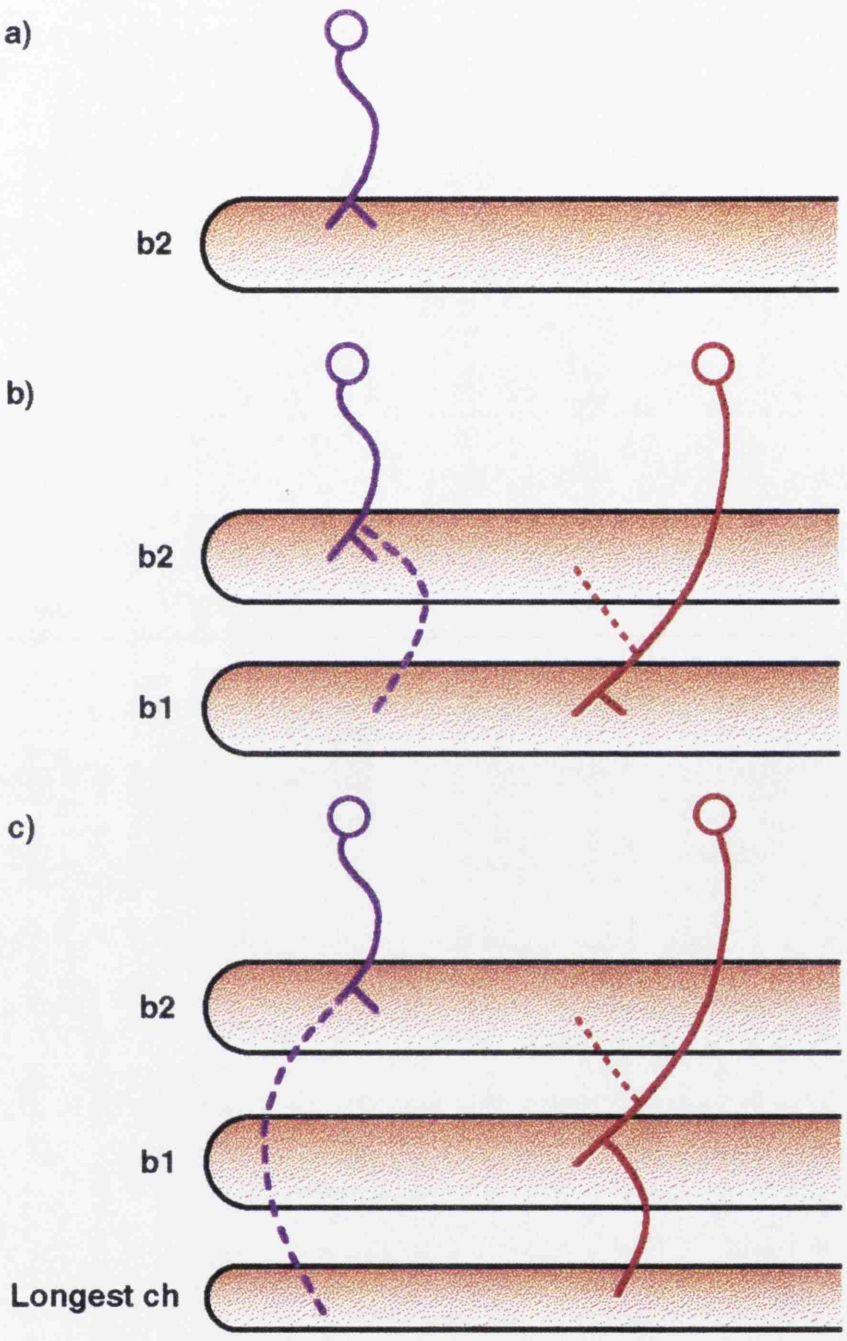
The initial contacts of motor axons might be considered to be guided chemically and so the motoneurone must be responsive to a particular substance (or substances) in which muscle type (e.g. fast, slow, b1 or b2 or chain) it is targeting. This hypothesis is favoured by Landmesser (1984) and Miller and Stockdale (1987) in avian muscle. However work on the developing innervation of fast and slow extrafusal muscle fibres in mammals (Thompson 1983,1986: Jones et al 1987a and b) favours first random innervation then re-arrangement of motor innervation during the period of synapse elimination. Motoneurone and muscle activity subsequently decides which motor endings will then go on to mature and develop the characteristic indented and deeply folded appearance of the extrafusal motor ending.

Motor innervation of intrafusal fibres may also be initially random with subsequent re-organisation of connections from 12dpn. This study has shown that while the numbers of motor axons remained constant throughout, the numbers of endings rose to a peak at 12dpn and then declined. There may have been motor axons which had specific targets, but these may have been masked by the non-selective ones which had a peak number of motor endings at 12dpn (fig. 21). However when considering b1 fibres the numbers of motor endings remained constant but there was an increase in the numbers of terminals of each axon, throughout the study. This suggests that some motor axons contacting b1 fibres are always selective to that fibre while other non-selective axons will leave the fibre surface with replacement of motor terminals by the selective dynamic motor axon.

A scheme is proposed in figure 30 for the separate innervation of b1 and b2 fibres. At a time when only b2 fibres were present (prior to 38df), all motor

Figure 30

Diagram of a possible mechanism of selective innervation of the bag 1 and bag 2 intrafusal fibres



Static motoneurone and axon is purple, dynamic motoneurone and axon is red.

Dashed lines indicate non-permanent connections.
(See text for details).

axons terminated on this fibre. (A). When the b1 fibre is formed (38-41df) axons may fleetingly innervate both b1 and b2 fibres (B). Some of these axons will be influenced by the b1 fibre to make strong connections with it and remove attachments from the b2 fibres (dashed lines, fig. 30).

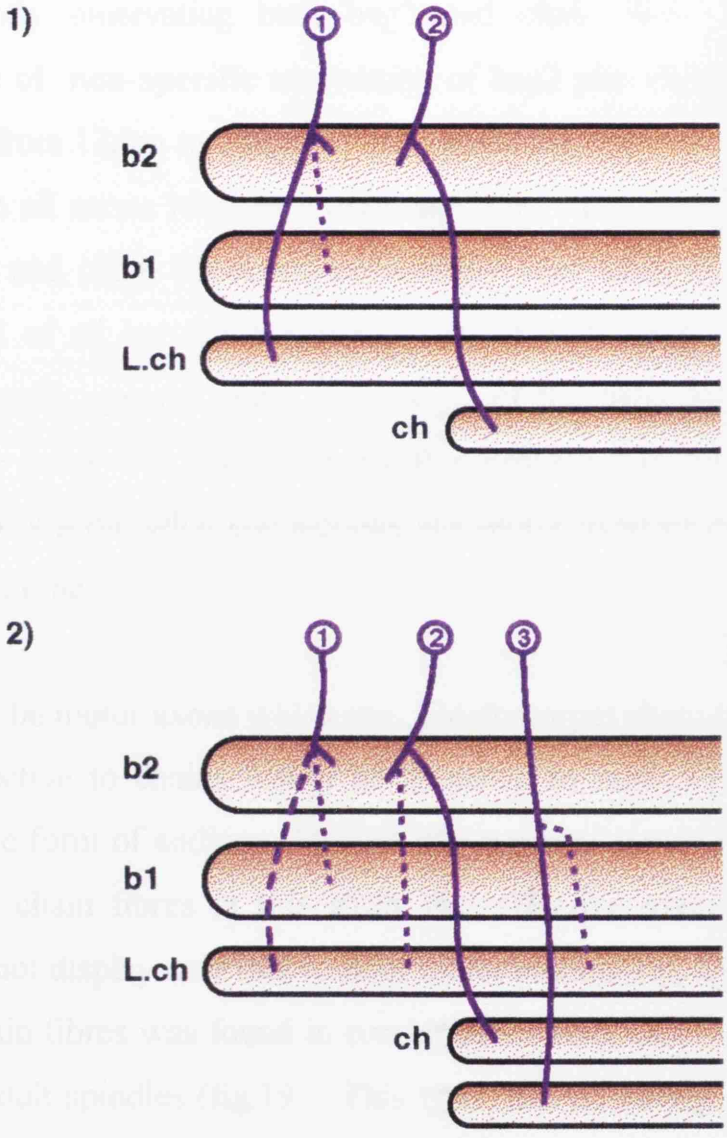
The first chain to be formed is the longest and sometimes actually becomes as long as the bag fibres and is known as 'the' long chain fibre. When this fibre is formed (41-43df), motor axons innervating either b2 or b1 can make connections with this nascent chain fibre (fig. 30C). Presumably some definitive dynamic gamma axons could retain their innervation of this fibre into adult spindles. Since there seems to be support in this study for dynamic gamma innervation to be determined early, the entirely non-specific axons ought to be gamma static.

Banks (1991) has shown that static γ axons with the slowest conduction velocities innervate chain fibres. Sequential decrease in conduction velocities of static motor axons may parallel the developmental order of intrafusal fibre innervation; chain fibres being formed and therefore innervated last.

Consider the static motor axons arriving in a timed sequence during development (fig. 31 numbers in circles indicating the order in which three gamma static motoneurones send out axons to the intrafusal fibres). Motoneurone 1 makes contact with the bag2 fibre (with or without a long chain fibre); subsequently, axons of motoneurone 2 make contact with bag2 and chain fibres (fig.31 (1))while even later, axons of motoneurone 3 innervate only typical chain fibres (fig. 31(2)). Any of these static motor axons may make brief contacts with the bag1 fibre as was seen in fig. 30 but not drawn here. The sequence of fig.31 indicates that the static gamma innervation has

Figure 31

Diagram of possible mechanisms of selective innervation of typical chain intrafusal fibres



As in Fig. 30, static innervation is purple. Numbers in the static motoneurones indicate a timed sequence in which their axons make connections with the intrafusal fibres.
(See text for details).

three parts from around 50df: a specific group of static motor axons targeting the bag2 fibre, another group specifically targeting chain fibres and a third group jointly innervating both bag2 and chain fibres at random. The percentage of **non-specific** innervation of bag2 plus chain fibres was greatly increased from 12dpn to 28dpn to 34% while the numbers of endings to chain fibres from all axons reached a peak at 12dpn. The contacts of motor axons with bag2 and chain fibres are presumably very labile during this time and withdrawal of all but the occasional contact with single chain fibres would lead to specific gamma static innervation of the bag2 fibre. Un-innervated chain fibre poles will also occur by this process. In this study it was not possible to observe what connections any motor axon made in more than one spindle at a time.

There may be motor axons which **specifically** target chain fibres but all motor axons selective to chains would be expected to make connections with the same single form of ending. At least two types of motor endings were found on typical chain fibres in this study and selective axons innervating chain fibres did not display only one form of motor ending (fig. 22A). Innervation of typical chain fibres was found in combinations with other intrafusal fibres not found in adult spindles (fig.19). This work had no strong evidence for motor axons specifically innervating chain fibres nor is there physiological evidence to support this.

A **specific** group of static motor axons targeting the b2 fibre may occur. A decrease in the numbers of motor endings occurred towards 28dpn on this fibre and it is reasonable to assume that some of the remaining contacts would be selective to the b2 fibre. Whether this was by direct targeting of the b2 fibre initially (fig. 31(1)) or subsequent removal of inappropriate contacts

cannot be determined here. Like motor innervation of b1 fibres (fig. 30) there may be a balance between the two mechanisms. However, recent work by Dickson et al 1993 has shown physiologically afferent recordings that indicate inhibition of chain fibres while b2 fibres were recruited by stimulating a single site in the midbrain. The possibility of static motor axons which specifically target the bag2 fibres seems highly likely.

It is possible that the development of the gamma static innervation depends more on attempts to make co-ordinated movements, and therefore full maturity is achieved later than in the gamma dynamic system.

Alternatively the evidence from this work could be interpreted as suggesting that motor innervation of the developing intrafusal fibres is by entirely random contacts until 12dpn, after which time a sorting process is in progress which is still incomplete at 28dpn.

General conclusion.

As is found in the development of extrafusal fibres, there were many more axon terminals on kitten intrafusal fibres than in the adult but unlike the extrafusal fibres during development there was no true polyneuronal innervation of each motor end-plate during the period studied. There was a reduction in numbers of these axon contacts and also readjustments of connections between mismatched axons and their target muscle fibres. Whether this process is triggered by the motoneurone with some form of recognition process between pre- and post-synaptic membranes or by muscle activity cannot be determined in the present study from a detailed analysis of changes in innervation patterns. Re-adjustment of inappropriate connections

in the kitten muscle spindles was still not complete by 28days after birth. Therefore, while all the components of the muscle spindle were present, including the motor innervation, prior to birth, the adult pattern of selective innervation of b1, b2 and chain fibres as well as co-innervation of b2 and chain fibres was not achieved even by 28days after birth. The ability of the kitten to walk or run is still fairly limited at a month after birth and therefore trials of co-ordinated muscle activity may also be required to determine the final appropriate connections of motoneurone with its target muscle.

Possibilities for future work

As the present work did not reach the point where the pattern of motor innervation to the spindle fibres had reached the adult pattern of innervation, an extension of the time scale of the present work would be necessary.

However, the time of most change in the developmental period studied here was immediately following 12dpn. At this point the numbers of motor endings was at its maximum. After this time there was a hint of increasing selectivity by the motor innervation of the intrafusal fibres. Work by Zelena et al (1957) indicates that the motor innervation is not required for muscle spindles to develop. Motor innervation would be expected to continue to develop after an initial delay or interruption. Use of a post synaptic receptor blocking agent such as α bungarotoxin which prevents the functional connection of motor axon with its muscle fibre could be used to ascertain if the toxin would inhibit, temporarily or permanently, the re-adjustment of the motor terminals towards the adult state.

In order to accomplish this, it was decided to try to allow release of α bungarotoxin onto the peroneal muscles of one hind limb by attachment of silastic strips impregnated with the toxin, in 12dpn kittens. (Peroneal muscles are more accessible than tenuissimus muscles). The kittens would be allowed to recover from the surgery and it was planned to sacrifice the kittens by anaesthetic overdose after another 14 days at which time their peroneal muscles would be prepared for electron microscopy as described in this thesis. The peroneal muscles of the other hind limb in each kitten would act as a control.

Due to Home Office intervention the α bungarotoxin had to be administered in mini-pumps inserted beneath the skin of each kitten's back. The α

bungarotoxin was therefore delivered from the pump to the lower limb muscles via a non-allergenic fine silastic tube sutured to the peroneal muscles of one leg. The procedure was carried out in two kittens, 11days old. One kitten died within the first day, the other kitten survived for six days.

Two main problems were encountered:

a) it was not clear how to find the appropriate dose of toxin relative to impregnated silastic strips.

b) retraction of the end of the silastic tubing occurred as the animal grew even although extra tubing was included.

I think this would still be an important project to pursue as observations of the peroneal muscle spindles in what would be kittens of almost 28dpn would indicate whether the pattern and number of connections of motor nerves to intrafusal fibres had remained in a 12dpn condition thereby indicating whether interruption of the sorting process at 12dpn caused the spindle to remain in a permanent state of early post-natal development.

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Appendix 1

Tissue Preparation And Embedding

Materials

Primary Fixative

Buffer

1M. NaCacod. = 50ml.
Sucrose. = 8.5g.
1M. CaCl. = 0.5ml.
Dist. Water = 350ml.

Adjust to pH 7.4, bring to 460ml with dist. water.

Fixative

Buffer = 115ml.
25% Glut. = 1x10ml Vial.

Buffer Wash

Cacodylate buffer wash.

Post Fixative

Cacodylate/OsO4

i.e. Buffer = 3 parts.
4% OsO4 = 1 part.

Araldite

Araldite	= 10ml.	= 25ml.	= 50ml.
Cy212.			
DDSA.	= 10ml.	= 25ml.	= 50ml.
BDMA or Dmp30.	= 0.4ml	= 1ml.	= 2ml.

Mix Thoroughly.

Procedure for Fixation And Dehydration

1.) Primary Fixative. = 120 Minutes.
2.) Buffer Wash. = 3 x 20 Minutes.
(Overnight at 4°C if necessary.)
3.) Post Fixative. = 90 Minutes.
4.) Buffer Wash. = 5 Minutes.
5.) Slow dehydration in graded alcohols (70% , 90% and absolute) for 20 minutes each.
6.) Propylene Oxide as an intermediate solvent for 5 minutes.
7.) Propylene Oxide-Araldite overnight.
(Caps Off Bottles.)
8.) Embed In Fresh Araldite for 3-4 days.
9.) Polymerise at 45°C For 24hrs, then Polymerise at 60°C for 24hrs.
N.B. Sometimes if the specimen is larger it may need a longer dehydration schedule.

Post embedding stains.

1μm sections.

Toluidine blue/pyronin Y. (Ito and Winchester, 1963).

Materials

a)Sodium borate (borax)	0.8g
b)Distilled water	$100cm^3$
c)Toluidine blue	0.8g
d)Pyronin Y	0.2g

Method.
1. Dissolve borax in distilled water.
2. Add in order: c) then d).
3. Dissolve thoroughly and filter into a stock bottle.

Ultra-thin sections.

Lead Citrate (Reynolds, 1963).

Materials.

a) 1.33g lead nitrate
b) 1.76g sodium citrate
c) 30cm^3 freshly boiled distilled water.

Method

1. Place a), b) and c) in a clean 50 cm^3 volumetric flask with a glass stopper.
2. Stopper and shake vigourously for 1 minute, and subsequently for 30 minutes intermittently. The solution will be milky.
3. Add 8.0cm^3 of 1N sodium hydroxide freshly prepared with boiled distilled water. The solution will clear.
4. Dilute to 50cm^3 with freshly boiled distilled water. The solution should be around pH12 and is now ready for use.

This solution should not be exposed more than necessary to the air and should always be filtered before use using a filter attached to a syringe.

Uranyl Acetate.

Materials.

a) 1g uranyl acetate
b) 49 cm^3 distilled water.

Method.
1. Add the uranyl acetate to the distilled water.

2. Sonicate for about 20 minutes to dissolve.
3. Filter.
4. Store in brown, glass stoppered bottles.

This stain should also be used through a syringe fitted with a filter.

Appendix 2

Table A. Mean numbers (±standard deviation) of motor endplates for each motor axon and fibre type.
(um; unmyelinated axon: my; myelinated axon: mep; motor endplate.)

	50df	1dpn	12dpn	21dpn	28dpn
b1					
um	**1.00**±0.00	**1.50**±0.71	**3.50**±0.71	**2.00**±0.00	
my		**1.00**±0.00	**2.30**±1.53	**3.50**±1.73	
Total	**1.00**±0.00	**1.66**±0.58	**3.50**±0.71	**2.25**±1.26	**3.50**±1.73
b2					
um	**4.00**±0.00	**2.00**±1.40	**2.00**±0.82		
my			**1.00**±0.00	**1.00**±0.00	**1.00**±0.00
Total	**4.00**±0.00	**2.00**±1.40	**1.80**±0.84	**1.00**±0.00	**1.00**±0.00
ch					
um	**2.00**±1.53	**3.00**±1.83	**4.00**±1.41	**2.50**±0.71	
my					**1.50**±0.71
Total	**2.67**±1.53	**3.00**±1.41	**4.00**±1.41	**2.50**±0.71	**1.50**±0.71
b2+ch					
um	**4.00**±0.00	**6.86**±2.79	**7.67**±3.05	**5.50**±0.71	
my		**4.00**±0.00	**9.00**±0.00	**4.50**±2.12	**5.00**±1.73
Total	**4.00**±0.00	**7.25**±2.82	**8.00**±2.58	**5.00**±1.41	**5.00**±1.73
Other combination.					
um	**5.20**±3.83	**5.00**±2.03	**7.67**±5.61	**4.30**±0.58	**4.00**±0.00
my		**7.50**±3.54	**8.00**±0.00	**6.20**±2.86	**5.50**±3.56
Total	**5.20**±3.83	**4.95**±1.93	**7.69**±5.37	**5.75**±2.31	**5.13**±3.09
Total mean nos. of mep/motor axon.	**3.92**±2.78	**4.82**±2.69	**6.00**±4.62	**4.05**±2.33	**4.04**±2.54
Total no. axons.	12	35	26	20	25

Table B. Means lengths (μm) of motor endplates comparing those on unmyelinated and myelinated axons. (key as for Table A).

	50df	1dpn	12dpn	21dpn	28dpn
b1					
um	**5.76**± 4.85 n=21	**7.35**± 8.42 n=34	**18.86**± 13.19 n=28	**10.71**± 5.05 n=7	**16.00**± 14.14 n=2
my		**10.67**± 9.07 n=3	**8.00**± 3.46 n=3	**12.00**± 9.34 n=19	**14.55**± 8.40 n=22
b2					
um	**4.33**± 6.04 n=27	**7.60**± 8.87 n=35	**13.07**± 11.88 n=42	**9.75**± 7.01 n=8	**6.00**± 0.00 n=1
my		**11.75**± 10.34 n=4	**21.00**± 11.70 n=7	**14.60**± 13.90 n=10	**23.38**± 25.00 n=26
ch					
um	**3.71**± 2.51 n=24	**5.53**± 4.74 n=57	**8.90**± 6.31 n=60	**8.36**± 8.08 n=14	**6.00**± 0.00 n=1
my		**5.17**± 4.45 n=6	**8.50**± 4.34 n=8	**7.28**± 5.13 n=21	**13.39**± 10.91 n=44
L.ch (t)					
um		**7.83**± 7.91 n=6	**11.67**± 5.13 n=3	**5.00**± 1.41 n=2	**6.50**± 1.00 n=4
my			**22.00**± 21.21 n=2		**8.75**± 5.50 n=4
L.ch (l)					
um		**5.05**± 4.59 n=18	**9.83**± 11.96 n=6		
my		**4.33**± 2.88 n=6			

Table C. Percentage of each motor endplate type found for each intrafusal fibre type. (key as for Table A).

	50df	1dpn	12dpn	21dpn	28dpn
b1					
a	66.7	43.2	32.3	69.2	41.6
af	9.5	24.3	16.1	3.8	8.3
ab	9.5	13.5	35.5	19.2	20.8
abf					4.2
b	9.5	16.2	12.9	7.7	25
bf			3.2		
c	4.8	2.7			
b2					
a	81.5	79.5	71.43	77.8	66.6
af	7.4	17.9	14.3	22.2	11.1
ab	3.7		8.2		14.8
abf			4.1		
b	3.7		2.1		7.4
c	3.7	2.6			
ch					
a	66.7	53.2	48.5	51.4	27.3
af	8.3	20.6	38.2	31.4	31.8
ab	4.2	4.8	1.5	2.8	
b	8.3	3.2	1.5	2.8	
c	12.5	17.5	10.3	11.4	38.6
ac		1.6			2.3
Lch thin					
a		66.6	20	50	50
af		33.3	60	50	25
ab			20		
c				25	
Lch large					
a		66.6	50		
af		12.5			
ab		4.2	16.7		
abf		4.2			
b		12.5	33.3		

Table D. Mean distance (μm) of motor endplate from equator with endplate type and for each type of intrafusal fibre. (key as for Table A).

	50df	1dpn	12dpn	21dpn	28dp
b1					
a	153.9±60	336.3±122	414.8±137	349.0±70	296.9±92
af	203±50	407±63	444±138	340±0	458±4
ab	162±57	430±149	370±131	306±32	538±370
abf					375.0±0
b	196±96	288±77	546±122		820±382
bf					
c	160±0	148±0			
b2					
a	148±50	304±137	357±117	315±60	523±344
af	193±39	406±133	355±100	286±79	284±105
ab	94±0		373±134		420±221
abf			516±111		
b	142±0				1099±166
c	257±0	453±0			
ch					
a	141±41	228±109	344±118	313±110	572±362
af	164±100	263±96	339±83	307±45	448±352
ab	81±0	197±48			
b	203±72	202±3	357±0		
c	258±20	356±83	433±100	235±27	362±159
ac		110±0			515±0
L.ch (thin)					
a		252±51	472±0	243±21	196±29
af		227±0	348±35	228±0	259±86
ab			415±0		
c		255±0			120±0
L.ch (large)					
a		408±106			
af		367±130			
ab		438±0			
abf		320±0			
b		482±116			

Table E. Mean length (μm) of motor endplate for each type of motor endplate and intrafusal fibre. (key as for Table A).

	50df	1dpn	12dpn	21dpn	28dpn
b1					
a	6.2±5.7	8.7±11.2	14.9±13.0	9.9±6.0	9.8±6.3
af	5.0±0.0	4.4±3.1	10.0±5.1	14.0±0.0	16.0±5.7
ab	7.0±2.8	8.8±7.8	16.4±11.4	10.6±5.5	21.2±9.6
abf				30.0±0.0	
b	3.0±2.8	8.6±6.5	31.2±4.9	29.0±18.4	17.6±8.9
bf			48.0±0.0		
c	4.0±0.0	6.0±0.0			
Total	5.7±4.8	7.6±8.3	17.8±12.9	11.7±8.3	14.6±8.6
b2					
a	3.4±3.1	8.1±9.7	13.5±11.2	9.1±5.6	25.3±29.0
af	16.0±21.2	7.4±6.0	13.9±6.0	24.3±18.0	19.3±13.0
ab	1.0±0.0		18.5±24.3		9.0±4.8
abf		24.0±17.0			
b	3.0±0.0		2.0±0.0		32.0±8.5
c	5.0±0.0	11.0±0.0			
Total	4.3±6.0	8.0±8.9	14.2±12.1	12.4±11.4	22.7±24.8
ch					
a	3.6±2.7	4.2±3.7	7.1±4.3	8.1±7.6	6.8±2.9
af	6.5±2.1	5.2±3.7	10.1±6.6	6.5±3.9	14.1±10.9
ab	2.0±0.0	7.0±7.0	6.0±0.0	17.0±0.0	
b	3.0±2.8	3.5±2.1	6.0±0.0	5.0±0.0	
c	3.7±1.1	9.5±6.4	11.7±10.2	8.5±6.4	15.7±11.3
ac		13.0±0.0			42.0±0.0
Total	3.7±2.5	5.5±4.7	8.9±6.1	7.7±6.3	13.2±10.8
L.ch (thin)					
a		8.3±4.8	7.0±0.0	4.0±0.0	6.0±0.0
af		7.0±4.2	11.7±5.1	6.0±0.0	7.0±1.4
ab			37.0±0.0		
c					11.5±7.8
Total		7.8±7.9	15.8±12.6	5.0±1.4	7.6±3.9
L.ch (large)					
a		3.8±2.8	5.0±1.7		
af		8.3±4.2			
ab		2.0±0.0	34.0±0.0		
abf		18.0±0.0			
b		3.7±2.1	5.0±2.8		
Total		4.9±4.2	8.8±11.9		