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# Morphometric Analysis of Coated Vesicles in Developing Rat Muscle Spindles

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## Summary

The incidence of coated vesicles under sarcolemmal surfaces of equatorial, juxtaequatorial and polar regions in developing and adult spindles of the rat soleus muscle was examined by quantitative morphometry of transverse ultrathin sections. Coated vesicles were more numerous: 1) under primary sensory endings than under other types of neuromuscular contacts; 2) under the appositional sarcolemma between neighbouring intrafusal fibres than under free surfaces of the sarcolemma; and 3) in developing than in mature spindles. Factors such as location and age of the animal often interacted to produce an additive effect on the incidence of coated vesicles. Although there was a high incidence of coated vesicles at the postsynaptic surface under sensory terminals of $bag_2$ fibres in 18 and 19 day gestational embryonic rats, it peaked in 4 day postnatal animals. The high incidence of coated vesicles at sensory endings supports the view that coated vesicles mediate neurotrophic interactions between afferents and intrafusal fibres during the critical late gestation and early postnatal time period, as sensory axons first contact their target fibres and exert a maximal directing influence on the differentiation of intrafusal fibre types. In addition, the preferential localization of coated vesicles under appositional rather than free surfaces of developing intrafusal fibres in 0-4 day rats suggests that they play a role in the transport of active substances among intrafusal fibres exhibiting different stages of maturity.

### Key words

Muscle spindle - Development - Coated vesicles - Morphometry - Rat

## Introduction

Extracellular substances, such as hormones and growth factors, are internalized in clathrin-coated vesicles in a variety of tissues (Willingham and Pastan 1985, Brown *et al.* 1983). However, little is known about the distribution and function of coated vesicles in embryonic, neonatal and adult skeletal muscles (Bursztajn 1984). Coated vesicles are thought to mediate uptake of alpha-macroglobulin *via* rec-eptor-mediated endocytosis in skeletal muscles (Haye *et al.* 1986) although they may also be involved in exocytosis (Benson *et al.* 1985).

Muscle spindle morpho-genesis appears to be highly dependent on the lines of communication developing between sensory nerve terminals and nascent intrafusal fibres, and among the developing intrafusal fibres themselves. Because the arrival of primary afferents initiates spindle formation, they are likely to signal primitive myotubes to differ-entiate into nuclear $bag_2$ intrafusal fibres (Zelená 1957, Milburn 1973, 1984). The nuclear $bag_1$ and chain fibres assemble in close association with the existing $bag_2$ myofibre; their development is likely to be directed by messages conveyed either from the $bag_2$ fibre or directly from the primary afferents or from both of these sources. Although the mechanisms involved in these developing lines of communication have not yet been elucidated, receptor-mediated endocytosis of neuro-active substances in coated vesicles is a likely candidate (Zelená and Soukup 1973). Indeed, coated vesicles are present under the sarcolemma of sensory endings on intrafusal fibres in muscle spindles of foetal rats (Landon 1972). If coated vesicles exhibit the same endocytotic functions in muscle spindles as in other tissues, then the development of intrafusal fibres may be influenced by neurotrophic factors thought to be released from sensory terminals (Landon 1972, Zelená and Soukup 1973). The present study examines whether the relative distribution of coated vesicles at different cellular sites is consistent with the hypothesis that they mediate the transfer of chemical information among the constituent cells of developing rat muscle spindles.

We tested the hypothesis that the internalization of neurotrophic factors in coated vesicles may be involved in cellular communication at two sites in developing muscle spindles of the rat soleus muscle, namely between afferents and intrafusal fibres and among the different types of intrafusal fibres. If the hypothesis is correct, then one would expect that the incidence of coated vesicles would be higher: 1) in developing versus adult spindles because the ontogenetic influence of afferents would be expected to be greatest during the formation and differentiation of spindles; 2) under sensory rather than motor terminals because afferents rather than efferents primarily regulate the formation of spindles (Zelená 1976); 3) under apposed surfaces of intrafusal fibres where intercellular transfer of substances would be expected to take place, rather than under the free sarcolemmal surface. Some of the results have been reported in a preliminary form (Stephens *et al.* 1988).

## Material and Methods

*Histological procedures.* Male and female Sprague-Dawley rats of six groups were studied: 18-day foetal rats, 19-day foetal rats, 0-day postnatal, 4-day postnatal, 8-day postnatal and young adult (180-200 g body weight). The staging of the gestational rats was carried out as described previously (Kucera *et al.* 1988). The postnatal rats were anaesthetized with sodium pentobarbital (35 mg/kg i.p.) and their hindlimbs were perfused *via* the abdominal aorta for 10 min with 2.5 % glutaraldehyde and 1 % paraformaldehyde buffered to pH 7.35 in 0.1 M cacodylate. Soleus muscles were excised, pinned at their resting length to a strip of paraffin wax and fixed for an additional 2 h. The specimens were postfixed for 1 h in 1 % osmium tetroxide, dehydrated in ethanol and embedded in Epon 812. Transverse sections were cut using an LKB Nova ultramicrotome. Semi-thin sections were stained with 1 % toluidine blue for light microscopy, and ultrathin sections were stained with uranyl acetate and lead citrate for examination with a Philips 300 electron microscope. The semi-thin sections were used to locate spindles, to identify intrafusal fibre types and for orientation of ultrathin sections. The latter were used to identify coated vesicles under selected membrane surfaces.

*Identification criteria and terminology.* Coated membranes can appear as invaginations, pits or vesicles (Willingham and Pastan 1985). However, all are characterized by the presence of an electron-dense, regular bristle-like coating or thickening on their cytoplasmic surface, hence they were all grouped into one category for analysis, and are referred to as 'coated vesicles' or 'organelles'.

Three categories of intrafusal fibre were recognized in postnatal spindles: nuclear $bag_2$, $bag_1$ and chain. Bag fibres possessed a greater degree of equatorial nucleation and were thicker and longer than the chain fibres. The $bag_1$ fibre could be distinguished from the $bag_2$ fibre in mature spindles according to established morphological criteria such as fibre diameter and length as well as the arrangement of equatorial nuclei (Banks *et al.* 1977, Walro and Kucera 1985). Additional criteria were used in developing postnatal muscle spindles: 1) the $bag_2$ fibre was morphologically more mature than the $bag_1$ or chain fibres and 2) the $bag_1$ fibre separated from the $bag_2$ chain fibre ensemble in the polar regions (Milburn 1973). Only $bag_2$ fibres were observed in 18- and 19-day embryonic muscle spindles; the nascent $bag_1$ fibres were present as immature myotubes although both displayed sensory endings (Kucera *et al.* 1988).

Terminals were classified as primary sensory, secondary sensory or motor based upon their location along the axis of the spindle and their morphology (Walro and Kucera 1985, Kucera and Walro 1988). Nerve fibres which terminated in the equatorial and juxtaequatorial regions were identified as primary and secondary afferents, respectively, whereas those terminating in polar regions were recognized as motor. In addition, the presence of basal lamina in the cleft between axon terminals and muscle fibres was a feature used to differentiate motor from sensory terminals (Landon 1972).

*Qualitative analysis.* Three muscle spindles from each of three postnatal age groups (0-day, 4-day and adult) were examined. In each spindle, the incidence of coated vesicles under the sarcolemma at three membrane surfaces in three regions was evaluated for each of the three types of intrafusal fibre. The three membrane sites were defined as: 1) 'junctional'- sarcolemma beneath the sensory or motor endings; 2) 'appositional'- sarcolemma between adjoining intrafusal fibres; 3) 'free'- or non-appositional sarcolemma of the intrafusal fibre (Fig. 1). The three spindle regions were: 1) the equatorial, 2) the juxtaequatorial and 3) the polar region. These three subdivisions correspond to the termination zone of primary afferents, secondary afferents and motor neurones, respectively. In addition to the *complete* study of 3 surfaces in 3 regions of 0-day, 4-day and adult rats, the analysis of coated vesicle incidence was extended to 18- and 19-day embryonic muscles but was, of necessity, restricted to equatorial $bag_2$ intrafusal fibres; 8-day fibres were included as well in this *time course* analysis; only junctional surfaces under sensory terminals were compared to free sarcolemma.

The protocol used to determine the incidence of coated vesicles differed slightly for sensory and motor components of postnatal spindles. For sensory areas the equatorial and juxtaequatorial regions were each cut at 5 different transverse levels. At each level, sets of 18 serial ultrathin sections spanning approximately 1 $\mu$m of tissue were obtained; the sets

were separated by a series of nine 1 $\mu$m thick sections collected in sequential order. The ultrathin sections were mounted on 200 mesh thin bar nickel grids, which allowed viewing of more than 80 % of the section surfaces. For each level, the first three sections of a set with complete (not eclipsed by the grid bars) spindle cross-sections were photographed, thereby ensuring random sampling. Micrographs of each intrafusal fibre were printed at a magnification of X 16 000 and those coated vesicles within 50 nm of the sarcolemma were counted using a Zeiss Makro dissecting microscope at a magnification of X 10. The length of each membrane surface was determined by morphometric analysis using a Hewlett-Packard digitizer tablet and Bioquant (Nashville, Tennessee) software coupled to an Apple IIe. Values were expressed as the number of coated vesicles per 10 $\mu$m of sarcolemma.

Sampling protocol was modified slightly for examination in the motor region. Motor terminals are not uniformly distributed along the length of the intrafusal fibre surface. Thus the motor zone was first located by light microscopy of 1 $\mu$m thick sections; the motor endings as they appeared on different intrafusal fibres were then sampled in 10-40 ultrathin sections. The length of the motor zone varied from 5 $\mu$m in spindles from 0-day rats to 40 $\mu$m in the adult. Coated vesicles were rare under sarcolemmal surfaces of motor terminals in the adult; it was often necessary to thin-section most of the motor zone to obtain an appropriate sample; 3-8 sections from as many as 10 intervals had to be analyzed in order that the rarity of coated vesicles did not bias the results. In all other respects the protocols for the sensory and motor regions were identical.

Thus, the basic sampling procedure for postnatal spindles included: 3 age groups (0-day, 4-day and adult) x 3 muscle spindles per age group x 3 regions (equatorial, juxtaequatorial and motor) per spindle x 5 levels per spindle region x 3 sections per level x 4 intrafusal fibres (nuclear $bag_1$, $bag_2$, two chains) per spindle section x 3 surfaces per intrafusal fibre (junctional, appositional, free). The values for the two chain fibres in spindles from 4-day and adult rats were pooled. As many as 540 measurements were made for each surface.

The protocol for either the sensory or motor region was also occasionally adjusted for special circumstances associated with the development of spindles. Spindles from 0-day rats contained only three rather than the four intrafusal fibres found in spindles from 4-day or adult rats. Sarcolemma of intrafusal fibres in the polar regions of developing spindles, and in both central and polar regions of adult spindles, are infrequently apposed; hence, analysis of appositional surfaces was inappropriate. As mentioned above, coated vesicles were extremely rare under motor terminals of adult intrafusal fibres. Thus, more intervals had to be sampled.

*Statistical analysis.* The incidence of coated vesicles per 10 $\mu$m of intrafusal fibre membrane was analyzed among samples classified according to age of the rat, region of spindle, muscle fibre type and surface. Seventeen of 81 possible combinations of these four independent variables (age, region, fibre type, surface) had no values, hence a four-way analysis of variance (ANOVA) was not possible. Differences among groups were elucidated (BMDP7D, BMDP Statistical Software) for combinations of independent variables with the incidence of coated vesicles/10 $\mu$m of membrane as the dependent variable. Significant differences indicated by ANOVA were analyzed *a posteriori* using a Student Newman-Keuls test (Sokal and Rohlf 1969).

## Results

The muscle spindles of different age groups could be distinguished by the number of constituent intrafusal fibres and their spatial arrangement. The number of intrafusal fibres per spindle increased with age. Only $bag_2$ fibres were recognized in 18- and 19-day gestational rats (Kucera *et al.* 1988). Spindles from newborn (0-day) rats possessed three intrafusal fibres each, which were recognized as one immature chain myotube and two relatively mature bag myofibres (Fig. 1-4). Spindles from the 4-day group contained three myofibres ($bag_2$, $bag_1$, chain) and one immature chain myotube. Adult spindles contained one $bag_1$, one $bag_2$ and two chain myofibres. Furthermore, the separation of the intrafusal fibres increased with age; as a consequence, appositional surfaces among the fibres were common only in developing spindles. The myofibres of developing spindles were usually tightly apposed, especially in the equatorial and juxtaequatorial regions (Fig. 4a-g); their separation increased with increasing distance from the equator regions. In contrast, appositional surfaces between intrafusal fibres were infrequent in spindles of adults, although sarcolemma of bag fibres were occasionally apposed in the equatorial regions (Fig. 4 i,j).

The form of the sensory and motor endings differed between developing and adult spindles. The extent of the circumference of intrafusal fibres that could be covered by sensory terminals was limited in developing spindles because the fibres were still closely packed together (Fig. 1). In contrast, the primary and secondary sensory terminals in adult spindles usually encircle most of the circumference of the individual intrafusal fibres in the characteristic annulospiral pattern (Mayr 1969). In addition, the breadth of the primary or secondary region was smaller in developing than in mature spindles (Kucera *et al.* 1988). The motor endings from developing spindles were short, and narrow in comparison with those of the adult as observed by Ovalle (1972). They consisted of several

vesicle-laden profiles of terminal axons that were closely apposed to segments of intrafusal fibres that displayed little or no postsynaptic specialization (Fig. 3a-e). Only $bag_2$ or $bag_1$ myofibres exhibited motor terminals in the immature spindles. Motor terminals were not present on chain myotubes of spindles from 0-day rats but were developed on fibres from 4-day rats.

*Features and localization of coated vesicles.* Coated membrane in the developing and mature spindles assumed a wide variety of shapes, ranging from oval-shaped coated invaginations of variable length to vesicles (Fig. 2-4). All coated membranes bore an electron dense thickening of clathrin on the cytoplasmic face (Fig. 2-4), similar to findings for other tissues (Harrison and Kirchhauser 1983). The appearance of coated organelles was similar for immature and mature spindles. Diameter of vesicles did not vary with age of the animal, intrafusal fibre type, or spindle region. However, the diameter of coated vesicles did vary according to their position within the intrafusal fibres. The diameter of the coated vesicles immediately underneath the sarcolemma at sensory terminals was 102.3 ± 8.29 nm (n=10). The coated vesicles found in the Golgi region of the intrafusal fibres were smaller, measuring 50-75 nm in diameter; their incidence was not assessed. Although coated invaginations of membrane surfaces had the same spiky appearance as the vesicles, they varied in width from 200-1000 nm (Fig. 2-4). Coated vesicles were often located close to multivesicular bodies, lysosomes and endosomes (Fig. 2,4).

The coated vesicles were found at two principal sites in the intrafusal fibres regardless of the age group; under junctional surfaces of sensory or motor neuromuscular junctions; and at the appositional surfaces of intrafusal muscle fibres. Coated vesicles were rarely encountered on free surfaces or within the sarcoplasm of the intrafusal fibres except for the Golgi region. At junctional sites, the coated vesicles were observed immediately under the junctional postsynaptic sarcolemma of the intrafusal fibres and throughout the axoplasmic terminal (Fig. 2,3). They were often closely associated with finger-like axoplasmic projections of primary and secondary sensory axons into the intrafusal fibre sarcoplasm in developing muscle spindles (Fig. 2). Coated vesicles were located frequently at appositional surfaces where thin fingerlike interdigitations of one fibre invaginated its adjacent neighbouring developing spindles (Fig. 4).

**Table 1 and 2**

One and two way analysis of variance of the incidence of coated vesicles (per 10 $\mu$m). Underline indicates means which are not significantly different at 0.05 level by ANOVA and a Student Newman-Keuls test. In Table 2 the sample size is indicated in parentheses after the mean value. The coated vesicle incidence is compared according to age, region, fibre and surface (all other variables combined) in Table 1 and according to double variables (Table 2) such as age and surface or region or fibre, as well as region with fibre or surface and finally the effect of fibre and surface.

**Table 1**

Comparison of densities (per 10 $\mu$m membrane) of coated vesicles among different rat ages, different spindle regions, different fibre types and different fibre surfaces.

| | | | |
|---|---|---|---|
| Age | Adult | 0-day | 4-day |
| | 0.3 | 0.9 | 1.2 |
| N (group) | 62 | 79 | 80 |
| Region | Polar | Juxtaequatorial | Equatorial |
| | 0.5 | 0.8 | 1.0 |
| N (group) | 55 | 72 | 106 |
| Fibre | Chain | $Bag_1$ | $Bag_2$ |
| | 0.7 | 0.9 | 1.0 |
| N (group) | 86 | 67 | 80 |
| Surface | Free | Appositional | Junctional |
| | 0.3 | 1.2 | 1.2 |
| N (group) | 97 | 46 | 90 |

**Table 2**

Comparison of densities (per 10 $\mu$m) of coated vesicles analyzed simultaneously according to two factors.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adult free | 0-day free | 4-day free | Adult junctional | 0-day appositional | 0-day junctional | 4-day junctional | 4-day appositional | |
| Age x Surface | 0.2(32) | 0.4(27) | 0.4(32) | 0.5(30) | 0.8(28) | 1.5(30) | 1.6(30) | 1.7(18) | |
| | Adult polar | Adult equator | Adult juxta equator | 0-day polar | 4-day polar | 0-day juxta equator | 4-day juxta equator | 0-day equator | 4-day equator |
| Age x Region | 0.3(20) | 0.3(24) | 0.4(18) | 0.5(15) | 0.8(20) | 0.8(30) | 1.0(24) | 1.1(34) | 1.5(36) |
| | Adult chain | Adult $bag_1$ | Adult $bag_2$ | 0-day chain | 0-day $bag_1$ | 4-day chain | 0-day $bag_2$ | 4-day $bag_1$ | 4-day $bag_2$ |
| Age x Fiber | 0.3(26) | 0.3(18) | 0.4(18) | 0.6(27) | 0.9(26) | 1.0(33) | 1.0(26) | 1.2(23) | 1.4(24) |
| | Polar chain | Polar $bag_1$ | Juxta-equator chain | Polar $bag_2$ | Juxta-equator $bag_1$ | Equator chain | Juxta-equator $bag_2$ | Equator $bag_1$ | Equator $bag_2$ |
| Region x Fiber | 0.4(19) | 0.5(17) | 0.6(25) | 0.6(19) | 0.8(24) | 0.8(42) | 0.9(23) | 1.1(26) | 1.2(38) |
| | Polar free | Juxta-equator free | Equator free | Polar junctional | Juxta-equator appositional | Juxta-equator junctional | Equator appositional | Equator junctional | |
| Region x Surface | 0.2(31) | 0.3(27) | 0.4(39) | 0.8(24) | 1.0(18) | 1.0(27) | 1.3(28) | 1.5(39) | |
| | Chain free | $Bag_1$ free | $Bag_2$ free | Chain appositional | Chain junctional | $Bag_1$ appositional | $Bag_1$ junctional | $Bag_2$ junctional | $Bag_2$ appositional |
| Fiber x Surface | 0.3(37) | 0.3(26) | 0.4(34) | 0.9(18) | 1.0(31) | 1.2(14) | 1.2(27) | 1.4(32) | 1.5(14) |

In 0-day 4-day spindles coated vesicles were found budding inwards from or immediately underlying the sarcolemma of adjacent intra-fusal fibres. At appositional surfaces it was common to see coated vesicles side-by-side (Fig. 4d). Because the inter-digitations of the finger-like processes were so frequent, coated vesicles often seemed to have been caught in closed saccules of cytoplasm; these were most likely interdigitations whose connections to the surface were not visible (Fig. 4).

Coated vesicles were also present at appositional surfaces between myoblasts adjacent to intrafusal fibres (Fig. 4h) and within Schwann cell processes. Coated vesicles were still found under the appositional surfaces occasionally observed in adult spindles (Fig. 4i,j).

Extrafusal muscle fibres of adult muscle occasionally displayed coated vesicles that were similar in size and appearance to those encountered in spindles. Coated vesicles of the extrafusal fibres in developing muscle were typically located on the appositional surfaces between the primary and secondary myotubes (Fig. 4k). Coated organelles at the free surfaces of both types of extrafusal myotubes were rare.

*Incidence of coated vesicles in developing versus adult spindles.* The incidence of coated vesicles was compared between developing and adult spindles at each of the following sites: junctional sarcolemma of sensory endings, junctional sarcolemma of motor endings and non-junctional (free) sarcolemma of intrafusal fibres (Fig. 5, Tables 1,2).

In general, the density of coated vesicles was higher in developing versus adult spindles (Fig. 5, Tables 1,2). Frequency of coated vesicles under the free sarcolemma of intrafusal fibres was low and relatively independent of age. However, the incidence of coated vesicles under the junctional sarcolemma of sensory endings in developing spindles was significantly higher than that of adult spindles. The number of coated vesicles under sensory junctions in embryonic development could only be studied for $bag_2$ fibres; frequency of coated vesicles at 18 and 19 days of embryonic life was high, increasing at 0 days to peak at 4 days (Fig. 5). Note that the actual values for the incidence of coated vesicles under sensory terminals are higher in Figure 5 than those for junctional surfaces in Tables 1 and 2 because the numbers are grouped in the latter. The values for 4-day sensory terminals were as much as 20 times higher than those for adult free sarcolemmal membranes. The differences between the developing and adult groups were consistent for different types of intrafusal fibre.

*Incidence of coated vesicles under sensory versus motor terminals.* The incidence of coated vesicles under the primary sensory endings (equatorial region), secondary sensory endings (juxtaequatorial region) and motor endings (polar region) were compared within each age group. Different factors such as age of the animal as well as the location of the coated organelles interacted to produce an additive effect on their incidence. The combined incidence of coated vesicles in the equatorial and juxtaequatorial regions was higher (as much as two-fold) than under motor endings within each of the three age categories (Tables 1,2). The incidence of coated vesicles was higher at primary than secondary sensory endings. These relationships were true for each of the three types of intrafusal fibre examined, i.e. the incidence of coated vesicles at sensory versus motor endings was higher for $bag_1$, $bag_2$ and chain fibres (Table 2). There were no consistent differences in coated vesicle distribution among the three types of fibre, although the values were generally less for the more immature fibre (Table 2).

*Incidence of coated vesicles at appositional versus free surfaces of the intrafusal muscle fibres.* The incidence of coated vesicles was higher under appositional than non-appositional (free) surfaces within each of the age categories for each of the three types of intrafusal fibre (Tables 1,2). The incidence of coated vesicles was similar under appositional and junctional surfaces.

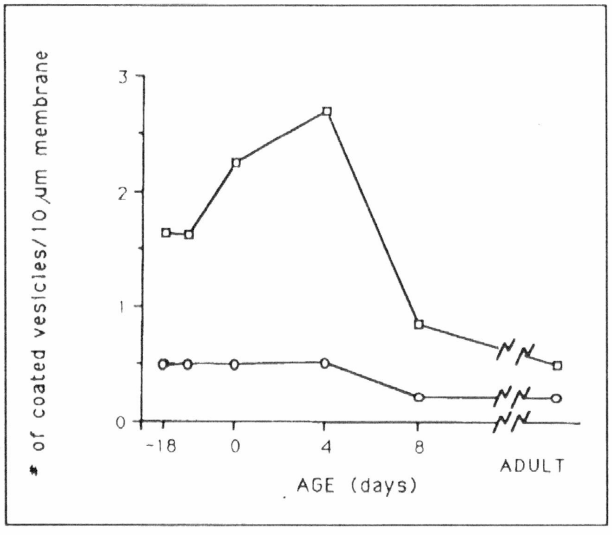



**Fig. 5**
Variation in the incidence of coated vesicles under sensory terminals of $bag_2$ intrafusal fibres (squares) versus free membrane (circles): effect of age. The standard error (not indicated) of the measurements was not more than 5 %. It was not possible to calculate standard errors in Tables 1 and 2 as the values were grouped.

## Discussion

*Features of coated vesicles and methodological considerations.* The coated vesicles found in the muscle spindle were similar in several respects to those

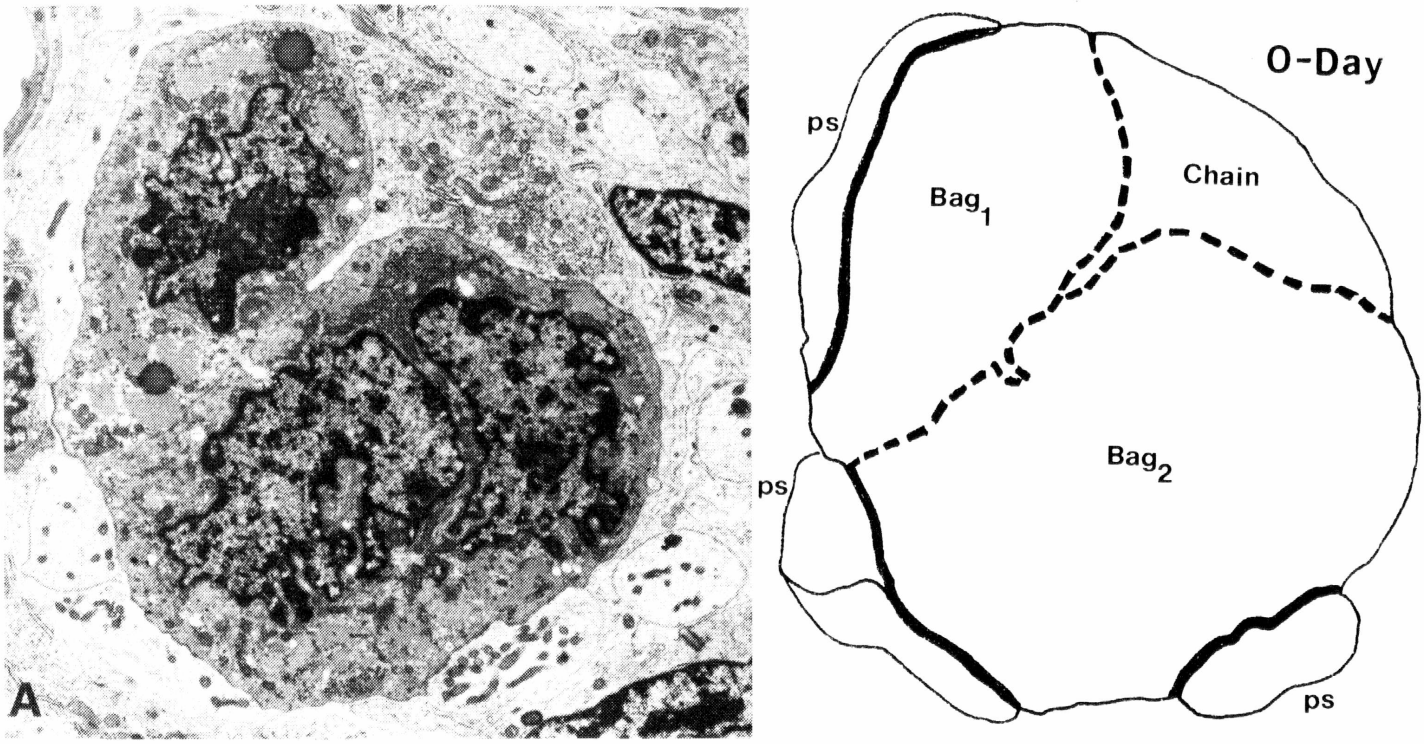
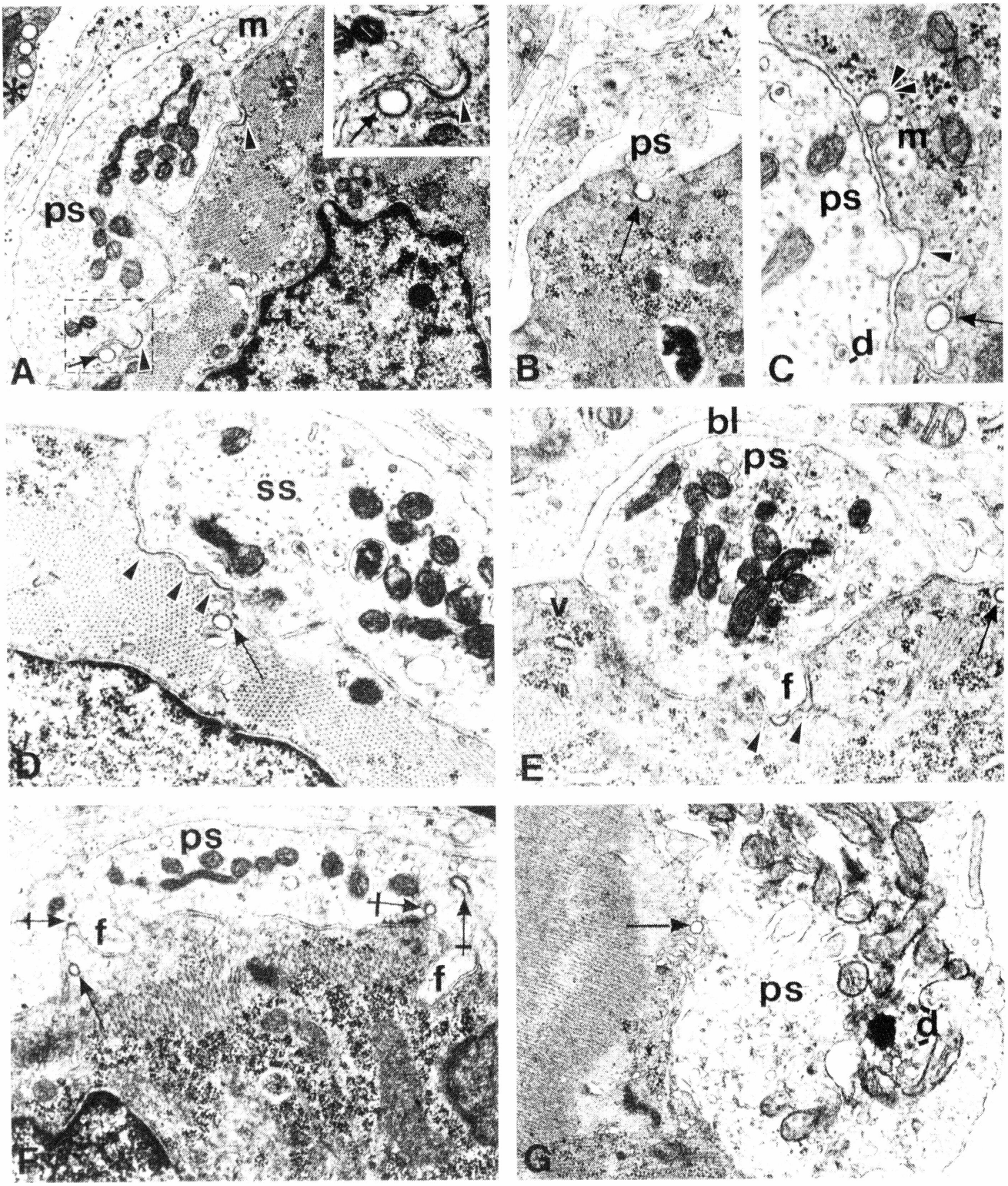



**Fig. 1**

Electron micrograph of a muscle spindle in the developing rat and schematic representation of the different sarcolemmal surfaces analyzed for the presence of coated vesicles. The equatorial region of a spindle from a 0-day rat soleus possesses three intrafusal fibres in this electron micrograph: a $bag_1$, $bag_2$ myofibre and an immature chain myotube. The $bag_2$ fibre contains two indented equatorial nuclei. Three sarcolemmal surfaces for each bag fibre are demarcated in the schematic overlay: i) the post-synaptic junctional surface, which is highlighted as a thick line, under the primary sensory axon (ps); ii) the appositional surface, a dashed line between the two bag fibres and between each bag fibre and the adjacent chain myotube which is closely sandwiched between the two bag fibres; iii) the free or non-appositional surface of remaining sarcolemma represented by a thin line. The chain myotube has two appositional (dashed line) sarcolemmal surfaces in addition to the remaining free or non-appositional sarcolemma (thin line). The chain myotube has no endings. These relationships were true for each of the three types of intrafusal fibre examined, i.e. the junctional surface in this transverse section. Original magnification of electron micrograph x 6000.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## Fig. 2

Junctional surfaces of sensory terminals from spindles of developing and adult rats.

A. 0-day rat soleus. Two coated invaginations (arrowheads) of the postsynaptic sarcolemma cover tiny axoplasmic fingers of a primary sensory axon (ps), which indent the surface of a $bag_1$ fibre in the equatorial region. A coated vesicle (arrow) has also formed immediately below the junctional surface. Three coated vesicles (*) are present in a connective tissue supporting cell. A multivesicular body (m) is seen in the axoplasm. x 25 000. Inset. Higher power view of the area outlined by dashed lines in a. illustrating the bristle-like periodicity on the cytoplasmic surface of the coated invagination and the coated vesicle underlying the sensory terminal in (a) x 25 000.

B. 0-day rat soleus. A tiny sensory terminal (ps) sits over a coated vesicle (arrow) lying under the postsynaptic sarcolemma of a $bag_2$ fibre sectioned in the equatorial region. x 30 000.

C. 0-day rat soleus. A coated vesicle (arrow), a coated pit (double arrowhead), a coated invagination (arrowhead) and a multivesicular body (m) are found beneath a primary sensory axon (ps). The latter contains a dense core vesicle (d). The coated vesicle is further than 50 nm from the postsynaptic sarcolemma; hence it would not have been included in the junctional count. x 40 000.

D. 4-day rat soleus. A series of three coated invaginations (arrowheads) molds the postsynaptic surface of a bag fibre cut transversely in the juxtaequatorial region. A coated vesicle (arrow) is found within the sarcoplasm.

A dense core vesicle is observed in the axoplasm of the secondary sensory axon (ss). x 20 000.

E. 4-day rat soleus. Two coated invaginations (arrowheads) of the postsynaptic surface of a $bag_2$ fibre are present at the bottom of a tiny axoplasmic finger (f) of a primary sensory (ps) terminal, covered by basal lamina (bl). A smooth vesicle (v) is seen underlying the sarcolemma on the left-hand portion of the figure, whereas a coated vesicle (arrow) is present on the free, non-appositional surface on the right-hand side. x 25 000.

F. 4-day rat soleus. Three coated invaginations (crossed arrows) are observed budding inwards from the presynaptic surface of a primary axon (ps) which covers a $bag_2$ fibre. A coated vesicle (arrow) is found immediately underneath the postsynaptic sarcolemma facing one of the presynaptic coated vesicles. Two axoplasmic fingers (f) are closely associated with the coated invaginations or vesicles in both the pre- and postsynaptic compartments. x 12 000.

G. Adult rat soleus. A coated vesicle (arrow) is present under the sarcolemma of a $bag_2$ fibre sectioned in the equatorial region. Dense core vesicles (d) are rare, as compared with sensory terminals in developing spindles. x 20 000.

**Fig. 3**

Junctional surfaces of motor terminals from spindles of developing and adult rats. Note that basal lamina is present between the axon terminal and intrafusal fibre.

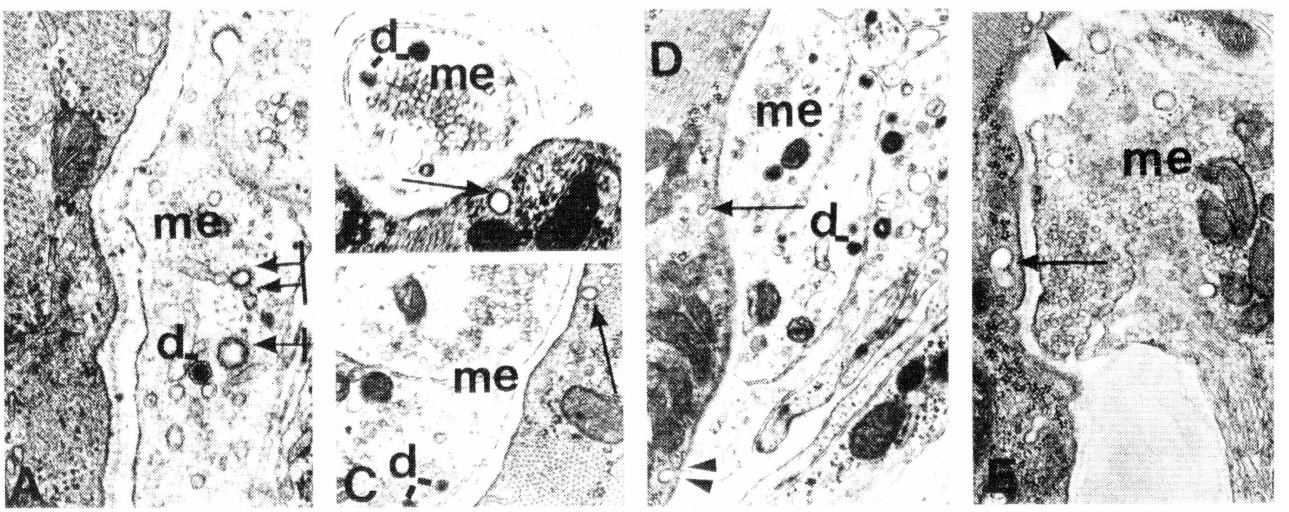
A. 4-day rat soleus. The motor ending (me) overlying a $bag_2$ fibre contains a coated vesicle (single crossed arrow), a small coated vesicle (double crossed arrows) budding from a smooth surfaced tubule, a coated invagination (upper right-hand corner) and a dense core vesicle (d). x 35 000.

B. 0-day rat soleus. A coated vesicle (arrow) lies immediately underneath the postjunctional sarcolemma of a $bag_1$ fibre. The motor ending (me) contains 2 dense core vesicles (d). x 25 000.

C. 0-day rat soleus. A coated vesicle (arrow) is observed in a $bag_2$ fibre underlying a motor ending (me). The second motor ending, which contains two dense core vesicles (d), does not make direct contact with the intrafusal fibre, hence the sarcolemmal membrane underneath would not be classified as junctional. x 15 000.

D. 4-day rat soleus. Two motor endings (me), containing both small clear vesicles and larger dense core vesicles (d), terminate on a $bag_2$ intrafusal fibre. Coated vesicles are found under the junctional membrane (arrow) and under free sarcolemma (two arrowheads). x 20 000.

E. Adult rat soleus. An invagination (arrow) is partially coated underneath a motor junction (me) on a $bag_2$ fibre. A coated vesicle (arrowhead) is seen under the free sarcolemma. A lightly stained elastic fibre is interposed between the ending and the $bag_2$ sarcoplasm at the bottom of the figure. x 19 000.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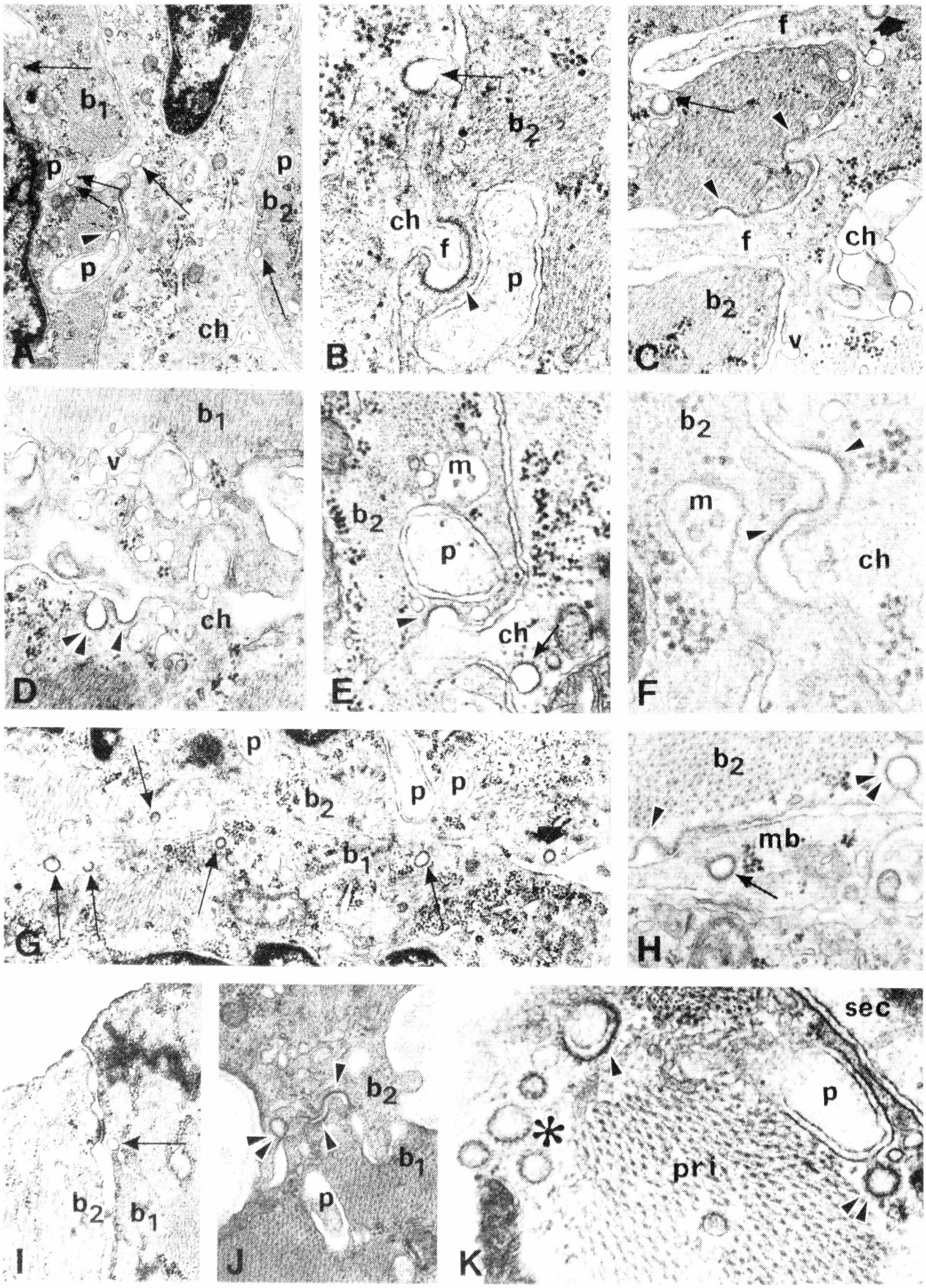
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**Fig. 4**

Appositional surfaces between constituent intra- and extrafusal muscle fibres of developing and adult rats.

A. Equatorial region of a spindle from a 0-day rat. A relatively immature chain (ch) myotube is sandwiched between a bag ($b_1$) and a $bag_2$ ($b_2$) intrafusal fibre. The appositional surfaces are infolded, resulting in finger-like processes (p). Coated vesicles (arrows) are commonly associated with the appositional surfaces, especially at these interdigitating sites. A coated invagination (arrowhead) is observed beneath one of these processes, whereas a coated pit in continuity with a smooth-surfaced tubule (double arrows) is found between two other processes. x 15 000.

B. Equatorial region of a spindle from a 0-day rat. A coated invagination (arrowhead) covers the $bag_2$ ($b_2$) sarcolemma opposite a finger-like (f) protrusion from the immature chain (ch) myotube. The light staining of the invaginated process (p) suggests it originated from the chain myotube. A coated vesicle (arrow) underlies the sarcolemma of the chain myotube. x 60 000.

C. Polar region of a spindle from a 0-day rat. Fingers (f) of the immature chain (ch) myotube penetrate within the neighboring, more mature $bag_2$ ($b_2$) intrafusal fibre. Two coated invaginations (arrowhead) and one coated vesicle (arrow) are found on the $bag_2$ sarcolemma. Several smooth-surfaced vesicles (v) are present on the chain side as well as one coated vesicle (short thick arrow). x 32 000.

D. Juxtaequatorial region of a spindle from a 0-day rat. Many smooth-surfaced vesicles (v) are found in close proximity to the appositional surfaces between a $bag_1$ ($b_1$) and a chain (ch) intrafusal fibre. A coated invagination (single arrowhead) and a forming coated pit (double arrowheads) are perched on the chain sarcolemma. x 35 000.

E. Equatorial region of a spindle from a 0-day rat. A coated invagination (arrowhead) forms on the $bag_2$ ($b_2$) fibre side, whereas a coated vesicle (arrow) appears to have invaginated from the sarcolemma of the chain (ch) myotube. Both finger-like processes (p) and multivesicular bodies (m) are associated with this appositional surface. x 40 000.

F. Juxtaequatorial region of a spindle from a 0-day rat. Coated invaginations (arrowheads) abut on either side of the appositional surface - "face-to-face" between a $bag_2$ ($b_2$) and a chain (ch) myofibre. A multivesicular body (m) is adjacent to the coated invaginations. x 100 000.

G. Equatorial region of a spindle from a 4-day rat. Many coated vesicles (arrows) line both the $bag_2$ ($b_2$) and $bag_1$ ($b_1$) appositional surfaces. However, fingerlike processes (p) probably from the $bag_1$ fibre, are observed only on the $bag_2$ side. A coated vesicle (short thick arrow) is actually underlying the free sarcolemma of the $bag_2$ fibre. x 20 000.

H. Equatorial region of a spindle from a 4-day rat. A coated invagination (single arrowhead) and a coated pit (double arrowheads) lie underneath the sarcolemma of a $bag_2$ ($b_2$) intrafusal fibre, adjacent to a myoblast (mb), which contains a coated vesicle (arrow). x 40 000.

I. Equatorial region of a spindle from an adult rat. A coated vesicle (arrow) underlies the $bag_1$ ($b_1$) side of the appositional surface adjacent to the neighboring $bag_2$ intrafusal fibre. x 20 000.

J. Equatorial region of a spindle from an adult rat. A coated pit (double arrowheads) and two coated invaginations -"face-to-face" (single arrowheads) line the appositional surface between a $bag_2$ ($b_2$) and a $bag_1$ ($b_1$) fibre. A cytoplasmic process (p) is present within the $bag_1$ sarcoplasm. x 30 000.

K. Extrafusal muscle from a 0-day rat. Many coated vesicles, a coated invagination (single arrowhead) and a coated pit (double arrowheads) are closely associated with the appositional surface between a primary (pri) and a secondary (sec) myofibre. The bristle-like coating of the coated pit, as it buds off a cytoplasmic process (p), as well as that of the coated vesicles (*) can be seen to advantage. x 60 000. @@@

described in cells from a variety of tissues, such as fibroblasts and endocrine cells as well as muscle fibres of extrafusal skeletal muscle (Willingham and Pastan 1985). First, their diameter was of the same order of magnitude, ranging from 50 to 200 nm (Nevorotin 1980; Willingham and Pastan 1985). Second, they exhibited the same bristle or "spokes on a wheel" coating of clathrin observed for coated vesicles in other tissues (Fine and Ockleford 1985). Third, other organelles known to be functionally associated with the coated vesicles, such as multivesicular bodies, lysosomes and endosomes (Brown *et al.* 1983; Geuze and Kramer 1974), were also visible in developing and mature spindles in the vicinity of coated organelles. A fourth morphological similarity between the coated vesicles in the spindle and those described in other tissues is their polymorphic character. The coated vesicles in muscle spindles exhibited a variety of shapes, such as coated pits, invaginations, etc. This polymorphy of the coated vesicle profiles, a common finding in many tissues, strongly suggests that they represented various transitional forms of coated membrane (Willingham and Pastan 1985).

Based on the morphological similarities between coated vesicles of the muscle spindle and other tissues, it can be argued that coated vesicles of intrafusal fibres are also functionally similar. They would, therefore, participate in receptor-mediated endocytosis as occurs in the uptake of macroglobulins by extrafusal fibres (Haye *et al.* 1986), rather than with secretory exocytosis which also takes place in the extrafusal muscle system (Benson *et al.* 1985). Exocytotic functions for coated vesicles in skeletal muscle fibres have been suggested, because they contain acetylcholinesterase (Benson *et al.* 1985). However, it is unlikely that the high incidence of coated vesicles under sensory terminals is due to exocytosis of this enzyme because there is little or no acetylcholinesterase beneath sensory terminals (Kucera *et al.* 1978). In addition, acetylcholinesterase in anchored in the basal lamina, which is also absent between intrafusal muscle fibres and sensory nerve terminals.

The functional significance of coated vesicles in extrafusal muscle also remains unclear. Heuser and Reese (1973) postulated that the recovery of vesicular membrane involves the formation of coated vesicles found at the neuromuscular junction (Zacks and Saito 1969). Therefore, one would expect an increased incidence of coated vesicles in axonal profiles following nerve stimulation, but this does not occur (Tremblay *et al.* 1986). The suggested role of coated vesicles in the transport of acetylcholine receptors has similarly been questioned (Matthew-Bellinger and Salpeter 1983, Bursztajn 1984, Fine and Ockleford 1985).

The short half-life and infrequent observation of coated vesicles on some surfaces posed sampling difficulties. Accordingly, we analyzed a great number of micrographs, so that the infrequent occurrence of the coated vesicles did not bias the results. In addition, only coated vesicles within 50 nm of the intrafusal fibre sarcolemma were counted because coated vesicles lose their clathrin coat as they move away from the plasma membrane. The calculated incidence of coated vesicles associated with sensory and motor terminals is probably an underestimation, due to the exclusion of vesicles located immediately adjacent to the junction. These coated vesicles may have moved laterally away from the junctional area under what we have termed the "free" surface of the sarcolemma. The incidence of coated vesicles associated with appositional membrane might also have been underestimated because those coated vesicles that were present on interdigitating fingers of adjoining membrane, detached from the surface under investigation, were not counted. Lastly, comparisons of the incidence of coated vesicles at different sites would be inexact if their half-life varied with their position or if their preservation varied with the rapidity of fixation. Data do not yet exist on the half-life of coated vesicles at different sites either for intrafusal or extrafusal muscle fibres.

*Differential distribution of coated organelles in muscle spindles*. The distribution of coated vesicles in both immature and adult spindles was site specific. They were distributed under the intrafusal fibre sarcolemma at both sensory and motor terminals, but their incidence was greater under the sensory endings. In addition, they were also found more frequently at the sites of apposition of neighboring intrafusal fibres compared to free, non-appositional sarcolemma. Regardless of the site, the incidence of coated vesicles was much higher in developing than in adult muscle spindles. The differential distribution of coated vesicles in spindles suggests that their high frequency in developing tissue is a specific event related to tissue development. The incidence of coated vesicles was highest at sites where two different cells interfaced, such as sensory or motor axons and intrafusal muscle fibres or between different types of intrafusal muscle fibre. The difference in frequency of coated vesicles in developing and adult spindles suggests that the functional role of coated vesicles is maximal during spindle formation and differentiation. The high incidence of coated vesicles at primary sensory compared to motor endings on the same fibre at the same time suggests that the occurrence of coated vesicles at neuromuscular junctions is specific for the type of axon, rather than being related to junctional specializations *per se*. The difference in distribution of the coated vesicles at appositional versus free sites of intrafusal fibres further supports the view that coated vesicles in spindles play specific roles at distinct sites along the intrafusal fibre.

*Are coated vesicles involved in neurotrophic communication?* Several authors have suggested that the morphogenetic effect of the primary afferent on the differentiation of muscle spindles (Zelená 1976, 1983) might be mediated by neurotrophically active substances released from sensory endings and capable of influencing gene expression. Smith (1971) proposed that the small dense cored vesicles of sensory nerve terminals may contain neurotrophic factors bound as a protein and available for release. Landon (1972) speculated that the coated vesicles associated with the sarcolemma opposite the area of sensory terminal contact represent the most likely candidate for the vehicle of transfer of such factors. Zelená and Soukup (1973) suggested that coated vesicles and invaginations found at both synaptic membranes were associated with the uptake of macromolecules from the synaptic cleft.

The present report is the first study to quantify the incidence of coated vesicles at different sites in muscle spindles and to test the hypothesis that coated vesicles may mediate information transfer within spindles. The greater incidence of these organelles in developing versus adult spindles of the rat is consistent with the release of neurotrophic agents from sensory terminals, because afferents are known to exert their greatest ontogenetic influence at early, critical stages of development of muscle spindles. Spindles disintegrate in muscles that are deprived of sensory innervation in early development, but not in adult animals (Zelená 1957, Kucera and Walro 1987).

In contrast, the role of motor innervation is less significant because spindles can differentiate in the absence of motor innervation (Zelená and Soukup 1973). Indeed, our finding that more coated organelles are present under sensory than under motor terminals is concordant with a greater influence of sensory versus motor innervation on the developing muscle spindle. The exact nature and mechanism of the neurotrophic influence of the sensory axon has not been explicitly delineated (Zelená 1976).

This greater incidence of coated vesicles under sensory as opposed to motor terminals suggests that axonal trophic factors would be released in greater quantity at equatorial rather than polar regions. Sensory axons are also more closely apposed to intrafusal fibres than motor axons, particularly where they send interdigitations within the intrafusal fibre sarcoplasm. Coated vesicles are closely associated with these axoplasmic fingers. A gradient of chemical information, similar to the morphogenetic induction of a retinoid acid gradient (Eichele and Thaller 1987) may be established originating immediately under the primary sensory axon and decreasing to either side. The decreased incidence of coated vesicles under sensory terminals in the juxtaequatorial region is consistent with this hypothesis. This information gradient may underlie the regional morphological and functional differences along the length of intrafusal fibres with increasing distance from the equator (Kucera *et al.* 1978).

The two-fold frequency of coated vesicles under primary sensory axons versus motor terminals might reflect the existence of several classes or subpopulations of coated vesicles - containing different neurotrophic factors. Both acetylcholine receptors and acetylcholinesterase are co-localized within coated vesicles (Fine and Ockleford 1985); thus it is not inconceivable that coated vesicles may also contain several neurotrophic factors. The presence of coated vesicles under arriving sensory axons, particularly at finger-like projection sites, may also be a mechanism for nerve-muscle recognition to establish appropriate neuromuscular specificity (Landmesser 1980). Although no axonal fingers penetrate the intrafusal fibre sarcoplasm under motor terminals, probably due to the intervening basal lamina, coated vesicles are nevertheless found with high frequency and could participate in internalization of neurotrophic agents. Coated vesicles may also be involved in nerve-muscle recognition at motor terminals overlying extrafusal fibres as coated vesicles are common in developing nerve-muscle contacts (Matthew-Bellinger and Salpeter 1983, Bursztajn 1984). At all of these junctional zones, chemical signals could be read by both the axon and muscle cell, perhaps coded by means of a concentration gradient, which would enable them to recognize each other and establish a feedback system. This would entail a two-way communication system; indeed, we observed coated vesicles under both the pre- and postsynaptic membranes of both sensory and motor terminals. The presence of coated vesicles at sensory and motor terminals of adult intrafusal fibres, although much less common than in developing spindles, would argue that they continue to participate in mature muscle as mediators of a continuing neurotrophic input. Differentiation of adult spindles affects their morphological and histochemical characteristics (Kucera 1980, Kucera and Walro 1987).

The high incidence of coated vesicles at appositional sites between intrafusal fibres suggests a role of coated vesicles as structural correlates of information transfer among the developing intrafusal fibres. Such intercellular communication would be most important for the muscle spindle during its formation, when the fibres are intimately connected by interlocking pseudopodial, filiform processes (Landon 1972). These projections mainly invaginate the surface of the more mature fibre (Landon 1972; Milburn 1973 and Fig. 4g, this study), consistent with the view that it is the more mature fibre ($bag_2$) that is directing the formation of the secondary generation myotubes ($bag_1$ and chain), in a manner analogous to the extrafusal system (Harris 1981). Indeed, coated vesicles are common at appositional surfaces between developing extrafusal muscle fibres, where it was postulated that

they may be involved in the fibre fusion process (Lipton and Konigsberg 1972). Our observations on the high incidence of coated vesicles at appositional surfaces of intrafusal fibres, which do not fuse, indicates that this is not necessarily the case. Rather, our finding that coated vesicles are also common at appositional surfaces between primary and secondary extrafusal myofibres suggests that they may be involved in communication between developing muscle fibres as we have proposed for intrafusal fibres. The primary and secondary intrafusal myotubes separate during development, and lose appositional surface as they mature. The ability of the muscle fibres to exist as independent entities attests to decreased importance of information exchange between muscle fibres concomitant with a decreased incidence of coated vesicles in adulthood. However, even in the adult, appositional surfaces exist to a limited degree. Coated vesicles there are a common finding, suggesting some communication continues to be of importance.

Variations on the distribution pattern of the coated vesicles that we have observed in muscle spindles suggest how intercellular communication between developing intrafusal fibres may be operating. The presence of coated vesicles which directly face one another at appositional surfaces of intrafusal fibres is highly suggestive of information exchanged between developing fibres rather than transfer solely from one fibre to another. In turn, a series of coated pits underneath the plasma membrane suggests that there may be a precipitation site or centre of crystallization from which coated vesicles are pushed or slid along. Coated vesicles are more numerous at points where interdigitating fingers of one intrafusal fibre penetrated into a neighbour than at linear portions of appositional membrane. This would indicate that the fingers participate actively in receptor-mediated endocytosis, as would be expected if they were sites involving exchange of chemical information rather than being merely anchorage points. The apposing membranes between intrafusal fibres are not tightly connected by desmosomal or other forms of junctions, hence, they could range extensively (Corjava *et al.* 1967; Mayr 1969). Such an environment would foster exchange of information during development; thus, concentration of coated vesicles at interlocking sites is predictable.

**Conclusions.**

Coated vesicles are differentially distributed in developing muscle spindles. Their high incidence at sensory endings supports the view that they mediate neurotrophic interactions between afferents and intrafusal fibres which may be important in early nerve-muscle recognition and subsequently in the differentiation of intrafusal fibres. In addition, their high frequency under appositional rather than free surfaces of developing intrafusal fibres suggests that they play a role in communication among intrafusal fibres exhibiting different stages of maturity. The nature of the substances that mediate the neurotrophic influence and the pathways which are followed by the trophic material within the cell after its incorporation remain to be ascertained.

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