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Scanning and light microscopic study of age changes at a neuromuscular junction in the mouse

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Summary

From previous work, it appears that synaptic transmission is well preserved at aging mouse neuromuscular junctions despite profound ultrastructural changes. Scanning and light microscopy have been used to determine whether expansion or sprouting of nerve terminals or postsynaptic reorganization play a role in this apparent compensatory mechanism. The number and length of nerve terminal branches in the extensor digitorum longus of young (7 months) and old (29 months) mice were studied with a combined silver–cholinesterase method. In aged animals, there were increases in nerve terminal length and number of intrasynaptic branches, with no change in muscle fibre diameter or numbers of axons entering the junction. Neither collateral sprouting nor collateral innervation, hallmarks of partial denervation, were present.

Motor endplates visualized by scanning electron microscopy appeared as slightly elevated, elliptical plateaux ('raised areas') with smooth surfaces into which the synaptic clefts were etched. In the aged endplates more than in young endplates, the primary clefts were often interrupted by narrow short outpouchings approximately perpendicular to the long axis of the primary cleft. In addition, oval primary cleft islets were more frequent and there was increased randomness and branching of secondary clefts.

Both light and scanning microscopy gave concordant quantitative evidence that nerve terminals and the underlying postsynaptic cleft are longer and more branched in aged mice. The increased length of synaptic nerve terminal approximately balances the loss of girth previously reported leaving nerve terminal volume unchanged. The observed expansion of the synaptic area in the aged neuromuscular junction may be compensatory, preserving neuromuscular function. The data also point to plasticity of adult neuromuscular synaptic structure.

Introduction

There is indirect evidence for ongoing structural remodelling of the neuromuscular junction (NMJ) in mature animals (Barker & Ip, 1966; Wernig *et al.*, 1980) but aging of the NMJ has received scant attention from this point of view. In fact, there are intriguing

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findings indicating that the organization of pre- and postsynaptic structures change with age. For instance, the average number of myelinated branches of the same terminal axon entering a junction has been reported to increase with age in cats (Tuffery, 1971), and the cholinesterase staining pattern is frequently broken or abnormal at junctions of old rats (Gutmann & Hanzlikova, 1965). Also, profound ultrastructural changes, such as reduction in synaptic vesicle density, were seen in aging junctions (Fahim & Robbins, 1982), contrasting with the finding in the same mouse strain that nerve terminals in aged animals release as much or more transmitter than terminals in mature animals (Robbins & Kelly, 1981). These observations raise the possibility that loss of synaptic vesicles in aging synapses may be compensated for by other structural changes such as an increase in synaptic contact area. Thus, detailed morphologic examination of the aging NMJ may be of interest as a particular case of synaptic plasticity.

In the present study, the three-dimensional organization of NMJs at the fine structural level was examined by scanning electron microscopy (SEM). The SEM technique gives high-resolution, quantifiable information about the surface appearance of the postsynaptic apparatus of NMJs not obtainable by other methods. To our knowledge, this is the first report of quantitative use of SEM at the NMJ. However, with the present method, SEM did not provide non-selective statistically valid data on *pre*synaptic changes. Therefore, in parallel with SEM, a light microscopic (LM) combined silver–cholinesterase histochemical staining method (Pecot-Dechavassine *et al.*, 1979) was used to delineate quantitatively the terminals at all junctions in a non-selective manner. In general, there was good agreement between the SEM and LM measures of junctional plasticity with aging.

Methods

Animals

In all experiments, male 'CBF-1' mice [$CB6F_1$(BALB/cNNia × c57BL/6NNia)] obtained from Charles River Laboratories were used, either 7 months (n = 5–7) or 29 months (n = 6) old. These particular ages were chosen for functional reasons discussed elsewhere (Fahim & Robbins, 1982).

Scanning electron microscopy (SEM)

The animals were anaesthetized with methoxyflurane (Pitman Moore) and the extensor digitorum longus (EDL) and soleus (SOL) muscles were exposed, care being taken to preserve the blood supply. EDL and SOL muscles were fixed *in situ* for 15 min by the drop-wise application of 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. After excision, the muscles were cut into longitudinal bundles and immersed in fresh fixative for 2 h. The muscle bundles were next washed for 1 h in 0.1 M phosphate buffer and stained for 2.5 min for cholinesterase according to the method of Tennyson *et al.* (1977). Areas of fibre bundles that contained cholinesterase-positive endplates were dissected free, postfixed for 1 h in unbuffered 2% osmium tetroxide, and rinsed in distilled water. The specimens were then treated with 8 N HCl for about 30 min at 60° C to remove tissue extrinsic to the muscle fibre (Desaki & Uehara, 1981), including

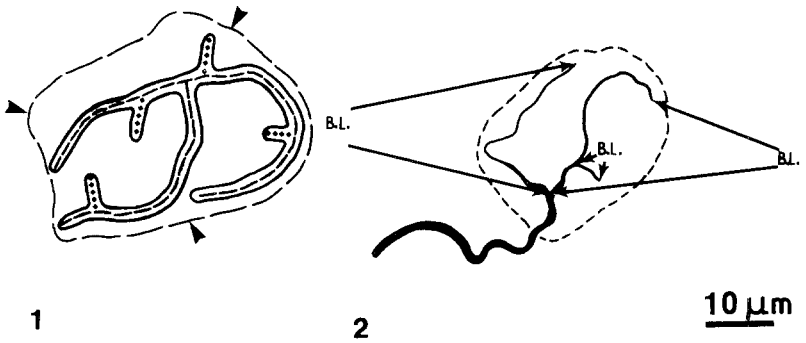


Fig. 1. Tracing of a scanning electron micrograph of the motor endplate in a 7-month EDL muscle, illustrating many of the parameters that were quantitatively measured: 1. 'raised area', the outermost dashed line, marked with arrowheads; 2. primary cleft perimeter, traced in a solid line; 3. primary cleft area, i.e. area within the perimeter; 4. number of primary cleft branches, each shown as a dashed or dotted line within the primary cleft perimeter; and 5. primary cleft length, i.e. sum of lengths of all branches.

Fig. 2. Camera lucida drawing of a silver–cholinesterase stained endplate from a 7-month EDL muscle, showing parameters measured quantitatively. The cholinesterase-positive area is contained within the dashed line. B.L., nerve branch length (ends of each branch indicated by arrows).

nerve terminals in most cases. The preparations were then washed in three changes of distilled water over 30 min, then dehydrated through a graded series of ethanol, critical point dried, sputter-coated with gold, and examined in an ETEC autoscan SEM at 20 kV.

Scanning electron micrographs at a magnification of 2500 were used in the morphometric analysis. A Neumonics Model 1224 electronic digitizer with a manually operated cursor was used to determine distances and areas in micrographs. Quantitative measurements, mostly illustrated in Fig. 1, included: 1. muscle fibre diameter; 2. 'raised area', measured as the integrated area within the outermost border of specialized endplate membrane (see Results for description); 3. total primary cleft length per junction; 4. total primary cleft perimeter per junction; 5. total primary cleft area per junction; and 6. number of primary cleft branches. Every junction encountered was used if at least 85% of the 'raised area' could be seen from the scanned surface. Scanning micrographs at a magnification of 10 000 were used to classify qualitatively the pattern of secondary folds as either 'more' or 'less' random. All microscopy and measurements were done double blind.

The degree of muscle contraction could affect the parameters but in fact, both young and aged muscle fibres were contracted to a similar degree. In 10 measurable samples of each age group, the mean (±S.E.) number of sarcomeres per 100 µm longitudinal length was 88 ± 2 in young and 86 ± 2 in aged muscle. In addition, there was no significant correlation between primary cleft perimeter and number of sarcomeres per unit length in either population.

Light microscopy (LM)

Intramuscular nerves and endplates were visualized by the combined silver–cholinesterase method of Pecot-Dechavassine *et al.* (1979). Experimental preparations were observed with a

Zeiss 100 × oil immersion objective, and camera lucida drawings were made of endplates and nerve terminal branches. Quantitative observations made from drawings (illustrated in Fig. 2) included: 1. muscle fibre diameter; 2. number of axons entering the endplate; 3. number of branches; and 4. lengths of nerve terminal branches within the cholinesterase-positive endplate region. The cholinesterase-positive area is reported only for purposes of comparison because the staining pattern was more diffuse than that obtained with other methods (e.g. Robbins *et al.*, 1980).

The measurements of muscle fibres and endplates were liable to error through shrinkage and deformation of the material during preparation (e.g. compare fibre diameters in Tables 1 and 2). Therefore, the quantitative data were mainly used for purposes of comparison between young and aged specimens. Student's *t*-test or the Mann-Whitney test (for non-normally distributed data) were used for statistical evaluation, and data are expressed as mean ± standard deviation.

Table 1. Scanning electron microscopic morphometric data* in young and old mouse extensor digitorum longus muscle.

Parameters	*Young*	*Old*	*% increase*
Number of fibres (number of animals)	68 (5)	58 (6)	
Fibre diameter (μm)	23.0 ± 5.9	23.1 ± 6.6	—
'Raised area'† (μm^2)	221.3 ± 89.5	243.4 ± 86.1	—
Total primary cleft length (μm)	58.1 ± 20.9	73.0 ± 20.2	26%‡
Total primary cleft perimeter (μm)	116.2 ± 40.2	147.3 ± 47.1	27%‡
Total primary cleft area (μm^2)	81.3 ± 39.7	100.2 ± 39.8	23%‡
Number of primary cleft branches	7.1 ± 2.6	15.0 ± 6.0	111%‡

*Results are expressed as mean (±S.D.) number per junction.
†See text for description of 'raised area'.
‡Significant difference between young and old ($P < 0.01$).

Table 2. Measurements from combined silver and cholinesterase stains of young and old endplates.

Parameters	*Young*	*Old*	*% change*
Number of fibres (number of animals)	77 (7)	69 (6)	
Muscle weights (mg)	15.6 ± 3.2	13.1 ± 1.3	—
Fibre diameter (μm)	34.8 ± 6.9	33.5 ± 6.5	—
Length of nerve terminals (μm)*	89.4 ± 37.8	141.2 ± 39.9	58%†
Acetylcholinesterase stained area (μm^2)	907.4 ± 371.2	1043.4 ± 361.7	—
Number of nerve terminal branches	5.3 ± 2.6	9.9 ± 3.7	87%†

*Results are expressed as mean ±S.D. per junction. In each animal, 8–15 fibres were examined.
†Significant ($P < 0.01$).

Results

ANIMALS AND MUSCLES

There was no indication of weight loss or muscle atrophy with age. Body weight and EDL muscle weights and fibre diameters were not significantly changed with age (Tables 1 and 2) and, as noted previously (Fahim & Robbins, 1982), there was no loss of muscle fibres. Body weights were 36.5 ± 5.4 g in young and 35.4 ± 3.9 g in old mice. The strain-characteristic pathology and criteria for exclusion of animals are described elsewhere (Fahim & Robbins, 1982). Except where indicated, all results described here are from the EDL muscle.

SCANNING ELECTRON MICROSCOPY

General description

Both young and aged endplates appeared as slightly elevated elliptical areas ('raised areas') that were smooth compared to the surrounding sarcolemmal surface (arrowheads, Figs. 3–5, 7, 8). The latter showed a repetitive pattern of circumferential corrugations corresponding to underlying sarcomeres (Figs. 3, 5, 8, 9). Primary clefts, usually denuded of nerve terminals with the present method, generally occurred within the raised area. The interior surface of the raised area was often deformed by bulges forming islands between the primary clefts (asterisks, Figs. 3, 6). The outermost primary clefts often formed a discontinuous ellipse (Fig. 4), frequently with short side branches ('s', Figs. 3, 4). In another common configuration, small, concave oval zones emanated from or were adjacent to the basic primary cleft (e.g. black arrows in Figs. 4, 8 and white arrow in Fig. 9). The floor of the primary cleft was formed by the branched or parallel ridges of secondary folds.

Comparison of endplates from young and old mice

The most striking difference between young and aged endplates was the arrangement of primary cleft branches and small concavities. In the aged endplates far more than in young animals, the primary clefts appeared partially segmented by indenting narrow ridges that were often perpendicular to the primary cleft long axis (Figs. 7–9), often giving a ragged appearance to the edges of the primary cleft (white arrow, Fig. 7). Between these ridges were small, oval concavities lined with secondary folds (black arrow in Fig. 8 and white arrow in Fig. 9). Frequently U-shaped primary cleft concavities appeared in the aged endplate (black arrow, Fig. 9).

Other differences between young and old endplates included: 1. an increased number of shorter primary clefts (compare Figs. 3–5 with 7–9); and 2. an increased branching and randomness of secondary cleft orientation (compare Fig. 6 with Fig. 10).

Routine SEM of nerve terminals was not possible in our hands, but in several cases remnants of the nerve terminals were preserved. In every such instance, the terminals entirely filled the primary clefts, including the small side branches (Figs. 5, 9) which were more prevalent in aging NMJs.

Morphometric SEM data

The 'raised area' was similar in young and aged junctions, but the total primary cleft length was significantly greater (Table 1). As evident from the histogram (Fig. 11), the increase in total cleft length was entirely due to a much larger number of short primary cleft branches. Furthermore, most of the endplates in aged mice exhibited this change: e.g. of the aged endplates, 79% had more than three primary cleft branches shorter than 2.5 μm compared to 21% in young endplates. Indeed, the number of primary cleft branches more than doubled with age (Table 1). Since primary cleft length, perimeter and area all increased in parallel, it appears that major shape changes did not occur with age (Table 1). Finally, in old animals (using double blind observer) secondary clefts were classified as 'more' randomly oriented in 52% of the population whereas only 23% of secondary clefts in young animals were so classified.

As expected from other studies using cholinesterase staining (e.g. Harris & Ribchester, 1979) the total primary cleft area per endplate, measured with SEM, was significantly correlated with muscle fibre diameter in both young and old mice. Thus, despite a larger primary cleft area in the aged NMJ, the relationship between fibre size and postsynaptic area was preserved.

In a parallel study on a small number (15 each, young and old) of soleus muscle fibres, the changes with age were similar to those in the EDL muscle. For instance, the number of nerve terminal branches was again increased more than two-fold with age.

Figs. 3 & 4. Scanning electron micrographs of endplates of 7-month EDL muscles. Note smooth-surfaced 'raised area' (borders marked with arrowheads in these and subsequent figures); side-branches (s) of primary clefts; insular bulges in raised areas (asterisks); and isolated oval primary cleft region (arrow). The white spots on the muscle surface are residual precipitate of cholinesterase stain, and the hole in the supporting film (Fig. 3) is part of a Nucleopore filter. Scale bar: 10 μm.

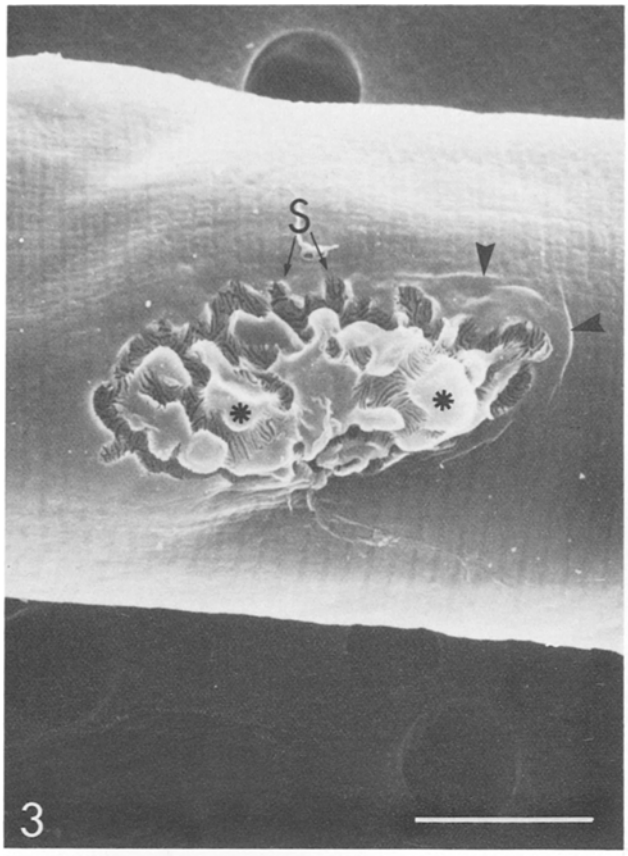
Fig. 5. A scanning electron micrograph of EDL muscle from 7-month mouse. Note remnants of intrasynaptic nerve terminals (clear curved arrows). Scale bar: 10 μm.

Fig. 6. Scanning electron micrograph of 7-month EDL muscle at higher magnification, showing the arrangement of secondary folds (clear arrows) and insular bulge in raised area (asterisk). Scale bar: 1 μm.

Figs. 7 & 8. Scanning electron micrographs of 29-month EDL muscles illustrating U-shaped (short arrow) and concave (long arrow) primary cleft regions. Scale bar: 10 μm.

Fig. 9. Scanning electron micrograph of 29-month EDL muscle showing nerve terminals attached to the outpouching regions of primary cleft (clear curved arrow) which characterized the aged neuromuscular junction. The short black arrow denotes the U-shaped primary cleft concavity. Scale bar: 10 μm.

Fig. 10. Scanning electron micrograph of 29-month EDL muscle at higher magnification, showing the increased randomness of secondary fold orientation (clear arrow) compared to that in Fig. 6. Scale bar: 1 μm.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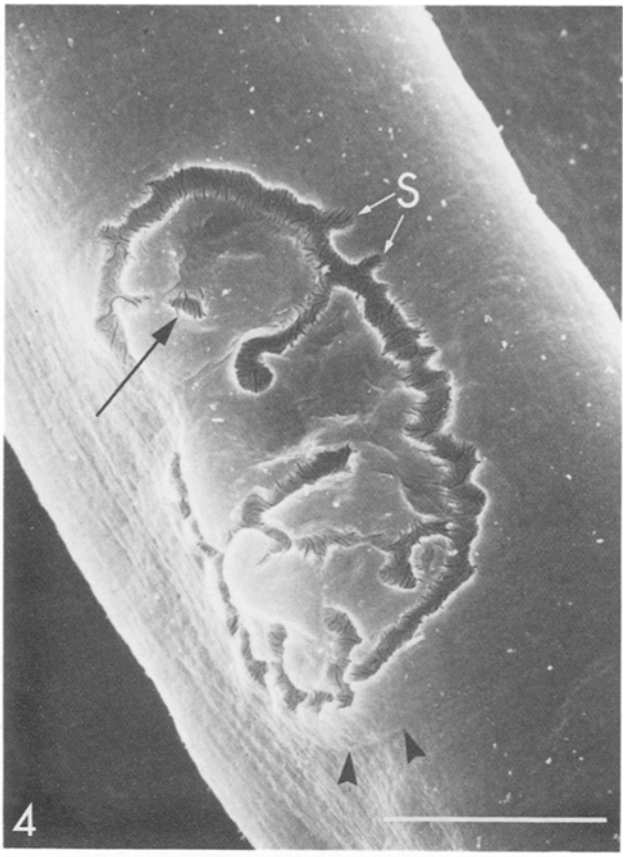
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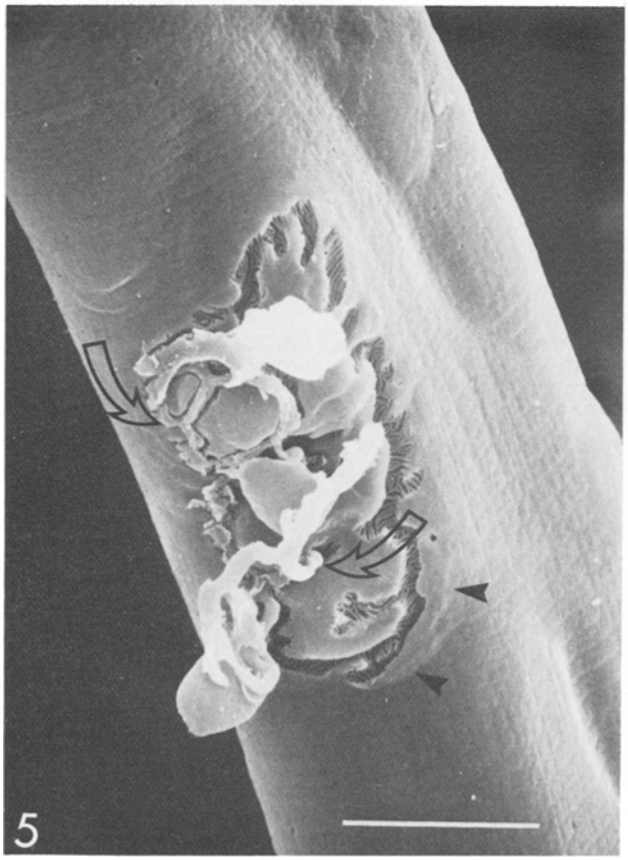
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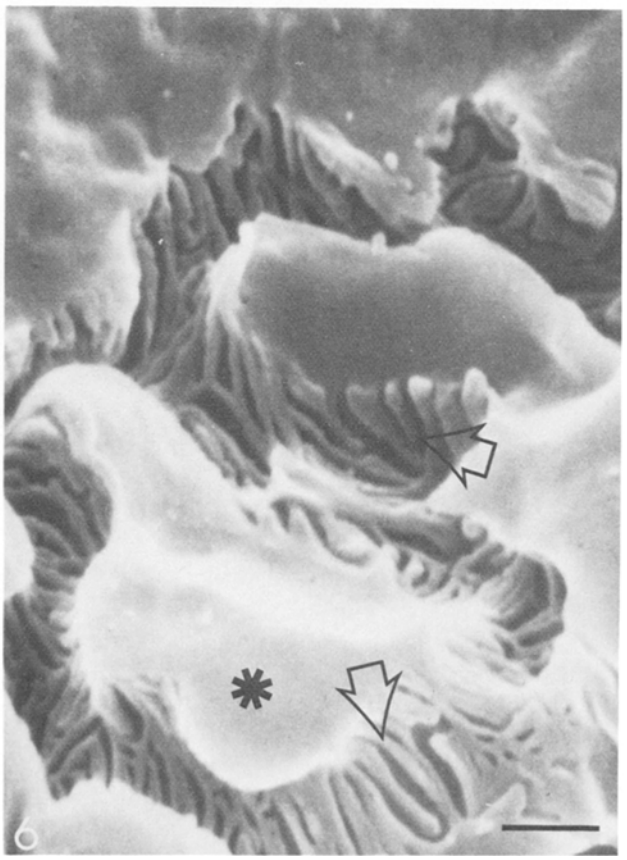
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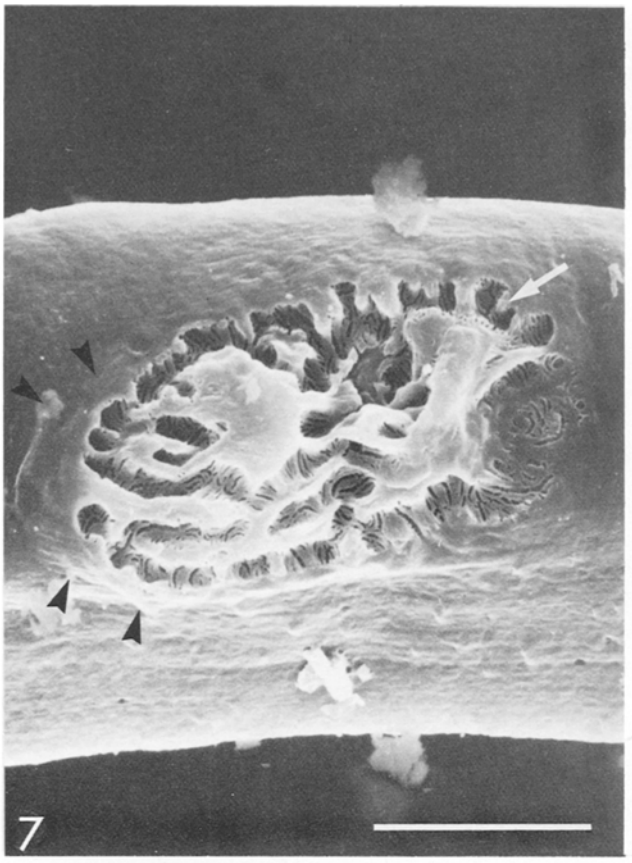
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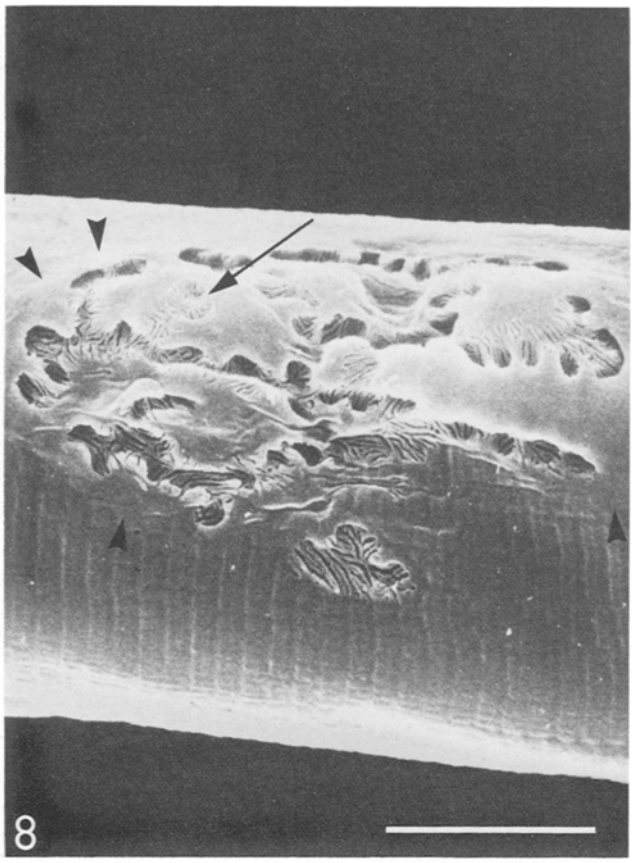
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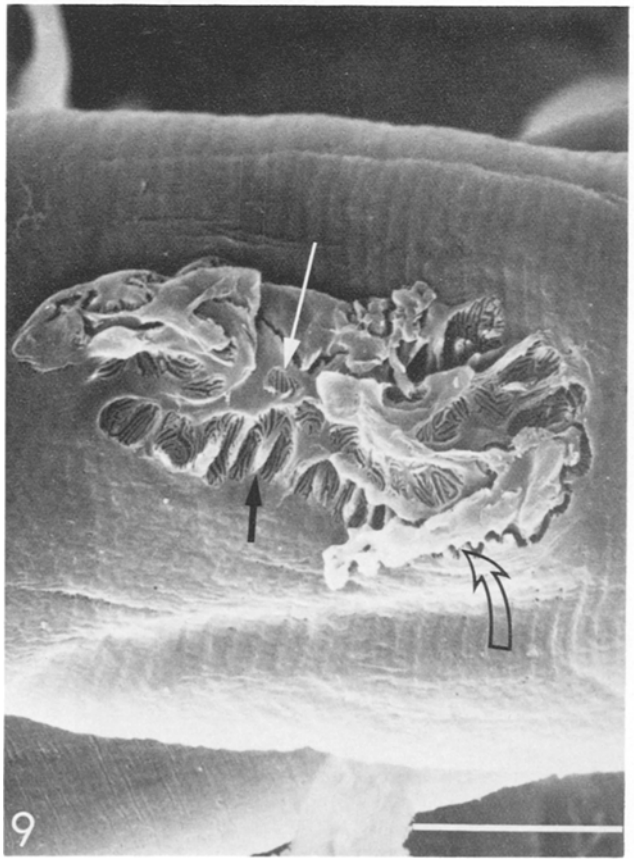
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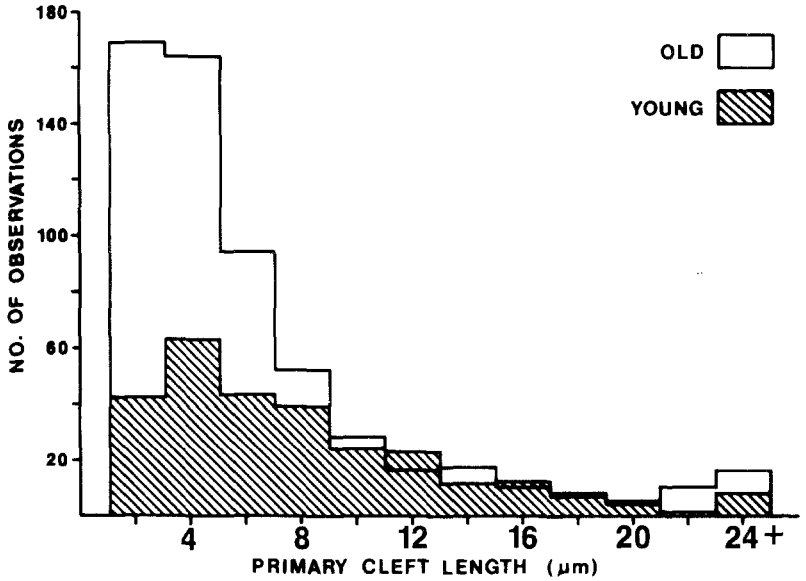

Fig. 11. Histogram showing the distribution of primary cleft branch lengths in young and old EDL muscles. Data are from 39 young and 39 old neuromuscular junctions. There is a marked increase in number of short primary cleft branches in old compared to young endplates, but no difference in lengths of branches over 9 μm long.

LIGHT MICROSCOPY

Nerve branching outside the junctional area

Combined silver-cholinesterase staining was used to determine the location and extent of branching of nerve terminals at junctional regions. The percentage of junctions innervated with two axons observed in aged animals (10%) was not significantly different from that of young animals (9%). Only one junction innervated with three axons was observed, in an old EDL muscle. 'Duplex' endplates (two myelinated branches of the same axon forming two closely spaced junctions on the same fibre, cf. Tuffery, 1971) were absent. Lastly, collateral branching of preterminal axons to endplates on other muscle fibres nearby, was not observed in either young or old muscles.

Nerve branching within the junctional area

There were two major differences in the results from young and aged mice (Table 2). First, in aged mice, nearly twice as many nerve terminal branches occupied the same junctional area as in young animals (compare Figs. 12 and 13). In terms of the population of fibres, more than 42% of aged endplates had more than ten branches compared to only 4% in young mice. The percentages of endplates with more than six branches was 78% in aged and 21% in young junctions. Thus, most of the aged endplates exhibited increasing branching to some degree. Second, the sum of the branch lengths per

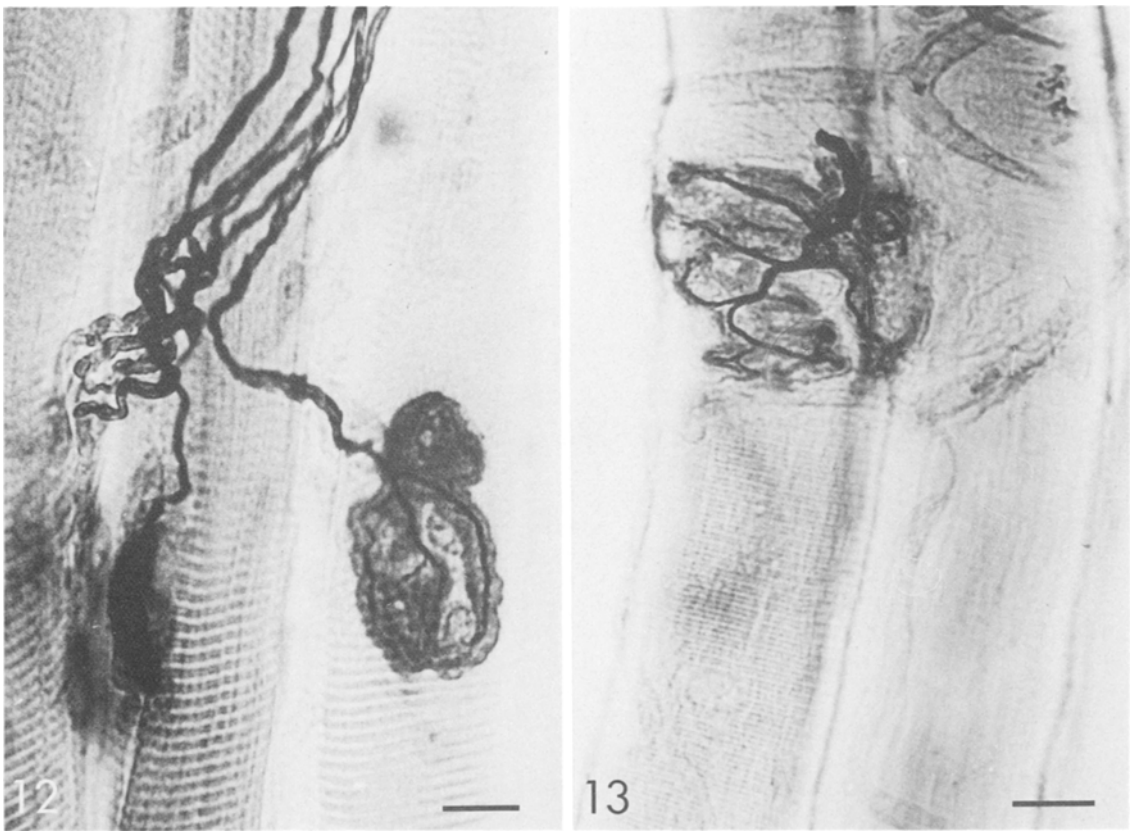

Fig. 12. Combined silver–cholinesterase stained endplates showing typical picture of an endplate from a 7-month EDL muscle. Scale bar: 10 μm.

Fig. 13. Neuromuscular junction in 29-month mouse after silver–cholinesterase staining. Note the increased number of terminal branches occupying the junctional area compared to that found in young animals (Fig. 12). Scale bar: 10 μm.

endplate was also increased at aged NMJs (Table 2). As in the corresponding SEM results, this finding accrued from an increase in short branches: in the aged endplates, 54% had nerve branches shorter than 3.5 μm compared to 21% in young endplates.

Discussion

Intrasynaptic nerve terminal branches in motor endplates of aged (29 months) EDL muscle fibres were more numerous than in young (7 months) mice. Correspondingly, on the postsynaptic side, there were significant increases in total primary cleft length, due mainly to numerous small outpouchings, which increased primary cleft perimeter, area and branching. The increase in outpouching and in oval regions of the primary cleft

probably account for the observation that aged rat NMJs show more areas of discrete acetylcholine receptor density than do young animals (Courtney & Steinbach, 1981). The circumference of the smooth 'raised area' characteristic of the NMJs in SEM, did not enlarge with age but, since the primary cleft area increased by only about 20 μm^2, the corresponding reduction in 'raised area' would be small. From transmission electron microscopy (TEM) information, this region probably contains nuclei, mitochondria and sarcoplasmic reticulum.

In general, there was good agreement between SEM and LM measures of junctional changes with aging. In young EDL, after taking account of shrinkage (one third more in SEM than after glutaraldehyde fixation alone), the shrinkage-corrected total nerve terminal length from LM measurements (2/3 of 89.4 = 59.6 μm) was almost identical to total primary cleft length measured from SEM (58.1 μm). However, in aged animals, the shrinkage-corrected LM nerve terminal lengths (2/3 of 141.1 = 94.0 μm) were 30% longer than the primary cleft length from SEM (73.0 μm). Several explanations for this discrepancy can be offered: 1. Because of the relatively large standard deviation in the measurements, this apparent difference may be in part due to sampling; 2. junctional tissue elements which may take silver stain, such as Schwann cell processes (Holmes & Young, 1942), may increase with age and become inadvertently included in the LM measurements of nerve terminal length; 3. nerve terminals may grow outside the primary clefts; and 4. primary clefts may be shared by more than one nerve terminal. Alternative 3 appears unlikely, since no nerve sprouts were seen outside primary clefts in a systematic TEM study (Fahim & Robbins, 1982). The same study gives some support to alternative 4 in that 10% of random cross-sections of aged (compared to 4% in young) junctions showed a double nerve terminal occupancy of a primary cleft.

Nerve sprouting from nodes of Ranvier or from nerve terminals to extrajunctional sites, or the presence of collateral innervation, the hallmarks of partial denervation (Brown *et al.*, 1981), were absent in EDL muscles from aged mice. Moreover, SEM of denervated rat soleus (unpublished data) frequently shows flattened primary clefts, but these were not present in the aged muscles studied here. The absence of signs of partial denervation confirms previous TEM (Fahim & Robbins, 1982), biochemical (Courtney & Steinbach, 1981) and physiologic data (Robbins & Kelly, 1981).

The absence of increase with age in numbers of preterminal axons per NMJ, as reported here, is consistent with other recent studies in rats (Tweedle & Stephens, 1981; Courtney & Steinbach, 1981) in which the number of such axons was found to increase during early development up until a few months of age, reaching a plateau thereafter. Tuffery (1971), however, reported more preterminal axons in aged than in young cats, but developmental rather than age changes may account for his findings. A similar developmental explanation may apply to another report (Pestronk *et al.*, 1980) of apparently increased *intra*synaptic nerve terminal branching, in which 10- and 18-month rat junctions were compared to those of 3-month (immature) animals. In the same paper, it was observed that junctions of 28-month rats showed *diminished* intrasynaptic nerve branching compared to younger animals in contrast to the present

study. However, in that study, the 28-month rats exhibited substantial weight loss and muscle atrophy, so that the results could reflect changes secondary to atrophy or extramuscular disease. Other problems in using standard laboratory rats in aging studies have been discussed elsewhere (Fahim & Robbins, 1982). Indeed, in the diaphragm muscle of the Fischer 544 strain, which does not show weight loss at 28 months, the number of intrasynaptic terminal branches per endplate is increased compared to that in 10-month rats (Smith & Rosenheimer, 1982). In the present study, the increase in number of intrasynaptic nerve branches occurred in the absence of weight loss or muscle atrophy. Further work will be necessary to determine whether this process of terminal expansion is continuous between 7 and 29 months or is only a late event.

A number of conclusions can be drawn about the aged NMJ by combining results from this and other investigations. First, the previously reported 55% diminution in nerve terminal cross-sectional area (Fahim & Robbins, 1982) combined with the 58% increase in total length of nerve terminal branches reported here, indicate that nerve terminal volume (area x length) was approximately preserved in aged NMJs. Second, physiologic studies in the same strain of aged mice revealed no deficit in transmitter release (Robbins & Kelly, 1981) despite substantial reduction in numbers of synaptic vesicles and mitochondria (Fahim & Robbins, 1982). Thus, the growth of functional nerve terminals within the junctional region may serve to compensate for possibly reduced transmitter release per site by providing more release sites. Alternatively, given that presynaptic 'active zones' are in register with secondary clefts (Heuser & Reese, 1973), the increased complexity and branching of such clefts in aged muscles may indicate that more presynaptic active zones are also present.

The growth in synaptic area reported here resembles that occurring in neuromuscular synaptic development, in which there is co-ordinated enlargement and expansion of the presynaptic nerve terminal, increasing transmitter release as the muscle fibre enlarges (review, Grinnell & Herrera, 1981). Matching of nerve terminal area and appropriate output also occurs in natural or experimental dual innervation. Similar mechanisms may maintain neuromuscular transmission with aging.

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