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Changes of macromolecular organizations in nonjunctional sarcolemmas after cross-innervation— a study of fast- and slow-twitch muscle fibres in rats

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ABSTRACT

The purpose of the present study was to analyse the changes of macromolecular organizations in nonjunctional sarcolemmas of different types of skeletal muscle fibres after cross-innervation. In normal rats the mean density of square arrays (6 nm particles organized in orthogonal arrays) was 9.02 / μm^2 for the nonjunctional sarcolemmas of fast-twitch extensor digitorum longus (control EDL, CE) muscle fibres and 0.34 / μm^2 for the nonjunctional sarcolemmas of slow-twitch soleus (control SOL, CS) muscle fibres. After cross-innervation between the fast-twitch EDL and slow-twitch SOL muscle fibres by slow and fast muscle nerves respectively for three months, the mean density was 0.45/ μm^2 for the nonjunctional sarcolemmas of the operated EDL (OE) and 8.3/ μm^2 for the nonjunctional sarcolemmas of the operated SOL (OS), This indicates that the cross-innervation causes a reciprocal transformation of the number and distribution of such macromolecular organizations in the electrically excitable nonjunctional sarcolemmas.

Key words: *Sarcolemma, square array, cross-innervation, freeze-fracturing, rat(Wistar).*

INTRODUCTION

Mammalian skeletal muscle fibres are distinguished into several types according

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to different classification schemes. Each type of muscle fibres has a set of specific characteristics. One well-known way to classify muscle fibres is to divide them into two broad types: slow-twitch or type I fibres, and fast-twitch or type II fibres. The former normally appears red and the later appears white. They possess wide differences with respect to physiological, biochemical, and morphological phenotypic characteristics, such as isometric contraction, the contraction speed with the time to peak tension and the time to half relaxation[l], the myofibrillar ATPase histochemical reaction[2], LDH isozyme[3], Z-band width[4], the mitochondrial density and sarcoplasmic reticulum features[5], and the postsynaptic folds at neuromuscular junctions[5, 6]. However, as hormones, activities or innervations imposed on fibres change, the fibre types might be rendered transformable accordingly[7-10]. It is because the motor neuronal influence has been considered as the most important factor in determining muscle fibre types that cross-innervation techniques are frequently employed by scientists to study the plasticity of mammalian skeletal muscle fibres. Extensor digitorum longus (EDL) contains relatively pure fast-twitch muscle fibres (type II fibres) while soleus (SOL) consists of a majority of slow-twitch muscle fibres (type I fibres)[6, 11, 12]. Both are calf muscles located close to each other in the hind legs of rats. By cross-innervation investigators are able to see at different levels to what extent one type of specific muscle fibres is transformed to its counterpart. Our previous studies were mostly focused on the transformed ultrastructural features of skeletal muscle fibres or neuromuscular junctions[l, 4, 6]; however, as freeze-fracture technique developed during the last few decades, it was applied in investigating the intramembrane molecular organizations which were exposed during cleaving process in various species[13, 14], and it offered a possible approach to realize and measure specific internal macromolecular architectures quantitatively. The earliest report that we could find using freeze-fracture techniques to analyze the split membranes from samples of fast-or slow-twitch muscle fibres is by Ellisman and his colleagues[15, 16], whose study revealed that in the electrically excitable sarcolemmas of fast-twitch muscle fibres, there was a kind of characteristic macromolecular organizations consisting of a variable number of 6 nm particles arraying orderly in an orthogonal (square) fashion, which distributed mainly over a specific portion of 0.5 mm to 2 mm departing from the neuromuscular junctions; while in samples of slow-twitch muscle fibres, little or no square arrays were identified in the split sarcolemmas. Therefore these findings add a new parameter that enables the two classes of fibres to be distinguished in the membranous molecular level. Because these orderly aggregated particles are arranged in electrically excitable membranes in close proximity to the neuromuscular junction, they are supposed to be responsible for the electrogenic properties of sarcolemmas[15, 16].

Ellisman and his colleagues also let slow muscles be reinnervated by fast nerves and one year after the operation they obtained the results that square arrays emerged in sarcolemmas[17].

In our experiments, we crossly innervated EDL muscle fibres and SOL muscle

fibres by soleus nerve and EDL nerve respectively. Three months later, animals were anesthetized and segments of control EDL(CE), control soleus(CS), operated EDL(OE), and operated soleus(OS) muscle fibre bundles were dissected at a distance of 0.5 - 2 mm from the neuromuscular junctions, fractured and replicated on a Balzers D-400 freeze-etching device. Our aim is to see if cross-innervation changes the number and distribution of macromolecular organizations in the electrically excitable nonjunctional sarcolemmas of fast and slow twitch fibers and how early the transformation can take place.

MATERIALS AND METHODS

Male Wistar rats weighing about 200 g were employed. Operations were performed under tribromoethanol anesthesia (200 mg/kg i. p.). One hind leg of the animals was scissored and scalpeled to expose both the SOL and the EDL muscles, the other one served as control. SOL muscles were crossly innervated by EDL nerves or EDL muscles were crossly innervated by SOL nerves to make ectopic neuromuscular junctions at a distance of 3-5 mm from the original ofies. The nerves of SOL or EDL muscles themselves were cut off as long as possible or they were implanted into other unrelated muscles to avoid re-innervating the original SOL/EDL muscles.

Three months after operation, the animals were sacrificed to provide 4 groups of samples, (1) the operated SOL(OS), (2) the control SOL(CS), (3) the operated EDL(OE), and (4) the control EDL(CE). A total of 30 animals were used in our experiments, half to provide CS /OS samples and the other half to provide CE /OE samples. Small bundles containing 5-10 individual striated muscle fibres were dissected, fixed in 4% glutaraldehyde in phosphate buffer (pH 7.4) for 4 h at 4℃. Rinsed thoroughly, muscles were stained by an improved Csillik method[6, 18] to reveal the motor end-plate. Selected portions of fibres at a distance of 0.5 -2 mm from the neuromuscular junctions (NMJs) were trimmed, immersed in 30% glycerol saline (cryoprotectant) for 1 h, then carefully placed onto brass mesh specimen supports, quickly frozen in liquid nitrogen, and fractured at -110℃ on a Balzers D- 400 freeze etching device. In our experiments, bundles of mammalian muscle fibres were not cleaved randomly, instead they were adjusted to be stroked by the knife to split the membranes along the myofibrillar axis. In this way a large area of splitting membranes was likely to be produced. Replicas were prepared by shadowing with a platinum- carbon gun. The thickness of platinum coat was regulated by an interlocked quartz crystal thin film monitor. After rinsing with chromic acid, replicas were then floated in distilled water and transferred to brass meshes, examined and photographed at a high voltage of 80 KV with an OPTON EM-902 electron microscope at original magnifications of 20,000 or 30,000. An approximately similar area of protoplasmic fracture face (PF) vs ectoplasmic fracture face (EF) was selected at random for statistical analysis. Photographs of replicas at a final enlargement of × 60,000 were used for counting particle densities and those at a higher enlargement (× 200,000) were used for measuring particle diameters. For each particle, an edge to edge measurement across its center was made twice and we take the average of the two measurements as one datum.

We used the density of square arrays (mean density) as a criterion indicating the changes of macromolecular architecture of the nonjunctional sarcolemmas.

RESULTS

At low magnifications, the membrane appeared smooth, marked with irregular cross-fractured openings (Fig 1, 2, 4, 5, 6, 8), which were hypothesized by Rash and Ellisman to be T-system tubules or cortical vesicles[15]. In the replicated P face of EDL fibre sarcolemmas, a large population of 10 to 20 nm particles were-found to

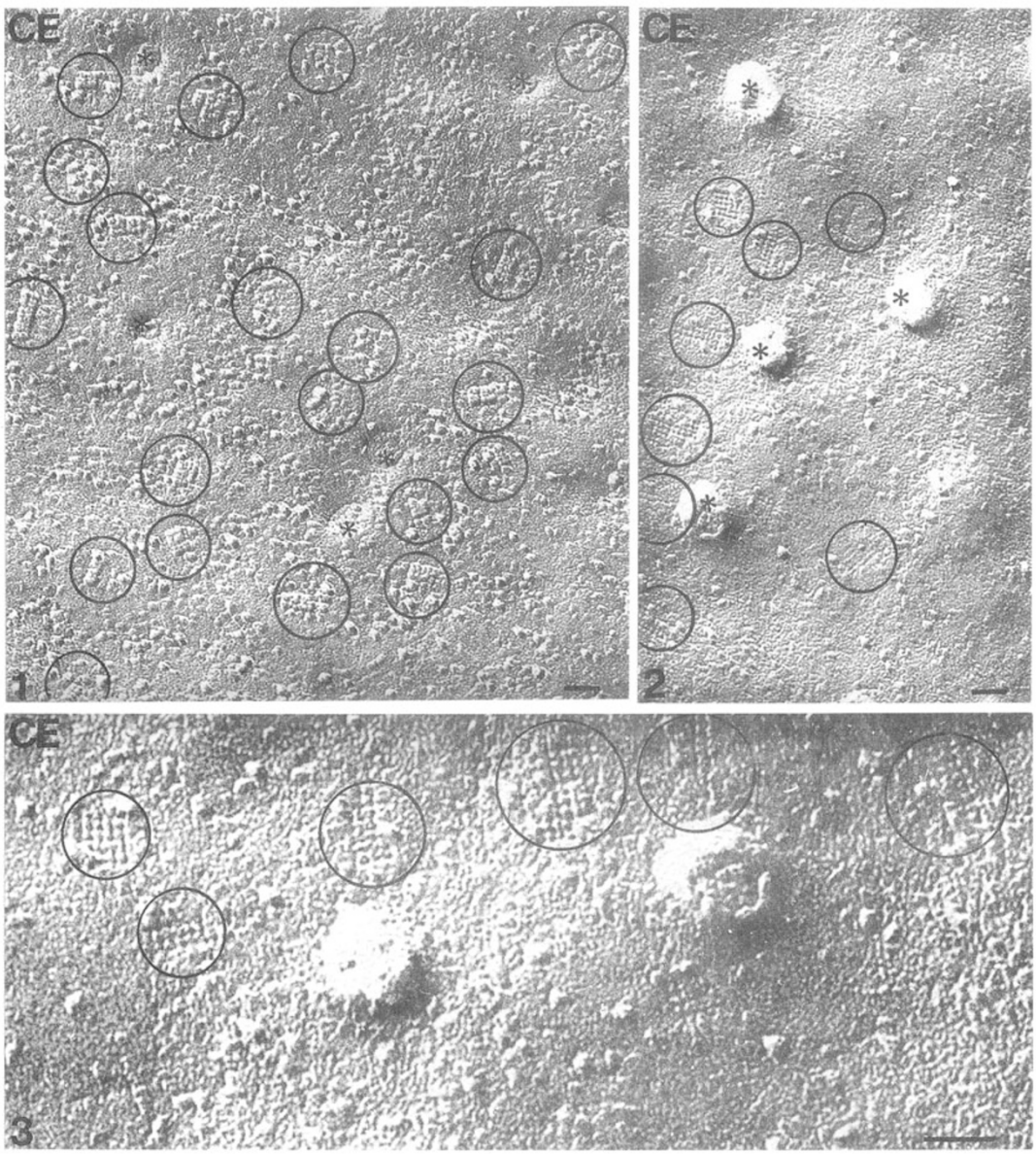

Fig 1. Protoplasmic (P) face of replicas from samples of control EDL(CE) skeletal muscle fibres in a portion of 0.5 to 2 mm apart from the neuromuscular junctions(NMJs) showing that distinct square arrays(circulated) were densely distributed over this specific area of sarcolemmas. Asterisks: Openings × 120,000 Bar=50 nm

Fig 2. Ectoplasmic (E) face of replicas from samples of control EDL(CE) skeletal muscle fibres in a portion of 0.5 to 2 mm apart from the neuromuscular junctions(NMJs) showing that subunits were pits alike and tightly packed in a square array(circulated). Asterisks: Openings × 120,000 Bar=50 nm

Fig 3. Fig 2 at a higher magnification showing that subunits in a square array(circulated) were arrayed orderly. CE: control EDL × 240,000 Bar=50 nm

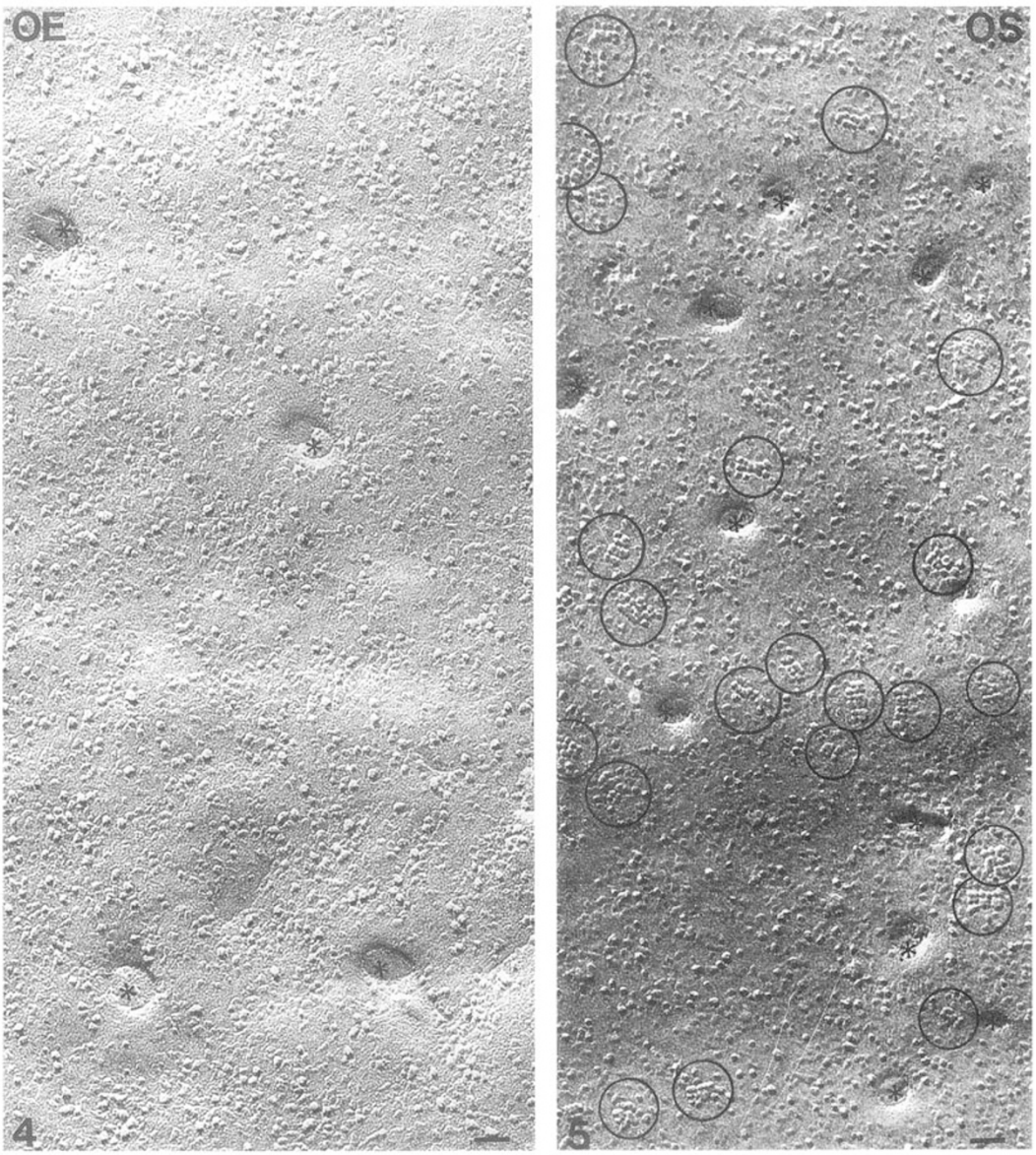

Fig 4. Protoplasmic (P) face of replicas from samples of operated EDL (OE) skeletal muscle fibres in a portion of 0.5 to 2mm apart from the neuromuscular junctions(NMJs), no apparent square arrays were detected. Asterisks: Openings × 120,000 Bar=50 nm

Fig 5. Protoplasmic (P) face of replicas from samples of operated soleus(OS) skeletal muscle fibres in a portion of 0.5 to 2 mm apart from the neuromuscular junctions(NMJs) showing patchily distributed square arrays(circulated) in this area of sarcolemmas. Asterisks: Openings × 120,000 Bar=50 nm

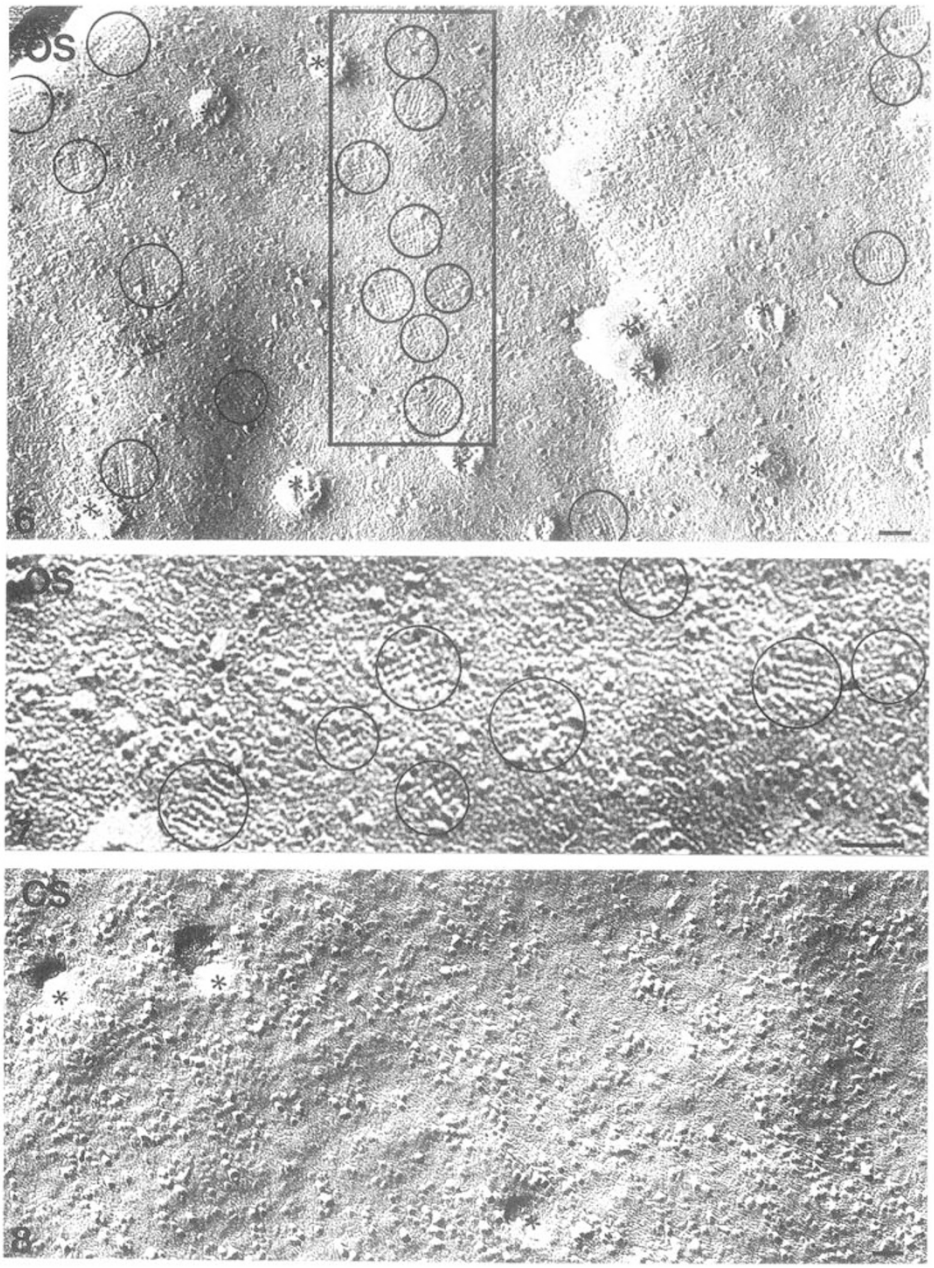

Fig 6. Ectoplasmic (E) face of replicas from samples of operated soleus(OS) skeletal muscle fibres in a portion of 0.5 to 2 mm apart from the neuromuscular junctions(NMJs) showing distinct square arrays(circulated) in this area of sarcolemmas. Asterisks: Openings × 120,000 Bar=50 nm

Fig 7. A portion of Fig 6 (rectangle) was enlarged to show that subunits(pits) in a square array(circulated) were regularly arranged, similar to those in Fig 3 in subuni t/array sizes and arraying fashions. OS: Operated soleus × 240,000 Bar=50 nm

Fig 8. Ectoplasmic (E) face of replicas from samples of control soleus(CS) skeletal muscle fibres in a portion of 0.5 to 2 mm apart from the neuromuscular junctions(NMJs) showing none apparent square arrays in this area of sarcolemmas. Asterisks: Openings × 120,000 Bar=50 nm

distribute randomly over the background (Fig 1). Whereas the small 6 - 10 nnl particles were observed to be packed tightly and arranged regularly in square arrays (Fig 1), distributing over a very confined range, 0.5 -2 mm away of the NMJs. Sometimes single or double small particles were examined in tile replicas, nevertheless, at least 4 particles laying orderly in an array fashion were considered as one "square array" in our statistical analysis. The number of replicated subunits in one array varied tremendously from a single or double row of particles to more than 30 ones. We have measured ten large square arrays with a length of 50 - 80 nm and a width of 35 - 50 nm. In our study, we utilized 5 to 6 square microns as one unit in counting particle numbers and a total area of 124.6 square microns was randomly selected from samples of replicated P face EDL fibres for statistical analysis. These particles aggregated highly patchily, and the value of each aggregate varied significantly. The array density was 8.37 /μm^2. However, none or very few arrays existed in the replicas of samples from soleus fibres; over an area of 300 square microns, only about 120 arrays were observed. While in the operated soleus (OS), a very similar kind of macromolecular organizations were discovered in the same portion of nonjunctional sarcolemmas, resembling square arrays in EDL fibres in particle size and arraying pattern (Fig 5). We extensively examined such distinct arrays from different samples and found that the distribution was very nonuniform. The density was 9.17/μm^2, based on the calculation from 133.9 square microns split membranes, randomfly selected from a large number of OS sarcolemmatic replicas. Contrary, in OE fibres, square arrays became dramatically sparse or absent (Fig 4). As we counted 300.9 square microns replicas, it revealed that the density dropped to as low as 0.77 /μm^2.

In the complementary surface of the split membrane (EF pits), we also observed well- organized macromolecular architectures, which extensively distributed over the EDL fibres, similarizing in density and appearance to those in PF (Fig 2, 3). Such characteristic arrays were variable in sizes. Large ones possessed approximately 30 - 40 subunits. 192.6 square micron replicas of samples from EDL fibres were counted, revealing a density of 9.67 /μm^2; while in the operated EDL fibres distinct square arrays almost absent from the examined sarcolemmas. The density was 0.12 /μm^2 for 330.4 square micron replicas analyzed. Similarly, in the replicated E face of soleus sarcolemmas, no apparent particle (pits) assemblies were detected during our examinations and very few were found in 312.7 square microns photographs (Fig 8). However, in the operated soleus fibres a vast number of arrays emerged, patchily distributed over the nonjunctional sarcolemmas (Fig 6, 7). Its density was 7.43/μm^2 (159.7 square microns). As we observed and measured the particle (pit) sizes, we found that those in PF replicas were relatively larger than those in EF replicas (see between Fig 1, 2). That may derive from the thickness difference of replicas between PF particles and EF pits, which causes different depths of focus during the process of photographing. Based on the following observations and analysis, we tended to consider arrays in PF and EF replicas as 2 opposite images reflecting

a single type of macromolecules. (1) They exist in the 2 complementary faces of split membranes while no other kind of regularly arranged particle assemblies are scanned over the two opposite planes; (2) the arrays on both faces distribute in a patchy pattern over a very confined range of sarcolemmas in EDL fibres; (3) subunits of the macromolecules are arranged similarly in an orthogonal or square fashion on both faces; (4) the densities of arrays on both faces are similar as observed from samples of EDL and SOL fibres; (5) the arrays on both faces respond conformably to muscle fibre transformation by nerves. We hope that experiments in progress using label- fracture techniques[13, 14, 19, 20] will give a better elucidation of these macromolecules.

Below we tabulate the data taken from two complementary faces together and summarize the number and distribution of square arrays in the nonjunctional sarcolemmas of EDL, SOL, OE, and OS fibres (Tab 1).

Tab 1. The distribution of square arrays in nonjunctional sarcolemmas of EDL and SOL fibres

	Density μm^2 of PF	Density μm^2 of EF	Mean density μm^2 of PF & EF
CE	8.37 (124.6)	9.67 (192.6)	9.02
OE	0.77 (300.9)	0.12 (330.4)	0.45
CS	0.40 (302.2)	0.28 (312.7)	0.34
OS	9.17 (133.9)	7.43 (159.7)	8.30

Explanations: (1) CE, control EDL fibres; OE, operated EDL fibres; CS, control SOL fibres; OS, operated SOL fibres; PF, protoplasmic face; EF, ectoplasmic face; NMJs, neuromuscular junctions
(2) Total areas in μm^2 are included in parentheses.

DISCUSSION

Our studies verify Ellisman and Rash's previous reports[15, 16] that the number and distribution of square arrays perhaps represented a quantitatively measurable parameter which might be of general significance in defining the membrane molecular differences. Based on these differences, two populations of fibres are distinguished at macromolecular levels. One population is Type II (fast-twitch) fibres which are associated with the nonrandomly distributed square arrays in nonjunctional sarcolemmas and the other population is type I (slow-twitch) fibres which possess virtually none (or very sparsely dispersed) distinct arrays. According to Ellisman and Rash's counts, there were none square arrays immediately surrounding the NMJs of fast-twitch muscle fibres, and several points in the nonjunctional sarcolemmas at different distances from the NMJs had varialle densities, i.e, at 0.5

mm, 50 /μm^2 ; at 1 mm, 15-30 /μm^2; at 2 mm, 10-20 μm^2; in addition, very sparse arrays scattered over the nonjunctional sarcolemmas more than 2 mm away of the NMTs[15, 16]. Our data are apparently lower than any of the above number. That is probably due to the following causes, (1) we let bundles of muscle fibres rather than individual fibres to be freeze-fractured, therefore it is impossible to align the NMJ of each single muscle fibre at exactly the same point, so some samples taken for statistical analysis might happen to be within the portion of 0 - 0.5 mm departing from the NMJs or beyond 2 mm away of NMJs; and (2) since the distribution of square arrays is highly asymmetrical, densities between the above 4 points might be of great differences from each of the above number obtained by point count. As we collect data from a large area of split sarolemmas, roughly 0.5 - 2 mm departing from NMJs, it is likely to get a lower mean density.

The significance of our results is that we firstly apply the cross-innervation technique to study the quantitative membrane properties of fast vs slow muscle fibre. We have chosen a series of time intervals from 3 months to 1 year after the operation and we find that 3 months is enough for the transformation. According to our observations, samples from EDL fibres(CE) possess a mean density of 9.02/μm^2, as operated (OE), it decreases to 0.45/μm^2, while samples from SOL fibres(CS) have a mean density of 0.34 /μm^2, which increases to 8.30 /μm^2 after operation (OS). Therefore, densities of CE and CS are almost identical with OS and OE respectively, as shown by Tab 1. Owing to the fact that each of EDL or SOL muscle comprises of a small portion of different fibres[6, 11, 12], the transformation of these membrane macromolecular components can be regarded as almost complete. The above results offer a new criterion for examining the effects of cross-innervation at molecular levels.

As our previous studies showed, motor neuron influenced muscle fibre type characteristics through impulse activities, i.e. regular tonic activity at low frequency overruled intermittent phasic activity at high frequency[21], and doubly innervated slow-twitch muscle fibres retained their original neuromuscular junction type, whereas the doubly innervated fast-twitch muscle fibres underwent a dramatic transformation of their neuromuscular junctions from the fast-twitch type to slow-twitch one, and none noticeable intermediate types were found[6]. It means that when SOL and EDL nerves coexist on the same muscle, the impulse activity of SOL nerve predominates over that of EDL nerves. While in the case of cross-innervation, the morphology of postsynaptic folds in rat soleus was transformed completely, similarizing to normal fast-twitch muscl fibres[22]. However, we are still not certain whether motor innervation controls the number and distribution of square arrays via impulse activities or through some specific neurotrophic factors. In order to have an insight into the molecular mechanism underlying fibre type transformations more precisely, we have also operated two groups of animals by double-innervation to see if the outcome conforms with our previous observations.

Sarcolemmas of skeletal muscle fibres are grossly differentiate into two portions,

the chemically excitable postsynaptic folds at the site of neuromuscular junctions (NMJs) and the electrically excitable nonjunctional sarcolemmas surrounding NMJs. The coupling of excitation- and contraction is triggered by the release of ACh (acetylcholine), which activates nicotinic ACh-receptors (nAChRs) to produce transient membrane depolarization or endplate potential-EPP. If EPP reaches the "threshold", action potentials are produced and propagated over sarcolemmas by continual activation of adjacent Na^+ channels. As impulses propagate into T-system tubules, they trigger the release of Ca^{2+} from sarcoplasmic reticulums and initiate the contraction of sarcomeres. Of all membrane integral proteins or known receptors, the structural and functional properties of nicotinic AChRs in the chemically excitable postsynaptic folds were most extensively studied during the last few decades[23, 24]. It was revealed that changes of innervation patterns or presynaptic input altered the number and distribution of nAChRs or influenced the channel properties of AChRs[25, 26]. Our current studies demonstrate that in the initial area of electrically excitable sarcolemmas, the number and distribution of square arrays are subject to change by motor innervations. This result indicates that apart from square arrays, ion channels, and Na^+ / K^+ ATPase which more directly correspond with the functional membrane properties of nonjunctional sarcolemmas perhaps also undergo transformation due to changing innervation patterns. Should it come true, the change of various significant macromolecular membrane components in structures, numbers and distributions over either chemically excitable postsynaptic folds or electrically excitable sarcolemmas may well explain the functional and phenotypic transformation of EDL and SOL muscles by cross- innervations.

Another question remaining open for discussion is the putative chemical nature and functional role of square arrays in membranes. Since square arrays are absent from the area immediately surrounding the NMJs which are electrically unexcitable but occupy the initial area of electrically excitable sarcolemmas[15, 16], it is feasible to hypothesize them to mediate the molecular events of excitability and impulse propagation. In fact, those characteristic particle assemblies are not uniquely bound with fast-twitch muscle fibres, instead, they distribute over a vast number of different cells in different organs of various species, i.e. the intestine epithelium[27], muscle fibres[15, 16, 28], brain astrocytes[29-31], and kidney collecting tubules[32]. And they are sensitive to chemical agents or cohl injuries[30]. Though we are still not certain of their chemical compositions, they may be of general functional significance tor specific membranes.

Conclusion: The asymmetrical distribution of square arrays in a definite range of nonjunctional sarcolemmas represents a new parameter in defining and discriminating two populations of skeletal muscle fibres, fast-twitch and slow-twitch muscle fibres at molecular levels. In accordance with the muscle fibre type transformation by nerves, the number and distribution of square arrays are rendered to be changed completely.

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