Morphological peculiarities of neuromuscular junctions among different fiber types: Effect of exercise

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Abstract

The aim of our research was to examine whether there are differences in the morphology of neuromuscular junctions of different types of muscle fibers in rodents, and after their adaptation to six weeks endurance exercise training. After 5-day acclimation, Wistar rats were subjected to run with the speed 35 m/min during 6 week, 5 days per week and the training volume reached 60 min per day. Muscle samples for ultrastructural studies were fixed, dehydrated and embedded in Epon-812. Ultra-thin sections were cut from longitudinally and transversely oriented blocs, using 4 blocks from each animal. The area of axon terminals on fast- twitch fibers is 1.5 time large ($p<0.001$) and the perimeter of terminals is 1.7 time large in comparison with slow- twitch oxidative fibers ($p<0.001$) in control group. There are correlation between cross-sectional area of different muscle fibers and length of axon terminals ($r=0.72$), between cross-sectional area and with of axon terminal ($r=-0.62$), and between turnover rate of contractile proteins and length of axon terminal ($r=0.75$). Fast remodeling of synapse on oxidative and oxidative-glycolytic muscle fibers during endurance training seems to guarantees the intensive renewal of the structures of muscle fibers with higher oxidative capacity.
Key Words: Neuromuscular junction, slow- and fast-twitch fibers, endurance exercise, remodeling of synapses.

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The neuromuscular junction (NMJ) is the interface between the motor nervous system and the skeletal muscle fibers. NMJ has shown consists of three cellular components, the nerve terminal, the postsynaptic muscle fiber, and the perisynaptic Schwann cells (PSCs). These cells all together create a chemical synapse that represents the neurological control of muscle contraction.[1] Slow- twitch (ST) fibers are innervated by frequently active motoneurons on low frequencies, they have slow conduction velocities, aerobic energy production, and can sustain tension for longer periods. Fast- twitch (FT) fibers fire at high frequency, have fast conduction velocities, but can maintain tension for only short periods and their fiber subtypes differ in energy metablism.[2-5] The end-plate region of the muscle fiber is regularly invaginated by postjunctional folds where nicotinic acetylcholine receptors (AChRs) are clustered at the top of these folds and are directly opposed to active zones.[6] PSCs have key role in contributions to chemical communication.[7,8] The space between the nerve terminal and the postsynaptic membrane is the synaptic cleft. Acetylcholine (ACh) diffuses across the synaptic cleft to active AChRs. Synaptic vesicles contain ACh, each vesicle contain 5000–10000 molecules of ACh.[9,10] Acetyl choline esterase in the basal lamina of the postsynaptic membrane and the synaptic cleft accelerates the disappearance of ACh from synaptic cleft, along with the diffusion of ACh out of the cleft.[11] Results about size of nerve terminals are conflicting. It has shown that the terminals of ST muscle fibers motor neurons are large and less varicose than those of FT fibers motor neurons,[12] yet they release fewer quanta per unit area than those of fast nerve.[13] Others had shown that the rat FT and ST muscle fibers terminals are of similar area, but the terminals of FT fibers are shorter.[14] The extent of postsynaptic folding is greater in NMJs on FT fibers,[15] although this has not been confirmed in all studies.[12] In some studies NMJ size and muscle fiber diameter were find positively correlated.[16] Exercise training enhanced nerve terminal branching without modifying endplate size. Both increased and decreased physical activity might result in reductions in the ratio between endplate area and length of nerve terminal branches, thus altering the pre- and postsynaptic relationship of NMJs.[17] It was shown that the morphology of the NMJ does change significantly in response to exercise training.[18] In addition, other authors have shown that the effect of endurance type of exercise

training on FT muscle fiber NMJs may reflect some transformation from fast to slow morphological characteristics.[3,14] During endurance training NMJs undergo a process of hypertrophy as a compensatory response,[17] as well differnt authors have demonstrate that morphology of NMJs does not change significantly.[18] Only in one study differences in morphology of NMJs of different fiber types during adaptation to endurance exercise training were described.[19]

The purpose of this study was to examine whether there are differences in the morphology of neuromuscular junctions in fast fatigue fast-twitch glycolytic, fatigue resistance fast-twitch oxidative-glycolytic and fatigue resistance slow-twitch oxidative muscle fibers, and after their adaptation to six weeks of endurance exercise training. We hypothesized that there exists relation between remodeling of neuromuscular junctions and oxidative capacity of muscle fibers. Our working hypothesis was a strict relation between the turnover of contractile proteins and the neuromuscular junctions morphology, specifically, during adaptation to endurance training.

Material and Methods

Animals were used in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and all procedures used in this study were approved by the Animal Experiment Committee of the Ministry of Agriculture, Tallinn, Estonia. All research and animal care was performed according to European guidelines and ethical standards.[20]

Animals

The animals used were 16-17 weeks old (at the beginning of the experiment) male Wistar rats. All animals were housed in identical conditions in polycarbonated type III cages, at 21 ºC. They received diet [SDS-RM-1(C) 3/8, Witham, Essex, UK] and water ad libitum. The rats were assigned to control group (n=18) and endurance exercise trained group (n=22).

Administration of labeled amino acid

L-[4,53H] leucine (170 Ci/mmol) was infused intraperitoneally, 1.0 ml for 2 hr, 200 µCi per 100 g b.w. before the muscle samples were collected.

Endurance exercise training

After a brief 5-day acclimation that consisted of treadmill running for 5-10 min, rats were subjected to run with the speed 35m/min during 6 week. Rats ran 5 days per week and the training volume reached 60 min per day.

Separation of muscle fiber types

For studies of fast glycolytic (FG) fibers and fast oxidative-glycolytic (FOG) fibers, the *quadriceps femoris* m. was dissected, liberated from fat and connective tissue and separated into superficial white portion and deep red portion. For further identification of muscle fiber types Cytochromes aa3 , Myoglobin and MyHC isoforms were used. Cytochromes aa3 and myoglobin were measured as described previously.[21]

Separation of myofibrillar protein

Frozen muscle tissue portions were thawed on ice, cut into small pieces, and washed with five volumes 20 mM Na Cl, 5mM sodium phosphate, 1mM EGTA (pH=6.5). Myosin was extracted with three volumes of 100 mM sodium hydrophosphate, 5 mM EGTA, 1mM ditiothreitol (pH=8.5), after 30 min of gentle shaking. Myofibrillar fraction was diluted with one volume glycerol and stored at -20 ºC.

MyHC isoforms separation

MyHC isoforms were separated by 7.2% SDS-PAGE using 0.75 mm thick gels. Myofibrils containing 0.5 µg of protein were loaded on the gel after being incubated for 10 min at 65 ºC in sample buffer containing 62.5 mM Tris. HCl, pH=6.8, 20% (v/v) glycerol, 5% (v/v) 2-mercaptoetanol, 2.0% SDS, 0.05% bromphenol blue. Electrophoresis lasted for 24 h at 120 V.[22] Gels were silver-stained by the method of Oakley et al.[23] Protein isoform bands were analyzed densitometrically by Image Master 1 D program,Version 3.0 (Amersham Pharmacia Biothech, USA) and the percentage distribution of various isoforms was evaluated.

Turnover rate of MyHC

The relative specific activity, which characterizes the turnover rate of MyHC protein fraction, was calculated as the ratio of the specific activity of the protein fraction to the specific activity of total muscle cell protein and expressed in percentages.[24] Specific activity is the ratio between 3H-radioactivity and protein.

Protein assay

Total muscle protein and myofibrillar protein was assayed by using the technique described by Bradford.[25]

Crossectional area

Crossectional area of muscle fibers was analyzed after labeled ATPase activity (modified method of Brooke and Kaiser.[26]

Ultrastructural studies

Muscle samples for ultrastructural studies were fixed in 2.5% glutaraldehyde, pos-tfixed in 1% sodium tetroxide, dehydrated in graded alcohol and embedded in Epon-812. Ultra-thin sections were cut from longitudinally and transversely oriented blocs, stained with uranyl acetate and lead hydroxide, using 4 blocks from each animal. In rats of the control group and of the endurance trained groups an electron microscopic examination of 140 axon terminals in each fiber type group, was studied.

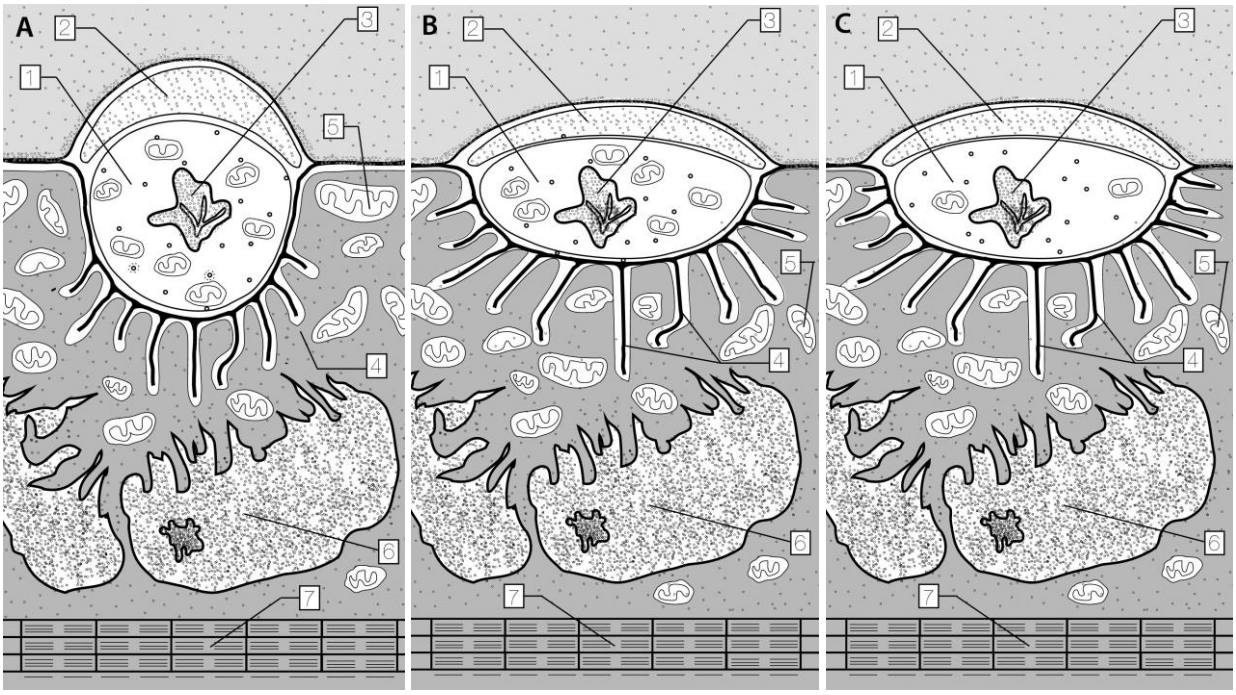


Fig. 1. Schematic representation of the synapse of oxidative (A), oxidative-glycolytic (B) and glycolytic (C) muscle fibers. 1 - axon terminal; 2 - Schwann's cell; 3 - autofagosome; 4 - postsynaptic folds; 5 - mitochondria; 6 - nucleus of the muscle fiber; 7 - myofibrils

Software

The imaging and analysis software (Cell* Soft Imaging System GmbH, Münster, Germany) was used.

Statistics

Means and standard errors were calculated from individual values using standard prcedures of Excel. The data were analyzed by R 2.12.2.[27] Pearson correlation coefficients were used for describing relationships between variables. Differences between groups were analyzed by the Wilcoxon rank sum (Mann-Whitney U) test. Probability distribution were compared using the Kolmogorov-Smirnov test. Differences were considered significant at $p<0.05$.

Results

MyHC isoforms relative content in differnt muscle fibers

In FG fibers myosin heavy chain (MyHC) IIb isoforms relative content was 97±6%, MyHC IId isoform relative content 3±0.3%; cytochromes aa^3 concentration was 9.4±0.9 ng/g muscle wet weight and myoglobin concentration 0.9±0.09 mg/g wet weight. In FOG fibers MyHC IIb isoform relative content was 23±2%, MyHC IId isoform relative content was 25±2%, MyHC IIa 44±4% and MyHC I isoform relative content was 8±0.8%. Cytochrome aa^3 concentration was 32±3 nm/g

Table 1. **Differences between axon terminal areas of different types of muscle fibers.**

Group	Number of animals per group	Total number of synapsees per fiber type	Axon terminal area (μm^2)		
			O	O-G	G
Control	18	216	259 ± 34	381 ± 48 *	326 ± 33
Endurance training	22	264	291 ± 26	420 ± 44 *	355 ± 34

O – oxidative type of muscle fibers; O-G – oxidative-glycolytic type of muscle fibers; G – glycolytic type of muscle fibers. * - $p<0.05$ in comparison with oxidative fiber;

***Table 2*. Remodelling of synapses according to changes of coated vesicles in terminals during endurance exercise training.**

Group	Number of synapsees per fiber type per animal	Number of coated vesicles per cut of terminal		
		O	O-G	G
Control	12	1.67 ± 0.2	1.70 ± 0.26*	1.35 ± 0.20
Endurance	12	5.06 ± 0.42 *** xxx	6.45 ± 0.44 *** xxx	2.05 ± 0.22 x

O – oxidative muscle fibers; O-G – oxidative-glycolytic muscle fibers; G – glycolytic muscle fibers.
*** - $p<0.001$ in comparison with glycolytiv fibers; xxx - $p<0.001$ in comparison with control group;
x - $p<0.05$ in comparison with control goup

and myoglobin concentration 4±0.4 mg/g wet weight. Slow oxidative (SO) fibers were separated from the *m. soleus*.

Turnover rate of MyHC in different muscle fibers

In ST O fibers protein turnover rate is 0.93±0.009; in FT O-G 0.90±0.01, and in FT G fibers 0.60±0.008.

Axon terminal morphology

Structures of neuromuscular synapses on different skeletal muscle fiber types are different. The axon terminals of rat ST O fibers are relatively short, round or oval shaped and are morphologically similar (Fig 1A). The sarcoplasm near the terminals contains a great number of mitochondria, which are full of cristae. The axon terminals of rat FT O-G fibers (Fig 1B, 2A) and FT G fibers (Fig 1C) are elliptical and 2.5 times longer than the terminals of ST O fibers ($p<0.001$). The postsynaptic folds of neighboring synapses on FT O-G and G fibers have linked with each other. In comparison with OG fibers the postsynaptic folds of G fibers are longer, more regular and they cover between them a much larger area of sarcoplasm. The area of axon terminals in this group is 1.7 time larger than the same area in ST O fibers ($p < 0.001$) in control groups (Table 1). Enduranc training increased the axon terminal area of about 10% in each fiber types groups but this change was not statistically significant. There are correlation between CSA of different muscle fibers and length of axon terminals (r=0.72), between CSA and width of axon terminal (r=-0.62), and change in turover rate of contractile proteins and width of axon terminal (r=0.75).

Remodelling of synapses

In terminal profiles of O (1.67±0.27) and O-G (1.70±0.26) fibers coated vesicles are present from 10-15% more than coated vesicles in terminal profiles of G fibers control group (Table 2).

Effect of endurance training on the morphology of synapses

Endurance training causes the heterogeneity of the structures of neuromuscular synapses which is clearly expressed in muscle fibers with higher oxidative capacity (O and O-G fibers). These fibers have faster turnover rate (subsequently 0.93±0.04 and 0.90±0.04) of muscle proteins as only a well developed synaptic apparatus guarantees intensive renewal of the structures of muscle fibers. The surface of the neighbouring neuromuscular contacts is smooth, the sarcoplasm near the terminals of the muscle fiber contains a great number of mitochondria which are full of cristae. After one week of endurance training as a result of branching terminals number increase (Fig 3B). One axon terminal may branch into 2-4 branches. The number of axon terminals of motor nerve endings on one cut may exeed ten. In some terminals lysosome like formations and myelin corpuscles number increase (Fig 2, 4B). It is

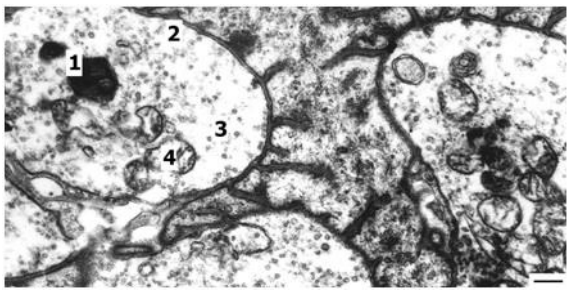


Fig. 2. Myelin figure in synapse of oxidative muscle fiber after six weeks of endurance training. 1. myelin figure; 2. synapse; 3. synaptic vesicles; 4. mitochondria; Bar 0.5 μm

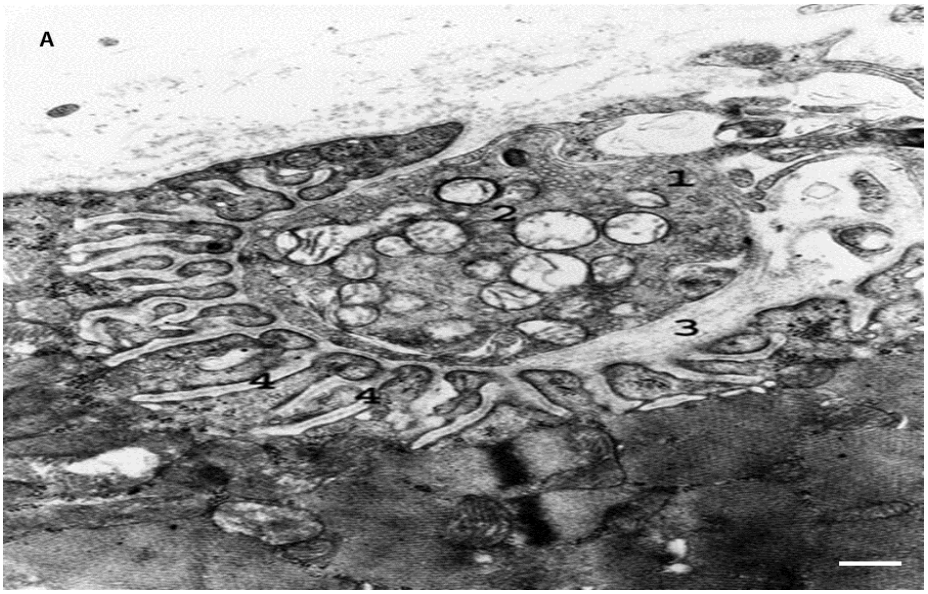


Fig. 3, A. Electron micrograph of synapse of oxidative-glycolytic muscle fiber. 1. axon terminal; 2. synaptic vesicles; 3. synaptic cleft; 4. postsynaptic folds. Bar 0.5 μm

typical of synapses of O-G fibers to have large postsynaptic folds area and as a result of endurance training the surface of nuclei on the side of terminals is covered with many extensions (Fig 4A). As a result of endurance training large complexes of mitochondria are located between the nucleas of muscle fibers and myofibrils, surrounding from each side the connection between the nerve and muscle (Fig 4A).

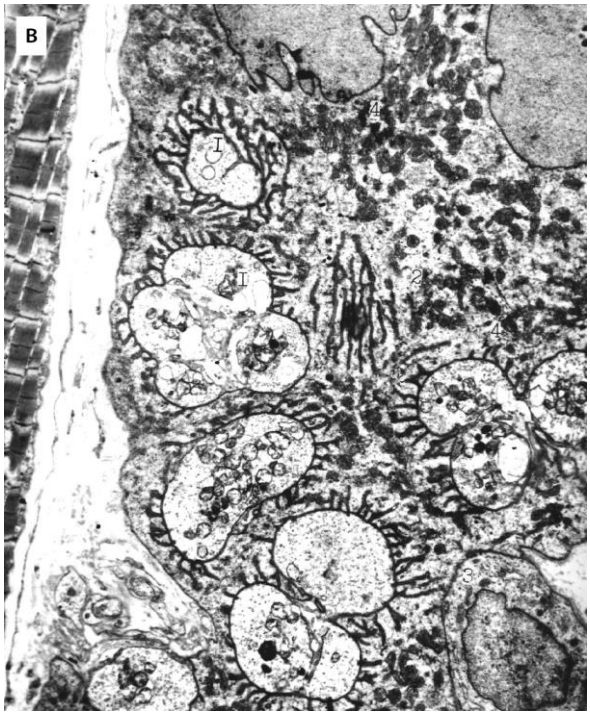


Fig 3, B. Effect of endurance training on structure of nerve synapse of oxidative-glycolytic muscle fiber after one week of endurance training. 1. neuromuscular synapse; 2. mitochondria in postsynaptic area; 3. satellite cell in synaptic area; Bar 1μm.

Effect of endurance training on the remodeling of synapses

The increase of the number of coated vesicles in terminals (Fig 4B) is connected with the resynthesis of Ach after the active functioning of the synapse. Coated vesicles appear mainly in the sarcoplasm of the presynaptic area of O and O-G fibers after endurance training and they are not only related to the resynthesis of Ach in nerve endings, but these vesicles also carry the proteins of choline receptors in the postsynaptic membrane. Remodeling of NMJs during 6 w of endurance training, according to change of coated vesicles in terminal (number of vesicles per profile of terminal) in comparison with control group increased about 300% (from 1.67±0.27 to 5.06±0.42; $p< 0.001$) in O fibers, 380 % (from 1.70±0.26 to 6.45±0.44; $p< 0.001$) in O-G fibers, and in G fibers 150% ($p<0.05$).

Discussion

Motoneuron type and not muscle fiber type determines the fast or slow character of NMJ. Not only size of the muscle fibers, but also the type and firing pattern of the motoneurons and the spatial constraints at preformed endplates influence the relation between junction size and muscle fiber diameter.[16] FT fast fatigued muscle terminals are active in short bursts at a high frequency,[28] while ST fatigue- resistant muscle with active terminals have longer periods of burst at low frequency.13 FT muscle terminals loos a higher proportion of their

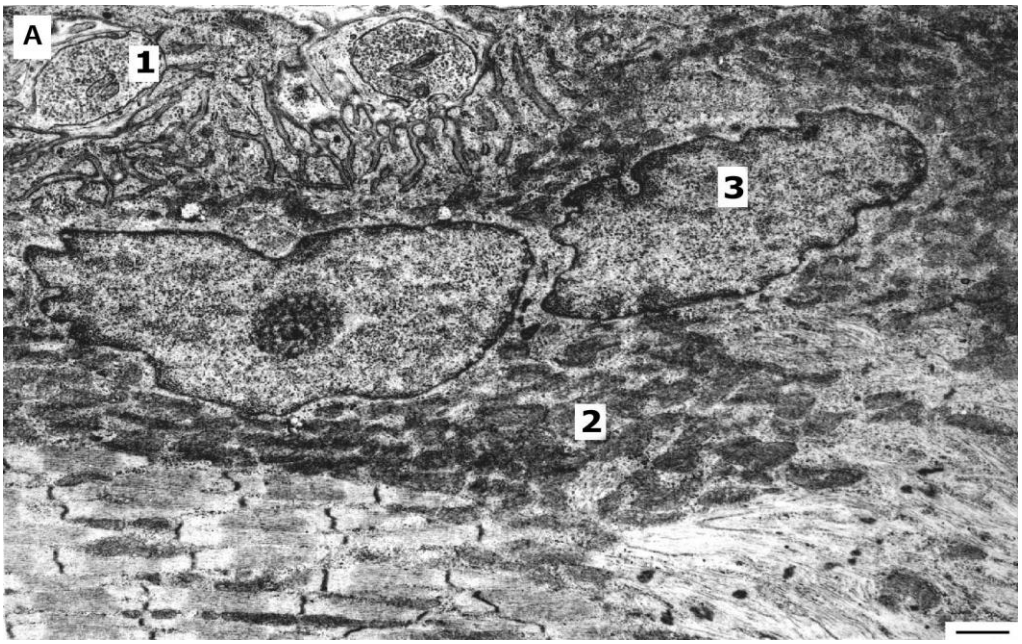
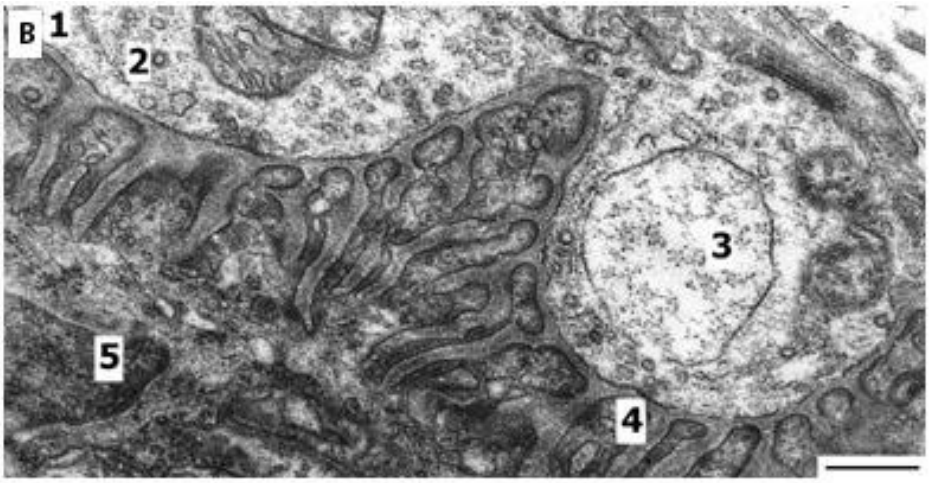


Fig. 4, A. Effect of endurance training on the structure of synapse of oxidative-glycolytic muscle fiber. 1. synapse, terminal is filled with synaptic vesicles; 2. mitochondria in postsynaptic zone; 3. nuclei of muscle fiber. Bar 1µm

vesicles per impulse and would, therefore, require that the vesicles return to the pool more rapidly in order to maintain transmitter release.[13] There are significant differences in synaptic vesicles trafficking in motor nerve terminals. ST muscle terminals support sustained quantal transmitter release much better than the terminals of FT muscle.[13] NMJs undergoes a continual process of remodeling and expansion during normal sedentary activity and exercise training.[17] Exercise training induces hypertrophy of the NMJs and nerve terminal branching independently of muscle hypertrophy and intensity of exercise.[29] Treadmill running increases the area of the nerve terminals, the corresponding muscle fiber diameter reducing, but significant differences between the trained group and the control group were obtained only in FT muscle.[14] If there is greater area nerve terminals in endurance trained group, it may be the result of intensive branching of terminals.[30] In some terminals there are lysosome-like structures, myelin corpuscles, synaptic



Fig, 4 B. Structure of synapse of oxidative-glycolytic muscle fiber after recovery from endurance training. 1. synapse; 2. coated vesicles in terminal; 3. large vacuole in axon terminal; 4. postsynaptic folds; 5. nucleus of muscle fiber. Bar 1µm

vesicles and numerous coates vesicles. Appearance of coated vesicles is connected with the resynthesis of ACh after the active function of the synapse. Endurance training causes heterogeneity of neuro muscular synapses on O and O-G muscle fibers. Some terminals of endurance trained rats are filled with a great amount of synaptic vesicles, the others have few of them, but both have lots of mitochondria. There are also completely ''clean'' terminals with reduced postsynaptic folds. It is typical of the synapses of O-G fibers to have a large postsynaptic area. At NMJs the most striking structural features of the postsynaptic region are the deep infolding of the sarcolemma. The chrests of the postsynaptic membrane infolding contain a very high density of AchRs,[6] which may explain the characteristic curvature of the membrane in this region. Between the nerve and muscle fibers there is a synaptic cleft about 50–100 nm wide. Especially large complexes of mitochondria are located between the nuclei as of muscle fibers and myofibrils, surrounding from each side the connection between the nerve and muscle after endurance training. A mechanism seems to link mitochondria and myofibrils in specific structures - intracellular energetic units, as was previously shown .[31,32] The reliability of neuromuscular transmission normally results from the release of more quanta of ACh than quanta required to initiate an action potential. The safety factor for neuromuscular transmission is used to describe this excess, i.e., a safety factor in terms of the number of ACh quanta actually released compared to the numbers needed to generate an action potential.[12] Due to the above mentioned morphological changes in NMJs, adaptive process in FT muscles shows high potential of recruitment of FT muscle fibers during endurance exercise.[33]

Axon terminals of ST O fibers are relatively short, round or oval shaped. The sarcoplasm near the terminals contains a great number of mitochondria. The axon terminals of FT O-G fibers and FT G fibers are elliptical and 2.5 times longer than the terminals of ST O fibers. In comparison with OG fibers the postsynaptic folds of G fibers are longer, more regular and they cover between them a much larger area of sarcoplasm. The area of axon terminals on FT OG and G fibers and the perimeter of terminals is are respectively 1.5 time and 1.7 time larger than the same in ST O fibers in control group. Increase of axon terminals area exceeds about 10% during 6 week endurance exercise training period when compared with G fibers in control groups. Endurance training causes the heterogeneity of the structures of neuromuscular synapses which is clearly expressed in muscle fibers with higher oxidative capacity. The surface of the neighbor neuromuscular contacts is smoother than the sarcoplasm near the terminals of the muscle fiber containing a great number of mitochondria full of cristae. Remodeling of NMJs during 6 w of endurance training, in comparison with control groups is in exceed of about 300% and 380% in O fibers and in O-G fibers respectively. Fast remodeling of synapse in O and O-G fibers during endurance training guarantees the intensive renewal of the structures of the muscle fibers.

List of acronyms

Ach - acetylcholine
AchRs - nicotinic acetylcholine receptors
FT - fast- twitch fibers
NMJ - neuromuscular junction
PSCs - perisynaptic Schwann cells
ST - slow- twitch fibers

Author's contributions

All authors performed the experiments, analyzed, designed and interpreted the data. Teet Seene drafted the article. All authors critically revisited the manuscript and approved its final version.

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Conflict of Interest

Neuromuscular junction, slow- and fast-twitch fibers, endurance exercise, remodeling of synapses.

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