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EFFECTS OF EXERCISE TRAINING ON NEUROMUSCULAR JUNCTION MORPHOLOGY AND PRE- TO POST-SYNAPTIC COUPLING IN YOUNG AND AGED RATS

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Abstract—The objective of this study was to determine whether pre- to post-synaptic coupling of the neuromuscular junction (NMJ) could be maintained in the face of significant morphological remodeling brought about by exercise training, and whether aging altered this capacity. Eighteen young adult (8 mo) and eighteen aged (24 mo) Fischer 344 rats were randomly assigned to either endurance trained (treadmill running) or untrained control conditions resulting in four groups ($N = 9$/group). After the 10-week intervention rats were euthanized and hindlimb muscles were surgically removed, quickly frozen at approximate resting length and stored at −85 °C. The plantaris and EDL muscles were selected for study as they have different functions (ankle extensor and ankle flexor, respectively) but both are similarly and overwhelmingly comprised of fast-twitch myofibers. NMJs were stained with immunofluorescent procedures and images were collected with confocal microscopy. Each variable of interest was analyzed with a 2-way ANOVA with main effects of age and endurance training; in all cases significance was set at $P \leqslant 0.05$. Results showed that no main effects of aging were detected in NMJs of either the plantaris or the EDL. Similarly, endurance training failed to alter any synaptic parameters of EDL muscles. The same exercise stimulus in the plantaris however, resulted in significant pre- and post-synaptic remodeling, but without altering pre- to post-synaptic coupling of the NMJs. Myofiber profiles of the same plantaris and EDL muscles were also analyzed. Unlike NMJs, myofibers displayed significant age-related atrophy in both the plantaris and EDL muscles. Overall, these results confirm that despite significant training-induced reconfiguration of NMJs, pre- to post-synaptic coupling remains intact underscoring the importance of maintaining proper apposition of neurotransmitter release and binding sites so that effective nerve to muscle communication is assured.

Key words: synapse, endplate, myofiber, aging, nerve terminal, NMJ.

INTRODUCTION

Previous research has demonstrated that the neuromuscular junction (NMJ) has the capacity to undergo considerable morphological remodeling as a result of increased activity presented in the form of exercise training. For example, endurance training has been shown to increase the total length of nerve terminal branching (Andonian and Fahim, 1988; Waerhaug et al., 1992), the area occupied by acetylcholine (ACh)-containing vesicles (Deschenes et al., 1993), the area occupied by ACh receptors (Cheng et al., 2013; Fahim, 1997; Gyorkos and Spitsbergen, 2014), and the dispersion of both pre-synaptic ACh vesicles and post-synaptic ACh receptors (Deschenes et al., 1993, 2000).

Similarly, it has been established that the natural process of aging also results in significant structural remodeling of the NMJ. Typically, aging is associated with adaptations such as increased pre-synaptic branching, post-synaptic endplate area, and increased separation of clusters of pre-synaptic vesicles and post-synaptic receptors, along with receptors that have been abandoned by previously linked nerve terminal branch points (see reviews by Gonzalez-Freire et al., 2014; Jang and Van Remmen, 2011; Rudolf et al., 2014).

More recently it has been reported that despite such extensive NMJ remodeling, the coupling of the pre- and post-synaptic components of the NMJ remains steadfast (Deschenes et al., 2013). In that investigation it was revealed that pre- to post-synaptic coupling remained constant despite the slow and progressive effects of the aging process, and the natural variation in activity levels characterizing different muscle groups (i.e., postural vs. non-weight bearing). To date, however, the impact of exercise training – which dramatically and abruptly increases neuromuscular activity – on pre- to post-synaptic coupling of the NMJ has yet to be determined. With that in mind, the present study was conducted to assess how endurance training affected NMJ morphology

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Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; BTX, α-bungarotoxin; NMJ, neuromuscular junction; PBS, phosphate-buffered saline.

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with a special emphasis on synaptic coupling of nerve terminal branches, ACh vesicles and post-synaptic ACh receptors.

EXPERIMENTAL PROCEDURES

Subjects

Eighteen young adult (8 mo old) and eighteen aged (24 mo old) male Fischer 344 rats were purchased from the National Institute of Aging Colonies. According to Turtorro et al. (1999) the average lifespan of male Fischer 344 rats is 25.5 mo while the average lifespan of men in the United States is 75.2 years (Arias, 2006). Accordingly, the rats used in the present study would roughly be the equivalent of 71-year-old men. Different muscles, i.e., soleus, from these same animals had been the subject of an earlier report from this laboratory (Deschenes et al., 2011).

Animals were provided standard rat chow and water *ad libitum* and were housed in a 21–22 °C environment with a 12-h light/dark cycle throughout the duration of the investigation. All procedures were approved beforehand by the Institutional Animal Care and Use Committee, which adheres to the National Institutes of Health Guide for the Care and Use of Laboratory Animals as revised in 2011. Throughout the study, all efforts were made to minimize the number of animals used and to alleviate their discomfort.

Exercise training

Rats from both age groups were randomly assigned to either endurance training, or control groups resulting in a total of four treatment groups each with $N = 9$. Endurance training consisted of a 10-week running program on a motorized treadmill (Accuscan Instruments, Columbus, OH, USA). The program began with training sessions of 15 min at a speed of 7.5 m/min at a 0% grade completed 5 days/week. Treadmill speed and duration of exercise sessions were gradually increased such that by the final week, young and aged rats ran for 60-min per session at a speed of 15 m/min, while maintaining a 0% grade, and still for 5 days/week. Increments in exercise speed and duration were determined by the tolerance of aged animals, and replicated by young ones, to ensure that both age groups completed the same training regimen. Exercise tolerance was subjectively determined by visual indications of physical fatigue such as decisively labored strides, heavy panting, and inability to maintain pace. Aged and young adult controls remained sedentary and stayed in their tubs throughout the 10-week intervention period.

Tissue preparation and storage

At the conclusion of the 10-week intervention period, animals were euthanized and hindlimb muscles were dissected out, cleared of fat and connective tissue, quickly frozen at resting length in isopentane chilled with liquid nitrogen and stored at −85 °C until analysis. For the present study, the plantaris and EDL muscles were used for analysis. Although both of these muscles are principally comprised of fast-twitch (type II) muscle fibers (Delp and Duan, 1996), they have different functions in that the plantaris is an ankle extensor while the EDL is an ankle flexor.

Cytofluorescent staining of NMJs

To visualize NMJs, 50-μm-thick longitudinal sections of the middle one-third of the muscle were obtained at −20 °C on a cryostat (Cryocut 1800; Reichert-Jung, NuBloch, Germany). To prevent contraction of sections, microscope slides were pretreated in a 3% EDTA solution as previously described (Pearson and Sabarra, 1974). Sections were washed 4 × 15 min in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Sections were then incubated in a humidified chamber overnight at 4 °C in supernatant of the primary antibody RT97 (Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:20 in PBS with 1% BSA. The RT97 antibody reacts with non-myelinated segments of pre-synaptic nerve terminals (Anderton et al., 1982). The next day, sections were washed 4 × 15 min in PBS with 1% BSA before being incubated for 2 h at room temperature in fluorescein isothiocyanate (FITC)-conjugated secondary immunoglobulin (Sigma Chemical, St. Louis, MO, USA) which had been diluted 1:150 in PBS with 1% BSA. Sections were then washed 4 × 15 min in PBS with 1% BSA before being incubated in a humidified chamber overnight at 4 °C in a solution containing rhodamine-conjugated α-bungarotoxin (BTX; Molecular Probes, Eugene, OR, USA) diluted 1:600 in PBS, along with anti-synaptophysin (MP Biomedicals, Solon, OH, USA) at a dilution of 1:50. BTX recognizes post-synaptic ACh receptors, while anti-synaptophysin binds to membranes of pre-synaptic vesicles containing ACh. Indeed, synaptophysin is the most abundant protein found in the synaptic vesicular membrane (Kwon and Chapman, 2011). The next day, sections were washed 4 × 15 min in PBS with 1% BSA before incubating them for 2 h in a humidified chamber at room temperature in AlexaFluor 647 (Molecular Probes, Eugene, OR, USA)-labeled secondary antibody to illuminate the anti-synaptophysin. Sections were then washed again for 4 × 15 min before being lightly coated with Pro Long (Molecular probes, Eugene, OR, USA) and having cover slips applied. Slides were then coded with respect to treatment group to allow for blinded evaluation of NMJ morphology and then stored at −20 °C until analysis. An example of this cytofluorescent staining of pre- and post-synaptic components of the NMJ is displayed in Fig. 1.

Pre-synaptic variables of NMJs assessed included: (1) number of branches identified at the nerve terminal, (2) the total length of those branches, (3) average length per branch, and (4) branching complexity which, as described by Tomas et al. (1990) is derived by multiplying the number of branches by the total length of those branches and dividing that figure by 100. Pre-synaptic vesicular staining was assessed as: (1) total perimeter, or the length encompassing the entire vesicular region comprised of stained vesicular clusters and non-stained regions interspersed within those clusters, (2) stained