Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise

Gregorio Valdez*a,1, Juan C. Tapia*b,1, Hyuno Kang*a,1, Gregory D. Clemenson, Jr.b, F. H. Gageb, Jeff W. Lichtmana,b,2, and Joshua R. Sanesa,b,2

*Department of Molecular and Cellular Biology and Center for Brain Science, Harvard University, Cambridge, MA 02138; and bLaboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA 92037

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The cellular basis of age-related behavioral decline remains obscure but alterations in synapses are likely candidates. Accordingly, the beneficial effects on neural function of caloric restriction and exercise, which are among the most effective anti-aging treatments known, might also be mediated by synapses. As a starting point in testing these ideas, we studied the skeletal neuromuscular junction (NMJ), a large, accessible peripheral synapse. Comparison of NMJs in young adult and aged mice revealed a variety of age-related structural alterations, including axonal swellings, sprouting, synaptic detachment, partial or complete withdrawal of axons from some postsynaptic sites, and fragmentation of the postsynaptic specialization. Alterations were significant by 18 mo of age and severe by 24 mo. A life-long calorie-restricted diet significantly decreased the incidence of pre- and postsynaptic abnormalities in 24-mo-old mice and attenuated age-related loss of motor neurons and turnover of muscle fibers. One month of exercise (wheel running) in 22-mo-old mice also reduced age-related synaptic changes but had no effect on motor neuron number or muscle fiber turnover. Time-lapse imaging in vivo revealed that exercise partially reversed synaptic alterations that had already occurred. These results demonstrate a critical effect of aging on synaptic structure and provide evidence that interventions capable of extending health span and lifespan can partially reverse these age-related synaptic changes.

Aging | neuromuscular junction | muscle | motor neuron | sarcopenia

Aging is accompanied by numerous functional alterations of both the central and peripheral nervous systems (1). Until recently, it was thought that many of these age-associated changes were secondary to neuronal degeneration. Recent studies show, however, that little neuronal death occurs in most areas of the aging nervous system (2). Although many possible explanations exist (3–6), a particularly attractive hypothesis is that some age-related alterations in mental function result from synaptic alterations. Supporting this idea, alterations in synapse number, spine densities, and synaptic plasticity have been documented in the brains of aging humans and experimental animals (1, 7, 8).

If synaptic changes underlie age-related defects in neural function, one might look to synapses as targets for treatments that minimize the decline. Two lifestyle regimens that have been consistently demonstrated to extend lifespan and mitigate age-related changes in neural function are caloric restriction and exercise (9). In that the cellular bases of age-related changes in mental activity are obscure, it is not surprising that the means by which exercise and caloric restriction attenuate these changes are also unknown. For both regimens, however, synaptic alterations have figured prominently among proposed mechanisms (9, 10).

An obstacle to progress in this area is the complexity and diversity of synaptic neuropil in the brain, which impedes detailed analysis of aging central synapses. It has therefore been difficult to determine whether the structure of synapses changes with age. In contrast, skeletal neuromuscular junctions (NMJs) are ideal for analysis of synaptic architecture: they are highly accessible, relatively simple, functionally uniform, and so much larger than central synapses that their size and shape can be assessed light microscopically (11). Moreover, several studies have noted differences in neuromuscular structure between young adult and aged rodents (12–16) and humans (17, 18). Here, we characterized and quantified these changes and determined their time course using transgenic mice in which motor axons were indelibly labeled with fluorescent proteins (19). We then assessed the effects of caloric restriction and exercise on these synaptic changes. Both of these interventions attenuate age-related declines in muscle function (20–24), but little is known about their effects on the NMJ (25). We show here that both conditions significantly blunt age-related structural alterations in the NMJ. Finally, we demonstrate that the beneficial effects of exercise do not result from prevention of age-related motor neuron loss or muscle fiber degeneration but rather reflect a partial reversal of structural alterations that have already occurred.

Results

Altered NMJs in Aged Skeletal Muscles. To begin this study, we assessed structural alterations in NMJs of aged mice. To optimize visualization of axons and nerve terminals, we used transgenic mice that express YFP in all motor axons (19). Acetylcholine receptors (AChRs), aggregated in the postsynaptic membrane, were labeled with fluorescently-tagged α-bungarotoxin (iBTX), a highly selective ligand for AChRs. We compared NMJs in tibialis anterior muscles of young adult (1–3 mo) and old (24–28 mo) mice. In young adult >99% of AChR-rich postsynaptic sites were apposed by terminal branches of a single YFP-labeled motor axon (Fig. 1A). At each junctional site, the preterminal axon was thick and relatively constant in caliber. AChRs aggregates formed continuous long branches, each precisely aligned with an axonal branch (Fig. 1C).

NMJs in muscles ≥24-mo-old mice differed in several ways from those in young adults. First, the AChR cluster on some muscle fibers was not contacted by an axon (Fig. 1 B arrows and D). More often, axons incompletely occupied a postsynaptic apparatus, leaving the AChR site partially denervated (Fig. 1 E and F). Second, aged junctional AChRs were often fragmented into small islands (Fig. 1 D–F). In some cases, the islands were only faintly visible, indicating decreased AChR density within them (Fig. 1E).


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1G.V., J.C.T., and H.K. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: jeff@mbz.harvard.edu or sanesj@mbz.harvard.edu.

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Third, preterminal and terminal axons were often misshapen. Some axons were much thinner than those observed at younger ages (Fig. 1G), whereas others harbored swollen varicosities, typically within 10–50 μm of an NMJ (Fig. 1H). Fourth, at some NMJs, two axons converged at the same postsynaptic site (Fig. 1I and Fig. S1), a pattern reminiscent of the polyneuronal innervation seen at all NMJs of neonates (11) but at <0.1% of NMJs of young adult mice. This multiple innervation could result

Fig. 3. Effects of caloric restriction on age-related changes in NMJs. (A and B) NMJs from tibialis anterior of 24-mo-old mice that had been fed ad libitum (Old-Ctrl) (A) or fed a calorie-restricted diet beginning at the age of 16 wk (Old-CR) (B). Muscles were stained with fBTX plus antibodies to neurofilaments and synaptotagmin-2. (C–F) Frequency of age-associated abnormalities in NMJs from the tibialis anterior (tib ant), medial gastrocnemius (gastroc), and gracilis (grac) muscles of 5- and 24-mo-old mice fed ad libitum and 24-mo-old calorically restricted mice. Each bar shows mean ± SEM from at least 100 NMJs from at least four mice. *P < 0.02 by t test. (Scale bar: 10 μm.)

Fig. 2. Time course of aged-associated abnormalities at NMJs. The frequency of age-associated abnormalities shown in Fig. 1 was quantified using criteria detailed in SI Materials and Methods. More than 100 NMJs from three animals at each age were analyzed.

Fig. 1. Age-related changes in the neuromuscular system. (A) Tibialis anterior muscle in a young adult mouse that expressed YFP in motor axons. In the region shown, two nerve bundles (yellow) innervated individual muscle fibers. AChRs were labeled with Alexa-594-BTX (red). Complete apposition between nerve terminals and AChRs is typical in young animals. (B) Tibialis anterior muscle in a 24-mo-old mouse. Denervated (arrows) and partially vacated AChR sites as well as prominent axonal swellings (arrowheads) are common. Young adult (C) and aged (D–J) NMJs are shown at higher magnification. Age-related alterations included complete denervation of the postsynaptic AChR site (D), fragmentation and faintly labeled patches of receptors (E), and receptors that are only partially covered by the nerve terminal (E–F). In some cases the innervating axons are very thin (G), bear large swellings proximal to the nerve terminal (H), multiply innervate one NMJ (I), or extend processes beyond the receptor area (J). (Scale bars: 50 μm (A–B) and 10 μm (C–J).)
from sprouting of axons to an NMJ that was already partially or completely denervated. In support of this possibility, we sometimes observed thin sprouts that extended beyond a postsynaptic site (Fig. L).

To determine the time course of these changes, we analyzed tibialis anterior muscles at several ages (Fig. 2). No significant structural differences were seen between NMJs of 1- and 6-month-old mice. At 12 mo, however, a few NMJs showed fragmentation, preterminal swellings, and partial denervation. Fully denervated and multiply innervated synaptic sites, terminal sprouts and axonal varicosities were present by 18 mo of age. By 24 mo of age, NMJs differed significantly from those of young adults in all categories analyzed. We also analyzed a small number of animals at 36 mo of age. By this age, all NMJs were fragmented, and other age-related changes were more prevalent than those seen in 24-mo-old animals (Fig. S2).

To test whether any of the age-related alterations observed arose as a consequence of the long-term expression of fluorescent protein in motor axons, we stained old wild-type mice with bTX plus antibodies to neurofilaments and synaptic vesicle proteins to label AChRs, axons, and nerve terminals, respectively. There was no difference in the frequency of any abnormality in immunostained NMJs of 24-mo-old wild-type animals compared with YFP-expressing animals (Fig. S3 and Table S1). These results are consistent with a previous report (26) that motor axonal defects are no more common in transgenic than in wild-type mice. Thus, prolonged YFP expression does not cause the synaptic changes described above.

**Caloric Restriction Attenuates Age-Related Changes in NMJs.** We next assessed NMJs of 24-mo-old mice that had been fed a diet in which caloric content was reduced by 40% starting at 16 wk of age (27). Because these mice, which were obtained from the National Institutes of Health, may have differed in background from those raised in our facility, we compared them to age-matched mice that were identical in background to the calorically restricted group but had been fed a standard diet. Muscles were stained with bTX and antibodies to neurofilaments and synaptic vesicle proteins as above.

Caloric restriction from 4 to 24 mo of age led to sparing of many NMJs in the tibialis anterior muscle (Fig. 3 A and B and Fig. S4 A–C). The frequencies of fragmented, faint, and denervated postsynaptic sites and of nerve terminals bearing terminal sprouts were all significantly lower in calorically restricted mice than in controls (Fig. 3 C–F). Similar results were obtained in gracilis and gastrocnemius muscles (Fig. 3 C–F and Fig. S5). Likewise, caloric restriction reduced the incidence of axonal atrophy and distended varicosities. Thus caloric restriction attenuates the deleterious effects of age on the structure of the NMJ.

**Exercise Attenuates Age-Related Changes in NMJs.** To assess the effect of exercise on the NMJ, we housed 22-mo-old animals with or without access to running wheels for 1 mo. Under these conditions, monitoring confirmed (28) that old mice ran approximately 4 km/d. Exercise reduced the frequencies of fragmented, faint, and denervated postsynaptic sites in the tibialis anterior, gracilis and gastrocnemius muscles (Fig. 4 A–D and Figs. S4 D–E and S6 A–B). Effects on presynaptic features were less striking: changes in denervation and sprouting were marginally significant (Fig. 4 E–F).

We also asked whether exercise affected the structure of NMJs in young adult mice. No significant effects of free running for 1 mo were observed when exercise was initiated at 4 mo of age (Fig. 4 C–F).

**Local and Systemic Effects of Caloric Restriction and Exercise.** To determine whether the effect of exercise on NMJ structure was systemic, we examined the triangularis sterni muscle on the inner surface of the rib cage, and the sternomastoid muscle in the neck. Neither muscle is likely maximally recruited during voluntary exercise (29). Age-related changes in the triangularis sterni and sternomastoid were similar to those documented above for limb muscles (Fig. 5 A–B and Fig. S7A), but exercise had no detectable effect on the structure of NMJs in either muscle (Fig. 5 C and E–H and Fig. S7B). Life-long caloric restriction significantly attenuated age-related changes in triangularis sterni, ruling out the possibility that this muscle was refractory to modulatory influences (Fig. 5 D–H). These results suggest that the effects of 1 mo of exercise on NMJ structure are confined to muscles directly involved in exercise.

**Distinct Effects of Caloric Restriction and Exercise on Degeneration of Motor Axons and Muscle Fibers.** Although little neuronal death accompanies aging in most neuronal populations, a substantial number of motor neurons have been reported to be lost in aging rodents and humans (20, 24, 30). Likewise, age-related sarcopenia involves degeneration of muscle fibers (31), many of which are rapidly replaced by regeneration of new fibers (32, 33). Because these changes might cause or result from alterations in the NMJ, we asked whether they were attenuated by caloric restriction or exercise.

To assess neuronal loss in aged mice, we compared the number of axons in the deep peroneal nerve, which innervates the tibialis anterior and other leg extensor muscles. The average number of myelinated axons in this nerve was approximately 30% lower in aged than in young adult mice (Fig. 6 A and B). Similar results
were obtained in the nerve that innervates the omohyoid muscle (72 ± 9 vs. 49 ± 6 axons; 32% loss; P < 0.002, n = 5). Because these nerves contain sensory as well as motor axons, we also examined ventral roots, which contain only efferent axons. The number of axons in the L1 ventral root was approximately 35% less in old animals than in young adults (245 ± 14 vs. 168 ± 18, P = 0.004, n = 3). The loss of motor axons is likely the result of motor neuron death, because high levels of activated caspase-3, an indicator of cell death, were observed in large neurons in the ventral horn of aging, but not of young adult, spinal cords (34). The incidence of cell death, because high levels of activated caspase-3, an indicator of cell death, were observed in large neurons in the ventral horn of aging, but not of young adult, spinal cords (34).

We used centrally located nuclei as an indicator of muscle fiber turnover; their presence signifies that the muscle fiber has died and another has regenerated in its place (34). The incidence of muscle fiber cross-sections with central nuclei was 5- to 10-fold higher in muscle fibers of old than young adult mice (Fig. 6 C and D). Centrally located nuclei were present in <2% of cross-sections through young adult muscle fibers in lower limb muscles, but in approximately 12%, 16%, and 18% of cross-sections through aged fibers in the gastrocnemius, tibialis anterior, and gracilis muscles, respectively.

Caloric restriction attenuated age-related loss of motor neurons and increase in muscle fiber turnover (Fig. 6 B and E), consistent with previous results (33, 35). In contrast, the exercise regimen we used had no effect on the number of axons or in the turnover of muscle fibers (Fig. 6 B and E). Although a longer period of exercise might attenuate axon loss or muscle fiber turnover, our results indicate that the beneficial effects of exercise on the NMJ are not secondary to rescue of axons or muscle fibers.

**Exercise Partially Reverses Age-Related Structural Alterations in the NMJ.** A comparison of the results in Figs. 2 and 4 suggests that 1 mo of exercise in aged mice decreases the fraction of NMJs with structural abnormalities to a lower level than was present at the beginning of the exercise period. This remarkable effect occurs in the absence of significant changes in motor neuron number or muscle fiber turnover (Fig. 6). How does exercise attenuate NMJ aging? Possible cellular mechanisms include loss of the most severely affected NMJs with selective retention of those less affected, dismantling of aberrant NMJs and assembly of new ones, and reversal of alterations that have already occurred within individual NMJs. To distinguish these possibilities, we applied time-lapse imaging protocols developed for the sternomastoid muscle (36, 37) to the extensor digitorum longus (EDL) of the hindlimb. Animals were anesthetized, the muscle was exposed, and AChRs were labeled with a subsaturating dose of fBTX. NMJs were imaged in 2- and 22-mo-old mice, and then the animals were returned to their cages. Half of the 22-mo-old animals were then given free access to a running wheel. They recovered within 1–2 d and exercised as vigorously as littermates that had not been imaged. One month later, NMJs were relocated and imaged again.

The shape, size, and topology of the NMJ changed little over the month in the EDL muscle of young adult mice (Fig. S9), consistent with previous results in the sternomastoid (37). In striking contrast, degenerative changes accumulated in many NMJs of sedentary old mice. Some AChR-rich branches broke into fragments, and some AChR-rich areas disappeared (Fig. 7 A–D). NMJs of exercised mice presented an intermediate picture: they were more stable than those in the sedentary old mice, but exhibited a degree of dynamism greater than that seen in young adults. Strikingly, during the month of exercise, some AChR-rich fragments fused to form continuous branches, and some new AChR-rich regions appeared (Fig. 7 B–D). Many NMJs exhibited both regressive (fragmentation, loss of AChRs) and progressive (fusion, appearance of new AChR-rich regions) alterations.

The extensive dynamism of old NMJs confounded attempts to quantify changes with simple metrics used previously (36). As an alternative, we presented unlabeled pairs of initial and final images of NMJs to four observers who had not taken part in the experiments and asked them to judge how the degree of AChR fragmentation changed over the month between views and whether addition of new AChR-rich regions outstripped loss of synaptic regions. The ratings of all four observers were in qualitative agreement: more NMJs in the sedentary than in the exercised group became more fragmented over the month between views (65% vs. 28%; P < 0.03), more NMJs in the exercised than the sedentary group became less fragmented (18% vs. 6%; P < 0.02), and net AChR addition was greater in NMJs of the exercised than the sedentary group (+17% vs. −16%; P < 0.005) (Fig. 7 E–G). We conclude that exercise can partially reverse age-related changes in NMJ structure.

**Discussion**

Beneficial effects of dietary restriction and physical activity on mental function have been amply documented, and both regimens are capable of blunting or delaying age-related declines in neural and neuromuscular capabilities (9, 10, 20, 23, 28). Here, we asked whether these regimens affected the tempo or nature of age-related alterations in synaptic structure. As a first step, we categorized and quantified age-related structural changes in the NMJ. In several muscles of the limb and thorax, a majority of postsynaptic sites are fragmented into small islands by the age of 24 mo. Many preterminal axons are abnormally thin or distended. Some NMJs are partially or multiply innervated, a configuration seen in <0.1% of young adult junctions. AChR levels are low at some postsynaptic sites. About one third of the motor axons are lost and about one-tenth of the NMJs are completely denervated. Many muscle fibers are atrophic and some show signs of degeneration and regeneration, both features of the clinically significant muscle wasting known as sarcopenia (31). Changes are apparent by 18 mo of age, and increase in severity over the next year, a period that extends far into old age for C57BL/6 mice, whose median lifespan is 26 mo (32). Use of XFP mice allowed us to provide a more quantitative...
and comprehensive view of age-related axonal changes than was available (11–14).

With these results as a foundation, we assessed the effects of caloric restriction and exercise on NMJ structure. Our principal result is that both regimens attenuate many age-related synaptic changes. For example, for the incidence of fragmented postsynaptic structures, dim AChR patches, and partial or complete denervation is lower in exercised or calorically restricted animals than in age- and strain-matched controls. On the other hand, the effects of the two regimens differ in several respects. First, the effects of exercise on axonal segments unopposed to synaptic sites are less striking than those of caloric restriction: the frequency of terminal sprouting, axonal atrophy, and swelling are decreased by the former but not the latter. Second, caloric restriction but not exercise blunts age-related loss of motor neurons and muscle fibers. These differences are noteworthy because they suggest that the effects of exercise on synaptic structure are not indirect consequences of sparing motor neurons or muscle fibers. Finally, caloric restriction affected all muscles examined, but exercise affected only those muscles that were exercised. This result suggests that the beneficial effects of exercise on the synapse result from local interactions. For example, increased activity in exercising muscles could lead to up-regulation of trophic factors from muscle that would, in turn, improve synaptic maintenance (20).

Although the different effects of exercise and caloric restriction on the NMJ suggest that they act through distinct mechanisms, two confounds make this interpretation premature. First, calorically restricted rodents are more active than those fed ad libitum (38, 39). Thus, some beneficial effects of caloric restriction could be secondary to increased activity and thereby mechanistically similar to exercise-dependent alterations. Second, the time course of the two regimens differed greatly, with caloric restriction being imposed in early adulthood but exercise offered for 1 mo in old age. Thus, motor neuron loss and muscle fiber turnover were likely well advanced by the time that exercise was offered, so there was no possibility for this regimen to have dramatic effects on these parameters. To disentangle the differences in regimen from the difference in time course, it will be important to vary the duration of each.

The dramatic effects of 1 mo of exercise on NMJ structure raised the possibility that this regimen did not merely decrease the tempo of age-related alternations, but actually reversed changes that had already occurred. Time-lapse imaging in vivo provided direct support for this idea. Although life-style regimens have previously been reported to improve behavioral performance (28, 40), there has not been, to our knowledge, evidence for reversal of age-related structural alterations that might account for this improvement. The NMJ promises to be a useful preparation not only for analyzing this reversal, which might also occur in the central nervous system, but also for assessing roles of molecules such as sirtuins and insulin-like growth factors, which might regulate or attenuate age-related synaptic changes.

Fig. 6. Loss of motor axons and regeneration of muscle fibers in aged animals. (A) Cross-section of the peroneal nerve stained with antibodies to neurofilament (NF) (red) and S100 (green) to mark axons and Schwann cells, respectively. (B) Number of axons present in the tibial nerve of 5-mo-old (Young-Ctrl) and 23- to 24-mo-old control mice (Old-Ctrl); 23-mo-old mice provided free access to a running wheel for their final month (Old-Ex) and calorically restricted 24-mo-old mice (Old-CR). Age-related loss of motor axon is attenuated in calorically restricted but not exercised mice. (C and D) Muscles from 5- and 24-mo-old mice were stained with DAPI to show nuclei. Central nuclei, which indicated degeneration and regeneration of the fiber, are indicated with arrows. (E) Percentage of muscle fiber cross-section with central nuclei. Age-related increase in frequency of centrally nucleated fibers is attenuated in calorically restricted but not exercised group mice. Each bar shows mean ± SEM from at least four mice. *P < 0.03 by t test. (Scale bar: 10 μm.)

Fig. 7. Time-lapse imaging reveals that exercise partially reverses age-related alterations in NMJ structure. (A–D) Pairs of micrographs of NMJs imaged twice at an approximately 35-d interval. (A) Control animal. Some AChR-rich regions disappeared (* or became fragmented (arrows) between views. (B–D) Animals exercised for 1 mo between views. New AChR-rich regions were added near (arrow in B) or between AChR clusters (small arrowhead in C and Left Insets). Some AChR fragments fused during the exercise period (large arrowheads in C and D). (E–G) Changes in NMJ morphology between views as rated by four individuals blind to which pairs of micrographs (such as those in A–C) were from control (Cont) and exercised mice (Ex). Thin lines show ratings of individuals, thick line is average of all four (n = 21 NMJs from four control and 32 NMJs from five exercised mice). (E) NMJs in which AChR-rich area increased or decreased (P < 0.005). (F) Percentage of NMJs in which fragmentation increased (P < 0.03). (G) Percentage of NMJs in which AChR-rich fragments fused (P < 0.02). P values are from paired t test. [Scale bars: 10 μm (A–C) and 6.7 μm (C Insets, D).]
Materials and Methods

Thy1-XFP transgenic mice were described previously (19). We obtained three sets of C57BL/6 mice from the National Institute of Aging: 4- to 5- and 22- to 24-mo-old mice that had been fed a standard diet ad libitum, and 24-mo-old mice that had been fed a calorically restricted diet (27). Some of the mice on a standard diet were given unlimited access to a running wheel for 30 d. Running distance was monitored electronically (Lafayette Instruments). Experiments were carried out under animal protocols approved by Harvard University and Salk Institute Animal Studies Committees.

For histological analysis, AChRs were stained with 5 μg/mL Alexa-594 conjugated BTX (Molecular Probes). Axons and nerve terminals were visualized by YFP or CFP expression in transgenic mice and by immunostaining with antibodies to axonal and synaptic vesicle components in wild-type mice. Sections of peripheral nerve were stained with antibodies to neurofilaments and S100. Sections of spinal cord were stained with antibodies to activated caspase 3. Axons in the peroneal nerve were analyzed by confocal microscopy. Methods for vital imaging of NMJs were modified from ref 37. Protocols and criteria for scoring age-related changes are detailed in SI Materials and Methods.

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