



Published in final edited form as:

*Exp Gerontol.* 2011 ; 46(2-3): 193–198. doi:10.1016/j.exger.2010.08.029.

## Age-associated alterations of neuromuscular junction

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### Abstract

Age-related loss of muscle mass and function greatly affects quality of life in the elderly population. Several hypotheses have been proposed but accumulating evidence point to alterations in neuromuscular system during aging as a key event that leads to functional denervation, muscle wasting, and weakness. Over the past few decades, age-associated degeneration of the neuromuscular junction (NMJ) and its components have been well documented. With advancing age, pre-terminal portions of motor axons exhibit regions of abnormal thinning, distension, and sprouting whereas postsynaptic endplates decrease in size, reduce in number, length, and density of postsynaptic folds. Although the exact underlying mechanisms are still lacking, recent studies provided direct evidence that age-associated increase in oxidative stress plays a crucial role in NMJ degeneration and progression of sarcopenia. Homozygous deletion of an important antioxidant enzyme, Cu,Zn superoxide dismutase (CuZnSOD, SOD1) leads to acceleration of age-dependent muscle atrophy, with a significant NMJ degeneration similar to that seen in old wild type sarcopenic animals. In this short review, we briefly summarize the current understanding of some of the cellular and molecular changes in the NMJ during aging and suggest a role for oxidative stress and mitochondrial dysfunction in age-related changes in the maintenance of neuromuscular innervation.

### 1. Introduction

Aging skeletal muscle is characterized by a progressive loss of muscle mass as well as a decrease in function (Narici and Maffulli, 2010). This age-acquired deficit known as sarcopenia, contributes profoundly to quality of life in the elderly and predisposes them to an increased risk of morbidity, disability, and mortality (Janssen et al., 2004). The etiology of sarcopenia is a multi-factorial process that involves both intrinsic and extrinsic factors. However, mounting evidence from both animal and human studies suggests that degeneration of motor neurons, followed by changes in structural and functional integrity of the neuromuscular junction (NMJ), functional denervation, and loss of motor units contribute significantly to the progression of skeletal muscle aging. Aging muscle fibers are

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known to undergo denervation and re-innervation cycles that lead to remodeling of the motor units. A preferential denervation of the fast-twitch fibers and re-innervation by axonal sprouting from slow motor neurons results in a conversion of type II fast fibers to type I slow fibers. When denervation outpaces re-innervation, a population of muscle fibers degenerates and atrophy occurs in the remaining fibers. Loss of muscle mass ultimately contributes to a compromised contractile function. Although the contribution of neuronal-induced alterations in aging skeletal muscle has been recognized for more than 40 years, the precise molecular mechanisms of age-associated deterioration in neuromuscular system have remained elusive. Recently, technical advances in imaging in combination with molecular genetics have identified key molecular players in development of age-associated alterations in the neuromuscular system and development of sarcopenia. In this short review, we will briefly summarize the recent literature on proposed mechanisms contributing to age-related changes in the neuromuscular innervation. We will also discuss the potential role of age-related changes in trophic factors, oxidative stress, and mitochondrial dysfunction during aging that might influence the maintenance of neuromuscular innervation.

## 2. Age-related changes in neuromuscular system

Age-associated degeneration of the neuromuscular junction (NMJ) is well documented in several animal model systems as well as in humans (Balice-Gordon, 1997; Courtney and Steinbach, 1981; Deschenes et al., 2010; Luff, 1998; McMullen and Andrade, 2009; Smith and Chapman, 1987; Wernig and Herrera, 1986). Early studies found that aging was associated with morphological changes that were confined primarily to nerve endings with little or no degeneration or loss of primary axons, suggesting that aging was associated with a functional denervation (Fujisawa, 1976; Gutmann and Hanzlikova, 1973). Subsequent studies revealed that the age-related changes in NMJ morphology vary among different muscle types and could potentially be related to muscle activity levels. For example, the number of nerve terminal branches per endplate and the number of terminal sprouts decrease in the extensor digitorum longus muscle (EDL) between 10 and 25 months of age in Fischer-344 rats (Rosenheimer and Smith, 1985) while this same group reported an increase in the number of endplates with ultra-terminal sprouts in the EDL (but not soleus or diaphragm) muscle in the old rats (Rosenheimer, 1990). These ultra-terminal sprouts originate from the endplate region and terminate on their own or in an adjacent muscle fiber and are often found associated with terminals that have characteristics similar to denervated fibers, such as an increase in endplate area per fiber and fewer nerve terminal branches. Another study in rat diaphragm from older rats (28 months) found no change in endplate size, but there were an increased number of nerve terminals per endplate, decreased sprouting, and an increased number of synaptic vesicles (Prakash and Sieck, 1998; Smith and Rosenheimer, 1982). Interestingly, while the nerve terminal area, endplate branching, and size were all increased in Type IIb and IIx muscle fibers of diaphragm in the 24-month-old rats, there was essentially no change in the NMJs on Type I and IIa muscle fibers with age in these rats, demonstrating that differences can also occur among fiber types within a single muscle. While there are considerable variations in age-related changes in postsynaptic endplate characteristics in different muscle types, changes in endplates also vary depending on the age of the animal examined, i.e., the relative percentage of the lifespan examined. For example, a study of endplate morphology in rat soleus muscle showed that endplates increased in size and complexity up to 18 months of age, but later (at 28 months of age and close to mean lifespan), size and complexity were dramatically decreased (Pestronk et al., 1980). Similar changes in NMJ morphology have been reported in mice during aging. In C57BL6 mice, it was reported that by 27 months only 40% of the NMJs appear normal compared to 85% in young mice (Ludatscher et al., 1985).

In the presynaptic terminal, age-related declines in synaptic vesicles, mitochondrial content, and nerve terminal area were reported in both soleus and EDL muscles from 29 month CBF-1 male mice compared to 7-month-old mice (Fahim and Robbins, 1982). In contrast, these investigators found an increased content of smooth endoplasmic reticulum (SER), an increase in the number of coated vesicles and increased cisternae in the old presynaptic terminals, which could be part of a compensatory response to increased vesicle recycling and to maintain adequate neurotransmitter levels. Alterations in these structures (i.e., mitochondria, SER and cisternae, and coated vesicles) could also potentially alter calcium sequestration and affect transmitter release (Fahim and Robbins, 1982). On the postsynaptic side, there was an increase in the number of branched subsynaptic folds, an increased number of subsarcolemmal vesicles, and increased lipofuscin deposits in the older mice. In the mouse EDL muscle, a comparison of 6- and 24-month-old C57BL6 mice showed an increase in the area, perimeter, and length of the nerve terminals, along with increased number of branches and increased sprouting (Fahim, 1993).

Along with changes in the morphology of the NMJ, age-related changes in the quantal content of neurotransmitter release have also been reported in mouse EDL and soleus muscle between 10 and 32 months of age in CBF-1 mice (Kelly and Robbins, 1983, 1986). These changes in quantal release, measured as increases in amplitude of evoked endplate potentials (EPP), were correlated with a decrease in the number of synaptic vesicles, a decrease in nerve terminal area, and a decrease in the number of post-synaptic folds lacking nerve terminals. The increase in transmitter release was associated with an increased turnover of neurotransmitter in muscle from old (28–30 month) compared to young (7–8 month) old mice and a 70% decrease in synaptic vesicle density in old CBF-1 mice (Fahim and Robbins, 1982; Kelly and Robbins, 1986).

It has been postulated that the age-related changes in the NMJ are also associated with alterations in axonal transport (Gutmann and Hanzlikova, 1973). A decline in axonal transport during aging could affect parameters such as availability of trophic factors as well as movement of organelles such as mitochondria that are critical for neuronal survival. Axonal transport was compared in young (7–8 month), middle-aged (19–20 month), and old (31–32 month) male Wistar rats by monitoring cholinesterase accumulation in sciatic nerve that was ligated upstream of the terminal. Cholinesterase accumulation was dramatically decreased in the older rats suggesting transport from the cell body was significantly reduced. The reduction in transport was not due to a reduction in axon number as determined by histological evaluation of axon numbers. Interestingly, the reduction in transport appeared to correlate with the extent of muscle atrophy seen in these rats (Gutmann and Hanzlikova, 1973; McMartin and O'Connor, 1979).

Changes in endplate morphology and NMJ remodeling are likely related to the changes in motor units that occur during aging. A study in soleus of 4- and 24-month-old rats found a significant age-related degeneration of endplates leading to a loss of muscle fibers and decrease in the size of motor units (Gutmann and Hanzlikova, 1966). A loss of muscle fibers and an increase in innervation ratio leading to a 30–40% loss in motor units and preferential loss of fast motor units were demonstrated in old rats as well as in humans (Edstrom and Larsson, 1987; Einsiedel and Luff, 1992a, b).

As imaging techniques have advanced over the last few decades, our understanding of neuromuscular synapse formation and molecular players that regulate its function have been greatly enhanced. Lichtman and Sanes have elegantly demonstrated the feasibility of *in vivo* live animal imaging, as opposed to imaging in fixed tissue, to monitor the neuromuscular junction in mouse sternomastoid muscle of over several months (Lichtman and Sanes, 2003; Rich and Lichtman, 1989). Balice-Gordon from same group applied these techniques to

monitor age-related changes in the same NMJs over time in C57BL6 mice (Balice-Gordon, 1997). They reported that following the developmental period of synapse elimination, NMJs were surprisingly stable over long period of time. However, when the animals reached the age of 12–18 months, a small percentage of NMJs started to display some loss of motor terminal branches and underlying acetylcholine receptors (AChR). In addition, the reported age-associated changes in NMJ morphology were remarkably similar to that of naturally occurring synapse elimination during development and synapse elimination following reinnervation (Balice-Gordon, 1997). Furthermore, these investigators found that motor axons appear to compensate for the loss of synaptic site by sprouting and adding new junctions. However this compensatory effect diminished dramatically as animals reached the age of 18 months and older. The newly formed junctions were also more vulnerable to degenerative changes and some of the junctions disappeared within weeks. By 24–36 months of age, the majority of NMJs were dissociated from presynaptic terminals and postsynaptic endplates were severely fragmented. In a single time-point study, our laboratory observed similar deteriorations in senescent (33 months) C57BL6 mice compared to younger (11 months) counterparts (Figure 1). By 33 months we found that over two-thirds of NMJs are either partially innervated or completely denervated with extensive sprouting of motor neurons. Moreover, we found that muscle atrophy and synaptic changes in aged muscles occur on a fiber-by-fiber basis, suggesting that denervation and alterations of NMJs are key factors in sarcopenia.

While progressive deterioration in both presynaptic motor neurons and postsynaptic muscle fibers likely contributes to denervation, other cell types such as terminal Schwann cells, which play an important role in axonal guidance and synaptic repair following denervation, may also contribute to a functional decline of the neuromuscular system in aging. Although there have been scarce reports suggesting degeneration of terminal Schwann cell during aging (Ludatscher et al., 1985), the exact mechanism of how terminal Schwann cells modulate denervation-reinnervation cycle in respect to aging have not been thoroughly investigated. It will be interesting to see if alterations in terminal Schwann cell and/or the trophic/growth factors secreted by Schwann cells contribute to functional decline in NMJ and skeletal muscle with age. In contrast to terminal Schwann cells, age-related changes in myelinated Schwann cells have been reported by several investigators. For example, collagen, lipid droplets, and oxidative damage in myelinated peripheral nerves have been reported to accumulate (Ceballos et al., 1999). Schwann cells associated with myelinated fibers decrease in number along with widening of the nodes of Ranvier during aging (Ceballos et al., 1999). Furthermore, two independent investigators have reported that the protein expression of major myelin proteins, P0, PMP22, and MBP significantly declined with age (Ceballos et al., 1999; Rangaraju et al., 2009). Interestingly, calorie restriction (CR), which has been shown to retard the aging process in multiple experimental model organisms, significantly attenuated age-related deterioration of peripheral nerve by up-regulating chaperones and lysosomal autophagic machinery (Rangaraju et al., 2009).

### **3. The role of trophic factors in maintenance of neuromuscular junction (NMJ) with age**

Interactions among presynaptic motor neuron, postsynaptic muscle, and Schwann cells play critical role in growth, maintenance, and survival of synapses. The exchange of trophic factors has been implicated in pre- and postsynaptic development as well as preserving neuronal and synaptic plasticity at the NMJ. Neurotrophins are known to participate in activity-induced modification of synaptic transmission (Schinder et al., 2000; Schinder and Poo, 2000). The exact role or the identity of neurotrophic and/or myotrophic factors that promotes survival and maintenance of presynaptic and postsynaptic apparatus at the NMJ in the context of aging have not been fully determined. However, recent studies indicate that a

variety of trophic factors such as brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), cytokines such as glial-derived neurotrophic factor (GDNF) and ciliary neurotrophin factor (CNTF), and other growth factors such as insulin-like growth factor (IGF-1 and IGF-II) and fibroblast growth factors (FGF) all play a modulatory role in neuromuscular system to a different extent during aging. For instance, BDNF has been shown to facilitate synaptic efficacy by increasing presynaptic depolarization at the NMJ (Huang and Reichardt, 2001). Furthermore, BDNF, NT-3, and NT-4 have been shown to be important factors for maintenance of AChR clustering in the NMJ (Belluardo et al., 2001; Gonzalez et al., 1999; Loeb et al., 2002). If neurotrophin signaling is interrupted or neurotrophin expression is suppressed, AChR is dispersed and NMJ is fragmented and affected muscles show increased fatigue (Gonzalez et al., 1999). Recently, Delbono's group using motor neuron-targeted IGF-1 tetanus toxin fusion proteins demonstrated that IGF-1 is a potent growth factor that can reverse age-related decline in specific force as well as improving morphological abnormalities at the NMJs (Payne et al., 2006). As mentioned earlier, loss of synaptic sites during aging remarkably resembles synapse elimination during development. Motor neurons are known to organize postsynaptic endplates by secreting two factors: a large proteoglycan called agrin and an epidermal growth factor (EGF) family protein, neuregulin. These proteins bind to their receptors on postsynaptic muscle fibers and induce clustering of acetylcholine receptor and gene transcription of specialized proteins that maintains synaptic transmission and structures (Sanes and Lichtman, 1999). Loss-of-function and gain-of-function studies using transgenic and knockout mouse in developmental synapse elimination have been characterized over the last two decades (Kummer et al., 2006; Sanes and Lichtman, 2001). However the exact functional role of the synapse regulatory proteins agrin and neuregulin in aging has been largely unknown. Interestingly, in recent years, neuregulin has been reported to be a myokine that exerts relevant effects on myogenesis and the regulation of muscle metabolism (Guma et al., 2010). The rapid and chronic metabolic effects of neuregulin appear to be related to muscle contraction and resemble those of exercise. Chronic exercise training has been shown to slow the aging process in muscle (Kim et al., 2008; Vandervoort, 2002). Therefore, it may be worthwhile to investigate neuregulin as therapeutic target against sarcopenia.

#### 4. Oxidative stress and mitochondrial dysfunction in neuromuscular innervation

The oxidative stress theory of aging predicts that the imbalance between oxidant generation and antioxidant defense mechanisms results in a steady-state accumulation of oxidative damage in a variety of macromolecules. Oxidative damage increases during aging, which results in a progressive loss in the functional efficiency of various cellular processes (Harman, 1956). Several laboratories, including ours, have tested the impact of oxidative stress on age-related muscle wasting (Jang et al., 2010; Mansouri et al., 2006; Weindruch, 1995). In order to test the direct cause and effect relation between oxidative stress and sarcopenia *in vivo*, we have utilized antioxidant enzyme deficient mouse models. In a recent report from our laboratory, we demonstrated that homozygous deletion of *Sod1* (Cu/Zn superoxide dismutase; CuZnSOD) leads to age-dependent muscle atrophy with alterations in NMJs that are similar to normal aging muscle but with a more frequent and earlier occurrence (Jang et al., 2010; Muller et al., 2006). The morphology of the NMJ at 11 months in *Sod1*<sup>-/-</sup> mice is comparable to NMJ in 33-month-old, wild-type mice (Figure 1), showing extensive sprouting, thinning of axon terminals, and fragmentation of postsynaptic AChRs. These data suggest that maintenance of NMJ during aging may be critically influenced by oxidative stress.

*Sod1*<sup>-/-</sup> mice exhibit several neuromuscular phenotypes that are similar to that of sarcopenia. For example, *Sod1*<sup>-/-</sup> mice exhibit a shift from fast to slow fiber type and a

grouping of slow fibers that may be the result of motor unit remodeling (Kostrominova et al., 2007). In addition, skeletal muscle of *Sod1*<sup>-/-</sup> mice shows age-associated mitochondrial dysfunction, which is also prevalent in senescent animals. Mitochondrial ROS generation is significantly increased compared to wild-type and further exacerbated with age in *Sod1* knockout muscle. H<sub>2</sub>O<sub>2</sub>, a stable membrane permeable ROS, has been shown to inhibit the quantal release of acetylcholine and the activity vesicle fusion protein SNAP25 at the NMJ is also inhibited by H<sub>2</sub>O<sub>2</sub> (Giniatullin et al., 2006). Moreover, both presynaptic terminal and postsynaptic endplate regions contain high concentrations of mitochondria. In presynaptic motor neuron, mitochondria travel along the microtubules using axonal transport to reach nerve terminals, whereas in postsynaptic muscle fiber, a subpopulation of mitochondria called subsarcolemma mitochondria reside around the NMJ and along the sarcolemma membrane. These mitochondria serve multiple functions such as energy support, regulation of calcium, synaptic transmission, and apoptosis. Not surprisingly, disruptions of mitochondria and their transport have been implicated in a wide variety of neurological diseases (Chan, 2006). Thus, it is plausible to hypothesize that mitochondrial dysfunction in the population of mitochondria associated with the NMJ may lead to altered calcium buffering and oxidative modification of key molecules in the NMJ and contribute to age-associated declines in neuromuscular innervation. In support of this, Zhou et al demonstrated that mitochondria adjacent to the AChR are selectively depolarized when muscle fibers are challenged by calcium in a mouse model of ALS, which exhibits significant neuromuscular degeneration (Zhou et al., 2010). In addition, other investigators have also shown that muscle-specific overexpression of uncoupling proteins (UCP1) significantly disrupted NMJ integrity. Furthermore, our laboratory has reported that isolated subsarcolemma mitochondria have significant deficits in ATP generation, oxygen consumption, and also generate more mitochondrial ROS compared to wild-type (Jang et al., 2010).

Mitochondria play a central role in buffering cytosolic Ca<sup>2+</sup> released by sarcoplasmic reticulum. Aging is shown to cause disruption in excitation-contraction coupling and a population of fast muscle fibers that become dependent on extracellular Ca<sup>2+</sup> to maintain tetanic force (Payne et al., 2004). Furthermore, increased cytosolic Ca<sup>2+</sup> may activate Ca<sup>2+</sup> dependent proteases such as calpain. Calpain has been shown to interact with the AChR clustering protein rapsyn and disrupt the AChR complex. Thus, the age-associated dispersal of AChR clusters maybe in part caused by Ca<sup>2+</sup> dysregulation and increased activation of calpain.

Oxidative stress may also exert its effect on peripheral motor axons. Indeed in a recent study, myelinated peripheral nerves were found to be oxidatively damaged during aging, and as a consequence, inflammation, increased accumulation of aggregated proteins, and lipofuscin adducts were found in the sciatic nerve of old animals. However, these age-related detrimental changes were significantly curtailed when rats were calorie restricted (Opalach et al., 2010). In our preliminary studies, we found similar protective effect against accelerated NMJ degeneration and muscle atrophy with lower levels of oxidative damage when *Sod1*<sup>-/-</sup> mice were maintained on a calorie restricted diet suggesting high oxidative stress *in vivo* directly affects neuromuscular maintenance.

## 5. Concluding remarks

Alterations in the neural control of skeletal muscle during aging are a crucial modifier and promoter of the muscle weakness and fatigue that occur in sarcopenia. Aging is complicated process involving multiple systems, but several lines of evidence, including our studies in the *Sod1*<sup>-/-</sup> mice mentioned above, point to an important role for oxidative stress in maintenance of NMJ during skeletal muscle aging. However, one needs to be cautious in

interpreting these data. In our study, we used conventional knockout mice that were created using homologous recombination. Therefore, the *Sod1* gene is deleted in every tissue and we cannot rule out the developmental defects due to high oxidative stress in these animals. In addition, it is unclear which cell type (motor neuron, muscle fibers, or Schwann cell) in the NMJ is most vulnerable to oxidative damage caused by *Sod1* deficiency. Currently, studies are underway in our laboratory to address these questions using tissue-specific and/or inducible *Cre-loxP* system that will allow us to direct the deletion to occur after development or in a tissue specific manner. Furthermore, studies aimed at determining whether oxidative stress *in vivo* alters the level of neurotrophic factors (i.e., BDNF, NT-4, and NT-3) or their receptors (p75 and Trk receptors) during aging will help us understand the mechanism of age-associated NMJ degeneration. Currently, very little is known about whether these trophic factors and their receptors vary with age at the NMJ. Interestingly, in the central nervous system (CNS) oxidative damage to neurotrophic factors have been implicated in age-related neurodegenerative disease such as Alzheimer and Parkinson's disease (Mattson, 2008). In summary, future studies addressing age-related changes in the regulation and maintenance of motor neuron terminals and neuromuscular junction components will allow us to develop and evaluate therapeutic strategies to attenuate, prevent, or ultimately reverse age-related muscle wasting and weakness.

## Acknowledgments

Supported by P01AG020591 (HVR) and a Julie Martin Mid-Career grant from the American Federation for Aging Research (HVR).

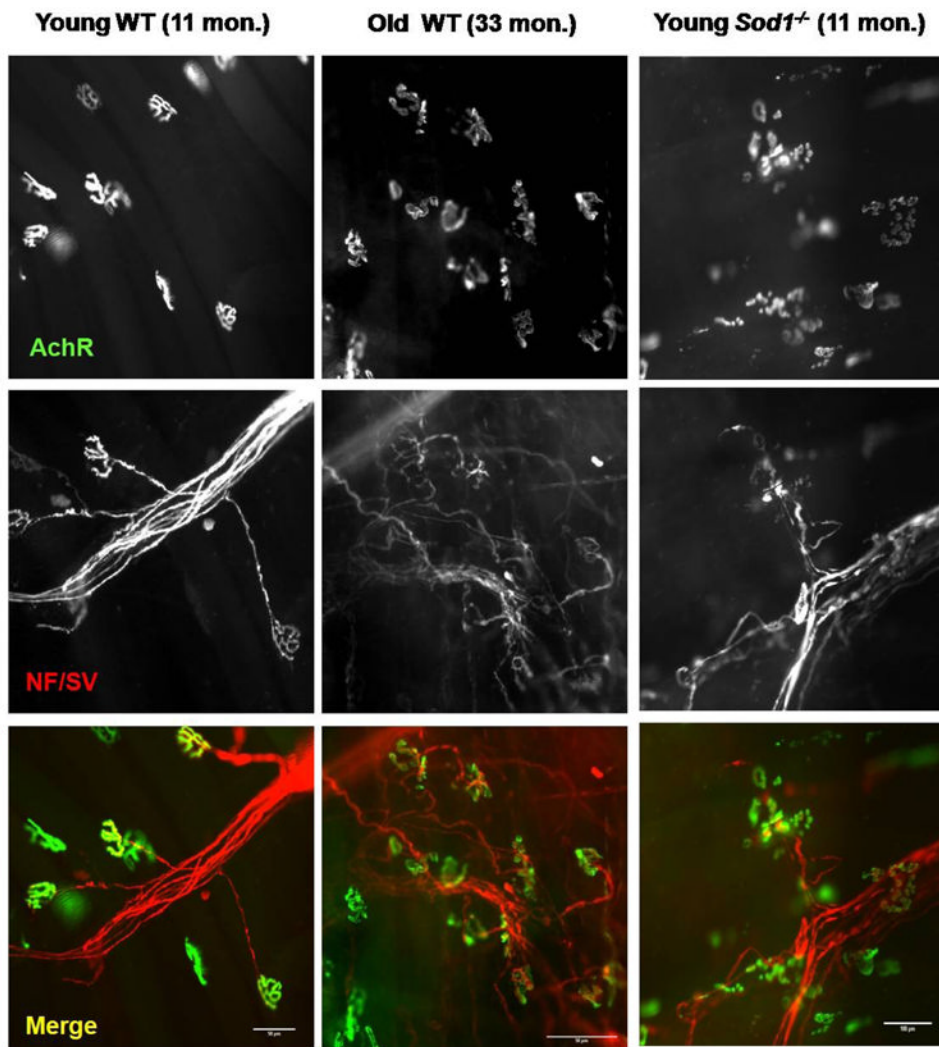
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**Figure 1.** A comparison of wild-type young (11 months), wild-type old (33 months), and young *Sod1<sup>-/-</sup>* (11 months) mouse neuromuscular junctions (NMJ) from gastrocnemius muscle. Motor axons and presynaptic terminals (NF/SV: pseudo-colored in red) were stained with anti-neurofilament and anti-synaptophysin then alexa-555 conjugated secondary antibodies. Acetylcholine receptors (AChR: pseudo-colored in green) were stained with alexa-488 conjugated  $\alpha$ -bugarotoxin (scale bar: 50 $\mu$ m).