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Expression and Regulation of Excitation-Contraction Coupling Proteins in Aging Skeletal Muscle

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Abstract

Functional and structural decline of the neuromuscular system is a recognized cause of decreased strength, impaired performance of daily living activities, and loss of independence in the elderly. However, in mammals, including humans, age-related loss of strength is greater than loss of muscle mass, so the underlying mechanisms remain only partially understood. This review focuses on the mechanisms underlying impaired skeletal muscle function with aging, including external calcium-dependent skeletal muscle contraction; increased voltage-sensitive calcium channel $Ca_v1.1$ β_{1a}-subunit and junctional face protein JP-45 and decreased $Ca_v1.1$ ($\alpha 1$) expression, and the potential role of these and other recently discovered molecules of the muscle T-tubule/sarcoplasmic reticulum junction in excitation-contraction uncoupling. We also examined neural influences and trophic factors, particularly insulin-like growth factor-I (IGF-1). Better insight into the triad proteins' involvement in muscle ECC and nerve/muscle interactions and regulation will lead to more rational interventions to delay or prevent muscle weakness with aging. The focus of this review is on the proteins mediating excitation-contraction coupling (ECC) and their expression and regulation in humans and rodent models of skeletal muscle functional decline with aging. Age-dependent changes in proteins other than those related to ECC, muscle composition, clinical assessment and interventions, have been extensively reviewed recently [1-3].

Keywords

Skeletal muscle; aging; sarcopentia; insulin-like growth factor 1; excitation-contraction coupling; denervation

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DISCLOSURE

It should be noted that the author has previously published much of the material covered in this review article in a book chapter titled 'Excitation-Contraction Coupling Regulation in Aging Skeletal Muscle' in the book titled 'Sarcopenia — Age-Related Muscle Wasting and Weakness'. Springer Science+Business Media B.V. ISBN: 978-90-481-9712-5. 113-134, 2011.

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DECREASED MUSCLE SPECIFIC FORCE IN MAMMALIAN SPECIES, INCLUDING HUMAN

The decline in muscular strength with age is caused largely by a loss of total muscle mass - but also a disproportionate loss of strength. Some studies in humans directly relate this diminished strength to muscle atrophy [4], while others find that it is greater than the decrease in muscle mass [5]. For example, the decline in normalized force (force/muscle mass, Nm/kg) in the knee extensors with aging has been found to follow a curvilinear relationship, starting at about 40 years and declining by about 28% from 40-49 to 70-79 years [5]. *In vitro* studies of single human muscle fiber contractility also reveal a decrease in specific force (force/cross-sectional area) with age [6]. Therefore, the intrinsic force-generating capacity of the skeletal muscle per contractile unit may be impaired in aging mammals, including humans. Postulated mechanisms include alterations to the excitation-contraction coupling (ECC) process [7-9] and decreased actin-myosin cross-bridge stability [10,11].

EXCITATION-CONTRACTION UNCOUPLING

The transduction of changes in sarcolemmal potential to elevated intracellular calcium concentration is a key event that precedes muscle contraction [12] (see Fig. 1). Electromechanical transduction in muscle cells requires the participation of the dihydropyridine receptor (DHPR) [13] located at the sarcolemmal transverse T-tubule. The DHPR is a multimeric voltage-gated L-type Ca^{2+} channel (dihydropyridine-sensitive). $Ca_v1.1$ (α1 subunit) activation evokes Ca^{2+} release from an intracellular store (sarcoplasmic reticulum, SR) through ryanodine-sensitive calcium channels (RyR1) into the myoplasm [14]. The functional consequence of the reduced number, function, or interaction of these receptors is reduced intracellular calcium mobilization and force development [15]. Calcium binds to troponin C, which by interaction with troponin I, T and tropomyosin, leads to crosslinkages between actin and myosin and sliding of thin-on-thick filaments to produce force [16]. Uncoupling of the excitation-contraction machinery is a major factor in age-dependent decline in the force- generating capacity of individual cells [17].

Aging muscle fibers exhibit less specific force than those from young-adult or middle-aged animals but similar endurance and recovery from fatigue [18-20]. Whether excitation-contraction uncoupling (ECU) results from altered neural control of muscle gene expression is not known. However, a series of studies support this concept. First, denervation results in a significant decrease in DHPR functional expression and alterations in ECC in skeletal muscle from adult rats [21]. Second, nerve crush leads to reduced levels of mRNA-encoding DHPR subunits and RyR1 in muscle [22], and studies show that both DHPR and RyR1 expression depend on skeletal muscle innervation [23,24]. Third, during development, DHPR mRNA levels change in relation to fiber innervation [25]. Fourth, myotube depolarization triggers the appearance of (+)-[^{3}H]PN 200-110 binding sites [26]. Finally, exercise and chronic stimulation *in vivo* increase DHPR expression in homogenates of soleus and extensor digitorum longus (EDL) muscles [27,28]. Thus, fiber-type composition, DHPR and RyR1, and ECC seem to depend on nerve stimulation and muscle activity.

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We are starting to understand how nerve stimulation of muscle activity influences muscle phenotype and the specific sarcolemmal-nuclear signaling pathways involved in muscle gene expression at different ages. Increasing evidence points to a decline in neural influence on skeletal muscle at later ages [29], leading to changes in muscle composition that result in ECU [11].

CHANGES IN SKELETAL MUSCLE INNERVATION AND PLASTICITY OF THE NEUROMUSCULAR JUNCTION WITH AGING INFLUENCE ECC

Muscle weakness in aging mammals may result from primary neural or muscular etiological factors or a combination [30]. Experimental muscle denervation leads to loss in absolute and specific force [31,32]. Although denervation contributes to the functional impairment of skeletal muscle with aging [33], its prevalence in human and animal models of aging remains to be determined.

Some studies have focused on the mechanisms underlying neuromuscular impairments in old age. Several aspects have been investigated: the phenomenon known as ECU [7,34], which leads to a decline in muscle specific force (force normalized to a cross-sectional area) [35]; the loss in muscle mass associated with a decrease in muscle fibers as well as fiber atrophy [36,37]; changes in fiber type [29, 38-40]; decreased maximal isometric force and slower sliding speed of actin on myosin [41,42]; and impaired recovery after eccentric contraction [43,44]. Identifying the triggers of these changes remains elusive. Some suggestions include decreased muscle loading [45], oxidative damage [46,47], age-dependent decrease in IGF-1 expression or tissue sensitivity [48-50], and decline in satellite cell proliferation [51].

Interaction between skeletal muscle and neuron is crucial to the capacity of both to survive and function throughout life. Thus, muscle atrophy and weakness may result from primary neural or muscular etiological factors or a combination. Growing evidence supports a role for the nervous system in age-related structural and functional alterations in skeletal muscle [52]. The number of motor neurons in the lumbosacral spinal cord of humans has been shown to decrease after the age of 60, and the number of large and intermediate-sized myelinated axon fibers decreases with age in the ventral roots with no change in small fiber numbers [30, 53,54].

Motor units decrease with motor neurons, as measured with electromyography in humans and *in situ* calculation in rats. As with motor neuron fibers, the loss of motor units seems to be greatest among the largest and fastest. A decline in the number and size of anterior horn cells in the cervical and lumbosacral spinal cord and cytons in motor neuron columns in the lumbar spinal cord in humans with age has been reported [55]. These studies found fewer large and intermediate-diameter cytons, which are the largest and fastest motor neurons [56,57]. In fact, aged motor units exhibit increased amplitude and duration of action potentials, supporting the idea that those remaining grow larger [33, 58]. Morphological evidence of this process can be found in the muscle. Fiber loss and atrophy with age is greatest among fast type-2 fibers, a finding that agrees with the loss of large and intermediate-sized motor neuron fibers and large motor units. Fiber type “grouping” has

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been found in human muscle with age, indicating a denervation/re-innervation process [30]. More direct evidence of a slow denervation process with aging is provided by the increased prevalence of old muscle fibers staining positive for neural cell adhesion molecule [59].

Overall fiber loss and a preferential decrease in type-2 fiber number and size in mixed fiber-type muscles, such as the vastus lateralis, is observed with aging (for a review see [30]). However, all lower limb muscles may not respond similarly to aging. The tibialis anterior, a predominantly type-2 muscle, has been shown to decrease, with compensatory hypertrophy in the remaining fibers to maintain overall muscle size (Lexell, unpublished results). Conversely, a recent report documents preferential atrophy of type-2 fibers in biceps brachii, an upper limb muscle, but not reduced numbers. This finding is consistent with clinical studies showing better preservation of upper limb muscle function with age [11].

Several groups have reported skeletal muscle denervation and reinervation and motor unit remodeling or loss in aging rodents or humans [57, 60-65]. Motor-unit remodeling leads to changes in fiber-type composition [66]. During development, muscle fiber-type phenotype is determined by interactions with subpopulations of ventral spinal cord motor neurons that activate contraction at different rates, ranging from 10 (slow fibers) to 100 (fast-fatigue resistant) or 150 Hz (fast-fatigue sensitive, limb muscles) [67-69]. Age-related motor-unit remodeling appears to involve denervation of fast muscle fibers with re-innervation by axonal sprouting from slow fibers [36, 58, 70, 71]. When denervation outpaces reinnervation, a population of muscle fibers becomes atrophic and is functionally excluded. Although denervation contributes to skeletal muscle atrophy and functional impairment with aging [33], its time course and prevalence in human and animal models of aging remain to be determined. Urbancheck *et al.* (2001) analyzed the contribution of denervation to deficits in specific force in skeletal muscle in 27-29-month (old) compared with 3-month (young) rats [59]. Contraction force recordings together with muscle immunostaining for NCAM (neural cell adhesion molecule), a marker of fiber denervation [72,73], showed a significantly higher number of denervated fibers in old rats. The area of denervated fibers detected by positive staining with NCAM antibodies accounts for a significant fraction of the decline in specific force [59].

We hypothesized that denervation in aging skeletal muscle is more extensive than predicted by standard functional and structural assays and asked whether it is a fully or partially developed process. To address these two questions, we combined electrophysiological and immunohisto-chemical assays to detect the expression of tetrodotoxin (TTX)-resistant sodium channels ($Na_V1.5$) in flexor digitorum brevis (FDB) muscles from young-adult and senescent mice. The FDB muscle was selected for its fast fiber-type composition (~70% type IIx, 13% IIa, and 17% type I) [74] and because the shortness of the fibers makes them suitable for patch-clamp recordings [75].

Two sodium channel isoforms are expressed in skeletal muscle, the TTX-sensitive $Na_V1.4$ and the TTX-resistant $Na_V1.5$. Both were originally isolated from rat skeletal muscle and denominated SkM1 [76] and SkM2 [77], respectively. To determine the status of denervation of individual fibers from adult and senescent mice, we took advantage of the following properties of the $Na_V1.5$ channel: (1) its expression after denervation but absence in

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innervated adult muscle; (2) its early increase in expression, recorded 24 h after denervation in hindlimb muscles [78]; and (3) its relative insensitivity to TTX [77, 79-81].

Sodium current density measured with the macropatch cell-attached technique did not show significant differences between FDB fibers from young and old mice. The TTX dose-response curve, using the whole cell voltage-clamp technique, showed three populations of fibers in senescent mice, one similar to fibers from young mice (TTX-sensitive), another similar to fibers from experimentally denervated muscle (TTX-resistance), and a third intermediate group. Partially and fully denervated fibers constituted approximately 50% of the total number of fibers tested, which agrees with the percent of fibers shown to be positive for the $Na_V1.5$ channel by specific immunostaining [75]. These results confirmed our hypothesis that muscle denervation is more extensive than that reported using more classical techniques.

Recovery from denervation implies nerve sprouting and re-innervation by the same or neighboring motor units. Different methods of inducing transient nerve injury and recovery have been employed with contrasting results. Slower regeneration and reinnervation in aged compared to young motor endplates was recorded in response to crush injury of the peripheral nerve [52, 82]. The difference in the time needed to recover was attributed to a transient failure in the spatiotemporal relationship between Schwann cells, axons, and the postsynaptic acetylcholine receptor regions during reinnervation in aged rats [82]; that is, nerve/muscle interactions contribute significantly to impaired recovery after nerve injury in the aged.

However, in apparent contrast, a comparable capacity for regeneration has been shown in muscles from very old compared to young rats [83]. Effects of age on muscle regeneration were studied by injecting the local anesthetic, bupivacaine, in fast-twitch muscles. It induced similar muscle fiber damage and reduced the mean tetanic tension in fast-twitch muscles from young adult (4-month) and old (32- and 34-month) rats. The same authors investigated muscle regeneration using heterochronic transplantation of nerve-intact EDL, a fast-twitch muscle. EDL muscles from 4- or 32-month-old rats were cross-transplanted in place of the same muscle in 4-month-old hosts. As a control, contralateral muscles were autotransplanted back into the donors. After 60 days, the old-into-young muscle transplants regenerated as successfully as the young-into-young autotransplants. Lack of nerve damage provided favorable conditions for muscle regeneration, together with an age-related effect of the local environment on the transplants [83].

As evidence of the importance of neural factors in nerve regeneration, the same group reported that when axons are allowed to regenerate in an endoneurial environment, there is no evidence of age-related impairment in muscle reinnervation [84]. Therefore, although old muscle can regenerate as successfully as young muscle, an intact nerve supply seems critical to recovery, together with less clearly defined factors associated with the local environment. We believe IGF-1 secretion and signaling is vital for the protection of nerve and muscle from age-related degeneration.

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Neural alterations occur at the ventral spinal cord motor neuron, peripheral nerve, and neuromuscular junction in aging mammals. Age-related changes have been documented in neuronal soma size [56, 85] and number [55, 57, 65] in the spinal cord and in peripheral nerve in tibialis nerves of mice aged 6-33 months [53], including accumulation of collagen in the perineurium and lipid droplets in the perineurial cells, together with an increase in macrophages and mast cells. From 6 to 12 months, numbers of Schwann cells associated with myelinated fibers (MF) decrease slightly in parallel with an increase in their internodal length, but then increase in older nerves in parallel with a greater incidence of demyelination and remyelination. The reported unmyelinated axon (UA) to myelinated fiber (UA/MF) ratio is about 2 until 12 months, decreasing to 1.6 by 27 months. In older mice, the loss of nerve fibers involves UA (50% loss at 27-33 months) more than MF (35%). In aged nerves, wide incisures and infolded or outfolded myelin loops are frequent, resulting in an increased irregularity in the morphology of fibers along the internodes [53].

In summary, adult mouse nerves (12-20 month) show several features of progressive degeneration, whereas general nerve disorganization and marked fiber loss occur from 20 months on [53]. The deterioration of myelin sheaths during aging may be due to decreased expression of the major myelin proteins (P0, PMP22, MBP). Axonal atrophy, frequently seen in aged nerves, may be explained by reduced expression and axonal transport of cytoskeletal proteins in the peripheral nerve [54]. The incidence and severity of the age-related peripheral nerve changes seem to depend on the animal's genetic background. Thus, histological examination conducted on isolated sciatic nerves and brachial plexuses revealed more pronounced axonal degeneration and remyelination in B6C3F1 and C3H than in C57BL mice [86]. Impaired nerve regeneration in animals and humans has been correlated with diminished anterograde and retrograde axonal transport [87], and retardation in the slow axonal transport of cytoskeletal elements during maturation and aging has been reported [88,89]. This reduced axonal transport could account for the inability of the motor neuron in old mice to expand the field of innervation in response to partial denervation [90].

Alterations of the neuromuscular junction in association with aging have been attributed to its "instability" [91]. The process of neuromuscular synapse formation and activity-dependent editing of neuromuscular synaptic connections is better understood [92] than the events leading to denervation in aging mammals. Apparently, after synapse formation, the terminals of the same axon, described as a *cartel*, exhibit heterogeneity in terms of acetylcholine release, which may contribute to nerve terminal selection in the developmental transition from innervation of each muscle fiber by multiple nerve endings to the adult one-on-one pattern. Activity plays a crucial role in synapse elimination during this period (for a review see [92]). These concepts prompt the interesting hypothesis that senescent mammals retain a similar mechanism for eliminating neuromuscular synapse. The level of physical activity among the elderly is highly variable and considered important for successful neuromuscular function. Endurance exercise modulates the neuromuscular junction of C57BL/6NNia aging mice [93]. When synaptic terminals occupying motor endplates in adult rats were electrically silenced by the sodium channel blocker tetrodotoxin or the acetylcholine receptor blocker α-bungarotoxin, regenerating axons that were both inactive and synaptically ineffective frequently displaced them. This study concludes that neither evoked nor spontaneous activation of acetylcholine receptors is required for competitive

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re-occupation of neuromuscular synaptic sites by regenerating motor axons in adult rats [94].

Experimental denervation of skeletal muscle from aging rodents leads to a series of changes, such as re-orientation of costameres (rib-like structures formed by dystrophin and β-dystroglycan) [95,96], proliferation of triadic membranes [97], decrease in charge movement (functional expression of the DHPR voltage sensor), and alterations in the SR calcium-release channel [9, 15, 21 98, 99]. The molecular substrate for these alterations is only partially understood. We hypothesize that age-related denervation may induce these structural and functional changes in mammalian, including human, muscle. Costameric proteins transmit mechanical lateral forces and provide structural integrity when mechanically loaded muscle fibers contract [100]. Muscle activity and muscle agrin, two orders of magnitude lower than the effective concentration of neural agrin, regulate the organization of cytoskeletal proteins in skeletal muscle fibers [95]. It would be interesting to explore these molecular changes in aging muscle and examine the potential beneficial effect of muscle agrin on costamere structure and force development. The studies reported above strongly involve neural alterations in the onset and progression of age-related decline in skeletal muscle function.

TROPHIC FACTORS REGULATE SPINAL CORD MOTOR NEURON STRUCTURE AND FUNCTION AND ECC

Target-derived neurotrophic factors, including the neurotrophin, and nerve growth factor have a well-established role in regulating survival of developing neurons in the peripheral and central nervous systems [101,102]. Some other studies point to a continued role for target-derived trophic factors in the plasticity of adult and aged neurons [103,104]. A series of studies suggest a role for neurotrophins, at least, in the adult neuromuscular system. Neural activity appears to contribute significantly to the trophic interactions between nerve and muscle at the adult neuromuscular junction. Neurotrophins regulate the development of synaptic function [105], and participate in activity-induced modification of synaptic transmission [106]. Potentiation of synaptic efficacy by brain-derived neurotrophic factor is facilitated by presynaptic depolarization at developing neuromuscular synapses [107,108]. Using a nerve/muscle co-culture in which neurotrophin-4 (NT-4) is overexpressed in a subpopulation of postsynaptic myocytes, presynaptic potentiation was restricted to synapses on myocytes overexpressing NT-4. Nearby synapses formed by the same neuron on control myocytes were not affected [109]. Furthermore, the production of endogenous NT-4 messenger RNA in rat skeletal muscle was regulated by muscle activity; the amount of NT-4 mRNA decreased after blocking neuromuscular transmission with alpha-bungarotoxin and increased during postnatal development and after electrical stimulation. Finally, NT-4 may mediate the effects of exercise and electrical stimulation on neuromuscular performance [110]. Thus, muscle-derived NT-4 appears to act as an activity-dependent, muscle-derived neurotrophic signal for the growth and remodeling of the adult neuromuscular junction.

These investigations of the complex role of neural activity in regulating nerve-target interactions have not extended to the aging neuromuscular junction. However, a close

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correlation between altered ligand-receptor expression(s) and axonal/terminal aberrations in senescence supports a role for neurotrophin signaling in age-related degeneration of cutaneous innervation [111]. An age-related decrease in target neurotrophin expression, notably NT3 and NT4, correlated with site-specific loss of sensory terminals combined with aberrant growth of regenerating/sprouting axons into new target fields [111].

The role of IGF-1 and related binding proteins in neural control of aging skeletal muscle ECC and fiber-type composition in mammals is under investigation. Systemic overexpression of human IGF-1 cDNA in transgenic mice resulted in IGF-1 overexpression in a broad range of visceral organs and increased concentrations in serum [112]. These mice exhibited increased body weight but only a modest improvement in muscle mass.

Because of the possible confounding effects of systemic expression, Coleman *et al.* targeted IGF-1 overexpression specifically to striated muscle [113] using a myogenic expression vector containing regulatory elements from both the 5′- and 3′-flanking regions of the avian skeletal α-actin gene. IGF-1 overexpression in cultured muscle cells causes precocious alignment and fusion of myoblasts into terminally differentiated myotubes and elevated levels of myogenic basic helix-loop-helix factors, intermediate filament, and contractile protein mRNA [113]. Transgenic mice carrying a single copy of the hybrid skeletal α-actin/hIGF-1 transgene had hIGF-1 mRNA levels that were approximately half those of the endogenous murine skeletal α-actin gene on a per-allele basis but conferred substantial tissue-specific overexpression without elevating serum levels of IGF-1. This localized, muscle-specific overexpression of human IGF-1 caused significant hypertrophy of myofibers, suggesting that IGF-1 is a more potent myogenic stimulus when derived from sustained autocrine/paracrine release than when administrated exogenously. Similar hypertrophy has been observed in response to simple intramuscular injections of IGF-1 in adult rats [114].

Effects of IGF-1 on muscle in aging animals have also been investigated. In old mice, muscle-specific overexpression of IGF-1 preserves skeletal muscle force and DHPR expression [8, 115], while viral-mediated, muscle-specific expression prevents age-related loss of type-IIB fibers [116]. There is evidence that the capacity of IGF-1 to induce muscle hypertrophy declines in adult and senescent mice [117]. However, its effects on fiber specific force are sustained until late ages [118], suggesting that the pathways it uses to control fiber size and to generate force diverge. Overexpression of the mIGF-1 isoform, corresponding to the human IGF-1Ea gene, resulted in sustained mouse muscle hypertrophy and regenerative capacity throughout life (Musaro *et al.*, 2001), indicating that this muscle-specific splice variant of the IGF-1 gene plays a different role in muscle molecular composition and function than the other IGF-1 splice variants.

We tested the hypothesis that target-derived IGF-1 prevents alterations in neuromuscular innervation in aging mammals [29]. We used senescent wild-type mice as a model of deficient IGF-1 secretion and signaling and S1S2 transgenic mice to investigate the role sustained IGF-1 overexpression in striated muscle plays in neuromuscular innervation. Analysis of the nerve terminal in EDL muscles from senescent mice showed that sustained overexpression of IGF-1 in skeletal muscle partially or completely reversed the decrease

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in cholinesterase-stained zones (CSZ) exhibiting nerve terminal branching, number of nerve branches at the CSZ, and nerve branch points. Target-derived IGF-1 also prevented age-related decreases in the postterminal α-bungarotoxin immunostained area. Postsynaptic folds were fewer and longer as shown by electron microscopy.

Transgenic overexpression of IGF-1 in skeletal muscle may also prevent the switch in muscle fiber-type composition recorded in senescent mice. The use of the S1S2 IGF-1 transgenic mouse model allowed us to provide morphological evidence for the role of target-derived IGF-1 in spinal cord motor neurons in senescent mice. The main conclusion of this study was that muscle IGF-1 prevents age-dependent changes in nerve terminal and neuromuscular junction, influencing muscle fiber-type composition and, potentially, muscle function [17, 115, 116].

The role of IGF-1 in motor neuron survival has been examined during embryonic or postnatal life [119] as well as in spinal cord pathology [120-122]. For example, in young rodents, IGF-1 expression is upregulated in Schwann cells and astrocytes following spinal cord and peripheral nerve injury, while IGF-binding protein 6 is strongly upregulated in the injured motor neurons [123]. In regions of muscle enriched with neuromuscular junctions, IGF-II was strongly upregulated in satellite and possibly glial cells during recovery from sciatic nerve crush [124] while IGF-1 showed less significant changes. In young animals, systemic administration of IGF-1 decreases lesion-induced motor neuron cell death and promotes muscle reinnervation [125]. It also promotes neurogenesis and synaptogenesis in diverse areas of the central nervous system, such as the hypocampal dentate gyrus during postnatal development [126], and increases proliferation of granule cell progenitors [127].

These studies suggest that IGF-1 might have beneficial effects on spinal cord motor neurons from senescent mammals. However, transgenic overexpression of IGF-1 in the central nervous system does not improve ECC or neuromuscular performance in the mouse [127,128]. In contrast to localized motor neuron expression, widespread IGF-1 may be deleterious for neuronal function or muscle innervation [128].

During embryonic and postnatal development, specific sets of CNS neurons show high levels of IGF-1 receptor gene expression combined with IGF-1 expression, while in hippocampal and cortical neurons, receptor and IGF-1 expression are localized in different cell groups [129]. These expression patterns suggest that IGF-1 exerts autocrine and paracrine effects in the CNS in addition to its previously described paracrine (muscle-derived) actions on spinal cord motor neurons. While these mechanisms contribute undoubtedly to the development of the appropriate neuronal phenotype and probably to its maintenance in adulthood, its involvement in aging processes remains substantially untested. Despite these uncertainties, an age-related decline in neuronal as well as muscle-derived IGF-1 combined with altered IGF-1 resistance through reduced expression or sensitivity of the receptor may contribute to the atrophy or death of motor and other CNS neurons in aging mammals. Through the previously described mechanisms, these changes may trigger a cascade of events leading to decreased skeletal muscle gene transcription.

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IGF-1 REGULATES SKELETAL MUSCLE ECC

IGF-1 may affect functional interactions between nerve and muscle by regulating transcription of the $Ca_v1.1$ gene [130]. Although the $Ca_v1.1$ subunit is critical to ECC, the basic mechanisms regulating its gene expression are unknown. To understand them, we isolated and sequenced the 1.2-kb 5' flanking-region fragment immediately upstream of the mouse DHPRα_{1S} gene [131]. Luciferase reporter constructs driven by different promoter regions of that gene were used for transient transfection assays in muscle C2C12 cells. We found that three regions, corresponding to the CREB, GATA-2, and SOX-5 consensus sequences within this flanking region, are important for DHPRα_{1S} gene transcription, and antisense oligonucleotides against them significantly reduced charge movement in C2C12 cells [131]. This study demonstrates that the transcription factors CREB, GATA-2, and SOX-5 play a significant role in the expression of skeletal muscle DHPRα_{1S}.

Whether IGF-1 regulates these transcription factors and subsequent expression of the $Ca_v1.1$ gene is not known. Using an approach similar to that described above [131], we investigated the effects of IGF-1 on various promoter deletion/luciferase reporter constructs. They were transfected into C2C12 cells, and IGF-I effects were measured by recording luciferase activity. IGF-I significantly enhanced DHPRα_{1S} transcription, carrying the CREB binding site but not in CREB core binding site mutants. A gel mobility shift assay using a double-stranded oligonucleotide for the CREB site in the promoter region and competition experiments with excess unlabeled or mutated promoter oligonucleotide and unlabeled consensus CREB oligonucleotide indicate that IGF-1 induces CREB binding to the DHPRα_{1S} promoter. We prevented IGF-1 from mediating enhanced charge movement by incubating the cells with antisense but not sense oligonucleotides against CREB. These preliminary results support the conclusion that IGF-1 regulates $Ca_v1.1$ transcription in muscle cells by acting on the CREB element of the promoter [130]. Confirming these results in skeletal muscle will be important as well as determining whether IGF-1/CREB signaling and the signaling pathway linking IGF-1R to CREB activation is preserved in aging mammals. We hypothesize that these effects are mediated by the direct action of IGF-1 on muscle cells, perhaps *via* activation of satellite cells (Barton-Davis *et al.*, 1998), but may involve neuronal access to muscle-derived IGF-1.

Muscle IGF-1 is known to have trophic effects on motor neurons [29], so its overexpression is effective in delaying or preventing the deleterious effects of aging in both tissues. Since age-related decline in muscle function stems partly from motor neuron loss, we created a tetanus toxin fragment-C (TTC) fusion protein to target IGF-1 to motor neurons. IGF-1-TTC was shown to retain IGF-1 activity as indicated by [^{3}H]thymidine incorporation into L6 myoblasts. Spinal cord motor neurons effectively bound and internalized the IGF-1-TTC *in vitro*. Similarly, IGF-1-TTC injected into skeletal muscles was taken up and transported back to the spinal cord *in vivo*, a process that could be prevented by denervation of the injected muscles. Three monthly IGF-1-TTC injections into muscles of aging mice did not increase muscle weight or fiber size but significantly increased single fiber specific force over aged controls injected with saline, IGF-1, or TTC. None of the injections changed muscle fiber-type composition, but neuromuscular junction postterminals were larger and more complex in muscle fibers injected with IGF-1-TTC compared to the other groups,

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suggesting preservation of muscle fiber innervation. This work demonstrates that induced overexpression of IGF-1 in spinal cord motor neurons of aging mice prevents muscle fiber specific force decline, a hallmark of aging skeletal muscle [132].

EXTERNAL Ca^{2+}-DEPENDENT CONTRACTION IN AGING SKELETAL MUSCLE

We have demonstrated that a population of fast muscle fibers from aging mice depends on external Ca^{2+} to maintain tetanic force during repeated contractions [133]. We hypothesized that age-related denervation in muscle fibers plays a role in initiating this contractile deficit and that preventing denervation by IGF-1 overexpression would prevent external Ca^{2+}-dependent contraction in aging mice, which was true. To determine whether IGF-1 overexpression affects muscle or nerve, aging mice were injected with a tetanus toxin fragment-C (TTC) fusion protein that targets IGF-1 to spinal cord motor neurons, and this treatment prevented external Ca^{2+}-dependent contraction. We also showed that injections of the IGF-1-TTC fusion protein prevented age-related alterations to the nerve terminals at the neuromuscular junctions. We conclude that the slow, age-related denervation of fast muscle fibers is responsible for dependence on external Ca^{2+} to maintain tetanic force in a population of muscle fibers from senescent mice [134].

More recently, we examined the role of extracellular Ca^{2+}, voltage-induced influx of external Ca^{2+} ions, SR Ca^{2+} depletion during repeated contractions, store-operated Ca^{2+} entry (SOCE), SR ultrastructure, SR subdomain localization of the ryanodine receptor, and sarcolemmal excitability in muscle force decline with aging. These experiments demonstrated that external Ca^{2+}, but not Ca^{2+} influx, is needed to maintain fiber force with repeated electrical stimulation. Decline in fiber force is associated with depressed SR Ca^{2+} release. SR Ca^{2+} depletion, SOCE, and the putative segregated Ca^{2+} release store do not play a significant role in external Ca^{2+}-dependent contraction. Note that a significant number of action potentials fail in senescent mouse muscle fibers subjected to a high stimulation frequency. These results indicate that failure to generate action potentials accounts for decreased intracellular Ca^{2+} mobilization and tetanic force in aging muscle exposed to a Ca^{2+}-free medium [135].

THE SR JUNCTIONAL FACE MEMBRANE PROTEIN JP-45 PLAYS A ROLE IN SKELETAL MUSCLE ECU WITH AGING

JP-45 has been reported exclusively in skeletal muscle, and its expression decreases with age [136]. It colocalizes with the Ca^{2+}-release channel (the ryanodine receptor) and interacts with calsequestrin and the skeletal muscle $Ca_v1.1$ [137]. We identified the JP-45 domains and the $Ca_v1.1$ involved in this interaction and investigated the functional effect of JP-45 on ECC. Its cytoplasmic domain, comprising residues 1-80, interacts with two distinct and functionally relevant domains of DHPRα1 subunit, the I-II loop and the C-terminal region. Interaction with the I-II loop occurs through the loop's α-interacting domain. A DHPR subunit, $\beta 1\alpha$, also interacts with the cytosolic domain of JP-45, drasically reducing the interaction between JP-45 and the I-II loop (Fig. 1).

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The functional effect of JP-45 on DHPRα1 subunit activity was assessed by investigating charge movement in differentiated C2C12 myotubes after overexpressing or depleting JP-45. Overexpression decreased peak charge-movement and shifted VQ1/2 to a more negative potential (−10 mV). Depletion decreased both the amount of DHPRα1 subunit and peak charge-movements. These results demonstrated that JP-45 is important for functional expression of voltage-dependent Ca^{2+} channels [137].

Another recent study demonstrates that deleting the gene that encodes JP-45 results in decreased muscle strength in young mice by decreasing functional expression of the $Ca_v1.1$ subunit, the molecule that couples membrane depolarization and calcium release from the SR. These results point to JP-45 as one of the molecules involved in the development or maintenance of skeletal muscle strength [138]. Whether JP-45 is modulated by neural activity and/or trophic factors is unknown.

In the last decade, a series of triad proteins have been identified, including mitsugumin-29 [139,140], junctophilin [141], SRP-27/TRIC-A [142-144], and junctate/hambug [145]. However, their role in ECC is only partially understood [146], and nerve-dependent regulation of their expression is unknown.

INCREASED CA_vB_{1A} EXPRESSION WITH AGING CONTRIBUTES SKELETAL MUSCLE WEAKNESS

Loss of specific force in old age [35, 147] is characterized in part by a deficit in Ca^{2+} release following depolarization [7, 148]. Excitation-contration uncoupling is not a result of decreased Ca^{2+} stores or RyR release function [148], and therefore may be caused by alterations in the functionality and expression of DHPR and its subunits with aging. The primary DHPR subunit in skeletal muscle is $Ca_v1.1$, previously known as $DHPR\alpha_{1S}$ [149]. $Ca_v1.1$ is a large transmembrane protein, which contains both the Ca^{2+} conducting pore and the voltage sensing S4 domain. Auxiliary subunits (α2/δ, β1a and γ) bind $Ca_v1.1$ to make up DHPR (for review, see [150]), with the most widely studied being the cytosolic $Ca_v\beta_{1a}$ subunit. $Ca_v\beta_{1a}$, a muscle specific member of the $Ca_v\beta$ family of proteins, binds to a region of the I-II intracellular loop of $Ca_v1.1$ known as the alpha interaction domain (AID) [151]. $Ca_v\beta_{1a}$ is classically described by its role in chaperoning $Ca_v1.1$ to the plasma membrane and regulating L-type Ca^{2+} current [152-155]. Most notably, E-C coupling cannot occur without $Ca_v\beta_{1a}$ [152]. $Ca_v\beta_{1a}$ binds to charged residues on RyR [156] and neutralization of these residues reduces ECC, suggesting a direct interaction with RyR. The correct organization of $Ca_v1.1$ into tetrads within the t-tubule membrane is also a specific function of the $Ca_v\beta_{1a}$ isoform [157].

Although classically known for augmenting the expression and function of Ca_v1 subfamily of calcium channels the $Ca_v\beta$ family of subunits may contribute to the down-regulation of Ca_v1 as well. A family of Ras-related G-proteins (RGKs) mediates the down-regulation of several Ca_v1 isoforms in a $Ca_v\beta$ dependent manner [158]. Additionally, the previously uncharacterized SH3 domain of $Ca_v\beta$ was shown to bind dynamin and mediate endocytosis of $Ca_v1.2$ [159]. As previous studies have shown that the $Ca_v1.1$ subunit declines in old rodents [128, 160, 161] and this causes an impairment of ECC [160], we wanted to

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investigate what effects aging had on $Ca_v\beta_{1a}$ expression, as this subunit is also critical for ECC.

Western blot analysis shows a substantial increase of $Ca_v\beta_{1a}$ expression over the full lifespan of FVB mice [162]. To examine the specific effects of $Ca_v\beta_{1a}$ overexpression, a $Ca_v\beta_{1a}$ -YFP plasmid was electroporated *in vivo* into young animals. The resulting increase in expression of $Ca_v\beta_{1a}$ corresponded to decline of $Ca_v1.1$ over the same time period. YFP fluorescence, used as a measure of $Ca_v\beta_{1a}$ -YFP expression in individual fibers, also showed an inverse relationship with charge movement, measured using the whole-cell patch-clamp technique. Specific force was significantly reduced in young $Ca_v\beta_{1a}$ -YFP electroporated muscle fibers compared to sham-electroporated, age-matched controls. siRNA interference of $Ca_v\beta_{1a}$ in young muscles reduced charge movement, while charge movement in old was restored to young control levels. These studies imply $Ca_v\beta_{1a}$ serves as both a positive and negative regulator of $Ca_v1.1$ expression, and that endogenous overexpression of $Ca_v\beta_{1a}$ during old age may play a roloe in the loss of specific force [162].

CONCLUDING COMMENTS

Age-related declines in the neuromuscular system are a recognized cause of impaired physical performance and loss of independence in the elderly. Epidemiological data associate these changes with increased risk of morbidity, disability, and mortality in the elderly [163-166].

We argue for the importance of neural factors in age-related impairment of mammalian skeletal muscle structure and function. Decreased local production of IGF-1 and/or neurotrophins and tissue resistance to these factors through altered receptor expression or responsiveness may result in loss and atrophy of spinal cord motor neurons. In fact, declining motor neuron function may be more extensive than that predicted by structural assays. Preliminary data support the concept that reduced IGF-1 synthesis may cause the failure of an IGF-mediated pathway to decrease CREB phosphorylation. In turn, reduced CREB phosphorylation may result in reduced DHPRα_{1S} transcription, ECU, and decreased muscle force.

The characterization of a number of triad proteins is shedding light on the molecular signaling involved in excitation-gene expression and ECC [167]. The role of neural factors in regulating the expression and function of these newly identified triad proteins is a necessary focus of research in the coming years. We hypothesize that neural factors (autocrine trophic factors, nerve activity and connectivity) play a vital role in preventing age-related ECU. Based on this hypothesis, we predict that interventions aimed at counteracting nerve loss will play an important part in ameliorating the loss of force exhibited in animal models of aging as well as in elderly humans.

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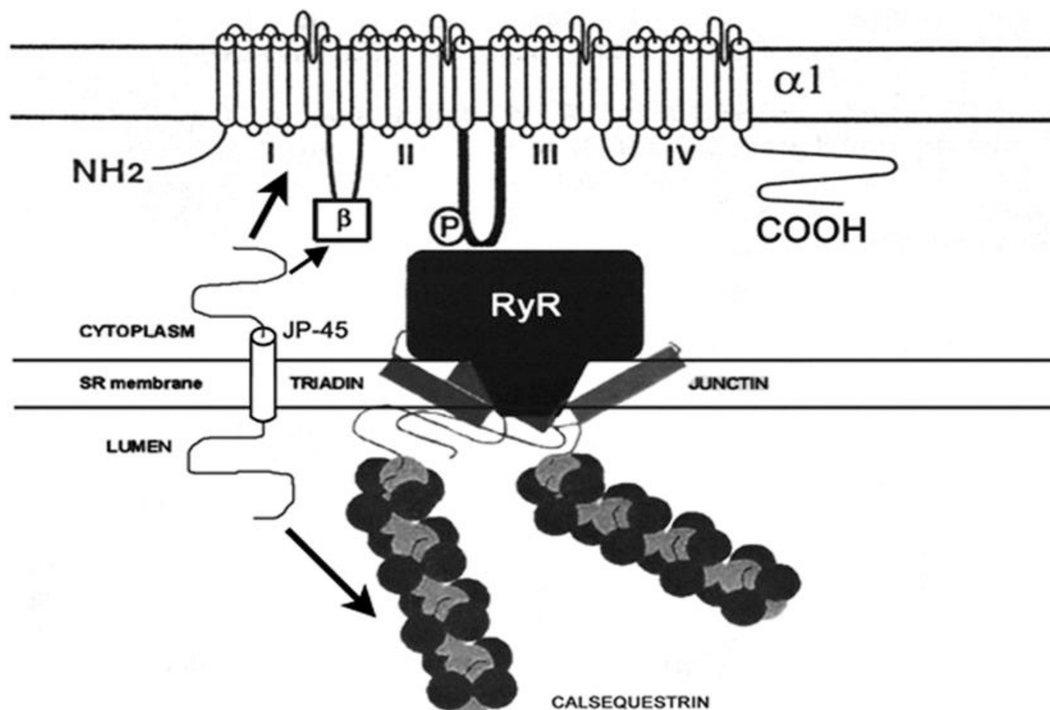

Fig. (1).

Organization of the triad junction emphasizing Ca_v1.1/DHPRα1 subunit, JP-45 and RyR interactions. JP-45 interacts with the Ca_v1.1/DHPR α1 and β1a subunits and calsequestrin (arrows). The ryanodine receptor (RyR) isoform-1 interacts with junction and triadin. Other triadic proteins such as calmodulin, FKBP, and protein kinases, have been omitted for clarity. Adapted from [168] and [169].

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