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Sarcopenia; Aging-related loss of muscle mass and function

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Running title: Sarcopenia

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ABSTRACT

Sarcopenia is a loss of muscle mass and function in the elderly that reduces mobility; diminishes quality of life, and can lead to fall-related injuries, which require costly hospitalization and extended rehabilitation. This review focuses on the aging-related structural changes and mechanisms at cellular and subcellular levels underlying changes in the individual motor unit: specifically, the perikaryon of α-motoneurone; its neuromuscular junction(s), and the muscle fibers that it innervates. Loss of muscle mass with aging, which is largely due to the progressive loss of motoneurones, is associated with reduced muscle fiber number and size. Muscle function progressively declines because motoneurone loss is not adequately compensated by re-innervation of muscle fibers by the remaining motoneurones. At the intracellular level, key factors are qualitative changes in post-translational modifications of muscle proteins and the loss of coordinated control between contractile, mitochondrial, and sarcoplasmic reticulum protein expression. Quantitative and qualitative changes in skeletal muscle during the process of aging also have been implicated in the pathogenesis of acquired and hereditary neuromuscular disorders. In experimental models, specific intervention strategies have shown encouraging results on limiting deterioration of motor unit structure and function under conditions of impaired innervation. Translated to the clinic, if these or similar interventions, by saving muscle and improving mobility, could help alleviate sarcopenia in the elderly, there would be both great humanitarian benefits and large cost savings for health care systems.

1. INTRODUCTION

A progressive loss of function, decreasing fertility and increasing mortality represent fundamental biological aging processes intrinsic to most cellular systems. The aging-related muscle atrophy is the most common type of muscle atrophy in man and is associated with significant impairment of function, such as slowing of movement and muscle weakness. The importance of aging-related changes in muscle function is increasing dramatically due to socioeconomic factors related to the demographic development in modern society with a growing proportion of old individuals due to improved living conditions, health care, etc. The process of random mortality caused by intercurrent disease (*e.g.*, infectious disease) has been replaced by mortality and morbidity caused by aging-associated injuries and diseases (*e.g.*, falls and fall-related injuries). There is accordingly significant need for an increased understanding of the mechanisms underlying the changes in skeletal muscle structure and function during aging as well as the design of specific intervention strategies for multiple reasons. First, the demographic evolution with an increasing proportion of elderly citizens with problems related to the aging-related motor handicap and dependency with subsequent dramatic social and economic consequences has resulted in an increased awareness of the importance of research efforts aiming at improving the quality of life in the aged. Second, it cannot be excluded that aging plays an important etiological role in the progression of specific neuromuscular disorders, such as amyotrophic lateral sclerosis, muscular dystrophies, critical illness myopathy, ventilator induced diaphragm muscle dysfunction, and the post-polio syndrome. Third, elderly fragile men and women need to use their maximum muscle power to rise from a chair and an additional small impairment may accordingly change their life from an independent to a dependent one (288). Fourth, falls and fall-related injuries are common among older individuals and women are at higher risk than men (385, 857). The cause of falls and fall-related injuries in old age are complex and involve impairments in the neuromuscular, peripheral, and central nervous system (664). The fall may be separaterd into two components, the initiation of the fall and the ability to restore disturbed balance. First, aging-related changes in somatosensation, vision, and vestibular function have a negative impact on maintaining postural balance, resulting in an increased risk of initiating a fall. Second, the ability to recover from a threthening fall is also impaired in old age, especially in old women, but is not related to an altered somatosensory process or the initiation of muscle contraction by the central nervous system (809). This process, on the other hand, lies primarily in events after depolarization of the muscle membrane, *i.e.*, in force generation and contractile speed (809).

The aging-related loss of muscle function involves quantitative and qualitative changes in skeletal muscle structure and function. This process is typically slow and the functional loss varies significantly among individuals, but is observed in all humans, *i.e.*, also in healthy, well-nourished, and physically active individuals (70). The decline in muscle mass and function probably represents the most dramatic and significant of all changes during the aging process (117), a process referred to as *sarcopenia* (117). Although the fundamental biological processes underlying aging remain obscure, it is clinically of great importance to have detailed knowledge about muscle tissue-specific mechanisms of aging. These efforts need to include both state-of-the-art basic research and clinical investigations. The study of sarcopenia is however complicated by several factors, such as muscle-specific differences in the response to aging, differences among species, and methodological errors related to scientific approach. This is probably, at least in part, why so many different and sometimes conflicting results have been reported in the literature.

This review focuses on current knowledge about how aging influences the different components of the motor unit, underlying mechanisms, intervention strategies, and the association between specific neuromuscular disorders and old age. We will refrain from lengthy discussions related to the numerous divergent results in the literature regarding sarcopenia and underlying mechanisms. Although there are species differences in response to aging, all mammals are characterized by sarcopenia and conflicting results are frequently secondary to study designs with their different inherent methodological limitations. Throughout this review, changes related to the aging process will be referred to as "aging-related", *i.e.,* a process which starts at the age of maturity and where old age is the age after an individual has passed the 50% survival age in the population (1000). On the other hand, "age-related" will be used to describe changes associated with the whole life-span, *i.e.,* including development and maturation in addition to aging. Further, sarcopenia will be referred to as its original definition (770), *i.e.,* the aging-related changes in skeletal muscle structure and function affecting mammals, including humans. Therefore, this review will pay specific attention to aging-related changes in skeletal muscle observed in both experimental models as well as in humans. Divergent or conflicting results observed in humans compared with experimental models may be related to species differences, but inherent errors in the design of the experimental model and limitations introduced in the study of sarcopenia in mammals with a long lifespan, such as humans, are more common.

2. EXPERIMENTAL MODELS

a. Methodological problems in the study of aging skeletal muscle

The study of sarcopenia in humans is complicated by the long duration of the aging process, large variability among individuals, and multiple factors affecting muscle that are not primarily related to aging *per se.* Studies of aging can be conducted using either a cross-sectional or longitudinal design, but neither are free from sampling errors (105, 514, 820). Many of the factors affecting skeletal muscle have changed dramatically in the past century, such as social-, nutritional-, and physical activity-related factors, adding a significant challenge in the interpretation of results in long-lived mammals such as humans. Cross-sectional studies are particularly vulnerable to these confounding factors and an additional weakness with the cross-sectional design is that very old subjects represent a specific cohort with a different rate of aging, resulting in the so called "reversible aging phenomenon", a phenomenon also observed in aging rodents (805, 834). Longitudinal studies have significant advantages, but are difficult to conduct in mammals with a long life-span, such as in humans as well as in rodent studies, where a large proportion of the total muscle mass needs to be analyzed at each timepoint. Premature aging offers a significant advantage logistically in studies on aging in humans, although aging represents a very complex biological system that is not perfectly mimicked in premature aging models. The problem with tissue sampling may, at least in part, be overcome in rodent studies by using genetically identical recombinant inbred mice strains sacrificed at different time points, offering a "longitudinal" study with a cross-sectional design (572-574). In addition, longitudinal studies in humans are not immune to the dramatic changes that have taken place during the past decades in nutrition and other environmental influences. Rodent models have frequently been used in the study of sarcopenia, offering a cost-efficient alternative. Mouse models have the advantage of efficient genetic engineering, but it is becoming increasing evident that the rat

is a superior rodent model for studies of both muscle disease and aging (416). This may in part be due to the fact that mice strains were originally selected for cancer research, while such selection has not occurred in rat strains primarily used for toxicological and physiological research (999). Further, the CRISPR interference technique has the potential to introduce cost-effective genetic manipulations of interest in aging research in rats. Finally, it is important to emphasize that many aging-related phenomena are closely related and impact on sarcopenia, adding to the complexity when interpreting results, such as endocrine, central and peripheral nervous system, social, nutritional, and central and peripheral circulatory influences and their impact on skeletal muscle (514).

b. Muscle-specific differences

Structural and electrophysiological changes in skeletal muscle are frequently reported first after the sixth decade in humans and followed by a progressive decline with increasing age (679, 847). However, aging-related changes in skeletal muscle power begin earlier according to observations in track and field Masters Athletes (321), *i.e*., muscle power (world records) begins to decline after the age of 30 and continues to decline linearly with advancing age (321). Although Masters Athletes represent a unique population, they have the advantage that they are both highly motivated and are used to performing at maximum or near maximum muscle power, separating them from the general population, where measurements of muscle function may be less reliable. Maximum power is of less importance for daily life and quality of life in old age, but it reflects aging-related changes in skeletal muscle function. Thus, aging-related changes in muscle power appear to precede detectable changes in various morphological and electrophysiological changes. This may be due to lack of precision or sensitivity of the methods used, or more likely that qualitative changes in regulation of muscle contraction go undetected by morphological and electrophysiological methods, which primarily quantify muscle mass and motor unit size/organization. However, the application of more sophisticated techniques will allow for the detection of progressive aging-related changes in skeletal muscle at a much earlier time and sarcopenia may be considered an epiphenomenon of cellular and molecular changes that start early in life.

In quadruped and biped mammals, the forelimb (arm) and hind limb (leg) appear to be affected differently by aging, with hind limb (leg) muscles being more severely affected than forelimb (arm) muscles (311, 375, 623, 679, 847). This is of significant importance since lower extremity function has a stronger impact on quality of life and independence in old age than upper limb function in humans. Furthermore, the cause of falls and fall-related injuries in old age has been reported to lie primarily in events after depolarization of skeletal muscle, *i.e*., in force-generation and contractile speed (809). In support of this, the decline in mobility and lower extremity disability have been reported most influential in predicting falls in the elderly (76, 596, 597, 761, 939), and the predictive value of the muscle weakness is further increased when muscle force is measured at a speed of movement resembling more functional limb velocities (939). Grip strength measurements have frequently been performed in the study of aging-related changes in skeletal muscle function, primarily due to the simple grip strength measurement procedure in both humans and rodents. However, it may be argued how well grip strength reflects the aging-related motor handicap and decreased quality of life in old age related to impaired mobility and risk of falls and fall-related injuries. Due to the strong impact of lower limb muscle function on falls and fall-related injuries and

the more pronounced changes in lower (hind) versus upper (fore) limbs, this review will focus on aging-related changes in lower (hind) limb muscles.

3. AGING-RELATED CANGES IN THE MOTOR UNIT

a. Aging-related changes in motor unit organization

The concept of the motor unit introduced by Lidell and Sherrington (568) and defined as the single alpha motoneurone and the muscle fibers it innervates was emphasized as the final functional unit in the motor system (250). Numerous attempts have been made to classify motor units into different types according to physiological characteristics, metabolic properties, or myosin heavy chain (MyHC) isoform expression in the motor unit fibers, *i.e*., the constituent muscle fibers within a single motor unit (645). Historically, the spatial organization of motor unit fibers was the subject of discussion for a long period and the scattered organization of motor unit fibers within the motor unit territory and the spatial overlap between different territories was not resolved until the single fiber EMG (852) and single motor unit glycogen depletion techniques were introduced. The glycogen depletion technique was originally proposed by Krnjevic' and Miledi (484) and successfully first reported by Edström and Kugelberg (257), Doyle and Mayer (240), Branstater and Lambert (98, 99), and Burke et al. (125). Originally, the spatial organization of motor unit fibers was considered to be random within the motor unit territory and metabolic properties to be uniform within single motor units, based on visual evaluation of glycogen-depleted motor unit fibers (124, 257, 662, 663). Albeit significantly smaller variation than among different motor units, microphotometric measurements of metabolic properties and computer-assisted measurements of spatial organization within the motor unit territory demonstrate heterogeneity in enzyme-activities and non-random organization of motor unit fibers (26, 79, 258, 358, 488, 516, 609, 610). In the large fast-twitch motor units in the rat tibialis anterior muscle, a gradient of the mitochondrial enzyme activity succinic dehydrogenase was observed along the superficial-deep axis of the muscle, indicating a biological phenomenon rather than a methodological error (516). The tibialis anterior muscle has a complex orientation of muscle fibers and the mechanical strain on muscle fibers both in the relaxed and activated strain may vary along to the superficial deep axis. This is of specific interest since mitochondria are mechanosensitive and respond to passive mechanical load (442, 443). In old age, a significant reorganization of motor unit fibers is observed, which precedes the fiber type grouping and grouped atrophy reported at an advanced age (24, 26, 258). The motor unit fiber reorganization is paralleled by an increased number of muscle fibers innervated by the α-motoneurone, a decreased total muscle fiber number, and a decreased number of large myelinated neurons in peripheral nerves and ventral roots, suggesting denervation due to loss of α-motoneurones and incomplete reinnervation of previously denervated muscle fibers. In the tibialis anterior muscle, motor units are organized according to the spatial organization along the superficial deep axis. That is, the largest motor units with the largest muscle fibers expressing the type IIb MyHC are located in the superficial region of the muscle and the small motor units expressing the type IIa MyHC isoform are located in the deep region of the muscle. In between these two motor unit types, motor units are localized having an intermediate size and fibers expressing the IIx MyHC isoform (**Fig. 1,** (524)). During aging, there is a fast to slow myosin transition, with a decreased amount of IIb MyHC fibers (522, 535) and motor unit fibers expressing the type IIx MyHC isoform are expressed in regions restricted to type IIb MyHC motor

unit fibers in young animals (521). Further, in old age, motor units have been identified including muscle fibers expressing different MyHC isoforms in contrast to motor units from young animals where motor unit muscle fibers are homogenous with regard to MyHC isoform expression (521) (**Fig. 2**). Such inhomogeneity has not been reported in motor units from in young animals and it may be speculated that this is secondary to an aging-related reinnervation but incomplete MyHC transition within the motor unit. However, muscle fibers co-expressing different MyHC isoforms are observed in both young and old animals and may increase in number in old age.

b. Motoneurone

During the aging process, the neuromuscular system is believed to undergo a continual reorganization of motor units due to a loss of motoneurones and reinnervation of denervated muscle fibers via collateral sprouting (265). This is supported by an aging-related decline in myelinated neurons in sensory and mixed peripheral nerves, in the mouse (126, 792), the rat (24, 265, 350, 376, 865, 916), the horse (938) and in humans (422, 858, 864, 882, 885), as well as in ventral roots from rats (24, 248) and humans (174, 319, 641, 753) (**Fig 3**). The loss of large anterior horn cells in the mouse (964); rat (376, 420) and humans (455, 456, 885) lend further support to an aging-related loss of motoneurones and the large α-motoneurones appear to be more affected by old age than the smaller γ-motoneurones (24, 374, 376). However, this is a slow gradual process including axonal, demyelinating and Schwann cell pathology, and the motoneurone loss is preceded by decreased metabolic capacity and structural changes at the motoneurone level, such as Wallerian degeneration, myelin sheet irregularities, myelin ovoids, infolded loops of myelin, macrophages overloaded with myelin debris, and myelin reduplication (439, 520). This condition is referred to as "sick" motoneurones which has significant consequences for the functional capacity of the motoneurone (265, 621). In parallel with the structural changes, there is a decreased rate of axoplasmic transport, speed of nerve regeneration and axonal conduction velocity (118, 444, 626, 705). The morphological changes indicating axonal degeneration have been reported more prominent in peripheral nerves than in ventral roots (24). This is suggested to be secondary to decreased protein synthesis in the neuronal cell body and/or the decreased rate of axonal transport in old age rendering the distal part of the neuron more vulnerable to these effects of aging (470, 475, 626). Axonal transport is highly dependent on intact microtubules (827) and the decreased axonal transport in experimental diabetes coincides with the glycation of tubulin (188). Reducing sugars target lysine residues during non-enzymatic glycosylation (glycation) and reactive lysine residues are essential for tubulin assembly into microtubules (188, 632). This offers a plausible mechanism underlying the slowing of axoplasmic flow in old age, since post-translational modifications of proteins and nucleic acids by non-enzymatic glycation are considered an important pathophysiological aging-related mechanism (115, 123). Ultimately, this process leads to a compromised ability of the old motoneurone to reinnervate via collateral sprouting, preceding the final aging-related motoneurone loss (265). Furthermore, the largest and fastest conducting neurons appear to be preferentially lost during aging with consequences for reinnervation capacity and muscle physiology (176, 641, 674, 677, 882). Thus, the loss of the largest motoneurones included in fast-twitch motor units, the impaired reinnervation capacity by "sick" motoneurones will contribute significantly to quantitative and qualitative changes of skeletal muscle structure and function in old age, such as muscle fiber loss and fiber type transitions (see(520)).

Mechanisms underlying the progressive loss of α-motoneurones during aging are highly complex and involve multiple factors. DNA damage and modifications accumulating throughout life eventually leading functional deterioration and cell death have been suggested to play an important role for the motoneurone loss in old age (197). This is supported by experimental studies in progeroid mice with a deficient DNA repair mechanism resulting in accumulation of DNA damage and a phenotype resembling the neuromuscular system observed in old age, *i.e*., degeneration of motoneurones and progressive disruption of the neuromuscular connectivity (197). Thus, accumulation of DNA damage and genotoxic stress have therefore been forwarded as a plausible mechanism contributing to the aging-related motoneurone loss (89, 197). Reactive oxygen species, malondialdehyde as well as non-enzymatic glycosylation may contribute to this process. Spinal motoneurones show selective vulnerability to intermediate products of glycation with neurotoxic effects, leading to apoptotic cell death (822). An inefficient glutathione defense system in old age will contribute to increased levels of glycated proteins and a progressive loss of spinal motoneurones, a pathophysiological mechanism also suggested to underlie the progressive loss of spinal motoneurones in patients with amyotrophic lateral sclerosis (ALS) (822). As discussed below, aging-related changes in the microcirculation of the spinal cord may contribute to the motoneurone loss in old age (see below).

c. Neuromuscular junction

There are clearly a bewildering array of deficiencies reported in the physiology, biochemistry and morphology of muscle fibers in aging animals. These have been covered extensively in numerous, excellent reviews of this subject (cf. (37, 107, 228, 421, 426, 763, 880, 897, 959)) and are covered elsewhere in this review. Given the close, reciprocal influence of nerve on muscle (for example, the activity patterns conveyed to the muscle by their nerves influence the size and contractile properties of the muscle fibers (cf. (34, 339)) and muscle and its activity on nerve (for example, the activity pattern of the nerve and muscle can influence the size and detailed morphology of the synaptic contact itself (749)s and disconnecting the two serves as a major stimulant for nerve growth (cf. (113)), it is not surprising that there are corresponding aging changes in the physiology, biochemistry, and morphology of the neuromuscular junction (NMJ) itself. There is also an equivalently bewildering array of manipulations to the neuromuscular system that can alter these properties both of the muscle and the NMJ that produce effects similar to those seen in aging; some of these are discussed below. It is undoubtedly the case that, given these complex interdependencies of the neuromuscular system and the number of manipulations that can cause alterations, that the mechanisms of aging are quite complex. Nonetheless, understanding what happens to NMJs during aging is important not only for the possible interventions that could be made to improve the quality of life but also for understanding the basic science of these synapses as well many diseases that impact the NMJ.

Developments allowing for vital imaging

One approach to understanding how NMJs age is to directly observe the changes as they occur. Such an approach was made possible by two developments. Firstly, the application of fluorescent stains that vitally label the synaptic components made it possible to expose a muscle through simple surgery, briefly image the synapse through a dipping microscope objective, allow the animal to recover, and then return at a later time to examine the same synapse. Much of the early efforts at

vital imaging were made by the use of a mitochondrial dye (4-Di-2-Asp) (595) combined with a fluorescent conjugate of a snake toxin alpha bungarotoxin (BTX, that binds irreversibly to the postsynaptic acetylcholine receptors (19). A second development, the advent of genetically encoded fluorescent proteins (cf.(283)), considerably expanded the effectiveness of labeling as well as the number of components that could be labeled at the same NMJ.

Initial efforts at imaging concentrated on use of the sternomastoid muscle of the mouse (567), an easily accessible muscle located just beneath the salivary glands in the neck, but these efforts have since expanded to additional muscles. One of the chief concerns about imaging like this is its vital nature. Neither the bungarotoxin applied nor the fluorescent proteins expressed should perturb synaptic transmission; any perturbation here will itself alter the NMJ. BTX is a competitive antagonist of acetylcholine at acetylcholine receptor. It binds essentially irreversibly and, while it blocks the receptor to which it is bound and would paralyze the muscle, BTX can be used at a dosage that blocks only a fraction of the receptors. Because NMJs operate at a huge safety factor (i.e. they release enough quanta of acetylcholine to produce an excess of the depolarization necessary to bring the muscle fiber to threshold), it is estimated that up to 70% of the receptors can be blocked without rendering transmission across the synapse ineffective (571).
Moreover, the BTX will persist in its bound state until the receptors to which it is bound turns over. As long as transmission remains intact, the turnover of these receptors proceeds normally (9). Hence, it is the case that the BTX can be applied once at low concentrations and the labeling of a fraction of receptors persists for some weeks (567). As which receptors are labeled is random, an image of the entirety of the postsynaptic acetylcholine receptor area (in the endplate) is visible. Although the turnover of receptors and the structure of the synapse is altered in the presence of a block of neurotransmission and action potential activity in the muscle (9), this does not occur if the number of BTX-bound receptors is insufficient to produce a block (45). After an initial imaging session, the incision can be closed and the animal recovered from anesthesia. At a subsequent imaging session, the receptor labeling can be repeated with another application of BTX, even one conjugated to a different fluorochrome, again with the caveat of not producing a block of transmission. This allows the visualization of changes that have occurred in the distribution of receptors since the last imaging session and in particular the identification of any new sites on the muscle fiber at which newly synthesized receptors have been added. Of course, it is essential that one be able to identify the same synapses at the different imaging sessions. This can be easily achieved in most cases because the junctions each have a unique pretzel-like morphology created by the aggregation of receptors underneath the branches of the nerve terminals at the sites in the muscle fiber where they come into contact. These receptors usually occur within depressions in the muscle fiber surface occupied by the nerve terminal called synaptic gutters. Each pretzel is said to be unique owing to the unique pattern of the nerve branches on the surface of the muscle fiber, making each junction uniquely identifiable. The nerve branches control the localization of the underlying receptors by inducing and stabilizing their presence. In addition to the pretzel shape of the junction of interest, each of the neighboring NMJs on adjoining muscle fibers within the so-called “endplate band” has its own unique pretzel shape and this can aid considerably in localization. Moreover, the presence of nerve branches and blood vessels and the rough position within the muscle help in re-identifying the junction originally imaged in a subsequent imaging session. Of course, this ability depends on how constant these structures remain over time, and the

observations to date presented briefly below show an amazing constancy except in certain circumstances where the usually stable junctions undergo morphological change ((43). However, even in these cases it is usually possible to reliably re-identify the same junction, especially as the pattern of the endplate band does not usually change and moreover the surrounding junctions have sometimes not changed dramatically.

Soon after their initial reports on how the nerve terminals at NMJs did not appear to change over time, Lichtman and colleagues were able to combine labeling of the terminals with labeling of the postsynaptic acetylcholine receptors with BTX, allowing the use of visualization through fluorescence of both pre- and postsynaptic components of the NMJs. In the initial years of their studies of NMJs, they relied heavily on the use of the 4-Di-2-Asp dye together with BTX (cf. (755). However, with the discovery of fluorescent proteins like GFP, the isolation of the genes encoding these fluorescent proteins, the discovery of promoters that could be used to drive gene expression in a restricted set of cells, and the developments in mouse transgenesis, investigators achieved the ability to vitally label individual cells in organisms. In particular, the so-called thy1 promoter was found to drive expression in neurons of the central nervous system (457) and could be utilized with the spectral variants of GFP to produce vital labeling of motorneurones, their axons within muscles and their nerve terminals present at the NMJ (283). Moreover, it was found that the same transgene, depending on its integration site in the mouse genome during the process of transgenesis, could produce lines of mice in which “subsets” of motoneurones (rather than whole motoneurone pools) were consistently labeled within each individual line (283, 910). These subset lines have been particularly useful in the same way Golgi stains were useful in the early stages of neuroanatomy: staining every cell or structure of one type makes it impossible to visualize individual cells; labeling of only a few makes individual components identifiable. This has been useful in the vital imaging of development of muscle innervation (cf. (75, 899, 900, 910)) and in investigating changes in innervation during pathologies (cf. (426, 803)). Since the development of the thy1-fluorescent protein (FP) mice, a number of other transgenics have been created, enabling, for example, the vital labeling of other structures at the NMJ, including glial cells (996, 998), the acetylcholine receptors in the postsynaptic muscle fiber (324), the cytoskeleton of the synapse and reactive glia (446) and resident macrophage (998). Essentially, the only limitation is the discovery of selective promoters (or alternately, the production of fusion proteins as knock-ins) and the cost and time involved in producing and maintaining lines of mice. While following up on successes in the imaging of neuromuscular synapses, the techniques have been adapted for imaging neuron-neuron synapses within the peripheral and central nervous system and these studies have added to our understanding of neural connectivity and plasticity.

Of course, there are limitations of what one can stain vitally within the muscle with the dyes available and there are cases where fluorescent transgenes are not readily available. In these cases, it is possible to vitally image the morphology of the synapse with the vital reagents one has available and then examine the same synapses *ex vivo* with reagents such as antibodies by the normal staining procedures (187). Once again, the constancy and uniqueness of the pretzel pattern of the receptors can serve to re-identify the synapse(s) studied *in vivo in vitro*. This has expanded the usefulness of vital imaging, although the investigator is limited to examining the status of the synapse at the endpoint of a series of vital images.

How constant is the morphology of the NMJ?

The question of how NMJs change during normal life is fundamental for how one interprets the results of aging and, for that matter, of disease as well. Purves, Lichtman, and colleagues used vital imaging of NMJs in the sternomastoid muscle of mice to examine whether these synapses are plastic and undergo morphological alterations during the normal life of mice, but did not look at the events of late aging. At the time of their studies there had been numerous studies involving static imaging of NMJs in mammals (cf. (53, 135, 136, 177, 275)) and particularly in frogs (cf. (27, 178, 187, 192, 471, 935)). It was clear that frog NMJs underwent seasonal adjustments in size by retraction and extension of nerve processes across the surface of individual muscle fibers. Furthermore, static observations in mammalian muscles (cats and rabbits) led to the conclusion that there was a significant amount of nerve sprouting that led to continual turnover of NMJs even in normal muscles (53). Therefore, it was surprising when the vital imaging results from mice suggested that their NMJs were mostly stable. There were occasional small deletions or additions to the pretzel and the pretzels grew in size as the muscle fibers themselves expanded in diameter as the animal itself grew. However, the overall message was one of stability. The growth in size of NMJs was described as intercalary (608), where the size of the synaptic contact changes but the overall morphology does not, a quite different picture from the malleability of the NMJs that then was prevalent. When androgen-sensitive, sexually dimorphic muscles in the rodent were caused to atrophy or hypertrophy by use of castration and/or administration of androgens, the muscle fiber diameters changed but the NMJs expanded or shrank without dramatic changes in their overall morphology (42). Similar imaging efforts by Wigston (944) chose to examine the morphology of the slow soleus muscle in the mouse (in contrast to the fast contracting sternomastoid utilized previously). Once again, the overall picture was one of constancy in morphology. But in contrast to sternomastoid, a significant fraction of the NMJs examined over time periods of months changed significantly in their morphology. Even most of those that changed still had significant remnants of the old, initially viewed morphology, having added or lost small portions of their synaptic contact. When these same experiments were repeated using the lateral gastrocnemius (945) (a muscle composed of fast contracting fibers like the sternomastoid), the observed number of changes were more in line with those observed in sternomastoid. There were a small number of instances where NMJs did undergo a substantial loss of receptor area. In these cases there was a loss of the originally observed receptor area where the nerve enters the junction (43). Along with the loss of receptor area, the portion of the nerve above the lost receptors had apparently become myelinated, suggesting that one of the glial (Schwann) cells of the junction had changed its phenotype from non-myelinating to myelinating and perhaps intruded between the nerve and the muscle at this site, disconnecting the two. Since studies of the arrangement of Schwann cells above the junction and the preterminal axon have shown lack of overlap of the cells above the junction and the myelinating cells along the axon (and even a tiling of the coverage of the terminal by individual Schwann cells of the junction) (101), such a change could easily occur without a total rearrangement of the glia at the synapse.

Secondly, it is also clear that whenever there is an interruption of the motor axon innervating a NMJ (experimentally by nerve section or crush, or even laser ablation) there can be a dramatic change in

the configuration of the synapse when the axon regenerates to reoccupy the old endplate site (754). This was beautifully demonstrated by Gutmann and Young in 1944 (361). Usually if the axon returns rapidly (which is the case after simple crush injuries since the axon can quickly regenerate down the old endoneurial tube filled with Schwann cells), the old sites containing high concentrations of acetylcholine receptors that remain even when the nerve is disconnected, are accurately reoccupied, usually by the very same axon that previously made contact (445, 667). However, if the nerve damage is more severe and creates a discontinuity in the old nerve sheaths that contained the motor innervation, reinnervation is much slower and it is unlikely that the nerve returns to each and every location on the fiber containing a high acetylcholine receptor density. Some of these unoccupied original sites turnover and some new sites are generated, likely due to the action of agrin secreted from sprouting nerve fibers (445, 667, 755).

An explanation of this receptor turnover during reinnervation has been provided by Balice-Gordon and Lichtman (45): they have shown that portions of nerve terminals that are rendered ineffective in synaptic transmission (by localized application of BTX to block the receptors underneath this portion of the nerve terminal) can lead to loss of the blocked receptors and withdrawal of that portion of the nerve terminal. However, such a loss occurs only if enough receptors are left unblocked in the synapse to allow effective transmission of excitation to the muscle fiber. This kind of loss does not occur if the entire synaptic site is blocked with BTX. Further, loss of receptors at the endplate does not occur if the entire junction is denervated and remains so (11). The implications of these experiments are profound. If some receptor sites are present that are not continually part of the excitation of the muscle fiber, these sites are lost. Thus, it is easy to see why asynchronous reinnervation of the synaptic sites on a muscle fiber could lead to remodeling of the synaptic site as portions would become active in advance of others, perhaps even before these other sites are reinnervated. Moreover, such a mechanism points to how important it is to maintain transmitter release across the synaptic site (even though the release of neurotransmitter from each active zone in the terminal is quite variable). Any long-term inactivity of a portion of the synapse would lead to remodeling, as would any unintentional damage during imaging. It is also easy to understand those cases where the preterminal axon becomes myelinated (43): the portion of the synapse whose apposing nerve becomes myelinated would be rendered effectively non-functional in transmission. It is understandable as well why prolonged denervation prior to reinnervation leads to increased asynchrony in restablishment of nerve contacts at the old synaptic sites and therefore more profound remodeling of this site.

A third example is the case of sprouting. Sprouting (the growth of new nerve processes from the terminal or axon) is a common phenomenon in muscle during reinnervation (840) and particularly during reinnervation following partial denervation (839). In partial denervation, when some of the axons innervating a muscle are damaged leaving other axons intact in their innervation, there is considerable terminal and nodal sprouting supposedly stimulated by the presence of the denervated muscle fibers. These sprouts can grow along glial cells to reinnervate the endplates on nearby muscle fibers (839, 840). In these cases, the morphology of the synapses formed can change. Some of this change has been attributed to polyneuronal reinnervation of some junctions with subsequent competition for the endplate site, displacement of one of the innervating axons and the

loss of receptors underneath the losing axon (755). However, from the arguments above and other studies, such remodeling can occur even in the absence of polyneuronal innervation (445).

Other investigators have used other methods of marking nerve terminals for vital imaging and they have come to somewhat different conclusions than those just presented. Robbins and collaborators labeled nerve terminals with fluorescently conjugated tetanus toxin C fragment (393, 394), a component of tetanus toxin that enters motor nerve terminals and is retrogradely transported but is otherwise non-toxic. Imaging the pectineus muscle in the mouse, they observed a remodeling of NMJs during their growth that was created by extension and withdrawal of nerve terminal processes with corresponding alterations in the receptor pretzel. The lack of stability of the NMJs in this study and ones previously discussed could be due to a number of factors: the different reagents for labeling nerves, the steps taken to limit phototoxic damage from imaging, the character or type of the different muscles investigated, the ability to approach the muscle without a reaction to the skin wound necessary to provide access to the muscle, and the time period in the life of the animal over which observations were made. However, it is important to recognize that the nerve terminal processes observed to be extended (and retracted) in these experiments were generally very small and many of them were resolved without any change in the postsynaptic acetylcholine receptor distribution. While some junctions were observed to change substantially over months, such changes have been observed even in the studies to be discussed below and have been attributed to the damage to muscle fibers that can occur during their normal use in contractions.

Despite the arguments from vital imaging made above that remodeling of the NMJ during most of the life of the animal is minimal, there have been very few muscles examined in this way and there likely are muscle-to-muscle differences based on the patterns of their usage. Similarly, few species have been carefully examined, and there could be differences here. While conclusions based upon static imaging are important (especially when the changes are profound), it seems most convincing if the examinations are made so that changes in individual synapses can be monitored over time. It is also clear that any situation where even temporary changes in synaptic transmission occur, the NMJ can be remodeled accordingly. So, any claim as to the plasticity present must carefully account for possible confounding changes that occur during the observations themselves.

Morphological changes to the NMJ during aging observed through static imaging

Whether or not NMJs are generally stable through most of life as argued above, something dramatic happens during advanced aging, although exactly what is happening and its consequences for synaptic function are less than clear and still controversial. Gutmann and colleagues reported on aged muscles of rats in the 1960's. They examined NMJs in 24 mo. old rats by electron microscopy, cholinesterase staining, and electrophysiology (359, 360). Despite atrophy of the muscles, and slowing of their contractile properties they reported no signs of obvious denervation. However, NMJs revealed by cholinesterase staining and some electron microscopy were reported as significantly changed. The cholinesterase staining was more "fragmented" and in electron micrographs there was some evidence of invasion of Schwann cells to surround some nerve terminals. Moreover, the frequency of miniature endplate potentials (mepps) was reported to be reduced (but see below). Histology of muscles and peripheral nerves were said to show a loss of

about a third of the muscle fibers in soleus but no obvious loss of motor axons (see 3b above and below for contrasting results). In general, these reports laid the groundwork for a host of subsequent studies on NMJs in old rodents. Cardasis and colleagues likewise reported in several papers an examination of NMJs, primarily in the soleus muscle of aged rats using cholinesterase staining in whole mounts and electron microscopy (134-136). The light micrographs showed enlarged but fragmented contacts on the muscle surface whose frequency increased with age. Many of these contacts were only partially occupied by nerve terminals. Electron micrographs of synaptic sites revealed large stretches of muscle fiber membrane where prominent junctional folds were present but that were unoccupied by nerve terminals or the nerve terminals were wrapped by Schwann cells. These observations led these investigators to propose that aging was a continuous process of withdrawal of nerve terminals from synaptic contact resulting in focal denervation followed by regrowth of nerves/sprouting and partial reinnervation. Robbins and colleagues (50, 275, 763) made similar findings in studying aging mice, employing zinc-iodide-osmium and silver/cholinesterase staining together with light microscopy and electron microscopy. Their findings were similar to those of investigators cited above (although they performed a much more thorough analysis of synaptic physiology), reporting as others have subsequentially, a remarkable preservation of transmission (see below). Their observations of morphology showed a number of pronounced changes (expansion of the perimeter of the junction along the fibers, increased and more complex branching of the nerve terminal within the synaptic site, fragmentation of the nerve contacts into small islets contained within the enlarged perimeter of contact) but no obvious complete denervation of individual fibers and no evidence of obvious collateral sprouting. Their assays of radioiodide-BTX binding suggested there was little change in the number of acetylcholine receptors. Other investigators have made similar observations with some exceptions in the details of the particular muscles affected, the particular fiber types affected, the species examined, and the methods for examining morphology (177, 678, 705, 772). However, the general picture emerging from all these studies is that there is a loss of innervation on some fibers, followed by a compensatory reinnervation/sprouting that replaces the loss in innervation. This reinnervation changes the synaptic contact in such a manner that the perimeter of NMJs on individual fibers is enlarged but the contacts of the individual nerve terminal boutons form smaller islands on the fiber surface. It is commonly suggested that these changes occur as a continuation of remodeling believed to occur constantly but that becomes enhanced/exaggerated during aging. Most investigators believe the morphological changes are an attempt to compensate for a gradual loss of motoneurones during aging by supplying new innervation to fibers that have lost it.

Vital imaging of changes in innervation during aging.

It seems that a clearer understanding of changes in NMJs during aging could be by repeatedly imaging the same synapse over time in an aging animal. The first effort at such vital imaging of aging NMJs was made by Balice-Gordon (41). The imaging was carried out by the protocols described above in the sternomastoid muscle of the mouse using 4-Di-2-Asp to label motor nerve terminals and BTX to label the acetylcholine receptors. By following individual junctions over time, Balice-Gordon demonstrated that the NMJs, in contrast to the junctions viewed repetitively in younger animals, were profoundly altered just as would have been predicted by the static imaging that had been performed previously. Unfortunately, most of the images were collected at intervals of

a month or longer, and, although the changes appeared to become more exaggerated with time, no new conclusions as to how the changes occurred could be made. By the time the next attempts at vital imaging were made, the technology had improved with the advent of fluorescent-protein-labeled motoneurone axons. Using transgenic mice in which motor axons were labeled blue with cyan fluorescent protein (CFP) and receptors could be labeled red at the time of the experiment with rhodamine-BTX, imaging of both structures was made possible by changing filter sets on a fluorescence microscope (see **Fig. 4**). Because of the spectral overlap between rhodamine-BTX and 4-Di-2-Asp and the relative intensity of their labeling, previous experiments had required separate labeling of each fluorophore; with fluorescent protein mice, this was no longer necessary. Li et al. (564) collected images from mice aged 19-26 months, taking images at much more frequent intervals than in the study of Balice-Gordon. They encountered two types of NMJs in the aged sternomastoid: those that had the pretzel-like appearance of "young" NMJs (**Fig. 4**) and junctions that had acetylcholine receptors that were fragmented into small islands (**Fig. 5**). The nerve terminals above the receptors had varicosities along their branches; these varicosities were apposed to the receptor islands. Reassuringly, the same two types of junctions were encountered in statically imaged junctions from these mice and the frequency of the aged phenotype increased with age. Li et al. had expected to observe junctions that underwent a slow transformation from young to fragmented and fragmented to more fragmented (564, 565). Surprisingly, this was not what they found. They saw that most of the small sample of NMJs they had previously imaged underwent almost no changes between imaging sessions. To insure that the imaging procedure itself did not change junctions, they had to limit their observations in each animal to a few junctions. Junctions that had a "young" appearance at the time of the first imaging session mostly remained young at subsequent imaging sessions. The same was true for fragmented junctions: they remained fragmented as in the first image acquired. However, a fraction of the junctions (something like 8%) underwent a remarkable transformation over as short a time as a few days. Further evidence that these junctions were undergoing a major transformation was from the labeling of the acetylcholine receptors. Normally, one application of a non-blocking dose of BTX labels a small fraction of the receptors at the NMJ. Typically in young muscles, some of the labeling achieved at the first imaging session remains when a second image is acquired of the same junction a week or so later because the binding is so avid and the receptors turn over slowly. Gradually over time, the labeling does disappear as the labeled receptors are replaced with new, unlabeled ones (9). While most of the junctions visualized in the aging muscles were like this, the junctions undergoing the transformation were not: they underwent a dramatic loss of the original label so it could barely be perceived (**Fig. 5**). Application of additional BTX in these cases labeled a set of receptors many of which were not in the original pattern of the previous image taken of this junction (**Fig. 5**). That these junctions were the same ones as previously imaged was obvious from the identification of neighboring junctions. Further, the site where the nerve entered the junction and some of the major terminal branches were the same. Li et al. concluded that these transformed junctions had undergone a major change in their synaptic structure: the original receptors present prior to the transformation event had been lost and been replaced by a new set of receptors that appeared fragmented. The nerve terminal had changed in its branching pattern and become more varicose. The Schwann cells at the junction had undergone changes as well (9). Two types of transformation were noted: young-appearing junctions with pretzel-like structure changed into the fragmented, varicose phenotype. Moreover, junctions that were already fragmented underwent a further transformation into an even more

fragmented phenotype. Li et al. concluded that the junctions in the aging muscle underwent an apparently stochastic transformation from the young to the old phenotype. To confirm their results without vital imaging they made use of the change in BTX labeling. They labeled the sternomastoid muscle in a set of aging mice under brief anesthesia with one fluorescent color of BTX. They then sacrificed the mice at an interval following this labeling, dissected the sternomastoid and labeled it with a second fluorescent color of BTX. They found junctions that were strongly labeled with the second color that were weakly labeled with the first color and these junctions were found in a state of fragmentation.

What causes this fragmentation of junctions? A possible clue comes from the location of nuclei in muscle fibers. Muscle fibers are multinucleate cells and the nuclei in a mature fiber are normally located peripherally just beneath the sarcolemma. However, in aging muscle many fibers have been reported to have strings of nuclei located not at the periphery but near the center of the fiber. These "central nuclei" were noted in some of the earliest studies of aging (446, 886). They are believed to be signs of "segmental necrosis" (457) in muscles, where a damaged muscle fiber degenerates, is phagocytosed by invading immune cells. Satellite (stem) cells of the muscle become activated, undergo mitosis, and differentiate into myoblasts that then fuse together to regenerate the original muscle fiber inside its basal lamina sheath. As in developing muscle fibers, these nuclei in regenerated segments are located, at least initially, centrally. In some cases it was possible for Li et al. to perform static imaging after observing such fragmentation of a NMJ *in vivo*; they then imaged the same fiber *in vitro* after staining with a nuclear dye (DAPI). Strings of central nuclei were found in some cases, but such examinations are typically difficult. A massive addition of nuclei occurs in the vicinity of the NMJ in aged animals and this obscures the location of the nuclei in the fiber; it is often difficult to positively determine whether any observed string of nuclei are in the fiber on which the junction of interest is located. One would predict that *in vitro* observations of fibers on which fragmented NMJs were located would all contain such central nuclei. However, this is not the case in static images of these fibers, suggesting several possibilities: the presence of central nuclei is unrelated to junctional fragmentation, the presence of central nuclei is transient and the nuclei in such fibers eventually move to the periphery (the junctions encountered in any muscle would vary as to when in the past fragmentation occurred), central nuclei are present somewhere along the fiber but do not extend through the region containing the junction. Whatever the case, this study shows that the morphological change in NMJs during aging is a sudden event rather than a slow, gradual process.

Damage to muscle fibers causes fragmentation of NMJs

A variety of experimental approaches that damage muscle fibers produce an aging-like phenotype in their NMJs. As aging muscles are more prone to contraction-induced damage and experience such damage (cf. (109, 110, 277), it is relevant to examine how muscle damage produces neuromuscular changes.

Deliberate damage to muscle fibers can produce changes in NMJs that mimic those in aging. Rich and Lichtman (754) conducted a study in which they deliberately damaged muscles in the sternomastoid muscle of mice by mechanical means and vitally imaged the effects on the NMJs

using 4-Di-2-Asp and BTX. They observed the degeneration of the underlying muscle fiber, the loss of most of the aceylcholine receptors, and the withdrawal of the nerve terminal from the muscle fiber. The nerve quickly returned and within 3 days, the junction had been dramatically transformed to have a new set of acetylcholine receptors that were in a fragmented configuration on the previously damaged fiber. Central nuclei were present, suggesting fiber regeneration. Rich and Lichtman (754) commented that the junctions had the appearance of aged junctions and concluded that the muscle fiber was necessary moment-to-moment to provide factors to maintain the nerve terminal. These results have been subsequently followed up by examining the consequences of postsynaptic injection of protein synthesis inhibitors (35, 620), showing that this manipulation can result in retraction of the nerve terminal. As another method of fiber damage, Van Mier and Lichtman (913) used a laser microbeam to ablate individual fibers by interrupting the sacrolemmal membrane outside of the NMJ. The result was the same as that in the case of Rich and Lichtman, only here by confining the damage to individual muscle fibers, they we able to detect a directional sprouting response from nearby undamaged NMJs.

Somewhat different results were obtained when Li and Thompson (565) utilized the Van Mier protocol to vitally image the consequences of ablating single muscle fibers in sternomastoid muscles of young mice with a laser microbeam. The laser microbeam was aimed *in vivo* at individual muscle fibers on which the NMJs were identified by labeling receptors with a non-blocking dose of rhodamine BTX. The microbeam was applied to the fiber on each side of the junction some 100-150 microns distant. The muscle fiber segment underneath the NMJ underwent necrosis. Indeed, within 1 day the site of the old muscle fiber was filled with phagocytic cells. The acetylcholine receptors originally labeled in the muscle fiber membrane became very dim within hours, suggesting these receptors had been mostly turned over during this interval, not surprising since the fiber itself was degenerating and being phagocytosed. However, beginning at least 3 days later new receptors could be identified by another application of BTX and a regenerating muscle fiber with sarcomeres could be identified underneath the old synaptic site. However, the nerve terminal during the period prior to the regeneration of the muscle fiber remained with all its branches, together with the associated Schwann cells and their processes (**Fig. 5**). These nerve terminals, likely attached to the basal lamina of the now absent muscle fiber, showed no signs of sprouting or terminal withdrawal, although the terminal area appeared to shrink, likely as a consequence of the collapse of portions of the basal lamina. When receptors began to reappear in the regenerating muscle fiber they were in a fragmented state and apposed by varicosities on old and new branches of the nerve terminal. When followed over months, the fragmented state remained and there was no sign the junction reverted to the pretzel configuration. These findings suggest that the damage produces an irreversible change in the configuration of the synapse. Moreover, the stimulus for new growth of the nerve terminal arises from regeneration of the muscle fiber, a conclusion also suggested by Van Meir and Lichtman, although in their case the growth was the formation of sprouts from adjoining muscle fibers. It is not clear what explains the difference between the findings of Van Mier and Lichtman and of Rich and Lichtman in the withdrawal of nerve terminals vs. their maintenance. One explanation might be the use of a mitochondrial dye rather than a fluorescent protein. It is possible the soluble fluorescent protein expressed transgenically in mice provides a more persistent labeling than the 4-Di-2-Asp or that the mitochondria labeled by this dye become redistributed or stain differently after loss of the muscle fiber. In this regard, it is interesting to make a comparison to the

frog. Here muscle fibers in the thin, flat cutaneous pectoris muscle can be removed without damaging the nerve terminals: the fibers are cut on each side of the endplate band and regeneration of these fibers prevented by irradiation (43, 797). Although a few of the terminals withdraw, most remain intact in contact with the basal lamina and do not sprout (27, 37, 247, 978). They even continue to release and recycle synaptic vesicles (19, 247) upon nerve stimulation, even at long periods after removal of muscle fibers. One change that is reported is that the terminals become enwrapped by the Schwann cells (37, 978).

Similar changes are observed in rodent muscle fibers in which muscle fibers are caused to degenerate by administration of myotoxic drugs (41, 42, 44, 178, 243, 431). Commonly these experiments use fractions of cobra venoms ("cardiotoxin") that are purported to have rather selective actions on muscle fibers, causing their necrosis, but do no damage to nerves, nerve terminals or blood vessels. These toxins produce rapid loss of the muscle fiber (and loss of most acetylcholine receptors) but are reported to leave the nerve terminal intact. New acetylcholine receptors appear on the surface of the muscle fiber as it regenerates from satellite cells within the old basal lamina sheath. The nerve terminal contacts this fiber. The nerve contacts are formed by varicosities in close vicinity to where the synaptic site was originally located. The acetylcholine receptors on the regenerating fiber appear in a fragmented pattern (like that seen in **Fig. 6, 7** after laser damage to individual fibers). Central nuclei are present within the regenerating fibers (like that seen in laser damage in **Fig. 8**), indicating that the damaged myofiber has been replaced. Thus, the events that follow the administration of myotoxins appear to mirror rather exactly the events following mechanical or laser damage to muscle fibers (cf. (42, 178)).

One of the well-known ways to damage muscle fibers either *in vitro* or *in vivo* is to cause them to undergo so-called "eccentric" contractions (30, 108, 724). In these cases, the muscle, instead of shortening during contraction, becomes lengthened (stretched) due to the activity of antagonist muscles. Use of membrane impermeant dyes or proteins normally enriched in the extracellular space show penetration of these components into fibers damaged by eccentric contraction, likely due to tears in the sarcolemma as the forces exerted on the molecules linking the cytoskeleton of the muscle and its cell membrane to the extracellular matrix in the basal lamina of the fiber itself exceed the ability to resist the strain (278, 303, 851). These tears can result in segmental necrosis. Interestingly, a muscle's susceptibility to damage from these types of contractions increases over the lifetime of the animal (30, 108, 110) and during certain disease states (see below). In both cases, it appears that the structure of the basal lamina and its linkage to the cytoskeletion of the muscle fiber become weakened. Some fibers unable to resist such eccentric damage even in young, normal animals consequently undergo fragmentation of receptors and the generation of central nuclei (564).

The primary example of purported eccentric damage to muscle fibers occurs in Duchenne muscular dystrophy (DMD). This disease results from mutations in the gene for the protein dystrophin, a large protein localized to the sarcolemma that links the actin cytoskeleton of the muscle fiber through the sarcolemmal membrane protein dystroglycan to proteins of the extracellular matrix. In addition, the dystrophin protein has several motifs that serve to localize important signaling molecules to the muscle membrane (e.g. nitric oxide synthetase) whose localization is defective in mutant fibers. This localization defect disturbs circulatory homeostatic reaction to active fibers, as synthesis and release

of nitric oxide normally stimulates the local dilation of blood vessels. Dystrophin-lacking myofibers are clearly more susceptible to damage from eccentric contractions. There are a number of other mutations to components of this "dystrophin glycoprotein complex" that increase the susceptibility of muscle fibers to contraction-induced damage (192). Taken together, all these cases of fiber injury point to the crucial role of linkages between the extracellular matrix and the muscle cytoskeletion in resisting mechanical damage to a muscle undergoing active contraction. Without these linkages, contractions generate increased strain on the sarcolemma (471, 744).

Molecular mechanisms underlying changes to aging NMJs

The precise molecular mechanisms that produce aging-associated fragmentation of NMJs remain poorly understood. One indication of the likely complexity of mechanisms here are experiments that show that the course of fragmentation can be profoundly influenced by exercise and by placing animals on a calorically restricted diet (cf. (908)). Moreover, a number of genetic manipulations result in structural changes like those in aging but occur without any evidence of overt fiber damage and regeneration. These results suggest the existence of additional mechanisms for aging of this structure. Several molecules involved in the formation and the maintenance of the NMJ, when absent, appear to produce pre- and/or post-synaptic alterations observed in aged muscles. Genetic deletion in adults by conditional knockout of the nerve supplied synaptogenic molecule agrin (114, 793) or its muscle receptor, Lrp4 (45, 52), results in dismantling of mature NMJs and features of the aged synapse. These include fragmentation of postsynaptic acetylcholine receptors, varicose motor axon terminals and in some cases partial or complete denervation of endplates. A similar change occurs after knockout of MuSK in the adult (391, 476). Further evidence of the importance of this pathway comes from results from the Burden laboratory (132), examining NMJs and synaptic transmission in mice overexpressing a mutant human superoxide dismutase transgene as a model of amyotrophic lateral sclerosis (ALS). In this model of ALS, components of the agrin signaling pathway were manipulated. Neural agrin binds Lrp4 which in turn activates a kinase in the muscle (MuSK) that has profound effects on synaptic differentiation. NMJs in this ALS model undergo denervation and reinnervation, both reported to occur in aging (145, 907). Burden et al. report that administration of an antibody engineered to activate the kinase MuSK, can ameliorate muscle denervation and improve synaptic transmission even after the advent of symptoms but has minimal effects on the life expectancy of the affected mice. Lastly, mice lacking ERK1/2 (134, 814) or having reduced trkB signaling (337, 495) generate NMJ fragmentation in the absence of central myonuclei, the usual criterion of muscle fiber damage and regeneration.

Precocious accumulation of aging -like NMJ morphologies are also seen in absence of synaptic basal lamina components laminin α4 (114, 793) and type IV collagen α5 (53, 300). These are required for expression of both synaptic collagen IV trimers, (α3)(α4)(α5) and $(\alpha5)_2(\alpha6)$ – where the initial formation and maturation occur normally. The absence of synaptic collagen IV trimers did not lead to a general disruption of muscle basal lamina or muscle fiber integrity as central myonuclei were rarely observed (53, 300). The muscle basal lamina and muscle fiber likely remain intact also for deletions of laminin α4, but this is presently unconfirmed.

Skeletal muscle fibers possess special mechanisms to ensure that proteins required to form and maintain a functioning synapse are synthesized by the ribosomes and nuclei located at the site of the synaptic contact. There are several muscle nuclei localized just postsynaptically beneath the synapse (the so-called sole plate nuclei) that aggregate here and carry out the transcription of so-called "synapse specific" genes (cf. (796, 828)). Among these genes selectively transcribed are those for acetylcholine receptors and the scaffolding proteins that allow the aggregation of these receptors at high density in the postsynaptic membrane. The molecular signaling allowing for this selective gene expression are largely known (390, 880) as are some of the mechanisms for the nuclear aggregation (267, 346). Genetic inactivation of Erm, a transcription factor that promotes gene expression by sole plate nuclei, also produces the fragmented postsynaptic appearance (395) and further implicates modulated expression of genes involved in NMJ formation in its aging-associated remodeling. An interesting recent paper by Liu et al. (578) reports a failure of this nuclear aggregation at the NMJ in aging muscles. The number of sole plate nuclei is drastically reduced and this reduction is mirrored by a decrease in the density of postsynaptic acetylcholine receptors, suggesting that aging muscles have a reduced ability to maintain the specialized synaptic contact. Several questions remain unanswered. Are the sole plate nuclei at aged synapses lost or is there some kind of failure in their maintained localization? Is the nuclear loss a general one occurring throughout the muscle fiber, or is it selective to the synapse? Aging adult muscle fibers following rounds of degeneration and reinnervation appear to be hypernucleated (Ryan Massopust, personal communication). However, Liu et al. (578) present evidence of some failure of generation of muscle stem cells in aging muscle to generate cells to renew or regenerate lost synaptic nuclei. Because the sole plate nuclei located underneath the synapse are specialized transcriptionally for the production of synaptic proteins, such a reduction in nuclei could produce alterations in synaptic proteins necessary for the maintenance of aged synapses. However, what causes the loss of the sole plate nuclei in these cases is unclear, as is also the role of muscle fiber damage. The purported failure of satellite cell generation is a controversial topic in aging and dystrophic muscle (7, 77, 169, 198, 327, 480, 598, 680, 810, 926).

Recent study also implicates glial involvement in producing aging-associated morphology. A transgenic increase in neuregulin1 expression by motor axons resulted in fragmentation of acetylcholine receptor aggregates and varicose appearance of presynaptic nerve terminal branches in every muscle in the animal (110, 549). This phenotype was a consequence of enhanced activity of terminal Schwann cells and occurred in absence of any muscle fiber damage. Moreover, recent evidence suggests that, in addition to neuregulin1, a set of molecules collectively known as "damage-associated molecular patterns" (DAMPs) or "alarmins" – released as a result of cellular damage or disease (50, 109, 155, 602, 878), can activate SCs (75, 136, 331, 902). Terminal SCs, therefore, may be recruited under various conditions (disease, muscle fiber damage, denervation, inflammation, etc) to produce synaptic fragmentation.

Absence of yet another synaptic basal lamina component, ColQ, leads to a fragmented postsynaptic morphology (282). However, unlike the molecules above, lack of ColQ may lead to synaptic remodeling as a consequence of muscle fiber necrosis (and regeneration) specifically at synaptic segments. This local muscle fiber damage likely results from the lack of acetylcholinesterase (282), whose acute inhibition results in such local muscle fiber degeneration (541).

Still another promising approach to understand the changes that occur at the NMJ in aging has been undertaken by Nishimune et al. (669). They have employed high resolution fluorescence microscopy of NMJs following immunolabeling of the molecule bassoon, a known component of the active zones where neurotransmitter is released from nerve terminals. Examination of the nerve terminals in mice has revealed a distribution of puncta of bassoon immunoreactivity in the synaptic membranes of nerve terminals that likely reflects the distribution of active zones in these terminals. They report a decrease in the density of these puncta in aging animals, suggesting a mechanism that could explain any diminution in neurotransmission that occurs at aging junctions.

Physiological changes in synaptic transmission at aging junctions

The physiological consequences of fragmentation are far more ambiguous. Careful studies of neuromuscular transmission at fragmented junctions have shown that, at least in the muscles studied, there is no change in the size of the mepps or in the quantal content of the endplate potentials, even when junctions known to be fragmented are studied physiologically, whether these junctions become fragmented in aging or in muscular dystrophy (243, 593, 947). There are several limitations to the interpretations possible from these studies, including the particular muscle(s) studied, the animal that was the source of the muscle, and the age of the animal. Most studies claim that the fragmentation in mice becomes most dramatic after around 20 months of age, even though some fragmented junctions can be found even in very young mice (9, 198). Most studies that claim to see a physiological difference have been conducted at advanced age. Another problem with these studies is that, in many cases, the evoked responses are studied only in response to single stimuli rather than the spike frequencies that occur *in situ*. That is, it is possible that there is a problem in transmission at fragmented junctions that occurs only upon repetitive stimulation, e.g. in the fatigue in transmission. Nonetheless, one can also point to a simple disease model in mice (mdx-the mouse model of Duchenne) where there is quite profound fragmentation of the receptors at NMJs without an obvious deficiency in synaptic transmission (593). Indeed, the mdx mouse is renowned for the lack of obvious behavioral deficits despite the rather extreme fragmentation of junctions that occurs early in the life of the animal (for example, after ca. 9 weeks of age) as a consequence of its muscle pathology.

So, is it possible to reconcile the obvious defects in muscle mass and contractile performance during advanced aging with the apparent effectiveness of NMJs when they are evaluated physiologically? Something is clearly hidden in the studies discussed to this point. One possibility is that atrophy and disappearance/necrosis of denervated fibers is rapid and they are not sampled in the various physiological assays. Another obvious possibility is that the defects in neuromuscular performance occur not at the level of individual synapses, but in the contractile performance of the fibers or alternatively in the way the synapses are utilized within the muscle. Compared to evaluating individual junctions, the evaluation of the ensemble of synapses made by individual motoneurones (i.e. motor units) is much more difficult. By various techniques, most commonly teasing filaments from ventral roots after laminectomy cf. (282, 487) it is possible to isolate bundles of axons that, if teased sufficiently small, contain among the several axons present, just a single axon innervating the muscle of interest. It is then possible to evaluate the contractile performance of that single motor

unit by monitoring the contractions of its unit fibers by tension recording. Moreover, it is possible to employ techniques that mark the utilization of metabolic energy by the activated fibers. Most prominent among these methods is the technique of glycogen depletion: activation of the fibers in the unit by stimulation of the motor axon can, under repeated stimulation in which efforts are made to ensure that neuromuscular failure does not occur, forces the active fibers to utilize their stores of glycogen. After staining muscle sections for glycogen, fibers activated during this procedure can be identified. What makes this procedure very tedious is that the depletion is usually conducted *in vivo* after extensive surgery and attendant difficulties of maintaining a living animal, there is just one unit identified in each experiment, and in slow-twitch motor units good glycogen depletion usually requires the imposition of anoxia during the stimulation. Despite these problems, the results obtained define some new major issues that would be missed by examining either the physiology of individual motor units or the morphology of individual junctions, even if these experiments are carried out with the most sensitive vital imaging techniques. Clearly, with the new technologies now available, including use of activity sensitive dyes (316, 390), optogenetics (303, 888), retrograde labeling of innervating axons with lipid soluble dyes injected at junctional sites (283, 291, 316, 317), the labeling of subsets of axons by transgenic expression of fluorescent proteins (283), and the tracing of individual labeled axons as they extend through the muscle by use of high resolution microscopy (299, 300, 396, 590) this situation will certainly change in the near future.

Glycogen depletion experiments have been performed on single motor units in individual muscles and the results compared between young and old animals. By far the most extensive of these experiments are those conduced in rats by Edstrom, Larsson, and colleagues (246, 247, 259, 520, 523, 524). Several profound conclusions can be made collectively from these experiments. First, there is clear evidence for a rearrangement of the distribution of fibers within motor units. The unit fibers, especially in the soleus muscle, that are normally spread in a random, scattered distribution in the muscle occupy an enlarged portion of muscle section, although very seldomly are fibers in a single unit adjacent to each other. This shows that there is a remodeling of the muscle innervation, but this remodeling differs from that seen after nerve damage leading to wholesale muscle denervation (or partial denervation) followed by reinnervation, in which case large, contiguous areas composed of adjacent fibers in the muscle can be innervated by single units (275, 487). The situation in old age could be explained by more moderate, gradual loss of innervation of individual fibers in a unit followed by sprouting of the axon losing the synapse or by neighboring motor axons (cf.(839). The conduits of the nerve basal lamina sheaths remaining behind would encourage such sprouting within the intramuscular nerves or even by terminal sprouts that enter these conduits and grow retrogradely along the pathway of the lost axon (Kang, Tian, and Thompson, unpublished observations). Such axons entering the previous endplate site might be indistinguishable from the normal innervation pattern (i.e. absent the sprouts from adjacent junctions in the muscle) or certainly the reformation of synapses upon regenerating fibers as argued above. Such events might explain the apparent absence of major sprouting in aging muscle. One might not expect to encounter such a change in the identity of individual motoneurones innervating individual fibers in aging muscle in vital imaging experiments in which the frequency of such events is low and the speed with which reinnervation of a fiber lacking innervation is rapid.

A second revelation of the glycogen depletion experiments is that the number of motor units is decreased (in some rats by as much as 25% at 20-25 months of age). Although there is a loss of muscle fibers, the average size of the remaining units is increased. This suggests there is a slow, gradual loss of motoneurones to the muscle, an observation supported by several other observations, including counts of axons in ventral roots and motoneurones in the ventral horn. The cause of this loss is not presently clear, but one idea that has received attention is that motoneurones, in face of continued, gradual loss of synapses are induced to sprout and this sprouting expands motor units and places additional stress on them for maintenance of an enlarged peripheral arbor. Indeed, it is very clear that even young motoneurones have a limit in their ability to expand their peripheral arbors (877) and that this ability is reduced in aged animals (705, 771). If the number of motoneurones is being reduced and the arbors of remaining motoneurones are expanding, any deficit in the ability of motoneurones to reinnervate the denervated fibers could lead to the atrophy of these fibers and their loss from the muscle (see above for further information)

A third revelation of the glycogen depletion experiments is that the motor unit fibers undergo a transformation in the differentiation of the contractile properties of their fibers. Evidence suggests that this transformation occurs unit by unit, likely due to changes in the pattern of activation of the fibers in the unit. As a motor axon comes to innervate additional fibers (or experiences a change in its pattern of activation), it is very clear that this axon can change the differentiation of the fibers that it innervates. This transformation is particularly prominent in muscles where the muscle fibers in individual muscle units shift away from fast a slower type (259, 521).

Taken together, these observations suggest that even if individual synapses apparently function well, the ability of the muscle to carry out different motor tasks is altered by a loss of motor units (which would be expected to decrease the fineness of motor control), a loss of muscle fibers (which would decrease the total strength of the muscle), and a change in fiber type within muscles (which would change their contraction kinetics and their ability to resist fatigue). What is really needed is a careful evaluation of how an individual muscle changes its performance and whether these changes can be accounted for by the changes that occur throughout the complement of units within the muscle.

Muscle Cell

Pathological changes at the motoneurone level preferentially affecting the largest motoneurones included in fast-twitch motor units, resulting in peripheral denervation and incomplete reinnervation by motoneurones included in slow-twitch motor units have been forwarded as the dominant mechanisms underlying the muscle wasting and loss of muscle function in old age (137, 265, 376, 420, 444, 709). A fast-to-slow myosin isoform (fiber type) switching was originally reported four decades ago in sedentary men ranging in age between 22 and 65 years (539) and repeatedly documented within the a same age-range as well as among older individuals (377, 415, 423, 644, 801, 825). This is in accordance with the aging-related decreases in fast MyHC mRNA levels in human limb muscles, while the slow MyHC mRNA levels were unaffected by aging (40, 102, 825, 928). Others, have not confirmed this fast-to-slow conversion during aging in humans (190, 423, 469, 644). This discrepancy may be secondary to the influence of differences in physical activity levels interfering with aging-related effects as previously discussed (130, 514, 515, 520). It is

therefore of specific interest to note that in the sternocleidomastoid muscle, a muscle which is considered to be less affected by aging-related changes in physical activity, a similar aging-related fast-to-slow MyHC isoform transition was recently reported as in human limb muscles (584). In experimental animal models, where secondary influences from environmental factors not related to the aging-process *per se* are more easily controlled, an aging-related fast-to-slow myosin isoform transition is more widely accepted (23, 25, 56, 85, 126, 258, 265, 444, 463, 482, 519, 521-523, 540, 586, 709, 855, 865). The fast-to-slow fiber type transition during aging may contribute to the slowing of movement as well as decline in maximum force since fast muscle fibers are considered to have a higher force generation capacity than slow fibers. However, it is important to emphasize that the relative changes in MyHC/fiber type proportions during aging in humans are relatively small (see (520)). Besides the aging-related motor unit loss, fast-to-slow muscle fiber shift, and fiber atrophy that accompany the progressive motoneurone loss, other intrinsic qualitative changes at the muscle fiber level as well as the loss of the coordinated expression of muscle proteins in old age may have a significant impact on overall muscle function. For instance, the coordinated expression of contractile *vs*. SR proteins, contractile *vs.* mitochondrial proteins and myosin heavy *vs.* light chain isoform expression observed at the single muscle fiber level in humans and in experimental animal models are at least partly lost in old age (520, 563, 791) and these changes have most probably far more reaching consequences for regulation of muscle contraction and muscle function in old age than aging-related small perturbations in fiber type/MyHC isoform expression.

Nuclear organization

Skeletal muscle fibers are the largest cells in the body. They may be several centimeters long and have a large cytoplasm. In order to support this large cytoplasm, muscle fibers are truly multinucleated cells and contain hundreds to thousands of nuclei (myonuclei). Myonuclei are post-mitotically fixed and typically located in the periphery of the muscle fiber, just beneath the plasmalemma, but central localization may be observed during transitional or pathological conditions. Myonuclei are evenly dispersed along the length of the fiber except for the neuromuscular junction and myotendinous ends, where nuclei are clustered and have specialized functions (185, 648). The even distribution of myonuclei along the major part of the fiber has been speculated to be the result of myonuclei repelling each other to minimize transport distances (120, 121) or the attractive interactions between microvasculature surrounding muscle fibers (especially in slow-twitch fibers) together with the repelling interactions between myonuclei and the intermediate filament desmin (739). Each myonucleus regulates the gene products in a defined cytoplasmic volume known as the myonuclear domain (MND) or DNA unit (12, 13, 150, 364, 507, 699). Myonuclei are not transcriptionally active simultaneously and regulation of transcription occurs in a pulsatile manner (665), but it remains elusive how transcriptional activities are coordinated between individual myonuclei.

The MND size is critical for the function and adaptability of the muscle cell. MND size may change in response to muscle hypertrophy/atrophy and aging (120, 121), and it is affected by various factors such as muscle fiber type (MyHC isoform) (12, 450, 778, 895), mitochondrial activity (576), and species (body size) (576). Slow-twitch oxidative muscle fibers have smaller MNDs than glycolytic fibers coupled to their higher protein synthesis and degradation rates than fast-twitch fibers (253, 553, 675, 917). Further, there is a tight coupling between muscle hypertrophy and the addition of

new myonuclei via satellite cell activation to maintain a similar MND size during muscle hypertrophy (119-121, 778). However, satellite cell activation and myonuclear addition may lag behind the initial phase of hypertrophy coupled to an increased transcriptional and translational activity (153). Single muscle fiber *in vivo* analyses of myonuclear organization show that myonuclei are not lost in proportion to atrophy after denervation, resulting in smaller MND size in response to atrophy which may serve as a "muscle memory" when later exposed to e.g. a hypertrophic stimulus (119). Further, there are significant differences in MND size between different mammalian species and a significant difference in MND size has been shown in single muscle fibers in six different mammalian species with a 100,000-fold difference in body mass (**Fig. 9**), presumably reflecting the lower metabolic demand and lower protein turnover rate in large vs. small mammals (576).

Conflicting results have been presented in the literature regarding the effects of how different muscle atrophy conditions, including sarcopenia, impact on myonuclei organization and MND size (119-121, 912). Aging has been reported to be associated with an increased (106, 441, 912) or unchanged (314, 605, 730, 856) MND size in both rats and humans. These differences may, at least in part, be related to methodological differences and inherent methodological limitations. Estimates of MND size during aging have frequently been based on measurements from single muscle cross-sections, *i.e*., counting the number of nuclei surrounding each fiber and assuming a constant shape and size of myonuclei as well as the exclusion of non-muscle fiber nuclei. However, myonuclei have different shapes and in human muscle the shape of myonuclei is affected by aging, i.e., myonuclei frequently present deviations from the rounded or elliptical appearances observed among young individuals with elongations and indentations of the nuclear enevelope in old age adding a systematic error when calculating aging-related changes MND size based on measurements from muscle cross-sections assuming constant myonuclei size and length (180). Further, the distinction between myonuclei and the numerous other nuclei located between muscle fibers is not trivial (95, 182, 576). To overcome these limitations, myonuclear organization has been investigated in 3D along the length of individual muscle fibers and this review will focus on 3D measurements of MND size and how it is affected by aging. Bruusgaard and co-workers (120) introduced a novel *in vivo* method to visualize myonuclei in intact muscle fibers from mice by microinjection of labeled DNA into single muscle fibers allowing longitudinal studies of myonuclear organization of myonuclei in single living cells and excluding satellite and non-muscle myonuclei. By using this model in young (2 mo.), middle-aged (14 mo.) and old (23 mo.) mice, the effects of aging on myonuclear organization was studied in muscle fibers from the slow-twitch soleus and the fast-twitch extensor digitorum longus (EDL) muscles (121). Significant aging-related changes were observed, such as an increased number of fragmented myonuclei, a less regular myonuclear shape, a decreased number of myonuclei in parallel with the preferential atrophy of fast-twitch muscle fibers as well as an impaired orderly distribution of nuclei in old age (121). The altered spatial organization of myonuclei in old age was suggested to be secondary to parallel changes in the microtubule network (121). Thus, these 3D *in vivo* analyses add significant novel information on aging-related changes in myonuclear morphology and organization which may have a significant impact on skeletal muscle function and adaptability. However, as discussed above, the mouse is not an ideal species for the study of human aging. In order to bypass experimental models, a 3D method combining confocal imaging and specific digital algorithms was introduced to determine the morphology and spatial organization of myonuclei along the length of individual single human membrane permeabilized muscle fiber

segments characterized according to muscle fiber size and MyHC isoform expression (180). In short, 3D confocal reconstructions of single human membrane permeabilized muscle fiber segments were performed after attaching both ends to 3-way positioners and setting optimal sarcomere length for force production. The spatial distribution of nuclei and *x,y,z* coordinates were determined and a custom made software calculated the cytoplasmic volume and size of MNDs corresponding to each myonucleus (**Fig. 10**) (180). In contrast to previous observations in rodents (120), myonuclear shape was not dependent on muscle fiber type, *i.e*., round or elliptical nuclei were observed in the same fiber independent on fiber type, age and gender. In old age, deviations from the rounded or elliptical shapes were frequently observed with indentations of the nuclear envelope as well as myonuclei aggregated in groove-like structures along the length of the fiber irrespective gender and fiber type. Analyses of 3D nearest neighbor distances in ~15,000 myonuclei using a custom made software confirmed aging-related aggregation of myonuclei (180) in accordance with previous observations in humans (95) and mice (121). This is consistent with a dynamic positioning of myonuclei in adult muscle (739). Aggregation of myonuclei has been reported in response to denervation (946) and the well-established long-term denervation reinnervation process during aging in both rodents and humans (520) may accordingly be an important factor triggering myonuclear aggregation in old age (180).

In muscle fibers expressing the IIa MyHC isoform, a small but significant decline in MND size was observed in old age. On the other hand, average MND size was not significantly affected by age in muscle fibers expressing the type I MyHC isoform, but there was an increased variation in MND size in old age independent of gender (**Fig. 11, 12**). The lower MND size in muscle fibers expressing the IIa MyHC isoform may represent a physiological adaptation to the preferential type II muscle fiber atrophy observed in old age (539, 801, 886), but it is unlikely that the increased variability in MND size in type I fibers was secondary to a denervation-reinnervation process since this process is not restricted to slow twitch motor units (520). The increased MND size variability in old age due to an altered spatial arrangement and aggregation of myonuclei may represent an important aging-related biological process (180) and it is consistent with an increased MND size variability in old mice (95). In old mouse muscle, the increased MND size variability appeared to be related to a loss of satellite cells and hindering of efficient nuclear replacement (95). The increased complexity of the spatial organization of myonuclei organization may impact on the efficiency of transcriptional activity with consequences for local protein synthesis and degradation along the length of the muscle fiber. In this context, it is of interest to note that accumulation of dysfunctional mitochondria and oxidative stress occur locally along the length of the muscle fiber and not homogenously in the muscle fiber (682, 918). Regions along the length of the muscle fiber characterized by large MND size may accordingly have a deficient protein turnover resulting in a lower concentration of contractile proteins with a negative effect on force generation capacity (183, 190, 529, 875) and/or post-translational modifications of proteins with a slow turnover such as contractile proteins in muscle fibers expressing the type I MyHC isoform (405, 529, 563, 740). Further, there appears to be a critical volume individual myonuclei can support to efficiently maintain function (732) and local areas within individual muscle fibers from old individuals may have exceeded this volume with negative consequences for muscle function.

Mitochondrial function, oxidative stress, heat shock proteins, post-translational protein modifications

Mitocondria are small intracellular organelles that play a crucial role in the life and death of cells. They produce most of cellular ATP through oxidative phosphorylation (OXPHOS), but are also essential for thermogenesis, for the control of the homeostasis of calcium, one of the main intracellular second messengers, and are the key effectors of the intrinsic pathway of apoptosis, the cellular pathway that leads to programmed cell death. All these functions are interconnected and are tightly regulated (129). Moreover, mitochondria host several other biochemical pathways such as the beta oxidation of lipids, portions of the urea cycle and several enzymes involved in amino acid metabolism.

Mitochondria derive from ancient Gram-negative bacteria, similar to modern-day Rickettsiae, which started an endosymbiontic process with the progenitor of the eukaryotic cells about two billion years ago (20). Mitochondria still retain many features of their bacterial progenitor. They are enveloped by two distinct membranes: a permeable outer membrane and a highly impermeable inner membrane (MIM). The MIM presents several introflexions named cristae, which expand the total area of the MIM, thereby enhancing its ability to produce ATP (266).

Mitochondrial genetics

Mitochondria have their own genome (mitochondrial DNA – mtDNA), which is distinct from the nuclear genome (nDNA) and is similar to bacterial chromosomes. In fact, mtDNA is circular, it is not organized into chromatin, genes are not interrupted by introns (and non-coding sequences comprise less than 5% of the genome) and is present in multiple copies per mitochondrion. The mitochondrial genetic code is also different from the universal code, therefore mitochondria have their own translation apparatus. These peculiar characteristics of mtDNA account for the unique laws of mitochondrial genetics, which are different from those of classical mendelian genetics and resemble more closely the laws of population genetics (234). First, mtDNA is transmitted only from the mother (paternal DNA is actively eliminated from the Zygote). Second, the presence of multiple genomes per mitochondria (a single muscle fiber can contain several hundreds of thousands copies of mtDNA) implies that whenever a mutation is present, wild type and mutant mtDNA may coexist at variable proportions, within individual mitochondria in a given cell, in distinct cells within a tissue, or in different tissues within the same patient. This phenomenon is called heteroplasmy. Strictly linked to heteroplasmy is the threshold effect. In fact, mtDNA mutations must affect a critical proportion of the mtDNA molecules within a cell in order to cause a biochemical phenotype. This threshold is in the range of 70-95%. In other words, the majority of mtDNA molecules in a cell must carry the mutation for the biochemical defect to become evident.

Last, the proportion of mutant versus wild type mtDNA may drift with cell divisions due to the random segregation of affected genomes at mitosis. In post-zygotic cells, selective replication of individual mitochondria with high mutational load may also change the proportion of mutant to wild type genomes, favoring the accumulation of defective mtDNA in individual cells (see below).

The respiratory chain

mtDNA encodes a total of 37 genes: the 2 ribosomal RNAs and 22 tRNA required for mitochondrial protein synthesis, and 13 structural genes, all of which are subunits of the respiratory chain. The respiratory chain (RC) is comprised of five multisubunit enzymatic complexes (C.I-C.V) and of two

electron carriers, coenzyme Q (CoQ) and cytochrome *c*. All complexes (except for C.II which contains only nDNA-encoded subunits) include both mtDNA and nDNA-encoded subunits. Moreover, nDNA provides the genes required for mtDNA maintenance and replication, the proteins involved in mitochondrial transcription and translation, and those required for the assembly of RC complexes and CoQ biosynthesis. Biogenesis of the RC requires a tightly regulated interplay between the two genomes and OXPHOS defects may derive from dysfunction of any of the two.

The first four complexes of the RC (C.I-C.IV) catalyse the transfer of electrons from the high energy compounds (NADH, $FADH_2$) generated by the reactions of the Kerbs cycle to oxygen. The energy liberated is used to translocate protons across the MIM generating the electrochemical gradient which is used by C.V to synthesise ATP (266).

RC complexes are not isolated structures, but are physically associated to form huge macromolecular structures termed “supercomplexes”. Supercomplexes continuously form and disassemble and are required for the optimal respiratory efficiency of the cells (4).

RC dysfunction, mtDNA mutations, ROS, and aging

Because their central role in cellular metabolism, especially in skeletal muscle, mitochondria have been considered key players in the aging process for more than four decades (372). In fact the RC is one of the main sources of ROS in cells and mtDNA is particularly susceptible to ROS damage due to the lack of histones and the relatively simple DNA repair system. The traditional mitochondrial free radical theory of aging postulates that ROS produced by the RC cause damage to mtDNA, resulting in mutations which progressively impair OXPHOS. This creates a vicious cycle, with more ROS produced by the dysfunctional RC, which result in further mtDNA damage (**Fig. 13**) (49).

However, in the last fifteen years it has become evident that the situation is far more complex and the relationships between ROS, mitochondrial dysfunction, aging, and sarcopenia are not as simple as previously thought (921). Furthermore, a key issue regarding the role of mitochondria in aging and sarcopenia is whether the observed mitochondrial alterations are primary events or are secondary to other extramitochondrial abnormalities.

A large series of studies have evaluated the changes in mitochondrial bioenergetics during aging in different models. Several approaches have been used from the (relatively) simple spectrophotometric assessment of RC enzymatic activities on muscle homogenates (54, 56, 224, 362, 408, 556, 592, 689), or polarographic studies on freshly isolated mitochondria (81, 133, 144, 342, 893), to the more complex *in vivo* spectroscopic measurements (170, 458, 509, 622). Results however are conflicting. Several factors have been invoked to explain the discrepancies in the different studies: technical issues (715, 846), confounding factors, like exercise or immobility, or, for example, altered blood flow in the case of *in vivo* spectroscopic studies (722), the parameters used for normalization of data (386), and species-specific features that make the situation in rodents different from that of humans (344). Even the type of muscle studied (fast *vs.* slow twitch) appear to be differently affected and the specific mitochondrial subpopulations (*i.e.*, the subsarcolemmal and intermyofibrillar mitochondria) may play different roles in the processes triggering muscle atrophy (544), adding more confusion to an already complex issue.

A good example of how methodological details may profoundly affect results is provided by the observation that the mitochondrial isolation procedure may exaggerate the impact of aging. When confronting mechanically separated mitochondria and saponin-permeabilized myofibers, Picard and coworkers showed that several parameters (maximal respiratory capacity, ROS production, RC activities) were significantly altered when determined in isolated mitochondria, but were normal or nearly normal in permeabilized fibers. The authors suggested that this may reflect the fact that aged mitochondria are more rigid, and reseal less efficiently during mechanical isolation, resulting in loss of mitochondrial matrix constituents, in contamination with cytoplasmic material, and in altered coupling (714). Moreover, some of the protocols used in the past to assay RC complexes activities, especially C.I and C.III, are questionable (846), and there is still no widely accepted method to reliably assay C.V spectrophotometrically (55).

Several studies have investigated the mtDNA alterations associated with aging. It should be noted that, rather than point mutations, skeletal muscle mtDNA accumulates large-scale rearrangements (deletions (451), but also duplications and more complex alterations (869) of the mitochondrial genome) during aging. The exact mechanisms generating these rearrangements are not completely understood but they involve polymerase slippage (59), homologous recombination or slip replication, as it has been hypothesized for the common 4.9 kb deletion which is mediated by a 13 base-pair direct repeat (808, 823), double strand breaks or inefficient repair mechanisms (122, 631). The abnormal genomes then begin to accumulate in mitochondria by a process termed clonal expansion and the affected fibers become filled with dysfunctional mitochondria. These fibers appear as ragged-red fibrers (RRF) on Gomori trichrome stain or ragged blue fibers on succinate dehydrogenase stain (SDH) (811). During aging, muscle becomes a mosaic of normal fibers, and of respiratory deficient fibers harboring very high levels of mutant mitochondrial genomes (>90% of total)(656). However only a minority of fibers appear to be respiratory deficient; this proportion varies in different studies from 5% to 14% (in most severely affected muscles) (100, 122, 785), hence the real impact of these alterations on muscle function is questionable. Moreover, these fibers do not seem to be smaller than those with normal mitochondria (775), therefore their contribution to atrophy is questionable. Finally, mtDNA deletions would cause reduced production of otherwise normal mtDNA-encoded RC subunits that would still function properly.

The issue of mtDNA point mutations is even more controversial. Disease-causing mtDNA mutations have been reported in aging skeletal muscle (440, 579, 654, 990), but the validity of these findings has been questioned (655, 690). Moreover, these mutations were mostly found at low levels (less than 2%), far below their pathogenic threshold. Although some point mutations may behave in a dominant fashion, where as little as 5% of mutant mtDNA in a cell is sufficient to impair OXPHOS (786), they appear to be rare, and have never been reported in aging muscle.

A mouse model harboring a mitochondrial DNA polymerase with a faulty proofreading activity (the “mutator” mouse) displays a marked increase in the rate of mtDNA point mutations with premature aging and muscle atrophy (892). The muscle phenotype of these mice is however different from that of normal aged mice. In fact mutator mice display reduced RC enzyme activitiyes, but a negligible increase in oxidative stress (494, 891), and increased mitochondrial fission and autophagy, whereas normal aged mice have normal RC enzyme activities, increased mitochondrial fusion, and decreased autophagy (640).

Moreover, patients with somatic mutations affecting the MT-CYB gene (encoding a component of RC complex III) in skeletal muscle display markedly reduced complex III activity, exercise intolerance, and myoglobinuria, with no sign of atrophy or sarcopenia (21).

More recently, attention has shifted from mtDNA point mutations to mtDNA copy number. An elegant study has evaluated mtDNA integritiy and function in three generations of women (grandmothers, mothers and daughters) from 18 families. No significant increase in mtDNA mutational load in the coding region was detected, but rather a progressive decline in mtDNA copy number which paralleled the decrease in mitochondrial function (382). The decline in muscle mtDNA copy number associated with aging has been reported by several groups in humans (510, 764, 824, 903), and in an equine model (557). Two studies also detected accumulation of mutations in the non-coding control region of mtDNA (382, 903) especially at position 408; however, there is no conclusive proof that these changes are affecting mtDNA replication. Increased oxidative damage has also been been invoked to explain the reduction of mtDNA copy number (824), however this explanation seems unlikely since there was a reduction also of nDNA encoded enzymes such as citrate synthase, which is usually elevated in case of mtDNA depletion syndrome (MDS) (603).

The concept of threshold applies also to mtDNA depletion, even though much less data are available than what is known about point mutations. Therefore copy number must fall below a certain level for the pathological phenotype to develop. This issue is critical to determine whether the reduction in mtDNA copy number is a primary event or if it refelects a more general impairment of mitochondrial biogenesis (see below). A study analyzing single muscle fibers of patients with the myopathic form of MDS, showed that fibers develop COX deficiency when their mtDNA content is less than 10% of that of controls (249). Moreover patients treated with antiretrovirals often display reduction in mtDNA copy number in muscle, sometimes with a partial reduction also of complex IV, but are clinically asymptomatic, and have normal oxygen consumption (584). Finally, conditions of severe neurogenic atrophy, as seen in patients with spinal muscular atrophy, may provoke a profound reduction in mtDNA copy number in muscle (70).

Another component of the RC, ubiquinone (coenzyme Q –CoQ) deserves a special mention. It is an electron shuttle from RC complexes I and II, to complex III, it is one of the principal cellular antioxidants, and is a modulator of the mitochondrial permeability transition pore, therefore controlling apoptosis (5). CoQ is also a cofactor of several other mitochondrial dehydrogenases, among which di-hydroorotate dehydrogenase (DHODH) and electron-transfer flavoprotein dehydrogenase (ETFDH), and of uncoupling proteins. CoQ deficiency is associated with decreased ATP production, increased ROS, abnormal lipid metabolism, and altered pyrimidine production (227, 583, 736, 890) (**Fig. 15**). Given the central role of CoQ in mitochondrial metabolism and homeostasis and the observation that muscle CoQ levels tend to decrease with aging (542), the impairment of CoQ biosynthesis has been considered an important player in the aging process. Consequently, CoQ administration has been proposed as a simple measure to counteract the aging process. Recent data however questioned this view and animal models (from nematodes to mice) with partial defects of CoQ biosynthesis actually display a longer lifespan and reduced markers of aging despite an increase in ROS and a decrease of ATP production (921). Mice in which the *Coq7* gene (encoding a hydroxylase essential for CoQ biosynthesis) was conditionally ablated at 2 months of age rapidly develop a multisystem disorder leading to death at age 9 months. However, if these

animals are treated immediately before death with 2,4-dihydroxybenzoate, which can bypass the *Coq7* defect and restore CoQ biosynthesis (972), they recover virtually all symptoms and their lifespan is normal (922).

Patients with mutations in genes involved in CoQ biosynthesis and low CoQ levels in skeletal muscle do not develop atrophy or sarcopenia (unless as a consequence of the severe encephalopathy which can be present in these cases) (226, 890). This holds true also for most other forms of mitochondrial encephalomyopathies.

A further proof of the controversial role of ROS comes from mice lacking one copy of *Sod2*, a mitochondrial matrix superoxide dismutase which is essential for protection against ROS. These animals display increased oxidative stress, decreased RC enzyme activities, and increased oxidative damage to both mtDNA and nDNA, but appear phenotypically normal and do not show premature aging (915).

Overall, these observations question the classical mitochondrial theory of aging and their role of ROS as the primary determinants of the aging process in muscle. Nevertheless, ROS may still play an important role in the process, and even if there is no hard evidence showing increased ROS emission with age, reduced endogenous anti-oxidant buffers and increased abdominal fat in older people can cause oxidative stress.

Mitochondrial dynamics and mitophagy

During the past 15 years other aspects of mitochondrial function have emerged that are not directly involved in OXPHOS, but have a crucial role in the functionality of these organelles.

Mitochondria continuously undergo a reshaping process with fusion and fission events which are controlled by a specific set of mitochondria-shaping proteins among which are OPA1, Mitofusin 1 and 2 (which promote fusion), DRP1 and FIS1 (promoting fission) (813) (**Fig. 15**).

All these aspects are tightly connected; in fact, cristae morphology modulates the organization and function of the OXPHOS system, with a direct impact on cellular metabolism (166). OPA1 controls RC supercomplex assembly, increasing the efficiency of respiration (167). This property has been exploited to ameliorate the bioenergetics defect of mouse models with C.IV deficiency (160). Cristae shape is also crucial for controlling apoptosis (159, 302).

Interestingly OPA1 is essential also for maintenance of mtDNA. Yeast lacking the OPA1 orthologue *MGM1* lose their mitochondrial genome (432), and patients harboring dominant-negative OPA1 mutations accumulate deleted mtDNA genomes in skeletal muscle (18).

Directly linked to mitochondrial dynamics is another process called mitophagy. Mitophagy is a specialized form of autophagy aimed at removing dysfunctional mitochondria. Small, fragmented, depolarized mitochondria are encapsulated by a double membrane and form the autophagosome, which is then fused to a lysosome (462) (**Fig. 15**). Mitophagy is designed to recycle whole mitochondria (785), but there is also a set of proteases which selectively degrade misfolded or damaged mitochondrial proteins. These mitoproteases are located in both the matrix compartment (Lon, ClpP, and m-AAA), and the intermembrane spaces (i-AAA, Yme1L1, HtrA2/Omi, OMA1, and

PARL) (767). They are also involved in controlling dynamics (by activating OPA1)(159, 381) and mitophagy (PARL modulates mitophagy by degrading PINK1)(430). The cytosolic ubiquitin-proteasome pathway is also involved in the degradation of several outer memebrane and intermembrane space proteins. Recently, an additional system was discovered which is involved in removing localized portions of mitochondria in response to oxidative stress. Mitochondria-derived vescicles (MDV) bud off from mitochondria in response to oxidative stress, are enriched in oxidized proteins, and are directed to lysosomes for degradation (843, 844). MDV are induced by the Pink-Parkin system but are independent from the fission machinery and from the classical autophagy proteins (767).

Overall, these processes are indispensable for mitochondrial quality control and prevention of atrophy. In fact mitophagy is essential for maintaining muscle mass. In mice, muscle specific knockout of an essential mitophagy gene, *Atg7*, resulted in muscle atrophy and aging-dependent decrease in force, and precipitated muscle loss during denervation and fasting. Affected muscles displayed accumulation of abnormal mitochondria, distension of sarcoplasmic reticulum, disorganization of sarcomere, and formation of aberrant concentric membranous structures (616). Accumulation of dysfunctional mitochondria may also contribute to atrophy by increasing pro-apoptotic signaling. Aged muscles, in fact, display an increased sensitivity to permeability transition (343) and apoptosis (615).

Ablation of Pink and Parkin reduces mitophagy and causes mitochondrial dysfunction, increased sensitivity to oxidative stress and muscle degeneration (74, 161, 694). On the other hand, excessive autophagy can become detrimental to muscle homeostasis. Overrexpression of FoxO3, Mul1 , Bnip3, or Nix triggers autophagy and induces muscle atrophy (582, 601, 766).

Defects of the proteolytic pathways have been reported in different tissues during aging, but data in muscle are scarce and only the Lon system was unambiguously shown to be altered (92), causing accumulation of carbonylated proteins in muscle mitochondria (93). Curiously, autosomal recessive mutations of the *LONP1* gene cause CODAS syndrome (850), characterized by developmental delay, craniofacial anomalies, cataracts, delayed tooth eruption, hearing loss, short stature with delayed ossification, but without prominent muscular symptoms.

Analysis of carbonylated proteins in normal, aged, and sarcopenic human subjects, showed that aging was associated with increased mitochondrial (but not myofibrillar or sarcoplasmic) protein carbonyl adducts, independently of sarcopenia. Mitochondrial protein carbonyl abundance negatively correlated with muscle strength, but not muscle mass. Levels of cytoprotective proteins, (HSP 27, 70 and CRYAB), were unaffected (67).

A well-regulated balance between pro-fusion and pro-fission proteins is also essential to maintain muscle mass. Electron microscopy analyses of murine aged muscles showed that sarcopenia is associated with larger and less circular subsarcolemmal mitochondria while intermyofibrillar mitochondria appeared longer and more branched. While total levels of shaping proteins were not changed, the Mfn2/Drp1 was altered, indicating increased fusion and/or decreased fission (545). Excessive fission (observed in mice overexpressing *Drp1*) also results in reduced muscle mass, compromised exercise performance, and severe remodeling of the mitochondrial network (887). In

mice, aging is associated with a progressive reduction in Mfn2 levels, while conditional ablation of *Mfn2* in muscle results in sarcopenia, impaired autophagy and accelerated aging (816).

However, among mitochondrial shaping proteins the role of OPA1 is probably the most important. In fact, its mild overexpression protects mice from muscle loss secondary to denervation (924). Denervation appears to be a key player in the development of muscle atrophy in old rats (776) and of octuagenerians (845) (see above). A recent work has further stressed the protective role of OPA1. A decline of *MFN1*/2, *OPA1* and *DRP1* transcripts was found in muscle biopsies of old sedentary individuals, while expression was maintained in muscle of senior sportsmen. Most importantly, reduction in muscle mass and force correlated with the in decrease in OPA1, but not with other shaping proteins. Data in aged mice recapitulated the human results; interestingly one week of exercise training could restore normal Opa1 expression in aged mice. Moreover, inducible *Opa1* ablation in muscle of adult mice caused muscle loss and weakness, but also a systemic phenotype (white hair, liver steatosis, hypogliycemia, and denervation) which was mediated by Fgf21 (870) (**Fig. 15**).

Mitochondrial biogenesis

Biogenesis of mitochondria requires a complex interplay between mtDNA and nDNA to ensure coordinated expression of proteins encoded by each genome (266). A specific transcription factor (peroxisome proliferator-activated receptor γ, coactivator 1 -PGC1α) appears to be the master regulator of the process (569). Specific metabolic sensors such as sirtuin 1 (SIRT1), mitogen-associated protein kinase (p38MAPK), and AMP-activated protein kinase (AMPK), activate PGC-1α in the cytoplasm inducing translation of PGC-1α to both the nucleus and mitochondria where it regulates the transcription of nDNA and mtDNA-encoded genes, as well as mtDNA replication (787).

SIRT1 is a member of the sirtuin family, a highly conserved group of NAD^+-dependent protein deacetylases. It is located predominantly in the nucleus but it is also found in the cytosol, and it can shulttle between these two compartments (570). Increases in NAD+ intracellular levels stimulate its activity allowing it to deacetylate (among its targets) PGC-1α (765). Phosphorylation of PGC-1α by AMPK (which in turn is activated by high AMP/ATP ratio (647) is a prerequisite for its deacetylation (131). SIRT1 is also involved in the deacetylation of FOXO3A in response to oxidative stress, promoting the transcription oxidative stress resistance genes among which SOD2 and catalase (117). SIRT1 is also involved in the mitochondrial quality-control machinery. Overexpression of SIRT1, increases the protein levels of Hsp60 and of the CLpP protease which are involved in the mitochondrial unfrolded protein response (651). Finally, SIRT1 appears to be a modulator of the permeability transition pore (562). Other sirtuins also have important roles in mitochondtial metabolism by controlling antioxidant defenses, mitochondrial dynamics, autophagy, and mitochiondrial stress responses (570).

Activated PGC-1α induces expression of nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) but it also directly interacts and coactivates the transcriptional function of NRF-1 on the promoter of mitochondrial transcription factor A (*TFAM*) (965). TFAM promotes transcription of mtDNA, but it is also important for mtDNA replication and packaging (447). Mutations in TFAMcause a severe form of hepatocerebral MDS (849) which affects also muscle mtDNA copy number.

Overexpression of PGC-1α causes an increase of mitochondrial mass (965), and its effect is not limited to proteins related to energy metabolism, but affects also mitochondrial proteins unrelated to energy metabolism (237). However, PGC-1α is not the only regulator of the process since exercise-induced mitochondrial biogenesis is still observed in mice with skeletal muscle-specific knock-out of PGC-1α (777). A closely related protein, PGC-1β, can also activate mitochondrial biogenesis, and deletion of both genes in skeletal muscle has a synergistic effect, causing profound impairment of mitochondrial function (29, 989).

Several studies have addressed PGC-1α levels during aging, but again results are conflicting (428). Because PGC-1α levels are reduced also in sedentary individuals, and physical training can increase its expression, exercise could represent a simple measure to counteract/reduce the effects of aging on mitochondrial biogenesis. In fact, another recent report confirms that aging-related muscle mitochondrial dysfunction can almost completely be rescued by aerobic exercise and the functional improvements are related to the reversal of muscle mitochondrial proteome alteration (764).

Besides its role in boosting bioenergetics, PGC-1α seems to be involved also in the maintenance of the neuromuscular junction (NMJ). It modulates denervation-induced mitophagy in skeletal muscle (906) and together with WldS it has a protective effect on neurons after axonal injury (672). Moreover, pyrroloquinoline quinone, a naturally occurring antioxidant with multiple functions including mitochondrial modulation, prevents denervation-induced muscle atrophy by increasing PGC-1α levels (498).

Other pharmacological stimulators of mitochondria biogenesis have been succesfuly used in murine models of mitochondrial myopathy. Nicotinamide riboside (which stimulates the SIRT1 cascade by increasing intracellular NAD^+ levels) corrected the myopathic phenotype of mice with mutations in Sco2 (142) and in Twinkle (459), while treatment with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMPK, resulted in partial correction of the COX deficiency of Surf1 and Cox15 knockout mice (954). Another compound, bezafibrate, was shown to induce expression of mtDNA-encoded subunits in both normal and *SCO2* patient cells, with an improvement of COX activity and of the bioenergetics status in patient cells (141). These compounds represent interesting candidates to improve mitochondrial biogenesis in aged individuals.

The role of denervation in causing mitochondrial dysfunction in aged humans was also investigated confronting cohorts of young or active aged individuals, with old, inactive subjects, and with a rodent model of sporadic denervation. Interestingly the main effect of denervation on mitochondria was shown to be increased sensitivity to mitochondrial permeability transition (845).

Despite all these advances, the precise role of mitochondria in aging and sarcopenia remains controversial. The classical mitochondrial theory of aging has been largely abandoned, but it has not been replaced by a novel formal set of hypotheses. It is still not clear whether the observed mitochondrial alterations are primary or secondary events and what is the main trigger that determines these changes. Paradoxically, even the exact nature of these alterations is still not completely clear. Apart from the methodological issues that we have discussed, some of the conflicting evidence could also be related to the animal models employed in many studies. In fact, overexpression or tissue-specific knockout of individual genes are situations which are not observed

in human mitochondrial diseases (most human genetic defects are hypomorphic and are not restricted to muscle), let alone during aging. Although these “extreme” situations may provide very useful information, they could also overemphasize some aspects that have marginal importance in the physiology of aging.

Nevertheless, mitochondria remain one of the main focus areas of aging research and altered mitochondrial homeostasis is likely to be one of the main players in the process. Given the tightly interconnected nature of the main mitochondrial functions (biogenesis, dynamics, mitophagy, bioenergetics, apoptosis), it is reasonable to think that they all participate to some extent to the physiological aging process, and that they could represent potential therapeutic targets.

Microvasculature

Sarcopenia is the result of an ever so slight, but prolonged, negative protein balance. Consequently, many investigators have focused their attention on signaling pathways and factors that may underlie the reduced rate of protein synthesis and increased rate of protein breakdown during aging (see below). Another factor that may contribute to sarcopenia is the gradual accumulation of incompletely repaired contraction-induced micro damage (277). Such repair requires activation of satellite cells (380) and it has been suggested that an aging-related decrease in their number, and ability to proliferate and differentiate, also contribute to sarcopenia (202). While all those factors undoubtedly play a causal role, little is known about a potential contribution of capillary rarefaction or capillary hypo perfusion to sarcopenia. This is surprising, as an adequate microvascular network is not only required for oxygen supply and removal of heat and metabolites, but also for the delivery of nutrients and (anabolic) hormones (966). The importance of an adequate blood supply for muscle size and function is readily seen in the ensuing atrophy during prolonged ischemia (230) and a dramatically hastened onset of muscle fatigue during a series of intermittent isometric contractions during acute ischemia (971), respectively. The impact of capillary rarefaction *per se* on skeletal muscle structure and function has hitherto not directly been investigated. However, in the heart a 10% arteriolar occlusion results in a large reduction in cardiac function and increase in size of hypoxic regions (379). This highlights the importance of local capillary supply for adequate muscle function and maintenance of muscle mass. Such and other observations have led some investigators to hypothesize that hypoxia contributes to sarcopenia (231). Given the above observations and the importance of the capillary bed for tissue oxygenation, removal of heat and metabolites, and delivery of nutrients and (anabolic) hormones, we will below explore how the capillary bed in the muscle is affected during aging and to what extent capillary rarefaction may or may not contribute to sarcopenia and skeletal muscle dysfunction in old age.

Relationship between capillary supply and size of a muscle fiber

Capillaries are often seen as the source of oxygen for the mitochondria in the surrounding tissue cells. In line with this, it has been found that muscles with a high oxidative capacity have a denser capillary network than those with low oxidative capacity, and even within a given muscle, regions with a higher oxidative capacity have a denser capillary bed (205, 210, 215-217, 347, 411). This relationship between capillary supply and oxidative capacity seems also to hold for individual fibers (65, 618). These latter comparisons, however, did not consider the influence of fiber size on this relationship.

The method of capillary domains (**Fig. 16**) calculates capillary domains (estimates of their oxygen supply area), defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent capillaries. This method allows one to calculate the capillary supply to individual fibers and in relation to their oxidative capacity and size, and to estimate the maximal oxygen demand supported by a capillary (91). Using this method, it has been shown that the capillary supply to a fiber depends more on the size (8, 216, 969, 970) than on the oxidative capacity (969, 970), or type (8) of the fiber (91), observations confirmed also with other approaches (424, 448). The importance of fiber size rather than oxygen demand is emphasized by the observation that the relationships between fiber size and capillarization during maturation are similar under conditions of reduced oxygen supply (maturation in hypoxia) or increased oxygen demand (treatment with thyroxin) to that seen during normal maturational growth (837). The intricate interrelationship between the size and the capillary supply to a fiber is also shown by the proportional increase in capillary number and fiber size in the quadriceps after 12 weeks resistance training in young men (349). Finally, the similar time course of muscle fiber hypertrophy and angiogenesis during overload (263, 719) even more forcefully suggests that fiber size and fiber capillary supply are tightly linked and controlled by common factors. This tight link between fiber size and capillary supply to a fiber lends further support to the idea that a potentially slow, but gradual capillary rarefaction during aging contributes to sarcopenia.

Aging-related changes in muscle capillarization

The capillary supply to a muscle is often described in terms of capillary density (CD in number of capillaries per mm^2) and capillary to fiber ratio (C:F). A reduction in C:F indicates capillary rarefaction, while an increase in C:F reflects capillary proliferation, assuming the fiber number is the same. Table 1 gives an overview of the observed aging-related differences in CD, C:F and fiber cross-sectional area (FCSA) in rodents, the Beagle and human.

Rodent studies

Most rodent studies do not report an aging-related difference in C:F or FCSA, suggesting there is neither capillary rarefaction nor atrophy during aging. Even an aging-related increase, rather than decrease, in FCSA has been reported in the soleus, plantaris and red, but not white, rat gastrocnemius muscle (389, 448). It should be noted, however, that in the majority of cases animals of less than 1 year old were compared to animals of 24 months or older. Such comparisons can be marred by maturational increases in FCSA, fiber type transitions and changes in function up to the age of around 9 months (204, 214, 591, 600) that are accompanied with capillary proliferation and increases in C:F (206, 757, 836). Too young animals in the young group may not have reached the largest FCSA and highest C:F, while those in the oldest group may not yet show signs of muscle aging (47, 204, 591). Consequently, any aging-related reduction in FCSA and C:F may be missed as illustrated in **Figure 17**. In support of this, it has been found that the FCSA and C:F were higher in the plantaris muscle of 13- than 5- and 25-month-old rats (215, 216) and that C:F In the soleus and EDL of rats decreased between 103 and 121 week of age (85). Other studies showing muscle fiber atrophy report, however, no change in C:F (111, 112, 274, 389), even when muscles from 12- and 24- were compared with those of 35-month-old rats (619). The latter suggests that aging-related muscle atrophy can occur without capillary rarefaction. This conclusion must, however, be taken with some caution as in theory capillary rarefaction can result in a maintained C:F when accompanied with

loss of fibers. For instance, in one study the C:F was higher in muscles of 28- than 6-month-old mice (193) which at first glance suggests capillary proliferation. It is more likely, however, that this increase in C:F is due to fiber loss, as reflected by the lower fiber count in the muscles of the old than the young mice (193). It is thus difficult to decide whether capillary rarefaction plays an important role in rodent skeletal muscle aging. It is recommended that future studies dealing with muscle fiber atrophy and capillary rarefaction in old age in rodents have 1) more than two age groups, 2) choose fully matured animals in the youngest and animals at >90% of average life span in the oldest group (47), and if possible 3) get an idea of the number of fibers in the muscle. Nevertheless, the current data suggest that capillary rarefaction does not play an important role in the aging-related muscle wasting in rodents.

While there does not appear to be a clear link between aging-related changes in capillarization and muscle fiber size in rodents, it is possible that the capillarization follows, or determines the aging-related changes in muscle oxidative capacity. As discussed above, oxidative muscles, muscle regions or muscle fibers have in general a denser capillary supply than non-oxidative muscle (fibers). The lower aerobic power in old (28-30 months) than young (8 months) rats with artificially matched convective oxygen delivery (387) could arise from a lower muscle capillarization. However, no significant aging-related reduction in C:F or CD was found, but rather a decreased oxidative capacity (389), suggesting that the old muscles had an excess capillary supply. Also in the soleus and EDL of 35- vs 12- and 24-month-old rats the decrease in mitochondrial volume was proportional to the decrease in FCSA, without any concomitant change the C:F ratio, thus resulting in a higher CD in old muscles (186, 619). This superfluous capillarization may serve to maintain an adequate oxygen supply during exercise in the face of reduced exercise-induced vasodilation and hence blood flow in the skeletal muscle of old rodents.

The superfluous capillarization in old rodent muscles may be the result of an accelerated rate of atrophy as a consequence of an accelerated denervation of fibers at advanced age and incomplete reinnervation due to motoneurone loss (209). Indeed, during the early stages of denervation, rodent (208, 729) and human (139) muscle appear to have a superfluous capillary supply. Interestingly, the regression of the capillary bed during denervation was slower in old than young-adult rat soleus, resulting in an increased CD in old denervated muscles only (208). The loss of muscle fibers that may ensue after motorneurone loss in the absence of capillary rarefaction will accentuate the superfluous muscle capillary supply, as observed in old mouse muscle (193).

Human studies

In Table 1 it can be seen that where in most rodent studies no aging-related fiber atrophy or reductions in C:F are observed, in human muscle often both atrophy and a reduction in C:F are seen. In most of these studies the loss of capillaries and reduction in fiber size are proportional, resulting in a similar CD in muscles from young and old people. The reduction in C:F can even occur without a reduction in FCSA (306, 784). In none of the studies an increase in CD was observed, suggesting that in contrast to what can occur in old rodent muscle there is no superfluous capillary supply in old human muscle and the coupling between fiber size and oxidative capacity with fiber capillary supply is maintained in old age (817).

It has been observed that the lower CD in old than young muscles was associated with a reduced oxidative capacity (164). A lower CD may partly be due to low activity levels as master athletes and young people matched for the 10-km run performance of the master athletes had a similar CD in the gastrocnemius muscles, even though the masters had a higher oxidative capacity (165). The higher C:F in the athletes than in the performance-matched young athletes was associated with larger fibers in the master athletes (165), supporting the concept that FCSA rather than oxidative capacity determines also in muscles of old people the capillary supply to a fiber.

Another factor that may contribute to a reduced capillarization of old muscle is insulin resistance, as it were particularly older (70 years) men with diabetes who had a lower C:F than young (24 years) men (356). In the diabetic older men, but not the healthy older men, also the muscle fiber atrophy was more pronounced and the CD less than in the young men (356). This is significant as insulin activates endothelial nitric oxide synthase (eNOS) and leads to vasodilation, where insulin resistance may result in an impaired post-prandial insulin-induced vasodilation (905). The consequent reduction in perfusion of the capillary bed may result in reduced shear stress and together with a reduced nitric oxide (NO) production leading to a lower vascular endothelial growth factor (VEGF) expression and impaired maintenance of the capillary bed.

In a 12-year longitudinal study a reduction in the C:F was found without a significant decrease in FCSA, even though the muscle size had decreased (306). The latter suggests fiber loss, considered an important factor contributing to the aging-related muscle wasting (555, 965) and implies, if fiber loss had occurred, an even larger capillary rarefaction in the 12-year period than suggested by the decrease in C:F. It is possible that the lower capillary supply to the remaining fibers, indicated by the lower C:F, creates a less than optimal environment for these fibers and precedes subsequent atrophy of the remaining fibers. In fact, it has been observed that in rats with heart failure endothelial apoptosis preceded muscle fiber apoptosis and atrophy (937). The authors suggested that the relative ischemia consequent to endothelial apoptosis causes an imbalance in fiber nutrition that in turn triggers fiber atrophy. Also during aging this may occur, as it has been observed in mouse gastrocnemius muscle, that the aging-related increase in apoptotic endothelial cell nuclei exceeds the apoptosis in satellite cells and myofibre nuclei by at least one order of magnitude (382). It is thus possible that the aging-related capillary rarefaction and hypoperfusion do contribute, and may even precede, sarcopenia.

Aging-related changes in the heterogeneity of capillary spacing

While CD and C:F give an idea of the quantitative capillary supply they do not tell us how the capillaries are distributed within a muscle. This is potentially important as an increased heterogeneity in capillary spacing may affect muscle oxygenation negatively (206, 212, 213, 264, 716, 898). It has been suggested, for instance, that heterogeneous stoppage of capillaries plays an important role in skeletal muscle pathology during sepsis (332). Yet, little is known about possible aging-related changes in the heterogeneity of capillary spacing, but there are some indications that it is elevated in old age. It has been reported that in the rat plantaris muscle the heterogeneity of capillary spacing was higher in 25- than 5-month-old rats, which was related to the increased heterogeneity in fiber size (211), which may cause an increased heterogeneity of muscle oxygenation (575).

Aging-related structural changes in the microcirculation

In addition to changes in C:F and CD during aging, other structural changes in the microcirculatory bed may affect delivery of oxygen and substrates, and removal of heat and waste products, and thereby have a negative impact on muscle function. Although a reduction in luminal diameter, as observed in rats after hind limb suspension (449), could be such a factor, no differences in capillary (448) or arteriolar (171) luminal diameter have been observed in aging rodents. Another factor is a decreased tortuosity and branching of capillaries that has been observed in the soleus but not EDL of 35- vs 12- and 24-month-old male FBN rats (619). This change is unlikely, however, to have a significant impact, as it did not result in a decreased capillary surface to fiber surface area or capillary length per fiber volume (619). Even though the capillary – fiber exchange area may stay the same, the aging-related increase in the periodic acid-Schiff (PAS) reactivity of the extracellular matrix and basement membrane of capillaries, arterioles and venules, may be reflective of a thickening and enhanced advanced glycation end-product formation in the basement membrane (838). Indeed, an aging-related thickening of the capillary basement membrane has been observed between the age of 28 and 63 years in men, that was, however, unrelated to glucose tolerance and readily reversible by endurance training (949). How such thickening of the basement membrane affects capillary function is unknown, but it is likely to increase the resistance to diffusion over the capillary membrane and to contribute to the impaired dilatory function of arterioles (701). In addition to these biochemical changes in the capillary and arteriolar wall, there may also be an increase in the segment length of arterioles as observed between 12 and 24 months in the cremaster muscle of male rats, implying reduced branching (171). Finally, in aging (25-month-old) BUFMna rats that suffer from muscle weakness and atrophy, discontinuous capillary segments with fenestrations may contribute to atrophy as they were particularly associated with bundles with small muscle fibers (225).

Aging-related changes in microcirculatory flow

The impaired vasodilatory response to adenosine (171), acetylcholine (829) and diazoxide may explain the reduced blood flow and fatigue resistance in old male Fischer344 rats (419) and indicates that the reduced dilatory response of arteries is most likely attributable to impaired endothelial function (229, 233). Endothelial dysfunction may underlie the lower exercise-induced blood flow and vascular conductance in older (55-68 years) men. Furthermore, the absolute leg blood flow and vascular conductance during exercise are less than in young (22-30 years) men working at the same absolute or relative workload (238, 726), while the venous oxygen pressure was lower. Not only the blood flow during submaximal exercise is reduced, but also the maximal mass-specific blood flow decreases with increasing age (478). The lower venous oxygen pressure suggests that the lower oxygen delivery due to the diminished exercise-induced blood flow is compensated, at least partly, by a larger oxygen extraction from the blood. A side effect is that average oxygen tension in the muscle tissue would be lower with muscle of the older person working in a somewhat locally hypoxic environment.

Endothelial dysfunction is also reflected by a reduced production of nitric oxide (NO) in aortic rings and impaired acetylcholine-induced vasodilation in old rabbits (759). The reduced availability of NO is not only due to a lower expression of eNOS, but also to oxidative stress and low-grade systemic inflammation where superoxide may capture NO and form peroxynitrite (233, 812, 904). To make matters worse during oxidative stress eNOS itself starts to produce more superoxide, making the bioavailability of NO even less (812). Together, the increased oxidative stress and lower expression

of VEGF, that has anti-apoptotic properties, will result in endothelial cell apoptosis (812), which has been suggested to precede muscle fiber apoptosis in heart failure (937) and aging (936). Lower flow, and hence shear stress, which is an important stimulus for angiogenesis and maintenance of the capillary bed (410) may thus underlie the capillary rarefaction and subsequent muscle wasting during aging.

Impaired vascular function is not limited to an impaired dilation, but also the arteriolar myogenic constriction (vasoconstriction in response to elevated blood pressure) has been reported to be impaired in old age (326). Where the vascular dysfunction in oxidative muscles is primarily related to changes in NO signaling, in glycolytic muscles it is a combination of impaired NO and prostanoid signaling (653). Such differential disturbances in vascular function may well cause the observed redistribution of blood flow from low-oxidative to highly glycolytic muscles during submaximal exercise in old compared with young rats (653, 658). This redistribution of blood flow in turn may result in a disturbed matching of oxygen demand and supply during exercise and contribute to the aging-related decrease in maximal oxygen uptake and exercise tolerance (199, 295, 363, 653).

Aging -related changes in angiogenesis and maintenance of the capillary bed

While capillary rarefaction does appear to occur during aging, this may be at least partly attributable to the aging-related decline in physical activity, a widespread phenomenon in aging organisms including humans (418). Disuse indeed does not only result in muscle atrophy, but also induces capillary rarefaction (437, 449, 901). In support of a role for reduced physical activity levels, the CD and C:F were higher in both young (21-30 years) and old (51-62 years) fit than sedentary people (727). Similarly, the gastrocnemius muscle of master athletes (63 years) had a higher C:F, muscle oxidative capacity and larger type I fibers, but similar CD as young runners (26 years) matched for 10-k run performance, while top 10% young runners had even higher C:F and CD than the master athletes (165). Although these observations do indeed suggest that part of the capillary rarefaction in older people is related to disuse, these were not training studies and therefore no conclusion can be drawn as to a similar or reduced angiogenic response in old age. What is known, however, is that in the vastus lateralis muscle of 65-74-year old men both endurance and resistance training can induce (330, 388, 933), although not always (835), angiogenis that in the case of resistance training is proportional to the increase in fiber size (388). However, the more pronounced capillary proliferation in young (20 years) than old (62 years) endurance trained men suggests an attenuated angiogenic response in old age that may even be associated with muscle fiber atrophy (221). Also in mice, the blunted hypertrophic response in old age was associated with impaired capillary proliferation (47). Further, evidence for an attenuated angiogenic response has been observed in ischemic hind limbs of old rabbits and mice (274, 759).

The significance of the capillary bed for subsequent resistance exercise-induced hypertrophy is further reflected by the observation that older men with a poorer capillary supply to type II fibers than those with a higher capillary supply to type II fibers develop less hypertrophy in response to resistance training (835). Interestingly, those individuals with a lower capillary supply to type II fibers not only showed little to no hypertrophy, but also did not exhibit satellite cell proliferation (835) reinforcing the ideas that 1) there is a cross-talk between endothelial cells and satellite cells and 2) capillary rarefaction may precede the aging-related muscle wasting.

Potential causes of capillary rarefaction and attenuated angiogenesis in old age

The question arises as to what may cause capillary rarefaction and the impaired angiogenesis in old age. Vascular endothelial growth factor (VEGF) is a potent endothelial mitogenic factor (284) that is increased in expression under conditions with elevated flow (636) and hypoxia (407), where blocking VEGF abolishes skeletal muscle angiogenesis induced by overload or shear stress (948). VEGF is also important for the maintenance of the capillary bed, as supported by the positive correlation between pre-exercise interstitial VEGF protein and capillary contacts per fiber (322). Indeed, targeted inhibition of VEGF expression in mice led to capillary rarefaction and apoptosis in skeletal muscle (866). It is thus possible that an aging-related reduction in expression, ability to increase VEG, and/or impaired response to VEGF expression underlie an impaired maintenance of the vascular bed and/or angiogenesis in old age.

Both lower basal levels of vastus lateralis muscle VEGF protein (184, 222, 784) and an attenuated acute exercise-induced increase in interstitial VEGF protein have been observed in old (65-72 vs 21-32 years) men and women (184, 322). Yet, the increases in VEGF and angiogenesis were similar in young (25 years) and old (64 years) men after 8 weeks endurance training (322). It is likely, however, that the endurance training-induced angiogenesis occur

ed somewhat slower in old age because of the lower basal levels of VEGF and attenuated exercise-induced increase in VEGF expression. In old rabbits and mice, a lower induction of VEGF and impaired endothelial function contributed to the impaired angiogenesis in the ischemic hind limbs, where the angiogenic response was rescued to some extent by administration of recombinant VEGF (759, 821).

In cell cultures of aortic smooth muscle cells isolated from young and old rabbits it was subsequently found that the hypoxia inducible factor-1α (HIF-1α) DNA binding, important for transcription of VEGF, is less in old than young cells, suggesting that the impaired angiogenesis in old age is related to impaired HIF-1α activity (758). This reduced activity may be due to an aging-related reduction in HIF-1α expression and a concomitant increase in the oxygen-dependent prolyl-4 hydroxylase domain proteins that play a role in the breakdown of HIF-1α (452). In addition to the reduction in HIF-1α activity, an aging-related reduction in eNOS has been shown to underlie the impaired angiogenic response in old ischemic mouse muscles (274). Finally, an impaired angiogenesis, and consequent reduced maintenance of the capillary bed, may also be due to reduced secretion and sensitivity to growth factors and an impaired ability to break down the extracellular matrix by matrix metalloproteinases that is crucial for angiogenesis (812).

Impaired angiogenesis may not only be caused by a reduction in the ability to increase VEGF expression, but also to an impaired angiogenic response to VEGF. Indeed, it has been found that the angiogenic capacity of VEGF was impaired in mice with an endothelium-specific SIRT1 knock-out. That such a situation may occur in old age was reflected by the fact that the capillarisation in old mice treated with nicotinamide mononucleotide, a precurosor of NAD+, that activates SIRT1, restored the capillarisation and endurance and hence rejuvenated the muscle (903).

Mechanisms controlling both capillarization and muscle fiber size

Above we have discussed the close link between muscle capillarization and fiber size and that part of the muscle atrophy during aging is associated with capillary rarefaction, at least in humans. Here we will discuss the potential mechanisms controlling both capillarization and fiber size. One common factor is that not only muscle fibers and satellite cells (298), but also endothelial cells act as mechanotransducers not only of high shear (e.g. hyperaemia), but also strain (e.g. muscle stretch) (262). The association between angiogenesis and fiber growth is reinforced by the increased capillary density, myogenic cell hyperplasia and transient rise in VEGF protein during 25% fiber hypertrophy after voluntary treadmill running in 18-month-old mice treated with an NO donor, but not when exercising only (551). This suggests that NO is important for the induction of VEGF, angiogenesis and fiber hypertrophy (551).

Satellite cells are in close proximity of capillaries (661) and it appears that activated satellite cells stimulate angiogenesis and *vice versa* that endothelial cells stimulate myogenesis (158). In co-cultures where satellite cells and endothelial cells were in separate layers without physical contact, the stimulation of satellite cell proliferation must have been due to factors released from the endothelial cells (158). Among these factors was VEGF. Neutralizing VEGF by VEGF antibodies reduced the satellite cell proliferation induced by endothelial cells by at least 41%, and even by 90% if a host of antibodies against factors released by the endothelial cells was used, while recombinant VEGF administration stimulated satellite cell proliferation (2, 158). VEGF probably acts via the VEGF receptors Flk-1 and Flt-1 on satellite cells where it stimulates satellite cell migration and inhibits satellite cell apoptosis (325). These observations indicate that VEGF has a direct effect on satellite cell proliferation, migration and survival. In turn, satellite cell conditioned growth medium stimulated the formation of connected endothelial cells *in vitro*, indicating a reciprocal interaction between endothelial and satellite cells (158). The close proximity of capillaries and satellite cells in the muscle facilitates the coordinated angio-myogenesis. Loss of capillaries may thus be associated with a loss of niches for satellite cells. In old human muscle the distance between capillaries and satellite cells in particularly type II fibers are greater than that in muscles from young people (661). The resulting impaired diffusion-based interaction between satellite cells and capillaries may underlie the impaired regenerative capacity and plasticity of old muscle. In line with this, conditions with capillary loss, such as amyopathic dermatomyosytis, are associated with a proportional loss of satellite cells, and *vice versa* the increase in capillaries in resistance-trained individuals was associated with an increase in satellite cells (158).

Angiogenesis and VEGF expression can be stimulated independent of HIF-1α via Akt, both during fiber hypertrophy and in ischemic hind limbs (863). It is therefore possible that the attenuated hypertrophy in older people that was associated with reduced satellite cell proliferation (707) finds its origin in impaired angiogenesis. In support of a role of both satellite cells and angiogenesis in muscle plasticity, it has been found that the blunted hypertrophic response in old mice is associated with both a lower satellite cell number (48) and impaired angiogenesis (46) in their muscles. Finally, it has been suggested that part of sarcopenia is attributable to incomplete repair of accumulated contraction-induced damage (277) and the importance of the microcirculation for muscle plasticity, repair and regeneration may well be underestimated. In support of this, it has been found that muscles from myostatin knockout mice that also overexpress estrogen related receptor gamma have a better regenerative capacity than those from myostatin knockout mice even though they have fewer satellite cells, but indeed a denser capillary network (685). In this context it is significant to note that an

adequate vasculature is crucial for muscle maintenance and repair, as indicated by the slower recovery from muscle damage that also includes damage to the neurovascular system than recovery from damage not affecting the neurovascular system, particularly in older (22-month-old) female C57BL/6J mice (546).

Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) not only plays an important role in mitochondrial biogenesis, but also the release of VEGF from myocytes (28) and it is suggested that a defect in this pathway may underlie the ageing-related capillary rarefaction (903). This pathway may underlie the higher capillary density in oxidative than glycolytic regions of the muscle and reflects that the cross-talk between muscle cells and endothelial cells is not limited to regulating the match between fiber size and capillary supply to the fiber, but also matching to some extent the aerobic metabolism with the capillary supply of the fiber.

Capillary supply in the nervous system

A significant part of the aging-related loss of muscle mass is considered to be due to loss of motoneurones, where reinnervation of the denervated muscle fibers is incomplete (209, 258, 279, 520, 521). As a consequence of the compensatory, though incomplete, reinnervation the size of the remaining motor units increases (209). The increase in motor unit size may reduce the efficacy by which contractile activity stimulates perfusion of the capillary bed in the muscle (310) and contribute to muscle dysfunction in old age apart from the muscle wasting induced by motoneurone loss. Little attention, however, is given to the cause of this loss of motoneurones, but here we suggest it is at least partly attributable to vascular disruptions in the spinal cord. Indeed, in a mouse model of amyotrophic lateral sclerosis (ALS) the loss of motoneurones appears to be preceded by a decrease in the capillary density (642). It is thus possible that the aging-related reduction in capillary density in the spinal cord (733) contributes to loss of motoneurones in old age.. Even in the absence of capillary rarefaction motoneurone loss may occur in the presence of reduced spinal cord perfusion (688). In the latter study, this was achieved by deletion of the HIF-binding site of the VEGF promotor, and was associated with weakness and signs of denervation-reinnervation in the muscle (688). Above we discussed that the aging-related impairment in the HIF-VEGF pathway may underlie the impaired angiogenic response during ischemia in skeletal muscle and similar changes at the spinal cord level may contribute to the aging-related loss of motoneurones.

Also peripheral nerves are sensitive to loss of capillaries, as reflected by the positive relationship between sciatic nerve degeneration and conduction failure with the degree of capillary embolization with microspheres in rats (461), similar to that observed in mice with deletion of the hypoxia-response element in the VEGF promotor (688). The latter is significant and similar to the cross-talk between satellite cells and endothelial cells, there is also such a cross-talk between Schwann cells that produce VEGF (652) and endothelial cells that produce brain-derived neurotrophic factor (BNDF), important for development, survival and repair of peripheral nerves (659). It is thus possible that the regression of myelinated fibers in old (21 months) compared to young-adult (7 months) rats is a consequence of the reduced diameters and branching of capillaries in the peripheral nerves (788). It is noticeable that there is not yet significant muscle atrophy in rats aged 21 months, and these observations thus suggest that the capillary rarefaction and morphological changes in peripheral nerves precede the aging-related loss of muscle mass.

Thus, there is considerable evidence, particularly in human studies, that sarcopenia is associated with capillary rarefaction. It is not yet clear, however, whether capillary rarefaction precedes or follows sarcopenia. Whatever the sequence muscle fiber capillary supply and fiber size appear to be closely linked and controlled by common factors. There is a considerable cross talk between satellite cells and endothelial cells, where in particular the endothelial mitogenic factor VEGF appears to play a key role in this cross talk. Finally, it is recommended to explore further the potential importance of aging-related changes in the microcirculation of the spinal cord and how this may contribute to the aging-related loss of motoneurones and sarcopenia.

Satellite cells and regenerative capacity

Mitotically quiescent satellite cells are residing in a specialized niche between cell membrane and basal lamina. The satellite cell niche includes extracellular matrix proteins, growth factors, myofibers and muscle resident non-myogenic pro-fibrotic cells, macrophages and regulatory T cells (for refs see (14)). The satellite cells represent a muscle-specific adult stem cell population and muscle repair/regeneration processes are primarily mediated via the activation of satellite cells, resulting in satellite cell entrance into the cell cycle giving rise to proliferating myogenic precursor cells which differentiate and fuse with existing muscle fibers or form new muscle fibers (960, 981). The transcription factor paired box 7 (PAX7) is a marker of satellite cells and an important regulator of the repair process (357, 815, 961). Quiescent satellite cells express PAX7 but not MYOD or other markers associated with proliferation. Proliferating satellite cells maintain PAX7 expression and induced expression of MYOD. In a subset of these satellite cells, myogenin expression is induced and PAX7 down-regulated and these cells are committed to differentiation. Another subset of activated satellite cells self-renew by inhibiting MYOD to reinstate quiescence to replenish the quiescent satellite pool to ensure a sufficient number quiescent satellite cells which can be activated during future rounds of muscle repair. Complex transcriptional mechanisms regulating satellite cell quiescence involve approximately 500 uniquely expressed genes, elegantly reviewed by Almada and Wagers (14). The membrane-associated inhibitor of receptor Tyr kinase signaling, Sprouty 1 (SPRY1), expressed in slow cycling satellite cells appears to be required for the return to the quiescent state after muscle repair (146, 341). In addition, the forkhead box protein 3 (FOXO3) transcription factor is involved in the return to the quiescent satellite cell pool (338). Interestingly, FOXO3 appears to be directly linked to NOTCH signaling and suggested to play a key function in the maintenance of NOTCH receptor expression, essential for the maintenance of the quiescent state. MYOD and MYF5 have been forwarded as the master transcriptional factors orchestrating the activation of satellite cell genes and both these transcription factors are under both transcriptional and post-transcriptional control (for refs see (14)). It has been demonstrated that p38α/β MAPK, activated by fibroblast growth factor receptor (FGFR) tyrosine kinase, induces MYOD by inhibiting a *Myod* mRNA-destabilizing protein (179, 232, 378, 433, 434, 453, 894). Further, miRNA-31 has been shown to post-transcriptionally repress *Myf5* mRNA in quiescent satellite cells, an inhibition lost during satellite cell activation (179). Recent results from zebrafish show that there are different populations of satellite precursor cells playing different roles in the initiation of fiber formation and fiber growth during muscle repair (717). Satellite cells expressing *pax7a* or *pax7b* gene reporters have different specific roles during muscle repair with *pax7a* preferentially initiate nascent muscle fibers while *pax7b* expressing cells more frequently fuse to repair and grow muscle fibers (717).

Adult stem cells have turnover rates and differentiation programs which are tissue-specific as well as common properties independent on tissue. However, independent of properties being intrinsic to the tissue or common to all stem cells, these traits are affected by aging and consequently impacting on tissue repair and regeneration capacity (96). In skeletal muscle, there are both quantitative and qualitative aging-related changes in satellite cells affecting muscle regenerative capacity, such as a ~65% decline in satellite cell number (95, 175), an increased DNA damage, a decline in antioxidant capacity and an altered gene expression (for refs. see (96)). The impaired skeletal muscle regeneration capacity in old age has been associated with intrinsic deficits in satellite cell function (141, 142, 175). However, no difference in muscle muscle fiber size and number, muscle fiber specific force or myonuclear number were reported between control old and old genetically manipulated mice with an inducible depletion of satellite cells resulting in loss of satellite cells to the degree sufficient to impair regeneratioin from adult throughout their lifespan (131). Thus, in mice not challenged with a muscle injury during aging, satellite cells appear not to influence the loss of muscle mass and function, i.e., sarcopenia. On the other hand, the increased amount of muscle collagen and fibrosis associated with aging was significantly increased in mice depleted of satellite cells during aging, suggesting that the aging-associated loss of satellite cells contributes to the muscle fibrosis in old age (131). Syndecan-3 is a transmembrane proteoglycan which is expressed in satellite cells and is involved in signaling to maintain satellite cells quiescent. In mutant mice lacking syndecan-3, improvements in muscle regenerative capacity and reduced fibrosis were observed in old as well as in dystrophic mice (474). Manipulating syndecan-3 was accordingly forwarded as as a promising target in the development of therapies enhancing muscle regeneration and reducing fibrosis in aged muscle (474).

Muscle satellite cells are sensitive to systemic soluble factors in serum, inflammation and local growth factors which all change during aging with an impact on satellite cell function (for refs see (14, 96)), resulting in an aging-related impairment or delay in the repair and regeneration process in response to a damaging insult to skeletal muscle (for refs see (14, 96)). It is of specific interest to note that neutralization of Wnt and TGFβ signaling pathways, both antagonizing Notch signaling critical for satellite cell activation, improved the muscle repair mechanism in old age (for refs see (96)). The increased TGFβ levels in old age has also been suggested to have a negative impact on satellite cell function via promoting pro-fibrotic muscle resident stem cell types known to cross-talk with satellite cells (96). The chronic low-grade “sterile” inflammation considered a hallmark of aging is associated with geriatric frailty, decreased muscle strength and incapacity to respond to stress (634). This chronic low-grade inflammation is closely associated with the senescence-associated secretory phenotype, comprising pro-inflammatory cytokines and chemokines (974). It has recently been shown by two independent groups that elevated levels of inflammatory cytokines regulate satellite cell function via the JAK-STAT3 pathway and inhibition of this pathway markedly improved satellite cell function, offering a promising therapeutic intervention for the treatment of sarcopenia (725, 879). In addition, the JAK-STAT pathway also plays an important role in cytokine production and JAK inhibitors decrease cytokine production in pre-adipocytes, aging-related adipose tissue accumulation, systemic inflammation as well as frailty (974). Thus, senescent cells contribute to the aging-related low-grade inflammation and inhibition of the JAK-STAT pathway may blunt the senescence-associated secretory phenotype as well as inhibit the direct negative effects of the pathway on satellite cell function (725, 879, 974). Further, elevated activity p38α/β MAPK signaling

pathway has been implicated in aging-related functional decline in satellite cell proliferative capacity and the inhibition of this pathway rescued the self-renewal capacity of satellite cells in old age (71, 175). The rescue of the deregulated FGFR- p38α/β MAPK pathway offers an interesting therapeutic intervention strategy in the treatment of sarcopenia (71, 175).

Although the aging-related loss of satellite cells may not be a prerequisite for sarcopenia, different interventioin strategies aiming at improving or altering satellite cell function may ameliorate aging-related impairments in skeletal muscle regenerative capacity upon repeated injury and reduce the aging-related muscle fibrosis. As discussed above there is experimental evidence suggesting that this may be achieved by targeting (*i*) syndecan-3, (*ii*) Notch signaling, (*iii*) aging-related low-grade inflammation or the JAK/STAT pathway directly, or (*iv*) activity p38α/β MAPK signaling pathway. However, it needs to be emphasized that these interventions are primarily forwarded based on observations in genetically manipulated mice and extensive analyses of efficiency and side-effects need to be completed in both experimental and clinical studies prior to the translation to clinical research and practice.

Regulation of muscle protein synthesis/degradation and intracellular signaling pathways

Muscle atrophy is secondary to the decrease in cell size involves the net loss of proteins, organelles and cytoplasm. The amount of organelles and proteins in the cell depend on the balance between biogenesis/biosynthesis and removal/destruction. This equilibrium defines the organelle and protein turnover and greatly affects the size and performance of cells including skeletal muscles. Growth of adult musculature occurs when enhancement of protein synthesis exceed rates of protein degradation. Few pathways have been shown to control protein synthesis and most of them converge to IGF1-PI3K-Akt/PKB-mTOR or TGFβ/myostatin/BMP signaling. Conversely, atrophy occurs when the main cellular degradation pathways, the ubiquitin-proteasome and the autophagy-lysosome systems, overcome protein synthesis. In sarcopenia, this dogma has been challenged by recent findings that put the quality more than the abundance of proteins and organelles as a critical factor for force generation and muscle function.

Protein synthesis

IGF1/AKT1/mTOR signaling

The IGF1-PI3K-Akt/PKB-mTOR signaling pathway is considered the key controller of protein synthesis in muscle providing the means for potent control of S6, a ribosomal protein, and factor 4E binding protein 1 (4EBP1), an inhibitor of the ribosomal eukaryotic translation initiator factor 4E (eIF4E) (513). Simultaneously, this pathway suppresses protein breakdown because Akt negatively controls FoxO family of transcription factors that are sufficient and required for the expression of rate limiting enzymes of the ubiquitin-proteasome and autophagy-lysosome systems (635). Indeed, muscle specific insulin and IGF1 receptors double knockout mice display a tremendous muscle loss that is FoxO dependent (673). Therefore, this pathway has a unique function that is to enhance protein synthesis and simultaneously to reduce protein breakdown. The critical factor that controls protein synthesis is the mTOR kinase that however belongs to two different complexes named mTORC1 and mTORC2. While mTORC2 is related to glucose and lipids homeostasis (497, 505, 684), mTORC1 regulates several anabolic processes including protein synthesis, ribosome and

mitochondria biogenesis. Interestingly, inhibition of mTORC2 complex in mice reduces life span only in males but the mechanistic insight of this effect remains to be identified (504). The mTORC1 effect on translation relies on its activating action on S6K1 and inhibitory effect on 4EBP1. The mTORC1 complex is critical for muscle hypertrophy induced by exercise, feeding and growth factors and is the target of the drug rapamycin (806). Although inhibition of IGF1/Akt/mTOR pathway has been found in several catabolic conditions characterized by muscle wasting, its involvement in aging sarcopenia is contradictory. In the adult mammals, IGF-1 is synthesized predominately in the liver under the growth hormone action, and acts as a systemic growth factor. However, IGF-1 is also produced in extrahepatic tissues where it plays a predominantly autocrine/paracrine role (703). The muscle isoform but not the circulating one is critical for muscle mass maintenance and growth in adulthood (657). While in rodents the muscle-specific IGF1 expression declines during aging and its reactivation in old mice counteracts sarcopenia (657, 795), in humans this reduction was not found but, surprisingly, an upregulation of IGF1Eb isoform was detected in elderly subjects (795). Controversial data have been also reported for Akt1 activity in old mice while data on mTOR are more consistent. Indeed, most of the studies show the persistence or even an increase of mTOR signaling during aging in humans, rats and mice (320, 464, 607, 698, 795, 941). These findings argue against the idea that inhibition of protein synthesis is the cause of sarcopenia. However, growth-promoting stimuli such as exercise or nutrients do not efficiently activate mTOR pathway in elderly people and this anabolic resistance is believed to be an important factor in the reduced muscle recovery after an injury (312, 320, 696). Interestingly, acute and transient activation of Akt1/FoxO axis has been reported to be beneficial in mice and elderly people (10, 683).

TGFβ Superfamily and signaling

The TGFβ superfamily comprises more than 30 secretable ligands with differing selectivity for specific receptor subtypes. The complexity of the TGFβ signaling network is related to the different biological actions executed within a cell, depending on the combination of ligands and receptors that are utilized. Downstream these ligands, two major signaling pathways are activated that impinge on two classes of transcription factors belonging to Smad family. Activins, some growth and differentiation factors (GDF) such as Myostatin/GDF8 and GDF11, and the TGFβ proteins promote activity of Smad2 and 3 and muscle loss. In contrast, other GDF ligands and the bone and morphogenetic proteins (BMP) rely on Smad 1, 5, and 8 to regulate target genes that control muscle growth. The manner in which the two subfamilies of ligands recruit different Smad proteins is a function of the different combination of type II and type I receptors with which ligands preferentially engage (798).

The Activin/Myostatin/TGFβ group bind plasma membrane associated activin type IIB and type IIA receptors (ActRIIB/IIA) and TGFβ receptors (TGFβRII). Ligands binding with ActRIIB/IIA/TGFβRII lead to recruitment and activation of activin receptor like kinase (ALK)-4, -7 and -5, and serine/threonine type I receptor kinase (617). ALK4/7 and ALK5 phosphorylate Smad2/3, to promote formation of an heterotrimeric complex with Smad4. Increased nuclear retention of an activated Smad2/3-Smad4 complex facilitates interaction with other transcription factors / co-activators / repressors, to modulate the transcription of target genes that either control atrophy or block hypertrophy (799, 889).

The other ligand group comprising BMP/GDF members preferentially binds to a combination of type II receptor that include BMP type II receptor (BMPRII), ActRIIA and ActRIIB, before promoting recruitment of type I receptors such as BMPRIA (ALK3), BMPRIB (ALK6) and ActRIA (ALK2). These ligand/TypeII/TypeI receptor complexes promote phosphorylation and heterotrimerisation of Smad1/5/8 with Smad4 to effect transcriptional upregulation of still unidentified hypertrophy genes and suppression of atrophy-related genes such as MUSA1 (800, 950).

Myostatin is the most famous member of this superfamily in muscle field because of the profound hypermuscularity of Myostatin knockout mice (629, 630). Despite the observation that myostatin inhibitors are sufficient to promote muscle growth in mice and humans (63), Myostatin does not seem to be involved in the onset of sarcopenia. Indeed, several recent studies reported no changes or even decreased myostatin levels in serum of elderly people (260, 720, 745, 795, 804) (**Fig. 18**). Consistently, myostatin transcript and protein did not change in muscle of both old physical active and inactive people (795, 927, 930).

Another TGFβ family member that recently attracted the attention as a potential culprit of sarcopenia and frailty syndrome is GDF11. GDF11 and myostatin are very similar in aminoacid sequence and structure and can be confused by each other when antibodies are used to check their expression level (260, 804). Despite an initial observation suggesting that GDF11 expression declines with ageing in mice (581), recent data in humans and rats do not confirm this finding and actually show the opposite. Indeed, GDF11 blood levels have been reported to increase during aging and importantly, to correlate with frailty syndrome (260, 804) (**Fig. 18**).

Very few data are available on BMPs and therefore, the role of this pathway in sarcopenia is unknown. Recent data have shown a dominant anabolic function of BMPs over myostatin in mice (800, 950) and a transcriptomic study have found a suppression of BMP5 in elderly subjects (930) suggesting that this signaling might play a role in the control of muscle mass during aging. Interestingly, MUSA1, a BMP target that is negatively regulated, has been reported to increase during aging in rats (416) (**Fig. 18**). Further studies are required to identify the members of the TGFβ superfamily that are involved in sarcopenia and frailty.

Protein breakdown

The discovery of the atrophy-related genes was a major advance in the field of muscle wasting. The comparison of the gene expression profiling of muscles from tumor bearing mice with fasted, diabetic and uremic animals led to the identification of a common set of genes that were named atrogenes. Since all these diseases have muscle atrophy in common, these genes are thought to be important for protein loss. Among these atrophy-related genes there were several belonging to the major cellular degradation systems, the ubiquitin proteasome and autophagy-lysosome. The transcriptional-dependent induction of these atrogenes is required to replenish the enzymes/proteins that are lost during the enhanced protein breakdown. The ubiquitin ligase, the rate-limiting enzyme during the ubiquitination process, undergoes auto-ubiquitination (80) therefore, an increased ligase activity during catabolic conditions will inevitably amplity their auto ubiquitination and their proteasomal-dependent degradation. Therefore, the transcriptional upregulation is particularly important mostly to replenish the loss of the ubiquitin ligase proteins. Similarily, the autophagy-related proteins (LC3, Gabarap, p62, NBR1, Optineurin, PINK1, Parkin, BNIP3 and BNIP3l/Nix),

which are critical for membrane commitment, cargo delivery and selective removal of damaged mitochondria, are entrapped into the autophagosome when the vesicle is formed and therefore are destroyed upon fusion of autophagosome with lysosome. This concept should be considered when protein expression does not match with transcript levels. Finally, recent findings suggest that the signalling pathways that control muscle loss vary between different muscles (103).

Ubiquitin-proteasome system

Proteasome activity was found decreased (36, 168, 285, 737), unchanged (90) or increased (16) in aged rats. However, since proteasome breaks ubiquitinated proteins, the *in vitro* measurement of proteasome activity does not mirror the level of protein breakdown inside muscles that instead is dependent on protein ubiquitination. In fact, in the ubiquitin-proteasome system, proteins are targeted for degradation by the 26S proteasome through covalent attachment of a chain of ubiquitin molecules. Different classes of enzymes, named E1, E2, and E3, are involved in protein ubiquitination. The E1 is the ubiquitin activating enzyme ~~E1~~ and is encoded by only one gene. The ubiquitin ligase enzyme, or E3, binds the protein substrate and catalyzes the movement of the ubiquitin from the E2 enzyme to the substrate. This is the rate-limiting step of the ubiquitination process, which affects the subsequent proteasome-dependent degradation. Once the protein is ubiquitinated it is docked to the proteasome for degradation, unless the polyubiquitin chain is removed by the de-ubiquitinating enzymes. Different E2-E3 pairs function in the degradation of different proteins, and the specificity of the E3s for specific groups of proteins provides exquisite selectivity to this degradation process. The human genome contains around one hundred E2 and almost one thousand E3. This great heterogeneity is involved in the precise regulation of different cellular processes. The content of different E2s and E3s varies between tissues and with different physiological conditions, but is still not known which specific E2s and E3s normally work in muscle.

The two most induced atrogenes belong to the ubiquitin ligases superfamily and were named atrogin1/Mafbx and MuRF1. While these E3 enzymes are induced at the transcript level in many muscle wasting condition, they are not upregulated in muscles of aged rats or elderly people (795, 929, 943) and in some studies were found to be suppressed (223, 242, 256). Consistent with the concept that sarcopenia is caused by an impairment of the degradation systems more than an hyperactivation, atrogin-1 and MuRF1 knockout did not show any beneficial effect in terms of muscle mass and force during aging. Importantly, both these knockout are weaker than age matched controls (795) and life span of atrogin1 deficient mice is shortened due to the onset of hypertrophic cardiomyopathy in adulthood (987). Interestingly, MuRF1 knockout mice experience a higher decay in muscle strength during aging compared to controls despite the fact that muscle mass is preserved (413, 795). A novel ubiquitin ligase named MUSA1, which was recently shown to be necessary for muscle loss after denervation (800), is upregulated during aging in rats (416). Whether MUSA1 contributes to sarcopenia remains to be addressed in future studies. In conclusion, the literature suggests that the ubiquitin-proteasome system is not enhanced during aging but instead is reduced/impaired or unaffected. Therefore, there is no evidence that sarcopenia is caused by excessive protease-dependent degradation and data suggest the contrary.

Autophagy-lysosome system

Autophagy is an evolutionarily conserved catabolic process through which damaged organelles and macromolecules are degraded and recycled within the cell. There are three different forms of autophagy named microautophagy, chaperone mediated autophagy and macroautophagy. A detailed description of the different types of autophagy and their role in muscle has been recently reviewed (82). The role of microautophagy and chaperone mediated autophagy in muscle and in sarcopenia remains to be unraveled. Instead macroautophagy, hereafter named autophagy, has been shown to be critical for muscle maintenance. Moreover, an extensive literature from yeast to mammals has linked tissue aging with the decline of autophagy (512, 594). The process of autophagy proceeds through several mechanistically distinct steps that include: i) induction, ii) membrane commitment and elongation to form the autophagosome, iii) engulfment of cellular components, the cargo, iv) autophagosome–lysosome fusion, v) degradation of sequestered components and recycling of aminoacids, lipids and glucose, vi) lysosomal rejuvenation and biogenesis. Each of these steps involves a number of conserved autophagy-related genes (Atg) that are transcriptionally regulated, reviewed in (512). Some of these Atg especially the ones related to membrane commitment and elongation (LC3, Gabarap), cargo recognition (p62, Bnip3) for selective degradation and lysosomal enzyme (CathepsinL) belong to the atrogenes and are under the regulation of FoxO transcription factors (635). Although autophagy was initially considered a non-selective degradation pathway, the presence of selective forms of autophagy is becoming increasingly evident. Indeed, autophagy can trigger the selective removal of either specific organelles such as mitochondria or proteins including protein aggregates. The specificity is elicited by some adaptor proteins such as p62, Nbr1, Optineurin, Bnip3 that simultaneously bind autophagosomes via LC3 and the dysfunctional organelles/damaged proteins/protein aggregates that usually, but not exclusively, are labeled by a poly-ubiquitin chain. In muscle disease an impairment of the selective removal of dysfunctional mitochondria, named mitophagy, results in an enhancement of aspecific autophagy that contributes to muscle loss and weakness (599). Interestingly, autophagy related genes and especially mitophagy-related genes are downregulated in elderly frail women (242) suggesting an impairment of the selective mitochondrial removal. Importantly, muscle specific and inducible muscle specific autophagy deficient mice display a muscle phenotype that recapitulates all the features of sarcopenia such as increased oxidative stress, accumulation of dysfunctional mitochondria, neuromuscular junction fragmentation and myofiber denervation, muscle atrophy and severe weakness that lead to a premature death (138, 580, 616). In aged rats, LC3II, a marker of autophagosome, and p62, the autophagy adaptor that is consumed by autophagosome-lysosome fusion, have been reported to accumulate in muscle (789, 941, 958) suggesting that autophagosome clearance (autophagy flux) is reduced during aging. Other studies have found reduction of LC3II, accumulation or no changes of p62 and/or reduction in the regulatory proteins like beclin1 suggesting that autophagy induction is impaired (138, 436, 627, 783). The reduction of autophagy leads to accumulation of dysfunctional mitochondria that generate reactive oxygen species (ROS). The increased oxidative stress contributes to precocious senescence of muscle stem cells and weakness in adult myofibers (138, 141, 580). Importantly, despite the finding that oxidative stress does not affect muscle atrophy, ROS-dependent oxidation of myosin and actin greatly reduces force production and contributes to aging-dependent weakness (138, 561, 585). In humans, a decrease of Atg7, the rate limiting enzyme of autophagosome formation, and LC3II has been shown in muscle biopsies of sarcopenic people confirming the concept of an aging-dependent autophagy decline (138). However, exercise and caloric restriction are sufficient to reactivate this

system in muscles of elderly rodents and humans (138, 940, 958, 976). Finally, reactivation of autophagy in aged mice by expressing Atg7 in skeletal or cardiac muscle ameliorates muscle mass and cardiac function (73, 138). In conclusion, most of the studies agree with an aging-dependent suppression of autophagy and in particular mitophagy in muscles supporting a direct contribution of an autophagy defect to sarcopenia.

Exercise and caloric restriction in sarcopenia affects similar but not identical pathways to improve muscle function

Exercise and caloric restriction have been repetitively shown to improve life span and attenuate aging-dependent disease onset and organ functional decline. Both these conditions impinge on the same molecular patterns that control glucose and lipid homeostasis, proteostasis, mitochondrial function/shape and energy balance. Molecularly, these life styles activate, in muscle, the energy stress sensor AMPK to improve glucose homeostasis, ameliorate mitochondrial function/biogenesis via transcriptional- and non transcriptional-dependent program, activate FoxO transcription factors to induce genes related to proteostasis (552, 977). However, despite these similarities it has been recently reported that exercise differs from caloric restriction. While both improved glucose uptake only exercise ameliorates mitochondrial content, mitochondrial electron transport, fatty acid oxidation (633) and supercomplex formation (351). In fact, chronic caloric restriction does not induce transcriptional-dependent changes and mitochondrial biogenesis but it minimizes oxidative damage to DNA and proteins by decreasing oxidant emission (511). Thus, exercise seems to be more effective than caloric restriction in ameliorating muscle function of sarcopenic muscles. Consistently, it has been recently found that while resistance exercise training improves muscle force of obese aged persons both at the single myofiber level and in whole muscle, caloric restriction does not enhance such improvements (923). The lack of an additive effect may be related to the behavioral changes induced by chronic caloric restriction that cause high burst of physical activity within short time. This high intensity physical activity may account for the beneficial effect of the diet in terms of muscle force/performance and would explain why caloric restriction does not improve the exercise-mediated effect (914). An alternative explanation considers the fact that chronic caloric restriction does not enhance the nutrient- and insulin/IGF1-dependent circadian rhythms, which has been found to be critical to counteract sarcopenia and aging (697). Time-restricted feeding is much more potent than chronic caloric restriction in enhancing insulin and nutrients oscillation and, consequently, FoxO and mTOR activity that, ultimately, modulates autophagy genes expression. This periodic fluctuation of genes and proteins better sustain autophagy in aged mice and adapt the organism to the daily metabolic changes by preventing aging-dependent energy imbalance, dyslipidemia and glucose intolerance (611). These findings suggest that exercise or intermittent fasting by acting as acute stress stimuli are more potent in preserving muscle function and counteracting sarcopenia. Several recent studies show that the beneficial effects of physical activity impinge on mitochondrial quality control system and mitochondrial energetics (219, 236, 988). In fact, there is a close relationship between muscle strength, walking performance and mitochondrial energetics (236, 988) so that mitochondrial dysfunction is considered a predictor of sarcopenia (219, 870). For instance, mitochondria ADP sensitivity is impaired during aging leading to an increased mitochondrial ROS emission and oxidative stress (401). Mitochondrial function is controlled by several mechanisms including mitochondrial dynamics (e.g. fusion and fission), mitophagy and mitochondrial proteostasis. In sarcopenic people the genes and proteins that control mitochondrial

fusion and fission are dramatically suppressed (219, 416, 870) as well as autophagy (138) leading to mitochondrial dysfunction and oxidative stress. Acute and muscle specific inhibition of the fusion protein OPA1 or autophagy is sufficient to induce a sarcopenia and premature tissue senescence in adult mice (138, 870). Exercise counteracts most of these aging-related mitochondrial quality control changes by reactivating the transcription and translation of fusion/fission and autophagy-related genes. Physical activity also improves ADP sensitivity and mitochondrial bioenergetics normalizing the fraction of electron leak to ROS and oxidative stress (401) and activate transcriptional-dependent programs to promote mitochondrial fusion and optimize mitochondrial respiration. The transcriptional dependent action are mediated by at least three different mechanisms which include: 1) the transcriptional co-activator PGC1α, which is a physical activity sensor being upregulated by exercise and suppressed by sedentary life, 2) the PGC1α downstream targets such as PPARγ and δ, and 3) the transcription factor TFEB, which is activated by calcium-calcineurin pathway and controls PGC1α expression, glucose homeostasis and metabolic flexibility during training (604). The metabolic changes of these factors not only affect muscle, but they systemically reverberate modulating the function of multiple tissues. For instance, the expression of PGC1α in muscle of aged mice is not only sufficient to improve mitochondrial function and muscle endurance but also to maintain balance and motor coordination (328). The beneficial effects of exercise are not only transcriptional dependent. In fact, exercise prevents also the activation of DNA-PK, a protein kinase that senses DNA double-stranded breaks. During aging DNA-PK is more active in muscle and inhibits AMPK function by blocking the chaperone HSP90α (695). Inhibition of DNA-PK increases AMPK, prevents the decline of mitochondrial function and of physical activity in middle-age mice and protects against type 2 diabetes (695). However, the different modalities of exercise do not impinge on the same molecular patterns and therefore, they slightly diverge in terms of functional effects in elderly people. While aerobic (high Intensity aerobic interval), resistance or combined (aerobic and resistance) exercise induce transcriptional adaptations, enhance insulin sensitivity and lean mass in aged people, only aerobic or combined improved mitochondrial function and aerobic capacity while only resistance or combined increases muscle strength (710, 764). Interestingly, only aerobic exercise induced the biggest changes of gene expression regardless of age while both aerobic and resistance training enhanced proteins involved in the translational machinery irrespective of age. The effect on protein synthesis by resistance exercises are potentially mediated by a relocalization of mTOR which moves from lysosome to plasma membrane in regions close to the blood capillary endothelium where it interacts with eIF3f and Rheb, two positive modulators of the translation machinery (841). Since, exercise and nutrients affect the same signaling pathway, although via slightly different mechanisms, they potentiate each other because of their synergistic action. Protein supplementation in combination with resistance exercise reduce aging-related muscle loss probably because both activate mTOR and translation machinery (97, 268). In the future, it would be interesting to combine exercise with fasting-mimetics to determine whether they can synergize. For instance it has recently been shown that chronic administration of Nicotinamide monucleotide that enhances NAD+ production, improves mitochondrial respiration and prevents aging-associated gene expression changes in muscle (637). Interestingly, NAD+ level typically increases during energy stress conditions and is a potent activator of sirtuins (SIRT) and especially of SIRT1, a longevity-related factor that controls the acetylation and activation status of many stress-dependent genes such as PGC1a and FoxOs (345, 986).

4. REGULATION OF MUSCLE CONTRACTION AT THE MUSCLE, MOTOR UNIT, MUSCLE CELL AND MOTOR PROTEIN LEVELS

Muscle function

Since Quetelet´s (734) pioneering study almost two centuries ago, aging-related changes in human muscle function have for many years received only sporadic scientific interest. However, the demographic development in modern society, rapidly increasing health care costs secondary to aging-related impairment in motor function, and concomitant consequences for quality of life and aging-related morbidity have triggered an increased scientific attention related to aging in general and the effects of aging on the motor system in particular. It is of interest to note an improved maintenance in hand-grip and back muscle strength in more recent studies compared with earlier studies, presumably related to the improvement of general living conditions, accounting also for the increased life expectancy (for refs see (514)). However, as discussed above (Experimental models, Muscle specific differences), hand-grip strength measurements are of limited importance in predicting fall and fall-related injuries and a maintained hand-grip strength has been reported up to the age of 60 (for refs see (514)), while profound changes in body composition with an increase in body fat and decrease in muscle mass resulting in decreased muscle strength and mobility have been reported in association with advancing age (57, 116, 189, 289, 307, 525, 671, 982).

The loss of maximum force in old age is related to quantitative changes in contractile material, i.e., a total loss of muscle fibers related to motor unit loss and incomplete reinnervation as well as muscle fiber atrophy (see (520)). In humans, the loss becomes prominent first at the end of the fifth decade and lower body muscle mass is preferentially affected during aging (427, 525). At the muscle fiber level, an atrophy is observed in old age and muscle fibers expressing fast myosin isoforms show a more pronounced decline in cross-sectional area than slow muscle fibers as originally reported four decades ago in humans (539, 886) and confirmed in multiple later studies (102, 182, 529, 554). More recently, a gender-related difference was reported regarding the preferential atrophy in fibers expressing fast myosin isoforms, i.e., the preferential type II fiber atrophy was only observed in men and not in women despite a significant aging-related decline in maximum voluntary force independent of speed of movement and gender (985). Further, the preferential type II fiber atrophy in sedentary men was diminished after a 3-month physical exercise intervention improving muscle strength irrespective subject age, but there was a persistent aging-related decline in maximum voluntary force irrespective speed of movement (517). Thus, it is unlikely that the preferential atrophy of the fibers included in fast-twitch motor units with a high threshold for activation plays a dominant role in the aging-related loss in muscle force. Based on whole muscle function measurements in humans, it remains controversial whether skeletal muscle undergoes qualitative aging-related changes in force generation capacity, i.e., a decreased maximum force normalized to the cross-sectional area of the contractile material (specific tension) or not (for refs. see (116, 520, 603)). However, the more pronounced decline in muscle strength at fast compared with slow speeds of movement indicates that the loss in force is not only due to loss in muscle quantity (525, 985). Several attempts have been made to normalize *in vivo* muscle force to the average muscle cell cross-sectional area in muscle biopsy specimens or total muscle cross-sectional area determined

from limb circumference, ultrasound, CT- or MRI-scans (57, 116, 289, 307, 525, 671, 982). However, these estimates of contractile material cross-sectional area do not take into account the gradual replacement of contractile proteins by connective tissue reported in old age in rodents (15, 23, 311, 482, 483, 523, 643) and in humans (301, 417). Cross-sectional muscle areas and muscle weights will accordingly overestimate the amount of contractile material in old age (520). Further, analyzes of muscle cell cross-sectional areas do not take into account the relative contribution of different muscle cell types during maximum voluntary contractions and aging may affect specific types of muscles, motor units and muscle cells differently (for refs. see (514, 520)).

In addition to the muscle atrophy and decreased force, slowing of movement is also a hallmark of sarcopenia with significant negative consequences for fall and fall-related injuries in old age due to an impaired capacity to quickly correct for loss in balance. However, the slowing of movement may be secondary to multiple factors ranging from the initiation of contraction in the central nervous system to mechanical properties of joints and tendons. However, the difficulty in rapid recovery from an impending fall in old age is primarily due to inherent aging-related changes in muscle tissue itself (809). At the muscle level, a fast-to-slow myosin isoform (fiber type) switching have been reported in both humans and in experimental animal models (see above). The fast-to-slow fiber type transition during aging may contribute to the slowing of movement as well as decline in maximum force since fast muscle fibers are considered to have a higher force generation capacity than slow fibers. However, the relative changes in fiber type proportions or myosin isoform expression in aging human muscle are relatively small and it may be questioned whether these changes will have a major impact on overall motor performance.

Motor unit function

Decreased speed of contraction and loss of force represent significant phenotypic characteristics of sarcopenia. At the single motor unit level, speed of contraction refers to the duration of the isometrich twitch, i.e., the duration of contraction and half-relaxation times and both being highly dependent on motor unit type with fast-twitch units having shorter contraction and half-ralaxation times than slow-twitch units irrespective age (258). During aging, there is a slowing of the isometric twitch in various mammalian species, including humans (23, 66, 126, 128, 194, 258, 360, 523, 735) and the reduced speed of contraction precedes the aging-related loss in muscle mass (23, 523). An aging-related loss of fast-twitch motor units or altered properties of the contractile machinery, or both, have been suggested to underlie the slowing of the isometric twitch in old age (23, 128, 258, 523, 666, 709, 735, 861). The slowing of the isometric twitch observed in both fast- and slow-twitch muscles may accordingly be related to an aging-related shift within fast-twitch motor units or from fast- to slow-twitch motor units. However, the slowing of the isometric twitch at the motor unit level, irrespective motor unit type, indicates significant aging-related changes in the function of the contractile apparatus and are forwarded as the dominant mechanism underlying the decreased speed of contraction (258, 521, 537). A number of different factors may influence the duration of the isometric twitch ranging from the transmission of the action potential, the Ca^{2+} handling by the sarcoplasmatic reticulum (SR), Ca^{2+} binding to regulatory proteins and the initiation of contraction to the function of contractile proteins. The capacity of the SR for Ca^{2+} release and recapture and the expression and function of the contractile proteins are the two key factors determining contractile speed. In adult mammalian motor units, there is a close co-ordinated expression of SR and

contractile proteins (104, 245, 489, 791), but this close co-ordination may be lost under different experimental conditions (104, 294) as well as during aging (791). A strong relationship has been reported between the contraction time of the isometric twitch and the SR Ca^{2+} uptake activity (104, 383), suggesting that altered SR properties underlie the slowing of the isometric twitch in old age. This is supported by the impaired structure, function and biochemical properties of the SR in old age as well as the correlation between the prolonged isometric twitch in single motor units and the impaired SR Ca^{2+} uptake activity in single membrane permeabilized muscle fibers from the same muscle (195, 537, 952). In this context it is interesting to note that the decreased speed of isometric contraction observed in cardiomyocytes from old rats has been reported to be coupled to a diminished rate SR Ca^{2+} uptake (501).

In glycogen depleted single motor units characterized according to type and motor unit fiber morphology, maximum twitch and tetanus forces have been reported to be maintained or increased in moderately old rats (20-24-month) (258, 521), but decreased in very old 27-month old rats (444). The increased or maintained maximum force in moderately aged anilmals at the same time as total muscle fiber number and muscle force are lower than in young animals is suggested to be secondary to the increased number of motor unit fibers in individual motor units caused by the ongoing denervation-reinnervation process related to the aging-related α-motoneurone and motor unit loss (26, 258, 520, 521, 524). The decline in single motor unit force in the very old (27-month old) rats coincides with ultrastructural evidence of myofibrillar loss and increased myofibrillar space (22) in 27-month old rats as well as the preferential loss of the molecular motor protein myosin observed in very old rats and humans (183, 875). Specific tension measurements in glycogen depleted motor units is complicated by the impact of pennation angle on the force recording, all muscle fibers in a motor unit can typically not be identified in one single muscle cross-section and additional aging-related changes in neuromuscular transmission may introduce incomplete glycogen depletion of all motor unit fibers in old age. Single muscle fiber preparations offer a superior alternative for specific force measurements, i.e., a preparation where fiber orientation, sarcomere length and temperature can be controlled during maximum force recordings.

Single muscle cell contractility

The maximum velocity of unloaded shortening (V_0) varies considerably between different muscle cells and V_0 is often used to characterize and separate muscle cells, motor units and muscles into different types (163). Muscles develop their maximum power at a shortening velocity of approximately one-third of V_0 and V_0 is therefore considered to be one of the most important design parameters of skeletal muscle (see (392, 625, 768)). The ability to generate power optimally over a wide range of movements is important for avoiding falls in the elederly and myosin isoforms are the major determinants for the wide range of shortening velocities under which muscle cells are operating. Studies at the single muscle fiber as well as motor protein levels have shown that there is a close relationship between V_0 and myosin heavy chain (MyHC) isoform composition (see (650)). A modulatory influence of essential myosin light chain (MyLC) isoforms on V_0 have a been documented in single skinned muscle fibers from small mammals such as the rat and rabbit (94, 348, 563, 859) as well as in *in vitro* motility assays using avian myosins (588). In humans, on the other hand, the modulatory influence of MyLC isoforms on V_0 has not been confirmed (526, 529, 530, 534), indicating a species difference in the regulation of muscle contraction.

Whole muscle or fiber bundle preparations are of limited usefulness when studying V_0 since the behavior of individual muscle cells is obscured by several independent factors, such as: (a) differences in intramuscular fiber orientation, (b) differences in the mechanical leverage provided by the bony anatomy of the joint, (c) the elasticity of the muscle and the muscle tendons, (d) patterns of motor unit recruitment, and (e) activation of antagonist muscles. Even if these difficulties were not present, multicellular preparations have the disadvantage that the roles played by different muscle fiber types in overall contractility are difficult to evaluate (see (392, 397, 398)).

The confounding influences of protein heterogeneity between muscles fibers or intercellular connective tissue in multicellular preparations are overcome in single muscle fiber preparations, allowing detailed studies of regulation of muscle contraction in a muscle fiber with an intact myofilament lattice. In humans, the skinned fiber preparation offers the advantage that several muscle cells can be studied from a single biopsy under near physiological conditions (292, 518, 534). The ability to investigate muscle fibers of different type (MyHC isoforms) is of crucial importance in studies on aging-related changes in muscle contractility since aging appears to have a differential influence on different muscles and muscle cell types. Further, the skinned fiber preparation has been found very useful for the study of regulation of muscle contraction under controlled conditions in human muscle fiber segments in both normal (292, 518, 532-534, 538, 848) and pathological muscle tissue (305, 508, 528, 531, 536, 560, 670).

Aging-related changes in regulation of muscle contraction at the single muscle fiber level have been investigated in both humans (181, 190, 220, 477, 529, 985) and rodents (218, 252, 463, 540, 781, 794, 842, 874, 984) and a decline in V_0 in muscle cells expressing the β/slow (type I) and fast (type II) MyHC isoforms have been observed in both humans (102, 181, 190, 477, 529, 985) and rodents (218, 463, 540, 874, 984). A discussed above, there is a close relationship between V_0 and MyHC isoform composition at the single-fiber level and the composition of MyHC isoforms determines the myosin ATPase activity (see (650)) irrespective species and essential MyLC isoforms have a modulatory influence on V_0 in muscle fibers expressing fast MyHC isoforms (94, 348, 563, 859), at least in small mammals. It cannot be completely ruled out that our knowledge of MyHC isoforms is incomplete and that novel MyHC isoforms are expressed in old age causing the decreased speed of shortening at the single muscle fiber level. This is supported by the identification of multiple β/slow (type I) MyHC isoforms in mammalian skeletal muscle by using S1 nuclease mappings and immunocytochemical and electrophoretic techniques (280, 315, 367, 412, 425) as well as the existence of additional MyHC isoforms in fibers so far considered homogeneous regarding their MyHC isoform composition (see (807). To date, eight different MyHCs have been identified in mammalian striated muscles, *i.e.,* β/slow (I), α-cardiac, embryonic, fetal, IIa, IIx (IId), IIb and a super-fast extra ocular myosin isoform. A ninth slow MyHC isoform has been identified in mammalian extra ocular and intrafusal muscle fibers which is antigenically different from the β/slow or type I MyHC observed in limb and trunk muscles (see (399, 807)). Furthermore, the observation by Smerdu and co-workers (833) of the presence of additional fast MyHC components in adult human skeletal muscle cells is of specific interest in this context. The sarcomeric MyHCs are encoded by three highly conserved multigene families and the human MyHC gene family includes several genes that code distinct isoforms. Our knowledge of the MyHC genes may not be fully understood (see (708)) and there are indications of as many as 31 MyHC genes in the chicken (762). Although relative changes in the MyHC isoform expression has been reported during the aging process in

mammalian skeletal muscle, including humans, there is to our knowledge no results demonstrating an increased expression of specific MyHC isoform in old age not expressed in adult skeletal muscle.

Aging-related changes in the molecular motor protein myosin can affect contractile speed by different mechanisms: (a) The reduced rate of protein synthesis in old age will affect enzymatic activities negatively (746). In accordance with this, the decreased rate of MyHC synthesis in old age (38) may have a negative effect on the myosin ATPase activity resulting in a slowing of contractile speed. (b) In adult laboratory mammals, MyLC isoform composition has been shown to have a modulatory impact on the primary influence of MyHC isoform composition on V_0 (94, 348, 563, 589, 650) and the aging-related slowing in muscle cells may accordingly be related to an altered modulatory influence of MyLC isoforms. (c) Finally, aging-related changes in other thick and thin filament proteins or in cytoskeletal elements cannot be completely ruled out as a mechanisms underlying the aging-related slowing in contractile speed.

Proteins undergo a number of posttranslational modifications during aging which influence enzymatic activity, stability and digestibility (see (646)). Aging-related myosin posttranslational modifications induced by e.g. reactive oxidative species may accordingly have significant impact on regulation of muscle contraction. Peroxynitrite ($ONOO^-$), formed under conditions of simultaneous generation of superoxide and nitric oxide, represents a potentially harmful reactive oxygen species due to its high reactivity with lipids and proteins leading to oxidation, hydroxylation and to ortho-nitrotyrosine (eg. (728)). Thus, reactive oxygen species have been forwarded as important factors inducing muscle cell protein modifications which affect skeletal muscle function (472, 750). During aging, 3-4 fold higher amounts of nitrotyrosine have been reported on the Ca-ATPase of the sarcoplasmic reticulum in muscle cells expressing the β/slow MyHC isoform (952), confirming periodical exposure of skeletal muscle to peroxynitrite. Thus, the slow Ca-ATPase isoform appears to be more susceptible than the fast Ca-ATPase isoform to this modification, possibly related to a less efficient antioxidant defense system of aged organisms (953)

Non-enzymatic glycation of proteins has been regarded as one of the biochemical basis underlying the pathophysiology of diabetes and aging (115). Non-enzymatic glycosylation (glycation) of proteins occurs by a chemical reaction of reducing sugars with primary amino groups in proteins to form Schiff´s base linkage (925). That is, the aldehyde groups of free, unbound sugars react with free amino groups of proteins, forming Schiffs´s bases, which further undergo various rearrangements to generate advanced glycation end products (502). This reaction of proteins is a common post-translational modification of proteins. Glycation of myosin has been sporadically reported by workers in different muscle preparations, viz., actomyosin (925) and myosin ATPase (862). The effects of myosin posttranslational modifications during aging and the effects on myosin function are discussed below.

The association between the aging-related V_0 decline and MyLC isoform shifts has been forwarded as a mechanism underlying the aging-related slowing within specific muscle fiber types (463, 681). This is confirmed by the parallel fast-to-slow transition of the regulatory MyLC and MyHC isoform expression (681) and the shift in essential MyLC isoform expression in single muscle fibers from old rats expressing fast MyHC isoforms (463). This is further supported by the attenuation of the aging-related slowing after recombinant adenovirus gene transfer of $MyLC_{3f}$ (463). However, the aging-

related essential MyLC shift and the attenuation of the decreased speed of contraction was mainly confined to muscle fibers expressing the type IIb MyHC isoform (463). This is an interesting finding, although muscle fibers expressing the IIb MyHC isoform are not expressed in normal skeletal muscle from larger mammals such as humans and the slowing in muscle fibers expressing the β/slow (type I) MyHC is not accompanied by a MyLC isoform switching (218). Further, the modulatory influence of the $MyLC_{3f}$ on V_0 observed in young animals was not confirmed at the single muscle fiber level in old animals and the coordinated expression of MyHC and essential MyLC isoform expression observed in young animals were not observed in old age (563). A the filament level this may be secondary to the independent incorporation of MyHCs and MyLCs into the thick filament backbone and the differential turnover rates of MyHC and MyLC isoforms (442). This is of specific interest since a loss in the coordinated expression of MyHC isoforms and mitochondrial enzyme activities (521), and between MyHC isoforms and SR proteins (791), and between MyHC and MyLC isoform expression (563) have been reported in rodents and humans. Thus, the loss in the coordinated expression of contractile, SR and mitochondrial proteins may play a more important role for the overall aging-related impairment of muscle function than changes in individual proteins.

As discussed above, other factors than quantitative changes related to total muscle fiber number and muscle fiber size may play a significant role for the aging-related decline in muscle force. A significant dissociation between muscle mass and strength have been reported during aging, hormone treatment, exercise and inactivity (reviewed in (780)) and suggested to be secondary to central factors rather muscle specific changes (780). Central factors may play a significant role for aging-related changes in motor function, but as mentioned above factors distal to depolarization of the muscle membrane are the primary determinants underlying the impaired capacity to correct an impending fall in old age with consequences for falls and fall-related injuries (809). Furthermore, studies showing discrepancies between muscle mass and function during aging, hormone treatment, exercise and immobilization are frequently based on measurements of muscle mass using imaging methods such as computerized tomography, force measured during maximal voluntary contractions under isometric or isokinetic conditions and central activation estimated by measuring the amplitude of the surface EMG, i.e., methods which are all associated with significant methodological limitations. As an example, long-term immobilization by bedrest shows modest decline in muscle mass and a greater decline in muscle strength (69) together with concomitant altered surface EMG amplitudes, this may be interpreted as central factors playing a dominant role for the inactivity induced decline in muscle function (69). However, when the intrinsic properties at the single muscle fiber level were evaluated in the same individuals, a significant decline in muscle fiber force generation capacity (maximum force normalized to muscle fiber cross-sectional area, specific force) were observed which were closely associated a significant loss of contractile proteins which exceeded the decrease in muscle fiber size (527). Thus, the study of aging-related loss in muscle function needs to go far beyond the study of muscle mass and voluntary force and focus specifically on regulation of muscle contraction at the cell and subcellular levels.

Single muscle fiber results indicate intrinsic changes in regulation of muscle contraction with a decline in single muscle fiber specific force (maximum force normalized to muscle fiber cross-sectional area) in old age in both rodents and humans (102, 190, 309, 335, 529, 532, 586, 587, 874, 883, 991). The decreased specific force in old age has been attributed to both quantitative changes in the number of force generating cross-bridges (183, 190, 875) as well as the function of individual

cross-bridges (561, 586, 587, 991). In line with this, a preferential loss of the molecular motor protein myosin has been observed during aging in both men and women (183) as well as in rodents (874). However, other intracellular factors prior to activation of contractile proteins have been suggested to play a more important role for the aging-related decline in specific force than quantitative and qualitative changes in contractile proteins. Events prior to activation of the contractile proteins are affected by aging such as neuromuscular transmission (discussed above) and from muscle membrane depolarization to the Ca^{2+} release from the sarcoplasmic reticulum (SR) also known as the excitation-contraction (EC) coupling (149). The events leading to SR Ca^{2+} release includes multiple steps involving the dihydropyridine receptor (DHPR) L-type Ca^{2+} channel and the SR ryanodine receptor (RR) for Ca^{2+} release (748). The depolarization of the muscle membrane and the propagation of the action potential along the muscle fiber and deep into the fiber via the transverse tubular system induces a conformational change in the DHPR acting as a voltage sensor, activation of the RR and Ca^{2+} release from the SR and activation of the contractile proteins. In addition to receiving a signal from the DHPR, the RR also generates retrograde signaling to the DHPR of importance for channel activity (51, 660). In old age, peak intracellular Ca^{2+} transients have been reported decreased (270, 336) due to an impaired SR Ca^{2+} release (429), resulting in decreased muscle fiber force generation capacity (270, 752). Changes in EC coupling in old age has been shown to be associated with the aging-related denervation process suggesting that the motoneurone loss may underlie the alterations in EC coupling in old age (700).

Myosin and actin interaction

The ability of skeletal muscle to generate force and motion can be attributed to the mechanical interaction between the two contractile proteins, myosin and actin. Different *in vitro* methods allow direct measurements of myosin-actin interaction in the absence of secondary influence from structural and regulatory proteins (370, 485, 818, 819). Since the introduction in the 1980s, the *in vitro* motility assay has been continuously improved. In the early studies, the movement of single fluorescently labeled actin filaments on a myosin-coated glass surface was followed in a flow cell where myosin had been extracted from muscle homogenates and randomly adhered to nitro-cellulose coverslips. Recent improvements include measurement of average force per cross-bridge by measuring the deflection an ultracompliant glass microneedle to which an actin filament was attached (468). In the technically challenging optical trap system, the interaction between a myosin molecule and a single actin filament can be measured allowing force and displacement measurement with pN and nm precision, respectively (290).

In the study of aging-related changes in myosin function, the single muscle fiber *in vitro* motility assay offers the opportunity to conduct detailed studies of the catalytic properties (motility speed) and force generation capacity of specific myosin isoforms or combinations of isoforms extracted from a single muscle fiber segment with a known myosin isoform expression (403, 404, 406, 558-561, 740, 741). In addition, myosin function can be investigated in detail in any mammalian species including humans (559). The single fiber *in vitro* motility assay has become a critical method for the study of myosin function, *i.e*., catalytic properties (motility speed) and force generation capacity, during aging and the viscous drag produced by the solution in the experimental chamber when fluorescent-labeled actin filaments are driven by myosin is negligible compared with the force produced by the motor protein. Therefore, *in vitro* motility speed is, under most conditions, a good

analogue for the maximum velocity of unloaded shortening (V_0) in single cells measured with the slack test (see (402)). The aging-related slowing of V_0 at the single muscle fiber level (see above) has been confirmed at the motor protein level in both rodents and humans in muscle cells expressing the type I and IIa MyHC isoforms (404, 405, 526, 561). According to X-ray diffraction single muscle fiber analyses, allowing detection of structural alterations of contractile proteins with high temporal and spatial resolution (481), filament lattice spacing was not altered in muscle fibers from old humans but actin-myosin lattice ordering was altered leading to a disrupted actomyosin interaction during muscle contraction in old age (561). This observation was supported by mass spectrometry analyses demonstrating posttranslational modifications of the myosin rod domain in old age, i.e., a region essential for the packing of myosin molecules into myosin filaments (127, 561, 676). Oxidative stress related to accumulation of reactive oxygen and nitrogen species and the compromised ability to handle this stress in old age is the most probable cause underlying the increased amount of protein modifications in old age (149).

In response to oxidative stress, aldehyde groups of reducing sugars react preferentially with free lysine residues to form Schiff´s base adducts, which may undergo further Amadori rearrangements and free-radical-mediated oxidation to form irreversible advanced glycation end products (AGEs) (502). Non-enzymatic glycation of proteins is a common post-translational modification affecting the structure, function and digestibility of extracellular proteins and has been regarded as one biochemical basis underlying the pathophysiology of diabetes and aging (115, 502, 925). The effects of glycation of long-lived extracellular proteins, such as collagen, have been described in detail during aging (896). However, intracellular muscle proteins are also affected by glycation (743). This is of specific interest, since myosin is a long-lived protein, it has a decreased turnover rate in old age, and it is lysine rich in critical regions of the motor protein, i.e., the actin binding site and in the catalytic domain (39). In line with this, glycation of actomyosin (925) and myosin ATPase (862) have been reported in aging muscle. Furthermore, exposure of myosin isoforms to a reducing sugar results in a significant impairment of myosin function linked to structural modifications of myosin at the catalytic domain (740) and the antioxidant glutathione protects myosin from the negative effects of a reducing sugar on myosin function (742).

Thus, significant qualitative and quantitative changes take place at the motor unit, muscle fiber and motor protein levels during aging significantly affecting skeletal muscle structure and function some of which may be affected by intervention strategies.

5. INTERVENTION STRATEGIES

Aging is accompanied by an imperceptibly slow progression of deterioration of tissue and organ function that ultimately results in death. Skeletal muscle is, however, highly adaptable and interventions that reverse or delay the progressive aging-related loss of muscle function will extent the number of years with a good quality of life.

Caloric restriction

Caloric restriction is an effective means to increase the life span in a wide variety of organisms ranging from yeast to primates and probably even humans (235, 384, 934). Caloric restriction in progeric mice even led to a staggering 3-fold increase in life expectancy (934). This is the consequence of redirecting resources to maintenance rather than growth and reproduction (934) to overcome periods of diminished food supply that severely reduces the chances of survival of offspring (467). Among the adaptations is a shift from glucose to fatty acid metabolism and a reduction in IGF-I and insulin (384, 934). As IGF-I is important for muscle growth (269) one may expect that a caloric restriction-induced reduction in IGF-I has a negative impact on muscle strength, but no such effect was seen in caloric restricted mice (934). Even so, any negative effects may be more than compensated for by the reduced mitochondrial leakage and ROS production (384) resulting in reduced oxidative modifications as indeed seen in caloric restricted mice with a mutation in a DNA-repair gene (934). As seen in isolated mitochondria, the diminished mitochondrial ROS generation during caloric restriction may be a consequence of a reduced mitochondrial membrane potential caused by a non-esterified fatty acid-induced increase in protonophoric activity of adenine nucleotide translocase (32).

The reduced oxidative stress may also explain the absence of an aging-related increase in expression of genes involved in the stress response, allowing resources to be channeled to growth, as reflected by the shift in gene expression toward increased protein turnover and decreased macromolecular damage (547) during caloric restriction. This increased protein turnover, decreased macromolecular damage and reduced ROS production may well explain why caloric restriction was associated with a decrease in Hsp27 and the DNA repair enzyme oxoguanine glycosylase 1 in the ventral horn of the spinal cord (157).

The caloric restriction-induced reduction in DNA damage in mice with deficiencies in DNA-repair genes not only tripled their life expectancy, but also improved maintenance of motoneurones and full motor function (934). Also in normal mice, life-long caloric restriction attenuated motoneurone loss and decreased the abundance of pre- and post-synaptic abnormalities in 24-month-old mice (907). Part of the attenuated loss of motoneurones can be ascribed to reduced caspase-dependent apoptosis (157). It may well be that the caloric restriction-induced attenuation of motoneurone loss underlies the prevention of muscle fiber loss during aging in rats that had been subject to 40% caloric restriction since the age of 4 months (624).

Caloric restriction not only exerts its beneficial effects on skeletal muscle mass by attenuating the aging-related decline in motoneurone numbers and alterations in the neuromuscular junction, but also via reducing the low-grade systemic inflammation observed in rats (712). The reduction in low-grade systemic inflammation diminished apoptosis, at least in the fast superficial vastus lateralis muscle in the rat (712).

There are some potential pittfalls in applying caloric restriction to humans, such as lowered blood pressure, slower wound healing and a danger of malnutrition (235). In addition, indications in older people with sarcopenic obesity that caloric restriction may lead to undesired loss of muscle mass has led to the suggestion that ‘weight loss by caloric restriction in individuals with sarcopenic obesity should likely be avoided’ (570). Overall, however, these observations bear great promise for dietary restriction to prevent and maybe even reverse some of the aging-related changes in skeletal muscle.

They also highlight the potential detrimental effects of present-day calorie-rich diets in the developed world that may accelerate skeletal muscle aging. Indeed, there are indications that the aging-related rate of muscle wasting is faster in obese and overweight people (647), and, in contrast to the above reservation, that caloric restriction normalised the inflammatory profile in the adipose tissue of obese people (162).

Activity/inactivity

While life-long caloric restriction appears to be a potent intervention to extend the life expectancy by a staggering 30% (235), the benefits are likely to become less when started later in life. In addition, continuing caloric restriction demands a great deal of will power and is in practice rarely, if at all, achievable. It is therefore desirable to explore other routes that can reverse or attenuate the aging-related decrements in muscle mass and function. In one study, the investigators showed with time-lapse imaging that 1-month wheel-running exercise reversed, similar to - though to a lesser extent than - life-long caloric restriction, some of the aging-related changes in the morphology of the NMJ in 22-month-old mice (907).

A striking characteristic of skeletal muscle is its ability to adapt to altered functional demands. Examples are the hypertrophy and associated increase in muscle strength in response to a repeated overload stimulus, such as delivered during a resistance-training program, and the increased aerobic capacity and fatigue resistance of a muscle after an endurance-training program or chronic electrical stimulation (790). Likewise, a reduced use of skeletal muscle, such as occurs during hospitalization, paralysis, or bed rest, leads to muscle atrophy, weakness and a reduction in the oxidative capacity and fatigue resistance of the muscle (203).

The effects of reduced use on skeletal muscle resemble those seen during aging (203) and the lower levels of physical activity and increased sedentary behavior in old age (418, 628, 967) may thus well be the prime cause of the aging-related loss of muscle mass and strength. Given these observation it is no surprise that significant attention has been paid to the potential of exercise to improve the quality of life and independence, and a substantial number of studies have shown significant muscle hypertrophy and strength gains in elderly men and women (eg. (58, 271-273, 288, 308, 517)). The potential of maintaining high levels of physical activity for daily-life exercise capacity is exemplified by master athletes and a case of a 97-year-old man who cycled more than 5,000 km a year, even at that age (154).

It is well known that muscle activity increases the rate of myofibrillar protein synthesis, resulting in muscle hypertrophy (e.g.,(333). Muscle hypertrophy *per se* is only valuable if it also leads to improvement in the ability to perform daily life activities, and it has been shown that progressive strength training does just that; it did improve gait velocity, stair-climbing power and spontaneous activity levels in elderly men and women (58). Proteomic analyses have indicated that this is associated with an increased expression of contractile and structural, metabolic and stress response proteins (651).

A rapid decrease in protein synthesis rate followed by an accelerated rate of protein degradation is typically observed in response to inactivity, resulting in weakness and muscle atrophy. Different models to simulate the unloading induced muscle atrophy during space flight have been introduced,

such as limb suspension and bed rest (see (83, 203), but the mechanisms underlying this loss of strength in response to unloading are not well understood (see (68, 244, 254)). While muscle atrophy is a significant cause of the force loss during unloading it cannot be the sole explanation, since the decrease in force is proportionally larger than that in fiber size or muscle cross-sectional area (e.g. (68, 244). The extent to which the loss of voluntary force is related to neural factors or hormonal changes is a matter of controversy (244, 254), where some studies have shown that motor unit activation is not impaired in response to short-term unloading (68) and others observed a reduced ability to voluntary activate the muscle (454).

There is evidence that at least part of the proportionally larger loss of force than muscle atrophy in response to unloading or reduced physical activity levels is attributable to a reduced specific tension of the muscle fibers. For instance, it has been observed in rat (751) that after hind limb suspension the specific tension in isolated muscle fibers was 12-15% lower. A more pronounced decline in specific tension was reported by Gardetto et al. (318), who found a 20% and 28% decrease in type I fibers from the gastrocnemius and soleus muscles, respectively, in response to 2 weeks of hindlimb suspension.

Specific tension is determined by the number of force generating cross-bridges per cross-sectional fiber area and the force generated per cross-bridge. The parallel decrease in specific tension and myofibrillar protein concentration in humans in response to 6 weeks bed rest (527) indicates that the reduction in specific tension is primarily related to a decreased number of force-generating cross-bridges. It has been shown in rats that the protein loss in response to hind limb suspension follows a triphasic time course, involving pre-translational, translational and post-translational control mechanisms: 1) an initial rapid reduction of protein synthesis, 2) a delayed increase in proteolysis, and finally 3) a decreased rate of myofibrillar protein degradation resulting in a new balance between rates of protein synthesis and degradation (see (84, 872)). The time-course of protein loss varies between sarcoplasmic, thick and thin filament proteins during hind limb suspension (84, 872).

Computed tomography (417) has not only shown an aging-related decrease in muscle cross-sectional area, but also a decreased density of lower limb muscles in humans, indicating both a loss of muscle mass and replacement of contractile material with fat and connective tissue, similar to the observations after hindlimb unloading in rats. This replacement of contractile material with fat may be a consequence of a decreased mixed muscle protein synthetic rate in the elderly (age>65 yr) compared to the young (age<35 yr) (931, 979, 980), where in particular the myosin synthesis rate was affected (38, 40). In fact, the aging-related decline in muscle strength per unit muscle mass correlated with the decreased rate of myosin synthesis. This suggests a compromised ability of skeletal muscle to maintain the quantity and quality of the molecular motor protein myosin in old age. The negative effect of inactivity on protein synthesis may accordingly add to the aging-related decline in myosin quantity and quality making the elderly more vulnerable to a period of immobilization.

The reduced myofibrillar protein synthesis rate makes the elderly not only more vulnerable to the detrimental effects of immobilization, but may also underlie the common clinical experience that the rehabilitation process is significantly slower in elderly than in young patients who have been immobilized for a long time because of various disabilities or diseases. This has a significant impact

on the quality of life and the health care system, where the older patient will need longer care during recovery and some even never fully recover from a period of immobilization. This effect of immobilization is at least partly related to the additive effects of inactivity and the normal aging-related impairments of neuromuscular function.

The reductions in muscle mass and strength induced by immobilization are paradoxically less in the old than in the young. For instance, both the absolute and the relative denervation-induced atrophy in rat soleus and gastrocnemius muscles was less in old than in young animals (17). A similar situation was seen after 2 weeks of unilateral leg casting, where the decline in muscle volume was less in old than young men (854). This was even reflected at the fiber level where in young men fibers of each type atrophied by 15-30%, while in the old men type IIa fibers only atrophied by just 13% (414). Yet, the rate of force development was decreased more in old than young men, and where in young men the muscles had fully recovered after 4 weeks retraining this was not so in the old men (414, 853). Even so, in older people post-operative resistance training resulted in a better recovery of muscle mass and strength and a shorter hospital stay after unilateral hip replacement (854).

It is intriguing that, as described above, the loss of muscle mass during immobilization is less in older, including humans, than in younger organisms. This observation has led to the idea that there may be a 'default' muscle mass (208) beyond which normally no further atrophy (can) occur(s). It could also be a reflection of a lower protein turnover in the old, where a reduced rate of protein synthesis is accompanied with an unaltered or even reduced rate of protein breakdown, as suggested by the lower specific activity of the ubiquitin proteasome pathway in muscles from old rats (285).

The slower rate of protein turnover is probably contributing to the accumulation of oxidized proteins in, and the lesser atrophy and slower recovery of, old muscles after a period of immobilization. This apparently negative characteristic of old muscle may, however, be turned into an advantage. If it results in a slower atrophy, as has been observed, one may expect that it also will result in a slower atrophy of muscles that had hypertrophied after a period of loading! That this is not just wild speculation was shown in an elegant study where indeed the loss of patagialis muscle mass after a period of loading was significantly less in old than in young quail (832). In fact, retraining may be even facilitated by a previous period of regular loading, due to the persistence of newly acquired myonuclei during the training regimen, as seen in temporarily overloaded mouse muscles (119). These observations may have significant clinical implications as, if this also applies to humans, pre-operative resistance exercise would then significantly shorten the duration of post-operative hospital stay, particularly when also followed by post-operative resistance exercise.

It has been observed that motor unit numbers are better preserved in muscles of the upper body than muscles of the lower body in both humans (313) and rodents (909). Given that both the activity and the mass of locomotor muscles decreases more than that of the arm during ageing(427), it may well be that maintained activity levels prevented motor unit loss in arm muscles. In support of a role of regular activity, it has been found that master athletes had a larger number of motor units than age-matched controls (723), with evidence of better reinnervation as reflected by larger motor units (713), fewer angulated fibres - indicative of denervation -, and a larger percentage of fibre type grouping (649).

This larger prevalence of fibre type grouping may, however, not be a reflection of better reinnervation, but rather a chance observation due to the larger proportion of type I fibres (our unpublished data), something also alluded to in a previous paper (649). In support of this, studies with larger numbers of participants observed an age-related reduction in the number of motor units (241). Importantly no evidence for preservation of motor units in master athletes was observed (713). Also in rodent muscles, there is no evidence that motor unit survival is enhanced by regular physical activity (1). There is thus little evidence that regular exercise prevents motor unit loss, or motor neuron death.

Overall, it appears that a significant part of the aging-related muscle wasting and weakness is attributable to reduced levels of physical activity. Even though somewhat attenuated compared to young adults, regular exercise has significant benefits for muscle strength and endurance in old age. This adaptability of the muscle may well be exploited in pre- and post-operative resistance exercise regimes that may prove to be a potent novel approach to reduce the length of hospital stay of the older patient. Surprisingly, this potential application of resistance exercise in a clinical setting has so far received little scientific attention.

Hormone therapy

While caloric restriction and exercise programs clearly have significant benefits for the older person and their muscles, they require considerable motivation and are not always possible because of frailty and co-morbidities (353). When considering other options it is important to recognize that many circulating anabolic and sex hormones show an aging-related reduction (503, 831). This does not need to be so when it comes to the concentrations of androgenic hormones in skeletal muscle. For instance, the muscle concentrations of dehydroepiandrostrone and androstenedione were similar while the estradiol and testosterone concentrations were even higher in post- than pre-menopausal women, the latter probably reflecting fat infiltration in the muscle (721).

Given the aging-related decrements in circulating sex hormones and their association with muscle wasting and weakness during aging, hormone replacement therapies or treatment with other anabolic factors has received considerable attention as a means to combat aging-related muscle wasting and as an alternative or enhancer of exercise-induced improvement in muscle mass and strength, particularly for the frail older person.

Anabolic steroids on their own may, however, not be enough to induce hypertrophy. For instance in old rats, nandrolone decanoate on its own did not induce hypertrophy (548) and the β_2 -adrenergic agonist clenbuterol did not prevent atrophy during hind limb suspension (151, 152, 975). Only when nandrolone decanoate was combined with 7 days of functional overload did the soleus, but not the plantaris, muscle develop hypertrophy (548). Similarly, clenbuterol combined with intermittent weight bearing in young rats prevented the hind limb suspension-induced atrophy of the solues, but not that of the extensor digoitorum longus muscle in rats (975). The beneficial effect of nandrolone decanoate during an overload stimulus in old age has been shown to be associated with suppression of cytokine gene expression in the old, but less so in the young overloaded soleus muscle (876). These data indicate potential of combined overload and anabolic hormone treatment that appear, however, to be limited to slow fibers/muscles only.

Also in humans, hormone replacement therapy has benefits for skeletal muscle mass and can even be effecitive without concomitant exercise in hypogonadism. For instance, continuous testosterone replacement via a gel over the skin (50 mg/day) for 6 months preserved muscle thickness in intermediate-frail and frail hypogonadic elderly men (33) and a meta analysis showed improvements in muscle mass in hypogonadic elderly men (409). In addition to these benefits on muscle mass, testosterone treatment also improved insulin resistance in hypogonadal frail elderly men, all stimulating further study on the effectiveness of testosterone treatment to improve the quality of life in hypogonadal frial ederly men (672).

In post-menopausal women, however, hormone replacement therapy increased strength without a change in muscle size (711, 963). Studies on postmenopausal homozygotic twins have shown that at least part of the attenuation of the loss of muscle power during estrogen-based hormone replacement therapy is attributable to a better preservation of specific tension of muscle fibers (731). Prevention of the reduction in muscle power during menopause by hormone replacement therapy can also be associated with an attenuated loss of muscle mass, particularly when the therapy is combined with exercise (831). Although this indicates that exercise training adds to the effects of hormone replacement therapy in post-menopausal women, the effects of training on muscle strength have been reported to be blunted in those with, compared with the gains in those without hormone replacement therapy (686). This, suggests that exercise without hormone replacement therapy is preferable and that adding hormone replacement therapy to an exercise programme does not elecit further gains.

While in women estrogen-based hormone replacement therapy improves muscle strength mainly by an increase in specific tension, in older men with low testosterone levels the increased force of single fibers after 5-month testosterone treatment resulted from an increase in fiber size, rather than in specific tension or alterations in cross-bridge kinetics (293). Similar to animal studies with clenbuterol and nandrolone decanoate, the benefits of testosterone treatment were particularly evident in type I fibers (293). Interestingly, the effects were more pronounced during weekly treatment with testosterone than a treatment with alternating months without testosterone administration (293). In women the benefits of hormone replacement therapy remained evident several years after cessation of the therapy (687). Although these observations are promising, care has to be taken about the dose because of the potential detrimental effects on the cardiovascular system (860).

The benefits of hormone replacement therapy may be genotype dependent. For instance, the gain in muscle strength in post-menopausal women was larger in those with the angiotensin-I converting enzyme insertion allele (ACE-I) than those without this allele (963). While hormone replacement therapy may thus have benefits for skeletal muscle function, it is questionable whether this should be advised to any woman, as it is associated with an increased risk of cardiovascular disease, thrombosis and cancer (765, 860).

Growth hormone

Growth hormone (GH) is the primary pituitary controller of skeletal muscle growth that stimulates the release of insulin-like growth factors (IGFs) by the liver into the blood. GH does not only affect muscle mass via IGF-I, but can also have a direct effect on skeletal muscle (see (297)). After the age

of 50, the secretion of GH declines and 50% of citizens over 65 have been reported to be partially or totally GH deficient (779, 782). In aging rodents (842) and humans (296) there is a loss of pulsatile secretion of GH. The associated lowering of circulating IGF levels suggests that GH therapy of the aged might reverse the aging-related changes in skeletal muscle (779, 782).This is supported by rodent studies showing that GH deficiency causes muscle atrophy, GH treatment stimulates muscle cell hypertrophy (61, 479), reverses aging-related changes in protein synthesis rate and membrane conductance (196, 842), and has ben shown to have beneficial effects in elderly humans (435, 606, 782). While GH and IGF-I replacement to adults with growth hormone deficiency has proven beneficial, this is not an unequivocal finding, possibly due to differences in dosage, treatment time and method of dosing (955). Given these equivocal observations and the potential risk of cardiovascular complications, particularly when the recipient is not growth hormone deficient, more studies are required to elucidate the risks and benefits of treatment, but overall it seems people with GH deficiency do benefit from GH and IGF-I treatment.

Thyroid hormone

The aging-related reduction in muscle power is not only attributable to a loss of force generating capacity, but also a slowing of the muscle. While considerable attention has been given to ways to increase muscle force generating capacity in the older person by interventions such as anabolic steroid treatment and hormone replacement therapies with or without exercise training, little attention has been given to combatting the other component of power: the slowing of the muscle.

Thyroid hormone is a nuclear hormone that via its interaction with the thyroid hormone receptor acts as a transcription factor that binds to the promoter regions of the myogenic regulatory factors that play an important role in regulation of the expression of muscle specific genes (983). Treatment with thyroid hormone induced a significant slow-to-fast transition in fiber type composition in the soleus muscle and an increase in the shortening velocity of the fibers in both male and female old rats (984). Thyroid hormone treatment is thus a promising avenue to help restore muscle power in the older person. However, while thyroid treatment in rats induced a transient improvement in cardiac function, continued treatment caused a transition to pathological cardiac hypertrophy with impaired cardiac function (207). Also in humans hyperthyroidism is detrimental for cardiac health (957), but treatment with a single intravenous bolus of thyroid hormone had a beneficial effect on cardiovascular function in patients with congestive heart failure (369). As thyroid hormone treatment is already used in clinical practice and has, at least transiently, a beneficial effect on cardiac function, the use of thyroid hormone to regain muscle power in old age by the induction of a slow-to-fast transition is something that deserves further attention. It would be particularly important to assess the save dose and duration of treatment.

Thus, caloric restriction appears to be a potent intervention to delay the aging process and does result in significant increases in life expectancy. It is maybe not so much a tool to regain lost muscle mass and power, as it has potential to delay the aging-related loss of muscle mass and function. However, results obtained in experimental studies in rats raised in a pathogen free environment may not be translated to the human situation.

Overall, muscle plasticity is maintained into old age even though the response to altered functional demands is slower in old muscle. However, rather than seeing this as a problem, new ways can be

developed to capitalize on this characteristic of skeletal muscle to accelerate recovery from hospitalization. Hormone replacement therapies appear to work best in combination with exercise, particularly in those suffering from hypogonadism. Older non-hypogonadic people are advised not to be given hormone replacement therapy because of the detrimental side-effects, such as increased risk of cancer and cardiovascular complications. Thyroid hormone bears promise as 1) it induces a slow-to-fast transition and 2) is already used in the clinic to treat cardiovascular conditions. However, before implementation to combat muscle weakness, clarification is required on the save dose and duration of treatment as prolonged treatment and too high doses of thyroid hormone have severe adverse effects on *e.g.* cardiac function.

Low grade inflammation and interventions

In 1999, Ferrucci et al. studied the association between the development of disability and serum IL-6 levels in 1,029 subjects aged 71 years or older without impairment in mobility and activities of daily living. Four years later, it was found that subjects with elevated IL-6 levels were more likely to develop disability (286) and high circulating levels of both IL-6 and C reactive protein (CRP) were also associated with higher mortality (373). Since then, other secondary muscle wasting conditions associated with cancer or chronic diseases (*e.g.* COPD and diabetes), have been shown to be closely associated with elevated levels of TNF-α, IL-1, and IL-6 which activate the ubiquitin proteasome proteolytic pathway (639, 968). Thus, it was hypothesized that the low-grade systemic inflammation observed in old age plays an important role in the pathogenesis of sarcopenia (202, 496). The mechanisms underlying the aging-associated low-grade inflammation have still not been fully understood, but dysregulation of the immune response, sedentary lifestyle, subclinical pathological conditions, such as increases of adipose tissue, presence of undetected infections, chronic health disturbance, and altered nutrition may all contribute. However, it is well established that older people exhibit the pro-inflammatory condition characterized by slightly elevated cytokine levels that lie far below the levels of acute infections (668). In this section, most research results were mainly reviewed from studies of aging healthy subjects, but results related to aging-related diseases and disabilities have also been reviewed.

Observational studies

Multiple cross-sectional and longitudinal studies have revealed that inflammatory markers, such as IL-6, TNF-α and CRP, increase during aging and correlate with disability, mortality, and the decline in muscle mass, muscle strength, physical performance and physical function (60, 287, 371, 490, 830, 956). Both CRP and IL-6 have been reported to be positively associated with total fat mass and inversely associated with fat-adjusted appendicular lean mass. Even when adjusted for sarcopenia, obesity remained significantly associated with high CRP and IL-6 concentrations (143). High IL-6 (>5 pg/mL) and CRP (>6.1 μg/mL) were reported to be associated with a 2 to 3 fold greater risk of losing more than 40% of muscle strength in 986 men and women aged 74.6±6.2 years after adjustment for confounders (802). Elevated CRP was documented to associate with reduced hand grip strength and chair stand performance in women but only chair stand performance in men in a study with 1,926 men and 2,260 women aged 65.3±9.0 years (368). In a clinical study of 270 adult patients with newly diagnosed biopsy-proven myopathy, 18% of patients were aged 70 years and older, and half of those patients had been diagnosed with inflammatory myopathies, which is significantly higher

than the proportion of 28% in patients aged 18-69 years. Thus, inflammatory myopathies were the dominant type of myopathy in the elderly compared with the non-elder group, indicating that low-grade inflammation plays an important etiological role in sarcopenia (251). In an experimental study, significantly higher plasma levels of fibrinogen and α_2-macroglobulin were observed in old male Wistar rats aged 22 months compared with 8-month-old rats, and a negative correlation between fibrinogen and albumin plasma concentrations in old rats revealed a low grade inflammatory state (692).

In addition, the molecular signature of sarcopenia identified increased expression of genes involved in mediating cellular responses to inflammation and apoptosis, including complement component C1QA, C/EBP-β, and FOXO3A in a study of human subjects (329). In an experimental study of rats aged 6, 12, 18, 21, 24 and 27 months, the global gene expression profiles in muscle demonstrated a progressive and rapid decline in muscle mass after the age of 18 months, which was associated with an up-regulation of the E3 ligase MuRF1, down-regulated mitochondrial energy metabolism, perturbed neuromuscular junction patency, increased absolute neutrophil count and serum globulin concentrations. Taken together, the loss of mitochondria and increased inflammation are presented upon initiation of sarcopenia, and restoring mitochondriogenesis and reducing inflammation may accordingly alleviate the sarcopenia phenotype (416).

Molecular pathways of low-grade inflammation in sarcopenia

Pro-inflammatory cytokines, such as TNFα, TWEAK, or IL-1, signal into two established pathways: the NF-κB pathway and the p38 MAP kinase. These two signaling mediators are required to up-regulate the expression of the key E3 ligases, MuRF1, which mediates sarcomeric breakdown, and atrogin-1/MAFbx, which control protein synthesis by ubiquitination of eIF3c. MuRF-1 and atrogin-1 are over-expressed in catabolic and muscle wasting conditions (80, 261, 334, 566, 774, 881). For a detailed review of proteolytic pathways involved in sarcopenia see section 'Regulation of muscle protein synthesis/degradation'.

In old mice, significantly higher total protein concentration, phosphorylation and ubiquitination of IκBα have been observed, preceding NF-κB activation (760). IκBα may also have roles beyond acting as an inhibitor of NF-κB, such as acting as a cotranscription factor affecting the transcription of various histone deacetylases (HDACs) that regulate muscle atrophy through histone acetylation regulation (64, 760). On the other hand, elevated levels of TNF-α decrease the mRNA abundance of PGC-1α, leading to an increased muscle protein degradation, since PGC1-α inhibits NF-κB and FoxO3 (614). Pro-inflammatory cytokines increase the synthesis of prostaglandin-E2 (PGE2) by inducing the activation of cyclooxygenase 2 (COX2), a rate-limiting enzyme in the synthesis of PGE2 that regulate skeletal muscle protein synthesis negatively (704, 942).

The aging-related low grade inflammation offers different intervention strategies aiming at alleviating sarcopenia. The pentametric CRP (pCRP) may undergo dissociation to a proinflammatory, monomeric CRP (mCRP) which via its proinflammatory effects (induced ROS generation as a major component of inflammatory tissue damage) may amplify inflammation and contribute to aggravation of tissue damage. It has been demonstrated that mCRP induces IL-8 secretion in human neutrophils via intracellular peroxynitrite signaling following the activation of NF-κB and activator protein-1, being

a major source of nitrosative stress. By blocking the dissociation of pCRP to mCRP, the mCRP proinflammatory activity can be prevented (460, 871).

Both TNF-α mRNA and protein levels have been reported elevated in skeletal muscle from frail elderly men and women, TNF-α mRNA and protein levels decreased in response to 3 months resistance training in the old, but did not change in the young control group. In addition, muscle protein synthesis rate in the old exercise group was inversely related to levels of TNF-α protein (352). In a randomized controlled trial, Thompson and co-workers confirmed significant effects of resistance training in old subjects, *i.e*., serum IL-6 decreased 29% and 33% after 12 and 24 weeks exercise, respectively, and increased after detraining although IL-6 levels remained lower than baseline by 13% even after 2 weeks detraining (873).

Ibuprofen, a commercially available NSAIDs, which inhibits cyclooxygenase activities, has additional anti-inflammatory properties due to modulation of leucocyte activity by reducing cytokine production or inhibition of the NF-κB signaling pathway (738). Chronic administration of Ibuprofen has been found to prevent low grade inflammation in old rats and result in a maintained anabolic effect of food intake and a significant decrease in the aging-related loss of muscle mass (756). An aging-related increase in fibrinogen levels coupled to an increase of α2-macroglobulin (4.3 fold) and a 15% decrease of plasma albumin levels were observed in old rats, but ibuprofen alleviated these effects on fibrinogen, albumin and α2-macroglobulin levels. In addition, ibuprofen decreased plasma IL6 and IL1β levels by 60% and 46%, respectively, and increased muscle mass significantly (756). Further, ibuprofen increased food intake and muscle protein synthesis, indicating that the treatment restored protein synthesis by inhibiting the low-grade inflammation (702). On the other hand, Ibuprofen activates the PGC-1α pathway that inhibits apoptosis and oxidative damage and counteracts the activity of NF-κB (31, 148). NSAID users have been reported with a lower risk of sarcopenia compared with non-users after adjusting for potential confounders, indicating that long-term NSAID use might have a protective effect against the loss of muscle mass and function, after adjusting for potential confounders (506).

Targeting senescent cells

Cellular senescence refers to the essentially irreversible growth arrest that occurs when cells experience potentially oncogenic insults or damage, including inflammatory or metabolic stress (543, 868, 992). Senescent cells remain viable and metabolically active. Some senescent cells secrete pro-inflammatory cytokines, chemokines, proteases, and inducers of stem cell dysfunction, termed the senescence-associated secretory phenotype (SASP)(172, 173). A number of inducers, including DNA damage, oncogenic mutations, dysfunctional telomeres, fatty acids, ceramides, ROS, mitogens, and cytokines (491-493, 867) can act alone or in combination to push cells into the senescent cell fate through pathways involving $p16^{Ink4a}$/Rb (retinoblastoma), p53/p21, and possibly other pathways. These contribute to widespread changes in gene expression and chromatin remodeling which underlie senescence-associated growth arrest, the SASP, and changes in morphology. In these respects, cellular senescence is effectively a cell fate reminiscent of differentiation, replication, or apoptosis. Intracellular autocrine loops reinforce progression to irreversible replicative arrest, heterochromatin formation, and initiation of the SASP over a matter of days to weeks. In addition to removing cells from the progenitor and stem cell pool, senescence may

contribute to local and systemic tissue dysfunction and chronic disease predisposition through the SASP and associated chronic sterile inflammation and extracellular matrix degradation. By these mechanisms, senescence appears to contribute to aging-related dysfunction and multiple chronic diseases (438, 465, 543, 691, 867, 868, 992). Diseases in which senescent cells accumulate at sites of pathology include atherosclerosis, Alzheimer's and other neurodegenerative diseases, renal disease, osteoarthritis, osteoporosis, diabetes, obesity, chronic lung disease, retinal disease, and cancers, among many others (71, 146, 175, 338, 340, 341, 400, 638, 920, 973). Cellular senescence in humans is associated with physical dysfunction: abundance of senescent cells in thigh subcutaneous adipose tissue in older women with reduced grip strength, 400 meter walk time, and 4 meter gait speed is higher than in older women with less physical dysfunction (439).

Genetic targeting of senescent cells

Eliminating senescent cells enhances healthspan and blunts aging-related declines in activity, sarcopenia, fat loss, and cataracts in progeroid mice expressing a drug-inducible suicide gene, ATTAC, driven by a senescence-induced promoter, the $p16^{Ink4a}$ promoter (INK-ATTAC mice)(894). Senescent cells can be cleared from INK-ATTAC mice using AP20187, an agent that activates the apoptosis-inducing caspase-8 moiety in the ATTAC fusion protein, which is only expressed by the $p16^{Ink4a}$-positive senescent cells in these mice and that does not affect normal cells. We found that senescent cells can be cleared from not only progeroid, but also naturally-aged INK-ATTAC mice by AP20187 (973). This alleviated aging-related metabolic dysfunction, enhancing insulin sensitivity. Decreasing senescent cells in naturally-aged INK-ATTAC mice alleviates cardiovascular dysfunction and other aging-related phenotypes, as well as improving cardiovascular function in atherosclerotic, high fat-fed INK-ATTAC mice (341, 378). These studies provide strong evidence that targeting senescent cells can alleviate a range of aging- and chronic disease-related disorders.

SASP inhibitors

Several classes of drugs reduce expression of SASP components, including JAK1/2 inhibitors (*e.g.*, ruxolitinib) (973, 974), glucocorticoids (232), rapamycin (499), and metformin (179). These agents may not inhibit secretion of every SASP factor: at least in the case of rapamycin, secretion of a subset of these factors is decreased. Administration of rapamycin to middle-aged or ruxolitinib to even very old mice reduces frailty and restores activity and strength (281, 974). Furthermore, ruxolitinib alleviates aging-related stem cell and metabolic dysfunction (974). Ruxolitinib alleviated frailty-like "constitutional" symptoms in older subjects with myelofibrosis, including improved 6 minute walking time, which can depend on muscle strength, compared to placebo (500), even though the ruxolitinib did not appear to improve the underlying myeloproliferative disorder, as assessed by bone marrow clonal analysis. Thus, SASP inhibitors appear to counter some of the effects of senescent cells in mice and humans, including muscle weakness and frailty. However, actually eliminating senescent cells may prove to be a better strategy for long-term pharmacological treatment of dysfunction caused by accumulation of senescent cells.

Senolytics

Senolytic agents are drugs that specifically eliminate senescent cells (486). To discover these drugs, a hypothesis-driven approach was used (227). Senescent cells are resistant to apoptosis (304, 919),

protecting them from the pro-apoptotic microenvironment resulting from their own SASP and their active DNA damage responses and heightened metabolic flux. It was reasoned that pro-survival pathways may be active in senescent cells, presenting a target that could, if interfered with, allow senescent cells to “commit suicide”. From proteomic analyses, survival pathways were identified that could confer resistance to apoptosis to senescent cells. Indeed, targeting these pathways by RNA interference caused selective removal of senescent cells. Based on this information, drugs that inhibit these pathways were selected. These agents induced apoptosis in mouse and human senescent cells, but not non-senescent cells in culture. The first senolytic agents identified were dasatinib, an inhibitor of tyrosine kinase anti-apoptotic pathways, and quercetin, a flavonoid that inhibits AKT-, Bcl-2-, and p21/serpine-related pro-survival pathways. Subsequently, based on the senescent cell pro-survival pathways identified earlier, it was demonstrated that navitoclax, a Bcl-2 family inhibitor, is senolytic (147, 994), as were A1331852, another Bcl-2 family inhibitor, fisetin and other agents (466, 510, 993). The senolytic drugs identified so far tend to be cell-type specific, reflecting differences in the pro-survival pathways activated in senescent cells originating from different cell types (994, 995). Therefore, combinations of agents, for example dasatinib plus quercetin, can be used to target a broader range of senescent cell types than individual agents. When administered to mice, this combination reduced senescent cell burden and alleviated a range of diseases and disorders, much as when senescent cells are eliminated genetically from INK-ATTAC mice. These aging- and disease-related disorders included decreased mobility, frailty, and osteoporosis in progeroid mice, impaired mobility following radiation damage to a leg in young mice, cardiac dysfunction in old mice, and vascular hyporeactivity and calcification in high fat-fed ApoE$^{-/-}$ mice, among others (769, 995). Since muscle-related dysfuntion is prominent among the phenotypes enhanced by genetic removal of senescent cells in INK-ATTAC mice, by pharmacologically removing senescent cells with senolytics in wild-type mice, and by SASP inhibitors in wild-type mice, targeting senescent cells may be a therapeutic option for aging- and disease- related loss of muscle function, provided clinical trials show these agents also clear senescent cells, are safe, and are effective in humans. Such interventions have the advantage of potentially simultaneously alleviating the range of co-morbidities typically encountered in elderly subjects who present with muscle dysfunction,weakness, and immobility. Hopefully, clinical trials designed to test this possibility will start soon.

Evidence for an irreversible aging process

Aging is associated with a decline in physiological functions, including that of skeletal muscle, ultimately resulting in death. The origin of aging is an evolutionary enigma (201), but there are multicellular organisms, such as the hydra (718) and the jellyfish *Turritopsis dohrnii* (612), that have been reported to be immortal. Cells have an extensive machinery to repair damage, where already 150 DNA-repair genes were known in 2005 (962), giving the impression that ‘Organisms are programmed for survival, not death’ (467). In fact, there are investiogators who expect that in the future we will be able to develop interventions that stop the ageing process (604, 906, 954), or can reset the aging clock (870), thus suggesting that ageing is reversible. However, our experience till now is that we age and ultimately die and the question thus arises whether there is anything that can at least slow down the aging process, and in particular for the context of this review, muscle aging.

The aging process is often said to result in a 1% decline in muscle mass per year after the age of 50. This does, however, not consider that in comparison to the muscle mass in the previous year this decrease amounts to an exponential, rather than a linear, increase in percentage loss of muscle mass year by year **(Fig. 19**; (200)) and explains why in longitudinal studies the rate of muscle wasting or other physiological functions often exceeds 1% per year. Such observations do not apply to muscle alone, but seem to be an inherent phenomenon affecting all organ systems, as reflected by similar aging-related patterns in swimming, running and chess performance (72).

It is often stated that muscle plasticity is maintained in old age and that gains of 30% muscle mass or aerobic capacity can be achieved at any age. It should be noted, however, that the absolute increase decreases with increasing age and a hundred-year old can never attain the muscle mass (s)he had when (s)he was twenty (illustrated in **Fig. 19A**). In fact, in old humans (706, 932) and rodents (46, 48, 78) the hypertrophic response is attenuated and may even result in an impaired rather than improved force generating capacity (204). The impaired muscle plasticity is not limited to an impaired hypertrophic response, but also the muscular adaptations to chronic electrical stimulation are delayed in old rats (911). In addition, it has been found in rats that lifelong exercise started in old age resulted in a reduced, rather than extended life expectancy in rats (255) corresponding with the observation that improvements in survival in humans over the last century are negligible, or even absent, after the age of 100 (239). This also illustrates that the 'rejuvenating' effect of exercise decreases with increasing age (**Fig. 19A**). These observations suggest that there is 1) a threshold age beyond which strenuous exercise becomes detrimental, rather than beneficial and 2) that there is an inherently irreversible aging process (**Fig. 19A**; (200)).

6. AGING AND NEUROMUSCULAR DISORDERS, ADDITIVE AGING EFFECTS AND AGING AS AN ETIOLOGICAL FACTOR

Aging represents an extremely complex biological process where intrinsic changes are accumulating with time resulting in an increasing probability of death and the development of disease (999). The distinction between normal aging and muscle disease is however difficult, since if the changes in muscle structure and function observed in all humans between the age of 30 and 90 would be considered pathological if observed in a young person over a six-month period. It may accordingly be argued that aging represents a disease in itself (473), but general changes in skeletal muscle structure and function during aging will be considered a physiological phenomenon rather than a disease in this review as stated in the introduction and named *sarcopenia*. Pathological changes observed in old age have been divided into different groups (999): *i) Aging-dependent changes/diseases* affecting all individuals to varying degrees, such as osteopenia and ostheoarthritis. *ii) Aging-related diseases* with a very long latency period and becoming manifest at old age such as some neoplastic diseases, *iii) Intercurrent diseases* which are observed at all ages, but are more common in old age due to a decline in homeostatic mechanisms, such as severe pneumonia, *iv) Wear-and-tear disease and remnants of incomplete healed former disease* such as old cardiac infarction and hearing loss, and *v) Age-dependent chronic non-lethal disease* which develop at an early age and remains present at old age such as chronic skin disease. However, the distinction between these four categories is not trivial for many diseases where old age may be an etiological or contributing factor to disease or disease progression. In this section we will focus on

diseases affecting different parts of the motor unit where aging is playing an important role, *i.e.*, diseases primarily affecting the motoneurone, neuromuscular junction and muscle cell.

Motoneuron disease

Post-polio syndrome

The muscle weakness and atrophy in acute poliomyelitis are proportional to the motoneurone loss caused by viral invasion of the nervous system and anterior horn cell destruction. The initial motoneurone loss will be, at least partially, compensated for by extensive collateral sprouting if there are remaining intact motoneurones reinnervating previously denervated muscle fibers and motor unit reorganization. The "post-polio syndrome" (PPS) is characterized by recurrence of weakness, atrophy and fatigue in muscles previously affected by poliomyelitis (*e.g.,* (6, 191, 366)). This condition is only observed in patients with a history of poliomyelitis, with a complete or partial recovery of muscle function after the acute disease, and with at least a 15-year period of stable muscle function prior to the onset of symptoms that affect daily activities. PPS was described for the first time more than a century ago *(e.g.,* (140, 747)), and has received increasing scientific attention due to the increased number of patients with PPS, caused by epidemic poliomyelitis in the early 1950s (for refs. see (365)).

PPS has been suggested to be secondary to failing compensatory and adaptive mechanisms in response to chronic overuse, premature motoneuron aging, or a consequence of the motoneurone loss associated with normal aging (see (6)). Due to the extensive collateral sprouting by intact motoneurons in severely affected muscles, remaining motor units are recruited excessively during activities of daily living. This "overuse" of motor units has been suggested to be an important etiological factor underlying PPS (6). The overused motor units typically show increased territories, type grouping, type I muscle fiber predominance, muscle fiber hypertrophy, frequent fiber branching of large size fibers, and an increased number of muscle fibers with internal nuclei (see (86-88, 354, 355, 365, 531, 884), altered motoneurone firing frequencies, lower capillary densities, and increased diffusion distances, along with altered contractile and metabolic properties in the overused motor units may lead to a shortage of substrate during muscle work. This in turn could explain the muscle fatigue and pain in patients with PPS (86, 355).

More than half of the motoneurons of an extremity muscle may be lost before the patient experiences any muscle weakness (see (86)). However, in a patient with a prior polio lesion, even a relatively modest loss of motor units may have a dramatic effect on muscle function and muscle size, due to the small number of very large and overused remaining motor units. Since the onset of PPS is typically found in middle-aged or older patients with a previous polio lesion, the relatively sudden reappearance of muscle weakness may be related to the combined effect of a small number of remaining very large and overused motor units and the normal aging-related loss of motor units. Although the aging-related loss of motor units is relatively small in quantitative terms, it will have a dramatic effect on muscle function in muscles with few remaining motor units containing a large number of muscle fibers.

Amyotrophic Lateral Sclerosis (ALS)

ALS is a rapidly progressive disease with muscle wasting, spasticity and hyperreflexia resulting from the degeneration of upper and lower motoneurones. Recent studies have suggested that post-translational modifications of proteins and other cellular components play an important role in several neurodegenerative diseases. Specific interest has been focused on modifications by oxidation, nitration, and glycation of proteins and neurofilaments in the pathophysiology of ALS (for refs. see (822)). It is of specific interest to note that spinal motoneurones show higher susceptibility to the neurotoxicity induced by reducing sugars, the accumulation of reactive oxygen species (ROS) and advanced glycation endproducts (AGE) than do spinal non-motoneurons. Further, the accumulation of proteins modified by oxidative stress has been reported in the spinal cord of patients with ALS. This progressive disease, which typically has its onset in middle age, may be associated with a defective or inefficient defense system against oxidative stress (62, 822). Glutathione (GSH) levels have been reported to be reduced in specific regions in the central nervous system in patients with ALS, while other cellular enzymatic defense mechanisms remain intact. This is of specific interest since glutathione has been reported to protect against the dose-dependent neurotoxicity induced by reducing sugars and the concomitant formation of AGE.

It has been suggested that the increased sensitivity of spinal motoneurons may be related to a relatively inefficient GSH system due to a less efficient detoxification of GSH, reduction by GSH reductase, or transportation of oxidized GSH to the extracellular space in spinal motoneurons (822). The effectiveness of the natural antioxidant glutathione has been established in the prevention of AGE in collagen or lens crystallins (550) as well as in the reversal of glycation- induced impairment of myosin function (741). It has been suggested that the prevention by this AGE formation inhibitor will decrease as this compound becomes increasingly oxidized during the aging process. Thus, an aging-related decrease in the prevention of AGE formation by antioxidants, such as glutathione, may provide one aging-related factor of importance for the expected age of onset of ALS as well as of sarcopenia. As discussed above, the aging-related changes in microvasculature may also play significant role for the progressive motoneurone loss in patients with ALS.

Myopathies

Myosinopathies

The coding sequence for the adult MyHC isoforms is among the most heavily amplified in mammalian species, and the large size of the 17p13.1 locus within the MyHC gene would predict a prominent role for MyHC mutations in human myopathy (826). There is a significant need for improved molecular diagnostic tools together with structure function analyses of myosin for screening patients with myosinopathies, since a large proportion of patients evaluated for skeletal muscle weakness still fail to receive specific diagnosis. Experimental animal studies have shown distinct patterns of muscle dysfunction in IIx and IIb MyHC null mice, while heterozygous mice are clinically normal (3). Missense mutation in the β/slow MyHC gene causes a familial hypertrophic cardiomyopathy-like disease in heterozygous mice, indicating that missense mutations are more deleterious than nulls (323). In humans, central core disease is seen in type I fibers in patients with dominantly inherited mutations in the β/slow MyHC gene (276). An autosomal dominant myopathy with a missense mutation in the type IIa MyHC inform has been reported, i.e., a mutation in a highly conserved region of the motor domain in the myosin head, resulting in the replacement of a glutamic

acid with a lysine amino acid (613). This myopathy is characterized by a mild myopathy in childhood and adolescence with a progression in middle age. The mechanism underlying the late progression of this myopathy is not known. However, it is interesting to note that the missense mutation results in the substitution of one glutamic acid with a lysine. Lysines are the primary targets for posttranslational modifications of proteins by glycation (for refs. see (740)). Thus an impaired ability of the natural antioxidant glutathione discussed above may accordingly have a negative impact on the structure and function of the IIa MyHC isoform in old age and contribute to, or cause, the late onset and progression of this specific myopathy.

Acquired muscle wasting in critically ill intensive care unit (ICU) patients

Intensive care and ICUs have undergone significant development during the past 65 years due to improvements in medical technology, progress in therapeutics, and improved understanding of pathophysiology and pathogenesis. Furthermore, evidence-based medicine has resulted in significant changes in the treatment of critically ill ICU patients, moving towards fewer and less invasive interventions and more humane care (951). Today, critical care is one of the fastest growing hospital disciplines. Because of the growing need for critical care, ICUs have been predicted to occupy one third of hospital beds by 2020 (951). In parallel with lowered mortalities, neuromuscular dysfunction induced by ICU-treatment has become increasingly apparent. The most common and important types of neuromuscular dysfunction are acquired paralysis of limb muscles (critical illness myopathy, CIM) and impaired respiratory muscle function (ventilator induced diaphragmatic dysfunction, VIDD) with enormous negative consequences for patient quality of life and health care costs. The two factors that most strongly predict morbidity and mortality in the ICU are old age and muscle wasting, which are likely synergistic. Furthermore, the molecular characteristics of CIM resemble those of sarcopenia. In addition, intensive care is one of the most costly disciplines in modern health care. In particular, costs for patients suffering from CIM and VIDD are enormous. For instance, CIM is observed in ~30% of ICU patients mechanically ventilated for ≥5 days. CIM is associated with 3-fold higher ICU costs (not including post-ICU costs). Mechanical ventilation can be lifesaving and ~40% of ICU patients are mechanically ventilated for a median duration of 5-7d, but weaning from mechanical ventilation is time-consuming, comprising ~40% of the time spent on the ventilator. Additional problems in weaning are observed in 20-30% of these patients due to VIDD, resulting in prolonged intensive support with an increased risk of secondary pulmonary complications and mortality, with elderly ICU patients being highly overrepresented (997). These patients account for ~30% of overall ICU costs or a staggering $64 billion in the US annually. Currently, there is no effective treatment for CIM other than supportive care and intensive rehabilitation. Several observations suggest risk for CIM is likely related to senescent cell burden. CIM is associated with: 1) older age, 2) inflammation, which is a risk factor for CIM, with increased circulating IL-6 and other SASP factors, 3) protein aggregation, and 4) insulin resistance. Similarly, cellular senescence increases with aging, is associated with inflammation through the SASP that is partly driven by IL-6 as well as protein aggregation, and predisposes to insulin resistance, muscle weakness, and wasting. Thus, targeting senescent cells offers a promising intervention strategy in ICU patients suffering from CIM and VIDD.

7. SUMMARY AND FUTURE PERSPECTIVES

The impaired muscle function during aging is related to both quantitative and qualitative changes in muscle structure and function. The ongoing denervation and incomplete reinnervation of skeletal muscles have been described in multiple experimental and clinical studies since half a century, although the mechanisms underlying the aging-related motoneurone loss are complex, include multiple factors which are far from fully understood. However, aging-related changes in microvasculature, oxidative stress, and protein modifications with negative effects on motoneurone transport mechanisms, structure, function and survival have been forwarded as significant etiological factors. The aging-related denervation-reinnervation process has a strong impact on quantitative changes in muscle such as muscle fiber loss and atrophy, resulting in overall muscle wasting and loss of muscle function in old age. However, qualitative changes in skeletal muscle affecting protein function, repair processes, the loss in the co-ordinated expression of contractile, SR and mitochondrial proteins, and the overall decreased resilience to stress in old age most probably have an even more important role for the impaired muscle function associated with old age. Thus, the impact of aging on skeletal muscle structure and function represents an extremely complex biological process that is not easily targeted by a singular intervention strategy; there are currently a number of interventions strategies which have the potential to improve muscle function and significantly improve quality of life among the growing population of elderly citizens, such as interventions targeting the low grade inflammation, interventions improving the proliferative capacity of satellite cells, and the elimination senescent cells. All these interventions together with less sedentary life style and optimal nutrition have the potential to improve muscle function with significant positive effects on mobility, reducing the risk of fall-related accidents, improving quality of life, albeit having little effect on overall life span. In this context, drugs targeting senescent cells secreting pro-inflammatory cytokines, chemokines, proteases, and inducers of stem cell dysfunction, termed the senescence-associated secretory phenotype (SASP) represent a new and highly promising intervention strategy in experimental models and being close to the introduction in clinical trials.

8. ABBREVIATIONS

ActRIIB/IIA=activing type IIB and type IIA receptors
AGE=advanced glycation endproducts
ALK=activing receptor like kinase
ALS=amyotrophic lateral sclerosis
ATP=adenosine triphosphate
BDNF=brain-derived neurotrophic factor
BMP=bone and morphogenetic proteins
BN=brown Norway
CD=capillary density
C:F=capillary to fiber ratio
CIM=critical illness myopathy
CoQ=coenzyme Q
COPD=chronic obstructive pulmonary disease
COX2=cyclooxygenase 2
CRP=C-reactive protein

CS=corticosteroids
DHOD=di-hydroorotate dehydrogenase
DHPR=dihydropyridine receptor
EC=excitation-contraction
EDL=extensor digitorum longus
EM= electron microscopy
EMG=electromyography
ETFDH=electron-transfer flavoprotein dehydrogenase
ExICU=experimental intensive care unit
FCM=fuzzy c-means
FCSA=fiber cross-sectional area
FGFR=fibroblast growth factor receptor
FOXO3=forkhead box protein 3
GEC=general elliptical cylinder
GDF=growth and differentiation factors
GH=growth hormone
HIF-1α=hypoxia inducible factor-1
HSP=heat shock protein
ICU=intensive care unit
IGF=insulin growth factor
IL=interleukin
JAK/STAT=janus kinase/signal transducers and activators of transcription
MAPK=mitogen-activated protein kinases
mCRP=monomeric C-reactive protein
MDV=mitochondria-derived vesicles
MND=myonuclear domain
mtDNA=mitochondrial DNA
MuRF=muscle-specific RING-B-box
MUSA=muscle ubiquitin ligase of the SCF complex in atrophy (also known as Fbxo30)
MyHC=myosin heavy chain
MyLC=myosin light chain
MYOD=myogenic differentiation
NBR1=neighbor of BRCA1 gene 1 protein
nDNA=nuclear DNA
NMB=neuromuscular blockade
NMBA=neuromuscular blocking agents
NMJ=neuromuscular junction
NO=nitric oxide
NSAID=non steroidal anti-inflammatory drugs
PAX7=transcription factor paired box 7
pCRP=pentameric C-reactive protein
PGC1α= peroxisome proliferator-activated receptor γ, coactivator
PPS=postpolio syndrome
QoL=quality of life

RC=respiratory chain
ROS=reactive oxygen species
RR=ryanodine receptor
SASP=senescene-associated secretory phenotype
SIRT1=sirtuin1
SPRY1=sproutty 1
SR=sarcoplasmic reticulum
TGFβ=transforming growth factor β
TNF=tumor necrosis factor
tRNA=transfer RNA
VEGF=vascular endothelial growth factor
VIDD=ventilator induced diaphragm dysfunction
3D=three dimensional

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Table 1: Aging-related changes in muscle capillarisation

Species	Muscle	Age	C:F	CD	FCSA	Reference
Mouse (C57BL/Icrfa) ♂	Sol EDL	6 vs 28 months	↑	↑		(193)
Mouse (C57BL/6) ♂	Gastrocnemius	3 vs 24 months	=	ND	↓	(274)
Rat Wistar ♀	Plantaris	5 vs 13 vs 25 months	↓ (5=13 > 25)	↓ (5>13>25)	↓ (IIb only 5<13>25)	(215, 216)
Rat Wistar ♀	Soleus	5 vs 25 months	=	=	=	(208)
Rat F344 ♀	Gastrocnemius	6 vs 24 months	=	ND	ND	(773)
Rat Wistar ♂	Soleus Plantaris	3 vs 19 months	↑ ↑	= =	= =	(448)
Rat F344 ♂	Flexor digitorum longus	6-8 vs 26-28 months	=	=	=	(911)
Rat Long Evans ♀	Sol EDL	9 vs 27 months	=	ND	=	(112)
Rat F344FBN (sex not given)	G_w G_R Sol	8 vs 28-30 months	= = =	↑ ↓ =	↓ ↑ ↑	(389)
Rat F344FBN ♂	Sol	12 & 24 vs 35 months	=	↑	↓	(619)

	EDL			↑	↓	
Beagle	Gastrocnemius, Semitendinosus, Triceps	2-3 vs 10-13 years	↓	↓	FCSA type II ↓ FCSA type II = FCSA type II ↓	(363)
Human ♂ & ♀	Gastrocnemius	24 vs 64 years	↓	↓	FCSA type II ↓	(164)
Human ♂	Gastrocnemius	26 vs 66 years	=	=	=	(156)
Human ♂	VL	21-30 vs 51-62 years	↓	=	↓	(727)
Human ♂	VL	22 vs 62 years	↑	=	↑	(221)
Human ♂	VL	24 vs 64 years	↓	=	↓	(322)
Human ♂	VL	21 vs 65 years	↓	=	=	(784)
Human ♂	VL	24 vs 70 years	↓	↓	↓	(356)
Human ♂	VL	21 vs 73 years	↓	=	↓	(693)
Human ♂	VL (longitudinal)	65 vs 77 years	↓	ND	=	(306)
Human ♀	VL	22 vs 70 years	↓	=	FCSA type II ↓	(184)
Human ♂	VL	26 vs 72	↓	↓	FCSA type II ↓	(933)

FGURE LEGENDS

Figure 1. *Spatial organization of motor unit fibers in young animals.* Camera lucida tracings of glycogen-depleted cross-sections of fast-twitch motor unit fibres from young animals, classified according to their MyHC composition, in 21 cross-sections of tibialis anterior muscle (each section is from a separate rat). The superficial part of the muscle is facing the top of the figure. The type IIa, type IIx and IIb MyHC units are identified by filled circles, open circles, and filled triangles, respectively. The horizontal bar represents 1 mm (the graph is modified from Larsson et al. 1991 (521, 524)).

Figure 2. *Spatial organization of motor unit fibers in old animals.* Camera lucida tracings of glycogen-depleted cross-sections of fast-twitch motor unit fibres from old animals, classified according to their MyHC composition, in 16 cross-sections of tibialis anterior muscle (each section is from a separate rat). The superficial part of the muscle is facing the top of the figure. Motor units including type IIa, type IIx and IIb MyHC muscle fibers are identified by filled circles, open circles, and filled triangles, respectively. Motor units containing more than one type of muscle fiber are identified by two filled circles. The horizontal bar represents 1 mm (the graph is modified from (521, 524)).

Figure 3. *Myelinated neurones in peripheral nerves and ventral roots from young and old rats.* Toluidine semithin transverse sections of the soleus motor nerve from a young (**a,** 5 months) and an old (**b**, 24 months) rat and from the L5 ventral root from a young (**c**, 4 months) and an old (**d**, 22 months) rat. In d, note myelin ovoids (black arrows), myelin reduplication (unfilled arrow), splitting of myelin sheets (asterisks) thinly myelinated fibers and clusters of regenerating fibers. Calibration bars 1μm (a,b) and 20 μm (c,d). redrawn from(24)

Figure 4. *Repeated, vital images of a mouse sternomastoid muscle NMJ illustrate the the utility of vital imaging and the stability of the synapse.* Transgenic expression of cyan and green fluorescent proteins (CFP and GFP) labels motor axons and Schwann cells, respectively. The AChRs were labeled with a nonblocking concentration of rhodamine-conjugated α-BTX (red fluorescence). The axon enters the synaptic site from the left and arborizes above pretzel-shaped AChRs on the surface of the target muscle fiber. Several terminal Schwann cell somata (round, brighter staining objects) are present above the NMJ that extend processes to cover the nerve terminal. No discernable changes are observed in the images taken after a 30-day interval (lower row compared with upper row). Image courtesy of Hyuno Kang and adapted from (445).

Figure 5. *Abrupt morphological changes occur in a small fraction of NMJs over any given interval in aging mice.* Repeated vital examination of NMJs in the sternomastoid muscle of aging mice reveals most junctions undergo no changes. However, abrupt morphological changes occur at a small fraction of junctions between each imaging session. NMJs that show such changes are found to have lost most of the AChRs that had been labeled with α-BTX in the prior imaging session. Newly synthesized AChRs then appear that are revealed by the reapplication of α-BTX, "new AChR"). The new AChRs are, however, found in altered locations demonstrating a change in NMJ morphology. (A) Within a period of two weeks, a young-appearing junction – with contiguous AChR-rich gutters and matching terminal motor axon branches, a "pretzel" – loses its pre-existing complement of AChRs and comes to have new AChRs whose fragmented appearance becomes more apparent as the fiber grew and

the endplate expanded in size (”new AChR”). Concurrently, the presynaptic nerve terminal becomes varicose. (B) Fragmented, old appearing junctions can also undergo further remodeling – a loss of the existing AChRs, insertion of significant amounts of new receptors – and additional fragmentation of the AChR-rich area occurs in this case. Scale bar = 20μm. Images courtesy of Yue Li and adapted from (564).

Figure 6. *NMJs on the regenerated muscle fibers undergo remodeling.* Repeated vital imaging of AChRs (red), SCs (green) and nerve terminal (blue) immediately following fiber ablation of a muscle fiber (Day 0) using a laser microbeam and after 12 days of recovery. Immediately following the injury to the muscle fiber, the AChR-rich aggregate has a “pretzel”-like appearance apposed by matching terminal branches of a motor axon. Upon regeneration of the ablated muscle fiber (Day 12) and earlier (not shown), the AChR aggregate becomes fragmented and the nerve terminal branches become varicose. This altered morphology is maintained for the remainder of the animal’s life. Scale bar = 10μm. Images courtesy of Yue Li and adapted (565).

Figure 7. *“Fragmentation” of postsynaptic AChR aggregates occurs in aging and in a variety of cases of damage to sternomastoid muscle fibers.* AChRs at normal mature, mouse NMJs occur in mostly contiguous “gutters” that resemble “pretzels”. In constrast, a large proportion of postsynaptic AChR aggregates in an aged (22M) muscle display a fragmented morphology. Similar fragmentation of postsynaptic AChR aggregates is observed in muscles of dystrophic (mdx) mice (in this case 1M old), in wild-type 1M old muscle 7 days after cardiotoxin-induced damage, and in a young muscle where a single muscle fiber was ablated with a laser microbeam applied on each side of the NMJ. Scale bar = 10 μm. Images courtesy of Robert Louis Hastings (CTX) and Yue Li (laser ablation).

Figure 8. *Fragmentation of AChRs at NMJs occurs after muscle fiber damage, fiber degeneration and regeneration.* NMJs in a 6 mo. old mouse sternomastoid muscle. More central, large image is a maximum projection of a confocal stack. A “young” NMJ (left bottom) and an “old” fragmented junction (top right) are both present. The fragmented NMJ is associated with a string of nuclei located centrally within the muscle fiber (see in YZ virtual section to the right and compare with the outline of the fiber). These central nuclei are hallmarks of muscle fiber segments that have undergone damage/degeneration and have subsequently regenerated from myoblasts created by satellite cells. The myonuclei associated with the young NMJs are peripherally located (seen in XZ virtual section). The vertical and horizontal yellow dotted lines indicate the location of YZ and XZ virtual sections, respectively. Scale bar = 10μm.

Figure 9. *Myonuclear domain (MND) size in muscle fibers from mammalian species with a body mass ranging from 25g to 2500 kg* Myonuclear domain size is measured in muscle fibers expressing the type I, IIa, IIax, IIx, IIxb and IIb MyHC isoforms. The MND size in muscle fibers expressing the type (β/slow) MyHC isoform is shown in the inset. Values are means + SD (from (577)).

Figure 10. *Modelling of fiber and myonuclear domains.* I. The different steps of modelling the fiber with a parametric general elliptical cylinder (GEC). (A) The needed model parameters are the center point c, the lengths of the major, and minor axes a, and b, and the angle θ between the major axis, and the x-axis. Here they schematically overlaid on an original

image slice. (B) The result of the fuzzy c-means (FCM) clustering of the pixels, where bright pixels denote likely fiber pixels, and dark pixels denote likely background pixels. (C) A slice of the weight volume G, that is created based on the parameters extracted from the FCM thresholded image slice. (D) A slice of the GEC model of the fiber surface (red), overlaid on the original image slice. The bar length denotes 50 μm. II. (A) A GEC model of a fiber segment. The centroids of the myonuclei are shown as blue spheres merged with the surface rendering. (B) The myonuclear domains extracted using the GEC, and the nuclei centroids. The representation of each myonuclear domain at the muscle cell surface is indicated by specific colors (from (180)).

Figure 11. *Confocal microscopy images of myonuclei in individual muscle fibres.* **I.** (A) Ordered myonuclei organisation in a type IIa fibre from a young man. (B) Increased variability in myonuclei distances in a type IIa fibre from an old woman. (C) Myonuclei aggregation in a muscle fibre from an old subject (detail from B). (D) Variation in the spatial organisation of nuclei in the lower (D) and upper (F) part of a single muscle fibre expressing the type I MyHC isoform from a young man. (F) Deep groove like structures on the fibre surface harbouring myonuclei from an old subject. Rhodamine phalloidin (red) was used to stain for actin and DAPI (blue) to stain for the DNA (nuclei). All pictures were captured using a Plan-Neofluar 20x/0.5 objective. The horizontal bar denotes 100 μm. **II.** Myonuclear domain sizein type I (A) and type IIa (B) fibersfrom young men (YM), young women (YW), old men (OM) and old women (OW) (from (180))

Figure 12. *Increased variability in myonuclear organization in old age.* **I.** Confocal images at the bottom of single muscle fiber segmants from a young (A) and an old subject. Nuclei are DAPI labeled (a), the centroids of the nuclei are shown asw spheres (b), the basal lamina protein laminin is labeled in yellow (c), and double labeling of nuclei centroids and laminin (d). The horizontal bars denote 50 μm. **II.** Box plots showing the standard deviation (SD) of the nearest neighbor (NN) distances between myonuclei from individual muscle fibers expressing the type I (A) and type IIa (B) MyHC isoforms in young men (YM), young women (YW), old men (OM) and old women (OM). The boxes represent the 25^{th} and 75^{th} percentiles, and the median value is indicated in the box. The horizontal bars denote the 10^{th} and 90^{th} percentiles, and data outside this range are represented as dots (from (180)).

Figure 13. *The postulated vicious cycle driven by ROS.* The classical model of mitochondrial damage by ROS postulates that ROS leaking from the RC cause mutations in mtDNA, which in turn determine the synthesis of dysfunctional RC protein subunits, thus increasing ROS production in a vicious circle.

Figure 14. *The central role of CoQ in mitochondria homeostasis.* CoQ is essential for energy metabolism (at the level of the RC and of the Krebs cycle) lipid and pyrimidine metabolism, controls thermogenesis and apoptosis, and is a major antioxidant.

Figure 15. *Cellular and biological process controlled by mitochondrial dynamic*. Mitochondrial fusion is mediated by mitofusin1/2 (MFN1/2) and OPA1 to produce an elongated mitochondrial network. Elongated mitochondria optimize energy production, prevent apoptosis and reduce mitophagy. Mitochondrial fission is controlled by DRP1, Mff and Fis1 to generate a fragmented mitochondrial network. Mitochondrial fission promotes mitophagy to remove the dysfunctional mitochondria but also induces ROS production that cause Endoplasmic Reticulum (ER) stress and activation of Unfolded Protein Response

(UPR). UPR induces the ATF4-dependent upregulation of FGF21 that is secreted by the muscle and causes a premature senescence of epithelial tissues.

Figure 16. *The relationship between muscle capillary supply area and aerobic capacity.* Frozen sections of human vastus lateralis muscle biopsies stained with (A) lectin (Ulex europaeus) to localise capillaries and (B) succinate dehydrogenase as an index of mitochondrial activity. (C) Capillary domains represent the area of tissue closer to a given capillary (red dots) than neighbouring capillaries. (Ci, Cii) Overlap of domains with fast and slow fibres, respectively. (Ciii) Magnified region to illustrate overlap of domains (blue outlines) and slow fibres (pink outlines). I: type I fibres; II: type II fibres; arrows indicate corresponding capillary locations in each panel; asterisks identify the same fibre in each panel. Scale bars: 100 μm (from (91)).

Figure 17. *Effects of aging on fiber size and capillary supply.* **A**) Fiber cross-sectional area (FCSA) and **B**) local capillary to fiber ratio (number of capillaries supplying a fiber) in normal (blue line) and hypertrophied (red line) plantaris muscles from female Wistar rats of different ages. This picture illustrates 1) maturational increases in FCSA and LCFR between the age of 5 and 13 months, 2) comparing 5- and 25-month-old rats would have shown no age effect on FCSA and LCFR, 3) and that at all these ages there is a maintained hypertrophy and angiogenic response (215, 217).

Figure 18. *Depicted principal pathways studied in sarcopenia.* The red arrows underline which and how the different factors have been shown to change in sarcopenia.

Figure 19. *Effects of physical exercise on physiological parameters.* **A**) Illustration of the impact of aging and training on a physiological parameter (e.g. muscle mass). Note the linear aging-related decrease in the parameter at a rate of 1% per year of the value at 30 years (this is similar to the often-reported annual 1% loss of muscle mass). The vertical arrows indicate a 30% improvement in e.g. aerobic function, muscle mass or muscle strength in response to a training program, where in absolute terms the improvement that can be gained decreases with age, also causing a decreasing 'rejuvenation' with increasing age, as illustrated by the horizontal arrows. This thus shows that 1) even when the relative gains do not change with age the 2) absolute gains and 3) attainable rejuvenation decrease with age. **B**) The data in panel **A** are here expressed as a percentage decline in the physiological parameter from the value it had in the year before. This exponential pattern can be explained by a random accumulation of damage in remaining tissue (from (200)).