Final work report for grant K-115461

The aim of the current project was to investigate the role of extracellular calcium entry – store-operated (SOCE) and excitation-coupled (ECCE) – in the regulation of skeletal muscle calcium homeostasis. It has been generally accepted that excitation-contraction (EC) coupling in skeletal muscle does not require external calcium. During fatiguing stimulation or under pathological circumstances external calcium influx is thought to be essential to maintain proper sarcoplasmic reticulum (SR) calcium filling and, consequently, appropriate calcium release from the internal stores. This could be achieved by the activation of either SOCE or ECCE.

During the year of 2016, we investigated the calcium homeostasis of skeletal muscle during long term activation in two special circumstances. In the first case, the connection of endocannabinoid system and the regulation of intracellular calcium concentration of skeletal muscle was studied. It was shown previously that marijuana can cause muscle weakness, although it is unknown whether it affects the muscles directly or modulates only the motor control of the central nervous system. The presence of CB1 cannabinoid receptors (CB1R), which are responsible for the psychoactive effects of the drug in the brain, have recently been demonstrated in skeletal muscle. It is unclear at the moment how CB1R-mediated signaling affects the contraction and Ca^{2+} homeostasis of mammalian skeletal muscle. In our study, we demonstrated that *in vitro* CB1R activation increased muscle fatigability and decreased the Ca^{2+}-sensitivity of the contractile apparatus, whereas it did not alter the amplitude of single twitch contractions. In C2C12 myotubes, CB1R agonists neither evoked, nor influenced inositol IP_3-mediated Ca^{2+} transients, nor did they alter excitation–contraction coupling. Contrary, in isolated muscle fibers of wild-type mice, CB1R agonists significantly reduced the amplitude of the depolarization-evoked transients in a pertussis-toxin sensitive manner, indicating a Gi/o protein-dependent mechanism. Concurrently, on skeletal muscle fibers isolated from CB1R-knockout animals, depolarization-evoked Ca^{2+} transients, as well as Ca^{2+} release flux via ryanodine receptors (RyRs), and the total amount of released Ca^{2+} was significantly greater than that from wild-type mice.

It is worth noting that the expression of the main Ca^{2+} binding protein calsequestrin which is localized inside the Ca^{2+} store was mostly unchanged in CB1R-KO muscles. Similarly, the expression of STIM1, the Ca^{2+} sensor of the SR, and key activator molecule of the store-operated Ca^{2+} entry mechanism, and Orai1, the main store-operated Ca^{2+} channel, was also unaltered in CB1R-KO muscles. These observations suggest that CB1R-mediated signaling most probably does not interfere with Ca^{2+} storage functions of the SR. Our results show that

CB1R-mediated signaling exerts both a constitutive and an agonist-mediated inhibition on the Ca^{2+} transients via RyR, regulates the activity of the SR Ca^{2+} ATPase and enhances muscle fatigability. This might decrease exercise performance, thus playing a role in myopathies, and therefore should be considered during the development of new cannabinoid drugs. This study was presented in a research article in the Journal of Physiology in 2016 (**impact factor: 4.731**).

In the second study we provide novel insights into the effects of dietary selenium on skeletal muscle functions. As an essential trace element, selenium plays a significant role in many physiological functions of the organs. It is found within muscles as selenocystein in selenoprotein N, which is involved in redox-modulated calcium homeostasis and in protection against oxidative stress. Since RyR, SERCA, STIM1 and Orai1 activity are altered by oxidation, the antioxidant selenium could have an impact on EC-coupling and SOCE by modifying intracellular calcium homeostasis.

To explore the effects of selenium on muscle properties of mice we examined the effects of two different selenium compounds (selenate and NanoSe in 0.5 and 5 ppm concentration for two weeks) by measuring *in vivo* muscle performance, *in vitro* force in *soleus* (SOL) and *extensor digitorum longus* (EDL) muscles and changes in intracellular Ca^{2+} concentration in single fibers from *flexor digitorum brevis* (FDB) muscle. Western-blot analysis on muscle lysates of EDL and SOL were used to measure the selenoprotein N expression.

While the grip force did not change, 5 ppm selenium diet significantly increased the speed of voluntary running and the daily distance covered. Both forms of selenium increased significantly the amplitude of single twitches in EDL and SOL muscle in a concentration dependent manner. Selenate increased fatigue resistance in SOL. The amplitude of the calcium transients evoked by KCl depolarization increased significantly from 343 ± 44 nM (in control) to 671 ± 51 nM in the presence of 0.5 ppm selenate in FDB fibers. In parallel, the rate of calcium release during short depolarizations increased significantly from 28.4 ± 2.2 to 45.5 ± 3.8 and 52.1 ± 1.9 μM/ms in the presence of 0.5 ppm NanoSe and selenate, respectively. In 0.5 ppm concentration both selenium compounds increased significantly the selenoprotein N expression only in EDL muscle.

As selenium supplementation augments calcium release from the SR it thus improves skeletal muscle performance. These effects are accompanied by an increased selenoprotein N expression in the muscles which could result in increased oxidative stress tolerance in case of long lasting contraction. Future research should aim to establish the complete set of SOCE controlling molecules, to determine their redox-sensitive residues, and to understand how

intracellular Ca^{2+} stores dynamically respond to oxidative stress. Mapping the precise nature and functional consequence of key redox-sensitive components of the pre- and post-translational control of SOCE machinery and of proteins regulating SR calcium content will be pivotal in advancing our understanding of the complex cross-talk between redox and Ca^{2+} signaling. This study was presented in a research article in Nutrition and Metabolism in 2016 (**impact factor: 3.28**).

In 2017 we investigated the role of extracellular calcium entry – SOCE – in the regulation of skeletal muscle calcium homeostasis. SOCE is a Ca^{2+} entry process activated by the depletion of intracellular stores and has an important role in many cell types. In skeletal muscle, it was suggested to have a role in replenishing the SR during sustained muscle contractions, however, its role during physiological muscle activation has been controversial. Impairment of SOCE activity was proposed to be involved in several diseases associated with muscle dysfunctions, although findings on its role in aged muscle are rather controversial. These observations raised the question: is the relatively faster kinetics of SOCE in skeletal muscle enough to account for any significant contribution of SR Ca^{2+} refilling during a single contraction-relaxation cycle?

To address this question, in this study we examined the properties and physiological role of SOCE in adult skeletal muscle fibers using the *Cmpt* mouse model (naturally occurring mutation in the myostatin gene) where the improper expression of myostatin resulted in excessive muscle mass but reduced overall endurance and increased susceptibility to fatigue. Former observations pointed out the involvement of SOCE in skeletal muscle diseases, where altered muscle performance and fatigue resistance were associated with modified SOCE activity. These observations lead to the assumption that decreased SOCE could lead to decreased specific force and rapid fatigue of the *Cmpt* skeletal muscle fibers.

When examining the key proteins of SOCE by Western blot, expression of Orai1 and both splice variants of STIM1 (STIM1L and STIM1S) were found to be reduced 40 and 27%, respectively, compared to WT, concomitant with decreased SOCE activity in *Cmpt* fibers estimated based on the changes of the intracellular Ca^{2+} concentrations.

To elicit Ca^{2+}-release from the SR of FDB fibers either a Ryanodine receptor agonist (4-chloro-meta-cresol, 4-CmC) or depolarizing pulses were used. Since in muscles from *Cmpt* mice endogenous protein levels of STIM1 and Orai1 were reduced, consequently, SOCE following 4-CmC-induced store depletion was suppressed. While the voltage dependence of SR calcium release was not statistically different between WT and *Cmpt* fibers, the amount of releasable calcium was significantly reduced by 26% in the latter, indicating smaller SR Ca^{2+} content

(Figure 1). To assess the immediate role of SOCE in replenishing the SR calcium store, the evolution of intracellular calcium concentration ($[Ca^{2+}]_i$) during a train of long-lasting depolarizations to a maximally activating voltage were monitored. *Cmpt* mice exhibited a faster decline in calcium release suggesting a compromised ability to refill the SR. We created a simple model that incorporates a reduced SOCE as an important partner in regulating immediate calcium influx through the surface membrane. Decreased external calcium influx readily accounts for the steady-state reduction of $[Ca^{2+}]_i$ and the more pronounced decline following calcium release in SR calcium content.

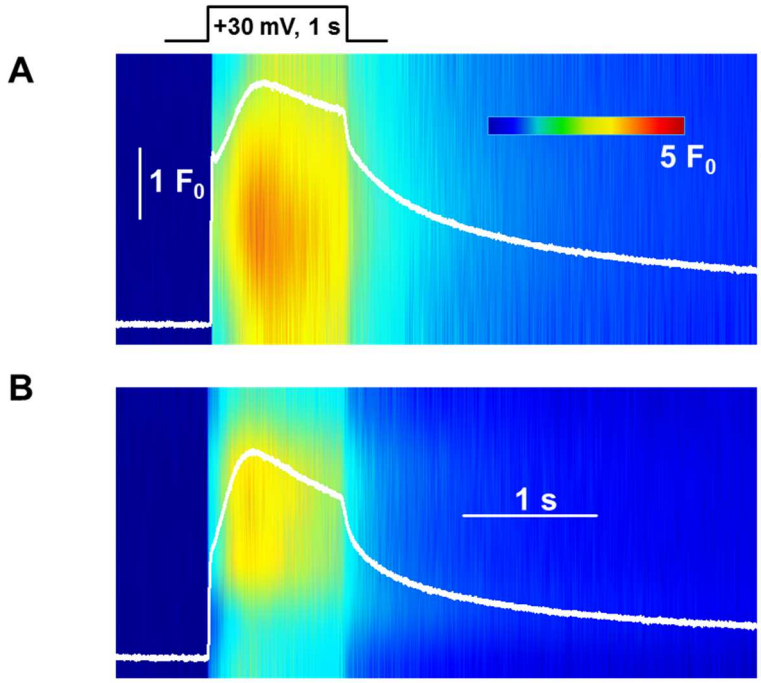

Figure 1. Suppressed Ca^{2+} transients in *Cmpt* fibers. Line scan images normalized to baseline fluorescence F0(x) in WT (A) and *Cmpt* (B) fibers subjected to a large, long-lasting (1 s) depolarization. The white curves represent the normalized fluorescence averaged over x.

Based on our observations, the most likely explanation for how the myostatin gene mutation leads to this reduction is the reduced expression of the SOCE partner proteins and the concomitant lower SOCE activity. We found SOCE having a role in maintaining and refilling SR Ca^{2+} stores not only in repetitive tetanic stimulation, as it was previously reported, but on an immediate basis in agreement with latest observations. This result was published in the Biophysical Journal (**impact factor: 3.656**).

Since muscle relaxation depends on the activity of the SR calcium pump (SERCA) we investigated its hypoxia and follistatin dependent expression. In ischemic conditions, like atherosclerosis which caused by a build-up of fatty plaques and cholesterol in the arteries, the

lumen of the vessels is obliterated resulting in restricted blood supply to tissues. This could lead to increased cytosolic Ca^{2+} level of skeletal muscle, indicating the alteration of Ca^{2+} removal mechanisms. Ca^{2+} is transported from cytosol into the SR by Ca^{2+}-ATPase (SERCA. Significantly increased (246±69%) SERCA1a expression (isoform expressed in adult muscle) was detected under ischemic conditions compared to healthy tissue. The SERCA2a expression (isoform expressed in slow skeletal muscle) did not change. In addition, in primary cultures derived from hypoxia affected tissue the diameter and fusion index of myotubes were significantly increased (30±1.6 μm vs. 41±2.4 μm and 31±4% vs. 45±3%, respectively) compared to cell cultures originated from healthy muscle samples. Based on these results we proposed that the increased SERCA1a expression indicates the existence and location of compensating mechanisms in ischemic muscle. This work was published in the journal Physiology International (**impact factor: 0.571**).

To increase the knowledge about SERCA the effects of follistatin was also studied. It is a high affinity activin-binding protein, neutralizing the effects of the Transforming Growth Factor-beta (TGF-β) superfamily members, as myostatin. Follistatin-treatment increased the fusion index, the size of terminally differentiated C2C12 myotubes, and transiently elevated the expression of the calcium-dependent protein phosphatase, calcineurin, at the beginning of differentiation. On the other hand, significantly decreased Ca^{2+}-uptake capability was determined by calculating the maximal pump rate (332±17 vs. 279±11 μM/s, in control and follistatin-treated myotubes, respectively). Similarly the expression and ATPase activity of the neonatal sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA1b) were decreased. Our results suggest that the follistatin controlled myotube growth is paralleled with the tight regulation of cytosolic calcium concentration, and the decline of SERCA1b appears to be one of the key components in this process. The results of these experiments were presented as an oral lecture at the annual Hungarian Physiological Society Meeting, in Debrecen and published in the Journal of Muscle Research and Cell Motility (**impact factor: 2.052**).

We examined the calcium homeostasis in another skeletal muscle disease model, too. Mutations in the gene encoding dynamin 2 (DNM2) are responsible for the autosomal dominant centronuclear myopathy. We studied the functional properties of Ca^{2+} signaling and excitation-contraction coupling in muscle fibers isolated from a knock-in (KI) mouse model of the disease, using microscopic (confocal imaging) and electrophysiological (voltage-clamp) techniques. The transverse-tubule network organization was unaltered in the diseased fibers but its density was reduced by ~10% as compared to that in control fibers. The density of Ca^{2+} current through

CaV1.1 channels and the rate of voltage-activated sarcoplasmic reticulum Ca^{2+} release were reduced by ~60% and 30%, respectively, in KI vs control fibers. In addition, Ca^{2+} release in the KI fibers reached its peak value 10-50 ms later than in control ones. Activation of Ca^{2+} transients along the longitudinal axis of the fibers was more heterogeneous in the KI than in the control fibers, the difference being exacerbated at intermediate membrane voltages. KI fibers exhibited spontaneous Ca^{2+} release events which were virtually absent from control fibers. Overall results demonstrate that Ca^{2+} signaling and EC coupling exhibit a number of dysfunctions that are likely to contribute to muscle weakness in DNM2-related autosomal dominant centronuclear myopathy. This work was presented as a poster at the Gordon Research Conference on Muscle EC-coupling in Switzerland and in a research paper published in the Journal of Physiology (London) (**impact factor: 4.737**).

During the year of 2018, we developed a completely new method to isolate striated muscles from the honey bee (*Apis Mellifera*). In a French collaboration we isolated enzymatically single muscle fibers from the leg of bees and measured for the first time elementary calcium release events (ECRE) and calcium waves. In fibers showing spontaneous activity, ECRE's frequency was calculated to be 2.20±0.47 kHz/mm^2. Homemade automatic image analysis program calculated the characteristic parameters of ECRE. Their average spatial spread at half maximum was 3.71±0.02 and 3.28±0.02 µm parallel with and perpendicular to the fiber axis, respectively. The mean amplitude of the events was 0.220±0.001. They looked 'wider' and their frequency is much higher than events (sparks, embers) detected previously in cardiac myocytes, batrachian and mammalian skeletal muscle fibers. This work was presented at the Biophysical Society in San Francisco (USA) and at the European Muscle Congress in Budapest (Hungary) as a poster.

Ion transport mechanisms in the plasma membrane (Na^+/H^+ exchange (NHE), Na^+/bicarbonate cotransport, and lactate/H^+ cotransport) play the key role in regulating the intracellular proton concentration in mammalian skeletal muscle. NHE system is a major mechanism for proton extrusion in skeletal muscle. We investigated in a Australian collaboration whether an NHE system is functional in the t-system of fast-twitch mammalian skeletal muscle fibers and, if so, determine its role in handling proton fluxes across the tubular membrane in the absence of confounding proton fluxes associated with the Na^+/bicarbonate cotransport and lactate/H^+ cotransport systems. For this, we used a novel approach in which we loaded dominant exogenous pH buffers within the t-system of intact fast-twitch muscle fibers before the t-system was sealed off by removing the surface membrane by microdissection, a procedure known as mechanical skinning. Using this approach, we show that the t-system of fast-twitch skeletal

fibers displays amiloride-sensitive NHE, which decreases markedly at alkaline cytosolic pH and has properties similar to that in mammalian cardiac myocytes. We observed mean values for NHE density and proton permeability coefficient of 339 pmol/m^2 of t-system membrane and 158 μm/s, respectively. We conclude that the cytosolic pH in intact resting muscle can be quantitatively explained with respect to extracellular pH by assuming that these values apply to the t-system membrane and the sarcolemma. These results were published in the Journal of General Physiology (**impact factor: 3.68**).

During the year of 2019 we continued the investigation of calcium homeostasis in aged skeletal muscle during long term activation. In aging decreased physical activity and reduced muscle mass (sarcopenia) leads to impaired muscle force and increased fatigability accompanied by a decline in sarcoplasmic reticulum (SR) calcium release. As an essential trace element selenium plays a significant role in muscle functions, as in selenium deficiency skeletal muscle disorders manifesting in muscle pain, fatigue, proximal weakness, and serum creatine kinase elevation could develop. We examined *in vivo* physical activity and i*n vitro* force of control, selenium treated and myostatin deficient (*Cmpt*) hypermuscular mice. Selenium supplementation and training significantly increased the maximal twitch and tetanic force of EDL compared to control (Figure 2).

The rate of KCl depolarization-evoked calcium release was also significantly greater in selenium supplemented (0.69±0.10 mM/s) and trained (1.12±0.07 mM/s) than in control (0.48±0.03 mM/s) animals. Western-blot analysis revealed no change in the expression of the dihydropyridine receptor and SERCA pump in aged animals while that of the ryanodine receptor declined with aging which was reversed by long-term training. Our results describe, for the first time, the positive effects of selenium supplementation on SR calcium release and muscle force in old age associated muscle weakness and also confirm the beneficial effects of training. On the other hand, the increased muscle mass of *Cmpt* mice during their lifespan doesn't improve their physical performance in old age. The results of these experiments were summarized in a manuscript which was accepted for publication in December 2019 and published electronically in February 2020 in Scientific Reports (**impact factor: 4.122**).

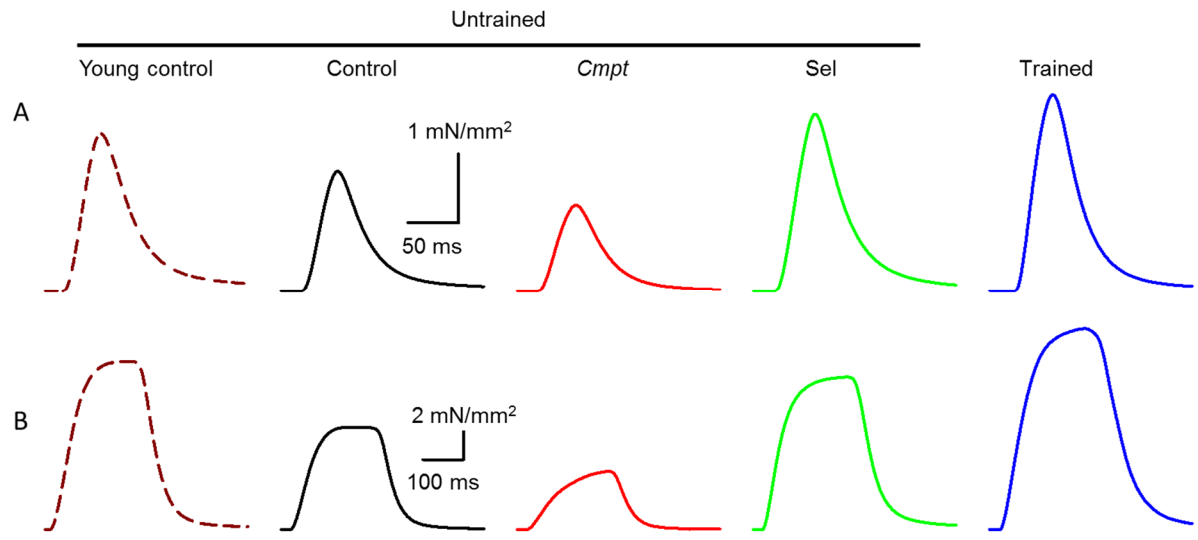

Figure 2. Isometric force in EDL muscle. Twitch (A, C) and tetanus (B, D) in EDL muscles of 4-month-old untrained control (brown, dashed line), 20-month-old untrained control (black), Cmpt (red), selenium fed (green), and trained control (blue) mouse at room temperature (24°C).

We continued the investigation of a special muscle disease, myotubular myopathy in a French collaboration. Skeletal muscle deficiency in the 3-phosphoinositide (PtdInsP) phosphatase myotubularin (MTM1) causes myotubular myopathy which is associated with severe depression of voltage-activated sarcoplasmic reticulum Ca^{2+} release. We showed that MTM1-deficient muscle fibers exhibit a partial loss of the normal control of ryanodine receptor Ca^{2+} channel activity. The diseased muscle fibers at rest exhibit spontaneous elementary Ca^{2+} release events at a frequency 30 times greater than that of control fibers. Twenty percent of the events take either the form of lower amplitude, longer duration Ca^{2+} release events or of a combination thereof. The events occur at preferred locations in the fibers, consistent with presence of areas of disrupted transverse (t-)tubule network where control of ryanodine receptor channel activity by t-tubule voltage is lost. Overall results demonstrate that opening of Ca^{2+}-activated ryanodine receptors is promoted both at rest and during EC-coupling in MTM1-deficient muscle fibers, which is likely playing an important role in the associated disease situation. This work was described in a manuscript published in the journal Cell Calcium (**impact factor: 3.718**).

We published a review article in Oxidative Medicine and Cellular Longevity (**impact factor: 4.936**) with the title “Changes in redox signaling in skeletal muscle during aging”. In collaboration with colleagues from Szeged (Hungary) we summarized our knowledge about oxidative stress and aging in skeletal muscle. The increased oxidative stress in aged muscle can lead to altered excitation-contraction coupling and calcium homeostasis. Furthermore,

apoptosis-mediated fiber loss, and atrophy of the remaining fibers, dysfunction of the satellite cells (muscle stem cells) and concomitant impaired muscle regeneration are also the consequences of increased oxidative stress, leading to a decrease in muscle mass, strength, and function of aged muscle (Figure 3). We summarize the possible effects of oxidative stress in aged muscle and the benefits of physical activity and antioxidant therapy.

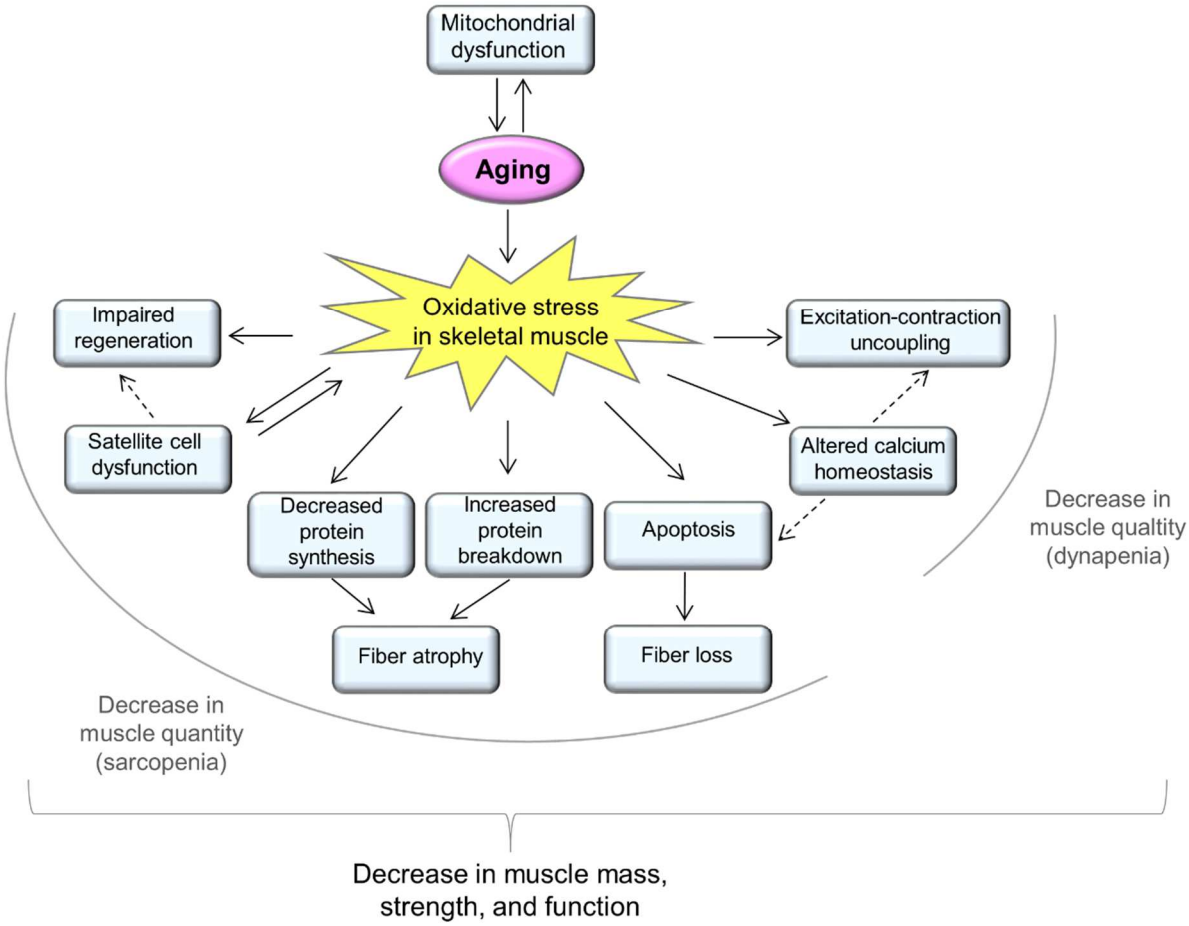

Figure 3. Schematic summary of the effects of oxidative stress in aged skeletal muscle.

We were asked to write a review article to Journal of Muscle Research and Cell Motility (**impact factor: 1.702**) with the title "Septins, a cytoskeletal protein family, with emerging role in striated muscle". We summarized our knowledge about septins, as the fourth component of the cytoskeleton. Appropriate organization of cytoskeletal components are required for normal distribution and intracellular localization of different ion channels and proteins involved in calcium homeostasis, signal transduction, and contractile function of striated muscle. Proteins of the contractile system are in direct or indirect connection with the extra-sarcomeric cytoskeleton. A number of other molecules which have essential role in regulating stretch-, voltage-, and chemical signal transduction from the surface into the cytoplasm or other

intracellular compartments are already well characterized. Sarcomere, the basic contractile unit, is comprised of a precisely organized system of thin (actin), and thick (myosin) filaments. Intermediate filaments connect the sarcomeres and other organelles (mitochondria and nucleus), and are responsible for the cellular integrity. Interacting proteins have a very diverse function in coupling of the intracellular assembly components and regulating the normal physiological function. Despite the more and more intense investigations of a new cytoskeletal protein family, the septins, only limited information is available regarding their expression and role in striated, especially in skeletal muscles. In the review we collected basic and specified knowledge regarding this protein group and emphasize the importance of this emerging field in skeletal muscle biology. The manuscript was accepted for publication in December 2019 and published electronically in January 2020.

In a collaboration with Italian researchers we investigated the lack of Ankyrin 1.5 in skeletal muscle. Small Ankyrins (sAnk1) are muscle-specific isoforms generated by the Ank1 gene that participate in the organization of the sarcoplasmic reticulum (SR) of striated muscles. Accordingly, the volume of SR tubules localized around the myofibrils is strongly reduced in skeletal muscle fibers of 4- and 10-month-old sAnk1 knockout (KO) mice, while additional structural alterations only develop with aging. To verify whether the lack of sAnk1 also alters intracellular Ca^{2+} handling, cytosolic Ca^{2+} levels were analyzed in stimulated skeletal muscle fibers from 4- and 10-month-old sAnk1 KO mice. The SR Ca^{2+} content was reduced in sAnk1 KO mice regardless of age. The amplitude of the Ca^{2+} transients induced by depolarizing pulses was decreased in myofibers of sAnk1 KO with respect to wild type (WT) fibers, while their voltage dependence was not affected. Furthermore, analysis of spontaneous Ca^{2+} release events (sparks) on saponin-permeabilized muscle fibers indicated that the frequency of sparks was significantly lower in fibers from 4-month-old KO mice compared to WT. Furthermore, both the amplitude and spatial spread of sparks were significantly smaller in muscle fibers from both 4- and 10-month-old KO mice compared to WT. These data suggest that the absence of sAnk1 results in an impairment of SR Ca^{2+} release, likely as a consequence of a decreased Ca^{2+} store due to the reduction of the SR volume in sAnk1 KO muscle fibers. These results were published in the International Journal of Molecular Sciences (**impact factor: 4.183**).

To continue the experiments on bee muscle we started to explore the effects of pesticides in mammalian muscle. Very recently, the diamide insecticide chlorantraniliprole was shown to induce Ca^{2+}-release from sarcoplasmic reticulum (SR) vesicles isolated from mammalian skeletal muscle through the activation of the SR Ca^{2+} channel ryanodine receptor. As this result

raises severe concerns about the safety of this chemical, we aimed to learn more about its action. To this end, single-channel analysis was performed, which showed that chlorantraniliprole induced high-activity bursts of channel opening that accounts for the Ca^{2+}-releasing action described before. We published these results in the General Physiology and. Biophysics (**impact factor: 1.479**).

We finished 2019 with writing a review article to Oxidative Medicine and Cellular Longevity (**impact factor: 4.936**) with the title "Astaxanthin, a potential mitochondrial targeted antioxidant treatment in diseases and with aging". Astaxanthin, a xanthophyll carotenoid, is the most abundant carotenoid in marine organisms and is one of the most powerful natural compounds with remarkable antioxidant activity. Here, we summarized its antioxidant targets, effects, and benefits in diseases and with aging. The manuscript was accepted for publication and published electronically in November 2019.

Debrecen, 6th of February, 2020.

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