Defective excitation-contraction coupling and mitochondrial respiration precede mitochondrial Ca^{2+} accumulation in spinobulbar muscular atrophy skeletal muscle

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Article

Keywords: Polyglutamine disease, Androgen receptor, Muscle atrophy, Calcium, Excitation-contraction coupling, Mitochondrial respiration

Posted Date: January 27th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1261384/v1>

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Version of Record: A version of this preprint was published at Nature Communications on February 6th, 2023. See the published version at <https://doi.org/10.1038/s41467-023-36185-w>.

Abstract

Polyglutamine (polyQ)-expanded androgen receptor (AR) causes spinobulbar muscular atrophy. Skeletal muscle is a primary site of polyQ-expanded AR toxicity, however it remains to be established what the early pathological processes are and how they unfold during disease progression. Using transgenic, knock-in SBMA mice and patient-derived muscle biopsies, we show that polyQ-expanded AR alters the generation of intrinsic muscle force prior to denervation. The pattern of expression of genes encoding key components of the excitation-contraction coupling (ECC) machinery is altered in the muscles of presymptomatic mice and patients. Altered mitochondrial respiration is another early event followed by stimulus-dependent accumulation of calcium into mitochondria during disease onset and structural organization alterations of the muscle triad. Surgical castration and AR silencing prevented early pathological processes. These observations show that androgen-dependent deregulations of ECC and defective mitochondrial respiration are early but reversible events followed by altered muscle force, contraction dynamics, and calcium dyshomeostasis.

Introduction

Spinobulbar muscular atrophy (SBMA, OMIM 313200), also known as Kennedy's disease, is a genetic neuromuscular disease caused by expansions over 37 of a microsatellite CAG trinucleotide tandem repeat encoding a polyQ tract in exon 1 of the *AR* gene¹. SBMA affects 2-5/100,000 subjects, which is probably an underestimation due to misdiagnosis and lack of disease-specific biomarkers. SBMA is a progressive late-onset disease characterized by the selective degeneration of lower motor neurons (MNs) and weakness, fasciculations, and atrophy of skeletal muscle². The disease is androgen-dependent, with full manifestations in males. AR is the main mediator of androgen signaling. Upon androgen binding, AR undergoes a conformational change that leads to nuclear translocation and activation, all necessary steps to toxicity, thus explaining the sex-dependent nature of SBMA³. The neuromuscular phenotype, sex dependence, and peripheral symptoms are well recapitulated in mouse models of disease⁴⁻⁷.

In the last decade, clinical and experimental evidence has established that in SBMA skeletal muscle atrophy is not only secondary to MN dysfunction, but it also results from the primary cell-autonomous toxicity of polyQ-expanded AR in the myofiber^{3,8}. Early muscle pathology and clear signs of myopathy are present in patients, including elevated levels of serum creatine kinase, myofiber degeneration, and areas without mitochondria similar to central cores, all detected before or at the onset of clinical symptoms⁹⁻¹². Similarly, signs of myopathy and reduced intrinsic muscle force generation precede spinal cord pathology in transgenic and knock-in SBMA mice^{7,13}. Among the primary and secondary pathological processes responsible for muscle atrophy in SBMA, there is evidence that deregulation of androgen signaling is *necessary* and *sufficient* to cause functional alterations in the motor unit. Indeed, overexpression of an AR with a normal polyQ tract size selectively in muscle elicited a phenotype that in several aspects resembles SBMA, showing that AR gain of function in muscle suffices to cause atrophy¹⁴. As a reverse of the coin, ablation of polyQ-expanded AR expression selectively in muscle prevented

disease manifestations, unequivocally showing that mutant AR expression in skeletal muscle is necessary to cause an SBMA-like phenotype in mouse ¹⁵. Two clinically relevant aspects underline the importance of skeletal muscle in SBMA. First, blood biomarkers of muscle damage correlate with the severity of the disease ¹⁶. Second, skeletal muscle is a valuable target tissue for therapy development. Indeed, pharmacological intervention to silence polyQ-expanded AR expression with antisense oligonucleotides (ASO) in peripheral tissues ¹⁷, as well as strategies to promote polyQ-expanded AR degradation while stimulating muscle hypertrophy, such as insulin-like growth factor 1 and the beta-agonist clenbuterol ^{18,19}, all ameliorated the phenotype of different SBMA mouse models. These observations support the idea that skeletal muscle is central to disease and represents a key target tissue for therapy development. Skeletal muscle accounts for 30-40% of body weight. Pathological processes that occur in skeletal muscle impact the homeostasis of the entire body, thus contributing to the progression/outcome of the disease and offering considerable therapeutic potential, which calls for a better understanding of its pathophysiological basis.

Skeletal muscle contraction is triggered by the action potential in the MN through a process known as *excitation-contraction coupling* (ECC), which links the electrical events that occur in the sarcolemma with myofiber contraction and force generation at the level of specialized structures, namely triads, which are formed by invaginations of the sarcolemma (T tubules) that face the sarcoplasmic reticulum (SR). Fast and sustained muscle contraction requires calcium (Ca^{2+}) and adenosine triphosphate (ATP). Ca^{2+} is stored in the lumen of the SR, and ATP is produced by mitochondria located close to the T tubules. Ca^{2+} links muscle contraction with ATP production. Upon arrival of the action potential at the neuromuscular junction, membrane depolarization leads to a conformational change of dihydropyridine receptors (DHPR) and ryanodine receptors (RYR) that results in Ca^{2+} release from the SR into the myoplasm. Ca^{2+} concentration in the sarcoplasm is remarkably controlled both in resting state and during myofiber contraction/relaxation by the combined action of proteins responsible for high buffer capacity, i.e. SR-resident calsequestrin 1 (CASQ1) and CASQ2, sarcoplasmic parvalbumin (PV), and SR Ca^{2+} -ATPase (SERCA) 1 and SERCA2 that pump Ca^{2+} back into the SR ²⁰. Another important player in *Ca^{2+} dynamics* is the SERCA inhibitor, sarcolipin (SLN) ²¹. Under physiological conditions, mitochondrial Ca^{2+} regulates oxidative phosphorylation and ATP production ²², and deregulation of Ca^{2+} homeostasis often results in pathological conditions ²³. We have previously shown that polyQ-expanded AR affects muscle metabolism and causes mitochondrial depolarization and mitophagy in SBMA mice and patients ^{5,9,24}, and this was rescued by ASO-mediated knockdown of polyQ-expanded AR ¹⁷.

Using constitutive and inducible transgenic SBMA mice, together with knock-in mice and human muscle biopsy samples, we provide the first evidence that mutant AR alters the pathways that represent the foundations for muscle function and structure (triad), mitochondrial respiration, Ca^{2+} dynamics, and the kinetics of muscle contraction.

Results

Disruption of muscle contraction dynamics precedes motor dysfunction in SBMA mice

Here, we investigated the pathological processes that occur in SBMA skeletal muscle as a function of disease progression. A unique feature of SBMA among other neurodegenerative diseases is that SBMA is tightly androgen-dependent. Serum androgen levels increase after birth and then drop down till puberty when they rise again. Mice reach sexual maturity between 6-8 weeks of age, and this is associated with an increase in androgen levels that peaks at 12-15 weeks of age²⁵. We recently generated and characterized a transgenic mouse model expressing human AR with an expanded polyQ tract (AR100Q)⁵. These mice recapitulate the main characteristics of the disease, including muscle atrophy and weakness in adulthood (8 weeks of age), progressive body weight loss, and motor dysfunction, followed by signs of denervation and neuromuscular junction degeneration at 12 weeks of age. Thus, we assigned a color code to distinguish three stages, *green* for 4-week-old mice – before onset of motor dysfunction, *magenta* for 8-week-old mice – disease onset, and *black* for 12-week-old mice – late stage of disease (Fig. 1a). Consistent with previous findings in SBMA patients²⁶ and mice²⁷, 8-week-old SBMA mice showed a dramatic reduction in the amplitude of the compound muscle action potential (CMAP) in the *tibialis anterior* (TA) muscle starting at 8 weeks of age (Fig. 1b, left and central panel) and, in the *gastrocnemius* muscle starting at 4 weeks of age (Fig. 1b, right panel). Since the defects in CMAP are consistent with both a neurogenic and a myogenic origin, we measured intrinsic muscle force by direct *ex vivo* electrical stimulation of the *extensor digitorum longus* (EDL), a fast-glycolytic muscle that shows signs of severe atrophy, and *soleus*, a slow-oxidative muscle that is less severely affected in SBMA mice^{5,24}. Although normal at 4 weeks of age, the production of specific maximum twitch force (P_t) and tetanic force (P_o) decreased by approximately 75% and 71% at 8 weeks of age in the EDL (Fig. 1c), but not in the *soleus* (Fig. 1d). Next, we investigated functional defects of muscle contraction dynamics. We found an increase in fiber contraction and half-relaxation time in the EDL muscle of 4- and 8-week-old AR100Q mice, indicating slower contraction kinetics (Fig. 1e), consistent with previous findings in SBMA patients²⁸ and mice^{13,29}. Importantly, slower relaxation time also caused a shift to the left of the normalized force-frequency curve (Fig. 1f), showing an anticipated fusion of stimuli and the maximum tension reached at 60 Hz for the AR100Q EDL muscle compared to 120 Hz for the WT EDL muscle at 8 weeks of age. This phenomenon was not observed in soleus. The shift in the normalized force-frequency curve was already established in the EDL at 4 weeks of age (Fig. 1f), before sexual maturity and alterations in the production of the maximum twitch force (P_t) and the tetanic force (P_o) (Fig. 1c). These findings show functional alterations of contraction dynamics in fast-twitch SBMA muscles. This aspect of SBMA muscle pathology is not due to decreased neurotransmission and occurs even before reduction of muscle force and motor dysfunction in this mouse model of SBMA⁵.

Early altered expression of genes involved in muscle contraction in SBMA mice

To elucidate the molecular processes underlying structural and functional changes responsible for muscle force reduction, we took an unbiased approach and performed a transcriptomic analysis. To avoid confounding effects due to AR overexpression, we performed microarray analysis in the quadriceps

muscle of knock-in mice expressing a humanized AR with 113 CAG repeats⁷. As control, we used mice expressing a humanized AR with 21 CAG repeats. To identify *early* gene expression changes, we performed our analysis in 12-week-old mice, which in knock-in mice corresponds to the presymptomatic stage as defined above, i.e. the age that precedes motor dysfunction^{7,24,30}. We identified 1233 differentially expressed genes (DEGs), of which 571 were up-regulated and 662 down-regulated ($p < 0.5$) (Supplementary Table 1). The analysis of gene ontology (GO) based on the 'cell component' revealed that the pathways enriched with DEGs are I band, Z disk, sarcomere, myofibrils, and contractile fibers (Fig. 2a-b, Supplementary Table 2). Similar results were obtained by analyzing a transcriptomic profile previously performed in the quadriceps of 24-week-old knock-in mice (GEO accession number GSE68441) (Supplementary Fig. 1a)^{24,31}. GO analysis based on 'Biological processes' showed early changes in metabolic pathways, including glucose and lipid metabolism, which is again consistent with previous transcriptomic analysis in older mice (Supplementary Fig. 1b)²⁴. Finally, the GO analysis based on 'Molecular functions' identified pathways, such as 'Receptor regulator activity', 'Extracellular matrix structural components', and 'Sulfur compound binding' (Supplementary Fig. 1c). These results highlight the involvement of early changes in the pattern of expression of gene involved in muscle contraction and structure in SBMA mice.

Altered expression of key ECC genes in SBMA skeletal muscle in a presymptomatic stage

In our transcriptomic analysis *Slh* was the gene most up-regulated in 24-week-old knock-in mice²⁴. By real-time PCR analysis, we found that the transcript levels of key ECC genes, including *Cacna1s*, which encodes a subunit of DHPR, *Ryr1*, *Atp2a1*, *Casq1* and *Pv*, were significantly decreased, while those of *Casq2* and *Slh* were up-regulated in the quadriceps, EDL, and *flexor digitorum brevis* (FDB) muscles of 8-week-old transgenic SBMA mice (Fig. 3a, Supplementary Fig. 2a-b). Importantly, changes in *Atp2a1*, *Casq1*, and *Pv* expression preceded (4 weeks of age) intrinsic muscle force defects (8 weeks of age). These abnormalities in gene expression were attenuated or absent in the soleus muscle (Supplementary Fig. 2c). In the quadriceps muscle, we did not detect any change in the expression of other genes that participate in the ECC process, including SH3 and cysteine-rich domain 3 (*Stac3*), Triadin, Junctin, and FK506 binding protein 12 (*Fkbp12*) (Supplementary Fig. 2d). We previously showed that postnatal (week 6) expression of mutant AR suffices to induce key manifestations of the disease, including muscle atrophy, body weight loss, and premature death⁵. By treating inducible AR100Q (iAR100Q) transgenic mice with doxycycline from weeks 6-to-8, we found that the transcript levels of specific ECC genes mirrored those observed in AR100Q mice, indicating that acute induction of polyQ-expanded AR expression in adulthood is sufficient to affect the expression of ECC genes (Fig. 3b). Importantly, we confirmed our microarray findings in knock-in SBMA mice, indicating that these gene expression changes are not the result of AR overexpression (Fig. 3c). By Western blot analysis, we verified that changes in mRNA levels correlate with changes at the protein level in transgenic mice and importantly, in the muscle of SBMA patients, highlighting the relevance of these findings to the disease (Fig. 3d). These observations revealed that in SBMA fast-twitch muscles, the expression of ECC genes is altered, and this is an early phenomenon that precedes a decrease in intrinsic muscle force generation.

Defects in myofiber respiration prior to motor dysfunction in SBMA mice

Mitochondria are located close to the triad and are required for ATP production and Ca^{2+} buffering during muscle contraction³². Skeletal muscle mitochondria are depolarized in 8-week-old AR100Q mice⁵ and 24-week-old knock-in AR113Q mice²⁴, and then degraded through mitophagy⁹. By immunofluorescence analysis of transverse sections of quadriceps of AR100Q mice, we observed areas deprived of RYR1, the major RYR isoform expressed in skeletal muscle, exclusively in fast-twitch fibers at 8 but not 4 weeks of age (Fig. 4a and Supplementary Fig. 3a), which resembles the muscle pathology of SBMA patients⁹. By Seahorse analysis, we found that the basal and maximal oxygen consumption rate (OCR) was reduced in myofibers isolated from FDB muscles of 8-week-old SBMA mice compared to WT mice (Fig. 4b). Importantly, defects in OCR were detected as early as 4 weeks of age, indicating that they precede mitochondrial membrane depolarization and RYR1 deprivation⁵. Interestingly, the basal and maximal OCR in SBMA myofibers were similar, suggesting that basal respiration is already at the maximum level in these muscles. Consistent with OCR, the activity of the respiratory chain complex I, II, and III was significantly higher in the quadriceps muscle of 8-week-old AR100Q mice compared to WT mice (Fig. 4c). Serum lactate levels were normal in AR100Q mice (Supplementary Fig. 3b), and mitochondrial pyruvate carrier 2 (*Mpc2*) and carnitine palmitoyltransferase I (*Cpt1*) transcript levels were both upregulated in the skeletal muscle of AR100Q mice compared to WT mice (Supplementary Fig. 3c). The expression of key mitochondrial genes is regulated by nuclear respiratory factor 1 (*Nrf1*) and *Nrf2*, which control the expression of several genes involved in mitochondrial biogenesis and function, such as succinate dehydrogenase [ubiquinone] flavoprotein subunit (*Sdha*) and mitochondrial transcription factor A (*Tfam*)³³. Interestingly, the transcript levels of *Nrf1* were downregulated, whereas those of *Nrf2*, *Sdha*, and *Tfam* were upregulated in the skeletal muscle of 8-week-old AR100Q mice compared to control mice (Fig. 4d).

Altered mitochondrial respiration and dysfunction are often associated with protein quality control, such as *Nfe2l2*, which induces the expression of target genes involved in the oxidative stress response, such as NADPH quinone oxidoreductase (*Nqo-1*), and genes involved in glutathione synthesis, such as glutathione-disulfide reductase (*Gsr*) and glutathione synthetase (*Gss*)³³. The transcript levels of *Nfe2l2*, *Nqo-1*, *Gsr*, and *Gss* were up-regulated in the quadriceps muscle of 8-week-old AR100Q mice (Fig. 4e). These results indicate that dysfunctional myofiber respiration is an early event (4 weeks of age), which precedes mitochondrial membrane depolarization and mitophagy (8 weeks of age)⁵. Defective myofiber respiration is followed by up-regulation of expression of gene involved in oxidative stress response, suggesting a causal link between early and late events.

Altered Ca^{2+} dynamics and activity-dependent accumulation of Ca^{2+} into SBMA mitochondria

The findings described above prompted us to analyze Ca^{2+} dynamics in SBMA myofibers. Using enzyme-dissociated FDB fibers and the ratiometric Ca^{2+} dye, Fura-2, we found that upon single-twitch stimulation the kinetics to reach the peak in cytosolic Ca^{2+} were prolonged in AR100Q mice, suggesting an altered balance between SR Ca^{2+} release and reuptake (Fig. 5a). Additionally, we observed a significant delay in

the decrease in the Fura-2 signal in AR100Q mice, suggesting slow kinetics of myoplasmic Ca^{2+} clearance (Fig. 5b-c). Interestingly, a similar phenotype was observed in mice lacking *Pv*³⁴. By monitoring cytosolic Ca^{2+} dynamics upon tetanic stimulation, we found that the increment of cytosolic Ca^{2+} that normally occurs after delivering multiple impulses at high frequency are significantly smaller in AR100Q compared to control myofibers ($\Delta R = 0.08$ vs 0.12 in WT mice, $p < 0.0001$) (Fig. 5c). Importantly, we also detected slower kinetics of decay time to return to the basal level after tetanic stimulation (67.1 ms in AR100Q vs 55.4 ms in WT mice) in AR100Q mice (Fig. 5d). Although these changes in cytosolic Ca^{2+} increments did not cause a significant alteration in the amount of cytosolic Ca^{2+} levels in the AR100Q myofiber (Fig. 5e), the impaired balance of cytosolic Ca^{2+} entry and clearance in between tetanic stimuli led to a redistribution of cytosolic Ca^{2+} levels (Fig. 5f), which is consistent with the alterations observed in the dynamics of muscle contraction (Fig. 1). Notably, the slower cytosolic Ca^{2+} entry in the initial phase (first 10 ms of Fig. 5f) led to slower contraction of the myofibers, whereas the prolonged cytosolic Ca^{2+} decay time caused a delay in the time needed for muscle relaxation. Similarly to *Pv* null mice³⁴, we did not find any change in cytosolic Ca^{2+} levels under basal conditions and in response to caffeine stimulation in 8-week-old mice (Fig. 5g). Using a mitochondrial Ca^{2+} -sensitive probe³⁵, we found a significant increase in mitochondrial Ca^{2+} accumulation upon caffeine stimulation in 8-week-old AR100Q mice (Fig. 5h), and in 16- and 24-week-old AR113Q knock-in mice (Fig. 5i). Notably, stimulus-dependent accumulation of Ca^{2+} into mitochondria also occurs in the *Pv* null mice³⁴. These findings show that SBMA mice have impaired Ca^{2+} dynamics in the different subcellular compartments during intense myofiber activity, which is associated with late activity-dependent accumulation in mitochondria.

Myofiber organization is disrupted in SBMA mice at the late stage of the disease

To establish whether the molecular and functional defects described above are associated with structural changes, we analyzed the organization of the myofiber by immunofluorescence. RYRs are positioned at the junctional SR (J-SR), which is the region of the terminal SR cisternae that faces transverse tubules (t-tubules) in triads in between longitudinal SR (L-SR) (Fig. 6a). By confocal microscopy and fluorescence intensity profile of RYR1 and CASQ, we found that in TA myofibers the regular doublet pattern of Ca^{2+} -release units was completely disrupted in 12-week-old SBMA mice (Supplementary Fig. 4). Mitochondria are in close proximity to the SR and form tight association areas between the two facing organelle membranes, known as mitochondria-associated membranes (MAM). By analysis of translocase of outer mitochondrial membrane 20 (TOM20) and RYR1, we found an altered pattern of distribution of the mitochondria in the TA bundle fibers of 12-week-old AR100Q mice compared to age-matched control mice (Fig. 6b). Similar results were obtained by analyzing the distribution of actinin, a microfilament protein that links actin filaments to the Z-lines necessary for the coordinated movement of sarcomeres (Supplementary Fig. 5). All these abnormalities were not detected at 4 weeks of age and were mild or absent at 8 weeks of age. Together, these observations reveal a progressive alteration of myofiber organization in the skeletal muscle of SBMA mice.

Suppression of androgen signaling normalizes mitochondrial respiration, myofiber structural changes, and ECC gene expression

Surgical castration in animal models and pharmacological treatment in patients to reduce serum androgen levels improve the SBMA phenotype^{4,6,7,36,37}. Therefore, we asked whether the early muscle pathological processes described above are androgen dependent and reversible. To address these questions, we performed surgical castration at 4 weeks of age and analyzed mitochondrial respiration and ECC gene expression at 8 weeks of age. Surgical castration of SBMA mice restored OCR back to normal levels (Fig. 7a) and reduced the changes in ECC gene expression observed in SBMA muscle (Fig. 7b). Amelioration of these early pathological processes was associated with an improvement in muscle pathology (Supplementary Fig. 6) and myofiber structure (Fig. 7c, Supplementary Fig. 7), suggesting a causal link between early dysfunctional processes involving the main components of the triads and structural/functional properties of striated muscles.

A gene therapy approach for GOF diseases currently under investigation consists of silencing of the disease gene. This approach has been pursued in SBMA with the use of antisense oligonucleotides (ASO) in knock-in AR113Q mice¹⁷. Treatment of knock-in SBMA mice with antisense oligonucleotides (ASO) to silence the *Ar* gene reduced changes in ECC gene expression in knock-in AR113Q mice (Fig. 7d). These observations indicate that the early pathological processes that occur in the SBMA muscle are reversible *in vivo*.

In conclusion, here we provide evidence that early changes in the expression pattern of ECC genes and mitochondrial respiration are novel early components of SBMA muscle pathology and may represent good therapeutic targets for patient treatment.

Discussion

Here, we show that deregulation of ECC gene expression and decreased mitochondrial respiration are the two pathological processes that occur early in SBMA muscle and are followed by mitochondrial Ca^{2+} overload and, later on, by altered sarcomere structure. Importantly, these pathological processes were pharmacologically reverted by therapeutic intervention known to ameliorate SBMA phenotype and were detected in skeletal muscle biopsies derived from SBMA patients, highlighting the relevance of these findings to disease.

Our microarray analysis in the skeletal muscle of early-stage (before motor dysfunction) knock-in SBMA mice revealed that the expression of genes encoding sarcomere contractile proteins and proteins involved in the ECC process is dysregulated long before muscle atrophy, weakness and motor dysfunction. Muscle expression of the ECC genes was altered not only in knock-in mice, but also in transgenic mice with constitutive and inducible expression of polyQ-expanded AR, and importantly in muscle biopsy samples derived from SBMA patients. The observation that induction of polyQ-expanded AR expression in adulthood was sufficient to trigger changes in gene expression indicates that these alterations are

unlikely to be the result of secondary pathological processes occurring during muscle development. Rather, these alterations are tightly linked to the expression of the disease protein and to the presence of androgens. In fact, both the silencing of the *AR* gene and the surgical castration restored the pattern of ECC gene expression back to normal. Another interesting aspect is that these defects were more evident in glycolytic muscles than in oxidative muscles, with some variability between different types of muscles likely due to the electrophysiological properties of each muscle type. We have previously shown that glycolytic muscles are more severely affected than oxidative muscles²⁴, and altered expression of ECC genes, a phenomenon that occurs early, may be causative or contribute to the progressive deterioration of SBMA skeletal muscle.

Alteration of the ECC machinery results in a wide range of myopathic conditions, spanning from myotonia to weakness, paralysis, and muscle wasting, and are classified as triadopathies^{38,39}. Mutations in the *CACNA1S* and *RYR* genes lead to malignant hyperthermia and specific forms of congenital myopathy, such as central core disease^{40,41}, which are characterized by muscle weakness, centrally located cores, and lack of mitochondria and oxidative enzymes with a disorganized contractile apparatus⁴². The pattern of muscle pathology in patients with SBMA resembles a central core disease, with muscle fibers that present central cores deprived of mitochondria and oxidative enzyme activity⁹. Importantly, similar muscle pathology was detected in AR100Q transgenic mice. *RYR1*-related myopathies are often associated with reduced expression or LOF mutations of *RYR1*, leading to impaired Ca^{2+} release, altered ECC process, and reduced muscle force generation. Altered expression and function of RYR and DHPR are likely related to muscle aging. Decreased levels of DHPR, biochemical alteration of RYR, and DHPR-RYR1 uncoupling correlate with loss of intrinsic muscle force in aged muscles^{43,44}. In SBMA muscles, we found decreased expression of *Cacna1s* and *Ryr1* at the onset of motor dysfunction. These gene expression changes may be due to pathological processes associated with aging. Nonetheless, they may contribute to dysfunctional dynamics of muscle contraction and relaxation and reduced force generation.

The expression of other ECC genes, i.e. *Atp2a1*, *Casq*, *Pv*, and *Sln*, was significantly altered before the onset of motor dysfunction in SBMA mice. Mutations in the *ATP2A1* gene that result in increased degradation or decreased activity of SERCA1 are associated with Brody myopathy, a disorder characterized by muscle weakness, delayed muscle relaxation, and stiffness due to impaired SR Ca^{2+} reuptake. Alteration of SERCA function has also been associated with myotonic dystrophy and hypothyroid myopathy, and decreased activity/level of this protein results in selective atrophy of fast-twitch myofibers⁴⁵. Interestingly, myotonia-like symptoms have also been reported in patients with SBMA⁴⁶. In skeletal muscle, SERCA activity is regulated by SLN. Genetic manipulation of SLN has remarkable effects on muscle. SLN overexpression reduced muscle force, slowed contraction and relaxation rate, and altered Ca^{2+} reuptake into the SR⁴⁷, while its ablation had the opposite effect⁴⁸. SLN levels must therefore be tightly regulated in muscles for proper contraction, force generation, and muscle metabolism. Indeed, the expression of the SERCA2 and SLN protein is up-regulated in several diseases, such as Duchenne muscular dystrophy (DMD)⁴⁹, dysferlinopathies, nemaline myopathy^{50,51}, and SBMA, as shown here. A gene therapy approach to decrease SLN levels has been shown to have beneficial effects

in DMD mice, thus showing that SLN is a potential therapeutic target for muscle-related diseases.⁵² Possibly, a gene therapy approach to modulate the expression of SLN and other ECC genes may be applied to SBMA and possibly other neuromuscular diseases with compromised ECC process.

Ca²⁺ homeostasis in muscle relies on additional proteins that buffer the amount of ions present in the sarcoplasm and inside the SR. These proteins are PV and CASQ1/2. PV and CASQ1/2 levels must be tightly regulated in the muscle. Several disease conditions are caused by LOF mutations of *PV* and *CASQ1/2*. Patients with *CASQ1* mutations show a late-onset exordium with slow progression, and symptoms include elevated serum CK levels, fibrofatty substitution, exercise intolerance, mild and age-dependent muscle weakness and atrophy, and the presence of vacuoles reactive to SR Ca²⁺-regulating proteins⁵³. Reduced PV expression was reported in the arrested development of righting response (*adr*) mouse, an animal model of ADR myotonia characterized by abnormally prolonged muscle contraction and half-relaxation time⁵⁴. Genetic manipulation of these genes in mice supports the idea that their expression is key to muscle function. *CASQ1* ablation altered muscle contractility and increased susceptibility to spontaneous mortality and heat/anesthesia-induced malignant hyperthermia-like episodes^{55,56}, while ablation of *PV* expression resulted in a fast-to-slow fiber-type switch⁵⁷. Several of these symptoms were found in patients with SBMA^{12,58-60}. Interestingly, the incidence of lethal hyperthermia is higher in male mice compared to female *Casq1* null mice, suggesting a pathogenic link between the ECC process, sex steroid hormones, and their receptors⁶¹.

Each cycle of skeletal muscle contraction and relaxation is based on two factors, ATP availability and Ca²⁺ transients. Skeletal muscle mitochondria are located in close proximity of Ca²⁺ release units and supply the myofibers with ATP. In physiological conditions, Ca²⁺ enters mitochondria through the voltage-dependent anion channel VDAC and reaches the mitochondrial matrix through the mitochondrial Ca²⁺ uniporter (MCU)^{62,63}. MCU is required for mitochondrial Ca²⁺ uptake under physiological conditions⁶⁴, and it controls skeletal muscle trophism and metabolism^{65,66}. Ca²⁺ is then rapidly extruded through the Na⁺/Ca²⁺ (NCLX) and H⁺/Ca²⁺ (mHCX) exchangers. An unprecedented key finding here is that SBMA muscle is characterized by altered Ca²⁺ homeostasis. SBMA myofibers showed slower release of Ca²⁺ in the cytosol after stimulation and slower kinetics of myoplasmic Ca²⁺ clearance, a necessary step for muscle relaxation. These changes may be at least in part responsible for the longer time needed for muscle contraction and the slower relaxation of the fast SBMA muscles. We detected an accumulation of mitochondrial Ca²⁺ in response to RYR1 stimulation by caffeine. An enhanced accumulation of Ca²⁺ in mitochondria may result from excessive Ca²⁺ flow through the MCU or reduced Ca²⁺ flow through the ion exchanger systems. It is possible that dysfunction of these systems causes mitochondrial Ca²⁺ overload in the SBMA muscle. In physiological conditions, mitochondrial Ca²⁺ controls oxidative phosphorylation by activating three rate-limiting enzymes of the Krebs cycle, and it controls the respiratory chain and ATP production by stimulating the function of both the ADP transporter and complex V, thus linking Ca²⁺ transients during muscle contraction to ATP production through aerobic respiration^{22,67,68}. Due to its important regulatory role, the level of Ca²⁺ matrix must be tightly regulated. The relevance of

maintenance of Ca^{2+} homeostasis is underscored by pathological conditions characterized by mitochondrial Ca^{2+} overloads, such as ischemia-reperfusion of the heart, metabolic syndrome and diabetes and excitotoxicity in NDDs. Mitochondrial Ca^{2+} overload triggers the opening of the permeability transition pore (PTP) ⁶⁹, which in turn leads to dramatic changes in mitochondrial membrane potential, mitochondrial swelling, hampered ATP production, and activation of death pathways and autophagy, all aspects previously described in SBMA muscle ^{9,24}.

Mitochondrial dysfunction is often associated with oxidative stress ³³. NFE2L2 is an oxidative stress sensitive transcription factor that belong to the cap 'n' collar family of basic leucine zipper transcription factors family of transcription factors and is responsible for regulating the expression of many antioxidant genes involved in the synthesis of glutathione. NFE2L1 is another member of the same family of transcription factors and works as a master regulator of expression of genes involved in protein quality control. Expression of Nfe2l1 and UPS target genes has been shown to be altered in the muscle of knock-in SBMA mice, thus contributing to muscle atrophy ³¹. Here we show that *Nfe2l2* and its target genes are upregulated in SBMA muscle. Importantly, *Nfe2l2* upregulation occurs early, concomitant with defects in mitochondrial respiration, while upregulation of genes involved in oxidative stress response occurs at disease onset. Interestingly, sequestosome (p62), which is upregulated and accumulates in SBMA muscle ⁵, has been shown to activate NFE2L2, suggesting crosstalk between oxidative stress response and autophagy pathways in SBMA muscle ⁷⁰. Pharmacological strategies to enhance *Nfe2l1* and *Nfe2l2* function in SBMA mice had benefits ⁷¹, suggesting that upregulation of these transcription factors is an adaptive response to accumulation of polyQ-expanded AR in the form of aggregates and inclusion bodies occurring throughout disease progression ⁵. In addition to *Nfe2l2*, we found that the expression of two transcription factors involved in the expression of mitochondrial genes were altered in SBMA muscle. *Nrf1* was downregulated, whereas *Nrf2* was upregulated, and these expression changes occurred at disease onset. We have previously shown that peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha is upregulated in SBMA muscle ^{5,24}. Interestingly, PGC-1alpha has been shown to control mitochondrial biogenesis through both NFE2L2 and NRF1/2 transcription factors ³³, suggesting crosstalk between these two signaling pathways. Possibly, early mitochondrial dysfunction elicits a protective response that on a long way becomes toxic, leading to oxidative stress, mitochondrial membrane depolarization, mitophagy and core-like pathology, as observed in mice and patients ⁹.

By combining information obtained in knock-in and transgenic mice as well as patient-derived muscle biopsies, we propose the following cascade of events (Fig. 8). Early (before onset of motor dysfunction) events involve altered skeletal muscle contraction dynamics, dysregulation of ECC gene expression and mitochondrial respiration. These early pathological processes are followed by (at the onset of motor dysfunction) altered Ca^{2+} dynamics with accumulation of the ion in mitochondria in response to stimulation of fiber contraction and reduced force production. These processes occur together with AR aggregation and inclusion body formation, up-regulation of atrogenes, metabolic changes, fiber-type

switch, and mitophagy⁵. The late phase of events is characterized by dismantled NMJs, up-regulation of denervation markers, severe muscle atrophy, cachexia, and ultimately death.

Materials And Methods

Animals

Animal care and experimental procedures were carried out according to the ethics committees of the University of Trento, the University of Padova, and the University of Michigan, and were approved by the Italian Ministry of Health. Mice were housed in filtered cages in a temperature controlled room with a 12-hour light/dark cycle with *ad libitum* access to water and food. Inducible mice (iAR100Q), transgenic mice (AR100Q), and knock-in mice (AR113Q) were previously characterized^{5,7}. Castration was performed at 1 month of age. An incision of 5 mm was made in the abdomen at the level of the hind legs. The testes were removed and the incision was closed with absorbable sutures. For ASO treatment, mice were injected from 6 weeks to 26 weeks of age once a week subcutaneously with either ASO or vehicle, as previously described¹⁷.

Human samples

Anonymized control and patient biopsy samples were obtained from the Telethon Network of Genetic Biobanks and Orthopedics and Orthopedic Oncology (University-Hospital of Padua). The tissue collection was approved by the local Ethics Committee and written informed consent was obtained from each patient. Information on the age, muscle, clinical status of controls, and Q tract length of SBMA patients is provided in Supplementary Table 3.

Biochemical analysis

Frozen tissues were pulverized using pestle and mortar on dry ice, and then homogenized in RIPA buffer [150 mM NaCl, 6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 2 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 1% Na-deoxycholate, 0.5% TritonX-100, 2% sodium dodecyl sulfate (SDS)] and protease inhibitor cocktail (Sigma). Muscle lysates were then sonicated and centrifuged at 15,000 rpm for 15 min at room temperature (RT). Protein concentration was measured using the bicyclonic acid (BCA) assay method. For Western blot, equal amounts of protein extracts from tissues or cell lysates were boiled in 5X sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol). Proteins were separated in 7.5% Tris-HCl SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for AR, 3-8% NuPAGE Tris-Acetate gels for RYR1, and 4-12% NuPAGE (Thermo Scientific) BisTris gels for the other ECC proteins. Gels were blotted overnight on 0.45 m nitrocellulose membranes (Bio-Rad, 162-0115). The following primary antibodies were used:

Calnexin (ADI-SPA-860, 1:5000), RYR1 (# MA3-925, 1:2000), CASQ (VIID12; 1:3000), SERCA1 (VE121G9, 1:50000), SERCA2 (N19; 1:1000), PV (ab11427, 1:50000), SLN⁵². Protein signals were detected using the Li-Cor Odyssey infrared imaging system or the Alliance Q9 Mini Chemidoc system (Uvitec, Cambridge) with appropriate secondary antibodies. Quantifications were performed using ImageJ 1.45 software. Mitochondrial depolarization and mitochondrial respiratory chain enzymatic activities were performed as previously described²⁴. For lactate measurement, blood was collected from the tails and lactate levels were measured using the Stat Strip Xpress measuring system following the manufacturer's instructions (Nova Biomedical). For mitochondrial complex activity, total muscle lysates were extracted and the enzymatic activities of the respiratory chain complexes I–IV were assayed, as previously described²⁴.

Immunofluorescence and microscopy

Muscles were isolated from mice of the indicated age and genotype and immediately fixed in 4% paraformaldehyde (PFA) for 15 min at RT. Skeletal muscles were further dissected into muscle bundles of about 20 myofibers each. The samples were quenched in 50 mM NH₄Cl for 30 minutes at RT and then saturated for 2 hours in blocking solution [15% vol/vol goat serum, 2% wt/vol bovine serum albumin (BSA), 0.25% wt/vol gelatin, and 0.2% wt/vol glycine in phosphate-buffered saline (PBS) containing 0.5% Triton X-100]. Incubation with primary antibodies anti-TOM20 (sc-11415, 1:200), RYR1 (MA3-925, 1:2000), CASQ (VIID12; 1:3000), AR (H280; 1:1000), and ACTININ (mouse monoclonal 1:5000, Sigma-Aldrich,) was carried out for at least 48 h in blocking solution. The muscles were then thoroughly washed and incubated with a secondary antibody conjugated with Alexa-555 or Alexa-488 diluted in blocking solution. After this incubation time, we added the 4,6 diamidino-2-phenylindole (DAPI) solution (D3571, Invitrogen) for 5 minutes. Images were collected with a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an objective of 20X HC PL APO 20X / 0.75 CS2. The laser excitation line, power intensity, and emission range were chosen according to each fluorophore in different samples to minimize bleed-through. Images were analyzed with ImageJ program using the 'plot profile' function on a determined area of the sample. For the transverse sections, tissues were flash frozen in isopentane precooled in liquid nitrogen and embedded in an optimal cutting temperature (OCT) compound (Tissue Tek, Sakura, Mestre, Italy). Cross sections (10 µm thick) were cut with a cryostat (CM1850 UV, Leica Microsystems, Wetzlar, Germany) and processed for BF-F3 (MyHC-IIb; 1:300), AR (H280, 1:200), RYR1 (MA3-925, 1:200), and WGA (1:500, W11261). The images were captured with a Leica DFC300-FX digital charge-coupled device camera using Leica DC Viewer software and morphometric analyzes were performed using ImageJ.

Quantitative real-time PCR analysis

Total RNA was extracted with TRIzol (Thermo Fisher Scientific), and RNA was reverse transcribed using iScript Reverse Transcription Supermix (1708841 Bio-Rad) following the manufacturer's instructions.

Gene expression was measured by RT-qPCR using the SsoAdvanced Universal Sybr green supermix (1725274 Bio-rad) and the C1000 Touch Thermal Cycler - CFX96 Real-Time System (Bio-Rad). Gene expression was normalized to actin. The complete list and primer sequence are provided in Supplementary Table 4.

Single-fiber electrical stimulation

Single digitorum brevis (FDB) fibers were isolated and transfected as previously described⁷². The transients of the fura-2 ratio R were recorded during 2 seconds of stimulations at 60 Hz, with a sampling rate of 1 ms, together with the excitation signals. Data analysis was automated with an ad hoc Matlab (R) script. After interpolation of the raw data using the built-in interp1 function, the following information was extracted. Basal levels were defined as the average of R in a 500 ms time window before the first impulse of the 60-Hz train. Since different cells required a slightly different number of stimuli to reach steady-state R , we defined the first peak as the maximum value within the first five impulses. The amount of Ca^{2+} released to the cytosolic space was estimated through the integral of R within the first 100 stimuli (about 1.6 seconds). For the transient decay after the last stimulus, R was fitted with a single exponential function, in a time window of 600 ms, using the built-in Curve Fitting toolbox, and the inverse of the rate of decay (the time constant) was reported in the text. Even if the noise in each trace between two stimuli was too high to give information, yet the recording of the stimuli in time in our set-up allowed for the alignment of each stimulus at its onset to obtain their ensemble average. The differences in increments of 61 of the ensemble averages of each cell showed statistical significance between the groups, and the different behaviors can be observed with the mean value of the ensemble averages for each group.

Cytosolic and mitochondrial Ca^{+2} measurements

For cytosolic Ca^{+2} measurements, the FDB muscles were digested in collagenase A (4 mg / ml) (Roche) dissolved in 1 ml of Tyrode salt solution (pH 7.4) containing 10% FBS. After 1 hour on ice and 40 minutes at 37 ° C, the single fibers were mechanically dissociated and plated on glass coverslips coated with laminin in DMEM with HEPES, supplemented with 10% FBS, 1% penicillin (100 U/mL), and 1% streptomycin (100 µg/mL). The day after, the fibers were loaded with 2 µM Fura-2/AM (F1221, Thermo Fisher Scientific) diluted in Krebs Ringer-modified buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 20 mM HEPES, 1 mM MgSO_4 , 0.4 mM KH_2PO_4 , 1 mM CaCl_2 , 5.5 mM glucose, pH 7.4) containing 0.02% pluronic acid for 20 min at 37 ° C and then washed with Krebs Ringer-modified buffer in the presence of 75 µM BTS (1576-37-0, Merk) to avoid fiber contraction. Ca^{2+} release was induced with 40 mM caffeine (C0750, Merck). Ca^{2+} signals were recorded using a Zeiss Axiovert 200 microscope equipped with a 40 × /1.3 N.A. PlanFluor objective. Excitation was performed with a DeltaRAM V high-speed monochromator (Photon Technology International) equipped with a 75 W xenon arc lamp. Images were captured with a high-

sensitivity Evolve 512 Delta EMCCD (Photometrics). Data were collected by alternatively exciting the fluorophore at 340 and 380 nm and fluorescence emission was recorded through a 515/30 nm bandpass filter (Semrock). Acquisition was performed at binning 1 with 200 EM gain. Data were analyzed by ImageJ after background extraction. The mean fluorescence was subtracted from the background and then $R_{340/400}$ was calculated. Data are presented as R or R/R_0 . For mitochondrial Ca^{+2} measurements, FDB fibers were enzymatically dissociated and plated as described above. The fibers were loaded with 2 μM mt-fura-2³⁵ and processed as described above. Ca^{2+} release was induced with 40 mM caffeine (C0750, Merck). Images were collected by alternatively exciting the fluorophore at 340 and 400 nm.

Compound motor action potential (CMAP)

Mice were anesthetized and the sciatic nerve was exposed at the sciatic notch and a small piece of parafilm was slid under the nerve, which was kept moist by a drop of PBS. Using a mechanical micromanipulator (MM33, FST, Germany) a pair of stimulating needle electrodes (Grass, USA) were then advanced until they gently touched the exposed sciatic nerve, approximately 0.5 mm below the sciatic notch. We used two needle electrodes for electromyography recording (Grass, USA): recording electrode was inserted halfway into the considered muscle (gastrocnemius or tibialis) while the indifferent electrode was inserted into the distal tendon of the muscle. CMAP was recorded during supramaximal stimulation of the sciatic nerve (15 V amplitude, 0.4 ms duration, and 0.5 Hz frequency), using a stimulator (S88, Grass, USA) through a stimulus isolation unit (SIU5, Grass, USA) in capacitive coupling mode. We amplified electromyographic signals with an extracellular GrassP6 amplifier (Grass, USA) and digitized on a PC using an A/D interface (BNC-2110, NI, USA). Data were recorded with WinEDR software (Strathclyde University, UK), and analyzed offline using pClamp10 software (Axon, USA).

Ex vivo electrical stimulation of muscles

The EDL and soleus were collected and mounted between a force transducer (KG Scientific Instruments, Heidelberg, Germany) and a shaft controlled by a micromanipulator. Muscles were immersed in a chamber in which oxygenated Krebs solution was continuously circulated at 25 ° C, and the length of the strips was adjusted until the production of maximal force. The electrical stimulation was carried out using a GRASS S44 module. The stimulation current was transmitted to the bath using platinum electrodes, the stimulation was performed at 30V and increasing pulse frequency (ranging from 1 Hz to 150 Hz). The single pulse duration was set to 1 ms, while a single train duration was set to 300 ms. The cross-sectional area was measured from optimal length and muscle weight.

Measurements of the oxygen consumption rate (OCR)

FDB muscles were digested in collagenase A (4 mg/mL) (Roche) dissolved in Tyrode's salt solution (pH 7.4) (Sigma-Aldrich) containing 10% FBS (Thermo Fisher Scientific). Single fibers were isolated, seeded in laminin-coated XF24 microplate wells and cultured in Dulbecco's Modified Eagle Medium (DMEM, D5030 Sigma-Aldrich), supplemented with 1 mM Na pyruvate, 5 mM glucose, 33 mM NaCl, 25 mM HEPES, and 1 mM L-Glutamine. The fibers were kept for 2 hours in culture at 37 ° C in 5% CO₂. The rate of oxygen consumption was evaluated in real time with the Seahorse XF24 Extracellular Flux Analyzer (Agilent). Fibers were plated as reported above. We performed a titration with the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), in order to utilize the FCCP concentration (0.6 µM) that increases OCR maximum, we loaded the fibers with 2 µM Calcein for 30 min, and we normalized the results for the fluorescence of Calcein (Sigma-Aldrich). We measured fluorescence using a PerkinElmer EnVision plate reader in well scan mode using a 480/20 nm filter for excitation and a 535/20 nm filter for emission.

Microarray hybridization and scanning, data acquisition, and analysis

The quality of the RNA was analyzed by microfluidic gel electrophoresis on a RNA 6000 Nanochip using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA). Cyanine-3 (Cy3) labeled cRNA was prepared from 0.2 µg RNA using the One-Color Low Input Quick Amp Labeling Kit (p/n 5190-2331, Agilent Technologies) according to the manufacturer's instructions (Agilent Technologies, USA), followed by RNeasy column purification (QIAGEN, Valencia, CA). Dye incorporation and cRNA yield were analyzed with the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, USA), and 1.65 mg of Cy3-labeled cRNA was fragmented at 60 ° C for 30 minutes in a reaction volume of 55 µl containing 1x fragmentation buffer and 2x GE (Gene Expression) Blocking Agent (Gene Expression Hybridization Kit, p/n 5188-5242, Agilent Technologies) following the manufacturer's instructions. At the end of the fragmentation reaction, 55 µl of 2x Hi-RPM hybridization buffer were added to the fragmentation mixture and hybridized to the Agilent Mouse GE 4x44K V2 microarray kit (G4846A, Agilent Technologies) for 17 hours at 65 ° C in a rotating hybridization oven. After hybridization, the microarrays were washed 1 minute at RT with GE Wash Buffer 1 and 1 minute at 37 ° C with GE Wash buffer 2 (Gene Expression Wash Buffer Kit, p/n 5188-5327 Agilent Technologies). Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505C, Agilent Technologies) using the AgilentHD_GX_1Color Profile (Scan Area: 61x21.6 mm; Scan resolution: 5 µm, dye channel set to 100% Green PMT) of Agilent ScanControl software 8.1.3 (Agilent Technologies). The scanned images were analyzed with Feature Extraction Software 10.7.3.1 (Agilent Technologies) using default parameters (protocol GE1_107_Sep09). Expression data was normalized using quantile normalization. DEGs were computed using eBay test from the limma R package (v3.48.0). Genes with a false discovery rate (FDR) ≤ 0.05 were considered significant. Gene set enrichment analysis (GSEA) was performed using the clusterProfiler R package (v4.0.0) to identify sets of related genes altered in each experimental group.

Statistical analysis

To compare measures across groups, the two sample t-tests of the student and the one-way analysis of variance (ANOVA) tests followed by Tukey's Honest Significant Difference post hoc tests were used for two and more than two group comparisons, respectively. For all tests, the significance threshold was set at $p < 0.05$.

Declarations

Acknowledgements

The authors thank Carlo Reggiani and Paolo Bernardi for insightful discussions. This work was supported by Fondazione Telethon (GGP19128 to M.Pe.), Association Française contre les Myopathies (22221 to M.Pe. and M.B.), PRIN-MIUR Italy (2017F2A2C5 to M.Pe.), PROGRAM RARE DISEASES CNCCS-Scarl-Pomezia (to M.Pe.), Kennedy's Disease Association Research Grant (U-GOV PIRA_EPPR19_01 to M.Pi and to E.Z.), US National Institutes of Health (R01 NS055746 to A.P.L.), and Fondazione Umberto Veronesi Fellowship (to E.Z.). S.N. is supported by a Rackham Pre-doctoral Fellowship and C.R. is supported by a Wellcome Trust Clinical Research Career Development Fellowship (205162/Z/16/Z). GJB was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, US National Institutes of Health (NIH) grant [AR069107]. The Neuromuscular Bank of Tissues and DNA samples, member of the Telethon Network of Genetic Biobanks (project no. GTB12001), funded by Telethon Italy and of the EuroBioBank network, provided us with biopsy specimens. The HTS and Validation Core Facility at CIBIO (University of Trento, Italy) performed the microarray experiment. EP and GS are members of the European Reference Network for Neuromuscular Diseases – Project ID N° 870177. The scheme (Fig. 8) was created with BioRender.com.

Authors' contribution

CaM and MPe conceived the study. CaM performed behavioral evaluation, biochemistry and molecular biology experiments, data analysis, and interpretation. GZ and MPi contributed to the study design and histological analysis. GG performed seahorse analysis and contributed to calcium experiments. RA contributed to the analysis of gene expression. AMa provided the calcium probe. MCh prepared samples for microarray analysis. NS and LAP performed experiments in knock-in mice. NL, MCa, ML, and BB performed the analysis of muscle force and Ca^{2+} dynamics. FS performed the statistical analysis. EB, AP, CB, PR provided human muscle biopsies. GJB performed WB to detect SLN. PM and CR performed the bioinformatical analysis. MB contributed to the study design and data interpretation. CaM and MPe wrote the manuscript. MPe provided main financial support for this work.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Figures

Figure 1

Prolonged muscle contraction/relaxation time precedes defects in intrinsic muscle force generation in SBMA mice.

a) Scheme of disease progression in AR100Q transgenic mice. We assigned a color code to distinguish three stages of the disease: Green: 4 weeks of age - before the onset of motor dysfunction; magenta: 8

weeks of age - the onset of motor dysfunction; black: 12 weeks of age - late stage of the disease. Phenotype was previously described ⁵.

- b)** CMAP analysis in the TA and gastrocnemius muscles of 4-week-old (green), 8-week-old (magenta), and 12-week-old (black) WT and AR100Q mice (n = 2-18 measures in muscles from 3 mice/genotype).
- c)** Generation of intrinsic force in the EDL muscle of 4-week-old (green) and 8-week-old (magenta) WT and AR100Q mice (n = 3-5 mice/genotype).
- d)** Generation of intrinsic force in the soleus muscle of 8-week-old (magenta) WT and AR100Q mice (n = 4-6 mice/genotype).
- e)** Peak contraction and half-relaxation time in the EDL muscle of 4-week-old (green) and 8-week-old (magenta) WT and AR100Q mice (n = 4-6 mice/genotype).
- f)** Intrinsic muscle force/frequency in the indicated muscles of WT and AR100Q mice (n = 3-5 mice/genotype).

Graphs show mean $\pm$ SEM, student's t-test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2

Altered expression of genes involved in sarcomere organization and muscle contraction in pre-symptomatic SBMA mice.

a-b) Transcriptomic analysis and gene ontology analysis of differentially expressed genes in the quadriceps muscle of 12-week-old (before onset of motor dysfunction) knock-in AR113Q mice compared

to control mice (n = 3 mice/genotype).

Figure 3

Altered expression of the ECC genes in the muscle of SBMA mice and patients.

a-c) RT-PCR analysis of the transcript levels of the indicated ECC genes normalized to *beta-actin* in the quadriceps muscle of 4-week-old (green) and 8-week-old (magenta) WT and AR100Q mice (A), 8-week-old iAR100Q mice treated with doxycycline (1 g/L) (B), and 24-week-old knock-in AR113Q mice (C) (n = 3-6 mice/genotype).

d) Western blot analysis of the indicated ECC genes in the quadriceps muscle of 8-week-old WT and AR100Q mice (left, n = 3 mice/genotype), and control subjects and SBMA patients (right, n = 3-6 subjects/group). ECC genes were detected with specific antibodies, and calnexin (CNX) was used as a loading control. The quantification (total/CNX) normalized to each control set as 1 is shown at the bottom of each panel. Representative images are shown.

Graphs show mean $\pm$ SEM, student's t-test; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 4

Early defects in mitochondrial respiration precede oxidative stress response in the muscle of SBMA mice.

a) Immunofluorescence analysis of RYR1 (green) and MyHC type IIb (red) in the quadriceps of WT and AR100Q mice (n = 3 mice/genotype). Shown are representative images. Bar, 40 μ m. Arrowheads point to fibers with areas deprived of RYR1.

b) Analysis of oxygen consumption rate (OCR) in FDB-isolated fibers from 4- and 8-week-old WT and AR100Q mice (n = 4 mice/genotype). Oligomycin 2 μ M, FCCP 0.6 μ M, rotenone 1 μ M, and antimycin A 1 μ M.

c) Mitochondrial complex (C) I, II, III, and IV activity normalized to mitochondrial citrate synthase activity measured in the quadriceps of 8-week-old WT and AR100Q mice (n = 3 mice/genotype).

d-e) RT-PCR analysis of the transcript levels of the indicated genes normalized to *beta-actin* in the quadriceps muscle of 4-week-old (green), 8-week-old (magenta), and 12-week-old (black) WT and AR100Q mice (n = 3-4 mice/genotype).

Graphs show mean $\pm$ SEM, student's t-test; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 5

Stimulus-induced accumulation of Ca^{2+} in mitochondria in SBMA mice.

a-d) Analysis of Ca^{2+} levels in response to twitch stimulation (a-b) and tetanic stimulation (c-d) in FDB-isolated fibers of 8-week-old WT and AR100Q mice (n = 64-67 measures in myofibers derived from 3 mice/genotype).

e-f) Analysis of Ca^{2+} levels in response to tetanic stimulation in fibers isolated from FDB of 8-week-old WT and AR100Q mice ($n = 3$ mice/genotype). The amount of Ca^{2+} accumulated in the cytosol during the tetanic stimulation (a), calculated as the integral of the FURA_2 ratio in one second, did not show significant differences between WT and AR100Q mice. The representative ensemble average of the Ca^{2+} transients between consecutive stimuli (f) showed a faster increase as well as a faster decrease in WT compared to AR100Q mice.

g-i) Resting and peak Ca^{2+} transients in response to caffeine (10 mM) in the cytosol (cyt, g) and mitochondria (mt, h-i) in FDB-isolated myofibers from AR100Q mice (g-h) and knock-in AR113Q mice (i), and relative control mice (cyt $n = 12$, mt $n = 30$ measures in myofibers derived from 3 mice/genotype).

Graphs show mean $\pm$ SEM, student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

Disrupted mitochondrial organization at the Ca^{2+} -release units in the skeletal muscle of SBMA mice.

a) Schematic representation of *triads* and *mitochondria* in skeletal *muscle* fibers.

b) Immunofluorescence analysis of RYR1 (red) and TOM20 (green) in fibers isolated from the TA muscle of WT and AR100Q mice (n = 3 mice/genotype). Shown are representative images. Bar = 25 μ m, magnification bar = 10 μ m. Graphs show fluorescence quantification (described in the Methods section).

Figure 7

Surgical castration and silencing of polyQ-expanded AR in adulthood normalizes ECC gene expression, mitochondrial respiration, and sarcomere organization.

- OCR analysis in FDB-isolated fibers of sham operated and surgically castrated (Cx) 8-week-old WT and AR100Q mice (n = 4 mice/genotype). Oligomycin 2 μ M, FCCP 0.6 μ M, rotenone 1 μ M, and antimycin A 1 μ M.
- RT-PCR analysis of the transcript levels of the indicated genes normalized to *beta-actin* in the EDL muscle of sham operated and surgically castrated (Cx) 8-week-old WT and AR100Q mice (n = 3 mice/genotype).
- Immunofluorescence analysis of RYR1 (red) and TOM20 (green) in fibers isolated from the TA muscle of sham operated and surgically castrated 8-week-old WT and AR100Q mice (n = 3 mice/genotype). Shown are representative images. Bar = 25 μ m, magnification bar = 10 μ m. The graphs show fluorescence quantification (described in the Methods section).
- RT-PCR analysis of the transcript levels of the indicated genes normalized to *beta-actin* in the quadriceps muscle of 26-week-old AR113Q mice treated with either vehicle or AR-targeting ASOs (n = 3-5 mice/genotype).

Graphs show mean $\pm$ SEM, ANOVA followed by Tukey's post hoc tests; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 8

Schematic representaion of physiological condition and SBMA.

Scheme of the major events occurring in SBMA muscle as a function of disease progression.

Supplementary Files

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