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Muscle spindles exhibit core lesions and extensive degeneration of intrafusal fibers in the *Ryr1*$^{I4895T/wt}$ mouse model of core myopathy☆

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ABSTRACT

Muscle spindles from the hind limb muscles of adult *Ryr1*$^{I4895T/wt}$ (IT/+) mice exhibit severe structural abnormalities. Up to 85% of the spindles are separated from skeletal muscle fascicles by a thick layer of connective tissue. Many intrafusal fibers exhibit degeneration, with Z-line streaming, compaction and collapse of myofibrillar bundles, mitochondrial clumping, nuclear shrinkage and pyknosis. The lesions resemble cores observed in the extrafusal myofibers of this animal model and of core myopathy patients. Spindle abnormalities precede those in extrafusal fibers, indicating that they are a primary pathological feature in this murine *Ryr1*-related core myopathy. Muscle spindle involvement, if confirmed for human core myopathy patients, would provide an explanation for an array of devastating clinical features characteristic of these diseases and provide novel insights into the pathology of *RYR1*-related myopathies.

1. Introduction

Core myopathies that include central core disease (CCD), multiminicore disease (MMD) and nemaline rod myopathy (NM) typically present with muscle weakness, in association with a spectrum of other clinical features, such as congenital hypotonia, weak tendon reflexes, joint hypermobility, floppiness, delayed motor milestones and skeletal deformities [1–3].

Core myopathies are most commonly caused by mutations in the *RYR1* gene, which encodes the skeletal muscle isoform of the sarcoplasmic reticulum Ca^{2+}-release channel, a key protein in skeletal muscle excitation-contraction (EC) coupling [4]. Recent mechanistic models have largely explained how mutations in *RYR1* may lead to skeletal muscle weakness and produce characteristic lesions within myofibers [4–6]. However, the etiology of disease-associated hypotonia, delayed motor development and a spectrum of disease-related abnormalities related to stretching and balance have not been explained.

Muscle spindles are complex motor-sensory organs that are responsible for the maintenance of skeletal muscle tone and stretch reflexes. Their involvement in human muscle disease pathology has been suggested ever since their discovery in the late 19th century [7,8], but has never been definitely confirmed or refuted, largely because these tiny organs are rarely observed in routine examination of human muscle biopsies [9–11]. Muscle spindles are located deep within the belly of skeletal muscles and are formed by specialized intrafusal muscle fibers that are highly innervated by both motor and sensory neurons [12,13]. Intrafusal fibers in muscle spindles monitor changes in the extent and rate of stretching of surrounding skeletal muscle fascicles and relay this information to the central nervous system, which responds with immediate corrective adjustments to the tonicity of both agonistic and antagonistic muscle groups. Finely tuned performance of these organs is essential for posture maintenance and coordinated movement.

Each muscle spindle is formed of but a few intrafusal fibers that, in their extended polar regions, retain the structural organization and contractile activity of extrafusal fibers. However, their central equatorial regions are virtually devoid of myofibrils and contain

Abbreviations: CCD, central core disease; MmD, multiminicore disease; NM, nemaline rod myopathy; RyR1, ryanodine receptor type 1; TB, toluidine blue; WT, wild-type.

☆ This paper is dedicated to Dr. Ernesto Carafoli on the occasion of his retirement as Editor of *Biochemical and Biophysical Research Communications*. We trust that he will appreciate a novel contribution to his beloved journal as much as he will appreciate the praise that he so richly deserves for his own sterling research contributions, his exemplary training of so many students and fellows (including E.Z.) and for his unflagging efforts to advance the Ca^{2+} field through his organization of numerous, excellent meetings and his dedication to his many editorial duties over the past half-century.

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numerous nuclei that are either aggregated (in nuclear bag fibers) or form a beaded row (in nuclear chain fibers). Both nuclear bag and nuclear chain fibers are indispensable for the proper functioning of the muscle spindle as a motor-sensory organ [12,13].

The IT/+ mouse model generated in our laboratory [14] carries a knock-in of an Ile4895 to Thr mutation in the mouse *Ryr1* gene, corresponding to an Ile4898 to Thr mutation in the human *RYR1* gene. This mutation has been linked to severe cases of CCD and, in some cases, to transient presentation as MmD and NM. We have shown that our mouse model faithfully reproduces the disease phenotype of human core myopathies by exhibiting skeletal muscle weakness and progressive formation of characteristic lesions within myofibers [4,5]. The lesions present initially as focal myofibrillar hypercontractions (mini-cores), but later form extended areas of myofibrillar compaction and mitochondrial depletion (cores) and, ultimately, show complete myofibrillar disorganization and accumulation of electron dense remnants of Z-line material (nemaline bodies or rods). Assuming that the IT/+ mouse line might also be an excellent experimental model in which to study the structure and function of muscle spindles in the context of *RYR1*-related core myopathies, we investigated whether similar disorganization of the contractile apparatus might occur in the intrafusal fibers of muscle spindles in this mouse model.

2. Materials & methods

Experimental protocols for animal research conformed to University of Toronto guidelines and were approved by the Animal Care and Use Committee of the University of Toronto.

The generation and genotyping of $Ryr1^{I4895T/wt}$ (IT/+) mice was described previously [14]. For maintenance, IT/+ mice generated on Sv129 background, were crossed with WT 129S2/SvPasCrl mice (Charles River, Canada) for more than 20 generations. The IT/+ mouse line can be purchased from the Jackson Laboratories, USA: stock number 025199, strain 129S-Ryr1tm1.1Dhm/J [URL: http://jaxmice.jax.org/strain/025199.html].

2.1. Tissue harvesting

Six to ten month-old heterozygous IT/+ and control WT male mice (n = 6 in each group) were euthanized by cervical dislocation. The choice of the age group investigated was based on our earlier observations that mild morphological abnormalities can be discerned in IT/+ mice as young as 6 weeks and that these abnormalities progress to core lesions by 10–12 months, when hind limb paresis becomes obvious [5].

Specimens from four hind limb muscles: vastus lateralis, gastrocnemius, tibialis anterior, and soleus were dissected within 10 min post-mortem and processed for histological and electron microscopic analyses.

2.2. Histological analysis

For hematoxylin and eosin (HE) stained sections, dissected muscles were fixed in buffered formalin for 24 h, embedded in paraffin and 5 μm thick sections were made, starting at the mid-belly of each muscle. To increase the chance of muscle spindle detection, all muscles were analyzed in 6–12 serial sections. The sections were stained following a standard procedure.

For toluidine blue (TB)-stained semi-thin sections, dissected muscle samples were fixed as described in Ref. [5]. Four to six blocks of tissue samples were dissected from each muscle and embedded in a Quetol-Spurr resin mixture. Semi-thin longitudinal and transverse sections (0.6 μm thick) were prepared and stained with toluidine blue, as described in Ref. [5]. Images of TB-stained sections were captured using a Zeiss AxioImager Z1 microscope with a Hamamatsu Flash 4.0 camera, 100x/1.3NA Plan-Neofluar objective lens and Metamorph version 7.7 software.

2.3. Electron microscopy

Samples for transmission electron microscopy (TEM) were fixed and stained as described in Ref. [5]. They were viewed on a FEI Tecnai 20 microscope. The images were captured with an AMT 16000-S camera.

2.4. Morphometric analyses

The number of spindles per section and the number of intrafusal muscle fibers within each spindle were counted manually. Each spindle was examined in 6–14 consecutive sections. Size and distance measurements were performed manually [15] using Zeiss AXIOVISION software. Fibers of a larger diameter and with aggregated nuclei were classified as nuclear bag fibers, whereas smaller fibers with aligned nuclei were classified as nuclear chain fibers. No attempt was made to estimate changes in innervation of muscle spindles in this study.

3. Results

Muscle spindles in IT/+ and WT hind limb skeletal muscles were analyzed initially in 5 μm HE-stained sections (Fig. 1A). Gross morphology, location, and frequency of spindles in IT/+ samples were similar to those in WT samples. As in WT control samples (not shown), spindles in IT/+ muscles were scarce and were located deep within the muscle tissue. Consistent with earlier reports, the postural soleus and its antagonistic tibialis anterior muscle contained a higher number of spindles: three to four spindles could be found in each transverse section of these muscles whereas, in gastrocnemius and vastus lateralis, spindles were found only occasionally, at an average frequency of less than one per microscopic section. The number of intrafusal fibers in spindles from both WT and IT/+ samples ranged between 2 and 6, with 2 typically classified as nuclear bag fibers and up to 4 classified as nuclear chain fibers.

The only morphological abnormality detected in IT/+ spindles in HE sections was a widening of the gap between the spindles and the skeletal myofibers (Fig. 1A), whereas, in WT muscles (Fig. 1B and C), spindles were invariably located in such close contact with extrafusal myofibers that extrafusal and intrafusal fibers would be subjected to the same stretch conditions, a pre-requisite for the spindle to function as a stretch receptor.

A detailed analysis of IT/+ spindle morphology was performed in semi-thin 0.6 μm toluidine-blue (TB) stained sections (Fig. 2). As soleus and tibialis anterior yielded the highest numbers of spindles per muscle section, the analysis was preferentially performed on these two muscles. Consistent with our findings in HE stained sections, a large number of IT/+ spindles were separated from extrafusal fibers, the spaces being filled with increased layers of connective tissue. The frequency of “detached” spindles in 6 month-old mice was as high as 40% and it increased to 70–85% of all spindles in 10 month-old mice. The width of the gaps that separated spindles from extrafusal myofibers reached up to 15 μm in 6 month-old mice and up to 20 μm in 10 month-old IT/+ mice (not shown). Widening of the gaps was most likely due to increased endomysial and perimysial fibrosis, which is common in myopathies and neuromuscular diseases [15]. Widening of the gap between spindles and extrafusal myofibers was also observed in WT muscle samples from 14 months and older mice (not shown) and, in this case, was assumed to be a part of normal muscle aging.

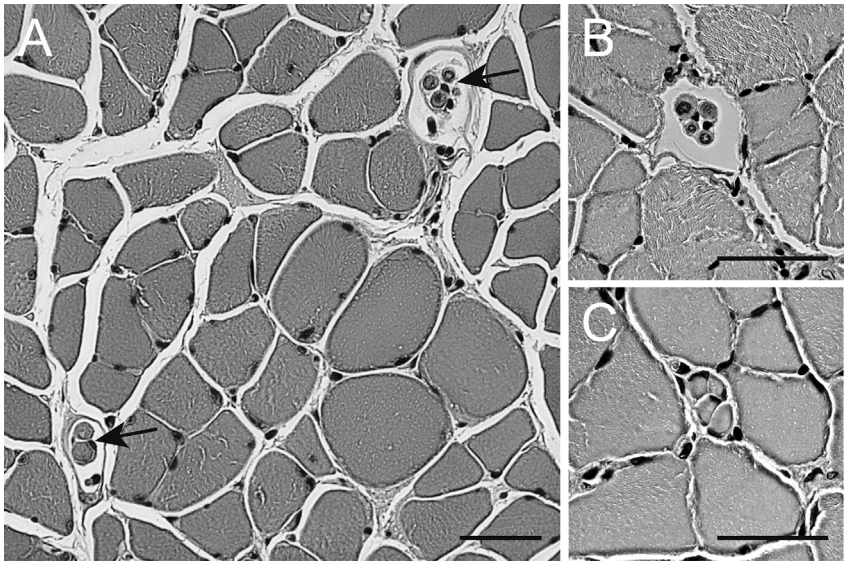

Fig. 1. Muscle spindles from 6 month-old IT/+ mice lose close association with extrafusal myofibers. A, a representative image of a transverse section from IT/+ soleus muscle showing two muscle spindles (arrows) surrounded by thickened layers of endomysial connective tissue. B and C, images of muscle spindles from WT soleus muscle. The images were enlarged to show the tight association of the spindles with extrafusal myofibers. HE staining. Bar, 20 μm.

The most striking feature of spindles observed in TB stained sections of IT/+ muscle was the overt degeneration of intrafusal fibers that manifested as a high incidence of focal myofibrillar shortening and hypercontraction (Fig. 2B–D). Features of fiber degeneration included dilation of the sarcoplasmic reticulum (Fig. 2B, D and F), recession of myofibrils and the appearance of myofibril-free subsarcolemmal areas (Fig. 2E). Nuclear abnormalities, including clumping of nuclear material and nuclear fragmentation, were commonly observed (Fig. 2D). The overwhelming majority of IT/+ spindles displayed focal hypercontractions of myofibrils in at least one intrafusal fiber. In some intrafusal fibers, there was complete disorganization and obvious collapse of the contractile apparatus to the extent that cross-striations could no longer be discerned (Fig. 2C–E). Degeneration involved both nuclear chain and nuclear bag fibers. Similar structural abnormalities were detected in spindles from IT/+ vastus lateralis and gastrocnemius muscles (not shown).

Table 1 provides a quantitative analysis of spindles in 4 hind limb muscles of IT/+ and WT 6 month-old mice. The highest concentration of spindles was found in postural anti-gravity soleus, followed closely by its antagonist, tibialis anterior. The number of spindles appeared to be increased in IT/+ mice compared to WT, but overall quantification of spindles in murine hind limb muscles was beyond the scope of this study. The structural organization of each spindle was analyzed in 6–14 serial sections. The percentage of spindles with abnormalities reached 89% for soleus and 73% for tibialis anterior. WT spindles did not show overt abnormalities in any of the muscles studied, except for the occasional slight shortening of the striation register in intrafusal fibers. Such changes were observed randomly in both WT and IT/+ fibers and were regarded as insignificant.

Ultrastructural abnormalities within IT/+ intrafusal fibers were also investigated by electron microscopy (Fig. 3). The intrafusal fibers showed varying degrees of sarcomeric shortening and misalignment. Both nuclear bag and nuclear chain fibers exhibited lateral compaction and focal hypercontraction of myofibrils, accompanied by massive displacement and accumulation of mitochondria, either in subsarcolemmal regions or among the disorganized and disintegrating myofibrils (Fig. 3B–D). Abnormal mitochondrial clumps within the intrafusal fibers often occupied substantial areas up to 5 μm in apparent diameter. Multilamellar autophagosomes (Fig. 3D) were often present in myofibril-free areas, suggesting that autophagy may play a role in IT/+ intrafusal fiber degeneration.

4. Discussion

In this study, we provide strong experimental evidence that muscle spindles undergo severe degeneration in our IT/+ mouse model of *RYR1*-related core myopathy.

The idea that muscle spindles must be involved in a neuromuscular disease phenotype has been voiced repeatedly [7,8] since the discovery of these organs almost 150 years ago, but has never been demonstrated. The assertion was based on specific and highly recurrent clinical features that often accompany neuromuscular diseases, and are especially persistent in congenital core myopathies, namely hypotonia, lack of tendon reflexes, poor coordination of movement and floppiness. However, the search for structural abnormalities in muscle spindles in various human neuromuscular diseases was unsuccessful [9–11,16–18]. Thus the idea has been generally accepted that muscle spindles are resistant to degeneration [10,19], perhaps because of different gene expression programs in spindles and in muscle fascicles. Nevertheless, it has also been widely acknowledged that the chances of finding these scarce and tiny organs within routine human muscle biopsy specimens are very low [9–11] and, consequently, the idea that muscle spindles were resistant to degeneration was based on the analysis of a very limited number of muscle spindles that were often harvested from unaffected muscles of patients.

The availability of our mouse model of an *RYR1*-related core myopathy allowed us to analyze the structure of spindles directly in disease-affected soleus and tibialis anterior muscles [5]. In so doing, we have revealed prominent abnormalities in muscle spindle organization, which include physical separation of spindles from muscle fascicles due to disease-associated fibrosis and structural disorganization and collapse of the contractile apparatus within the intrafusal fibers. Clearly, the same *Ryr1* gene defect had the same degenerative effects on the intrafusal fibers of the muscle spindles

Table 1
Cumulative data on muscle spindle detection and analysis in hind limb muscles of 6 month-old IT/+ and WT mice. n denotes the number of WT and IT/+ mice taken for each muscle group analysis.

Muscle group	Number of Spindles, IT (WT)	Number of Sections, IT (WT)	Number of Serial Sections per spindle	IT Spindles with abnormalities, %
Tibialis anterior, n = 6	11 (7)	102 (82)	12	73
Gastrocnemius, n = 4	4 (2)	58 (40)	6	50
Vastus lateralis, n = 4	3 (1)	27 (24)	6	66
Soleus, n = 6	45 (10)	171 (90)	14	89

as it had on the extrafusal fibers of the bulk of the muscle, as reported earlier [5].

Separation of muscle spindles from extrafusal fibers due to increased endomysial and perimysial fibrosis was observed in IT/+ mice as young as 6-months old (Fig. 1). We propose that separation of muscle spindles from muscle fascicles by layers of fibrotic connective tissue would disrupt the faithful recording by intrafusal fibers of the extent and rate of stretch experienced by adjacent extrafusal muscle fascicles. Moreover, the stretch properties of fibrous connective tissue would not match the stretch properties of extrafusal and intrafusal fibers, adding further complications. Spatial separation of muscle spindles would lead to an impairment of muscle-nerve communication and, ultimately, compromise the control and maintenance of muscle tone, balance and the stretch response.

Muscle spindle separation from muscle fascicles was also observed in aged 14 month-old WT mice, apparently as part of normal aging. We propose that physical separation of muscle spindles from extrafusal fascicles due to increased perimysial and endomysial fibrosis is an important, spindle-related pathological feature of both neuromuscular diseases and normal aging.

The more profound abnormality observed in IT/+ muscle spindles is severe deterioration of intrafusal fiber organization and the collapse of the contractile machinery, which recapitulates the pattern of core formation in extrafusal fibers [4,5]. However, a critical difference is that core lesions in intrafusal fibers are readily apparent in 6 months old IT/+ mice whereas comparable core lesions in extrafusal myofibers are readily apparent only in 8–10 months-old IT/+ mice. In other words, structural abnormalities in IT/+ muscle spindles precede the appearance of core lesions in adjacent extrafusal fibers.

In 6 month-old mice, spindles exhibited a wide array of abnormalities from focal myofibrillar hypercontraction (Fig. 2B) to massive collapse of striation in both nuclear chain and nuclear bag fibers (Fig. 2C–F, Fig 3). Electron microscopic imaging of IT/+ intrafusal fibers (Fig. 3) revealed Z-line streaming and loss of sarcomeric organization, accompanied by a highly unequal distribution of mitochondria among myofibrillar bundles that is characteristic of cores only in much older extrafusal fibers. The intrafusal fiber degeneration observed in 6 month-old IT/+ muscle samples was clearly sufficiently extensive to abrogate the stretch receptor function of such muscle spindles.

The fact that overt structural abnormalities in IT/+ muscle spindles precede the appearance of core lesions in the adjacent extrafusal fibers suggests that muscle spindles represent an important early target in *Ryr1*-related disease pathology. Moreover, muscle spindle degeneration appears to proceed independently from separation between spindles and fascicles, since intrafusal fiber abnormalities are observed in both detached and tightly associated spindles and some detached spindles show normal striation. Thus, problems resulting from an impaired stretch

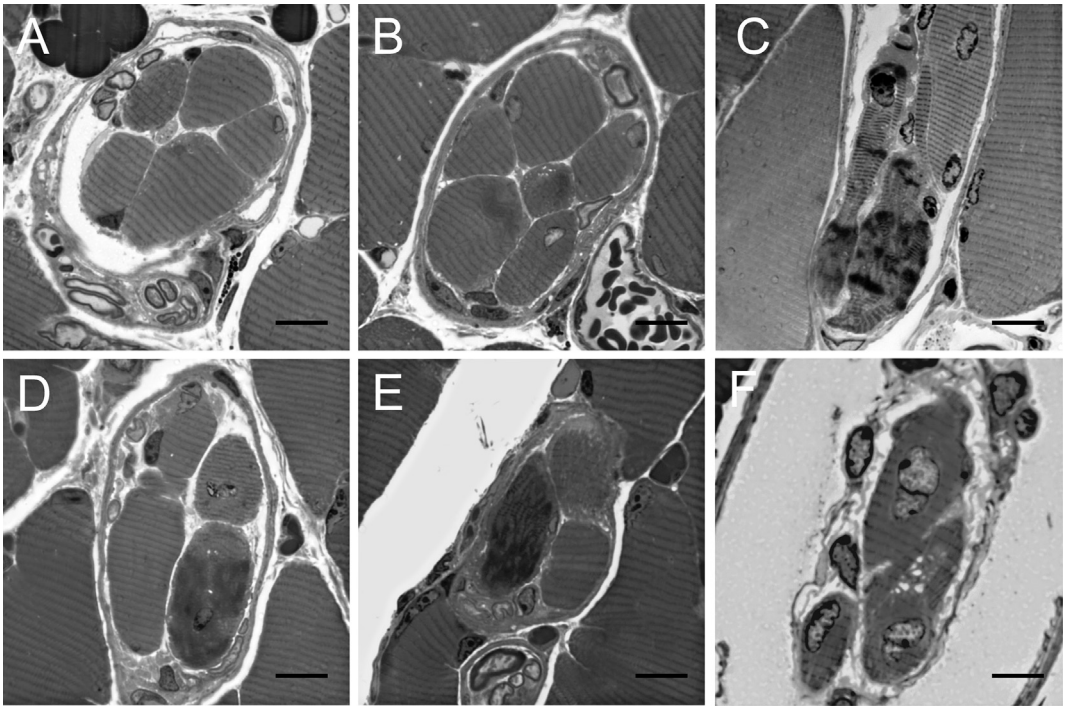

Fig. 2. Representative images of muscle spindles in semi-thin longitudinal sections of a soleus muscle from 6 month-old WT (A) and IT/+ (B–D) mice. A, Intrafusal fibers in a WT muscle spindle exhibit well-defined striations. B–D, intrafusal fibers in IT/+ muscle spindles show focal (B) and extensive (C–E) hypercontraction of myofibrils with a loss of the striation pattern. E, extensive myofibrillar detachment in subsarcolemmal areas. D and F, pyknotic and fragmenting nuclei. F, an extensive network of dilated sarcoplasmic reticulum. Toluidine blue staining. Bar, A–E, 10 μm, and F, 5 μm.

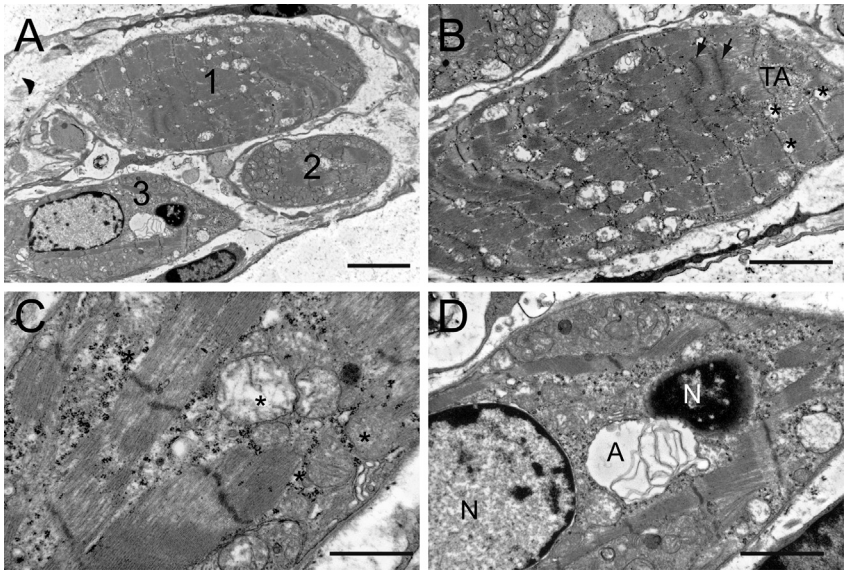

Fig. 3. Ultrastructural abnormalities in a muscle spindle from a 6 month-old IT/+ mouse soleus muscle. Representative TEM images of longitudinal sections from intrafusal fibers of IT/+ tibialis anterior. A, a low magnification image of a spindle with three intrafusal fibers (1–3). B–D, higher magnification images of fibers 1–3. B and C, areas of Z-line streaming, focal myofibrillar hypercontraction (arrows in B), tubular aggregates (TA), mitochondrial accumulation and swelling (asterisks in B and C). D, a perinuclear region in fiber 3 with pyknotic nuclei (N), swollen mitochondria and a gigantic, multilamellar, rosebud-shaped autophagosome (A). Bars, A and B, 5 μm; C, 1 μm; and D, 2 μm.

response may manifest before the onset of generalized muscle weakness.

In conclusion, our data show that muscle spindles undergo severe deterioration that may precede structural changes in extrafusal myofibers, in our animal model of *Ryr1*-related core myopathy. If our observations can now be confirmed in human core myopathy patients, the understanding of the basis for hypotonia and defects in the stretch response, in balance, locomotion and coordination that accompany or even precede generalized muscle weakness in human patients, including infants, will be transformed. Our observations now warrant a renewed, detailed investigation of the involvement of muscle spindles in human *RYR1*-related core myopathies and in other human skeletal muscle diseases and in aging.

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Conflict of interest

None declared.

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