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Workshop report

182nd ENMC International Workshop: *RYR1*-related myopathies, 15–17th April 2011, Naarden, The Netherlands

Heinz Jungbluth [a,b,*,1], James J. Dowling [c,1], Ana Ferreiro [d,e,1], Francesco Muntoni [f,1]

[a] *Clinical Neuroscience Division, IOP, King's College, London, United Kingdom*
[b] *Department of Paediatric Neurology – Neuromuscular Service, Evelina Children's Hospital, St. Thomas' Hospital, London, United Kingdom*
[c] *Division of Pediatric Neurology, Pediatric Neuromuscular Clinic, 5328 BSRB, 109 Zina Pitcher Place, University of Michigan Medical Center, Ann Arbor, USA*
[d] *INSERM UMR_582, Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 75013 Paris, France*
[e] *UPMC Univ Paris 06, UMR_582, 75013 Paris, France*
[f] *Dubowitz Neuromuscular Centre, UCL Institute of Child Health & Great Ormond Street Hospital for Children, London, United Kingdom*

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1. Introduction and overview

Twenty clinicians and basic scientists from 10 countries convened for the 182nd ENMC sponsored Workshop on *RYR1*-related myopathies from the 15th to the 17th of April 2011 in Naarden, The Netherlands. *RYR1*-related myopathies are a clinically and pathologically heterogeneous group of conditions caused by mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene, encoding the principal sarcoplasmic reticulum (SR) Ca^{2+} release channel (RyR1) with a crucial role in excitation–contraction (E–C) coupling. Myopathies with *RYR1* involvement have been the topic of several previous ENMC workshops, namely the ENMC workshops on Central Core Disease (CCD) in January 2001 [1], on Multi-minicore Disease (MmD) in May 2000 [2] and November 2002 [3], and on the various core myopathies in March 2007 [4], resulting in numerous productive collaborations between the various participants.

The focus of the present workshop on *RYR1*-related myopathies as a group reflects the ever expanding phenotypical spectrum of these conditions as well as the recognition of *RYR1* mutations as one of the most common causes of inherited neuromuscular disorders, often presenting with different histopathological features. Further emphasis was placed on the evaluation of common mechanisms of normal and abnormal intracellular Ca^{2+} homeostasis and E–C coupling both *in vitro* and in recently emerged animal models, with a particular view to potential future pharmacological interventions. Secondary alterations of RyR1 function as an important pathogenetic mechanism in other neuromuscular disorders as well as the role of RyR1 expression in non-muscle cells were additional topics discussed.

After the welcoming addresses by **Francesco Muntoni** (London, UK) and **Baziel van Engelen** (Nijmegen, The Netherlands), Research Director of the ENMC, the workshop was introduced by **Heinz Jungbluth** (London, UK), who gave a brief overview over the *RYR1*-related myopathies.

RYR1 mutations have emerged as one of the most common causes of inherited neuromuscular disease since complete sequencing of the large *RYR1* gene has become more widely available. The spectrum of clinical and pathological phenotypes associated with *RYR1* mutations is extremely wide. Associated muscle biopsy findings may be normal in the malignant hyperthermia susceptibility (MHS) trait, or comprise the histopathologic features typical of Central Core Disease (CCD), Multi-minicore Disease

Abbreviations: CCD, Central Core Disease; CNM, Centronuclear Myopathy; E–C, excitation–contraction; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptibility; MmD, Multi-minicore Disease; *RYR1*, skeletal muscle ryanodine receptor gene; RyR1, skeletal muscle ryanodine receptor; SR, sarcoplasmic reticulum

* Corresponding author at: Department of Paediatric Neurology – Neuromuscular Service, Evelina Children's Hospital, St. Thomas' Hospital, Westminster Bridge Road, London SE1 7EH, United Kingdom.
E-mail address: Heinz.Jungbluth@gstt.nhs.uk (H. Jungbluth).

[1] On behalf of the Core Myopathy Consortium.

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(MmD), Centronuclear Myopathy (CNM) and congenital fibre type disproportion (CFTD). Whilst dominant *RYR1* mutations associated with MHS and CCD have been well characterized at the functional level, the mechanisms underlying the predominantly recessively inherited *RYR1*-related myopathies – MmD, CNM and CFTD – remain only partially understood.

2. Delineation of *RYR1*-related myopathies – emerging clinical and pathological phenotypes

Central Core Disease (CCD) and the malignant hyperthermia susceptibility (MHS) trait due to dominant "hotspot" mutations were the first *RYR1*-related myopathies to be characterized. A spectrum of recessive *RYR1*-related myopathies with considerable clinical and histopathological overlap has emerged more recently and was the topic of the first session.

Nigel Clarke (Sidney, Australia) reported on the association between *RYR1* mutations and congenital fibre type disproportion (CFTD), characterized by consistently small type 1 fibres compared to type 2 fibres.

The four patients reported so far [5] had a wide range of clinical severity from neonatal death to remaining ambulant in late teenage years. The degree of fibre size disproportion varied between 50% and 84%. These patients resembled recessive *RYR1*-related core myopathies in both clinical features (frequent ophthalmoplegia, ptosis, scoliosis) and mutation pattern (all patients had compound heterozygous 'null' and novel missense mutations), suggesting similarities between *RYR1*-related CFTD and MmD. Ophthalmoplegia may be the most specific clinical sign to identify CFTD patients with *RYR1* mutations from other genetic causes. The molecular basis for the marked difference in fibre sizes, a frequent finding in other recessive *RYR1*-related myopathies, is currently unclear.

Heinz Jungbluth (London, UK) presented clinical and histopathologic features of congenital myopathies with central nuclei due to recessive *RYR1* mutations.

Centronuclear Myopathy (CNM) represents a clinically and genetically heterogeneous group of congenital myopathies with the common denominator of abundant central nuclei on muscle biopsy (for review, [6,7]). CNM has been associated with mutations in the myotubularin (*MTM1*) (X-linked recessive, XLMTM), the dynamin 2 (*DNM2*) (autosomal-dominant) and the amphiphysin 2 (*BIN1*) (autosomal-recessive) genes but many cases remain currently genetically unresolved.

The genotypes associated with *RYR1*-related CNM in the 17 patients reported to date [8] were characterized by compound heterozygosity for *RYR1* missense mutations and mutations resulting in reduced RyR1 protein expression, corresponding to other recessive *RYR1*-related myopathies which also share many clinical features, namely external ophthalmoplegia, bulbar and respiratory involvement. Recessive inheritance seems consistent in *RYR1*-related CNM but some mutations may be missed on routine sequencing, suggesting that the only previously reported case with a single *de novo* *RYR1* missense mutation [9] may in fact have been recessively inherited, due to an undetected second mutation. Although usually relatively mild and improving over time, *RYR1*-related CNM may mimic XLMTM in the most profoundly affected males (HJ, unpublished observation). Compared to other genetic forms of CNM, multiple internalized nuclei are often more common than strictly centralized nuclei. Core structures on oxidative stains are not typically seen at presentation but may appear over time.

Carsten Boennemann (Philadelphia, USA) presented data from the series of *RYR1*-related myopathies seen at the Children's Hospital of Philadelphia (CHOP). Among this group, based on histological data, 23 patients could be classified as "classic" CCD, five as severe neonatal CCD, three as MmD, four as congenital muscular dystrophy (CMD)-like and one CFTD.

Seven patients had early-onset severe disease, precluding ambulation in those old enough to assess. The phenotypic range seen in the this subgroup included CMD-like features with ophthalmoplegia, cleft palate, variable early kyphoscoliosis, arthrogryposis, adducted thumbs and respiratory failure. Out of the seven patients with early-onset severe disease, two patients had *de novo* dominantly acting *RYR1* mutations with central cores on muscle biopsy and five patients had recessively acting *RYR1* mutations. Histopathological appearance in the five patients with recessively acting *RYR1* mutations was variable, ranging from nonspecific myopathic and CMD-like with occasional core-like appearance to ubiquitous cores on oxidative stains. Recessive *RYR1* mutations were also identified in three other patients but with milder disease; of those, histological appearance was that of CNM and MmD, respectively, in one case each whilst one patient had not had a muscle biopsy. Clinically, only one of the patients with early-onset severe disease had ophthalmoplegia but this was more common in more mildly affected cases with recessive *RYR1* mutations. Overall, whilst clinical severity was variable even with the same mode of inheritance, dominant *RYR1* mutations were more commonly found in the mild whilst recessive *RYR1* mutations were more commonly seen in severe cases. Cores and minicores were seen in both dominant and recessive cases, whilst CFTD, CNM-like and CMD-like pathologies were only seen in the recessive cases. However, NADH irregularities and central nucleation were seen frequently throughout and could indicate *RYR1* involvement in genetically unresolved congenital myopathies.

3. *RYR1*-related myopathies and the malignant hyperthermia susceptibility trait

Luc Heytens (Antwerp, Belgium) reported on current concepts in Malignant Hyperthermia (MH) and the MH susceptibility (MHS) trait.

MHS is an autosomal dominant, genetically heterogeneous trait, manifesting as acute rhabdomyolysis on exposure to volatile anaesthetics and/or succinylcholine during general anaesthesia. There is considerable clinical variability of MH episodes due to anaesthesia-related, host, environ-

mental and genetic factors. Safe anaesthesia can be administered even in genetically susceptible individuals provided all triggering drugs are avoided. Anaesthetic guidelines recommend the anaesthesia machine to be decontaminated but prophylactic administration of Dantrolene is not recommended. Susceptibility to MH is diagnosed by *in vitro* contracture testing (IVCT). The IVCT requires a fresh skeletal muscle sample, which is exposed *in vitro* to incremental doses of different specific testing agents – caffeine, halothane, ryanodine and 4-chloro-m-cresol – and the contracture response measured. This test has been standardized across Europe by the European Malignant Hyperthermia Group (EMHG) and shows a high degree of sensitivity (99%) and specificity (93.5%). However, it is an invasive test and is not validated in children, hampering its widespread use as a screening instrument. Genetically, the MHS trait has an estimated prevalence of 1 in 3–10,000. Up to 80% of families demonstrate linkage to the *RYR1* locus, however, only 30 causative *RYR1* mutations are listed in the EHMG genetic testing guidelines (www.emhg.org), as the pathogenicity of the majority of the more than 200 MH-associated *RYR1* mutations has not yet been functionally assessed. At the present time, once there is confirmation of MH susceptibility and key family members have been biopsied and phenotyped by IVCT, the entire *RYR1* gene is sequenced to identify causative mutations.

Although the MHS trait and clinically manifest myopathies are considered distinct presentations of specific *RYR1* mutations, a continuum between these entities is indicated by the finding of central cores in patients with MHS, and of MHS in some patients with CCD. **Heinz Jungbluth** (London, UK) summarized myopathic manifestations occasionally seen in association with MHS mutations:

King-Denborough syndrome (KDS) is a rare syndrome characterized by the triad of dysmorphic features, a myopathy, and MHS. *RYR1* involvement had been previously suggested in one case with a *de novo* heterozygous dominant missense mutation [10]. More recently [11], heterozygous *RYR1* missense mutations, one of them novel and two previously reported in MH, were identified in a small cohort of three patients with suggestive features. In most of these cases, *RYR1* mutations were inherited from a parent with previous MH history but no or only little weakness. Further studies showed marked RyR1 protein reduction in index cases, suggesting the presence of allelic *RYR1* mutations not detectable on routine sequencing. *Exertional myalgia* was recognized early as a presenting feature in CCD [12] and isolated cases with additional episodic rhabdomyolysis have been recently reported associated with MH mutations [13]; the prevalence of this phenotype is currently unknown but may be underdiagnosed as affected individuals may have no associated weakness. An almost exclusively *axial myopathy* with late-onset has been reported in one isolated case [14] and two additional families in whom heterozygous dominant *RYR1* mutations were identified, either localizing to one of the MHS hotspots or previously associated with the MHS trait (HJ, unpublished observations).

4. *RYR1* genotype–phenotype correlations

Joel Lunardi (Grenoble, France) reported on genotype–phenotype correlations in structural congenital myopathies with cores associated with *RYR1* mutations identified by his group.

Three hundred and ninety-six familial ($n = 151$) or sporadic ($n = 245$) cases were investigated at the molecular level based on suggestive features of a "myopathy with cores". A *RYR1* mutation could be identified in 53% of all cases and in around 66% of cases where the *RYR1* gene was fully sequenced. Mutations responsible for AD forms of the disease that were mainly associated with central or unique cores mapped preferentially (95%) to the C-terminal domain of the RyR1 protein [15]. Full *RYR1* sequencing revealed that a significant percentage of sporadic cases proved to be recessively inherited and that *de novo* mutations represented almost 25% of the genotyped sporadic cases. Whilst most recessive mutations were missense mutations distributed throughout the *RYR1* gene, 1/3 of recessive mutations were "quantitative" mutations affecting the RyR1 protein level in muscle [16]. Recessive inheritance might thus result from the association of two "qualitative" mutations, of one "qualitative" and one "quantitative" or from two "quantitative" mutations. These different combinations might account at least partly for the large clinical and pathological variability seen in *RYR1*-related myopathies.

Genetic pitfalls included (i) occurrence of an additional *de novo* mutation in a family previously classified as an AD with variable penetrance, (ii) apparent homozygosity due to genomic deletion of the second allele, (iii) apparent absence of cDNA nonsense mutation resulting from mRNA decay and (iv) deep intronic mutations. These findings clearly indicate the complexity of *RYR1* mutation analysis.

Generally, findings in this large cohort demonstrate that *RYR1*-related congenital myopathies present (1) with a wide clinical spectrum ranging from moderate forms to severe and lethal neonatal cases; (2) with different modes of transmission; and (3) with different histological phenotypes that include classical cores (central cores, multicores, minicores), nemaline rods and cores or less specific pathological features such as fibre type disproportion, nuclear centralisation, type I fibre predominance and sarcomeric disorganisation. Histological phenotypes may evolve over time in the same patient and likewise, histological features may vary in patients harbouring the same *RYR1* mutation. These findings indicate that a "myopathy with cores" terminology might be more appropriate to describe the pathological phenotype rather than more specific designations such as CCD or MmD.

Francesco Muntoni reported data from a collaborative UK study on pathogenic *RYR1* mutations in 77 families, based on genomic sequencing of the entire *RYR1* coding regions including splice sites.

Within this cohort, 39 had dominant and 38 had recessive inheritance, although in some presumably recessive cases only one heterozygous mutation inherited from an asymptomatic parent was identified. 37 of 59 recessive

but only 6 of the 28 dominant *RYR1* mutations were novel: dominant mutations were more frequently located in one of the three previously recognized hotspot regions, whilst recessive mutations were distributed throughout the *RYR1* coding sequence. Dominant mutations were predominantly missense, whereas recessive genotypes frequently where a combination of a missense mutation with a second *RYR1* mutation expected to result in a reduced amount of functional RyR1 protein. Of the 37 novel recessive mutations, 22 were missense, six were nonsense, four were frameshift, three were splice site, and two were single amino acid deletions. We found the combination of a *RYR1* missense mutation with a nonsense mutation in 9/38, with a splice site mutation in 5/38 and with a frameshift mutation in 4/38 patients. 6/38 patients were found to be homozygous for a missense mutation. There was wide clinical variability in patients with both dominant and recessive inheritance: with the exception of few severe cases with *de novo* mutations, those with dominant mutations were generally more mildly affected than those with recessive inheritance. Extraocular muscle involvement was almost exclusively observed in the recessive group. In a number of these cases assigning pathogenicity was challenging and often had to rely not only on clinico-pathological findings, but also on muscle MRI (see below).

Jim Dowling (Ann Arbor, USA) presented *RYR1* genotype–phenotype data resulting from a collaborative effort among several US clinicians. Mutations and clinical characteristics from 29 patients with confirmed *RYR1* mutations (15 dominant and 14 recessive) as well as a database containing all previously published *RYR1* mutations were presented. In addition, he presented a detailed analysis of the recessive subgroup of patients from the unpublished US series and from the medical literature, focussing on the relationship between several parameters and clinical severity. No obvious association was found between mutation location or histopathologic subtype and severity, but an emerging trend toward association between reduced protein levels and clinical severity was observed. Further data and the inclusion of additional patients will be needed to confirm this potential association, emphasizing the need of an inclusive database of *RYR1* mutations and associated phenotypes for the use of all researchers.

Francesco Muntoni reported muscle MRI findings in *RYR1*-related myopathies, based on a large collaborative, predominantly UK study [17]. In this study, muscle MRI of 37 patients with dominant or recessive *RYR1*-related myopathies were assessed blindly and the pattern of selective muscle involvement compared to the pattern previously reported in *RYR1*-related myopathies [18], and to a group of 23 patients with congenital myopathies without *RYR1* involvement. Scans were classified as *typical* if they were identical to the reported pattern, *consistent* if it was similar to the reported one but with some additional features, or *different*. Scans with no or little changes were classified as *uninformative*. 21/37 patients with *RYR1* mutations had a *typical* pattern and in 13 the pattern was classified as *consistent*. Two patients had *uninformative* scans and only one had a *different* pattern. Compared to those with dominant mutations, patients with recessive mutations and ophthalmoparesis had a more diffuse pattern, classified as *consistent* in 6/8. In contrast, 10/11 with recessive mutations but without ophthalmoparesis had a *typical* pattern. All scans of 23 patients with other myopathies were classified as *different*. These results suggest that muscle MRI is a powerful predictor of *RYR1* involvement, especially if patients carry a dominant mutation or recessive mutations without ophthalmoparesis.

5. The biology of RyR1 and RyR1-associated proteins

Susan Treves (Basle, Switzerland) reviewed the relation between RyR1 and the proteins of the excitation–contraction (E–C) coupling machinery.

In striated muscle, activation of contraction is initiated by membrane depolarization, which triggers the release of Ca^{2+} stored in the SR by a process called E–C coupling. The core components involved in this process are the dihydropyridine (DHPR) receptors, ryanodine (RyR1) receptors and calsequestrin, which serve as voltage sensor, Ca^{2+} release channel, and Ca^{2+} storage protein, respectively. A number of additional small accessory proteins have been the focus of recent research, because of their potential roles in regulating E–C coupling and as potential candidates for currently unresolved neuromuscular disorders. So far mitsugumins, junctophilins, junctate, JP-45 and SRP27 have been characterized. Susan Treves provided evidence that SRP-35, a newly identified accessory protein, is an all-*trans* retinol dehydrogenase enriched in the longitudinal skeletal muscle SR. Retinol is the substrate of SRP-35 since its transient over-expression leads to an increased production of all-*trans* retinaldehyde. In skeletal muscle, SRP-35 may be involved in the generation of intracellular signals linking Ca^{2+} release (i.e. muscle use) with the activation of glycolytic metabolism.

Julien Faure (Grenoble, France) reported on RyR1 function and SR membrane morphology, focussing on protein partners of RyR1, namely triadin and caveolin, that could modify membrane structure of the SR and, indirectly, modulate RyR1 activity. During muscle contraction, activation of RyR1 by DHPR is mediated by physical coupling, which requires a precise localisation of each channel in its membrane. Recent data suggest that the membrane structure surrounding RyR1, and more specifically its interactions with phospholipids, also plays a role in Ca^{2+} release.

Triadin is a SR membrane protein known to interact with RyR1. Triadin knock-out mice have impaired muscle function and disorganized triad membranes [19]. *In vitro* studies showed that triadin has the property to modulate reticulum membrane morphology. Domains of the molecule responsible for membrane shape regulation have also been shown to be essential for triadin anchoring to triad in muscle. These findings suggest that multimerization of triadin in close proximity to RyR1 could allow specific SR membrane bending that would favour RyR1

Ca^{2+} release. *Caveolins* are scaffolding proteins known to organize subdomains of membranes. A pool of the muscle specific caveolin isoform (Cav-3) is associated with the calcium release complex of skeletal muscle via its interaction with RyR1 [20]. As demonstrated by studying *RYR1* mutations associated with structural core myopathies, the membrane part of RyR1 can directly interact with Cav-3, raising the possibility that caveolin-mediated SR membrane structuration in close proximity to RyR1 could also participate in the process of Ca^{2+} release.

Valerie de Crescenzo (Worcester, USA) reported on the role of RyR1 receptors in the peripheral and central nervous system. In neurons, RyR2 is the most common and RyR1 the second most common of the 3 RyR1 isoforms [21]. While RyR2 is implicated in the amplification of the Ca^{2+} signal due to influx from the extracellular space via "Ca^{2+} induced Ca^{2+} release", no specific role had been attributed to RyR1 in the brain. Recently, Valerie de Crescenzo and colleagues found spark-like transients in nerve terminals which they termed Ca^{2+} syntillas (*scintilla*, Latin for spark from a nerve terminal, a SYNaptic structure) [22]; they also found that depolarization of the plasma membrane in the *absence* of external Ca^{2+}, resulted in an increase in syntilla frequency and designated this process as voltage-induced Ca^{2+} release (VICaR) [23], a concept subsequently confirmed by others [24,25]. A role of RyR1 in neuronal VICaR was suggested by observations in the RyR1 I4895T KI mouse, a mouse model of CCD where there is not only muscle weakness but also loss of VICaR in nerve terminals. Moreover, whilst upon long depolarizations the Ca^{2+} transient is smaller in the mutant compared to WT, the plasmalemmal Ca^{2+} current is similar, suggesting that long depolarizations are able to induce VICaR. Furthermore, the maximum rate of Ca^{2+} release is significantly reduced in mutant nerve terminals compared to WT. Moreover, preliminary data show that elicited exocytosis is enhanced in the RyR1 I4895T KI mouse during long depolarization, even though the Ca^{2+} transient is greatly *decreased*, suggesting that syntillas inhibit exocytosis. These findings provide functional characterization of the RyR1 mutant phenotype at the neuronal cellular level and indicate a role of RyR1 in neurons, matching its widespread CNS distribution.

Susan Treves (Basel, Switzerland) presented an overview on RyR1 receptors in the immune system and other non-muscle cells based on work by her group.

In both excitable and non-excitable cells, generation of intracellular Ca^{2+} transients is due to the release of Ca^{2+} from intracellular stores via $InsP_3R$ or ryanodine receptors (RyRs) present on the endoplasmic/sarcoplasmic reticulum (ER/SR) membranes and opening of Ca^{2+} channels present on the plasma membrane. Generally, excitable cells, which need to respond to signals within milliseconds, are equipped with RyR Ca^{2+} channels. Though both B-lymphocytes and dendritic cells are not excitable cells and express $InsP_3R$, they also express RyR1. Both cell types can act as antigen presenting cells and initiate T-cell driven immune responses. The involvement of Ca^{2+} signalling events in dendritic cells maturation had been previously postulated and recent evidence suggests that RyR1 mediated Ca^{2+} signals can act synergistically with signals generated via Toll like receptors driving dendritic cells maturation. More importantly, activation of the RyR1 signalling pathway is both necessary and sufficient to cause the rapid release of an intracellular pool of MHC class II molecules onto the plasma membrane. Such a rapid signalling mechanism may be important in the very early phases of an immune response when T-cells and dendritic cells become intimately connected: engagement of T-cell receptors with the MHC class II molecules on the surface of immature dendritic cells rapidly activates T-cells to release factors stimulating an increase in the level of expression of MHC class II molecules on the surface of immature dendritic cells, possibly supporting very early activation steps of T-cells. As to its role in B-cells, pharmacological activation of RyR1 leads to rapid release of an intracellular pool of the pro-inflammatory cytokine IL-1β. Current studies focus on the effect of a MH-associated *RYR1* mutation in a knock in mouse model, in order to determine its effect on the immune system.

Francesco Zorzato (Ferrara, Italy) reported on abnormalities of excitation-coupled calcium entry (ECCE) in patients with *RYR1*-related disorders [26]. ECCE is defined as the entry of extracellular Ca^{2+} into muscle cells induced by prolonged depolarization of skeletal muscle cells and relies on the interaction between the 1,4-dihydropyridine (DHPR) receptor on the sarcolemma and the RyR1 on the SR membrane.

His group have investigated their hypothesis that decrease of SR Ca^{2+} load via leaky RyR1 channels and/or alteration of Ca^{2+} influx via store operated channels or excitation-coupled Ca^{2+} entry, may account for the phenotype of patients with CCD, including muscle weakness and abnormal secretion of inflammatory cytokines. They investigated the mechanisms activating Ca^{2+} influx by directly measuring ECCE applying total internal reflection fluorescence (TIRF) microscopy in human skeletal muscle myotubes harbouring *RYR1* mutations linked to MHS and CCD. They found that ECCE is strongly enhanced in cells from patients with CCD compared to individuals with MHS and controls. Furthermore, ECCE induced generation of reactive nitrogen species (RNS) and enhanced nuclear localization of NFATc1. The increase of NFATc1 nuclear translocation may in turn account for the enhancement of IL-6 secretion from myotubes derived from CCD patients.

6. Function and dysfunction correlations of RyR1: *in vitro* approaches

Gerhard Meissner (Chapel Hill, USA) provided novel biochemical and electrophysiological information on RyR1 pore structure, ion conductance and selectivity. The clinical relevance of the findings of his group is supported by mutations in the RyR1 transmembrane domain that are associated with aberrant muscle function in skeletal muscle such as CCD [27]. Following a brief overview, he described the construction of a homology model of the open RyR1 mem-

brane spanning domain (Ramachandran and Dokholyan, unpublished studies) using the structure of Kv2.1 as a template and cryo-electron microscopy images that show significant similarity in the transmembrane-spanning domains of RyR1 and Kv2.1 [28]. The raw homology model was refined using Chiron and Medusa for backbone and side-chain refinement, respectively. The activity and Ca^{2+} and K^{+} conductances and selectivity of single site mutants including several reported CCD mutants are currently determined in single channel recordings using the planar lipid bilayer method to test and refine the model (Chakraborty, Xu and Meissner, unpublished data).

Werner Melzer (Ulm, Germany) reported on anterograde and retrograde signalling [29] between DHPR and RyR1 receptors in wild-type and mutant RyR1 channels.

Ryanodine receptors (RyRs) in the junctional SR membrane are influenced by a large number of modulators of which the transverse tubular membrane potential is the most effective one. Two functionally distinct classes of RyR1 mutations have been identified [30], either leading to overactive RyR1 channels ("leaky channel mutations") or to RyR1 channels with diminished Ca^{2+} permeability ("EC uncoupling mutations"). In recent years, knock-in mice for each group have become available [31–33].

Studies of voltage step-induced Ca^{2+} release in muscle fibres from mice with the Y524S (YS/+) and the I4895T mutation (IT/+) (corresponding to human Y522S and I4898T mutations, respectively) showed a displacement in the voltage dependence of activation to more negative potentials with no change in maximal amplitude in the Y524S fibres and a decrease in release without alteration of voltage threshold [34,35] in the I4895T fibres, corresponding to the suggested opposite molecular changes induced by above mutations. Retrograde coupling from the RyR1 to the DHPR receptor appeared unchanged in YS/+ and IT/+ fibres [34,35], however, in YS/+ fibres the voltage dependence of slow inactivation was found to be shifted to negative potentials (−10 mV), leading to lower amplitudes of both Ca^{2+} release and Ca^{2+} current compared to WT fibres at conditioning potentials more positive than the normal resting potential [34]. This novel finding indicates a feedback from the mutant RyR1 to the DHPR enhancing DHPR inactivation, which can be viewed as a protective mechanism to counteract the effects of hyperactive RyR1 channels. The voltage dependence of activation and inactivation predicts a range of voltages in which Ca^{2+} release is permanently activated at low level. Indeed, we found a steady increase in the basal Ca^{2+} concentration in a window of voltages compatible with the prediction. In the YS/+ fibres this window is broader and shifted to more negative potentials. A rise in temperature by 10 °C caused a further substantial shift (ca. −20 mV), suggesting a role of window Ca^{2+} release in the heat-induced MH observed in these mice [34].

In summary, mutations altering RyR1 function in opposite ways lead to characteristic differences in the voltage dependence of Ca^{2+} release. The voltage modulation of release might contribute to resting Ca^{2+} destabilization in the presence of hyperactive RyR1 channels. A newly recognized means of potential pharmacological interest to prevent a dangerous rise in resting Ca^{2+} is enhancing DHPR inactivation by altering its voltage dependence.

7. Function and dysfunction correlations of RyR1: animal models

Jim Dowling (Ann Arbor, Michigan) presented data on an existing zebrafish model of RyR1-related myopathies. Zebrafish have two *RYR1* paralogues, *ryr1a* and *ryr1b*, (expressed in slow and fast muscle, respectively). Relatively relaxed (*ryr*) [36] is a spontaneous zebrafish mutant that harbours an insertion that, when homozygous, results in greater than 90% reduction in *ryr1b* levels. The *ryr* mutant is an excellent model of recessive *RYR1*-related myopathies, and shares some features with a recently published patient with MmD and additional periodic paralysis [37].

Jim Dowling also presented new unpublished data exploring the relationship between RyR1 dysfunction and oxidative stress. Microarray analysis on the *ryr* zebrafish (with confirmatory qPCR data) revealed abnormalities in several gene products associated with oxidative stress, whose overall levels were found to be significantly increased using both whole embryos and isolated myofibers. Moreover, treatment with N-acetylcysteine (NAC) reduced oxidative stress, improved the histopathologic changes, and improved motor function. In all, these data suggest that NAC may be a viable therapeutic option for *RYR1*-related myopathies.

Feliciano Protasi (Chieti, Italy) reported on characterization and temporal development of cores in a model of malignant hyperthermia (MH), generated by expressing a human *RYR1* mutation (Y522S) in mice. Heterozygous Y522S knock-in mice are characterized by SR Ca^{2+} leak, excessive oxidative stress, and MH susceptibility [38]. Electron microscopy (EM) analysis of skeletal muscle fibres, $RYR1^{Y522S/WT}$ knock-in mice revealed the presence of mitochondrial-deficient core regions evolving over time. Mitochondrial swelling/disruption, the first detectable structural change observed in young adult $RYR1^{Y522S/WT}$ mice (2 months), is initially confined to discrete areas termed *early cores*, where also sarcoplasmic reticulum (SR) and transverse-tubules (T-tubules) display clear signs of structural damage. At later stages (1 year) structural modifications affect larger fibre regions and were classified in two main classes: *contracture cores*, characterized by extreme sarcomere shortening and lack of SR and mitochondria, and *unstructured cores*, areas in which also contractile elements are severely compromised. These findings present many similarities with human CCD and suggest a model of core formation in which initial mitochondrial/SR disruption causes significant loss of local Ca^{2+} sequestration. In turn, these changes would cause Ca^{2+} accumulation in confined areas, which results in contracture formation and, ultimately, progressive degradation of the contractile elements [39].

8. Secondary pathologies associated with RyR1 dysfunction

Feliciano Protasi (Chieti, Italy) also summarized recent studies aiming to define the reciprocal positioning of mitochondria and Ca^{2+} release units (CRUs) in developing and adult skeletal muscle fibres [40]. Bi-directional Ca^{2+} signalling between mitochondria and intracellular stores (endoplasmic/sarcoplasmic reticulum) underlies important cellular functions, including oxidative ATP production; in striated muscle, this may require proximity between mitochondria and release sites. Using electron microscopy/tomography, small bridges (or *tethers*) were identified that link the outer mitochondrial membrane to the SR in the vicinity of CRUs. This mitochondria–SR association: a) results in mitochondria being less mobile in skeletal fibers and (b) is sufficiently strong that treatment with hypotonic solution fails to pull the two organelles apart. In addition, coupling of mitochondria to the SR is: (i) developmentally regulated; ii) involves a progressive shift from a longitudinal clustering at birth to a specific CRU-coupled transverse orientation in adult; and (iii) results in a change in the mitochondrial polarization state. These results suggest that tethers establish and maintain SR/mitochondrial association in adult muscle and likely provide a structural framework for bi-directional signalling between the two organelles. Interestingly, mitochondrial structure and their association to CRUs are challenged by mutations affecting E–C coupling proteins (see above), and also in conditions such as ageing and denervation. Interestingly in all these different situations, oxidative stress is higher than normal.

Michael Duchen (London, UK) reported on Ca^{2+} dysregulation and mitochondrial function as mechanisms of muscle disease. The primary role of mitochondria is the generation of ATP by oxidative phosphorylation. In addition, mitochondria also accumulate Ca^{2+} via an electrogenic uniporter in response to a local Ca^{2+} rise, especially in microdomains of high Ca^{2+} generated at sites of release from SR to ER (reviewed in [41]). There are a number of discrete mechanisms, potentially vulnerable to RyR1 dysfunction, by which intracellular Ca^{2+} signalling influences mitochondrial function and distribution:(1) *Calcium and oxidative phosphorylation:* at appropriate physiological levels, mitochondrial matrix Ca^{2+} promotes oxidative phosphorylation through several concerted mechanisms, coupling Ca^{2+} signals with enhanced oxidative phosphorylation [41]. Failure of these mechanisms in cells expressing mutant RyR1 channels may impair oxidative phosphorylation, contributing to impaired ATP homeostasis and muscle weakness and wasting. (2) *Calcium overload and mitochondrial damage:* conversely, mitochondrial Ca^{2+} overload may cause mitochondrial damage through opening of the mitochondrial permeability transition pore (mPTP), a large conductance, cyclosporine A sensitive channel which opens in the mitochondrial inner membrane to a combination of raised Ca^{2+} and oxidative stress (for review see [42]). mPTP opening disables oxidative phosphorylation and may trigger cell death leading to muscle atrophy. (3) *Calcium and mitochondrial biogenesis:* Ca^{2+} signaling in muscle induces Ca^{2+}-dependent mitochondrial biogenesis [43]; Abramov and Duchen, unpublished observation), increasing mtDNA copy number and also expression of nuclear encoded respiratory proteins. Thus, impairment of this pathway may impair mitochondrial oxidative phosphorylation. (4) *Calcium and mitochondrial movement, fission and fusion, and autophagy:* it is not clear whether cores develop because mitochondria move away from the core or whether mitochondria within the core are destroyed. In general, aspects of mitochondrial movement, fission and fusion in the cell are Ca^{2+}-regulated, however, almost nothing is known about the potential mobility or quality control of the mitochondrial population in muscle. Dysfunctional mitochondria are removed from the cell by the process of autophagy which may be activated by oxidative stress [44]. Ca^{2+} overload may increase mitochondrial free radical generation, while impaired oxidative phosphorylation or mPTP opening decrease mitochondrial potential, also triggering autophagic removal of mitochondria.

Thus several different mechanisms present themselves as potential contributors to the pathophysiology of *RYR1*-related myopathies. Characterizing and differentiating between these mechanisms will be an important first step in identifying novel therapeutic strategies.

John Lueck (Iowa, USA) presented work from Robert Dirksen's laboratory (in collaboration with Angela Dulhunty's group), focusing on altered RyR1 function and Ca^{2+} cycling in skeletal muscle during development and in myotonic dystrophy type 1 (DM1).

The *RYR1* gene undergoes alternative pre-mRNA splicing during development. One of the many pathomechanisms of DM1 is inappropriate expression of neonatal splice isoforms of many RNAs. The juvenile *RYR1* splice isoform (ASI(−)) lacking exon 70 (Ala^{3481}-Gln^{3485}) is the major splice isoform expressed in skeletal muscle of DM1 patients [45]. Effects of alternative *RYR1* splicing on steady-state intracellular Ca^{2+} homeostasis and voltage-gated Ca^{2+} release during E–C coupling were characterized following expression in myotubes derived from RyR1-null (dyspedic) mice. Expression of ASI(−) resulted in depolarization-induced Ca^{2+} release that is enhanced by >50% compared to that of the adult RyR1 isoform (ASI(+)), with no significant change in or voltage dependence of Ca^{2+} release, DHPR L-type currents or SR Ca^{2+} content. Consistent with results from bilayer experiments, the addition of the RyR1 agonist 4-chloro-*m*-cresol on ASI(−) expressing myotubes resulted in reduced Ca^{2+} release compared to ASI(+) expressing myotubes, suggesting reduced ASI(−) channel activity. These findings suggest that the ASI(−) region may contribute to an inhibitory module of RyR1 and enhance orthograde coupling, suggesting that aberrant *RYR1* splicing and subsequent downstream changes in Ca^{2+} homeostasis may contribute to muscle weakness and wasting in DM1 skeletal muscle. Furthermore, the ASI(−) splice variant is the predominant RyR1 isoform in skeletal myotubes; this ought to be considered when interpreting the effect of specific mutations on release channel function in murine and human myotubes as they are expressed primarily in the context of ASI(−).

Jim Dowling discussed the emerging interrelationship between *RYR1* and centronuclear myopathies (CNMs). In addition to *RYR1*, there are four other genes with mutations implicated in CNM, namely *MTM1*, *DNM2*, *BIN1*, and *MTMR14*. The respective gene products – myotubularin, MTMR14, dynamin 2 and amphiphysin 2 – are all regulators of membrane traffic; *MTM1* and *MTMR14* are specifically involved in the regulation of phosphoinositide metabolism, acting as 3-position phosphatases.

MTM1 knockdown in the zebrafish results in disorganization of the triad structure and in impaired E–C coupling [46]. Corroborating results in both the mouse and canine models of myotubularin dysfunction support the fact that *MTM1* is required for the proper formation of the T-tubule/SR interface as well as for normal E–C coupling [47,48]. Furthermore, triad abnormalities are present in human CNM biopsy samples of all subtypes [49]. In addition, recent studies on MTMR14 function in zebrafish and mouse reveal that it also is required for proper E–C coupling [50,51]. MTMR14 does not dramatically alter triad structure but is likely to disturb RyR1 regulation via abnormalities in the levels of PI3,5P2. Data, both published and unpublished, support the concept that direct RyR1-PI3,5P2 binding potentiates Ca^{2+} flux through the channel [51].

Future experimentation is geared toward determining the mechanism underlying the role of CNM proteins in the formation of the triad, and further exploring the relevance of the interaction of PIPs and RyR1.

Ana Ferreiro (Paris, France) discussed oxidative stress as a potential common pathomechanism and therapeutic target in *SEPN1*- and *RYR1*-related myopathies.

Previous work by her group established that absence of selenoprotein N (SelN) in *SEPN1*-related myopathies is associated with increased basal oxidant activity, susceptibility to exogenous oxidative stress and abnormalities in Ca^{2+} homeostasis in cultured human myotubes, which are compatible with redox dysregulation of RyR1. In addition, markers of oxidative stress and reduced SelN-devoid muscle cell survival in oxidant conditions are fully restored by *N*-acetylcysteine (NAC) [52]. The existence of a clinical and pathological overlap between *SEPN1*- and *RYR1*-related core myopathies and the proposed interaction between SelN and RyR1 suggest that both types of core myopathies share common pathomechanisms. One of these could be oxidative stress, a hypothesis supported by the fact that RyR1 is emerging as a paradigm of a redox-regulated ion channel [53]. Ana Ferreiro also reported on studies exploring oxidative stress as a therapeutic target in *RYR1*-related myopathies. Cultured primary muscle cells from eight patients with dominant or recessive *RYR1* mutations, associated with either CCD or MHS, are being analyzed using microarray, qRT-PCR, western blot and pharmacological studies to analyze production of reactive oxygen species by different Ca^{2+}-dependent sources. Preliminary data suggest that RyR1 defects are not associated with abnormal expression of selenoprotein or other antioxidant-protein encoding genes. Further studies, including *ex vivo* therapeutic tests, are currently in progress.

9. Potential therapeutic approaches in *RYR1*-related myopathies

Erwin Hauser, a 52-year-old Paediatric Neurologist affected by CCD due to a heterozygous *RYR1* missense mutation [G14582A] [54] reported his experience of self-medication with a number of pharmacological agents thought to affect muscle function and, more specifically, Ca^{2+} homeostasis. Therapeutic trials over 6 months with Diltiazeme, Carnitine and Creatine did not have any effects; however an increase in strength and endurance was noted following medication with the beta-adrenergic agents Clenbuterol, Salbutamol and Dantrolene.

Following commencement of Dantrolene at a dose of 25 mg twice daily (body weight 70 kg), the distance covered on a stationary exercise bike measured daily under standardized conditions increased significantly. There was no added effect when the Dantrolene was increased to 100 mg twice daily. A further increase of maximum cycling distance was noted when Clenbuterol was added at a dose of 40 mcg twice daily. There was also subtle functional improvement with respect to climbing stairs, walking, rising from a sitting position and decreased falls. Discontinuation of Dantrolene resulted in functional decline and transient increase in CK levels up to 2500 IU/l, followed by improvement after the medication was reinitiated. This personal trial with Dantrolene and Clenbuterol has now been continued for 5 years and resulted in sustained functional improvement without side-effects.

10. Natural history, outcome measure and clinical trial readiness

Francesco Muntoni (London, UK) reported on an UK registry for *RYR1*-related and other congenital myopathies. This project is being pursued in collaboration with the Muscular Dystrophy Campaign (MDC) and will parallel existing initiatives concerning Duchenne Muscular Dystrophy (see North Star Project http://www.muscular-dystrophy.org/how_we_help_you/for_professionals/clinical_databases). A prospective clinical assessment tool is being developed which will encapsulate both key medical and functional aspects relevant for ambulant and non-ambulant individuals. This initiative corresponds to ongoing efforts to establish a prospective assessment tool for congenital muscular dystrophies, developed by the London group in collaboration with the MDC, Cure CMD and NIH (Carsten Boennemann).

During the ensuing discussion it was also stressed that, in contrast to MHS-associated *RYR1* mutations, currently there is no single regularly updated repository for myopathic *RYR1* mutations. This complicates both the interpretation of clinical mutation analysis and is an obvious obstacle for translational research. As the first step to address this bottleneck, the clinical and genetic groups

attending the meeting discussed sharing their existing *RYR1* mutational databases. It was also discussed that efforts should be put in identifying a central and publically available repository for all myopathic *RYR1* mutations, such as for example the Leiden database (see http://www.dmd.nl/nmdb/home.php?select_db=RYR1).

11. Conclusions

The phenotypical spectrum of *RYR1*-related myopathies has continuously expanded. Recent work has resulted in important new insights into the diagnostics and pathophysiology of these conditions with strong potential for translational research and therapy development. In addition, secondary disturbance of RyR1 in other neuromuscular disorders and the impact of disturbed RyR1 function in non-muscle cells are emerging themes likely to attract ongoing interest. Development of patient registries and natural history studies will be important to support trial readiness in this common group of congenital myopathies.

12. Workshop participants

Carsten Boennemann (Philadelphia, USA)
Nigel Clarke (Westmead, Australia)
Valerie de Crescenzo (Worcester, USA)
James Dowling (Ann Arbor, USA)
Victor Dubowitz (London, UK)
Michael Duchen (London, UK)
Baziel van Engelen (Nijmegen, The Netherlands)
Julien Faure (Grenoble, France)
Ana Ferreiro (Paris, France)
Erwin Hauser (Vienna, Austria)
Luc Heytens (Antwerp, Belgium)
Heinz Jungbluth (London, UK)
John Lueck (Iowa City, USA)
Joel Lunardi (Grenoble, France)
Gerhard Meissner (North Carolina, USA)
Francesco Muntoni (London, UK)
Susan Treves (Basel, Switzerland)
Werner Melzer (Ulm, Germany)
Feliciano Protasi (Chieti, Italy)
Francesco Zorzato (Ferrara, Italy)

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