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Gene therapies for RyR1-related myopathies

Isabelle Marty, Mathilde Beaufils, Julien Fauré and John Rendu

Abstract

Myopathies related to variations in the *RYR1* gene are genetic diseases for which the therapeutic options are sparse, in part because of the very large size of the gene and protein, and of the distribution of variations all along the sequence. Taking advantage of the progress made in the gene therapy field, different approaches can be applied to the different genetic variations, either at the mRNA level or directly at the DNA level, specifically with the new gene editing tools. Some of those have already been tested *in cellulo* and/or *in vivo*, and for the development of the most innovative gene editing technology, inspiration can be sought in other genetic diseases.

Addresses

Univ. Grenoble Alpes, INSERM, Grenoble Institut Neurosciences, U1216, CHU Grenoble Alpes, Grenoble, France

Corresponding author: Marty, Isabelle (Isabelle.marty@univ-grenoble-alpes.fr)

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Keywords

Ryanodine receptor, Muscle contraction, Myopathy, Therapy.

Introduction

Type 1 ryanodine receptor (RyR1) is the main intracellular calcium channel in skeletal muscle. In association with the voltage-activated calcium channel dihydropyridine receptor (DHPR), it constitutes the core of the calcium release complex (CRC) responsible for the intracellular calcium release inducing muscle contraction [1]. RyR1 function is tightly regulated by post-translational modifications such as phosphorylation, S-nitrosylation, oxidation as well as by regulatory proteins, and by intracellular ion concentrations [2]. Mutations in the *RYR1* gene can directly alter the channel function of the protein (gain-of-function or loss-of-function), or induce a reduction in the amount of protein [1,3], which results in altered cytosolic calcium homeostasis. Many different myopathies have been associated with *RYR1* pathogenic variations such as Central Core Disease (CCD), Multi-mini core Disease (MmD), Centronuclear Myopathy (CNM), Congenital fiber type disproportion (CFTD), and they are now referred to as "RyR1-related myopathies" or RyR1-RM. Therapies for RyR1-RM are limited by a number of specificities of the *RYR1* gene and protein, among which the size of the gene (15 kb for the transcript) and the protein (more than 5.000 amino acids), forming a homotetramer of more than 2 MDa. Two therapeutic options are currently explored: pharmacological therapies, using chemical molecules, and gene therapy, schematically encompassing DNA- or RNA-directed therapies as classified by the European Medicines Agency and the US Food and Drug Administration. Both therapeutic strategies have their own specificities, which make their strength and weakness. Generally speaking, pharmacological therapy is performed usually with a small chemical molecule, provided regularly (every day or every week), orally or by intravenous injection. Pharmacological therapy targets part or all the downstream pathophysiological mechanisms. Gene therapy is usually performed with a large DNA/RNA molecule, given once or few times to the patients. Gene therapy targets directly the affected gene or its products, upstream of the different pathophysiological mechanisms, and therefore its action covers a large spectrum of consequences that can theoretically all be reversed by the same treatment. Pharmacological approaches are the only therapies currently in clinical trials for RyR1-RM. A randomized, double-blinded, placebo-controlled trial (phase I/II) has been recently completed with an antioxidant treatment (*N*-acetylcystein), which unfortunately did not reduce the previously identified elevated oxidative stress, nor significantly improve the physical activity of the patients [4]. An ongoing trial uses a so-called Rycal molecule (S48168) regulating the RyR1 channel function (ClinicalTrials.gov identifier NCT4141670, [5]), to reduce the calcium leak resulting from a subset of pathogenic variations. In addition to pharmacological therapy, gene therapy now appears as an attractive solution for those genetic diseases. Indeed, the use of pharmacology is appealing because it is easy to implement (for example when the molecule is provided orally such as NAC or S48168), easy to interrupt in case

of adverse side effects, and independent of a precise mutation. But it can present a low efficiency (possibly not all the pathophysiological mechanisms will be corrected) and the need for long term/permanent treatment. On the other side, gene therapy appears as a single, highly efficient (as the target will be upstream of all the consequences of the variation) and definitive treatment, but as such has to be fully mastered to ascertain its safety before being implemented in any human patient, as it cannot be reversed in case of adverse side effect. In addition, the biological risk has to be taken into account (for example with viral vectors). Gene therapy is in very rapid evolution, and this review is dedicated to the most recent developments in the field and their potential application to RyR1-RM. The first gene therapy products are now commercial for Spinal Motor Atrophy-SMA [6], and are in clinical trial for Duchenne Muscular Dystrophy-DMD [7]. Thus, inspiration for RyR1-RM gene therapy is sought in these two neurodegenerative diseases, all the approaches presented in this review for RyR1-RM being at best at the preclinical stage in mouse models. The denomination "gene therapy medicinal products" encompasses both strategies modifying mRNA, such as splice modulation and exon skipping, and therapies modifying the DNA, such as gene replacement, gene editing, and gene modulation. One important issue for neuromuscular diseases is the ability to target all the muscles of the body, and especially the respiratory muscles (diaphragm and intercostal muscles) which are among the less accessible to injection. This issue is currently overcame by the systemic delivery of viral vectors such as adeno-associated virus (AAV) but the systemic delivery requires the use of high viral vector doses thus increasing exposure/risk of treatment-emergent adverse events related to vector.

Therapy at the mRNA level

RYR1 being a very large gene, it is incompatible with the current viral vectors packaging ability. The first gene therapy approaches aiming to correct *RYR1* pathogenic variations were developed at the mRNA level, thanks to the use of siRNA or antisense oligonucleotides (AON).

A- depletion of mutant allele with siRNA

In two mouse models with dominant *RYR1* mutations, the specific *in vivo* knockdown of the mutant allele has been performed using mutation-specific siRNAs electroporated in the *flexor digitorum brevis* muscle [8]. Although successful, this approach is limited by the incorporation of the siRNA in all the targeted muscles, and thus restricted to the muscles accessible to injection and electroporation. In addition, the restoration of muscle function reflects the efficiency in the knockdown of the mutant allele, and requires optimization steps for each mutation/patient with patient-specific siRNAs.

B- splicing modulation with AON

Antisense oligonucleotides (AONs) have also been used to modulate *RYR1* splicing, a successful strategy in the case of pathogenic variation altering the splicing and responsible for the presence of an additional exon [9]. The AONs modulate the recognition of splice sites by binding at the level of the primary transcript by sequence complementarity. The resulting steric hindrance blocks the recognition and fixation of splicing effectors to the sites, thus preventing inclusion of the targeted exon in the final mRNA. For increased efficiency, the sequence of the AON was fused to the sequence of the U7 snRNP and integrated in a viral vector [9]. Such a strategy has been extensively developed for another myopathy, the DMD, and is among the most promising strategy for this disease to skip an exon containing a premature STOP codon and restore the reading frame, resulting in the production of a shorter but functional protein. A long term phase II clinical trial for the skipping of dystrophin exon 53 in eligible patients [10] has recently demonstrated the efficacy of this approach.

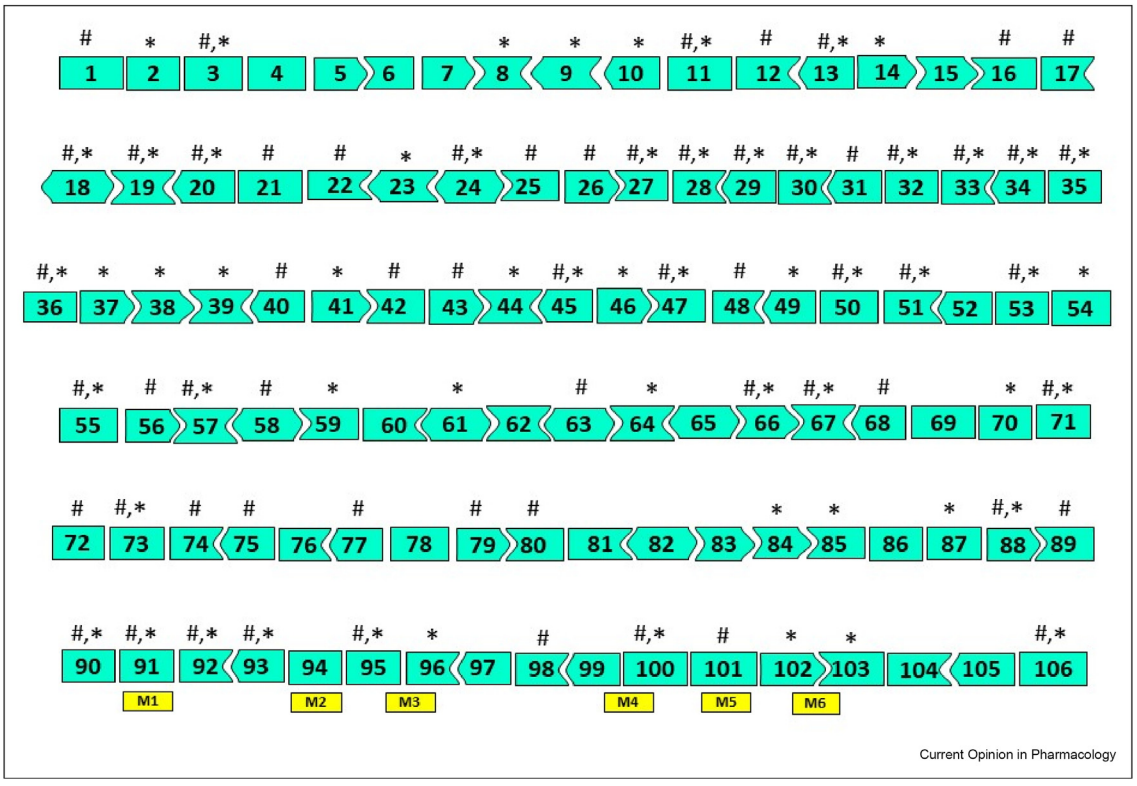
Unfortunately, a similar strategy can only be applied to RyR1-RM patients with the presence of an additional exon induced by the variation, because none of the 106 exons of the *RYR1* gene, beside the two alternative exons 70 and 83, is dispensable. In addition, the skipping of many of the exons will disrupt the reading frame or induce deletion of important functional/structural domains (Figure 1). To date, no truncated and nonetheless functional RyR1 has ever been identified.

C- exon retention

As exons can be skipped, a reverse approach was also developed to force the retention of targeted exons. When a variation abolishes a consensus donor splice site, a modified U1 snRNP tailored to bind the mutated sequence can force the splicing machinery to include the exon in the transcript. Because U1 snRNP fixation is the first step of the splicing process, its binding will trigger the recruitment of the whole spliceosome. This approach is only possible for variations in donor splice sites, and requires a specific design of the modified U1 for each variation [11]. Based on ClinVar *RYR1* specific database [12,13], donor splice site variations represent more than 53% of the splice variants, and constitute about 6% of the total variants (Figure 2).

Splice modulation therapy has also been developed to increase the recognition of splice sites by the use of AONs targeted to splicing regulatory elements such as silencers. The steric hindrance due to AON-mRNA duplex will prevent the recognition of the silencer element involving an increased exon splicing. This strategy is used for SMA to restore a correct transcript [14]. Future research will perhaps unveil this kind of mechanism for the *RYR1* gene but to date regulatory

Figure 1



Schematic representation of the reading frame of *RYR1* exons. The 106 exons are represented with a different shape depending on the splice site (after the first, the second or the third nucleotide of each codon). The transmembrane domains (M1 to M6) are represented by yellow squares. The exons containing STOP (*) or frameshift (#) variations (leading to disruption of the allele) identified in patients are represented respectively with an asterisk or hashtag above. In order not to disrupt the reading frame, exon skipping can only be considered if it leads to connection between exons with the same shape.

elements have not been identified nor studied in this gene.

Although promising initial results were obtained with AON and siRNA, no further studies have made use of these tools in RyR1-RM, in contrast with SMA and DMD where they constitute one of promising therapeutic options.

D- trans-splicing

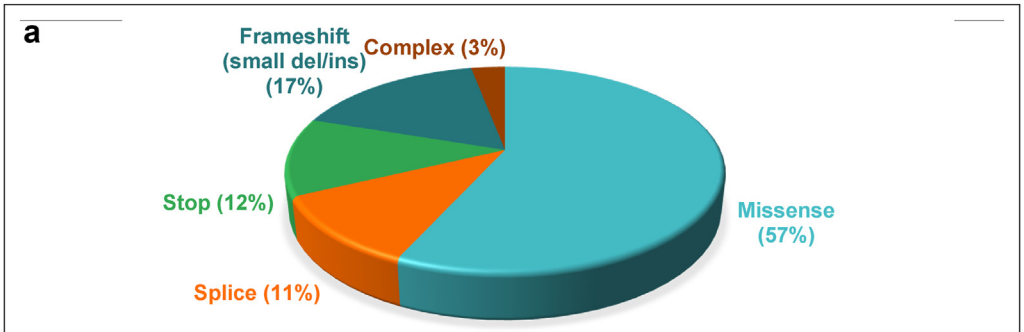
Another splice modification, based on a physiologically rare splicing process that occurs between two different mRNA molecules, is called trans-splicing. It occurs naturally in trypanosomes, *Caenorhabditis Elegans* and at a low rate in *Homo Sapiens* [15], and allows the joining of exons from more than one pre-mRNA transcript to form a single chimeric mRNA molecule. Thus in a therapeutic perspective, any mutated mRNA segment could be replaced by an exogenous non-mutated one (Figure 3). The major strength of trans-splicing is the possibility to target many patients with the same molecule, to correct all the variations present in the same region. Although encouraging results have been observed for DMD [16], its current use is limited by a very low efficiency.

Therapy at the DNA level

A- gene replacement

Gene replacement for RyR1-RM would consist in the introduction in all the muscles of a new functional copy of the *RYR1* gene, using a viral vector. Although very attractive, this strategy is facing a large number of issues specific to the *RYR1* gene. Due to the size of the *RYR*1 transcript and the encapsidation capacity of viruses used for therapy (AAV have a limited packaging capacity of 4.7 kb, [17]), it is currently not possible to consider integrating the full length *RYR1* coding sequence in any viral vector beside herpes simplex-derived vectors with a packaging capacity up to 150 kb [18]. Alternative strategies are however under development to express large genes in AAV [17]. Based on the ongoing clinical trial for DMD using a mini- or micro-dystrophin [19], the expression of a truncated RyR1 protein could be considered. Nevertheless no functional truncated RyR1 has ever been identified, and the reduction required in the *RYR1* sequence would be too large and damaging

Figure 2



b	allele disruption	exon retention or skipping	Trans splicing	gene replacement	base and prime editing
Missense	X (only for dominant variant)		X	X (recessive variant)	X
Splice		X	X	X	X
Stop			X	X	X
Frameshift			X	X	X (only for prime editing)
Complex			X	X (recessive variant)	X (size dependent)

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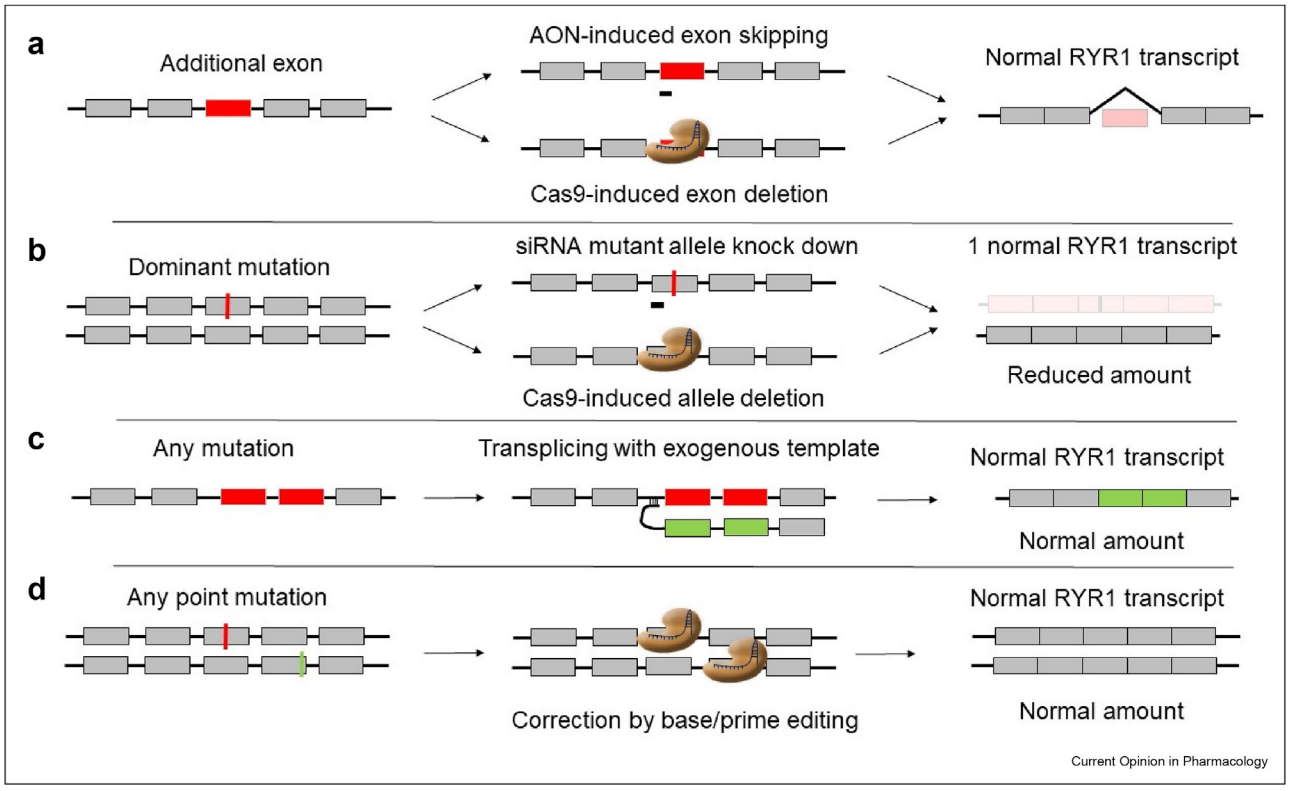
a- Pathogenic or likely pathogenic *RYR1* variant published in the databases. b- Gene therapy option(s) for each type of variation. As of June 2022, 766 variants were classified as pathogenic or likely pathogenic variants for RYR1 in the ClinVar and LOVD database. Among these, 435 were missense variants, 94 stop variants, 129 small deletions/insertions leading to frameshift, 85 splice variants (37 acceptor sites, 45 donor sites and 3 variations leading to pseudo exon inclusion).

(from 15 kb to 4.7 kb). Another alternative would be to split the *RYR1* sequence in multiple parts, each fitting an AAV, and rely on a fusion between the different segments by a trans-splicing mechanism, a strategy that has been successfully adapted to dystrophin in DMD [16,20,21]. This could be a promising approach, if the targeting efficiency of the viral vectors is high enough to ascertain that the different AAVs are all present within the same muscle fiber. In parallel to the development of optimized sequences for gene transfer, an active field of research is thus dedicated to the optimization of viral vectors. A recent breakthrough has been the development of the so-called Myo-AAV, which targets with high efficiency skeletal muscle, while its affinity for the liver is reduced [22], allowing the use of reduced amounts of viral vectors compared to AAV9. This tool could be the missing link for the development of an efficient gene therapy with reduced side effects (an ongoing clinical trial has been stopped after the death of 3 patients affected by X-linked myotubular myopathy, following hepatotoxicity related to high dose of AAV8, [23]). Another bottleneck in gene replacement therapies for RyR1-RM is to question the expected benefits in presence of dominant mutation because the dominant negative effect of the mutant allele will not be erased. Thus such a strategy could only be interesting for recessive null variants, resulting from frameshift, nonsense or splice variants leading to nonsense mediated decay (NMD), and hypomorph missense mutant (leading to reduction in the amount of RyR1 protein). Altogether it could target at least 43% of pathogenic or probably pathogenic *RYR1* variants in the ClinVar and LOVD database (Figure 2), even more if recessive missense variants are taken into account, and therefore this approach is worth the energy put into its development.

B- gene editing

With the ground breaking discovery of CRISPR/Cas9 [24], gene editing, allowing for the deletion, modification, insertion, or replacement of a DNA segment, has rapidly emerged as the method of choice for genetic diseases, and specifically for RyR1-RM for which therapeutic options are limited. A number of steps still have to be fully mastered before using CRISPR/Cas9 gene editing as a safe and efficient therapeutic approach, but

Figure 3



The gene therapy options for the main classes of *RYR1* variations. The possible therapeutic interventions are presented for variations leading to an additional exon (**a**), dominant mutations (**b**), or any point mutation (recessive and dominant, **c** and **d**). Other mutations types can also be corrected by prime editing and are not represented in this figure for simplicity.

this research field is in rapid evolution and offers great promise for neuromuscular disorders [25]. As for gene replacement, the major issue is to integrate the CRISPR/Cas9 tools (the Cas9 nuclease and the guide RNA) into the muscle fibers, which will benefit from the improvement of viral vectors for muscle therapy [26]. As *Cas9* is much smaller than *RYR1*, its integration in muscle should be improved by the use of Myo-AAV [22], or by engineered virus-like particles [27].

Gene/allele disruption

The nuclease Cas9, once introduced into the cell, will induce a double strand break on a specific localization determined by the guide RNA (gRNA). This double strand break in muscle fiber will eventually lead to insertion-deletion by a repair mechanism so-called "Non Homologous End Joining (NHEJ)" resulting in the silencing of the targeted gene or in exon skipping in the targeted gene (the repair by the Homology Directed Repair-HDR mechanism is not discussed here because inefficient in the non-dividing muscle fiber).This approach has successfully been used in an *in vivo* proof of concept in mice for exon skipping in the dystrophin gene, leading to the deletion of mutant exon 23 causing the disease [28–30]; review by Chemello et al. [25]. As mentioned previously, exon skipping in the *RYR1* gene could only apply to additional exon(s), and thus this strategy could be used for a limited number of patients. In addition, it can be used to delete a mutant allele in presence of a dominant mutation, similar to what has been done with siRNA [8].

Base and prime editing

Base editing is an upgraded version of gene editing that enables the direct conversion of one target DNA base into another in a programmable manner, which is ideal for point mutation correction. It is performed with a base editor instead of a nuclease, allowing the editing of one base pair instead of the production of a double strand break (DSB), therefore DNA repair mechanisms are not involved [31,32]. Base editors exist in different subtypes: cytosine base editor, able to change a C for a T, and adenine base editor, able to change an A to a G [33]. Technological improvements have rapidly allowed the *in vivo* application of base editing to neuromuscular disease such as DMD [34,35]. The most recent improvement in the field has been the prime editing technology [36], which allows all base changes in

addition to small insertion and deletions [33]. The improvement in the prime editing system is progressing at such a pace that the therapeutic application can be foreseen in a very near future [37]. RyR1-RM could benefit of such technology, and theoretically 97% of the *RYR1* variations could be corrected by prime editing (Figure 2), with only large size deletion or insertion impossible to correct to date.

Conclusion

After the explosion of genetic screening methods to identify variations in disease causing genes, the new research era is that of gene therapy. Initially sparse in part because of the size of the *RYR1* gene and its products, the number of therapeutic options for correction of variations responsible for RyR1-RM is rapidly expanding. Variations in the *RYR1* gene are spread all along the sequence, responsible for dominant or recessive disease, and only a few of them are recurrent (based on the LOVD and the ClinVar databases [12,13,38,39]). The question is not only to identify which therapy for which variation, but also how to move forward toward a less personalized medicine for all these private variations. Indeed, the neuromuscular diseases for which gene therapy is getting in clinical phases are the diseases with mutation hot spots or recurrence, such as SMA or DMD, i.e. pathologies in which one treatment can apply to many patients. Figure 3 depicts the gene therapy solutions for the most frequent variations types in the *RYR1* gene. Overall, a gene therapy could be considered for at least 97% of these variations, if not 100% (Figure 2).

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Conflict of interest statement

Nothing declared.

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** of outstanding interest

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