Trojan triplets: RNA-based pathomechanisms for muscle dysfunction in Huntington's disease

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In the early 1990s, several genetically inherited disorders were found to result from trinucleotide repeat expansions (increases in the length of normally short regions of trinucleotide CNG repeats, e.g., CAG, CTG, CCG, and CGG). Huntington's disease (HD; CAG expansions), fragile X syndrome (CGG expansions), fragile XE syndrome (CCG expansions), myotonic dystrophy type I (DM1; CTG expansions), spinal-bulbar muscular atrophy (CAG expansions), and spinocerebellar ataxia (CAG expansion) were all shown to originate from the expansion of a single CNG triplet in either coding or noncoding regions of the affected gene (Budworth and McMurray, 2013). In each case, the affected gene carries a low number of trinucleotide repeats in nonaffected individuals. However, disease symptoms appear when expansion of the repeat length increases above a certain threshold because of repeat instability and errors in genomic maintenance. Thus, in analogy to a Trojan horse, triplet repeat expansions covertly localized within the genome are uniquely positioned to unleash their toxicity from deep within the cell. Moreover, because of repeat instability, the number of repeats increases throughout the lives of affected individuals, and the increased repeat length can then be passed on to the next generation. The phenomenon of an increase in the number of expanded repeats, resulting in an acceleration of disease onset and an increase in symptomatic severity in successive generations, is referred to as genetic anticipation. In this issue, Miranda et al. quantify changes in ion channel expression in adolescent skeletal muscle from a mouse model of HD containing a CAG repeat expansion. They find that CAG expansion causes a shift in gene expression and mRNA splicing toward embryonic/neonatal forms, consistent with a defect in postnatal muscle maturation in these mice.

Expansion of a normally short CAG repeat in the 5′ coding region of the *huntingtin* (*HTT*) gene beyond the nonsymptomatic number of 36 repeats results in a polyglutamine expansion that leads to detrimental effects in cells that express the mutant gene (MacDonald et al., 1993). Although HD is normally categorized as a neurodegenerative disorder because of its profound negative impact on cognitive and motor functions, the *HTT* gene is expressed widely in mammalian cells (Sharp et al., 1995). HD is characterized by a restlessness of skeletal muscle during early stages of the disease that later progresses to include more severe motor dysfunctions, including chorea, rigidity or rigor, and dystonia. R6/2 mice, which are transgenic for the 5′ region of the human *HTT* gene and contain an expansion of ∼120 CAG repeats, exhibit a progressive neurological phenotype that mimics many of the features of juvenile-onset HD in humans, including chorea, involuntary movements, tremors, and epileptic seizures. Skeletal muscle fibers from R6/2 mice display alterations in gene expression (Luthi-Carter et al., 2002) that affect metabolism, mitochondrial integrity, and normal muscle differentiation, which leads to a loss of strength and muscle atrophy (Zielonka et al., 2014). In addition, muscle fibers from R6/2 mice at an advanced stage of disease progression exhibit increased muscle fiber excitability, caused by marked reductions in resting membrane chloride and potassium conductances (Waters et al., 2013). The reduced conductances were shown to originate from the reduced expression of muscle chloride channels (ClC-1 or the *Clcn1* gene) and inward rectifier potassium channels (Kir or the *Kcnj2* gene). In the same study, similar drug-induced reductions in ClC-1 and Kir conductances increased membrane excitability to levels sufficient to result in spontaneous reexcitation of the membrane after stimulation, resulting in myotonic runs of action potentials. Although down-regulation of *Clcn1* mRNA in muscle occurs shortly after denervation (Klocke et al., 1994), this is unlikely to account for the reduced muscle chloride conductance (G_{Cl}) in R6/2 mice, as motor neurons in R6/2 mice exhibit normal innervation and the ability to regenerate (Ribchester et al., 2004).

DM1 is caused by a CTG repeat expansion located in the 3′ untranslated region of the dystrophia myotonica protein kinase (DMPK) gene (Budworth and McMurray,

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2013). DM1 pathogenesis is widely thought to represent an RNA-transdominant mechanism whereby DMPK transcripts containing expanded CUG repeats accumulate in the nucleus and sequester critical protein regulators of RNA processing (e.g., muscleblind-like protein 1 [MBNL1]) that are required for proper splicing of a specific subset of developmentally regulated genes (Mankodi et al., 2002). Consistent with this pathomechanism, transgenic mice that exhibit high and muscle-specific expression of mRNA for human skeletal actin (HSA), harboring ∼250 CUG repeats in the 3′ untranslated region of the gene (HSA^{LR} mice), exhibit prominent myotonia, sequestration of MBNL1 proteins into nuclear inclusions, and aberrant splicing of *Clcn1* pre-mRNA. The major *Clcn1* splicing change is the inclusion of a cryptic exon (exon 7a) that causes a frame shift and premature termination in the ClC-1 protein and accelerated decay of the mRNA (Lueck et al., 2007). As a result, ClC-1 expression/function is markedly reduced, and membrane excitability is increased in muscle fibers from these mice. In DM1, expanded CUG repeats in the DMPK mRNA form hairpin structures that sequester MBNL1 proteins, thereby functionally disabling these proteins from executing developmentally regulated splicing events (Mankodi et al., 2002). Similar to the aberrant inclusion of exon 7a in DM1, an increase in *Clcn1* mRNA containing exon 7a, and a parallel decrease in ClC-1 function, is also observed in muscle fibers from end-stage R6/2 mice (Waters et al., 2013). The similar findings regarding altered *Clcn1* splicing and ClC-1 function from mouse models for two separate trinucleotide repeat expansion disorders suggest a common pathogenic disease pathway (or at least overlap) between HD and DM1.

Effects of reduced G_{Cl} on muscle excitability in R6/2 HD mice

Muscle fibers from late-stage R6/2 mice exhibit an ∼60% reduction in G_{Cl} (Waters et al., 2013). Although this reduction in G_{Cl} results in an increase in membrane impedance, and thus membrane excitability, the reduction alone is unlikely to be sufficient to produce myotonia. Results from both experimental data and mathematical modeling indicate that G_{Cl} must be reduced by >80% to result in a myotonic phenotype. Consistent with this finding, there are no studies of myotonia in the R6/2 mice or from HD patients, indicating that the observed hyperexcitability is not sufficient to cause myotonia. Also, to preserve muscle excitability during continuous action potential firing, G_{Cl} is reduced by as much as 70% in working muscle (Pedersen et al., 2016) without resulting in myotonia. About 80% of the resting membrane conductance is contributed by G_{Cl} in skeletal muscle. This large G_{Cl} serves to dampen increased excitability during repetitive action potential firing, which results from potassium flux from the myoplasm into the transverse tubules (t-tubules) through voltage-gated potassium channels during repolarization. Because muscle fibers have an extensive network of narrow, diffusion-limited t-tubules of small volume, K^+ can accumulate to levels sufficient to shift the potassium equilibrium potential (E_K) to a more positive value. Because approximately four fifths of the total membrane area of skeletal muscle is contributed by t-tubules, the impact of K^+ accumulation on E_K during repetitive firing is substantial. To counteract the impact of this shift in E_K on membrane potential, Cl^- moves into the muscle fiber through ClC-1 channels to short circuit the depolarization in E_K, and thus stabilize the membrane potential.

In working muscle, a consequence of the increased excitability during repetitive action potential firing is the inactivation of some voltage-gated Na^+ channels. To maintain sufficient excitability under these conditions, an activity-dependent reduction in G_{Cl} increases muscle excitability to counteract sodium channel inactivation. In this way, excitability is maintained during repetitive firing, whereas sufficient repolarization between each action potential is achieved by balancing G_{Cl} to levels that allow the repeated firing of action potentials but are still large enough to combat spontaneous activity (Pedersen et al., 2016). In line with this, contractile endurance in rat muscle is optimal at a reduction in G_{Cl} of ∼70% (de Paoli et al., 2013), but becomes myotonic if G_{Cl} is reduced by >80%.

Reductions in G_{Cl} and G_K in muscle precede motor symptom deficits in R6/2 mice

In this issue, Miranda et al. (2017) significantly extend the findings of Waters et al. (2013) by carefully quantifying changes in *Clcn1* and *Kcnj2* mRNA levels with ClC-1 and Kir2.1 current density in muscle throughout adolescent development in R6/2 mice. The results show that increased aberrant *Clcn1* mRNA splicing (i.e., increased exon 7a inclusion) and reduced ClC-1 and Kir function precede the onset of detectable motor symptoms. Muscle fibers from presymptomatic and late-stage R6/2 mice were also found to exhibit a marked reduction in membrane capacitance that likely reflects a reduction in the contribution of the t-tubule system. In addition to the observed defects in chloride and potassium membrane conductances (G_{Cl} and G_K, respectively) and membrane capacitance, maturation of fast-twitch tibialis anterior muscle was reduced in late-stage R6/2 mice, as evidenced by significant decreases in mRNA levels for fast myosin IIx and IIb that occurred in parallel with an increase in the expression of the neonatal (and possibly embryonic) myosin heavy chain isoform. Interestingly, a modest increase in *Clcn1* exon 7a inclusion, reduction in ClC-1 conductance, and decrease in the expression of fast myosin IIb were also observed in muscle from 1-yr-old Q175 mice, a late-onset

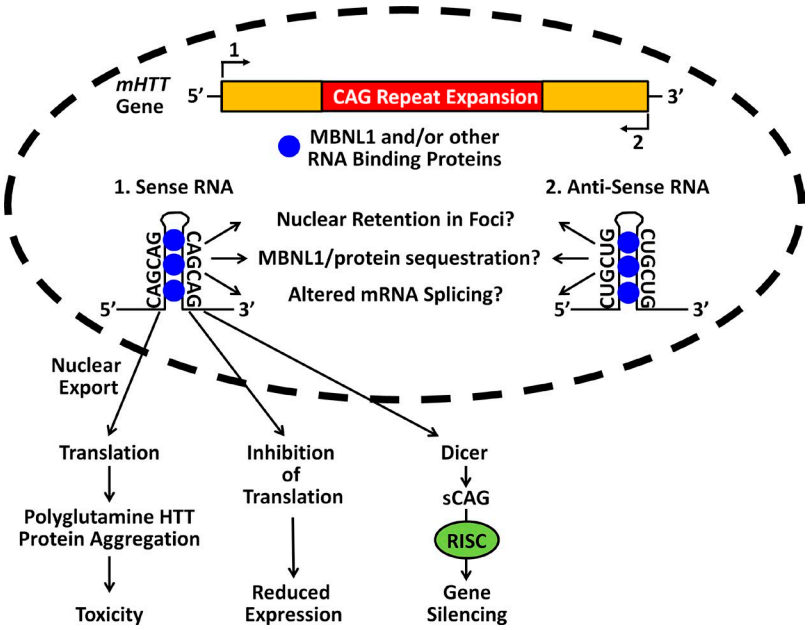

Figure 1. **Schematic summarizing potential protein aggregate and RNA-based mechanisms for muscle dysfunction in HD.** RISC, RNA-induced silencing complex. This figure was adapted from Martí (2016).

mouse model of HD. Collectively, these observations present a picture in which the expression of expanded CAG repeat–containing transcripts in skeletal muscle promotes a shift in muscle gene expression and mRNA splicing toward a more embryonic/neonatal gene program, consistent with a defect in postnatal muscle maturation in R6/2 mice.

As discussed above (see the first paragraph of the previous section), the ∼60% reduction of G_{Cl} in muscle fibers from late-stage R6/2 mice (Waters et al., 2013; Miranda et al., 2017) alone is unlikely to be sufficient to produce myotonia. In addition, given the central role of an intact t-tubule system and K^+ accumulation for hyperexcitability, the observed reduction in membrane capacitance would further serve to reduce the likelihood for myotonia in R6/2 mice. However, assuming the absence of compensatory mechanisms that limit the normal activity-dependent reduction in G_{Cl} that occurs upon the onset of activity (Pedersen et al., 2016), the reduced resting G_{Cl} (and G_K) in R6/2 muscle coupled with normal activity-dependent reduction in G_{Cl} may together be sufficient under some conditions to set the stage for myotonia. Future studies are needed to assess the susceptibility of different skeletal muscles of R6/2 mice for developing activity-dependent myotonia.

Possible RNA-based mechanisms for altered muscle function and maturation in R6/2 mice

Muscle from R6/2 mice exhibits reduced ClC-1 and Kir conductances (Waters et al., 2013; Miranda et al., 2017), as well as increased expression of neonatal/embryonic myosin isoforms (Miranda et al., 2017). The reduction in ClC-1 conductance is caused in part by increased inclusion of the same embryonic exon 7a as that observed in HSA^{LR} mice (Lueck et al., 2007). Thus, similar to DM1, RNA-based posttranscriptional mechanisms may also contribute to the muscle phenotypes observed in R6/2 mice. Consistent with this, expanded CAG repeats bind and sequester MBNL1 proteins (Ho et al., 2004), and nuclear foci containing MBNL1 have been identified in fibroblasts from HD patients (Mykowska et al., 2011). Although transgenic expression of extended CAG repeats (200) in mouse muscle results in myopathic changes (including internalized nuclei, fiber splitting, and loss of fiber-type distinction), as well as nuclear inclusions containing Mbnl1 and expanded CAG repeat RNA, no Mbnl1-dependent splicing changes were detected, including *Clcn1* mRNA (Hsu et al., 2011). One of the more interesting findings in the study by Miranda et al. (2017) is that, whereas muscle from R6/2 mice exhibits nuclear foci of Mbnl1 and CAG repeat RNA, the two rarely colocalize. This is entirely different than what is found in DM1, where Mbnl1 and CUG repeat RNA almost perfectly colocalize within nuclear foci. This apparent discrepancy in the muscle of R6/2 mice could be explained by recent evidence for bidirectional transcription producing antisense transcripts with expanded CUG repeats from the *HTT* gene loci in HD patients (Chung et al., 2011). Although the antisense CUG repeat transcripts are expressed at lower levels, their higher affinity for binding Mbnl1 proteins could potentially explain the lack of colocalization of Mbnl1 and CAG repeat RNA in muscle from R6/2 mice (Fig. 1).

Additional pathomechanisms could also contribute to muscle dysfunction in HD patients and R6/2 mice (Fig. 1). Because the CAG repeat expansion is located in the coding region of the huntingtin protein, potential abnormal functions produced by the mutant protein and its aggregates cannot be ruled out. Indeed, polyglutamine aggregates exhibit several toxic gain-of-function

effects, including transcriptional regulation, mitochondrial energy production, and calcium regulation. In addition to MBNL1 sequestration, other RNA-based pathomechanisms could also be in play. For example, expression of a transcript with an expanded CTG repeat in the 5′ untranslated region of a reporter gene was found to inhibit translation (Raca et al., 2000). If the expanded CAG repeat in R6/2 mice also inhibited translation, this could contribute to the reduction of functional ClC-1 and Kir channel expression that occurs in the absence of a decrease in pre-mRNA levels, as reported by Miranda et al. (2017). Finally, uninterrupted expanded CNG repeats that form hairpin structures are substrates for Dicer endonuclease–mediated cleavage into short double-stranded CNG repeat RNAs of 20–25 nucleotides in length. Indeed, short CAG repeat RNAs (sCAGs) have been detected in HD patients and are neurotoxic (Bañez-Coronel et al., 2012). Dicer-generated sCAG can also be loaded into the RNA-induced silencing complex (RISC) to silence complementary CUG-containing transcripts. However, the relative impact of expanded CAG repeat RNA on translation and gene silencing in the muscle of HD patients and R6/2 mice remains unknown.

What's next?

The results presented by Miranda et al. (2017) in this issue of *The Journal of General Physiology* indicate that detectable changes in *Clcn1* splicing, total membrane capacitance, and ClC-1 chloride and Kir potassium conductances occur in muscle of R6/2 mice even before the development of motor symptoms. At later stages, changes in myosin isoform expression, including increased expression of the neonatal myosin, are also observed in muscle from R6/2 mice. Although an increase in exon 7a inclusion in *Clcn1* mRNA likely contributes to the observed reduction in ClC-1 function in muscle fibers from R6/2 mice, the observed fractional increase of exon 7a in the muscle of even late-stage R6/2 mice is relatively modest (~12% of total) compared with that observed in the HSA^{LR} DM1 mouse model (~50%; Lueck et al., 2007). Thus, additional mechanisms for the observed reduction in G_{Cl} may be involved in R6/2 mice. Questions that remain to be answered include: What is responsible for distinct nuclear and myoplasmic Mbnl1 aggregates in R6/2 muscle? Is nuclear Mbnl1 sequestered by CAG or by antisense CUG repeat transcripts? What proteins are present in nuclear CAG foci? Do therapeutic interventions that have been successfully used in DM1 mouse models (e.g., exon-skipping morpholinos, Mbnl1 overexpression, small molecule inhibitors of Mbnl1 binding to expanded CUG repeat RNA, and knockdown of mutant transcript) rescue one or more of the different muscle phenotypes of R6/2 mice (e.g., Mbnl1 aggregation in nuclear foci, increase in exon 7a inclusion, reduction in G_{Cl}, G_K, and membrane capacitance, increase in excitability, and/or switch in myosin expression)? In this regard, Rué et al. (2016) recently found that treatment with antisense oligonucleotides complimentary to the CAG expansion reduced RNA toxicity, increased markers for striatal neurons, and reduced motor deficits in R6/2 mice. Importantly, many of the muscle phenotypes in R6/2 mice identified by Miranda et al. (2017) were found to appear before the development of motor deficits. Although HD, like most trinucleotide disorders, exhibits anticipation and an increased disease severity that is proportional to repeat burden (i.e., number of repeats and expression level), there are still large variations in the onset of symptoms. Thus, reliable early biomarkers of HD are needed to properly guide patient diagnosis, treatment, and care. As skeletal muscle is an abundant and readily accessible tissue from which biopsies are relatively easily obtained, muscle biomarkers of presymptomatic HD could serve as a powerful means of predicting disease onset and progression in high-risk individuals. By facilitating early detection of the presence and imminent danger posed by the "Trojan triplet" contained within, future treatments may be guided to maximize benefit to the patient. Finally, the findings presented by Miranda et al. (2017) add to the growing body of literature demonstrating that HD is not only a neurodegenerative disorder, but is also a systemic disease that involves both protein- and RNA-based pathomechanisms, and thus will likely require more than one line of treatment.

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