



Published in final edited form as:

Neuron. 2013 October 16; 80(2): 257–258. doi:10.1016/j.neuron.2013.10.010.

C9orf72 associated FTD/ALS: When less is more

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Abstract

Hexanucleotide repeat expansions in *C9ORF72* cause neurodegeneration in FTD and ALS by unknown mechanisms. A new report finds that these repeats trigger a pathogenic gain-of-function cascade that can be corrected by suppressing expression of the repeat transcript, paving the way for therapeutic strategies aimed at eliminating the toxic RNA.

Frontotemporal dementia (FTD) and Amyotrophic Lateral Sclerosis (ALS) are fatal neurodegenerative disorders that share overlapping pathologies, genetic causes and a lack of disease-modifying treatments(1). Precisely two years ago, adjoining papers in *Neuron* identified large intronic GGGGCC repeat expansions in a gene of unknown function, *C9orf72*, as a common genetic cause of both FTD and ALS (C9FTD/ALS)(2, 3). The discovery sparked great interest in the field, partly because the *C9orf72* expansion looked a lot like something scientists had seen before: the CUG and CCUG repeats that cause the common dominantly inherited muscle disease, Myotonic Dystrophy (DM).

Work over the past 20 years has demonstrated that repeat expansions in two genes, a CUG repeat in *DMPK* (in DM1) and a CCUG repeat in *ZF9* (in DM2), elicit dominantly inherited disease through a “toxic RNA” gain-of-function mechanism: the expanded repeats as RNA bind to splicing factors, inhibiting their normal functions. In DM1, for example, the expanded CUG repeat binds Muscleblind-Like (MBNL) RNA binding proteins, sequestering them in nuclear foci and causing abnormal splicing of key transcripts in muscle and brain. Mice lacking MBNL1 or MBNL2 recapitulate disease features of DM1, and conversely, boosting MBNL protein expression suppresses CUG repeat-associated toxicity in model systems (reviewed in(4)). These findings laid the groundwork for successful preclinical trials using antisense oligonucleotides (ASOs) to eliminate the toxic CUG repeat RNA in mouse models (5), with plans for a follow-up clinical trial in DM1 patients soon.

By contrast, how the GGGGCC repeat expansion triggers C9FTD/ALS is less clear for at least three reasons. First, the case for RNA toxicity in C9FTD/ALS is incomplete. Although GGGGCC RNA foci are present in disease tissues, it remains uncertain whether proteins are bound by the RNA repeat to a degree that would impair normal functions. Experiments that rescue disease features by overexpressing specific sequestered proteins or recapitulate disease features by knocking down the same sequestered proteins have not been reported. Second, expression of *C9orf72* mRNA in C9FTD/ALS patients is reduced by ~50% (3, 6) and the expanded repeat and neighboring CpG islands are hypermethylated(7), consistent with transcriptional silencing of the mutant allele and a potential loss of function model of disease pathogenesis. Third, a major cellular pathological hallmark of C9FTD/ALS, cytoplasmic aggregates that stain positively for the P62 protein, appear to result from protein translation through the hexanucleotide repeat (8, 9) via a recently discovered process known

as RAN (Repeat Associated Non-AUG) translation (14). RAN translation generates unconventional protein products from some disease-causing repeats, including at least CAG and CUG repeats in Spinocerebellar Ataxia Type 8 and CGG repeats in Fragile X-associated Tremor/Ataxia Syndrome (10, 11). In C9FTD/ALS, the GGGGCC repeat in all three reading frames generates dipeptide repeat-containing proteins that presumably are prone to aggregate.

So what is the toxic mechanism in C9ALS/FTD? Too much toxic RNA, too much RAN translated protein, or not enough C9orf72 protein? In this issue, Donnelly and colleagues (12) address this question using induced pluripotent stem cells (iPSC) derived from C9ALS/FTD patients and marshaling a wide range of techniques. They first established that C9ALS/FTD iPSC derived neurons exhibit three important pathologic features present in patients: decreased C9orf72 mRNA, nuclear and cytoplasmic GGGGCC RNA foci, and expression of at least one RAN product (Gly-Pro dipeptide), consistent with a previously published report(13). C9 iPSC derived neurons also exhibit enhanced sensitivity to glutamate excitotoxicity (long suspected as a contributor to selective neuronal vulnerability in ALS), and an altered transcriptional profile that partially overlaps with transcriptional changes observed in iPSC neurons derived from mutant SOD1 ALS patients and in C9 FTD autopsy tissues. (An intriguing peripheral observation is that the vast transcriptional differences in C9ALS/FTD neurons versus SOD1 neurons suggest that these two forms of ALS are quite different molecular beasts.)

In parallel, the authors used proteome arrays to identify 19 proteins that can associate with GGGGCC repeats *in vitro*, then focused on one identified protein, ADARB2, as a potential RNA target. ADAR proteins are intriguing candidates because they participate in RNA editing and are highly expressed in the nervous system. ADARB2 co-localizes with GGGGCC RNA foci in C9 iPSCs and in patient samples, and ADARB2 knockdown results in a decrease in RNA foci, suggesting that ADARB2 and the RNA repeat functionally interact *in vivo*.

The best insights into pathogenesis emerged when a series of ASO's were used to suppress C9orf72 RNA expression. Both repeat and non-repeat targeting ASOs led to significant reversals in many of the observed phenotypes in C9 iPS neurons, including suppression of glutamate-induced toxicity, reduction in RNA foci formation, and correction of a small subset of the observed transcriptional changes. Importantly, for at least one non-repeat associated ASO, these improvements occurred despite a further decrease in detectable C9orf72 mRNA expression.

Overall, these findings suggest that the expanded GGGGCC repeat triggers toxicity predominantly through a toxic gain of function rather than a loss of C9orf72 protein function. Consistent with this view, a recent study reported a patient homozygous for the C9 mutation who, outside of enhanced P62 inclusion burden and markedly decreased C9orf72 RNA expression (~25% of normal), displayed a FTD clinical phenotype resembling heterozygous carriers in the same family (14). Together, these studies support a model in which the expanded GGGGCC repeat, as RNA, and with or without associated RAN-translated proteins, is a driving force in C9 FTD/ALS disease pathogenesis. A critical

implication is that therapeutics targeting elimination of the repeat RNA in C9FTD/ALS patients are likely to be beneficial, though the impact of markedly and chronically lowering C9orf72 expression *in vivo* still remains to be determined.

Despite these advances, significant work remains. Although iPSCs offer significant advantages as models, the lack of *in vivo* context potentially can skew results and assumptions which still require validation in animal model systems. Similarly, the impact of C9orf72 loss over a longer time and in control neurons will be important next steps in the validation of ASO based therapeutic approaches. Moreover, the potential pathogenic role of RAN-translated peptides remains an open question. Although Donnelly et al demonstrate rapid resolution of RNA foci yet the continued presence of RAN-translated protein signal in C9 iPS neurons treated with ASOs, this result does not preclude a role for continually produced RAN products in ongoing neurotoxicity. Indeed, whether newly synthesized soluble oligomers versus higher order aggregates are toxic to neurons remains unresolved in many neurodegenerative diseases, and is only now being addressed for RAN translated proteins. Further, while several groups have identified GGGGCC repeat associated RNA binding proteins (15-17), and the ADARB2 studies represent an encouraging step, the field now needs to demonstrate that sequestration of specific factors is necessary and sufficient to recapitulate aspects of the clinical syndrome. Finally, these types of iPSC models from patients with ALS or FTD may allow us to answer a decades old question related to what factors drive selective and differential neuronal vulnerability. Use of different classes of iPSC neurons derived from different clinical phenotypes within the same family may provide a route forward.

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