Loss of Function of C9orf72 Causes Motor Deficits in a Zebrafish Model of Amyotrophic Lateral Sclerosis

Sorana Ciura, PhD,[1] Serena Lattante, PhD,[1] Isabelle Le Ber, MD, PhD,[1,2] Morwena Latouche, PhD,[1] Hervé Tostivint, PhD,[3] Alexis Brice, MD, PhD,[1,4] and Edor Kabashi, PhD[1]

Objective: To define the role that repeat expansions of a GGGGCC hexanucleotide sequence of the *C9orf72* gene play in the pathogenesis of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). A genetic model for ALS was developed to determine whether loss of function of the zebrafish orthologue of C9orf72 (zC9orf72) leads to abnormalities in neuronal development.
Methods: C9orf72 mRNA levels were quantified in brain and lymphoblasts derived from FTLD and ALS/FTLD patients and in zebrafish. Knockdown of the zC9orf72 was performed using 2 specific antisense morpholino oligonucleotides to block transcription. Quantifications of spontaneous swimming and tactile escape response, as well as measurements of axonal projections from the spinal cord, were performed.
Results: Significantly decreased expression of C9orf72 transcripts in brain and lymphoblasts was found in sporadic FTLD and ALS/FTLD patients with normal-size or expanded hexanucleotide repeats. The zC9orf72 is selectively expressed in the developing nervous system at developmental stages. Loss of function of the zC9orf72 transcripts causes both behavioral and cellular deficits related to locomotion without major morphological abnormalities. These deficits were rescued upon overexpression of human C9orf72 mRNA transcripts.
Interpretation: Our results indicate C9orf72 haploinsufficiency could be a contributing factor in the spectrum of ALS/FTLD neurodegenerative disorders. Loss of function of the zebrafish orthologue of zC9orf72 expression in zebrafish is associated with axonal degeneration of motor neurons that can be rescued by expressing human C9orf72 mRNA, highlighting the specificity of the induced phenotype. These results reveal a pathogenic consequence of decreased C9orf72 levels, supporting a loss of function mechanism of disease.

ANN NEUROL 2013;74:180–187

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are prevalent neurodegenerative disorders that share common clinical, pathological, and genetic characteristics. ALS is the most common motor neuron disease characterized by progressive paralysis caused by the gradual loss of upper and lower motor neurons.[1] FTLD is the second most common form of degenerative dementia, affecting individuals younger than 65 years.[2] FTLD comprises the progressive atrophy of the frontal and temporal lobes of the brain and is distinguished by deteriorations in language, behavior control, and emotional management. Although traditionally considered as 2 separate etiologies, emerging clinical commonalities between ALS and FTLD have brought forth the concept of an ALS/FTLD disorder. Clinically, approximately 15% of FTLD patients have motor dysfunction meeting the criteria of ALS,[3,4] and 15 to 18% of ALS patients have FTLD.[5] In addition, the

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.23946

Received Jan 11, 2013, and in revised form Apr 4, 2013. Accepted for publication May 17, 2013.

Address correspondence to Dr Kabashi, Centre de Recherche de l'Institut du Cerveau et de la Moelle Epinière - CRICM, INSERM UMR_S975, CNRS UMR 7225, Université Pierre et Marie Curie, Hôpital de la Salpêtrière, 47, Boulevard de l'Hôpital, 75651 Paris cedex 13, France.
E-mail: edor.kabashi@icm-institute.org

From the [1]Research Center of the Institute for Brain and Spinal Cord CRICM, National Institute of Health and Medical Research Inserm UMR_S975, National Center for Scientific Reseach CNRS UMR_7225, Pierre and Marie Curie University UPMC Paris 6, F-75013, Paris, France; [2]2AP-HP, Hôpital de la Salpêtrière, Reference Centre for Rare Dementias, F-75013, Paris, France; [3]Evolution of Endocrine Regulation, CNRS, UMR_7221, National Museum of Natural History, Paris, France, and; [4]AP-HP, Hôpital de la Salpêtrière, Department of Genetics and Cytogenetics, F-75013, Paris, France.

Additional supporting information can be found in the online version of this article.

majority of ALS and FTLD cases share a common pathological background, consisting of TDP-43–positive inclusion bodies found throughout the central nervous system.[6] The ALS/FTLD link was finally consolidated with the recent discovery of the *C9orf72* gene, mapping at the locus 9p21, as the most common genetic cause for both ALS and FTLD.[7,8]

Three different transcripts of C9orf72 have been reported to date, encoding for 2 protein isoforms of unknown function. The gene contains a stretch of up to 20 hexanucleotide (GGGGCC) sequences in the control population mapping to the first intronic region, or to the putative promoter region, depending on the C9orf72 transcript variant. This sequence has been found to be abnormally expanded in ALS/FTLD patients, with repeat numbers ranging from >60 to several hundreds.[7,8] A number of cross-sectional studies confirm that in different populations *C9orf72* repeat expansion accounts for 10 to 50% of familial and 5 to 7% of sporadic ALS cases and about 12 to 25% of familial and 6 to 7% of sporadic FTLD patients.[9,10] Clinical characteristics of ALS patients carrying the *C9orf72* expansion include a higher rate of psychotic symptoms, more frequent bulbar onset, associated FTLD, and shorter disease duration than patients with other known ALS mutations.[11–13]

The pathogenic mechanism associated with the hexanucleotide expansion of this gene remains unclear to date. Initial reports have shown that patients carrying the repeat expansion have lowered levels of C9orf72 transcripts,[7,8,14] suggesting that haploinsufficiency of this gene could be a pathological factor in disease. In parallel, RNA foci containing the hexanucleotide repeat were identified in patients carrying pathological numbers of the GGGGCC repeats, suggesting the possibility of a toxic gain of function.[7] In addition to RNA inclusions, 2 independent reports have recently identified abnormal peptide inclusions associated with the *C9orf72* hexanucleotide repeat expansion.[15,16] Unconventional translation of the GGGGCC repeat results in the formation of aggregates of insoluble polypeptides specific to patients carrying the hexanucleotide repeats. Although the pathogenicity of these peptide aggregates is still unclear, it is possible that they carry toxicity by sequestering essential cellular factors.[15–18] Alternatively, it has also been suggested that this type of unconventional translation could interfere with the normal processing of the C9orf72 transcripts, resulting in decreased levels of C9orf72.[15] Discerning the different mechanisms of toxicity associated with the *C9orf72* hexanucleotide repeat expansion is currently impeded by the lack of animal models for this genetic abnormality.

Here we report reduced levels of *C9ORF72* gene expression in a French cohort of ALS/FTLD patients harboring the hexanucleotide expansion. Interestingly, we also determined lowered C9orf72 transcript levels in samples from sporadic cases of FTLD and ALS/FTLD carrying normal hexanucleotide repeat lengths. Using the zebrafish as a vertebrate genetic model, we investigated whether a decrease in C9orf72 levels is sufficient to induce neuropathological processes. We show that blocking the translation of the zebrafish C9orf72 orthologue using antisense morpholinos results in specific locomotor deficits and axonopathy of the motor neurons, suggesting that *C9orf72* could play an essential role in the functioning and viability of these neurons and could represent a converging pathological mechanism in the ALS/FTLD spectrum of neurodegenerative disease.

Materials and Methods

Methodology for experiments described here are available in the Supplementary Data.

Results

Reduction of C9orf72 mRNA Expression in ALS/FTLD Patients Carrying the Hexanucleotide Repeats

We investigated the levels of C9orf72 transcript in a group of patients selected from a French cohort of unrelated probands with ALS/FTLD, previously identified as carriers of the expanded hexanucleotide repeat in the 5′ regulatory region of the *C9orf72* gene.[10,11] The pathogenic hexanucleotide repeat expansion is found in the first intron of the short C9orf72 variants, and in the putative promoter region of the longer variant (Fig 1). Repeat primed polymerase chain reaction (PCR) was used to confirm the presence of the pathogenic expansion in all the patient samples. A minimum of 60 repeats were detected in patient DNA, whereas normal-sized repeats, from 3 to 15, were observed in our control sample group. Transcript levels of C9orf72 were measured by real time (RT) PCR using an inventoried hydrolysis probe from Roche (Mannheim, Germany) that detects all 3 C9orf72 mRNA transcripts. C9orf72 mRNA transcripts were significantly lowered in ALS/FTLD patients ($n = 7$) compared to age-matched controls ($n = 8$) in postmortem frontal cortex tissue ($p = 0.002$). The quality of the mRNA extracted from these samples following significant neurodegeneration and variable postmortem times could be a confounding factor in interpreting these results. To address this issue, we performed RT-PCR on mRNA obtained from patient and control lymphoblastoid cell lines. As shown in Figure 1E, a similar decrease of the total C9orf72 transcript levels was observed in

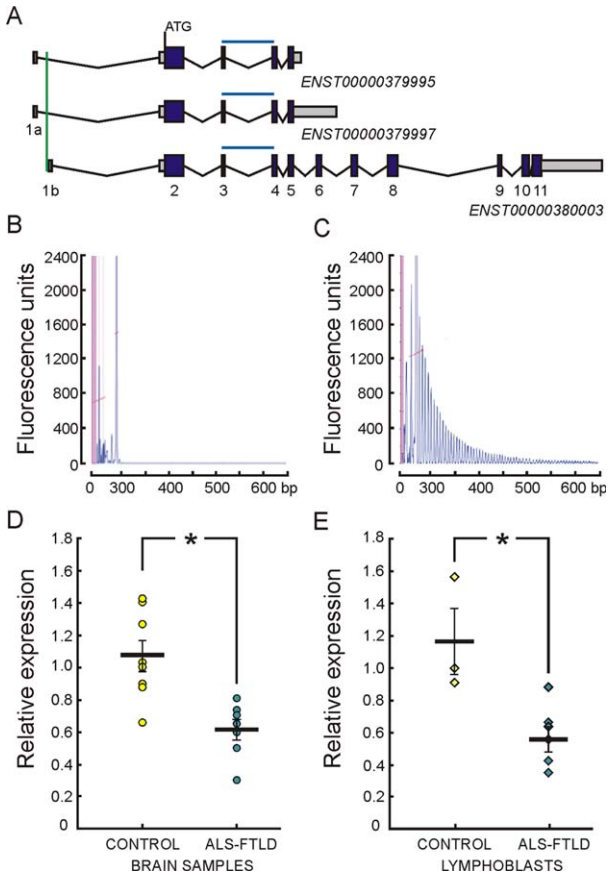

FIGURE 1: Hexanucleotide expansion in the *C9orf72* gene leads to reduced levels of C9orf72 transcripts in brain and lymphoblasts of amyotrophic lateral sclerosis (ALS)/frontotemporal lobar degeneration (FTLD) patients. (A) Schematic of the main human C9orf72 protein-coding transcripts indicating the location of the hexanucleotide expansion *(green bar)* and the binding of the quantitative polymerase chain reaction (PCR) probe *(blue bars)*. (B, C) Electropherograms of repeat primed PCR probing for GGGGCC repeats in control (B) and patient carrying the *C9orf72* hexanucleotide expansion (C). The repeat expansion produces a typical sawtooth pattern, with a peak periodicity of 6 base pairs. (D) Quantification of C9orf72 transcript levels in frontal cortex tissue obtained from ALS/FTLD patients carrying the hexanucleotide repeats as compared to controls. (E) Quantification of C9orf72 transcript levels in lymphoblast cell lines obtained from ALS/FTLD patients as compared to controls. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

lymphoblast cell lines from ALS/FTLD patients carrying the hexanucleotide repeat expansion (n = 6) versus controls (n = 3, $p = 0.01$). Because the pathogenic repeat expansion is found in the promoter region of the long transcript variant of C9orf72, we tested whether the levels of this transcript are directly affected by the expansion. Using a probe that specifically recognizes the long transcript, we noted a significant decrease in the expression of this transcript in lymphoblast samples from patients (n = 6) compared to control (n = 3, $p = 0.04$; Supplementary Fig 1A).

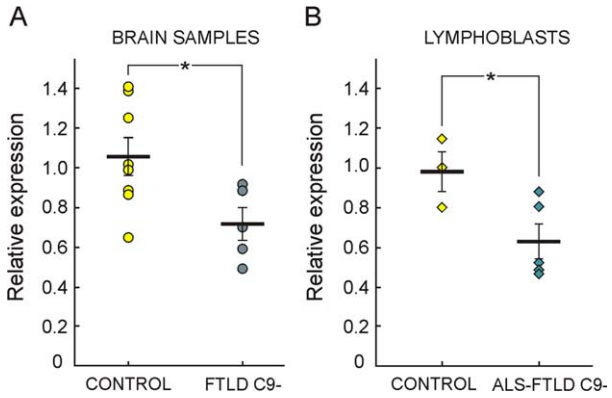

FIGURE 2: C9orf72 transcript levels are reduced in brain and lymphoblasts of amyotrophic lateral sclerosis (ALS)/frontotemporal lobar degeneration (FTLD) patients with normal-size hexanucleotide repeats. (A) Quantification of C9orf72 transcript levels in frontal cortex tissue obtained from sporadic FTLD patients with a normal range of C9orf72 hexanucleotide repeats as compared to nonaffected controls. (B) Analysis of C9orf72 transcript levels in lymphoblastoid cell lines from ALS/FTLD patients with nonpathogenic hexanucleotide repeats compared to nonaffected controls. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

We next investigated whether the decrease in C9orf72 mRNA is specific to patients who carry the hexanucleotide repeat expansion or whether it represents a general feature of the ALS/FTLD spectrum disorders. A set of frontal cortex and lymphoblast samples from sporadic cases of FTLD and ALS/FTLD were analyzed using repeat primed PCR to confirm the absence of the C9orf72 hexanucleotide repeat expansion. RT-PCR analysis revealed a significant decrease of the total C9orf72 transcript levels in FTLD brain samples (n = 5) relative to controls (n = 8, $p = 0.03$; Fig 2A). In a set of 3 ALS/FTLD brain samples, the levels of C9orf72 were highly variable, and overall no decrease was detected when compared to the control set (n = 8, $p = 0.71$; data not shown). This result could be due to the suboptimal nature of the postmortem tissue combined with the limited sample size. RT-PCR analysis of lymphoblast cells obtained from ALS/FTLD patients (n = 5) revealed a significant reduction in C9orf72 mRNA levels when compared with control (n = 3, $p = 0.04$; see Fig 2B). A similar decrease was also observed in the levels of the long C9orf72 transcript in lymphoblast samples of sporadic ALS/FTLD patients when compared to controls ($p = 0.02$; see Supplementary Fig 1B). In conclusion, we found decreased C9orf72 levels in patients affected with the ALS/FTLD spectrum disorder in the presence or absence of the hexanucleotide repeat expansion, indicating that the *C9orf72* gene could play a wider role in the etiology of this neurodegenerative condition.

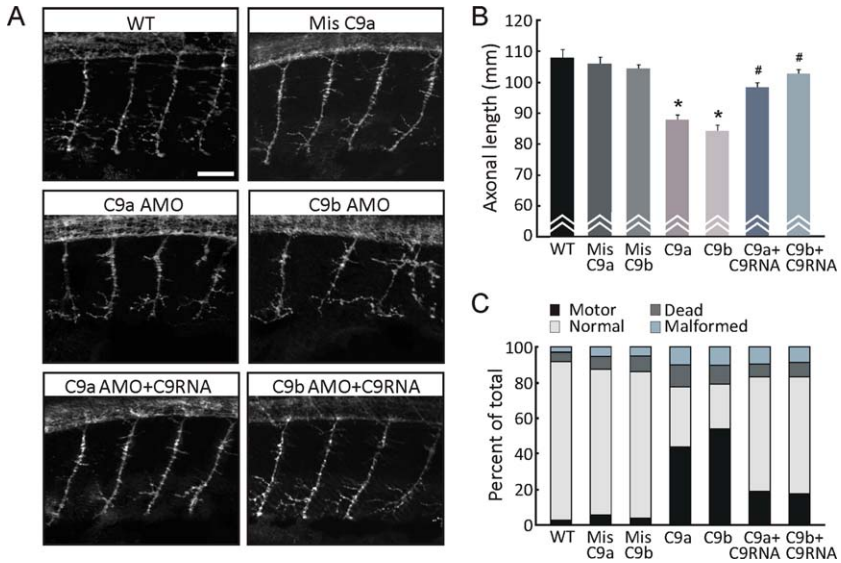

FIGURE 3: Axonopathy and motor phenotype associated with z*C9orf72* knockdown in zebrafish. (A) Representative images of motor neurons visualized with anti-ZNP immunohistochemistry in the different treatment conditions showing severe axonopathy in the C9orf72 knockdown using antisense morpholino oligonucleotides (C9a and C9b AMOs) when compared to noninjected wild-type (WT) controls or to mismatch (Mis C9a/b) AMO-injected fish. Significant rescue was obtained by introducing human C9orf72 RNA (C9a/bAMO + C9RNA). (B) Quantification of motor neuron axonal length demonstrates a significant decrease of axonal length in 48 hours postfertilization (hpf) zebrafish larvae injected with z*C9orf72* AMOs. The phenotype was absent in mismatch injected fish and was significantly rescued by human C9orf72 mRNA. (C) Bar graph illustrating the distribution of common phenotypes seen in 48-hpf zebrafish larvae showing increased percentage of zebrafish displaying motor deficits (Motor) in the z*C9orf72* knockdown conditions and consequent rescue by coexpression of human C9orf72 mRNA. *$p < 0.001$ compared to the mismatch (Mis) AMO control; #$p < 0.01$ when compared to the respective C9 AMO alone. Scale bar = 50μm. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

Knockdown of the zC9orf72 Orthologue in Zebrafish Leads to Axonal Deficits

To investigate the consequences of lowered C9orf72 levels in vivo, a zebrafish knockdown model was developed by using antisense morpholino oligonucleotides (AMOs) to block translation of the orthologue of the human *C9orf72*. Zebrafish has only 1 *C9orf72* orthologue (z*C9orf72* or *C13H9orf72*) that is highly conserved, having 76% nucleotide identity to the human gene (Supplementary Fig 2). Similarly to the human C9orf72 protein-coding transcripts, the zebrafish also possesses 3 protein-coding transcripts (see Supplementary Fig 2A), giving rise to 2 C9orf72 protein isoforms of different size. Whole-mount in situ hybridization of zebrafish embryos shows C9orf72 mRNA expression in the forebrain and hindbrain regions as well as light labeling in the spinal cord at 24 hours postfertilization (Supplementary Fig 3A), as previously reported.[19] Reverse transcription PCR revealed expression of C9orf72 at 18 and 24 hours postfertilization with primers specific for the 3′ and 5′ ends (see Supplementary Fig 3B). At adult stages, zC9orf72 mRNA is present in a number of zebrafish organs and tissues (see Supplementary Fig 3D), with notable expression levels observed in the forebrain, midbrain, and hindbrain, as well as in the spinal cord.

Two different specific AMOs were designed, C9a AMO and C9b AMO, directed against 2 separate translation initiation sites for all 3 protein-coding zC9orf72 transcripts (see Supplementary Fig 2A). Interestingly, we observed a decrease in mRNA levels following injections with both C9a AMO and C9b AMO, suggesting that this treatment has an impact on mRNA stability that could be additive to the translation blocking effect (see Supplementary Fig 3C). Dose-dependent toxicity curves were performed to determine the working concentration of both ATG-blocking AMOs resulting in minimal levels of death and developmental abnormalities (Supplementary Fig 4). Two mismatch morpholinos (misC9a for C9a AMO and misC9b for C9b AMO), with 5 nucleotides modified to block binding to the ATG sites, were designed and used as controls in the following experiments (all AMO sequences are provided in the Supplementary Materials and Methods).

Previous genetic models of ALS in zebrafish, including TDP-43, FUS, and SOD1, have described behavioral and cellular deficits associated with the disease-specific mutations, including shortened and overbranched axonal processes from motor neurons.[20–22] Similarly, knockdown of z*C9orf72* with both C9a and C9b AMOs resulted in disrupted arborization and shortening of the motor neuron axons when compared to noninjected or mismatch AMO-injected fish (Fig 3A, B). To test the specificity of the z*C9orf72* knockdown axonal

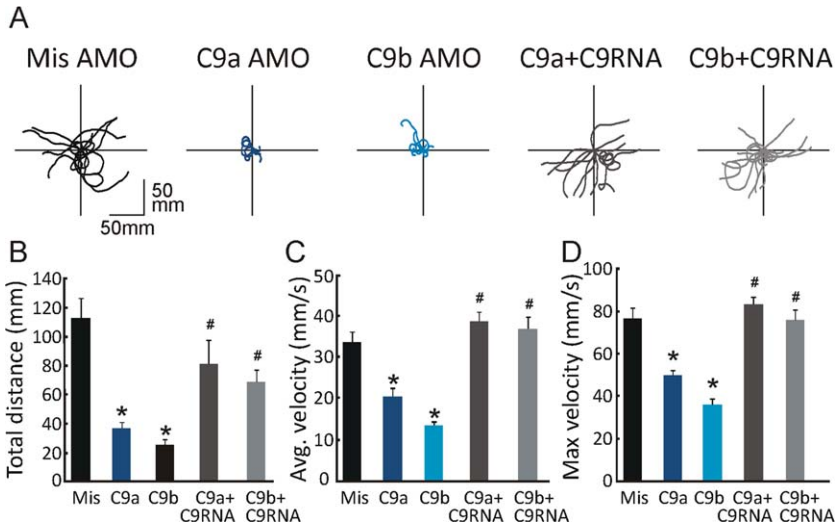

FIGURE 4: Deficient evoked swimming in z*C9orf72* knockdown zebrafish. (A) Tracing of the swimming paths of 48 hours postfertilization larvae following light touch. A minimum of 10 traces were used per condition. (B–D) Quantification of the touch-evoked swimming distance (B), average velocity (C), and maximum velocity attained (D) shows significant functional impairment of the zebrafish injected with antisense morpholino oligonucleotides (AMOs) against z*C9orf72*. mRNA coding for human *C9ORF72* significantly rescued this deficit. *$p < 0.01$ when compared to mismatch (Mis) controls; #$p < 0.01$ when compared to the respective C9 AMO alone. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

phenotype, we coinjected mRNA encoding the human C9orf72 long transcript (C9RNA) with the translation-blocking AMOs and obtained significant rescue of the axonopathy. Gain as well as loss of function of TDP-43 is associated with an axonopathy in zebrafish,[20–22] reminiscent of the zC9orf72 knockdown phenotype described here. To determine whether zC9orf72 knockdown had any effects on the levels of zTDP-43, we performed semiquantitative RT-PCR analysis that revealed no change in the levels of TDP-43 transcript (Supplementary Fig 5).

Knockdown of the C9orf72 Orthologue in Zebrafish Leads to Quantifiable Abnormalities of Spontaneous and Evoked Swimming

Escape swimming response following a light tactile stimulation was used to assess the extent of motor deficits associated with the axonopathy caused by zC9orf72 knockdown. Both C9a and C9b AMOs caused a significant increase in the percentage of zebrafish displaying a deficient touch-evoked escape response (TEER) in the absence of gross morphological abnormalities, as represented by image sequences in Supplementary Figure 6. The percentage of zebrafish larvae with motor deficits are shown as black bars in Figure 3C and detailed in the Supplementary Table. Injections of either the long or the short (C9RNA-S) transcripts of human C9orf72 alone had no deleterious effects (see Supplementary Fig 4 and Supplementary Table), but coexpression of these transcripts together with the C9a and C9b AMOs resulted in a significant reduction of the percentage of zebrafish with TEER (motor) deficits (see Fig 3C and Supplementary Table). Individual swimming episodes following an escape response were traced, with representative traces for each condition shown in Figure 4. Quantitative analysis of the TEER demonstrated that zC9orf72 knockdown led to major deficits, as significant reductions were observed in the total distance, average and maximum velocity of the locomotor behavior. Each of these parameters was significantly rescued upon coexpression of C9RNA or C9RNA-S (Supplementary Fig 7A).

To determine whether zC9orf72 knockdown leads to quantifiable differences in spontaneous swimming, we employed a semiautomated assay detecting movement of 4-day-old zebrafish larvae in a 96-well plate format (Fig 5). Motility analysis revealed reduced distance, average velocity, and overall mobility (percentage of time spent swimming) upon zC9orf72 knockdown using C9a and C9b AMOs. Significant rescue was again obtained by coexpression of human C9orf72 transcripts C9RNA or C9RNA-S (see Supplementary Fig 7B).

In addition to the 2 ATG-blocking morpholinos, we also designed a splice-blocking AMO (C9 spAMO) as an alternative and quantifiable method of altering the C9orf72 transcript. Injection of C9 spAMO caused TEER deficits, as represented by traces of escape response in Supplementary Figure 8A and quantified in Supplementary Figure 8B. These TEER deficits were observed in an increased percentage of larvae with motor phenotype (see Supplementary Fig 8). The motor deficits were

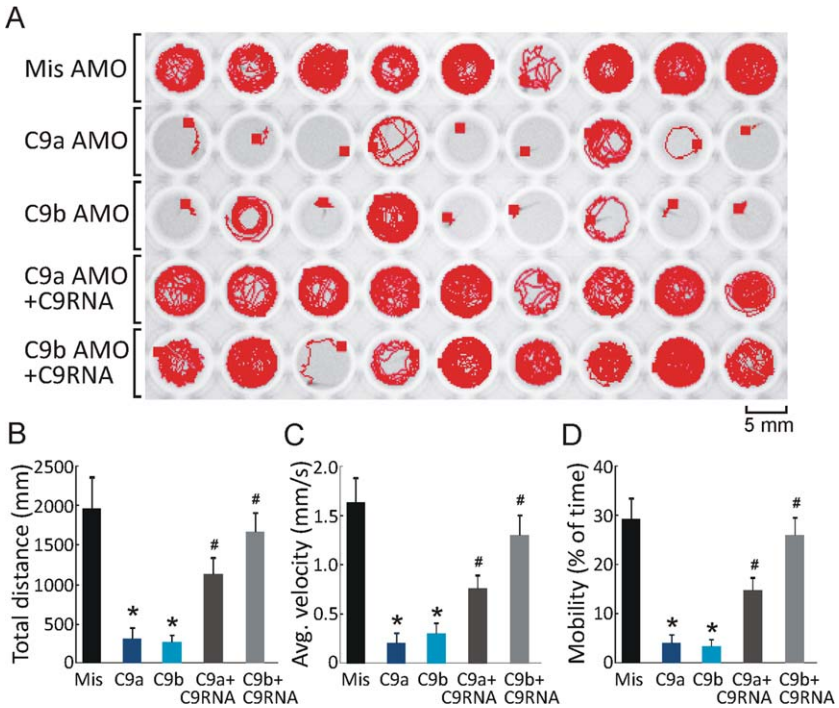

FIGURE 5: Reduced spontaneous swimming persists upon z*C9orf72* knockdown in zebrafish larvae. (A) Detection of freely swimming zebrafish larvae in 96-well plates. (B–D) Quantification of spontaneous swimming parameters showing deficits associated with z*C9orf72* knockdown and rescue with C9orf72 RNA in 4-day-old zebrafish larvae. *$p < 0.01$ compared to mismatch (Mis) antisense morpholino oligonucleotide (AMO) control; #$p < 0.05$ when compared to the respective C9orf72 AMO alone. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

specific, as they could be rescued upon coexpression of C9orf72 mRNA. Alternative splicing of the transcript was observed with increasing doses of C9 spAMO, indicating that altering the splicing of zC9orf72 does bear some pathogenic effects, even if the overall levels of the zC9orf72 mRNA remained unaltered.

Discussion

The recent discovery of the hexanucleotide repeat expansion in the noncoding region of the *C9orf72* gene as the major genetic cause of both ALS and FTLD holds great promise for the better understanding of these disorders and the development of future therapeutic applications. A possible pathogenic mechanism mediated by this repeat expansion is the reduction of C9orf72 transcript levels,[7,8,14] triggering neurodegenerative processes due to haploinsufficiency. Here, we show that a probe designed to detect all 3 C9orf72 transcript levels assessed a significant reduction both in lymphoblast cells (~49%) as well as in frontal cortex samples (~42%) of ALS/FTLD patients carrying the hexanucleotide repeats as compared to nonaffected controls (see Fig 1D, E). Interestingly, a significant but less drastic reduction (~33% in brain and 36% in lymphoblasts) of C9orf72 transcript levels was observed in frontal cortex samples of FTLD patients, as well as in lymphoblast cell lines from ALS/FTLD patients with normal-sized repeats when compared to nonaffected controls (see Fig 2). The decrease in total C9orf72 mRNA was paralleled by a specific decrease in the levels of the long transcript in lymphoblastoid cell lines from ALS/FTLD patients with normal-size or expanded hexanucleotide repeats, similar to previous reports.[7,14,16] These results raise the possibility that depletion of C9orf72 levels is a key process that triggers neurodegeneration in a wider proportion of patients than previously thought. Such a decrease can be directly caused by the presence of the pathogenic hexanucleotide repeat expansion in patients carrying this mutation, or by unknown genetic and cellular processes in the case of other patients. Future studies on larger cohorts of patients are needed to establish whether decreases in C9orf72 transcript levels are commonly associated with ALS/FTLD spectrum disorders.

An alternative interpretation of these results is the possibility that C9orf72 depletion is not a causative factor in the etiology of the disease but a consequence of toxic cellular processes involved in neurodegeneration. In the case of the hexanucleotide repeat expansion, such neurotoxic processes could be triggered by the accumulation of repeat transcripts into nuclear RNA foci,[7] or by cytoplasmic inclusions containing repeat-encoded dipeptides resulting from unconventional translation of these

repeats.[18] Two recent studies have shown that expanded repeats are translated through repeat-associated non-ATG translation in pathological tissue samples from patients carrying the C9orf72 repeat expansion.[15,16] Dipeptides encoded by these repeats were found to aggregate into cytoplasmic inclusion bodies that could carry a pathological toxicity in neurons.[15,16] Interestingly, whereas the gain of function hypothesis would predict that longer repeat lengths produce higher toxicity, and progressively more severe clinical parameters, such correlation was not found in several clinical studies to date.[23,24] It is also possible that a combination of loss and gain of function of C9orf72, as appears to be the case for TDP-43 and FUS, is at play in orchestrating a complex and devastating neurotoxic effect.[25,26]

To address the loss-of-function hypothesis, we developed a genetic model of C9orf72 haploinsufficiency via knockdown of zC9orf72 transcripts in the vertebrate model organism, zebrafish. *C9orf72* has only 1 orthologue in zebrafish displaying high homology (76%) to humans. We found that expression of this orthologue is enriched in the developing nervous system at the embryonic and larval stages and is present in most major organs at the adult stage. Knockdown of the zebrafish z*C9orf72* orthologue by targeting either the common ATG for all 3 transcripts, or an upstream ATG using specific AMOs, causes both behavioral and cellular deficits, characterized by largely reduced motility and axonopathy in zebrafish. This phenotype was rescued upon overexpression of human C9orf72 mRNA transcripts, highlighting the specificity of the induced phenotype. Upon injection of the 2 ATG-blocking AMOs, we observed reduced transcript levels of the zC9orf72, indicating that the binding of the morpholinos could result in decreased stability of the transcript and could complement the effects that these morpholinos have on translation. Due to unavailability of specific antibodies, we were unable to determine the degree of reduction in zC9orf72 protein levels in zebrafish following this treatment. As an alternative method of altering the integrity of the zC9orf72 transcript, we developed a splice morpholino designed to bind at the 5′ end of the transcript. This results in a milder, but significant motor phenotype that was partially rescued by the human C9orf72 mRNA. Interestingly, whereas the splicing at the 5′ end of the mRNA was altered by the C9spAMO, there was no decrease in the overall level of the gene transcript as measured by semiquantitative PCR on the 3′ end of the transcript, possibly explaining the lack of a more pronounced motor phenotype. We conclude that the motor deficits observed in our zebrafish model using each of the 3 AMOs described in this study were dependent on the transcript downregulation/modification, as these abnormalities were rescued upon coexpression of the human C9orf72 mRNA variants.

The combined results presented here suggest that loss of function of *C9orf72* due to the GGGGCC repeat expansion is sufficient to cause motor neuron defects in an in vivo setting. A toxic gain of function mechanism, which is common for other coding as well as noncoding expanded repeat disorders,[27] could also contribute to C9orf72-related neurodegeneration.[28] To further elucidate the contribution of C9orf72 gain and loss of function to the pathogenicity of ALS and FTLD, transgenic models expressing expanded hexanucleotide repeats as well as stable deletion or knockdown transgenic lines for C9orf72 will need to be designed in a number of animal models.

Zebrafish models of disease present the advantage of a vertebrate organism amenable to large automated screens, extensively used to discover therapeutic targets for a number of health disorders. More recently, zebrafish models have been increasingly used for genetic and pharmacological screens to identify key modifiers of neurodegenerative processes[22,28,29] as well as to identify pathophysiological mechanisms involved in motor neuron diseases.[30,31] Here we describe a genetic model of *C9orf72* loss of function presenting a robust behavioral phenotype, which lends itself to semiautomated detection and quantification that could be used in the future for drug screening and physiological characterization to identify appropriate therapies for patients with ALS and FTLD, disorders that are currently untreatable.

Acknowledgment

This work was funded by the Atip/Avenir from the National Institute of Health and Medical Research, a Bernice Ramsay Discovery Grant from ALS Canada, the Robert Packard Foundation, AFM-Téléthon, the Association for Research on Amyotrophic Lateral Sclerosis, the French Agency for Research, the Plan Alzheimer Foundation, and a Career Integration Grant from the Marie Curie Actions (E.K.) and from the program "Investissements d'Avenir" (ANR-10-IAIHU-06). S.C. is supported by a postdoctoral fellowship from the European Molecular Biology Organization; S.L. is supported by postdoctoral fellowships from the School of the Neurosciences (Paris) and the Cognacq-Jay Foundation.

We thank the patients for taking part in this work; the GIE Neuro-CEB (Pitié-Salpétrière Hospital, Paris, France) and the DNA and cell bank of IFR70 (Pitié-Salpétrière Hospital) for generously providing human tissues; Dr C. Duyckaerts, S. Leclere-Turbant, and A. Camuzat (Pitié-Salpétrière Hospital) for providing patient samples; and J. Gomez, Dr A. Schorscher-Petcu,

F. Quan, B. Chereau, S. Katz, and V. Bercier for technical assistance.

Potential Conflicts of Interest

I.L.B.: grants/grants pending, French Agency for Research (ANR), Hospital Research Program (PHRC). A.B.: consultancy, Wolfson Foundation; grants/grants pending, ANR, PHRC, European Union. E.K. grants/grants pending, ANR, European Research Council (ERC).

References

1. Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci 2006;7:710–723.
2. Harvey RJ, Skelton-Robinson M, Rossor MN. The prevalence and causes of dementia in people under the age of 65 years. J Neurol Neurosurg Psychiatry 2003;74:1206–1209.
3. Lomen-Hoerth C, Anderson T, Miller B. The overlap of amyotrophic lateral sclerosis and frontotemporal dementia. Neurology 2002;59:1077–1079.
4. Burrell JR, Kiernan MC, Vucic S, Hodges JR. Motor neuron dysfunction in frontotemporal dementia. Brain 2011;134:2582–2594.
5. Ringholz GM, Appel SH, Bradshaw M, et al. Prevalence and patterns of cognitive impairment in sporadic ALS. Neurology 2005; 65:586–590.
6. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 2006;314:130–133.
7. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 2011;72:245–256.
8. Renton AE, Majounie E, Waite A, et al. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron 2011;72:257–268.
9. Majounie E, Renton AE, Mok K, et al. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. Lancet Neurol 2012;11:323–330.
10. Le Ber I, Camuzat A, Guillot-Noel L, et al. C9ORF72 repeat expansions in the frontotemporal dementias spectrum of diseases: a flowchart for genetic testing. J Alzheimers Dis 2013;34:485–499.
11. Millecamps S, Boillée S, Le Ber I, et al. Phenotype difference between ALS patients with expanded repeats in C9ORF72 and patients with mutations in other ALS-related genes. J Med Genet 2012;49:258–263.
12. Cooper-Knock J, Hewitt C, Highley JR, et al. Clinico-pathological features in amyotrophic lateral sclerosis with expansions in C9ORF72. Brain 2012;135:751–764.
13. Snowden JS, Rollinson S, Thompson JC, et al. Distinct clinical and pathological characteristics of frontotemporal dementia associated with C9ORF72 mutations. Brain 2012;135:693–708.
14. Gijselinck I, Van Langenhove T, van der Zee J, et al. A C9orf72 promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study. Lancet Neurol 2012;11:54–65.
15. Ash PE, Bieniek KF, Gendron TF, et al. Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. Neuron 2013;77:639–646.
16. Mori K, Weng SM, Arzberger T, et al. The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. Science 2013;339:1335–1338.
17. Rademakers R, Neumann M, Mackenzie IR. Advances in understanding the molecular basis of frontotemporal dementia. Nat Rev Neurol 2012;8:423–434.
18. Taylor JP. Neuroscience. RNA that gets RAN in neurodegeneration. Science 2013;339:1282–1283.
19. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 2008;3:59–69.
20. Kabashi E, Lin L, Tradewell ML, et al. Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. Hum Mol Genet 2010;19:671–683.
21. Kabashi E, Bercier V, Lissouba A, et al. FUS and TARDBP but not SOD1 interact in genetic models of amyotrophic lateral sclerosis. PLoS Genet 2011;7:e1002214.
22. Van Hoecke A, Schoonaert L, Lemmens R, et al. EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. Nat Med 2012;18:1418–1422.
23. Rutherford NJ, Heckman MG, Dejesus-Hernandez M, et al. Length of normal alleles of C9ORF72 GGGGCC repeat do not influence disease phenotype. Neurobiol Aging 2012;33:2950.e5–e7.
24. Beck J, Poulter M, Hensman D, et al. Large C9orf72 hexanucleotide repeat expansions are seen in multiple neurodegenerative syndromes and are more frequent than expected in the UK population. Am J Hum Genet 2013;92:345–353.
25. Mackenzie IR, Rademakers R, Neumann M. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. Lancet Neurol 2010;9:995–1007.
26. Lee EB, Lee VM, Trojanowski JQ. Gains or losses: molecular mechanisms of TDP-43-mediated neurodegeneration. Nat Rev Neurosci 2011;13:38–50.
27. Pearson CE. Repeat associated non-ATG translation initiation: one DNA, two transcripts, seven reading frames, potentially nine toxic entities! PLoS Genet 2011;7:e1002018.
28. Vaccaro A, Patten SA, Ciura S, et al. Methylene blue protects against TDP-43 and FUS neuronal toxicity in C. elegans and D. rerio. PLoS One 2012;7:e42117.
29. Kabashi E, Champagne N, Brustein E, Drapeau P. In the swim of things: recent insights to neurogenetic disorders from zebrafish. Trends Genet 2010;26:373–381.
30. Armstrong GA, Drapeau P. Calcium channel agonists protect against neuromuscular dysfunction in a genetic model of TDP-43 mutation in ALS. J Neurosci 2013;33:1741–1752.
31. McGown A, McDearmid JR, Panagiotaki N, et al. Early interneuron dysfunction in ALS: insights from a mutant sod1 zebrafish model. Ann Neurol 2013;73:246–258.