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C9ORF72 interaction with cofilin modulates actin dynamics in motoneurons

Running title: C9ORF72 and actin dynamics

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Abstract

Intronic hexanucleotide expansions in *C9ORF72* are common in ALS and FTLD, but it is unknown whether loss of function, toxicity by the expanded RNA or dipeptides from non ATG-initiated translation are responsible for the pathophysiology. We determined the interactome of C9ORF72 in motoneurons and found that C9ORF72 is present in a complex with cofilin and other actin binding proteins. Phosphorylation of cofilin is enhanced in C9ORF72 depleted motoneurons, in patient derived lymphoblastoid cells, iPS cell derived motoneurons and post-mortem brain samples from ALS patients. C9ORF72 modulates the activity of the small GTPases Arf6 and Rac1, resulting in enhanced activity of LIMK1/2. This results in reduced axonal actin dynamics in C9ORF72 depleted motoneurons. Dominant negative Arf6 rescues this defect, suggesting that C9ORF72 acts as a modulator of small GTPases in a pathway that regulates axonal actin dynamics.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common form of motoneuron disease in the adult. Both sporadic and familial forms exist, and with the discovery of a GGGGCC (G4C2) intronic repeat expansion in the *C9ORF72* gene, a common genetic cause of familial ALS has been identified [1, 2]. The same intronic repeat expansion has also been discovered in patients with frontotemporal dementia (FTLD) [2], the second most common form of presenile dementia after Alzheimer´s disease. Interestingly, ALS and FTLD often co-occur in families and sometimes present in the same patients (FTLD-ALS), with similar clinicopathological features [1-4]. In healthy individuals, the number of repeats ranges from 2 to 23. In patients with ALS and FTLD, sixty to several hundred G4C2 repeats are found in the *C9ORF72* gene [4, 5]. The mechanism by which G4C2 repeat expansions cause neurodegenerative disease is still not clear. Three main hypotheses are currently discussed [6]. The first proposes that G4C2 repeat transcript as well as the antisense repeat transcript may accumulate in RNA foci which sequester RNA-binding proteins, leading to disturbed RNA metabolism that causes neurodegeneration [7-9]. The second hypothesis builds on observations that the long repeat gives rise to proteins consisting of dipeptides by non-ATG translation (RAN translation) [10]. These dipeptide repeat proteins can then aggregate and cause neurodegeneration in affected brain regions [11]. The third hypothesis suggests that the expansion of repeats leads to haploid insufficiency of *C9ORF72* expression, thereby resulting in loss of function [2]. Which of these different mechanisms predominates or whether they act together in the pathophysiology of ALS and FTLD is currently unknown. In particular, little is known about the effects of loss of function of the protein encoded byC9ORF72, because its physiological role is still unclear.

Loss of function is a possible pathomechanism as patients with *C9ORF72* repeat expansion show a 50% reduction in mRNA levels of both long and short transcripts [2, 3]. Recent studies revealed that reduction of C9ORF72 levels in both cortical and motoneurons does not affect cellular survival *in vitro* [10] or *in vivo* [12]. However, zebrafish models show reduced axon length in motoneurons and reduced locomotion after antisense morpholino-mediated reduction of C9ORF72 levels [5]. In contrast to the results in *C. elegans* and zebrafish, studies in mouse have so far not revealed conclusive results for C9ORF72 loss of function as a possible cause of FTLD/ALS [13-16]. Administering antisense oligonucleotides (ASOs) targeting mouse C9ORF72 by stereotactic intracerebroventricular (ICV) injection reduced C9ORF72 mRNA levels to 30-40% of control levels in the spinal cord and brain [17]. C9ORF72 depletion in these mice was well tolerated and did not result in any behavioural or motor impairment. A mouse with a conditional knockout allele for C9ORF72 was generated using the Cre/loxP system. These mice were crossed to Nestin-Cre mice, which express Cre recombinase in neurons and glia starting at E10.5 and continuing into adulthood [18]. Cre-mediated inactivation of C9ORF72 in neurons and glia did not cause loss in motoneuron numbers or in motor function, including motor performance and grip strength [19]. Hallmarks of ALS pathologies such as ubiquitinated TDP-43 aggregates and gliosis were not detected either. Thus, in two different mouse models, loss of C9ORF72 function is not sufficient to cause neurodegeneration and FTLD/ALS-related phenotypes.

However, these finding do not preclude the possibility that loss of C9ORF72 function contributes to neurodegeneration when combined with toxicity of dipeptides generated via RAN translation or dysregulated RNA metabolism. Loss of C9ORF72 in Neuro-2A cells has been shown to disturb the function of a complex involving WDR41 and SMCR8 which modulates GTPase function of Rab8a and Rab39b,

leading to impaired autophagy in neurons [20]. To characterize the function of C9ORF72 in motoneurons, we investigated the interactome of C9ORF72 with quantitative proteomics and found that it immunoprecipitates with cofilin and other actin binding proteins. Functional analyses in isolated mouse motoneurons and human iPS cells as well as post-mortem analyses of patient brain tissues revealed a role of C9ORF72 as a modulator of actin dynamics via a pathway involving phosphorylation of cofilin through Arf6, Rac1 and LIMK1/2 activity. The resulting loss of actin dynamics could contribute to the capacity of neurons to maintain axons and axon terminals in the cellular pathology underlying ALS and FTLD

Results

C9ORF72 modulates axon growth in cultured motoneurons

To understand the cellular function of C9ORF72, we used lentiviral vectors (Suppl. Fig. 1A,B) to overexpress or knockdown C9ORF72 in primary mouse embryonic motoneurons. Immunostaining (Fig. 1A,B), RT-PCR (Suppl. Fig. 1C) and immunoblots (Supplementary Fig. 1D) showed that C9ORF72 levels were reduced (Suppl. Fig. 1C) or enhanced (Suppl. Fig. 1D,E) by about 50%, respectively, in primary mouse motoneurons cultured for 7 days. Overexpression or knockdown of the protein did not affect the survival of the cultured motoneurons (Suppl. Fig. 2A). Interestingly, compared to controls axons were significantly longer in C9ORF72-overexpressing motoneurons at 7 days in culture (Fig. 1C,D, Suppl. Fig. 2B), and significantly shorter after knockdown . The knockdown effect could be rescued by overexpression of human C9ORF72, indicating that is not due to off target effects (Suppl. Fig. 2C). C9ORF72 overexpression also led to increased growth cone size at

day 5 in culture while knockdown reduced growth cone size compared to control (Fig. 1D). These results indicate that C9ORF72 modulates axonal growth and growth cone size in spinal motoneurons.

C9ORF72 interacts with Cofilin

To investigate the mechanism by which C9ORF72 acts on axon growth and differentiation, we determined interacting proteins by mass spectrometry (MS)-based proteomics. Due to the lack of suitable antibodies against mouse C9ORF72 protein, immunoprecipitation of endogenous protein was not possible. Therefore, we overexpressed HA-tagged human C9ORF72 protein and immunoprecipitated interacting proteins from NSC-34 cells (Suppl. Fig. 3A,B,C). Interacting proteins were then analyzed by quantitative interaction proteomics as described [21, 22]. Key regulators of actin dynamics such as cofilin, Arp2/3 and coronin were among statistically significant interaction partners of C9ORF72 (Fig 2A, Suppl Excel Sheet 1). For further analysis, we first focussed on cofilin, of which we identified two family members (Cfl1 and Clf2) as significant C9ORF72 interactors, because cofilin plays a central role in the regulation of actin dynamics and could therefore be responsible for reduced axon growth [23] and reduced size of axonal growth cones [24]. Immunoprecipitation of C9ORF72-HA from primary mouse motoneurons confirmed the interaction with cofilin by immunoblot analysis (Fig. 2B, Suppl. Fig. 4A,B). Endogenous cofilin pulldown revealed even stronger binding both of overexpressed C9ORF72-HA (Fig. 2B) and endogenous C9ORF72 (Fig. 2C, right panel), probably because cofilin is more abundant in motoneurons and NSC-34 cells than the C9ORF72 protein [25]. This interaction was further confirmed by immunostaining in cultured mouse motoneurons, showing that C9ORF72-HA co-localizes with cofilin in

the cell body, axon shaft (Fig. 2D) and in particular in the axonal growth cone (Fig. 2E, Suppl. Fig.4C,D).

C9ORF72 depletion enhances phosphorylation of cofilin

Cofilin is a ubiquitous actin-binding factor required for the reorganization of actin filaments. It promotes actin assembly or disassembly depending on its concentration relative to actin and relative to the concentration of other actin-binding proteins [26]. The activity of cofilin depends on phosphorylation at Ser3 which inactivates its function in F-actin assembly [27]. We found that overexpression of C9ORF72-HA protein reduced phosphorylation while knockdown increased cofilin phosphorylation (Fig. 3A) without any change in total cofilin levels (Fig. 3A,B). To test whether actin dynamics is also altered in human cells with *C9ORF72* intronic expansion, we investigated immortalized lymphoblasts from three age-matched control and three *C9ORF72* repeat expansion-carrier ALS patients. Immunoblots of lymphoblastoid cell extracts from these patients showed an upregulation of cofilin phosphorylation (Fig. 3C,D). In order to study whether these cell culture experiments are representative of patients at disease stage, we investigated post-mortem brain tissue from three ALS patients with *C9ORF72* intronic expansion and three age related controls. Using protein extracts from post-mortem cerebellum, we found reduced levels of endogenous C9ORF72 ($p=0.0002$, unpaired student´s t-test) in the patient samples, and a more than 3-fold upregulation of cofilin phosphorylation ($p=0.0002$, unpaired student´s t test) in the absence of altered cofilin levels (Fig. 3E,F). The results from cultured mouse motoneurons, patient derived lymphoblastoid cells and post-mortem brain tissue together demonstrate that cofilin phosphorylation is enhanced after

depletion of C9ORF72 and under the genetic conditions of *C9ORF72* intronic hexanucleotide expansion in patients.

Actin dynamics is disturbed in C9ORF72 depleted or mutated motoneurons

We next tested whether C9ORF72 has an influence on actin dynamics and cloned cytoplasmic actin linked with GFP into a shRNA lentiviral vector for C9ORF72 repression (Suppl. Fig. 5A). This allowed us to study actin dynamics by live cell imaging of cultured mouse motoneurons lacking C9ORF72. A scrambled sequence was used for the control GFP-actin construct. The expression of both constructs was confirmed by Western blotting and immunostaining (Suppl. Fig. 5B,C), which showed colocalization of GFP-actin with phalloidin. We performed live cell imaging with mouse motoneurons at 5 days in culture because at this stage axon extension appears with highest speed and dynamics [28] (Fig. 4A, Suppl. Movie 1-2). The number of newly generated actin filaments was reduced in C9ORF72 depleted motoneurons (Fig. 4B). C9ORF72 knockdown led to a significantly reduced velocity of actin movement in axonal growth cones compared to motoneurons transduced with scrambled control constructs (Fig. 4C,D). The dynamics of growth cone movement, as measured by the area into which the growth cone expanded over time was also significantly reduced (Fig. 4E). Thus actin dynamics is impaired when C9ORF72 levels are reduced in motoneurons. We then investigated actin dynamics in iPSC derived human motoneurons from C9-ALS patients (Suppl.Fig.6, 7). Consistent with our observations in primary mouse motoneurons after C9ORF72 suppression, these induced human motoneurons showed reduced velocity of actin movement in axonal growth cones compared to control patient derived human motoneurons (Fig. 4 F-I). In parallel to the live cell imaging, we performed protein fractionation of F-actin and G-

actin, which confirmed that G/F actin ratios are altered when C9ORF72 is knocked down or overexpressed in mouse motoneurons (Fig. 5A-D).

C9ORF72 depletion leads to activation of Arf6

Cofilin ability to bind and depolymerize actin is abolished by phosphorylation of serine residue 3 [27, 29]. The kinases responsible for this phosphorylation are LIM-kinase 1 (LIMK-1) and LIMK-2 [30]. LIMK-1/2 can phosphorylate cofilin at Ser3, both *in vitro* and *in vivo* [31]. Therefore, we tested whether cofilin phosphorylation is regulated by LIMK-1/2 phosphorylation by analyzing phospho-LIMK-1/2 levels after overexpression or knockdown of C9ORF72 in primary mouse motoneurons after 7 days in culture. Overexpression of C9ORF72-HA protein reduced LIMK-1/2 phosphorylation while knockdown increased LIMK-1/2 phosphorylation (Fig. 6A) without any change in total LIMK protein levels. We then analysed cofilin and LIMK phosphorylation in patient iPS cells derived motoneurons matured for 14 days (Fig. 6B). Interestingly, a similar phenotype was observed as in C9ORF72 depleted mouse motoneurons. These results indicate that C9ORF72 influences the phosphorylation of LIMK which directly regulates the activity of cofilin.

Recent publication have shown that C9ORF72 protein has homology to the Differentially Expressed in Normal and Neoplasia (DENN) protein family, which function as guanine nucleotide exchange factors (GEFs) or modulators of guanine nucleotide exchange factors to regulate Rab GTPase activity [32, 33]. Therefore, we searched for potential small GTPases among the proteins found in the C9ORF72 interactome and identified ADP-ribosylation factor-1 (Arf1) and Arf6 (Fig. 6C,D). Arfs [34] constitute a family of Ras-related, low molecular mass (~20 kDa) GTP-binding

proteins that act as molecular switches for membrane and vesicle traffic as well as actin dynamics in cells [35]. In a first step, the interaction of C9ORF72 with these small GTPases was confirmed by co-precipitation analyses, using antibodies against Arf6 (Fig. 6C). This study was limited by the quality of available antibodies, and therefore complemented by experiments in which flag-tagged Arf1 and Arf6 were overexpressed in NSC34 cells (Fig. 6D). Pulldown with FLAG antibodies confirmed that C9ORF72 co-precipitates with Arf1 and Arf6 and thus might be present in the same complexes. Arf6 plays an essential role in membrane trafficking and cytoskeletal rearrangement both in neuronal [36] and non-neuronal cells [37]. We therefore analyzed the activity of Arf6 with gel shift assays to investigate whether C9ORF72 acts as a modulator of GTP/GDP binding to Arf6. The results show that C9ORF72 depletion in NSC-34 cells elevates the levels of GTP-bound Arf6 (Fig. 7A-C), indicating that C9ORF72 does not act as a GEF for Arf6, but that it is involved in modulating this pathway. Rac1 acts as a downstream mediator of Arf6 activation [38] in a pathway that modulates actin dynamics. In order to study this pathway, we performed active GTPase immunoprecipitation assays in which the GTP-found forms of Arf6 and Rac1 were pulled down. These data indicate that levels of the GTP-bound forms of these small GTPases are increased in the absence of C9ORF72 (Fig. 7D-F), thus suggesting a mechanism by which actin dynamics is disturbed. The reduced level of C9ORF72 leads to more active form of GTP-bound ARF6 which activates Rac1, thus leading to the phosphorylation of LIMK-1/2 and as a consequence to inactivation of cofilin by phosphorylation at Ser3. In order to test this hypothesis in more detail, we investigated whether knockdown of Arf6 could restore cofilin phosphorylation and altered axonal actin dynamics in motoneurons. Therefore we investigated Rac1 activation in iPSc cells derived motoneurons from C9-ALS patients. Under control conditions, enhanced Rac1 was observed, but knockdown of

Arf6 by shRNA rescued this phenotype (Fig. 8A,B). As a second line of evidence we investigated whether overexpression of constitutively dominant negative Arf6 (resembling Arf6-GDP) in parallel with knockdown of mouse C9ORF72 could rescue the axonal elongation phenotype. The results from (Fig 8C,D) demonstrate that the axonal elongation phenotype observed after knockdown of mouse C9ORF72 was rescued with overexpression of dominant negative Arf6.

Together our data provide evidence that C9ORF72 plays a cellular role in regulating actin dynamics via regulation of the GTPase activity of Arf6, cofilin phosphorylation and possibly other proteins shaping the actin cytoskeleton. Our data also explain the axonal phenotype observed in zebrafish after C9ORF72 suppression[5]. Thus, loss of function of C9ORF72 could contribute to the clinical phenotype in ALS and FTLD, because reduced actin dynamics influences synaptic strength [39] , maintenance [40] and axon stability [40].

Discussion

Several previous studies have shown that cells derived from *C9ORF72* patients have decreased levels of *C9ORF72* transcripts [5], raising the on the cellular function of C9ORF72 and whether loss of this function could contribute to the pathophysiology of ALS and FTLD [6]. C9ORF72 has been implicated in intra-cellular membrane trafficking [32, 33]. Our Mass spectrometry (LC-MS), immunoprecipitation analyses releaved that C9ORF72 is in a complex with key regulators of actin dynamics such as Cofilin, Arp2/3 and Coronin. Cofilin is an ubiquitous actin-binding factor required for the organization of actin filaments [26]. The activity of cofilin depends on

phosphorylation at Ser3 which inactivates its function in F-actin assembly [27, 29]. Recent studies have shown that cofilin plays an essential role for actin bundling in axons, and that augmented retrograde flow of actin filaments appears as an essential component of axon elongation [23]. The same study showed that cofilin knockout results in impaired axon growth, similar as in C9ORF72 depleted mouse motoneurons observed in our study. This emphasizes the importance of cofilin activity for axonal maintenance in motoneurons. The activity of cofilin is regulated by phosphorylation through LIMK-1/2. These serine threonine kinases are activated by effector kinases of small GTPases, in particular PAK1 and PAK4 [42] These effector kinases act downstream of Rac1 which itself is activated by Arf6 [38, 43] and Arf1 [44]. The observation that the GTP bound form of Rac1 and Arf6 are relatively increased when C9ORF72 is repressed in primary mouse motoneurons, or in iPS cell derived motoneurons strongly suggest that C9ORF72 plays an essential role for the GTP exchange activity in particular of Arf6. This conclusion is supported by the observation that knockdown of Arf6 expression of iPS cell derived motoneurons normalizes the enhanced levels of GTP bound Rac1. Expression of a dominant negative Arf6 [36] or depletion of Arf6 [45] enhances axon elongation which fully supports our observation that enhanced activity of Arf6 in C9ORF72 deficient neurons correlates with reduced axon growth, and that expression of a dominant negative Arf6 in C9ORF72 depleted motoneurons or in iPS cell derived motoneurons from C9-ALS patients rescues the axonal defects observed after C9ORF72 depletion.

Bioinformatic analysis has revealed that C9ORF72 contains a DENN domain that is characteristic of the family of DENN proteins, some of which showing activity as GDP/GTP exchange factors for Rab GTPases and possibly also other small GTPases [32, 33]. This appeared in line with the observation that C9ORF72 colocalized with Rab1, Rab5, Rab7 and Rab11 and that depletion of C9ORF72 inhibited

endocytosis and vesicle trafficking in neuronal cell lines [46]. A recent study identified C9ORF72 protein in Neuro-2A cells as component of a complex with SMCR8 and WDR3 exhibiting GEF activity for Rab8a and Rab39 [20]. In our mass spectrometric analysis, we did not observe a specific interaction of C9ORF72 with SMCR8 and WDR41. This is possibly due to the fact that the proteomes of Neuro-2A cells and primary motoneurons differ significantly [25], in particular with respect to proteins relevant for cytoskeletal organization and pre-synaptic differentiation. This previous study also did not find evidence that the C9ORF72 protein itself acts as a GEF for these Rab proteins. SMCR8 bound to Rab8 and Rab39 independently of C9ORF72, indicating that C9ORF72 itself does not function as a GEF in this context but rather as a modulator of the GEF activity of SMCR8. Thus, the question remains open whether small GTPases are modulated directly by C9ORF72, and how C9ORF72 achieves this function as a modulator of GDP/GTP binding [32, 33]. In our study, we identified enhanced GTP binding in Arf6 and Rac1 after C9ORF72 depletion. This effect was also observed in iPS cell derived motoneurons from C9-ALS patients, in lymphoblastoid cells and in the postmortem brain samples from C9-ALS patients. We do not know yet how C9ORF72 modulates the GTP binding of these small GTPases. This could be via action as a GEF for one or more upstream small GTPases, or via inhibition of other GEFs for Arf6 and Rac1 which then results in LIMK-1/2 and phosphorylation of cofilin. Such potential allosteric mechanisms that modulate GEF activity have been identified in the context of differential regulation of Ras and Rac activity by Sos. When Sos is bound to Abi1/E3B1, Eps8 and PI3K [47], it acts as a GEF for Rac. However, when Sos is bound to Abi1/E3b1, it cannot bind to Grb3, and thus its GEF activity for Ras is reduced [48]. Such a mechanism could explain the enhanced GTP binding of Arf6 and Rac1 when C9ORF72 levels are low in iPS cell derived

motoneurons, in postmortem brain samples for C9-ALS patients and in primary mouse motoneurons after lentiviral C9ORF72 knockdown.

The presence of Cofilin, Coronin and other actin modulatory proteins in complexes with endogenous C9ORF72 indicates that the modulation of actin dynamics could constitute an essential cellular function C9ORF72 in motoneurons. Enhanced phosphorylation of LIMK-1/2 and cofilin in iPSC derived neurons and in postmortem brain samples of C9-ALS patients indicates that this mechanism might contribute to the pathology in ALS and FTLD.

Data availability.

The data that support the findings of this study are available from the corresponding author upon request.

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Author Contributions:

R.S., C.D., M.S., D.H., F.M. and M.M. designed the experiments. R.S. developed lentiviruses, performed all motoneuron cultures and did all experiments to characterize the function of C9ORF72 in cultured motoneurons. C.D. and A.A.H. helped with the initial generation of viral vectors for C9ORF72-HA overexpression and knockdown. D.H. did the LC-MS experiments and D.H., M.F. and M.M. were responsible for the analysis of the LC-MS result. N.F helped with live cell imaging and analysis. A.H., X.L. and J.S. contributed iPS cells and performed experiments with iPS cells derived motoneurons. P.S. and P.I. collected and provided postmortem tissues from ALS patients. R.S. and M.S. wrote the manuscript. All authors read and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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