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Toxic gain-of-function mechanisms in *C9orf72* ALS-FTD neurons drive autophagy and lysosome dysfunction

Jimmy Beckers [a,b] and Philip Van Damme [a,b,c]

[a]Department of Neurosciences, Experimental Neurology, and Leuven Brain Institute (LBI), KU Leuven-University of Leuven, Leuven, Belgium; [b]VIB, Center for Brain & Disease Research, Laboratory of Neurobiology, Leuven, Belgium; [c]Department of Neurology, University Hospitals Leuven, Leuven, Belgium

ABSTRACT

Hexanucleotide repeat expansions in the *C9orf72* gene are the primary genetic cause for both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two related neurodegenerative diseases. Significant advances in the elucidation of the disease mechanisms responsible for *C9orf72* ALS-FTD have revealed both a toxic gain-of-function and a loss-of-function mechanism as possible underlying disease cause. As the differential contribution of both gain and loss of function in *C9orf72* ALS-FTD pathogenesis remains debated, we investigated disease mechanisms in motor neurons derived from both authentic human patient *C9orf72* ALS-FTD iPSCs as well as a *C9orf72* knockout iPSC line. We found that patient neurons presented with less motile and enlarged lysosomes, a decrease in autophagic flux and an increase in SQSTM1/p62 puncta and insoluble TARDBP/TDP-43 species. Importantly, we found that *C9orf72* knockout barely has any influence on these phenotypes and mainly results in impaired endosomal maturation. Together, our data suggest that toxic gain-of-function, rather than loss-of-function, mechanisms in *C9orf72* ALS-FTD impair the autophagy-lysosome system in neurons.

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Over a decade ago, a groundbreaking discovery in the field of ALS and FTD made it possible to ascribe the majority of familial and a significant proportion of sporadic ALS and FTD cases to "GGGGCC" hexanucleotide repeat expansions (HREs) in the *C9orf72* gene. Significant advances in the elucidation of the disease mechanisms responsible for C9orf72 ALS-FTD have put forward three hypotheses. The first two hypotheses are both toxic gain-of-function mechanisms that propose a toxicity from either RNA foci transcribed from these HREs or from the dipeptide repeat protein directly transcribed from HRE-containing RNA species. The third hypothesis suggests a loss-of-function mechanism marked by reduced levels of the native C9orf72 protein, which is best known for its role in the regulation of membrane trafficking events. Importantly, while these proposed mechanisms are not mutually exclusive, most recent studies emphasize the importance of toxic gain-of-function mechanisms as primary disease cause but attribute a synergistic role to C9orf72 loss of function. In ALS and FTD, a specific subpopulation of neurons degenerates. While the reason for this selective vulnerability is not entirely clear, the presence of large protein aggregates suggests a defect in the mechanisms that are meant to remove these inclusions, i.e., macroautophagy/autophagy. Moreover, mutations in several genes implicated in the autophagy-lysosome pathway such as *SQSTM1/p62*, *GRN*, *TMEM106*, *VCP*, *UBQLN2*, *TBK1* and *OPTN* have been linked to ALS-FTD.

In order to try and add one or more pieces to this complex puzzle, we generated motor neurons (MNs) and investigated pathogenic disease mechanisms from *C9orf72* patient-derived (C9-patient) iPSCs and a *C9orf72* knockout (C9-KO) iPSC line focusing on the autophagy-lysosome pathway in our recent study [1]. Using these C9-patient MNs, we were able to report the surprising finding that C9orf72 toxic gain-of-function mechanisms lead to dysfunction in multiple aspects of multiple features of the autophagy-lysosome pathway (ALP). These ALP-related phenotypes include, but are not limited to, abnormal lysosome transport, altered lysosomal morphology and disrupted lysosome homeostasis, SQSTM1 accumulation and autophagic flux inhibition. Interestingly, these defects coincided with or even preceded MN degeneration and TARDBP pathology in our C9-patient MNs, suggesting an active role for ALP dysregulations in the pathogenesis of *C9orf72* ALS-FTD. In our study, we found that C9-patient MNs harbor less mature $CTSD^{+}$ lysosomes and have an increased proportion of enlarged and dysfunctional lysosomes at least in part caused by defects in axonal transport of these vesicles as this process is indispensable for their maturation and functioning. Interestingly, these (endo)lysosomal defects are recapitulated at the functional level as we observed accumulation of both insoluble TARDBP species, an increase in SQSTM1-positive inclusions, a reduction in autophagic flux and an increased vulnerability to autophagic stress (Figure 1). Induction of autophagy, however, seems to be normal in these C9-patient MNs.

CONTACT Philip Van Damme philip.vandamme@uzleuven.be Department of Neurosciences, Experimental Neurology, and Leuven Brain Institute (LBI), KU Leuven-University of Leuven, O&N, V Herestraat, 49 - box 602, Leuven 3000, Belgium

This article has been corrected with minor changes. These changes do not impact the academic content of the article.

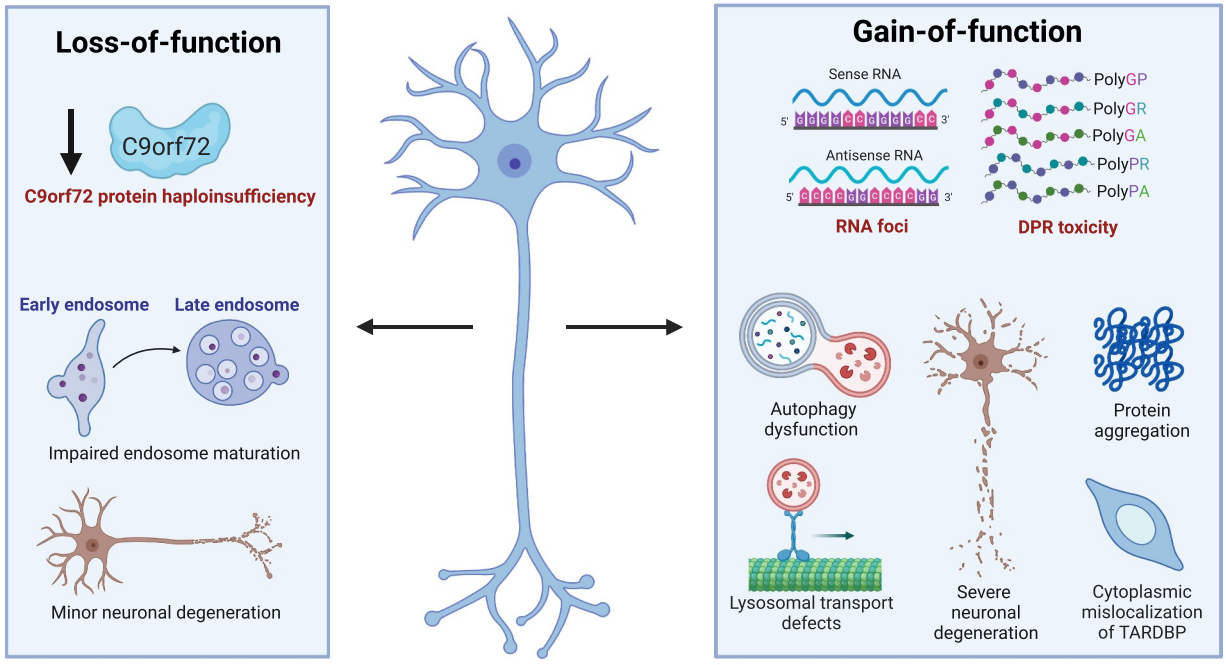

Figure 1. Overview of the phenotypes found in C9-patient and C9-KO MNs. (Right) Neuronal toxic gain-of-function mechanisms (RNA foci and/or dipeptide-repeat proteins [DPRs]) of the C9orf72 HRE result in autophagy dysfunction, lysosomal transport defects, protein aggregation, TARDBP mislocalization and a relatively high degree of MN degeneration. (Left) Neuronal loss of function of the C9orf72 protein mainly results in impairments of endosomal maturation and a lesser degree of neurodegeneration.

Although we observe a marked reduction in C9orf72 protein in *post-mortem* tissue from patients, we fail to detect this C9orf72 haploinsufficiency in C9-patient MNs. However, as C9orf72 expression is highly cell-type dependent and is mainly expressed in microglial cells, rather than in MNs, this finding could reflect physiological conditions. Despite our initial results supporting a role for toxic gain-of-function mechanisms rather than C9orf72 haploinsufficiency in ALP dysfunctions in MNs, we decided to contribute to the understanding of C9orf72 haploinsufficiency and assessed some of our phenotypes in C9-KO MNs. Surprisingly, complete loss of C9orf72 has only marginal or even no effects on lysosomal homeostasis and autophagy. However, endolysosomal maturation is clearly impaired in C9-KO MNs, which is not surprising as C9orf72 is suggested to regulate membrane trafficking events (Figure 1). Although this endosomal phenotype might ultimately affect the autophagy-lysosome pathway, our results highlight a more pronounced role for gain-of-toxic-function mechanisms in autophagy dysregulation in *C9orf72* ALS-FTD neurons. Finally, inspired by data from a recent study that links toxic gain-of-function mechanisms in *C9orf72* with *TBK1* and TARDBP pathology driven by endolysosomal defects, we also assessed TBK1 activity. Interestingly, whereas reduced TBK1 activity is suggested to cause endolysosomal defects, we observe hyperphosphorylation of TBK1 in C9-patient MNs. Despite activating TBK1, this hyperphosphorylation is thought to be a result of TBK1 sequestration and concomitant inactivation of the kinase by C9orf72 dipeptide-repeat proteins that lead to autophosphorylation of TBK1. While the mechanistic basis underlying these ALP defects observed in C9-patient MNs definitely warrants further investigation, our results highlight alterations in TBK1 function as being at least one of the important players in this process.

More and more studies point toward the pathogenic involvement of endolysosomal dysfunctions in both ALS and FTD. Although our data strengthen this hypothesis and highlight the ALP as a promising therapeutic target for multiple, if not all, ALS and FTD subtypes, it also raises some critical questions especially in the context of *C9orf72* ALS-FTD. For instance, recent studies in ALS and other diseases have uncovered defects in multiple stages of this pathway including autophagy induction, autophagosome formation, autophagosome-lysosome fusion, lysosome function, endosome recycling or autophagosome and lysosome biogenesis. However, it is crucial for future autophagy-targeted therapy development that we accurately define the exact step of this process that is defective in ALS-FTD given the importance of the delicate balance between the formation of autophagosomes and their lysosomal degradation on cellular health. In addition, the lack of full-blown (endo)lysosomal phenotypes upon complete ablation of C9orf72 protein levels in neurons, does question the involvement of subtle downregulations of C9orf72 in patient motor neurons. Nevertheless, we do not argue or claim that C9orf72 haploinsufficiency is not involved in the pathogenesis of *C9orf72* ALS-FTD. In fact, we strongly think that reduced C9orf72 protein levels cooperate with toxic gain-of-function mechanisms, albeit not in MNs as C9orf72 is expressed at a very low level in MNs, while it is highly expressed in microglia. We therefore suspect that

knockout of *C9orf72* (or C9orf72 haploinsufficiency) will have a more profound effect on autophagy, and possible other pathways, in other cell types. Investigation of the relative contribution of non-cell autonomous disease mechanisms caused by ALP dysfunctions in microglia and astrocytes on neuronal health are thus crucial in our understanding of *C9orf72* ALS-FTD pathogenesis.

Disclosure statement

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ORCID

Jimmy Beckers http://orcid.org/0000-0003-2484-6556
Philip Van Damme http://orcid.org/0000-0002-4010-2357

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