COMMENTARY

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RNA versus protein toxicity in *C9orf72* ALS/FTLD

Thomas Arzberger[1,2,3] · Martin H. Schludi[1,4] · Carina Lehmer[1,4] · Bettina Schmid[1,4] · Dieter Edbauer[1,4]

A $(G4C2)_n$ expansion with several hundred or thousand repeats in the first intron upstream of the *C9orf72* coding region is the most common cause of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), but the driver mechanism remains unclear [7]. Three main pathomechanisms have been proposed, but their relative role is vigorously debated because they require partially opposing therapeutic strategies. Three reports in this issue take a provocative stance strongly arguing for either RNA or protein toxicity in *C9orf72* pathogenesis (Fig. 1).

Sense and antisense transcripts of the repeat accumulate in ubiquitous small nuclear, and occasionally cytoplasmic, RNA foci. Many $(G4C2)_n$-binding proteins have been identified that are partially sequestered by the repeat RNA. Several of the trapped RNA-binding proteins are involved in alternative splicing and splicing abnormalities have been reported in *C9orf72* patients [22, 25]. However, a sophisticated study on 63 *C9orf72* cases shows no correlation of sense and antisense foci with neurodegeneration or clinical parameters [6], although antisense foci have been linked to TDP-43 pathology by others [5].

Repeat-associated non-ATG (RAN) translation of both sense and antisense repeat transcripts in all reading frames generates five co-aggregating dipeptide repeat (DPR) proteins: poly-GA/GP/GR from the sense transcript and poly-GP/PA/PR from the antisense transcript. The sense-strand derived DPRs are abundant throughout the neocortex,

hippocampus, thalamus and cerebellum, but scarce in brain stem and spinal cord. Although the DPR proteins co-aggregate predominantly in cytoplasmic and less frequently in intranuclear inclusions in neurons, the individual proteins have very different biophysical properties. Cryoelectron tomography shows that poly-GA forms twisted ribbons that interfere with proteasome function [9]. Poly-GR and -PR undergo liquid–liquid phase separation in vitro and interfere with the dynamics of the nucleolus and stress granules [13] and disturb nucleocytoplasmic transport [12]. The aggregate distribution of neither DPR species has been shown to correlate with TDP-43 pathology or neurodegeneration [16, 17, 27].

Finally, the repeat expansion interferes with transcription and/or splicing and leads to lower *C9orf72* expression, a protein that has recently been linked to autophagy [8]. Homozygous knockout of *C9orf72* causes a variable (lupus-like) immune phenotype due to strong expression of *C9orf72* in the myeloid lineage, but the mice show no overt neurodegeneration [1, 20]. However, *C9orf72* haploinsufficiency sensitizes patient-derived motoneurons to DPR toxicity and other stressors, suggesting it may contribute to neurodegeneration in *C9orf72* ALS/FTD [28]. Thus, lowering *C9orf72* expression to treat gain-of-function mechanisms may be detrimental.

Swinnen et al. [29] injected zebrafish embryos with sense and antisense *C9orf72* repeat RNA or synthetic genes encoding individual DPR proteins. This method is more rapid than transgenesis and allows dosage studies; however, only developing embryos can be analyzed. They focus their analysis on the length and branching pattern of motor axons during outgrowth. Consistent with severe toxicity in other models, $(GR)_{50}$ and $(PR)_{50}$ expression resulted in shorter axons with abnormal branching pattern, while the other individual DPRs had no effect. Expression of sense or antisense RNA had a similar effect starting at 35 or 70 repeats, respectively. In all cases, toxicity was dose dependent. Strikingly, repeat RNA injection did not result in significant DPR expression via RAN-translation in zebrafish embryos. Poly-GR/PR was not detectable by dot blot, although poly-GR/PR could still

✉ Dieter Edbauer
   dieter.edbauer@dzne.de

1   German Center for Neurodegenerative Diseases (DZNE),
    Munich, Feodor-Lynen-Str. 17, 81377 Munich, Germany

2   Center for Neuropathology and Prion Research,
    Ludwig-Maximilians-University Munich,
    Feodor-Lynen-Str. 23, 81377 Munich, Germany

3   Department of Psychiatry and Psychotherapy,
    Ludwig-Maximilians-University Munich, Nußbaumstraße 7,
    80336 Munich, Germany

4   Munich Cluster of Systems Neurology (SyNergy),
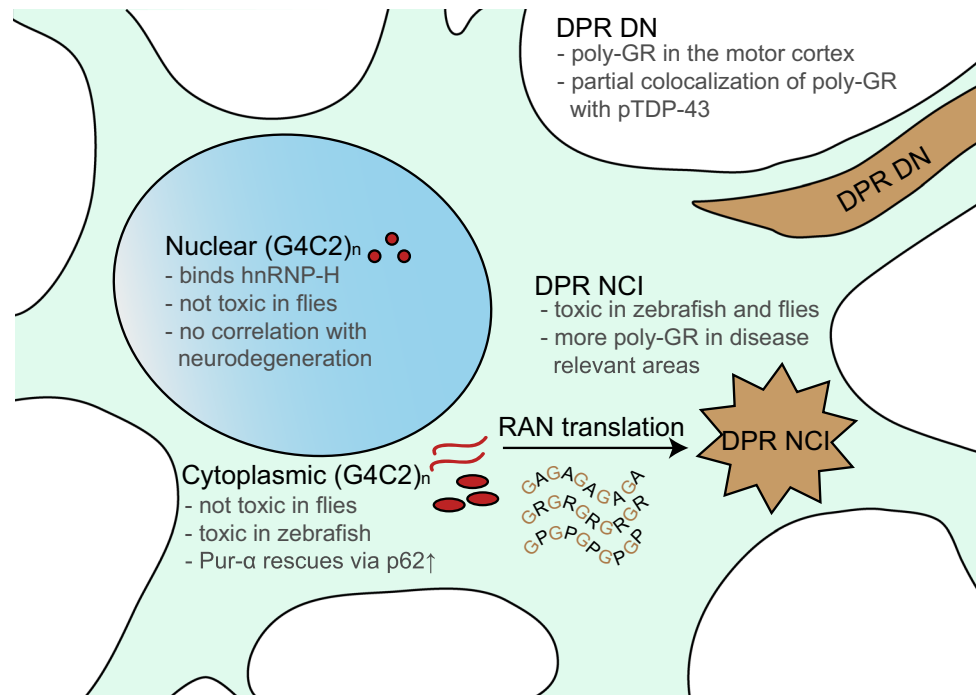   Feodor-Lynen-Str. 17, 81377 Munich, Germany

Fig. 1 Novel insights into C9orf72 pathomechanisms. Three reports in this issue dissect RNA and protein toxicity mediated by the $(G4C2)_n$ repeat expansion. The $(C4G2)_n$ antisense transcript has similar effects in drosophila and zebrafish and is translated into additional rare DPR proteins poly-PR and poly-PA (not depicted)

be detected at non-toxic levels in embryos injected with synthetic DPR constructs. In contrast to the fly model [18], injection of sense and antisense RNA interrupted by stop codons in all reading frames ("RNA-only") still reduced axon length, but had very little effect on branching. To identify mediators of toxicity of the RNA repeats and potential drug targets, Swinnen et al. analyzed RNA-binding proteins known to interact with the $(G4C2)_n$ repeat. Co-expression of Pur-alpha rescued RNA toxicity depending on the G-rich and PUR2 domain, although these domains are not required for $(G4C2)_n$-binding, which argues for an indirect effect. Other well-characterized repeat-binding proteins, such as hnRNPH and hnRNPA1 did not rescue, which highlights the role of Pur-alpha in repeat RNA toxicity. This effect depends on a ~ 50% upregulation of p62 by Pur-alpha expression, which is unexpected because p62 is the prototypical component of proteinaceous inclusions.

A caveat of this interesting model is the predominantly cytoplasmic localization of the injected repeat RNA, which is in contrast to the predominantly nuclear RNA foci seen in patients. In *C9orf72* post-mortem tissue, nuclear RNA foci show little correlation with neurodegeneration and disease parameters, but cytoplasmic repeat RNA has not been investigated so far [6].

Consistent with previous analysis in zebrafish, repeat RNA is clearly toxic in zebrafish upon RNA injection [14]

and transgene driven overexpression [21]. However, motor axons only seem to be affected upon RNA injection and are unaffected when derived from a transgene [21] potentially due to different timing of expression during development or different structure of in vitro versus in vivo generated RNA species. Poly-GA/GP/GR were not detectable by immunoblotting in these models, which also argues for a significant component of RNA toxicity. It is unclear, why RAN-translation is so inefficient in zebrafish, but the early developmental stage could be a key factor, because DPR expression is also barely detectable in most iPSC-derived neurons.

In the same issue, Moens et al. [19] analyzed RNA toxicity in drosophila comparing different length of sense and antisense RNA-only repeats targeted either to the nucleus using intronic expression or targeted to the cytoplasm using a polyA tail. The inducible transgenic system is very elegant because it allows direct comparison of each line with or without expression of the *C9orf72* repeat to exclude integration-specific effects and other genetic variabilities. Importantly, the expression is restricted to adult neurons and allows life-long phenotyping. Some lines contain over 1000 repeats confirmed by Southern blotting like most typical *C9orf72* patients. PolyA-tailed RNA forms cytoplasmic foci (similar to the zebrafish model), while intronic expression results in nuclear RNA foci that are predominant in patients. The interspersed stop codons in all reading frames

completely suppress DPR expression in most lines. The nuclear $(G4C2)_n$ foci also partially colocalized with Glorund, the fly ortholog of hnRNP-H, an RNA-binding protein consistently reported to bind the *C9orf72* repeat RNA. Quite strikingly, none of the RNA-only fly lines showed any toxic phenotype or survival deficits. In fact, two independent lines expressing $(G4C2)_{100}$ in an intron even survived significantly longer upon transgene induction, although only by a few days. The 1000-repeat lines had much more abundant foci, but no apparent behavioral or developmental phenotype. Thus, RNA foci of even large sense and antisense repeat transcripts cause no overt phenotype in Drosophila in the absence of DPR expression, although they bind at least one RNA-binding protein like in humans.

Therefore, in fly models, $(G4C2)_n$ toxicity is clearly predominantly driven by poly-GR toxicity [18, 30]. A possible explanation for the lack of RNA toxicity in this fly model is the pronounced formation of RNA foci which is in contrast to the more diffusely distributed cytoplasmic repeat RNA in zebrafish. Moens et al. and Swinnen et al. are the first models for antisense RNA toxicity. Unfortunately, some important details about the antisense transcript in patients are still unclear, such as the exact start site, splicing events, capping and poly-adenylationation. Therefore, we cannot tell which of the models reflects the patient situation best.

Saberi et al. [24] analyzed *C9orf72* haploinsufficiency and poly-GR toxicity through nucleocytoplasmic transport in a small cohort of *C9orf72* ALS cases with short post-mortem intervals. The authors focused on areas with clear clinical relevance (motor cortex, frontal cortex and the anterior horn of the spinal cord) and compared them to areas with no clear clinical relevance for ALS/FTLD (parietal cortex, occipital cortex and posterior horn of the spinal cord). To address the *C9orf72* loss of function component, they thoroughly validated a commercial antibody in mouse knockout tissue because raising good antibodies has been notoriously difficult. The reduction of C9orf72 protein in both frontal and occipital cortex extends previous findings [31] and supports a haploinsufficiency component, while arguing against its primary role.

Using home-made rabbit polyclonal DPR antibodies, they observed inclusions pathology largely consistent with previous observations. Strikingly, they found much higher levels of poly-GR but not of other DPR species in frontal and motor cortex compared to parietal and occipital cortex and detected dendritic poly-GR inclusions only in the motor cortex. 85% of these inclusions colocalized with phosphorylated TDP-43 (pTDP-43), although only 4% of dendritic pTDP-43 inclusions colocalized with poly-GR.

Both poly-GR and RNA toxicity has been linked to impaired nucleocytoplasmic transport in various model systems. However, the detailed analysis by Saberi et al. shows normal distribution of RanGAP and Lamin B1 in motor cortex or anterior horn of the spinal cord. In particular, neurons with poly-GR inclusions show similar RanGAP distribution as neighboring cells. The nuclear shape was slightly abnormal in both *C9orf72* and non-hereditary ALS cases. This is consistent with a parallel study showing that nuclear pore defects are not unique for *C9orf72* cases but are also directly connected to the TDP-43 pathology in ALS [4].

Thus, Saberi et al. support a causal role of poly-GR in *C9orf72* pathogenesis, while questioning a key pathway proposed to mediate its toxicity. Unfortunately, the cohort is rather small ($n = 4$–5) and does not contain pure FTLD cases. Also, the degree of DPR pathology in the frontal cortex has not been correlated with the degree of neurodegeneration or the presence of FTD-like symptoms. Therefore, the study cannot explain why some *C9orf72* patients develop pure ALS, FTLD or a mixture of both.

The different frequencies of both perinuclear and dendritic poly-GR inclusions in disease-related regions vs. disease-unrelated regions in this cohort are striking. In a previous study, we had reported similar levels of poly-GR inclusions in those areas [27]. Furthermore, the findings of Saberi et al. are in contrast to absent or very low neuritic poly-GR pathology detected in the motor cortex with two different monoclonal poly-GR antibodies [17, 27]. Re-evaluation of our own stainings confirmed our previous results that neuritic poly-GR pathology was generally rare with the highest frequency in hippocampal areas, but was not restricted to the motor cortex as observed by Saberi et al.

For poly-GR the specificity of the antibodies used in the different studies is decisive, because poly-GR shows complex post-translational modifications. Arginine methylation gives rise to four different species that likely occur in a complex mixture even within one molecule: non-methylated, mono-methylated, symmetrically di-methylated and asymmetrically di-methylated. De-imination of arginine to citrulline is another conceivable modification. Elucidating the exact specificity of the new antibody for the different poly-GR modification and replication in a larger and more diverse cohort will be crucial to draw mechanistic conclusions.

What does that mean for developing a therapy for *C9orf72* ALS/FTD? Not surprisingly, the papers conclude with opposing suggestions to either focus on the RNA or DPRs. Using the strength of the different available models wisely, is likely the best strategy to design and validate therapeutic approaches. Thanks to Moens et al., it is now clear that the *C9orf72* repeat in the current fly models predominantly invokes poly-GR/PR toxicity. It will be interesting to test long repeat models not interupted by stop codons to study the effects of DPR proteins of physiological length. In zebrafish embryos, RNA-mediated toxicity seems to dominate, but forced expression of DPRs can boost toxicity. BAC-transgenic and AAV-based mouse models recapitulate RNA foci, DPR inclusions, (nuclear) TDP-43 pathology and

neurodegeneration and argue for combination of RNA and DPR toxicity [2, 15], because at least the current DPR-only mice show less severe phenotypes and only faint hints of TDP-43 pathology [26]. However, strain-specific effects such as a different genetic background seem to affect the phenotype of both *C9orf72* BAC and knockout mice [10]. It is remarkable that only 6 years after the discovery of the C9orf72 mutation, the current mouse models are already closer to human pathology than Alzheimer mouse models. Human iPSC-derived neurons are widely popular, but DPR expression is very low in most differentiation protocols and robust TDP-43 pathology has not been reported. Thus, iPSC models may be most useful to study RNA toxicity and the consequences of reduced *C9orf72* protein expression [28]. Human neuropathology clearly remains the gold standard. A comprehensive quantitative analysis of proteins interacting the repeat RNA in nuclear and cytoplasmic RNA foci throughout the brain could be rewarding.

Until we know more about the pathomechanisms, the safest bet is to target both the repeat RNA and the DPRs, for example, by antisense oligonucleotides [11] that have been recently approved for the therapy of spinal muscular atrophy after a very successful clinical trials [3]. However, the long prodromal stage of *C9orf72* disease might require very early treatment [23], which is less feasible with the current invasive delivery method. Dissecting the mechanistic cascade of *C9orf72* disease will require continuous comparison of animal models with human pathology. Ultimately only an effective drug that targets only a subset of the proposed *C9orf72* mechanisms (e.g., immunotherapy) will reveal the crucial component for *C9orf72* ALS/FTLD.

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