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Review Article

Arginine-rich dipeptide-repeat proteins as phase disruptors in C9-ALS/FTD

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A hexanucleotide repeat expansion GGGGCC (G_4C_2) within chromosome 9 open reading frame 72 (*C9orf72*) is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD). This seminal realization has rapidly focused our attention to the non-canonical translation (RAN translation) of the repeat expansion, which yields dipeptide-repeat protein products (DPRs). The mechanisms by which DPRs might contribute to C9-ALS/FTD are widely studied. Arginine-rich DPRs (R-DPRs) are the most toxic of the five different DPRs produced in neurons, but how do R-DPRs promote C9-ALS/FTD pathogenesis? Proteomic analyses have uncovered potential pathways to explore. For example, the vast majority of the R-DPR interactome is comprised of disease-linked RNA-binding proteins (RBPs) with low-complexity domains (LCDs), strongly suggesting a link between R-DPRs and aberrations in liquid–liquid phase separation (LLPS). In this review, we showcase several potential mechanisms by which R-DPRs disrupt various phase-separated compartments to elicit deleterious neurodegeneration. We also discuss potential therapeutic strategies to counter R-DPR toxicity in C9-ALS/FTD.

Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are devastating neurodegenerative diseases that lie within a continuous clinical and pathological spectrum [1,2]. Most patients with ALS/FTD die within ~3–10 years from disease-onset, underscoring the vital need to find a cure [3,4]. The discovery of the hexanucleotide repeat expansion GGGGCC (G_4C_2) within the first intron of chromosome 9 open reading frame 72 (*C9orf72*) as a common genetic cause of ALS/FTD has massively stimulated research aimed at revealing the etiology [5,6]. It has been established that patients with C9-ALS/FTD can have hundreds to thousands of G_4C_2 repeats within hexanucleotide repeat expansions (HREs), in contrast to healthy individuals who carry between ~2 and 23 repeats [5–7]. Although many questions lie ahead, our partial understanding of the genetic mechanisms of C9-mediated disease has enabled a clearer path to potential therapeutics for C9-ALS/FTD.

C9-mediated toxicity is thought to arise from three non-mutually exclusive mechanisms [2,8]:

1. *Loss-of-function due to reduced expression of the C9orf72 protein* [5,6,9–11]. C9orf72 protein is a guanine nucleotide exchange factor (GEF) for Rab- and Rho-GTPases, and is believed to have a role in membrane trafficking and autophagy [12]. Low levels of C9orf72 protein have been observed in the frontal and temporal cortices of ALS/FTD post-mortem brain tissue [11]. Additionally, knockdown of *C9orf72* orthologs in zebrafish and *Caenorhabditis elegans* led to neuronal degeneration [13,14]. However, none of the mouse *C9orf72* knock-out models recapitulated ALS/FTD phenotypes, which initially suggested that while loss-of-function may contribute to disease, it is likely not the primary disease-driving mechanism [10,15]. However, more recent studies in mice indicate that reduced C9orf72 function can enhance G_4C_2 repeat-associated toxicity [16,17]. Moreover, loss of

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C9orf72 function can alter macrophage and microglia function [18] and exacerbate neural inflammation caused by gut bacteria [19,20], which can prompt immune responses that might also contribute to neurodegeneration.

2. *Toxic gain-of-function due to C9-RNA accumulation.* The discovery of the G_4C_2 repeat expansion was accompanied by the discovery of its bidirectional transcription, which leads to the production of sense and antisense repeat RNAs [5,21,22]. C9-RNAs can fold into stable secondary structures, including hairpins and G-quadruplexes that are capable of sequestering many essential RNA-binding proteins (RBPs) in nuclear and cytoplasmic foci [10,23–25]. This sequestration, in turn, is proposed to lead to aberrations in RNA-related processes, including splicing, translation, and RNA transport, majorly contributing to the pathology seen in several C9-ALS/FTD disease models [24,26,27]. Importantly, targeting the sense strand of the repeat with antisense oligonucleotides (ASOs) rescues disease phenotypes, and is considered a highly valuable therapeutic strategy [28].
3. *Toxic accumulation of dipeptide-repeat proteins (DPRs).* DPRs produced via non-AUG driven translation may be another mechanism associated with C9-toxicity, and is the major focus of this review [10,29]. Repeat-associated non-AUG (RAN) translation of the sense and antisense strands of the HRE has been observed [29–31]. While there is evidence that the production of DPRs can be detrimental to neurons, their relative contribution to neurodegeneration in ALS/FTD remains unclear and is debated.

Five different DPRs are produced by RAN translation: glycine-alanine (GA), glycine-arginine (GR), and glycine-proline (GP) are produced from the sense strand; proline-alanine (PA), proline-arginine (PR), and GP are produced from the antisense strand [30,31]. It is believed that RAN translation of the sense strand occurs more frequently than that of the antisense HRE, with GA aggregates being the most abundantly detected in post-mortem tissue [9,30,32]. Whether DPRs are toxic at pathophysiological conditions and concentrations is yet to be determined. Nevertheless, it has been established that GA is primarily visible in the brain and spinal cord of C9-ALS/FTD patients, has a strong propensity to aggregate, and its expression in neuronal cell cultures causes several pathological outcomes that render it moderately toxic [10,33–36]. Unlike PA and GP, the two other uncharged DPRs, GA expression results in increased ER stress, reduced dendritic branching, proteasomal inhibition, and induced apoptosis [10,34,35]. While in some contexts PA has been found to be toxic [36], PA and GP are generally thought of as the inert DPRs, since they are commonly nontoxic and have no known protein-binding partners [10]. However, emerging evidence has shown that GA aggregates in C9-ALS/FTD post-mortem tissue co-localize with and promote the seeding of GP inclusions [37]. Surprisingly, further investigation provided evidence that the predominant mechanism by which this seeding occurs is via the production of chimeric GA:GP DPRs, which is a newly described phenomenon that provides an additional level of complexity to our understanding of how DPRs collaborate in disease [37]. Finally, GR and PR, the highly charged, polar, and arginine-rich DPRs, are the most heavily studied DPRs due to the compelling evidence demonstrating their high toxicity in several disease models [8].

Arginine-rich DPRs (R-DPRs) have several features in common. When individual DPRs were expressed in yeast, mammalian cells, fly, zebrafish, or mouse models, the highest levels of toxicity were consistently observed with GR and PR [10,38–40]. Additionally, when overexpressed in cellular models, R-DPRs were both localized to the nucleolus, where they impaired rRNA synthesis and ribosome biogenesis [41,42]. Furthermore, proteomic analysis revealed that GR and PR have overlapping interactomes, with the majority of their binding partners being proteins harboring low-complexity domains (LCDs) and ribosomal proteins [40,43,44]. These common features put GR and PR at the forefront for therapeutic targeting, and therefore, a clear mechanistic understanding of their pathological pathways is vital. While there are several similarities between GR and PR, differences in their subcellular localizations, binding partners, and overall stability may provide valuable insights into how they can yield a variety of toxic outcomes in C9-ALS/FTD.

Initial mechanistic insights on GR/PR toxicity stem from proteomic studies [40,43–47]. LCD-containing proteins, which comprise the vast majority of the GR/PR interactome, are capable of undergoing liquid–liquid phase separation (LLPS), the physical process by which two liquids spontaneously separate [40,48–50]. The unstructured and flexible nature of LCDs allow them to engage in a series of multivalent interactions, favoring their dynamic assembly into thermodynamically stable, higher-order structures [50,51]. In a physiological context, phase separation is the driving force for the formation of membraneless organelles, including stress granules, Cajal bodies, and the nucleolus, all of which are 'hubs' of concentrated RNA and LCD-containing proteins that perform specialized functions [48,49,52]. A plethora of evidence shows that R-DPRs can

specifically bind to the low-complexity sequences of RBPs — the major constituents of membraneless organelles. Those interactions with GR/PR could potentially perturb the phase separation, formation, dynamics, and functions of membraneless organelles, which can subsequently lead to pathology [40]. In fact, using an RNAi-based genetic screen, several GR/PR-interacting, LCD-containing proteins were identified as significant genetic modifiers of toxicity in a C9-fly model [40]. These findings illuminate the interconnected nature of the different mechanisms leading to aberrant phase separation in neurodegeneration. On one hand, disease-linked mutations in RBPs, most commonly occurring in the LCD region (or their prion-like domain, PrLD [53]), directly promote aberrant phase separation behavior and accelerated aggregation of proteins [54–58]. On the other hand, the interactions of R-DPRs with RBPs indirectly lead to their aberrant phase separation [59]. Thus, two mechanisms of toxicity ultimately converge to result in the same pathological outcome.

While targeting R-DPRs is becoming an enticing therapeutic strategy for C9-ALS/FTD, we are still faced with several challenging roadblocks. To date, most *in vitro* and *in vivo* studies elucidating the toxic effects of R-DPRs employ overexpressed peptide sequences that are typically short in length. The overexpression of short R-DPRs in cellular models might not accurately reflect their length, concentration, and subcellular localization in C9-ALS/FTD patients, which represents an important caveat for the field [9,10,36,60]. Additionally, given the toxicity of R-DPRs, it is thought that most vulnerable neurons with the highest load of DPRs are likely long dead and gone at the time of autopsy, which hinders our ability to obtain post-mortem tissue to further investigate the pathological contribution of R-DPRs [9,32,61]. In fact, there can be no correlation between DPR load and neurodegeneration in post-mortem tissue [61], although perhaps the closest correlation is with GR [62]. Moreover, most studies focus on examining large insoluble inclusions, which undermines the burden of soluble DPRs. Furthermore, it is difficult to dissect the contribution of RNA foci from DPR toxicity when the RNA repeats themselves are the source of DPRs. In an attempt to tease out repeat RNA versus DPR toxicity, an earlier study expressing 'pure repeats' that can be RAN translated, or RNA-only repeats that cannot undergo RAN translation, showed that RAN-translated repeats are more toxic, which could be attributed to the production of DPRs [39]. While this was a successful approach, one caveat is that it remains unknown whether non-RAN-translated repeats behave similarly to 'normal' repeats in terms of their interactions with RBPs. Therefore, downstream effects with non-RAN translated repeats might be lessened due to the inherent differences between the two sequences and not due to the absence of DPRs or RAN translation [63,64]. Importantly, however, it is now clear that neuroprotection can be achieved by selective inhibition of RAN translation while allowing transcription of G_4C_2 repeats to continue [65]. Additionally, a recent study in transgenic fly models and rodent neurons suggested that the C-terminal extension outside the repeat sequence is another determining factor of DPR toxicity, which adds to the overall complexity of differential DPR behavior and pathogenesis [66]. Collectively, these findings suggest that DPR toxicity is a major driving force in C9-ALS/FTD. Nevertheless, the mechanisms that underlie DPR toxicity are still being elucidated. In this review, we will cover the mechanisms by which R-DPRs disrupt specific phases. We will then discuss potential therapeutic strategies to combat their deleterious effects in neurodegeneration.

Effects of GR/PR on membraneless organelles

Nucleolus

The nucleolus is a prominent membraneless organelle in eukaryotic cells. It is composed of three biophysically and functionally distinct liquid layers: the fibrillar center (FC), the dense fibrillar component (DFC), and the outermost granular component (GC) [67–69]. Each region hosts a specific subset of proteins and plays a unique role in rRNA synthesis and ribosome biogenesis. Previous studies using mammalian cells and neuronal cultures showed that nucleolar stress is associated with neurodegenerative disease, majorly due to the fact that the nucleolus is home to R-DPRs [38,40,42]. GR and PR are enriched in the GC region, evident from co-localization with nucleophosmin-1 (NPM1), a major constituent of the GC [40,42,70]. GR, but not PR, is also found in the DFC, where fibrillarin is a major constituent [70]. Finally, both R-DPRs are excluded from the innermost FC. Overexpression studies in mammalian cells, and *in vitro* biochemical assays demonstrated that the detrimental effects of GR and PR on nucleolar integrity and overall functions are largely attributed to their direct interactions with nucleolar components, like NPM1, and disruptions of nucleolar phase dynamics (Figure 1) [40,70].

NPM1 is a key protein in maintaining the nucleolar liquid-like properties. It has three acidic LCDs, allowing it to form multivalent interactions with arginine-rich motifs [71]. This property makes NPM1 a vulnerable

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target for R-DPRs. Indeed, both GR and PR directly interact with NPM1, *in vitro* and in cells, and cause an overall increase in GC rigidity [40,70]. Interactions with R-DPRs enhance the LLPS of NPM1 by reducing the critical concentration required to undergo phase separation. This effect, in turn, alters the interactions of NPM1 with other nucleolar components like SURF6. Interestingly, when R-DPRs are added in excess, they can dissolve preformed NPM1 or NPM1/SURF6 droplets [40,70]. More specifically, recent evidence from mammalian cells showed that R-DPRs not only induce NPM1 LLPS, but also sequester NPM1 away from its interacting partners, including rRNA, and can lead to its mislocalization from the nucleolus [70]. Additionally, R-DPRs can interact with and sequester rRNA, which directly impairs the most essential nucleolar function [70]. These events lead to the dissolution of the nucleolus and eventually cell death [70]. Notably, longer PR repeats had more adverse effects on NPM1 phase separation, suggesting that DPR length could be a determining factor in toxicity [70]. In conclusion, interactions of R-DPRs with NPM1 lead to harmful consequences: they deleteriously impact the biophysical properties of nucleolar proteins, dynamics, morphology, and overall functionality. Whether these exact toxic pathways operate in C9-ALS/FTD patients, however, remains to be established. However, evidence for nucleolar stress directly linked to R-DPRs, more specifically GR, in C9-FTD patient brains provides strong support for findings from experimental models [72]. Nevertheless, GR and PR inclusions do not localize to the nucleolus in C9-ALS/FTD patient brain tissue, which remains an important caveat for C9-model systems [73].

Cajal bodies and nuclear speckles

Other nuclear membraneless organelles targeted by R-DPRs include Cajal bodies and nuclear speckles, both of which house components of the spliceosome (Figure 1). Indeed, GR and PR appear to directly block spliceosome assembly and splicing, as shown in both patient-derived motor neurons and HeLa cells [45]. Many proteins associated with Cajal bodies, nuclear speckles, and spliceosomes were identified as GR/PR-interacting binding partners and as modifiers of toxicity in C9-fly models [40]. For example, SRSF7, a nuclear-speckle component and a splicing factor, interacts with both GR and PR in mammalian cells. PR specifically alters the phase separation of SRSF7 and reduces the dynamic assembly of nuclear speckles, which also contributes to aberrations in splicing [40]. Furthermore, GR/PR-overexpressing cells are devoid of Cajal bodies, suggesting that their assembly is directly impaired by DPRs [40].

Stress granules

Moving on to the cytoplasm, stress granules (SGs) rapidly assemble in response to transient insults that impair translation [74]. The main constituents of SGs are non-translating mRNAs and RBPs that can undergo LLPS, including G3BP1, DEAD-Box Helicase (DDX3X), and several heterogeneous ribonucleoproteins (hnRNPs) like hnRNPA1 [56,75–77]. Phase separation is key in maintaining the dynamic nature of SGs, since their rapid assembly and disassembly appears to be important for mounting and dampening stress responses [78,79]. In fact, alterations in SG dynamics are associated with neurodegenerative disorders [54,56,80]. Indeed, prolonged SG assembly can lead to TDP-43 proteinopathy, cell death, and inhibition of nerve regeneration [81–83].

Several lines of evidence establish that R-DPRs can directly interact with SG components and negatively impact their liquid phase (Figure 1) [10]. For example, in mammalian cells, overexpressed GR and PR can interact with G3BP1 and reduce the rate of its fluorescence recovery after photobleaching (FRAP). This finding suggested that GR/PR can enhance the multivalent interactions, making the liquid phase of G3BP1 less dynamic [40]. Additionally, G3BP1 and 2, both of which can be essential for SG assembly, were identified as strong enhancers of GR-mediated toxicity when depleted in fly models, perhaps implying their importance in countering toxic GR effects [40]. Both GR and PR have detrimental effects on SG dynamics as they are both seen in cytoplasmic inclusions in C9-patients [72,73]. However, when short repeats are overexpressed in mammalian cells, it is believed that GR is the dominant perpetrator given its predominant cytoplasmic localization. In contrast, PR mostly resides in the nucleus and nucleolus in cellular models. Consistently, several *in vivo* and *in vitro* studies have shown that GR co-localizes and directly interacts with ribosomal subunits and the translation initiation factor eIF3η [40,43,44,84]. Importantly, ribosomal proteins co-aggregated with R-DPRs inclusions in patient brain tissue, attesting to the vital role R-DPRs play in impairing translation and SG formation [44,84]. Generally, the induced expression of R-DPRs stimulates the spontaneous assembly of poorly dynamic SGs that are difficult to disassemble. The abnormal persistence of SGs make them 'hot spots' or seeding points for aggregation, since the propensity of their components to undergo aberrant liquid-to-solid transitions increases [40,54–56,80,84].

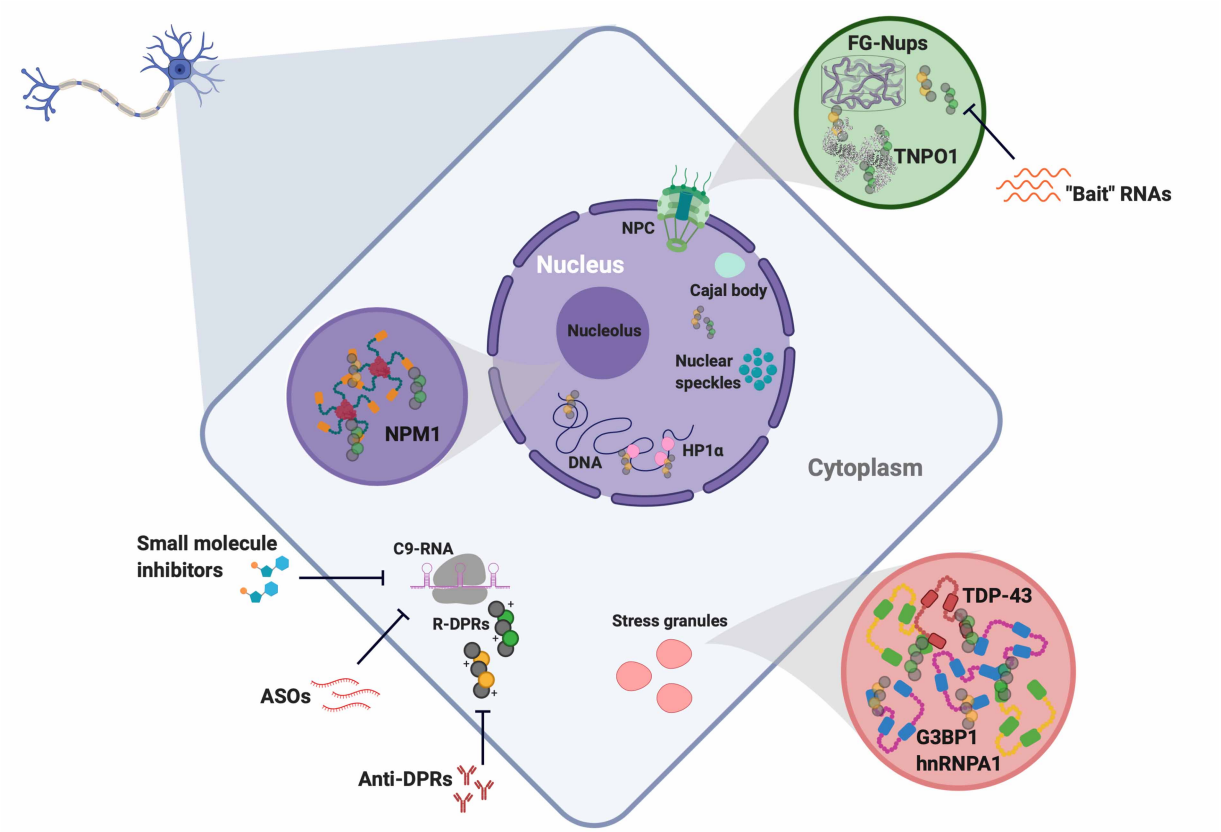

Figure 1. Arginine-rich dipeptide-repeat proteins (R-DPRs) disrupt various phases in neurodegenerative disease.
R-DPRs can directly interact with several LCD-containing proteins, leading to aberrations in phase separation and disruption of membraneless organelles. Direct interactions with the nucleolus, nuclear speckles, Cajal bodies, and stress-granule components negatively impact their phase separation and overall cellular functions. Disruption of the phase separation of heterochromatin protein 1α (HP1α) leads to irregularities in gene expression. The NPC is also impaired due to R-DPR interactions with nuclear-import receptors, like TNPO1. TNPO1 is sequestered from interacting with FG-rich nucleoporins (FG-Nups) leading to impaired nucleocytoplasmic transport (NCT). Potential therapeutic strategies to combat the deleterious effects of R-DPRs include the use of small-molecule inhibitors, antisense oligonucleotides (ASOs), antibody immunotherapy (anti-DPRs), and 'bait' RNAs.

Interestingly, with the aid of crowding agents, polyanions (e.g. RNA), or other LCD-containing proteins, high concentrations of GR and PR can undergo LLPS, in a temperature-dependent manner [85,86]. This process has been posited as another possible mechanism by which SG formation is perturbed [85]. Overall, the poorly dynamic, DPR-induced SG assemblies, are predisposed to undergo pathogenic fibrillization, leading to the accumulation of pathological inclusions that are no longer guarded by the quality control mechanisms of the cell.

Links between GR/PR and nucleocytoplasmic transport

The transport of proteins between the nucleus and the cytoplasm is heavily orchestrated by the nuclear pore complex (NPC). The human NPC is a large macromolecular assembly of ∼120 MDa in mass [87,88]. Its central channel is composed of phenylalanine-glycine repeat-containing nucleoporins (FG-Nups), which can undergo LLPS through the multivalent interactions of their low-complexity sequences [88,89]. The phase separation of FG-Nups is critical for the creation of what is described as a meshwork barrier [90], which may be the mechanism by which nucleocytoplasmic transport (NCT) is controlled [89]. Nuclear-import receptors (NIRs), such as importin β and transportin 1 (TNPO1), directly interact with FG-repeats to enable the passage of cargo

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through the NPC. Therefore, the phase dynamics of the nuclear pore channel are vital for maintaining proper trafficking across the nuclear membrane [89].

A panoply of proteins involved in NCT as well as components of the NPC were identified as potent modifiers of C9-toxicity [40,47,91]. However, the mechanism by which NCT is impaired in C9-ALS/FTD is debated. It has been established that R-DPRs can interact with FG-Nups and components of the nuclear-import machinery, including RanGAP1, importin β, TNPO1, and several importin alphas (Figure 1) [40,47,91]. Proteomic analyses, co-immunoprecipitations, and co-localization studies in mammalian cells have solidified those outcomes [40,47,91,92]. What remains disputed, however, is the downstream effects of those interactions and whether they directly contribute to neuropathology. Earlier studies using permeabilized cells suggested that the interaction of GR/PR with FG-Nups impairs the phase dynamics of the central channel and directly blocks trafficking through the nuclear pore [93]. On the contrary, other studies using intact cells demonstrated that nuclear transport is not directly blocked by R-DPRs, but instead suggested that DPRs might exert their effects indirectly through the spontaneous assembly of SGs, where essential nuclear transport machinery might be sequestered away from its function [92,94]. Indeed, there is evidence to support this notion that SGs can act as 'sinks'. The overexpression of R-DPRs results in the mislocalization of NCT factors and NPC components into poorly assembled SGs [92]. However, mechanistic discrepancies still remain. The most recent proposal indicated that the disruption of NCT is due to neither the sequestration of NCT factors nor the direct blockade of FG-rich NPCs. Rather, the main cause of NCT impairment was due to the soluble interactions of R-DPRs with importin β and TNPO1, which directly interfere with cargo loading onto import receptors [95]. Interestingly, PR was shown to be more potent than GR in those assays [95]. Another study done *in vitro* also demonstrated that PR directly interacts with TNPO1, sequestering it away from its RBP cargo, Fused in Sarcoma (FUS). However, since FUS inclusions do not co-exist with C9-associated pathology, the physiological implications of this study remain uncertain [96].

In summary, while the effects of R-DPRs on NPC phase separation are disputed, there is no disagreement around the fact that their presence is detrimental to NCT. There likely exists more than one mechanism of pathogenesis that involves R-DPRs, perhaps with indirect harmful consequences on NPC phase separation.

GR/PR interactions with LCD-containing proteins

Several lines of evidence show that R-DPRs interact with the LCDs of several proteins, altering their phase separation behavior [40]. Thus far, the general mechanistic understanding of this detrimental process is that the interactions with R-DPRs lowers the critical concentration required to undergo LLPS, and changes the material properties of the resulting condensates. Condensates become less dynamic, fewer fusion events are observed, and the 'wetting' effect is reduced when GR/PR are involved [10,40]. It is suggested that the protein aggregation observed in neurodegeneration may be due to the rapid, irreversible liquid-to-solid transition caused by R-DPRs [40]. However, R-DPRs can also directly disrupt preformed phases [97]. Known interactions and direct effects of R-DPRs on the LLPS of several LCD-containing proteins in C9-ALS/FTD are described below.

HP1α

Heterochromatin protein 1α (HP1α) plays an essential role in maintaining heterochromatin structure and regulating gene silencing (Figure 1) [98]. It has been proposed that HP1α LLPS allows it to maintain its function [99–101], although recent developments suggest that heterochromatin compartments are likely more complex *in vivo* [102–104]. Nevertheless, R-DPRs, more specifically PR, can directly interact with HP1α droplets and vigorously burst them [97]. Further investigation elucidated that the rupture of the liquid phase of HP1α results in its release from heterochromatin, making it vulnerable for degradation. This eviction of HP1α, in turn, allows PR to accumulate on heterochromatin and directly interact with DNA [97]. Additionally, the same study showed that the PR-induced rupture of HP1α liquid droplets results in lamin invaginations, which are not only detrimental to the integrity of the nuclear membrane, but also affect gene expression [97]. Indeed, increased expression of repeat expansions was observed in those cells [97]. Collectively, these findings provide a mechanistic link between PR and neurodegenerative disease, directly through alterations in HP1α phase separation [97].

Disease-linked RBPs

The majority of the GR/PR interactome is comprised of LCD-containing proteins, most of which are aggregation-prone RBPs implicated in neurodegenerative disease, including FUS, TDP-43, hnRNPA1,

hnRNP2B1, TIA1, Ataxin-2, and Matrin-3 [40, 53]. A thorough understanding of how R-DPRs interact with these RBPs is lacking. Likewise, whether their direct interaction leads to deleterious outcomes in C9-ALS/FTD remains unclear. However, given their attraction to LCDs, it is reasonable to speculate that R-DPRs could potentially accelerate the liquid-to-solid transition or fibrillization of several disease-linked RBPs. TDP-43, for example, undergoes LLPS via its prion-like domain [105]. Under prolonged stressful conditions, TDP-43 can undergo pathological phase separation in the cytoplasm [58,83,106]. Indeed, the mislocalization and aggregation of TDP-43 is a key pathological hallmark of ALS/FTD [107–109]. Evidence supports that GR/PR can associate with TDP-43-positive inclusions, like SGs or RNA granules [62,110]. These granules become poorly dynamic in the presence of R-DPRs, which could be attributed to the fibrillization of its protein components, like TDP-43. Consistently, co-immunoprecipitation and co-localization studies showed that GR can interact with TDP-43, and this warrants further investigation [40,62]. It was initially thought that R-DPR pathology does not correlate with TDP-43 burden, but shortly thereafter, it was postulated that TDP-43 pathology is actually downstream of C9-repeats/DPR burden [111]. Additionally, the deleterious effects of TDP-43 and R-DPRs on NCT is yet another testament to their convergent toxic pathways [106,112].

Another example of an RBP that is a target of R-DPRs toxicity is hnRNPA1. GR and PR promote the phase separation of hnRNPA1, through lowering the critical concentration required to undergo LLPS [40]. hnRNPA1 is a constituent of SGs that can interact with TDP-43 via its LCD, and it regulates the splicing of mRNAs [56,113]. Unlike TDP-43, however, hnRNPA1 inclusions have not been reported to co-occur in C9-patients. Nevertheless, perturbations in hnRNPA1 phase separation are detrimental to its protein and RNA interactions, SG assembly, and overall cellular functions [54,56]. A detailed account on how the phase separation of other RBPs are affected by R-DPRs is yet to be discovered.

Therapeutic strategies to combat GR/PR toxicity

Our growing understanding of the mechanisms behind R-DPRs toxicity has aided our ability to develop promising therapeutics to counter their damaging effects. One clever strategy is to selectively reduce transcription of the expanded G_4C_2 repeat, which can be achieved by knockdown of transcription elongation factor Spt4 [114] or PAF1C, a transcriptional regulator of RNA polymerase II [115]. Another clever strategy is to inhibit RAN translation of the C9-repeat, which can be achieved by knockdown of small ribosomal protein subunit 25 (RPS25) [65] or through up-regulation of DDX3X [116]. Several groups have also identified and utilized C9-targeting ASOs and were met with tremendous success [28,117–120]. In C9-ALS/FTD induced pluripotent stem cells (iPSCs) and mouse models, ASOs not only suppressed the formation of RNA foci, but also rescued NCT deficits and alleviated neurotoxicity (Figure 1) [28,117,118,120]. It has been reasonably postulated that the use of ASOs can lower the R-DPR burden, however, further studies are still needed. Another therapeutic strategy would be to directly target R-DPRs via antibody immunotherapy (Figure 1) [121]. The advantage of having relatively small antibodies is that they can penetrate the blood–brain barrier. High-affinity human antibodies against GA or GP were generated and tested in C9-mouse models. Remarkably, anti-GA worked to reduce GA, GP, and GR levels, and decreased overall neurodegeneration [121].

Using small-molecule inhibitors has also shown promise [122,123]. Several small-molecule inhibitors that can penetrate the blood-brain barrier were developed to target the secondary structure of the C9-repeat, which is another strategy to block the translation of DPRs (Figure 1) [124,125]. Other small molecules that target the ER-stress response, including the integrated stress response inhibitor (ISRIB), have also been shown to be useful in combating neurodegeneration in C9-ALS/FTD models [92]. Another strategy of interest is to increase the turnover rate of DPRs by inducing small heat shock proteins (HSPs). One study showed that inducing HSPB8 decreased the accumulation of DPRs [126]. Along the same lines (but at the RNA level), the induction of the nonsense-mediated decay (NMD) pathway, via the up-regulation of the eukaryotic translation termination factor 1 (eRF1) or upframeshift protein 1 (UPF1), targets C9-HRE RNAs for degradation, which halts the production of DPRs and mitigates toxicity [127–129]. Furthermore, inducing the symmetric di-methylation of R-DPRs reduces their phase separation, which poses as a promising strategy to alleviate toxicity [130]. In contrast, asymmetric di-methylation of GR has been shown to contribute to its toxicity, which can be rescued by type I protein arginine methyltransferase (PRMT) inhibitors [131].

More recently, utilizing RNA as 'bait' has become an exciting therapeutic avenue [58,95]. The vital role of RNA in maintaining the solubility of RBPs and their assemblies is widely appreciated. Therefore, it was hypothesized that the addition of RNA could mitigate deleterious protein–protein interactions. Indeed, when total cellular RNA was added to permeabilized cells, R-DPRs were sequestered away from interacting with

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NIRs, which subsequently rescued NCT (Figure 1) [95]. Earlier evidence *in vitro* supported that RNA can in fact interact directly with DPRs and induce their phase separation [85]. These outcomes support the notion that using 'bait' RNAs to safeguard neurons from detrimental interactions with DPRs is an encouraging approach. Finally, NIRs themselves have been utilized as protein disaggregases [132–134]. For example, TNPO1 (also known as Kapβ2) can interact with FUS and prevent its phase separation and aggregation [132–136]. Given that NIRs have been shown to directly interact with R-DPRs, in permeabilized cells, and *in vitro*, one could postulate that up-regulating NIRs in an intact system would sequester away DPRs the same way that 'bait' RNAs would [95]. In support of this hypothesis, earlier studies provided evidence that the up-regulation of importins reduces PR toxicity [40,47,91]. Several other protein-disaggregase systems might also be tailored to attack DPR inclusions [137].

In conclusion, there are several strategies by which we can counter the deleterious effects of R-DPRs. While we still need better biomarkers that would increase the efficiency of our therapeutic targeting, the results we have thus far are encouraging. Needless to say, we are in urgent need for finding a cure for C9-ALS/FTD, and must continue to build our understanding of the pathological mechanisms leading to R-DPRs toxicity.

Summary

- Arginine-rich DPRs alter the LLPS of several disease-linked RBPs.
- Phase-separation dynamics within membraneless organelles are negatively impacted by interactions with arginine-rich DPRs.
- Antibody immunotherapy, ASOs, 'bait' RNAs, and other mechanisms targeting RAN translation or DPRs are promising therapeutic strategies to combat DPR toxicity.
- Elucidating the mechanisms by which arginine-rich DPRs contribute to C9-toxicity will accelerate our path to finding a cure for C9-ALS/FTD.

Competing Interests

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Abbreviation

ALS, amyotrophic lateral sclerosis; ASOs, antisense oligonucleotides; DFC, dense fibrillar component; DPRs, dipeptide-repeat protein products; FTD, frontotemporal dementia; FUS, Fused in Sarcoma; GC, granular component; HREs, hexanucleotide repeat expansions; HSPs, heat shock proteins; iPSCs, induced pluripotent stem cells; LCDs, low-complexity domains; LLPS, liquid–liquid phase separation; NCT, nucleocytoplasmic transport; NIRs, nuclear-import receptors; NPM1, nucleophosmin-1; RAN, repeat-associated non-AUG; RBPs, RNA-binding proteins; SGs, stress granules

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