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Review Article

C9ORF72 hexanucleotide repeats in behavioral and motor neuron disease: clinical heterogeneity and pathological diversity

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Abstract: Hexanucleotide repeat expansion in *C9ORF72* is the most common genetic cause of frontotemporal dementia (FTD), a predominantly behavioral disease, and amyotrophic lateral sclerosis (ALS), a disease of motor neurons. The primary objectives of this review are to highlight the clinical heterogeneity associated with *C9ORF72* pathogenic expansion and identify potential molecular mechanisms underlying selective vulnerability of distinct neural populations. The proposed mechanisms by which *C9ORF72* expansion causes behavioral and motor neuron disease highlight the emerging role of impaired RNA and protein homeostasis in a spectrum of neurodegeneration and strengthen the biological connection between FTD and ALS.

Keywords: *C9ORF72*, frontotemporal dementia, amyotrophic lateral sclerosis, motor neuron disease, RNA, protein trafficking

Introduction

A GGGGCC hexanucleotide repeat expansion intronic to *chromosome 9 open reading frame 72* (*C9ORF72*) was identified in 2011 [1, 2] as the most common genetic cause of amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease) and frontotemporal dementia (FTD) with or without concomitant motor neuron disease (MND). The literature on *C9ORF72* has expanded greatly in the ensuing two years, leading to characterization of the frequency of pathogenic expansion carriers (C9+) in diverse populations and to putative molecular mechanisms underlying the pathogenicity of such expansions.

In addition to two forms of TDP-43 pathology (harmonized [3] Type A and B), C9+ is also characterized by Ub+/p62+/TDP-43- inclusions, most notably in cerebellum, thalamus and hippocampus; the latter pathology is unique to C9+ and, in some cases, this may be the only form of pathology [4-8]. These pathological findings are broadly mirrored by neuroimaging findings describing diffuse cortical and subcortical atrophy in all lobes of the brain, with cerebellar and thalamic atrophy emerging as distinguishing features of C9+ when compared to sporadic disease in FTD/FTD-MND (reviewed in [9]) and ALS [10, 11]. One notable observation has been the diversity of phenotype associated with C9+ patients [12], which will be highlighted below. The clinical heterogeneity associated with *C9ORF72* expansion is predicted to be a reflection of the well-established structural and pathological heterogeneity [13]. Identifying the molecular mechanisms responsible for the apparent morphological and pathological diversity will be critical for making predictions about clinical outcomes in carriers of this shared genetic risk factor.

Clinical features of *C9ORF72* expansion-mediated disease

Motor features

Pathologic expansion of *C9ORF72* is the most common genetic cause of ALS, estimated at around 34% of familial and 6% of sporadic ALS cases [13]. C9+ ALS patients may demonstrate more bulbar onset of symptoms (reviewed in [13, 14]). MND occurs concomitantly in about 30% of C9+ FTD [13]. C9+ may be a rare cause of other motor neuron disorders as well. One study investigating a large Dutch cohort found N=4 individuals with progressive muscular atrophy and N=1 patient with primary lateral sclerosis with expanded repeats [15] highlighting the variability of upper and/or lower motor neuron involvement associated with *C9ORF72* expansion.

Some C9+ patients also show Parkinsonism, with or without MND. Parkinsonism symptomatology usually appears after onset of FTD or ALS findings, and is likely explained by neurodegeneration of the substantia nigra in many C9+ cases [16]. Hexanucleotide expansion of *C9ORF72* has also been associated with a handful of cases with clinical diagnoses of idiopathic Parkinson's disease (PD) [17-19]. Intermediate repeat length has also been suggested as a risk factor for sporadic PD in two studies surveying large numbers of patients: for >20-30+ repeats in N=889 Caucasian PD or essential tremor plus Parkinsonism patients, and for ≥7 repeats in N=911 Han Chinese PD patients [20, 21]. Further investigations in diverse populations are required to confirm these findings.

Isolated cases of progressive supranuclear palsy, corticobasal, and olivopontocerebellar degeneration syndromes have also been reported, further expanding the spectrum of phenotypes associated with C9+ [19, 22]. Whether these rare cases are associated with C9+ pathology specifically in the basal ganglia, brainstem, and cerebellum remain to be determined.

Regions expressing the C9ORF72 mouse ortholog (discussed in more detail in the next section) include the striatum (a component of the basal ganglia), brainstem, and cerebellum [23]. Neuroimaging and pathological studies show that the cerebellum, which plays a critical role in motor control, is particularly affected in C9+ disease. The thalamus—which appears uniquely involved in C9+ compared to sporadic disease—participates in both the direct and indirect pathways linking the striatum and motor cortex, resulting in motor stimulation and inhibition, respectively [24]. Thus, pathological changes in cerebellum and thalamus have the potential to affect multiple aspects of motor control, which could lead to less common motor syndromes.

Behavioral features

Behaviorally, *C9ORF72* expansion is most commonly associated with a clinical syndrome of behavioral variant (bv)FTD, characterized by deficits in social behavior and executive function. Less common diagnoses include primary progressive aphasia (PPA), predominant amnestic, and psychiatric clinical syndromes. Some individuals also show deficits in visuospatial function (reviewed in [25]). In addition, cognitive and behavioral impairments appear to be more common in ALS patients with C9+ versus sporadic ALS [11]. Some cases of bvFTD associated with C9+ have remarkably slow progression and little to no visible neuroanatomical involvement [16, 26-28]. In addition to lack of frank brain atrophy, self-awareness of disease remains relatively intact, and patients are sometimes able to make behavioral modifications to compensate for the deficits imparted by disease [26]. This insight is in contrast to the majority of bvFTD patients, where there is marked lack of awareness into social and emotional deficits [29].

In the context of these broader clinical syndromes, specific psychiatric symptoms may further differentiate C9+ patients from other patients with sporadic or genetic forms of FTD. In particular, psychotic features may be enriched in *C9ORF72* expansion carriers, with delusions and hallucinations more common in C9+ versus matched sporadic cases [30, 31]. In one Swedish C9+ kindred, psychotic symptoms and somatic complaints were observed in the majority of affected individuals [32]. Anxiety and depressive symptoms [8] are also observed in C9+. These symptoms may relate to findings that *C9ORF72* expansion is associated with unique pathology in critical regions of the limbic system such as the thalamus and hippocampus [4-8]. Similarly, in Alzheimer's disease (AD), degeneration of the hippocampus may allow 'release' of its regulation of the amygdala, resulting in higher levels of anxiety and emotional contagion [33]. In addition, degeneration of the cerebellum could result in 'disconnection' of the emotion-regulating portions of this brain region from the cortex [34].

Occasionally, C9+ patients present clinically with an AD-like dementia; in a recent screen of

FTD genes in early-onset AD patients, two individuals were found to harbor *C9ORF72* expansions [35]. In these cases, neuroimaging may be particularly informative if findings are atypical of AD but instead show frontotemporal involvement [36]. Amnestic presentation may be related to the hippocampal sclerosis and/or p62+ pathology observed in the hippocampus of many C9+ patients [4-8]. In addition, episodic memory deficits in C9+ correlate with atrophy in the frontal, temporal and parietal cortices, including the posterior cingulate cortex, and are distinct from the regions correlated with episodic memory in sporadic bvFTD (i.e., medial prefrontal, medial and lateral temporal cortices) [37]. Finally, visuospatial deficits are in line with observed parietal lobe involvement in C9+ (reviewed in [9]). This further highlights how anatomic heterogeneity in *C9ORF72* expansion-mediated disease may contribute to a diversity of clinical symptoms. The diversity of clinical behavioral syndromes associated with *C9ORF72* expansion strongly suggests the presence of pathology in distinct areas of the brain across individuals.

Molecular mechanisms of *C9ORF72* disease

If *C9ORF72* expansion is associated with altered structural organization of the brain that culminates in a wide-spectrum of clinical disease, what molecular mechanisms might explain these changes? The three primary models accounting for *C9ORF72* expansion-mediated toxicity [38] are: (1) loss of C9ORF72 protein function [1, 2]; (2) accumulation of toxic RNA foci [39], which sequester RNA-binding proteins such as TDP-43, FUS, hnRNP A3 [40], and Pur α [41] and result in dysregulation of RNA splicing, trafficking and translation; (3) novel dipeptide aggregate formation resulting from non-ATG mediated (RAN) translation of the expanded GGGGCC hexanucleotide repeat [42, 43]. Additional mechanisms that could modify disease pathogenesis include differential expansion size of *C9ORF72* hexanucleotide repeats across different tissues and independent genetic modifiers that mediate any of the factors that lead to neuronal toxicity. Also of note, recent evidence suggests that C9+ toxicity may not necessarily occur cell-autonomously in neurons; any of the proposed mechanisms of toxicity may in fact occur first in astrocytes and subsequently spread to neurons [44]. Potentially, the large degree of clinical heterogeneity observed within the C9+ patient population could be a result of distinct pathogenic mechanisms (or combinations thereof) occurring in different individuals.

C9ORF72 haploinsufficiency

Loss of C9ORF72 protein function from reduced expression due to pathogenic expansion is one proposed mechanism of disease. The expanded copy of *C9ORF72* results in reduced gene expression due to histone trimethylation, as measured in blood [45, 46]. This gene is predominantly expressed in neural populations vulnerable in FTD and ALS. Specifically, the mouse ortholog of C9ORF72 is expressed in the hippocampus, dentate gyrus, striatum, thalamus, brainstem nucleus, cerebellum, throughout the cortex, and in the spinal cord, as well as several peripheral tissues. In mouse, expression appears to be limited primarily to gray matter [23]. Recent studies in both *C. elegans* and zebrafish indicate that loss of C9ORF72 function may be associated with motor neuron degeneration [47, 48].

The protein product of *C9ORF72* is predicted to be structurally similar to the Differentially Expressed in Normal and Neoplasia (DENN) family of guanine nucleotide exchange factors that activate Rab-GTPases (Rab-GEFs), which are important regulators of membrane traffic [49, 50]. The putative yeast ortholog of C9ORF72, Lst4p, prevents lysosomal delivery of cargo by redirecting endosome-localized proteins to cell surface [51]. If C9ORF72 similarly serves to sort endosomal cargo to the plasma membrane in neurons, then mutations reducing its function would be predicted to augment lysosomal degradation of particular cargo proteins. Intriguingly, the membrane protein TMEM106B, which has recently been shown to be a genetic modifier of both progranulin- and C9-mediated FTD, appears to influence both lysosomal morphology and dendritic trafficking of lysosomes within neurons [52, 53]. In addition, homozygous loss-of-function mutations in progranulin result in neuronal ceroid lipofuscinosis, a lysosomal storage disorder [54]. Dysfunctional degradation within the endolysosomal pathway may thus represent a common molecular pathology associated with altered levels of C9ORF72, progranulin and TMEM106B. Consistent with this scenario, accumulation of ubiquitinated proteins downstream of impaired lysosomal degradation could explain the Ub+/p62+/TDP-43- pathology that discriminates C9+ from other forms of FTD and ALS.

van der Zee and colleagues found decreased expression of C9ORF72 with an increased number of repeats at intermediate repeat numbers [55]. rGGGGCC (but not rCCCCGG) repeats form stable, tract length- and RNA concentration-dependent unimolecular and multimolecular RNA G-quadruplexes [56, 57], which can affect promoter activity, genetic instability, RNA splicing, translation and mRNA localization within neurites. The dose-dependence of stability of these structures suggests a mechanism by which increased repeat length would be more toxic. These RNA structures are potentially amenable to intervention with small molecules that break up G-quadruplexes [58-60]. This repeat (and how it folds) may serve as a mechanism by which splice variation occurs (given redistribution of *C9ORF72* splice variants with expansion); ASF/SF2 splicing factor can bind to this repeat [57].

One patient has been reported with a homozygous repeat expansion; this individual had an early onset of bvFTD but typical clinical and pathological presentation within the spectrum of C9+ heterozygous disease. The authors of this report suggest that this case provides evidence that haploinsufficiency is not the only mechanism of C9+ disease as one would expect a more severe or different clinical phenotype associated with homozygous loss of *C9ORF72* expression compared to heterozygous loss [61]. Toxic gain of function would be in line with an earlier onset but phenotypically similar form of C9+ disease, though it is also possible that presence of genetic or environmental disease modifiers play a role in this individual.

Sequestration of RNA-binding proteins into RNA foci

Another potential mechanism of toxicity involves the GGGGCC expansion itself, whereby toxic RNA foci are formed that sequester RNA-binding proteins and splicing factors such as TDP-43 and FUS, the latter of which was identified in rGGGGCC binding screen [40]. Both sense and antisense RNA foci have been identified via *in situ* hybridization, where they are most abundant in neurons of the frontal cortex, and to a lesser extent in astrocytes, microglia and oligodendrocytes [62]. Accumulation of expanded RNA into toxic foci is a disease mechanism implicated in other neurodegenerative expansion disorders such as several spinocerebellar ataxias and fragile-X associated with tremor/ataxia syndrome (FXTAS) [1]. Screening for point mutations in *C9ORF72* via sequencing of 389 ALS samples did not render any pathogenic variants, further suggesting that *C9ORF-72* pathogenesis is caused by a toxic gain of function due to RNA foci resulting from the non-coding expansion [63]. These RNA foci have the potential to sequester other RNA-binding proteins, which could result in widespread effects on transcriptional regulation and protein expression.

One RNA-binding protein critically linked to C9+ disease is TDP-43. As one of the main protein aggregates found in C9+ FTD/ALS, TDP-43 is a DNA- and RNA-binding protein that cycles between the nucleus and cytosol (though it localizes primarily to the nucleus) and plays numerous roles in RNA metabolism, including transcription and regulation of splicing, transport and translation, miRNA processing, and stress granule formation (reviewed in [38]). Mutations in *TARDBP*, which encodes TDP-43, cause ALS (reviewed in [64]). TDP-43 binds and regulates hundreds of RNA targets, including an enrichment of genes involved in neuronal development and synaptic function [65, 66]. TDP-43 is critical for early embryonic development of the central nervous system [67, 68] and plays an important role in the association and size of stress granules, which form transiently in response to cellular stress (e.g., [69]; reviewed in [38, 70, 71]). This suggests a possible mechanism by which early sequestration of TDP-43 could cause alterations in multiple proteins involved in neuronal development and function that could ultimately result in altered structural and/or network architecture that is vulnerable to diffuse cortical and subcortical damage. This would then be exacerbated by alterations in the cellular stress response due to altered stress granule dynamics.

Identification of specific RNA-binding proteins that bind the *C9ORF72* GGGGCC repeat expansion is currently underway. In a recent screen, Xu, *et al*. found that rGGGGCC binds the RNA-binding protein Pur α, and overexpression of

Pur α rescues rGGGGCC-mediated neurodegeneration in *Drosophila* [41]. Pur α is involved in modulation of gene transcription, translation, controls cell cycle and differentiation and is a component of RNA-transport granules [72, 73]. The putative disease mechanism would thus be a loss of function of Pur α due to binding to rGGGGCC. Of note, Pur α also binds the *FXTAS* GCC repeat [74]. This model of neurodegeneration in *Drosophila* would thus argue against a primary role for loss of C9ORF72 function in disease pathogenesis.

Another screen for rGGGGCC RNA-binding proteins identified hnRNP A3, which forms p62+/TDP-43- neuronal cytoplasmic and intranuclear inclusions in hippocampus, as well as cerebellum in a subset of C9+ [40]. hnRNP A3 cycles between the nucleus and cytoplasm and is involved in alternative pre-mRNA splicing, nuclear import and cytoplasmic trafficking of mRNA, as well as mRNA stability, turnover and translation [75]. Expressed primarily in the nucleus of neurons, it appears to be redistributed to cytosol in its pathological state, similarly to TDP-43 and FUS [76-79]. The hnRNP A3 finding was not replicated by Xu, *et al.* but this discrepancy could relate to differences in binding conditions and protein concentrations [41]. Also of note, a screen in *Drosophila* for *FXTAS*-repeat associated changes in miRNA expression identified miRNA-277; hnRNP A2/B1 can directly regulate miRNA-277, which modulates CGG repeat-mediated neurodegeneration in FXTAS [80]. In iPSCs derived from C9+ ALS patients, repeat-containing RNA foci colocalized with hnRNPA1 and Pur α [81].

The ability of RNA foci to sequester RNA-binding proteins and thus alter the processing and expression of hundreds of distinct genes in a stochastic nature [38, 39] could result in markedly diverse forms of disease across different individuals. With known genetic modifiers (*TMEM106B*, described in more detail below) and variability in the number of hexanucleotide repeats it is not surprising that *C9ORF72* expansion results in a diverse set of anatomical, clinical and pathologic phenotypes. Utilizing large datasets to identify patterns of RNA expression change across multiple C9+ individuals with the same clinical syndrome may be useful for dissecting the spectrum of changes that are most likely to predict a particular set of symptomatology. Targeting the cause of the expression changes—that is, reducing RNA foci formation—may prove beneficial for C9+ carriers with distinct clinical presentations. In support of this notion, antisense oligonucleotides (ASOs) targeting the *C9ORF72* transcript suppressed RNA foci formation and reversed gene expression changes and aberrant cell excitability associated with the pathologic expansion [81, 82] suggesting a potential therapeutic intervention.

RAN-dependent translation of GGGGCC expansions

Repeat-associated non-ATG (RAN)-dependent translation of dipeptides from both sense and anti-sense strands of the expanded hexanucleotide repeat in *C9ORF72* form insoluble aggregates [42, 43, 83]. RAN translation of the sense strand creates poly Gly-Arg (poly-GR), poly Gly-Pro (poly-GP), and poly Gly-Ala (poly-GA) dipeptides which are hydrophobic and aggregation-prone; anti-sense RAN translation results in Pro-Ala, Pro-Gly, and Pro-Arg dipeptides. Using an antibody binding the poly-GP dipeptides, Ash, *et al.* showed variability in pathological location [42]. Highest presence included hippocampal regions, motor cortex, temporal and frontal cortices, amygdala, anterior and lateral thalamus, and Purkinje cells of the cerebellum. RAN-translated dipeptides have been shown to colocalize with p62+ inclusions [42, 43] in granule cells of the cerebellum, cells in the dentate gyrus, and the CA4 of the hippocampus [84].

The presence of inclusion bodies of these dipeptides does not appear to correlate with clinical severity or neurodegeneration (whereas TDP-43 pathology does), and has been suggested by some to be a protective response to coping with large numbers of dipeptides rather than a driving force of neurodegenerative processes [85]. This evidence, however, does not preclude the possibility that soluble forms of the dipeptides, or variation in the distribution of the different types of dipeptides across brain tissue, could contribute to the clinical and/or pathological manifestations of C9+ disease.

Formation of RAN-translated dipeptides can also be partially ameliorated with ASOs in mouse models [86] and iPSC-differentiated neurons [82], however, ASO intervention in C9+ iPSCs appears to ameliorate gene expression and cellular deficits despite continued presence of RAN translated dipeptides [82], further suggesting that RAN-translated products may be a secondary or downstream mechanism which has less influence on pathology. Additional testing of this type of intervention in the context of clinical disease may help to determine the role that RAN-translated dipeptides play in *C9ORF72* expansion-mediated disease.

Notably, the amount of RAN translation that occurs could alter the availability of rGGGGCC repeats to sequester RNA-binding proteins, since RAN translation would be expected to reduce the binding of proteins such as TDP-43 and Pur α. Thus it is possible that the amount of RNA-binding protein sequestration versus RAN-mediated translation that occurs in each cell is variable, offering yet another source of disease heterogeneity. If indeed dipeptide aggregates are not toxic to the cell [82, 85], then it stands to reason that formation of dipeptides through RAN-mediated translation may be an adaptive mechanism by which the cell attempts to limit the formation of RNA foci and sequestration of RNA-binding proteins. In line with this theory, Gendron, *et al.* found that RAN-translated poly-GP peptides infrequently colocalized with RNA foci [83]. For neurons with long axons, such as motor neurons, alterations in RNA-binding proteins may be particularly problematic (e.g., myotonic dystrophy) [87]. Thus, the balance of RAN translation versus RNA foci formation in particular neuronal subtypes could potentially affect disease pathogenesis and thus clinical presentation.

Other variables that may play a role in C9+ disease

Expansion-size differences across tissue: The *C9ORF72* hexanucleotide repeat expansion length is highly variable and likely unstable due to surrounding genomic architecture ([55]; reviewed in [14]). C9 expansion size varies across different brain regions [88-90] and between monozygotic twins [89], and larger expansions may contribute to more potent pathology in the affected network of neurons. Three studies have investigated this with varying results. One study of blood samples found that C9+ length did not correlate with diagnostic group when comparing FTD, ALS, and other neurodegenerative phenotypes, but longer expansion correlated with older age of onset [90]. However, other studies showed that expansion length varies across tissues (e.g., blood versus brain [88, 89]) suggesting measures from periphery may not be representative of expansion size in the brain [13]. Another study found that *C9ORF72* expansion length did not correlate with FTD, FTD-MND or MND diagnostic groups in frontal cortex, cerebellum or blood samples; they found that longer frontal cortex expansion length correlated with older age of onset in FTD only, and that longer cerebellar expansion length was associated with reduced survival [88]. A third study did not find correlations between C9+ length in cerebellum and age of onset or disease duration, but found that cerebellar expansion length was higher in ALS versus FTD [89]. Thus, it remains unclear what role expansion length in different brain regions plays in C9+ disease.

Finally, evidence suggests that intermediate repeat expansion lengths that fall under the "pathologic" cutoff of 30 repeats but are above what is considered normal (less than 20) may serve as a risk factor for sporadic FTD [91], ALS [92], and PD [20, 21]. This is in line with evidence suggesting that intermediate repeat lengths are associated with reduced C9ORF72 expression, if protein haploinsufficiency plays a role in C9+ pathogenesis. Further work will be required to characterize the role of expansion length in pathological and clinical heterogeneity.

Genetic modifiers of C9ORF72 expansion disease: It is likely that genetic variation plays a role in modifying the pathological and clinical manifestation of C9+ disease. Mutations in other ALS-associated genes have now been found in C9+ carriers suggesting a two-hit model of disease (e.g., [93-96]), in line with the oligogenic theory of ALS, which suggests that harboring multiple risk variants in different ALS-associated genes is sufficient to cause disease (reviewed in [97-99]). C9+ patients that also carried deleterious variation in other FTD genes (*GRN* or *MAPT*) demonstrated early disease onset, bvFTD clinical presentation, and no motor neuron involvement suggesting a parallel two-hit model for FTD [100].

Common variation in other neurodegenerative disease associated genes may also contribute to clinical heterogeneity in C9+ carriers. This relationship has been observed in patients with *GRN* mutations, where carrying the AD risk allele *APOE*-ε4 resulted in exacerbated disease progression, amnestic syndromes and accompanying amyloid pathology [101]. For example, pathologic *C9ORF72* expansion coupled with a high-risk genetic polymorphism in an ALS gene could represent a risk mechanism predisposing some C9+ individuals to MND, whereas individuals without these additional risk variants may have a predominantly behavioral form of disease. This remains an untested hypothesis worth exploring in the context of disease-modifying risk genes.

In addition to exacerbating clinical presentation, genetic variation also has the potential to reduce disease risk. A recent study by van Blitterswijk, *et al.* identified variation in *TMEM-106B*, which was previously associated with protection from FTD with TDP-43 pathology (FTD-TDP) [102, 103] as protective in C9+ patients with FTD but not MND [104]. One study also found that variation in *TMEM106B* protected against cognitive change in ALS patients [105]. Taken together, these results suggest that TMEM106B may broadly modify behavioral/cognitive symptoms associated with TDP-43 pathology and may thus represent a robust therapeutic target [106].

C9ORF72 expansion as a disease of dysfunctional cellular trafficking

Recent studies in model organisms *C. elegans* and zebrafish provide compelling evidence that loss of C9ORF72 function is pathogenic to motor neurons [47, 48] and leads to motor deficits. While it is currently unclear if loss of C9ORF72 function contributes to disease in C9+ carriers, the observation that C9ORF72 transcript levels are reduced in patients with FTD and FTD-MND suggests that loss of protein function should be seriously considered as a disease mechanism.

What cellular consequences might be expected due to loss of C9ORF72 function? Sophisticated homology searches have revealed that C9ORF72 is a full-length homolog of the DENN family of Rab-GEFs, as noted above [49, 50]. While nothing is known about the cell biological function of mammalian C9ORF72, its yeast ortholog has been implicated in the sorting of endosome-localized proteins to the cell surface, such that they do not reach the lysosome. If this function is conserved in humans, reduced C9ORF72 levels might be associated with defects in the endo-lysosomal pathway. In addition, TMEM106B, the genetic modifier of both progranulin- and C9 expansion-associated FTD, has recently been implicated in lysosomal trafficking in neurons [52, 53]. In particular, TMEM106B appears to negatively regulate retrograde transport of lysosomes within dendrites, with reductions in TMEM106B associated with movement of lysosomes toward the neuronal soma [52]. Since TMEM106B influences lysosome function and modulates progranulin levels [52, 107], it is tempting to speculate that its protective role in C9+ carriers might similarly involve the endo-lysosomal pathway, providing a common link to two genetic forms of FTD. Finally, the finding that some C9+ carriers harbor unique Ub+/p62+/TDP-43- pathology further implicates dysfunctional autophagy, as p62 is a ubiquitin-binding protein which accumulates when autophagy is impaired [108]. Since lysosomal degradation is the ultimate endpoint of autophagy, defects in lysosomal trafficking or degradation would be expected to produce the observed Ub+/p62+/TDP-43- pathology that is seen in C9+ carriers. A mutation in the multivesicular body protein *CHMP2B* leading to familial FTD in a Danish pedigree further implicates dysregulation of the endo-lysosomal system as a pathological mechanism leading to FTD [109, 110].

C9ORF72 pathogenesis spreads through neuroanatomical networks

The underlying pattern of neurodegeneration in C9+ may be the best starting point for understanding how one type of genetic variant can result in such heterogeneous clinical presentations. The pattern of diffuse gray and white matter involvement observed in C9+ FTD/FTD-MND (reviewed in [9]) and ALS [10, 11] patients stands in contrast to the idea of neurodegenerative processes spreading through specific, clearly defined functional brain networks [111, 112]. Two intriguing hypotheses suggest how these patterns may fit into the 'selective vulnerability' framework: 1) the epicenter of vulnerability in C9+ neurodegeneration is highly and diffusely interconnected to both cortical and subcortical regions of the brain; 2) functional brain networks in *C9ORF72* expansion carriers are less strongly defined (i.e., there is more inter-network connectivity than intra-network connectivity).

The first theory proposes a 'central station' node that serves as a major hub for multiple different pathways throughout the brain such that degeneration of that network would result in a diffuse pattern of cortical atrophy and profound white matter integrity loss. One such centrally connected subcortical structure is the thalamus. Divided into numerous functionally distinct nuclei, the thalamus receives sensory and motor information from a variety of cortical, cerebellar, and brainstem efferent projections, and then relays it through afferent projections to the cortex for further processing and integration. Each nucleus has specific afferent and efferent projections associated with it, and the nuclei themselves are also highly connected (reviewed in [113]). One longitudinal study of C9+ patients found neuroimaging patterns consistent with spread through such a distributed subcortical network, with thalamic and cerebellar atrophy most prominent while cortical atrophy appeared diffuse and nonspecific [114].

Given the behavioral component of FTD, the dorsomedial nucleus is one tempting candidate given its interconnectivity with the prefrontal, cingulate, and association cortices, and its involvement could also contribute to memory deficits observed in a subset of C9+ carriers [115, 116]. Also, the pulvinar nucleus dominates the posterior portion of the thalamus and is highly interconnected with the occipital cortex, as well as adjacent areas of the parietal and temporal cortices. These two nuclei, along with the lateral posterior—which receives afferent projects from occipital cortex and projects to the parietal cortex—make up the 'associative' functional group of thalamic nuclei involved in high level cognition [34]. The ventral anterior and ventral lateral nuclei receive inputs from basal ganglia and cerebellum, and project to premotor and motor areas of the frontal cortex, respectively, and along with the ventral posterior nucleus compose the 'effector' group involved with movement and aspects of language [34]. Functionally and anatomically, these two groups of thalamic nuclei represent domains affected in the clinical syndromes associated with *C9ORF72* expansion thus far: bvFTD, ALS/MND and PPA. The role of the thalamus in C9+ disease remains to be elucidated through careful pathological dissection and characterization, and may benefit from studies of resting state connectivity seeded within specific thalamic nuclei and studies of thalamic microstructural connectivity [117, 118].

In contrast to the central node hypothesis, the second theory suggests that the diffuse pattern of neurodegeneration observed in C9+ patients may be a by-product of damage that is spreading throughout multiple functional networks rather than being isolated in a single, defined functional circuit, and implicates early systemic disorganization as the underlying cause of diffuse non-selective spread. Reduced network connectivity has been observed even prior to symptom onset in Huntington's disease (HD), another neurodegenerative disorder caused by DNA repeat expansion in the *HTT* gene. Pathogenic *HTT* expansion carriers show lower cortico-striatal functional connectivity as compared to controls, even prior to disease onset [119]. Early changes in brain organization have been suggested in a transgenic rat model of HD [120], with differential aging patterns observed in the brains of transgenic rats as compared to wildtype as early as the first year of life [121]. Microstructure alterations in brain regions relevant to HD were also seen in these transgenic rats during postnatal development [122], though further study is required to determine if similar changes occur in people.

Identifying early changes in brain structure and function in *C9ORF72*-expansion carriers may help to disentangle these two hypotheses, which are not necessarily mutually exclusive. For example, a highly connected node of C9+ neurodegeneration could be identified during prodromic stages of disease, with longitudinal follow-up demonstrating insidious spread across multiple, interconnected functional networks of the brain. On the other hand, early animal experiments established that retrograde degeneration of thalamic nuclei occurs when damage is inflicted upon the cortical area that specific nucleus projects to [123], suggesting a mechanism by which widespread cortical loss across multiple networks could result in thalamic neurodegeneration.

Contributions of *C9ORF72* expansion to clinical heterogeneity

In addition to the phenotypic heterogeneity highlighted in preceding sections, C9+ disease is also associated with other aspects of phenotypic variability. As suggested by slowly progressive C9+ bvFTD cases, there is a large variation in the length of disease course; some groups have suggested that C9+ patients demonstrate longer disease courses than matched sporadic cases (e.g., [124]) whereas others have observed shorter durations of disease (reviewed in [13]). Age of onset is also highly variable, ranging from the 20's – 80's [13], with 50% penetrance by age 58 and nearly full penetrance by age 80 [125]. One report, however, described two C9+ carriers with no cognitive impairments as of ages 80 and 84, suggesting that *C9ORF72* expansion has incomplete penetrance [126]. Whether slowly progressive forms of C9+ bvFTD and predominant psychiatric presentations are a result of reduced expansion size or other genetic or environmental modifiers remains to be established.

In sporadic neurodegenerative disease, there appears to be a sudden precipitous drop in cognitive function several years before a full clinical symptom manifests [127, 128]. However, in a genetically mediated adult-onset disease it is difficult to deny the neurodevelopmental aspect – how is the brain of a disease-causing gene carrier different from that of a non-carrier? Does the brain learn to 'adapt' to deficits, and only during the aging process—which weakens neural plasticity—does dysfunction become apparent? Is there slow, insidious accumulation of pathology in the neurons such that, only after 50+ years, it comes to the point where neurons are being killed? Or are there subtle signs that there is underlying dysfunction from the outset, but these go unrecognized until the symptoms become impossible to ignore?

Whether a prodrome of neurodegenerative disease exists remains unanswered; gene carriers may provide a unique opportunity to study disease in its earliest stages, prior to frank symptom onset. For example, early personality/behavioral changes have been described in some C9+ carriers [129]. In C9+ bvFTD patients, there is often emotional dysregulation reminiscent of cerebellar disconnection syndrome [8, 34]. If subtle alterations in emotional and/or physiological regulation reflect progressive neural dysfunction from a central node or due to systemic disorganization as proposed above, then measures of these features could provide a quantitative measure of these underlying pathological processes as they progress into a full clinical syndrome. Studies of sporadic bvFTD suggest that patients often have psychiatric diagnoses years before referral to the neurology clinic [130]. Whether these are simply misdiagnoses of an underlying neurodegenerative process or are, in fact, early manifestations of FTD remain to be determined.

If C9+ pathogenesis begins in the thalamus, then the molecular mechanism of spread through the interconnected networks of the thalamic nuclei could involve physical spread of toxic TDP-43 pathology in a seeded fashion [131], or functional spread whereby changes in synaptic activity in the thalamus could result in downstream neuronal dysfunction. In mouse, C9ORF72 is robustly expressed in the thalamus [23], and unique Ub+/p62+/TDP-43- pathology is often found in the thalamus of C9+ carriers, supporting a mechanism whereby molecular changes resulting from *C9ORF72* expansion could begin in this central subcortical region and then, over time, affect other regions of the brain through its interconnectedness with cerebellar and diffuse cortical structures.

Regardless of the mechanism, the fundamental leap to identifying effective biomarkers for making predictions of clinical prognosis and disease progression will require linking peripheral measures of disease with local pathological processes. This may include tracking changes in the expression of *C9ORF72* transcripts or other genes dysregulated (directly or indirectly) by hexanucleotide-generated RNA foci and/or RAN-translated dipeptides. Multimodal neuroimaging may also serve as a sensitive measure of C9-specific changes in gray and white matter structures over time [114, 132], even in presymptomatic carriers.

Concluding remarks

In summary, *C9ORF72*-mediated disease is characterized by heterogeneous clinical presentations of motor and/or behavioral syndromes of ALS, bvFTD, or FTD-MND, as well as less common diagnoses of PPA, primary amnestic presentation and psychiatric disease such as depression or bipolar disorder. Parkinsonism is also a common symptom accompanying these clinical diagnoses. Three main molecular mechanisms of C9+ disease have emerged as potential contributors to this observed clinical heterogeneity: haploinsufficiency resulting in a loss of C9ORF72 protein function, formation of RNA foci resulting in a toxic gain of function, and formation of dipeptide aggregates resulting from RAN-mediated sense and antisense translation of the hexanucleotide expansion. Variable expansion length across tissue types and brain regions as well as contributions of other genetic modifiers may provide additional sources of disease heterogeneity. In addition, C9+ diseases may be associated with alterations in cellular trafficking, particularly within the endo-lysosomal pathway. The mode of spread of one or more of these contributing pathological mechanisms could occur via a centrally located neural hub connecting multiple selectively vulnerable functional networks, or through multiple, interconnected networks converging on a common neuroanatomical region. The diversity of clinicopathology demonstrated by C9+ patients suggests a spectrum of disease manifestations that ultimately culminate in unique protein pathology (Ub+/p62+/TDP-43- in the cerebellum and hippocampus) and neuroanatomical damage (thalamic atrophy).

Elucidation of novel genetic and molecular modifiers of C9-mediated disease progression will provide the opportunity for development of therapeutic interventions. In addition, identification of biomarkers that predict future clinical syndrome will be critical for identification of candidates for clinical trials. Finally, gaining a better understanding of the preclinical manifestations of disease – whether they are behavioral, functional or physiological—will also provide deeper insight into the workings of the neuroanatomical system most vulnerable to *C9ORF72* expansion disease.

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Disclosure of conflict of interest

None.

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