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C9orf72-FTD/ALS pathogenesis: evidence from human neuropathological studies

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Abstract

What are the most important and treatable pathogenic mechanisms in *C9orf72*-FTD/ALS? Model-based efforts to address this question are forging ahead at a blistering pace, often with conflicting results. But what does the human neuropathological literature reveal? Here, we provide a critical review of the human studies to date, seeking to highlight key gaps or uncertainties in our knowledge. First, we engage the *C9orf72*-specific mechanisms, including C9orf72 haploinsufficiency, repeat RNA foci, and dipeptide repeat protein inclusions. We then turn to some of the most prominent *C9orf72*-associated features, such as TDP-43 loss-of-function, TDP-43 aggregation, and nuclear transport defects. Finally, we review potential disease-modifying epigenetic and genetic factors and the natural history of the disease across the lifespan. Throughout, we emphasize the importance of anatomical precision when studying how candidate mechanisms relate to neuronal, regional, and behavioral findings. We further highlight methodological approaches that may help address lingering knowledge gaps and uncertainties, as well as other logical next steps for the field. We conclude that anatomically oriented human neuropathological studies have a critical role to play in guiding this fast-moving field toward effective new therapies.

Keywords

C9orf72; Frontotemporal dementia (FTD); Amyotrophic lateral sclerosis (ALS); Dipeptide repeat proteins; RNA foci; TAR DNA binding protein of 43 kDa (TDP-43)

INTRODUCTION

A close kinship between frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) has been evident for years based on the overlapping clinical and pathological features of the two disorders [62, 80, 98, 141]. In 2011, this relationship was deepened further still when a hexanucleotide (GGGGCC) repeat expansion in *C9orf72* was identified as the major genetic cause of FTD, ALS, and FTD-ALS [27, 115]. The discovery showed that, at least in *C9orf72* expansion carriers, FTD and ALS represent variable and often admixed

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manifestations of the same underlying disease process. This breakthrough and related downstream findings have raised a host of key questions. What are the targeted brain regions and cell types? What are the most damaging pathogenic mechanisms? In what sequence do these mechanisms unfold and how, if at all, do they interact with each other? What causes one patient to develop FTD and another ALS, even within the same family? What do brain regions and cell types targeted in FTD have in common with those targeted in ALS? Does this shared biology render these regions and cell types more vulnerable than others? Answers to these questions may prove critical in the race to treat, cure, or even prevent *C9orf72*-FTD/ALS.

In the general population, the intronic GGGGCC expansion can range from 2–30 repeats [6, 50, 85, 107, 118, 153, 162], but in patients with *C9orf72*-FTD/ALS repeats typically number several hundred to thousands [5, 12, 27, 28, 55, 57, 149]. Somatic instability can produce repeat length variability detectable in blood and across tissue types and brain regions [5, 12, 36, 104, 149, 156]. Several studies have sought correlations between repeat length, assessed in blood or brain, and clinical variables, such as syndrome, age at symptom onset, or disease duration, but to date no clear consensus has emerged [5, 6, 29, 47, 104, 139, 149]. In contrast to Huntington's disease and other CAG repeat expansion disorders [reviewed in 138], in *C9orf72*-FTD/ALS anticipation is seen only in some families [111, 154].

Several candidate pathogenic mechanisms have emerged from careful study of *C9orf72*-FTD/ALS patient tissues. Expansion carriers show reduced *C9orf72* mRNA [27] and protein expression [39, 156, 163], resulting in a potential haploinsufficiency state. The repeat mRNA that is transcribed, in the sense or antisense direction, can form RNA foci within the neuronal nucleus or soma [22, 26, 30, 94]. Expansion-containing mRNA can also be translated, through repeat-associated non-ATG-dependent (RAN) mechanisms, into one of five aggregation-prone dipeptide repeat proteins (DPRs) [2, 96]. Finally, patients with *C9orf72*-FTD/ALS develop pathological aggregation of TAR DNA-binding protein of 43 kDa (TDP-43), accompanied by loss of nuclear TDP-43 within inclusion-bearing neurons [7, 137].

With a wide array of mechanistic candidates identified, model-based experimentation has raced forward, outpacing the human tissue-based follow-up studies, and not surprisingly. Modeling a genetic disease in yeast, flies, worms, zebrafish, or mice affords causal inference through experimental manipulation and often proves more attractive and feasible for the typical postdoctoral fellow. Quantitative human pathological studies are labor-intensive and may require that patient materials be newly collected or gathered and harmonized across centers and handling protocols. Additional challenges relate to inter-subject variability and tissue degradation, especially impactful for ultrastructural and transcriptomic studies. These barriers, however daunting, do not make the human neuropathology any less critical to the field. Models, despite their value, remain models, and we should view any model-generated hypothesis with caution unless the relevant human data exist and are at least consistent with the hypothesis. Ultimately, with the diversity of potential *C9orf72*-FTD/ALS mechanisms and pathways, the field will need a way to prioritize candidates for drug development. In our view, the most useful proof-of-concept will emerge through an iterative process in which human tissues undergo increasingly deep and sophisticated phenotyping, as modern

techniques have begun to allow, and are compared with existing models or new ones generated in response to the human findings. This two-way interaction may help the field determine which candidate mechanisms still look promising after they have been scrutinized across complementary approaches that span multiple species and experimental platforms, including quantitative human neuropathology.

Here, we provide an overview of the human *C9orf72*-FTD/ALS tissue studies to date, seeking to highlight the need for more comprehensive, well-controlled, and anatomically precise approaches to guide the whole field forward. We focus on the human pathological research because it is the literature we know best and because outstanding reviews of the model-based literature have been provided by leaders in the field [4, 38, 45, 48, 53, 168]. We present the literature from a systems neuroanatomical point of view, thinking critically about the brain regions examined and their relationship to the clinical syndromes under study.

In discussing candidate pathogenic mechanisms, we find it useful to distinguish between "*C9orf72*-specific mechanisms" (C9orf72 haploinsufficiency, RNA foci, DPRs) not observed in non-*C9orf72* FTD/ALS and "*C9orf72*-associated mechanisms," which include all *C9orf72*-specific mechanisms but also those, such as TDP-43 aggregation and loss-of-function, nuclear transport defects, and others, that also pertain to other forms of FTD/ALS.

CLINICAL AND ANATOMICAL FEATURES OF *C9orf72*-FTD/ALS

C9orf72-FTD typically manifests as the behavioral variant (bvFTD), presenting with some combination of apathy, disinhibition, compulsivity, overeating, loss of empathy, and executive dysfunction. Many patients also develop psychotic features [40, 133], such as delusions or hallucinations, but these features can also be seen in bvFTD without the expansion. Rarely, patients with *C9orf72*-bvFTD develop atypical non-epileptic spells of unclear etiology [155]. Anatomically, sporadic and *C9orf72*-bvFTD are more similar than they are different, with most patients showing early, prominent atrophy in anterior insula, anterior cingulate, amygdala, and striatum [108, 127], but *C9orf72*-bvFTD more conspicuously involves the medial pulvinar thalamus [72, 84, 131]. Morphometric deficits in this structure, which emerge as early as the 3rd decade in presymptomatic expansion carriers [73, 116], may produce targeted cortico-striato-thalamic network dysfunction [72]. Indeed, in some patients, the medial pulvinar thalamus appears to be the primary structure affected [155]. Accentuated cerebellar atrophy has also been reported in *C9orf72*-FTD and -ALS, though less consistently [56, 72, 84], and cerebellar Purkinje and granule cells showed no neuronal loss in one quantitative post-mortem study of *C9orf72*-ALS [143]. As for FTD, for ALS patients with the *C9orf72* expansion generally resemble those without, though *C9orf72*-ALS tends to have a shorter disease duration [148]. A higher frequency of bulbar onset has been reported in some studies [90] but not others [148]. Intriguingly, patients with *C9orf72*-ALS, like those with sporadic ALS and other familial forms, show primary motor cortex hyperexcitability in human electrophysiological studies [42].

C9orf72-SPECIFIC MECHANISMS

C9orf72 haploinsufficiency—Haploinsufficiency of C9*orf*72 protein is one feature sure to be present from birth, yet it remains understudied, perhaps in part because we still lack a

basic biological understanding of this protein and its functions. C9orf72 is a DENN-domain containing protein that likely acts as a GTP-GDP exchange factor (GEF) for Rab GTPases. C9orf72 has been shown to interact with numerous Rab proteins, including Rab1, Rab3, Rab5, Rab7, and Rab11 in human neuronal and neuroblastoma cell lines, mouse primary cortical neurons, and human spinal cord motor neurons [34, 39, reviewed in 164], and these interactions are proposed to regulate endosomal trafficking. Humans express two C9orf72 isoforms, a 222 amino acid short form (C9-S), encoded by exons 2–5, and a 481 amino acid long form (C9-L) encoded by exons 2–11. Researchers have begun to explore, using isoform-specific antibodies, whether C9-S and C9-L have differing cellular localizations or functional roles in healthy human neurons. Thus far, these studies suggest that C9-S may be localized along the nuclear membrane [163], but further studies are needed to confirm this finding given that more recent antibodies validated using *C9orf72* knockout mice failed to detect C9-S protein in human brain [39, 119]. C9-L is expressed as neuronal cytoplasmic granules or puncta that often co-localize with lysosomal markers [39, 163]. One of these studies [39] further showed that C9-L is enriched at presynaptic terminals in humans. Collectively, the findings to date suggest a possible yet unconfirmed role for C9-S in nucleocytoplasmic transport and a more established role of C9-L in lysosomal function and synaptic vesicle trafficking and/or release.

Zebrafish knockdown of the *C9orf72* orthologue, z*C9orf72*, results in motor and behavioral deficits partly rescued by z*C9orf72* overexpression [18]. *C9orf72* knockout mice, in contrast, develop a pro-inflammatory/autoimmune phenotype without neurodegeneration [105]. Not unlike the knockout mice, patients with *C9orf72*-FTD/ALS and other TDP-43-related disorders show an increased prevalence of autoimmune disease [92, 93, 147]. Whether and how the immune phenotype relates to neurodegeneration remains unclear. Patients with *C9orf72*-FTD/ALS show reduced *C9orf72* transcript levels in lymphoblasts [27, 156] and reductions in both mRNA and protein levels have been reported in brain [18, 27, 164]. Intriguingly, one study observed reduced *C9orf72* transcript in both sporadic (neuropathological diagnoses not specified) and *C9orf72*-associated FTD/ALS [18]. On the other hand, a recent study showed no difference between C9orf72 protein levels in frontal cortex (deemed disease-relevant) and occipital cortex (deemed non-relevant) [119]. Still other data showed increased C9-S but decreased C9-L in postmortem frontal and temporal cortices when comparing *C9orf72*-ALS to non-*C9orf72*-ALS. No C9-S or C9-L expression differences were observed between these groups in primary motor cortex or cerebellum, and no control subjects were included for comparison [163, 164]. A larger recent study using new and thoroughly characterized antibodies to C9-S and C9-L showed a 20% reduction of cerebellar C9-L levels in *C9orf72*-FTD/ALS (6 FTD, 6 MND, and 5 FTD-MND) carriers compared to neurological disease controls (5 FTD, 16 MND, and 6 FTD-MND) [39]. The studies to date have generally involved small samples and/or unevenly distributed clinical syndromes, as is often the case for pioneering work. In that light, the lack of consistency across studies is not surprising and suggests that larger, more comprehensive and anatomically guided studies may help resolve whether, where, and how specific C9orf72 protein isoforms are altered in disease.

Unique cases, deeply studied, can shed important light on controversies regarding disease pathogenesis. A recent case series from China screened *C9orf72* for pathogenic sequence

variants and identified one patient with seemingly sporadic ALS and some features of bvFTD [78]. The patient, who lacked a repeat expansion, carried a variant introducing a premature stop codon (p.I201fsX235). The patient's leukocytes had reduced mutant *C9orf72* mRNA transcript levels compared to the control sequence transcript, suggesting non-sense mediated decay of the truncated transcript. Unfortunately, no autopsy was performed, but the absence of a pathogenic expansion suggests that the neurodegenerative syndrome could have been driven by reduced C9orf72 function (or sporadic FTD/ALS in the presence of a coincidental non-pathogenic *C9orf72* variant). Additional similar patients, if described, would suggest an important role for C9orf72 haploinsufficiency, considering that these patients should lack other *C9orf72*-specific features like RNA foci and DPR inclusions. A recent report made a similar argument from a different point of view [89]. An asymptomatic 90-year-old man harbored an intermediate *C9orf72* repeat expansion in the blood but somatic mosaicism produced both small and large repeats in the body and brain. His two children, who inherited large expansions, developed *C9orf72*-ALS. At autopsy, the patient showed widespread RNA foci and DPR inclusions but normal *C9orf72* mRNA and protein levels and no TDP-43 aggregation or neurodegenerative changes. The authors argued that the patient's resilience may have reflected the lack of C9orf72 haploinsufficiency. Further research into the normal biological roles of the C9orf72 isoforms and how these proteins behave in patient tissues should help the field clarify what role, if any, C9orf72 haploinsufficiency plays in human disease pathogenesis.

Repeat RNA foci

RNA foci: regional, cellular, and subcellular distribution: Repeat-containing RNA foci were observed in patients with *C9orf72*-FTD/ALS at the time of initial gene discovery [27]. These foci may contain RNA transcribed in the sense or antisense direction and can be seen throughout the central nervous system [21, 30, 43, 74, 94, 175]. In most brain regions, neurons show a predominance of sense foci (Table 1). This pattern has proved consistent across the various frontal and other cortical regions studied, hippocampal dentate gyrus granule cells, and cerebellar granule cells. In cerebellar Purkinje cells and spinal or bulbar lower motor neurons, however, antisense RNA foci appear more prevalent [21, 26]. Foci also emerge in glia, including astrocytes, oligodendrocytes, and microglia, but at a lower frequency [94], as well as in peripheral blood leukocytes, in which predominantly sense foci are observed [175].

In neurons, RNA foci occupy the nucleoplasm or sit at the edge of the nuclear membrane or, less often, within the cytoplasm [22, 30, 94]. In addition, we and others have shown that some nuclear RNA foci, particularly antisense foci, adopt a "perinucleolar studding" pattern, lining up along the edges of the nucleolus [95, 155]. To further illustrate this phenomenon, here we extended our previous observations to four new cases (3 FTD-MND and 1 ALS) in whom we assessed RNA foci, using FISH, and p62 immunostaining in 8–20 spinal cord anterior horn cells per case (staining as previously described [155]). P62 was chosen to capture inclusions composed of any of the 5 DPR proteins or TDP-43. Manual counts performed on z-stacked confocal microscopic images confirmed that RNA foci are abundant in anterior horn cells, with antisense foci occurring in 73% (Table 1 and Fig. 1). Some neurons with RNA foci also contained p62-positive cytoplasmic inclusions, likely

representing TDP-43 aggregates given the scarcity of DPRs in the anterior horn. Nucleolar studding was particularly prominent in some lower motor neurons (Fig. 2c). In another *C9orf72*-FTD analysis, neurons with sense foci positioned at the nucleolar margin showed larger nucleoli than those with nuclear sense foci not lining the nucleolus [95]. Whether the number, type, or subcellular localization of RNA foci influences the integrity of individual neurons remains otherwise unstudied in human tissues.

RNA foci sequester RNA-binding proteins: Using *in vitro* techniques, researchers have shown that *C9orf72*-associated GGGGCC sense RNA forms hairpins and length-dependent G-quadruplex structures [35, 114]. CCCCGG antisense RNA can also form secondary structures; these C-rich sequences can form i-motifs [67] and quadruplexes [169]. *C9orf72*-ALS patient derived cell lines showed increased RNA G-quadruplex foci using an antibody against DNA/RNA G-quadruplex structures [20], however the existence of these expanded repeat-containing secondary structures in patient neuronal tissue remains unproven. A recent study showed increased R-loops (DNA-RNA hybrids) in spinal lower motor neurons from patients with *C9orf72*-ALS compared to controls [157], but this study could not confirm that the R-loops contained elements of the repeat expansion. Higher order RNA structures could enhance sequestration of various RNA-binding proteins, leading to loss of function. In brain tissue from patients with *C9orf72*-FTD/ALS, sense and antisense RNA foci have been shown to co-localize with various RNA-binding proteins, including hnRNP A1 [22, 123], hnRNP H/F [22, 74], SRSF2 [22], and ALYREF [22]. UV crosslinking assays confirm that these interactions are direct and specific for most of these proteins [21]. SRSF2, a core component of nuclear speckles [135], has shown the most frequent co-localization with sense and antisense RNA foci [21, 22] among the proteins studied to date, but this co-localization does not appear to deplete cells of SRSF2 entirely. Whether binding of RNA foci to various RNA-binding proteins compromises the integrity of individual neurons also remains unstudied in human tissues.

Are RNA foci pathogenic or protective in humans?: In myotonic dystrophy type 1 (DM1), which is caused by a CTG repeat expansion in *DMPK*, the evidence for RNA-mediated toxicity is substantial. Repeat-containing foci sequester RNA-binding muscleblind (MBNL) family proteins, leading to impaired binding of these proteins to their normal cellular targets in the nucleus, which causes widespread splicing abnormalities [33, 59, 86, 91]. Moreover, MBNL1 knock-out mice develop key features of human myotonic dystrophy and can be rescued by overexpression of MBNL1 [61]. Similarly, *C9orf72* expansion-related sense and antisense RNA foci could bind to and sequester RNA-binding proteins, leading to neurodegeneration. For example, in *C9orf72*-FTD/ALS, 70% of all sense foci observed in cerebellar granule cells co-localized with hnRNP H [74]. For many of the RNA-binding proteins examined, however, co-localization with RNA foci appears to be a low frequency event, detected in, at most, only a few foci per neuron. Often such co-localization has no effect on normal (nuclear) protein localization, raising questions about sequestration as the mechanism underlying RNA-mediated toxicity.

Relatively few studies have attempted to correlate the abundance and distribution of RNA foci with *C9orf72*-FTD/ALS clinical and anatomical features. The first study to do so

quantified the proportion of neurons with sense and antisense RNA foci, evaluating anterior frontal cortex, hippocampal dentate gyrus, and cerebellar granule cells in 7 patients with *C9orf72*-FTD [94]. Patients with more sense foci in frontal cortex had an *earlier* age at symptom onset. Antisense foci showed the same trend but did not reach statistical significance. A second, larger study examined associations between RNA foci and clinical features in 63 patients with *C9orf72*-FTD/ALS representing the full clinical spectrum [26]. The authors studied middle frontal gyrus (cortical layers 3–6), cerebellar granule cells, and Purkinje neurons using semi-automated segmentation of digitized FISH images. They examined a variety of measurements (proportion of neurons with sense or antisense foci, number of foci per neuron, etc.), seeking relationships to age at onset, repeat length, *C9orf72* transcript levels, poly-GA and poly-GP DPR levels (in the same brain regions), clinical syndrome, and disease duration. Of all of these associations, only one significant relationship emerged. Patients with antisense foci in a higher proportion of middle frontal gyrus neurons showed a *later* age at symptom onset. Thus, despite observing similar proportions of affected neurons and numbers of foci per neuron, the two studies reported contradictory findings with regard to age-of-onset. Although the second study was much larger and better suited to finding patient-level relationships, at this point it seems most reasonable to conclude that the abundance of RNA foci shows a weak relationship, if any, to the clinical features of *C9orf72*-FTD/ALS, at least when frontal, hippocampal, and cerebellar foci are examined. It remains possible, however, that studying brain regions and neuron types closer to the core of the bvFTD and ALS clinical syndromes could have led to different conclusions. Age at symptom onset and other patient-level clinical variables no doubt reflect multiple complex factors. Alternative readouts, focusing on the integrity of individual neurons, may provide clearer signals with regard to pathogenic relevance. For example, further studies could explore in more detail how RNA foci alter nucleolar function, perhaps especially in spinal cord anterior horn cells.

Dipeptide repeat proteins: prevalent, but relevant?

RAN translation in C9orf72 patient tissue: Prior to the discovery of the *C9orf72* expansion, human neuropathological observations in families linked to Chromosome 9 revealed ubiquitin- and p62-positive neuronal cytoplasmic inclusions (NCIs) that stained negatively for TDP-43 [9]. Once the expansion was identified, researchers astutely surmised and then demonstrated that the TDP-43-negative inclusions were byproducts of RAN translation [2, 97]. For the GGGGCC expansion, RAN translation is carried out across three reading frames in the sense and anti-sense directions, generating six distinct poly-DPR proteins: poly-GA, poly-GP, and poly-GR from the sense strand [2, 87, 96, 97], and poly-PA, poly-GP, and poly-PR from the antisense strand [43, 96]. In addition, poly-PR and poly-GP can be generated using the putative ATG from the antisense strand. RAN translation may extend beyond the repeat region, such that five of the six reading frames can generate unique c-terminal fragments [96, 175] that could themselves influence stability or toxicity.

Regional, cellular, and subcellular distribution of DPR inclusions in C9orf72-FTD/ALS: DPR inclusions are widely distributed throughout the brain in patients with *C9orf72*-FTD/ALS. Inclusions are most abundant in the cerebral cortex, hippocampus (especially CA3–4 and subiculum), amygdala, medial thalamus, and cerebellar granule cells and are

generally less frequent in basal ganglia, brain stem nuclei, and spinal cord [2, 49, 83, 124]. Of the different types, poly-GA, -GP and -GR inclusions are far more prevalent in neurons compared to poly-PA and -PR, which are rare to absent across the diverse brain regions studied [83, 87, 119, 124]. Adding complexity, individual neurons can develop inclusions containing more than one type of DPR. Even sense and antisense-translated versions of the same DPR have been observed within the same human cerebellar and hippocampal neurons [96]. Studies to date have failed to detect DPR inclusions in astrocytes, microglia, and oligodendrocytes, but they are present in some ependymal cells of the spinal cord central canal and ependymal and subependymal cells lining the lateral ventricle [124]. Moreover, DPRs can be detected in CSF and blood [44, 75], and poly-GP cytoplasmic and intranuclear inclusions were detected in testicular Sertoli cells [2].

In neurons, subcellular DPR staining patterns include stellate NCIs, intranuclear inclusions (NIIs), dystrophic neurites (DNs), and diffuse dendritic and/or cytoplasmic staining (Fig. 2) [2, 43, 83, 97, 119, 124]. Most neuronal DPR aggregates occur as compact round or stellate cytoplasmic inclusions. Diffusely DPR-stained neurons are rare, ~4% of poly-GR positive cells in one study (frontal cortex); these authors speculated, based on *in vitro* data, that diffuse DPR staining represents a transitional state preceding compact inclusion formation [172].

Each DPR has unique biophysical properties, which may determine its expression and aggregation patterns in brain tissue. For example, poly-GA is highly aggregation-prone, presumably due to its hydrophobic nature. Electron microscopy studies of cerebellar granule cells from a *C9orf72*-FTD/ALS patient showed that poly-GA NCIs contain 15–17 nm filamentous structures [174], but a more recent study using cryo-electron tomography showed that poly-GA aggregates in cultured neurons are made up of densely packed twisted 13–15 nm thick ribbons with varying length and width [52]. Further understanding of the ultrastructural featuresof DPR aggregates could shed light on toxicity mechanisms and guide therapeutic development. Arginine-containing poly-GR and poly-PR are positively charged [reviewed in 38], which could influence their interactions with other proteins. In cerebellar homogenates, most poly-GP is soluble, whereas the majority of poly-GA is insoluble [46]. DPR proteins also undergo post-translational modifications. Poly-GR NCIs in several brain regions of two patients with *C9orf72*-FTD/ALS were shown to have non-methylated and symmetrically or asymmetrically dimethylated arginines [96], and a recent study confirmed these findings in a larger sample [120]. Like RNA foci, poly-GA inclusions co-localize with, and may sequester, various proteins including Drosha, Unc119, and HR23B in neurons, often leading to protein mislocalization [88, 110, 124, 173]. Moreover, diffuse and aggregated cytoplasmic poly-GR was shown to colocalize with ribosomal proteins S6 and L21 and translation initiation factor eIF3η in frontal cortex from 3 patients with *C9orf72*-FTD/ALS [172]. A similar study also reported that poly-GR, and to a lesser extent poly-PR, co-aggregate with STAU2 and several ribosomal proteins including S6, S25, L19, and L36A [54]. One study suggested that 5–18% of cerebellar and hippocampal poly-GR NCIs co-localize with poly-PR or poly-PA NCIs in patients with *C9orf72*-FTD/ALS [96]. Poly-GA NCIs may even sequester other DPR proteins, with some model-based studies suggesting a protective effect [166], but the degree to which different DPR inclusions co-occur and

interact within individual neurons or undermine neuronal integrity has yet to be studied systematically in patient tissues.

The relationship of DPR inclusions to disease: clinico-pathological correlation studies: Are DPR inclusions responsible for cellular dysfunction and degeneration in *C9orf72*-FTD/ALS? Model-based studies have shown that specific DPRs, particularly the arginine-rich poly-GR and -PR, cause diverse forms of cellular pathophysiology including nucleolar dysfunction [70, 144], nucleocytoplasmic and other transport defects [37, 60, 88], impairment of protein translation and stress granule dynamics [54, 160, 172], endoplasmic reticulum stress [174], and DNA damage [81]. In patient brains, however, DPR inclusions are observed in both degenerating and non-degenerating brain regions and neuron types, raising concerns about the pathogenic relevance of these inclusions. Several clinico-pathological correlation studies have pursued this issue (summarized in Table 2). Taken together, these studies suggest that the distribution of most DPR inclusions has little if any relationship with regional neurodegeneration severity [24, 25, 82, 83, 124]. For example, if frontal cortex (typically unspecified) can be taken to represent a vulnerable region in bvFTD, and spinal cord anterior horn represents a vulnerable region in ALS, the data reveal abundant frontal yet only rare spinal cord DPR inclusions, for most DPR types, regardless of whether the patient had bvFTD, ALS, or both (Table 2). There have been few exceptions to this theme, often small effects detected amidst a large number of correlational hypothesis tests. For example, in frontal lobe upper cortical layers, poly-GA-immunoreactive DN correlated with local neurodegeneration severity [83], whereas, in another study [25], more abundant poly-GA NCIs in cortical, hippocampal, and motor neuron-containing regions were associated with earlier age at symptom onset. In yet another analysis, poly-GA inclusions in cerebellar granule cells were significantly more abundant in FTD compared with MND or FTD-MND [46, 124]. Using more quantitative approaches, a recent study based on 5 patients with *C9orf72*-ALS and new antibodies found that inclusions containing poly-GR, but no other DPR, were significantly more abundant in disease-relevant (frontal cortex, precentral gyrus, and anterior horn of spinal cord) than non-relevant (parietal cortex, occipital cortex, and posterior horn of spinal cord) brain regions [119]. In a larger cohort of 40 *C9orf72*-FTD/ALS cases, middle frontal gyrus poly-GR inclusions were strongly correlated with neurodegeneration in this brain region [120].

Few studies have attempted to relate the presence of DPR inclusions to the integrity of individual neurons. In one laudable effort, researchers found that frontal cortex neurons with poly-GR NCIs had almost double the nucleolar volume of neighboring neurons without these inclusions, even after controlling for nuclear volume [95]. Patients with *C9orf72*-FTD/ALS had reduced nucleolar volume overall, however, raising the possibility that poly-GR protects neurons against shrinkage rather than promoting nucleolar enlargement. Neurons from *C9orf72* expansion non-carriers showed a nucleolar size falling between the poly-GR-positive and GR-negative neurons from carriers.

With few exceptions [46], the human DPR studies to date used immunohistochemical stains that may be better suited to detecting aggregated, insoluble DPR protein inclusions. Therefore, some may consider whether undetected soluble DPR species will prove more toxic and correlate with cellular and regional degeneration. Despite this caveat, overall the

links between DPR inclusions and clinical features or neurodegeneration have been few and inconsistent across studies. Therefore, the relevance of DPR proteins to pathogenesis requires further study. Among the DPRs, the case for pathogenicity may be strongest, based on the human findings, for poly-GR, potentially a key interactor with TDP-43, which has consistently shown a much stronger relationship to clinical phenotype and local neurodegeneration severity, as described below [24, 82, 83].

C9orf72-ASSOCIATED MECHANISMS

TDP pathobiology: loss-of-function, aggregate toxicity, both, or neither?—In addition to the *C9orf72*-specific phenomena, nearly all patients with *C9orf72*-FTD/ALS show TDP-43 aggregation, taking the form of NCIs, neuropil threads, and in some cases glial cytoplasmic inclusions. TDP-43 is a widely expressed nuclear RNA-binding protein involved in multiple cellular functions (reviewed in [32]) including splicing regulation, repression of cryptic exons [1, 13, 58, 76, 109, 140, 142, 145, 146], and translational repression [106, 158]. Normal nuclear TDP-43 is depleted in nearly all neurons with cytoplasmic TDP-43 inclusions [23, 99], making it difficult to disentangle potential loss-of-function vs. aggregate toxicity effects. In patients with *C9orf72*-associated FTLD-TDP, the subtype is usually Type B but can be Type A or a pattern too sparse or blended to classify (Fig. 3a).

Human neuropathological studies suggest that TDP-43 inclusion burden strongly predicts regional neurodegeneration in *C9orf72*-FTD/ALS [24, 82, 83]. That is, the distribution of TDP-43 inclusions mirrors the patterns of regional degeneration in bvFTD and ALS, tracking much more closely than the DPR inclusion pattern [24, 82, 83]. Nonetheless, rare patients with bvFTD show DPRs and RNA foci but few or no TDP-43 inclusions [3, 155], suggesting that neurodegeneration can occur in the absence of TDP-43 aggregation, at least under some circumstances. In patients, DPR inclusions and RNA foci appear to arise before symptom onset[111, 155], followed by TDP-43 aggregation [155], but it remains unclear whether TDP-43 aggregation causes degeneration or merely reflects a degenerative process triggered by *C9orf72*-specific mechanisms. The infrequent co-occurrence of DPRs or RNA foci with TDP-43 NCIs would seem to argue against the “triggering” hypothesis, but results regarding co-localization or co-expression of TDP-43 and *C9orf72*-specific phenomena within individual neurons have been few and inconsistent to date. The stronger relationship between TDP-43 and clinical deficits echoes the widely replicated finding in Alzheimer’s disease (AD) research that tau neurofibrillary pathology correlates better with neurodegeneration than does amyloid plaque burden. Some have used this parallel to argue that DPRs, like the amyloid plaques in AD, may create the conditions in which TDP-43 aggregation can take hold and then drive the neurodegenerative cascade [31].

Depletion of nuclear TDP-43 may lead to neuronal dysfunction, degeneration, and death, even in the absence of cytoplasmic TDP-43 mislocalization or aggregation. *TARDBP* knockout mice show embryonic lethality [68, 130] and partial knockdown of TDP-43 results in motor deficits [68, 165] and motor neuron loss in mice [165]. Several recent cell-based studies have reported that TDP-43 knockdown leads to incorporation of cryptic exons [58, 76, 140, 142], also detected in FTD/ALS patient cerebral cortical areas with substantial

TDP-43 aggregation [76], in AD with TDP-43 proteinopathy [140], and in motor cortex, spinal cord, brainstem and occipital cortex from patients with ALS [146]. Incorporation of cryptic exons in neurons lacking nuclear TDP-43 may, in turn, reduce expression of essential proteins via nonsense-mediated decay of aberrant transcripts; this process is likely involved in both sporadic and *C9orf72*-FTD/ALS. Indeed, a recent cell-based study found expression of cryptic exon-containing *ATG4B* mRNA, accompanied by reduced ATG4B protein, in the context of *TARDBP* knockdown [146]. "TDP-43 depletion," the loss of TDP-43 from neuronal nuclei in the absence of a visible, well-developed cytoplasmic inclusion, is a recently described feature of *C9orf72*-bvFTD [155] and sporadic ALS [10, 11], and may occur early in the disease process. TDP-43-depleted neurons were reported, for example, in a surgical resection specimen removed 6 years prior to symptom onset [155]; although suggestive, this study could not resolve whether TDP-43 depletion in these neurons was due to *C9orf72*-related mechanisms or was a consequence of refractory epilepsy or surgery-associated neuronal stressors. TDP-43-depleted neurons have yet to be well characterized but provide an opportunity to separate the relative contributions of nuclear TDP-43 loss-of-function and TDP-43 inclusion formation in postmortem human tissue.

In summary, whether TDP-43 represents an early or tractable therapeutic target for *C9orf72*-FTD/ALS remains unclear, but the human literature clearly links TDP-43 aggregation and/or loss of nuclear TDP-43 to regional neurodegeneration and clinical deficits.

Nuclear import/export—Loss of nuclear TDP-43 in neurons with cytoplasmic TDP-43 aggregation has long raised the question of whether nucleocytoplasmic transport defects play a role in human FTD/ALS. Nucleocytoplasmic transport is tightly regulated; proteins larger than 40–60 kDa must be transported across the nuclear membrane through the nuclear pore complex [51], via binding to importins and exportins and using energy from the Ran GTP-GDP gradient. RanGAP1 and the Ran binding proteins together enhance the GTPase function of Ran in the cytoplasm, while RCC1, a RanGEF, is involved in RanGDP formation in the nucleus [69].

Data from model systems implicate poly-GA [63] and poly-PR [8, 60, 132] DPRs, as well as the GGGGCC repeat expansion itself [37, 170], in producing nucleocytoplasmic transport defects; these and other model-based studies have been reviewed in detail elsewhere [65, 113]. iPSC-derived neurons from patients with *C9orf72*-ALS show evidence of nucleocytoplasmic defects, with decreased nuclear/cytoplasmic Ran ratio [170] and decreased nuclear RCC1/RanGEF [60], as well as large RanGAP1-positive puncta that occasionally co-localize with RNA foci [170]. In addition, TDP-43 inclusions in HeLa cells may sequester proteins involved in nucleocytoplasmic transport [17], and knockdown of TDP-43 in cell models leads to altered expression of a large group of proteins regulated by TDP-43, including nucleocytoplasmic transport proteins [112, 136].

The evidence for nucleocytoplasmic transport deficits in patients with FTD/ALS, however, remains limited and inconsistent. In non-*C9orf72* FTD or ALS, a handful of early neuropathological studies reported abnormal nucleocytoplasmic transport. Anterior horn cells from patients with sporadic or *SOD1*-associated familial ALS have nuclear membranes with a wrinkled, shrunken appearance and loss of importin-β immunostaining [66]. A more

TDP-43-centered study found decreased levels of cellular apoptosis susceptibility (CAS) protein, an exportin, and karyopherin-α2, an importin, in the middle/superior temporal gyrus from patients with FTLD-TDP and increased levels of CAS and karyopherin-α2 in ALS spinal cord [102], while in *GRN* mutation-associated FTLD-TDP, neuronal nuclear Ran was decreased in inferior frontal gyrus neurons with cytoplasmic TDP-43 aggregation and nuclear clearing [159].

In patients with *C9orf72*-FTD/ALS, most postmortem studies investigating nucleocytoplasmic transport have been small, qualitative studies intended to provide support for animal or cell model findings. One *C9orf72*-ALS study [163] evaluated spinal motor neurons bearing TDP-43 inclusions and loss of nuclear TDP-43. The authors reported decreased nuclear immunostaining of C9-S protein, accompanied by loss of importin-β1 and decreased Ran staining. To corroborate *C9orf72* iPSC findings, another group described diffuse nuclear RanGAP1 and Nup205 staining and large RanGAP1 or Nup107-positive puncta, described as aggregates, which appeared to be associated with the nuclear membrane on brightfield microscopic images of primary motor cortex from patients with *C9orf72*-ALS [170]. Another recent study described Nup205-positive puncta around the nuclear membrane in *C9orf72*-associated ALS [17]. The authors also described large Nup205-positive puncta in sporadic ALS or ALS due to *TARDBP* mutations; the Nup205 puncta stained positively for phosphorylated TDP-43 in sporadic or *TARDBP* mutation-associated ALS but not *C9orf72* ALS [17]. Despite these intriguing early findings, Nup205 staining patterns, even with the same antibody, have varied between studies. Some show perinuclear staining, while others show diffuse cytoplasmic Nup205 in both control subjects and patients with *C9orf72*-FTD/ALS. Recently, using confocal images, a different group demonstrated that the apparent diffuse nuclear staining of RanGAP1 observed with brightfield microscopy in fact emanates from the surface of shrunken (possibly degenerating) nuclei [119]. In our hands, confocal imaging of Nup205 (Fig. 4f, g) and RanGAP1 (Fig. 4h) immunofluorescence staining supports this observation and further suggests that the perinuclear RanGAP1 or Nup205-positive “puncta” seen on widefield images (Fig. 4a-e) may be an illusion resulting from increased nuclear membrane folding in some neurons. In addition, inclusion markers and proteins associated with the nuclear membrane may show close proximity without true co-localization (Fig. 4h). For these reasons, we suggest that widefield images of nuclear membrane proteins should be interpreted with caution, especially in degenerating neurons. Highlighting this point, a recent immunohistochemical study examined nucleocytoplasmic transport proteins in five patients with *C9orf72*-ALS, three with sporadic ALS, and three control subjects. Fifty randomly selected layer 5 upper motor neurons and all surviving lumbar spinal cord lower motor neurons were visually categorized based on their RanGAP1, importin-β1, or lamin localization. This more rigorous approach revealed no differences in staining patterns between the patient and control groups [119]. There was also no change in the pattern of nuclear RanGAP1 staining between neurons with or without poly-GR aggregates [119]. Although described in *C9orf72* ALS iPSC-derived neurons [170], colocalization of RanGAP1 puncta with RNA foci has yet to be reported in patient tissues. A recent study observed nuclear depletion and cytosolic accumulation of KPNA4, a member of karyopherin-α family involved in the nuclear import pathway, in the frontal cortex of sporadic and *C9orf72*-FTD/ALS, with the phenotype being more pronounced in patients

with the *C9orf72* expansion [134]. This nuclear depletion was observed in neurons with and without sense-encoded DPR or phosphorylated TDP-43 inclusions.

Overall, then, the human tissue findings regarding nuclear transport defects remain inconsistent, at times contradictory, and require further study before firm conclusions can be drawn about the role this mechanism plays in *C9orf72*-FTD/ALS pathogenesis.

INTERACTIONS AMONG CANDIDATE MECHANISMS

How might the various *C9orf72*-specific and associated factors interact to produce disease? Despite the strong association of *C9orf72*-FTD/ALS with TDP-43 inclusion pathology, few model systems have shown a clear, prominent TDP-43 phenotype. Two complementary mouse models of *C9orf72*-FTD/ALS [15, 79] develop TDP-43 inclusions, in addition to DPRs and RNA foci, especially with advancing age, although many of the inclusions were nuclear, which is rarely a feature of *C9orf72*-FTD/ALS in humans. Phosphorylated TDP-43 aggregates have been reported in a SH-SY5Y cell model expressing only poly-GA DPR protein [103].

In human tissues, immunohistochemical evidence suggested that neurons with reduced C9-S are more likely to contain cytoplasmic TDP-43 aggregates, perhaps indicating that C9-S shuttles TDP-43 between the nucleus and cytoplasm [164]. This notion provides a potential direct link between C9orf72 haploinsufficiency and TDP-43-mediated neurodegeneration. RNA foci and DPR proteins may also somehow interact with TDP-43. One study investigated sense and antisense RNA foci distribution in the cerebellum (Purkinje and granule neurons), spinal cord anterior horn cells, and hippocampal dentate gyrus and CA4 in seven patients with *C9orf72*-ALS [21]. Antisense RNA foci were most abundant in anterior horn cells and were associated with loss of nuclear TDP-43, with 77% of antisense RNA foci-containing neurons showing loss of nuclear TDP-43, with or without an associated TDP-43 inclusion. By comparison, only 13% of neurons without antisense foci lacked nuclear TDP-43. Among the DPRs, a recent study showed that poly-GR NCIs and DNs co-localized with phosphorylated TDP-43 (pTDP-43) in primary motor cortex [119]. Although only a few poly-GR DN were present in each patient, 80% of those DN co-localized with pTDP-43, whereas less than 20% of poly-GR NCIs co-localized with pTDP-43. This observation seems compatible with a model in which poly-GR aggregation within distal dendrites occurs first and somehow cultivates TDP-43 misfolding. Poly-GR and pTDP-43 may then in some cases undergo a centripetal spread back to the neuronal perikaryon, culminating in an inclusion containing one or both proteins. Whether this process may then proceed into the axon, leading to transsynaptic spread between networked neurons, remains unknown.

DISEASE MODIFYING FACTORS

Methylation of the *C9orf72* pathogenic hexanucleotide repeat expansion

Epigenetic modification of the hexanucleotide repeat expansion has the potential to influence *C9orf72* expression and thereby alter the intensity of *C9orf72*-specific pathological processes. Hypermethylation of the CpG island in the *C9orf72* promoter region

results in reduced expression of the pre-mRNA transcript, with the potential to reduce formation of GGGGCC G-quadruplexes that promote RNA foci and DPR aggregation in model systems [161]. Consistent with this idea, CpG promoter hypermethylation is associated with longer disease duration, later age at death, and reduced DPRs and RNA foci in patient postmortem cerebellum [77, 117]. Could regional methylation differences predict whether an expansion carrier develops FTD or ALS? So far, the evidence argues against this hypothesis, with both syndromes showing comparable methylation status in frontal cortex and spinal cord [161]. In blood, the GGGGCC repeat itself is also methylated when expanded (>50 repeats) but not with small or intermediate (22–43) repeats. As with brain, in blood the degree of methylation does not appear to distinguish between clinical syndromes [161]. A recent study, however, utilized measures of methylation to identify an age-of-onset modifier located in a linked genetic region containing two overlapping genes, LOC101929163 and C6orf10 [171]. With each protective A allele of the top SNP tagging this linkage block, rs9357140, there was an associated 30% reduced hazard for disease conversion, with AA individuals showing a median age of onset 6 years later than those with the GG risk genotype. In addition, the G risk allele was associated with increased expression of the non-coding RNA LOC101929163 and HLA-DRB1, a class II MHC receptor important in initiating the adaptive immune response. These findings further support a role of pro-inflammatory responses in *C9orf72*-mediated disease.

TMEM106B and ATXN2: phenotype-modifying genes involved in lysosomal biology

Single nucleotide polymorphisms (SNPs) forming a haplotype encompassing *TMEM106B* on chromosome 7 had been associated with risk for FTLD-TDP in both sporadic and *GRN* pathogenic variant carriers [152]. Numerous studies since have replicated the association of *TMEM106B* SNP minor alleles with protection from FTD, particularly in the context of *GRN* or *C9orf72* pathogenic variation [101]. Patients with a *C9orf72* expansion who are homozygous for the protective *TMEM106B* variant (functional SNP rs3173615, p.T185S) appear less likely to present with an FTD syndrome (and therefore more likely to present with MND) [151]. The protective effect of p.T185S in TMEM106B may be due to increased degradation of the mutant protein isoform and subsequent reduction of total TMEM106B levels [100]. Cellular assays have shown that overexpression of TMEM106B results in C9orf72-dependent lysosomal defects [14, 41], corroborating a role for reduced TMEM106B expression as protective against FTLD-TDP.

In contrast to *TMEM106B*, *ATXN2* has emerged as a disease modifier that increases the risk of presenting with MND. In *C9orf72* expansion carriers, having an intermediate (>29 repeats) CAG expansion in *ATXN2* has been linked to presenting with clinical MND over FTD in some studies [71, 150] but not others [16]. Cellular studies suggest that intermediate expansions in *ATXN2* may synergize with *C9orf72* depletion to induce motor neuron death through disturbances in autophagy [129]. The contrasting effects of *TMEM106B* and *ATXN2* on *C9orf72*-associated syndrome may reveal important differences in the pathobiological factors that drive selective vulnerability within bvFTD- versus MND-vulnerable neurons.

NATURAL HISTORY CONSIDERATIONS

What is the sequence of pathogenic events in *C9orf72*-FTD/ALS? As difficult as it is to address this question in humans or to build confidence in clues provided by model systems, many authors have begun to assemble a natural history based on the limited available human data. It is now clear from multiple brain imaging studies that selective and disease-relevant deficits in brain volume, structural connectivity, and functional connectivity are detectable by the 30s [73, 116]. We are careful to refer to these abnormalities as deficits rather than losses so that we acknowledge the uncertainty regarding whether the deficits reflect abnormal neurodevelopment or incipient neurodegeneration, although these possibilities could also co-exist in the same patient.

Although rare patients have shown *C9orf72*-specific pathological changes but no TDP-43 aggregation [89, 111, 155], the reverse has yet to be reported. One extraordinary patient with *C9orf72*-FTD showed RNA foci and DPRs but essentially no TDP-43 inclusions in tissue obtained 5 years before symptoms (as part of a surgical resection for epilepsy). By the time of autopsy 13 years later, the patient had developed full-blown FTLD-TDP, Type B. Collectively, these findings suggest that at least one of the *C9orf72*-specific changes may account for the early life structural and functional network deficits observed in presymptomatic carriers. Whether these mechanisms exert their influence *in utero*, during a specific stage of childhood or adolescent development, or in early adulthood remains unclear. TDP-43 pathobiology may represent a second, degenerative phase that could emerge either as a cumulative result of aging, as the consequence of an unknown environmental stressor, or both. Importantly, this *C9orf72*-based framework raises the possibility of an undiscovered “first hit” at work even in patients with sporadic FTLD-TDP and ALS-TDP.

LIMITATIONS AND FRONTIERS IN THE HUMAN *C9orf72*-FTD/ALS LITERATURE

The human pathological literature on *C9orf72*-FTD/ALS is growing, but significant unanswered questions remain. Perhaps most notably, it remains unclear whether, how, and where *C9orf72*-specific changes interact with each other and with *C9orf72*-associated phenomena in humans. In model systems, *C9orf72*-specific pathological features prove sufficient to produce neurodegeneration, but so far few examples of mild, focal neurodegeneration without TDP-43 aggregation have been described in patients [155]. Emerging *C9orf72*-associated phenomena, such as RNA mis-splicing and DNA damage, have generated excitement but could reflect causes or effects of neurodegeneration, and, so far, these possibilities have not been resolved in humans or model systems. Finally, as highlighted throughout this review and in the next section, greater anatomical precision maybe needed to facilitate interpretation of human tissue studies. This issue receives widely varied treatment in the literature. Some studies have taken a carefully considered approach while others have chosen regions based on convenience or the anticipated density of *C9orf72*-specific phenomena, which can influence experimental ease and throughput. To improve the signal to noise ratio in neuropathological research, however, it may prove

critical to study brain regions—and even neuronal subtypes—that lie at the heart of the bvFTD and ALS anatomical patterns.

A PATH FORWARD: LEVERAGING SELECTIVE VULNERABILITY TO UNDERSTAND FTD/ALS

In ALS, upper and lower motor neurons provide a clear target for investigations of disease-specific processes in sporadic and *C9orf72*-associated disease. In bvFTD, human neuropathological studies have typically focused on unspecified “frontal cortex”. The heterogeneity of frontal regions and their neuronal subtypes, however, makes it difficult to draw clear conclusions about candidate mechanisms examined within a region so coarsely defined. For years, anatomical imprecision in bvFTD studies reflected our lack of a clear bvFTD pattern, but that knowledge gap has been filled. Converging research across multiple groups, cohorts, and studies has shown that the anterior cingulate and ventral anterior insular (i.e. frontoinsular) cortex represent the earliest and most consistently affected cortical regions in bvFTD [108, 125, 127, reviewed in 128]. At the microscopic scale, our group [64, 126] and now two others [121, 122, 167] have shown that the von Economo neurons (VENs), found almost exclusively within the anterior cingulate and frontoinsular cortices, are among the earliest-affected and most vulnerable neurons in bvFTD, whether the syndrome is due to FTLD-TDP, -tau, or -FUS [64, 121, 122, 126, 167]. The only dedicated study of these neurons in *C9ORF72*-bvFTD showed a ~40% decrease in anterior cingulate VEN density that did not reach statistical significance in a sample of 13 patients [167]. This finding appropriately places *C9orf72*-bvFTD toward the mild end of the bvFTD neurodegeneration severity spectrum. The study also built upon in vivo neuroimaging work to show that patients with *C9orf72*-bvFTD exhibit more striking atrophy of the medial pulvinar thalamus compared to controls, possibly augmenting the network destabilizing effects of the milder VEN loss observed.

VENs, like the giant Betz cells targeted in ALS, are large-diameter, layer 5b projection neurons with likely subcerebral targets [19]. We hypothesize that these two susceptible neuron types may share some aspect of their neuronal identity that confers selective vulnerability. More generally, we propose that focusing neuropathological studies on the most selectively vulnerable neuron types may provide clearer correlative signals with regard to candidate pathogenic mechanisms. Using this approach, which benefits from the greater homogeneity within each neuronal class, degeneration-related changes in cellular morphology or protein expression can be measured in lieu of a physiological outcome, mitigating a key limitation of postmortem human tissue studies. At a minimum, focusing region-level studies on brain structures most relevant to the clinical syndromes under study (Table 3) may help to elucidate the pathophysiology of these devastating diseases.

SUMMARY

These are exciting times in FTD/ALS research, and the discoveries regarding the *C9orf72* expansion and related pathobiological features have fueled new hope for treatments. Human tissue research has played a key role in identifying the various mechanistic candidates in *C9orf72*-FTD/ALS, but so far neither model-based nor human studies have provided

consistent, unambiguous information about which mechanisms are most damaging. In partnership with model-based experimentation, human studies have an essential role to play by continuing to test correlative hypotheses, in increasingly modern and nuanced ways, and by generating new hypotheses through discovery-based platforms. Focusing these efforts on the most vulnerable cell types and brain regions will no doubt speed researchers along the path toward therapeutic discovery.

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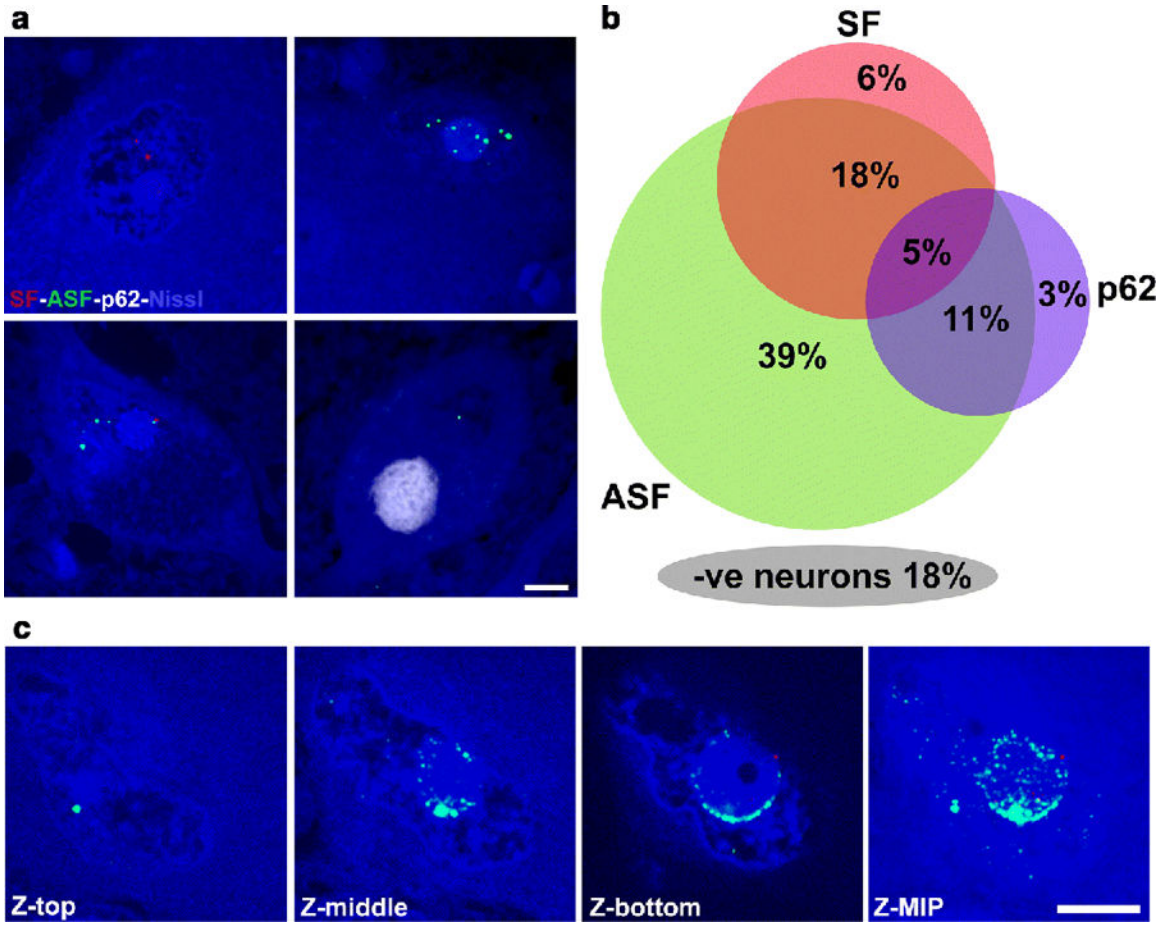

Fig. 1. RNA foci in spinal cord anterior horn cells in *C9orf72*-FTD/ALS.

(a) Anterior horn cells with RNA foci are shown. Lower right panel shows a motor neuron with a sense RNA focus and a p62-positive likely TDP-43 inclusion **(b)** Anterior horn cells stained for sense foci (SF), antisense foci (ASF) and p62 together showed a high rate of antisense foci, which often occurred with other inclusion types. Venn-diagram shows the rates of co-occurrence. Because DPR inclusions are so rare in the spinal cord, most of the p62-positive inclusions can be assumed to be TDP-43 inclusions. Overall, the rate of ASF in motor neurons was high, and most p62-positive inclusions co-occurred with ASF +/− SF. **(c)** Selected z-stack images through an anterior horn cell shows “perinucleolar studding” with ASF. MIP, maximum intensity projection. Scale bar represent 10 μm.

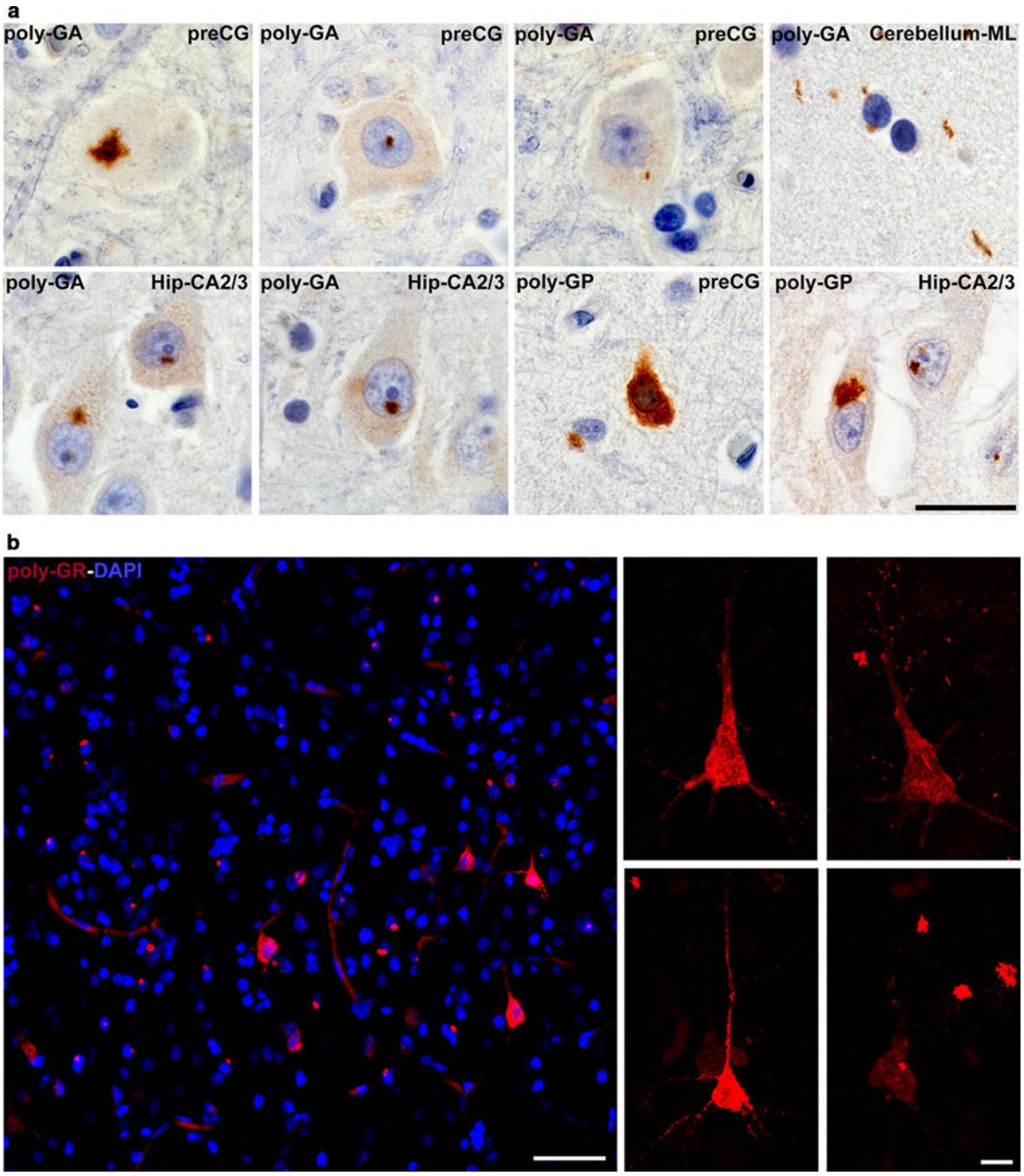

Fig. 2. Diverse DPR staining patterns observed in *C9orf72*-FTD/ALS.

(a) Most DPR protein aggregation takes the form of neuronal cytoplasmic inclusions (NCIs). Other patterns include neuronal intranuclear inclusions, dystrophic neurites, and diffuse proximal somatodendritic staining. **(b)** Immunofluorescence for poly-GR in the precentral gyrus of a patient with *C9orf72*-FTD/ALS shows numerous compact, often stellate NCIs spanning the cortical layers. Occasional diffusely stained neurons can be seen in which poly-GR fills the cytoplasm and enters the proximal dendrites. Scale bars represent 25 μm in (a) and 50 (left) or 10 μm (right) in (b).

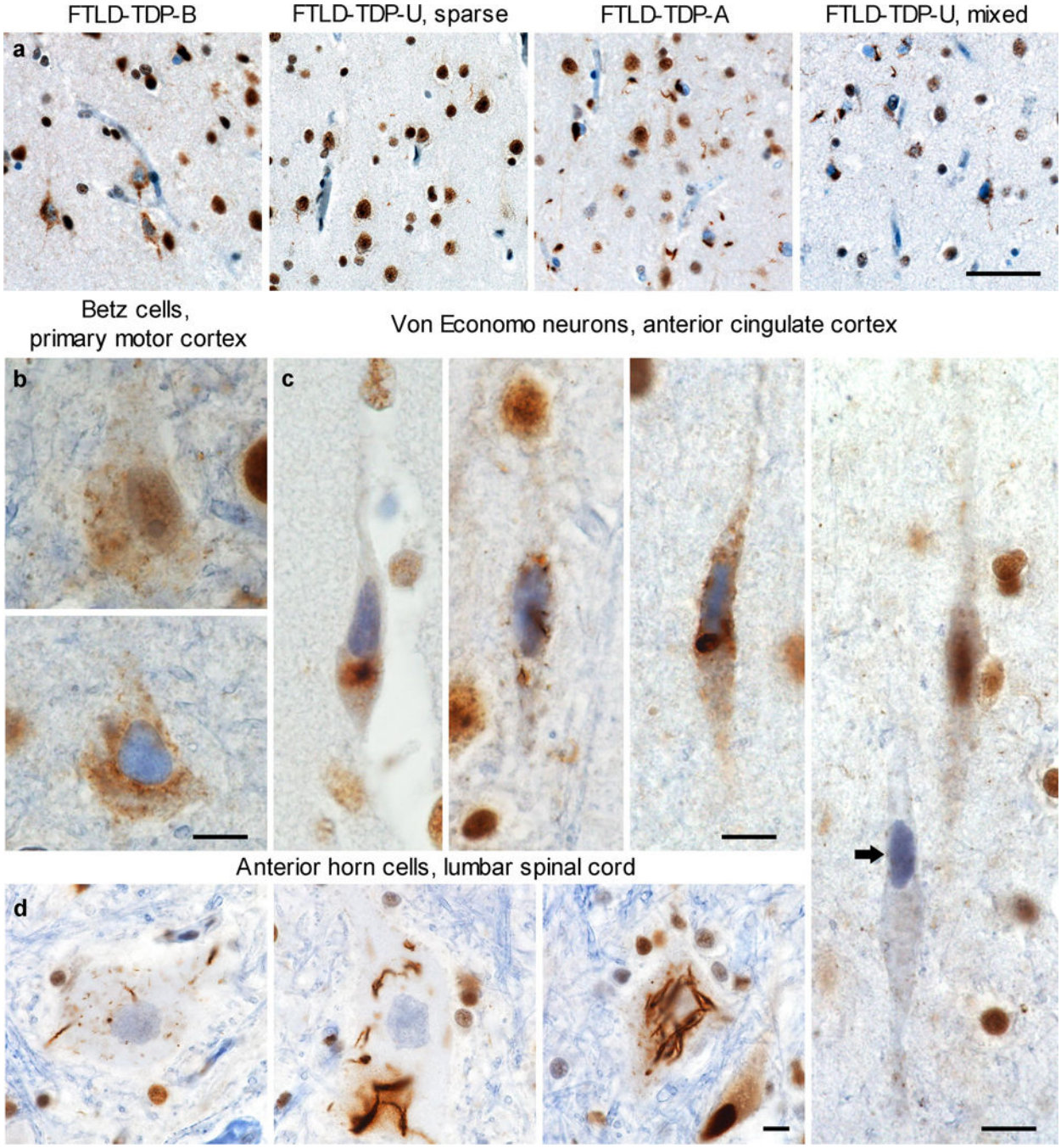

Fig. 3. TDP-43 pathobiology in *C9orf72*-FTD/ALS.

(a) Patients with *C9orf72*-associated FTLD-TDP most often show Type B inclusions (panel 1). Others may show TDP-43 inclusions too sparse to classify (unclassifiable, Type U, panel 2), Type A (panel 3), or Type U with a mixed pattern (panel 4). Betz cells **(b)**, von Economo neurons **(c),** and anterior horn cells **(d)** are prone to TDP-43 pathobiology, and may develop compact, granular, or skein-like TDP-43 inclusions, usually accompanied by a loss of nuclear TDP-43. A proportion of von Economo neurons, as well as Betz cells and other

neurons (not shown), show loss of nuclear TDP-43 in the apparent absence of a neuronal cytoplasmic inclusion (**c**, arrow). Scale bars represent 100 μm **(a)** and 10 μm **(b-d)**.

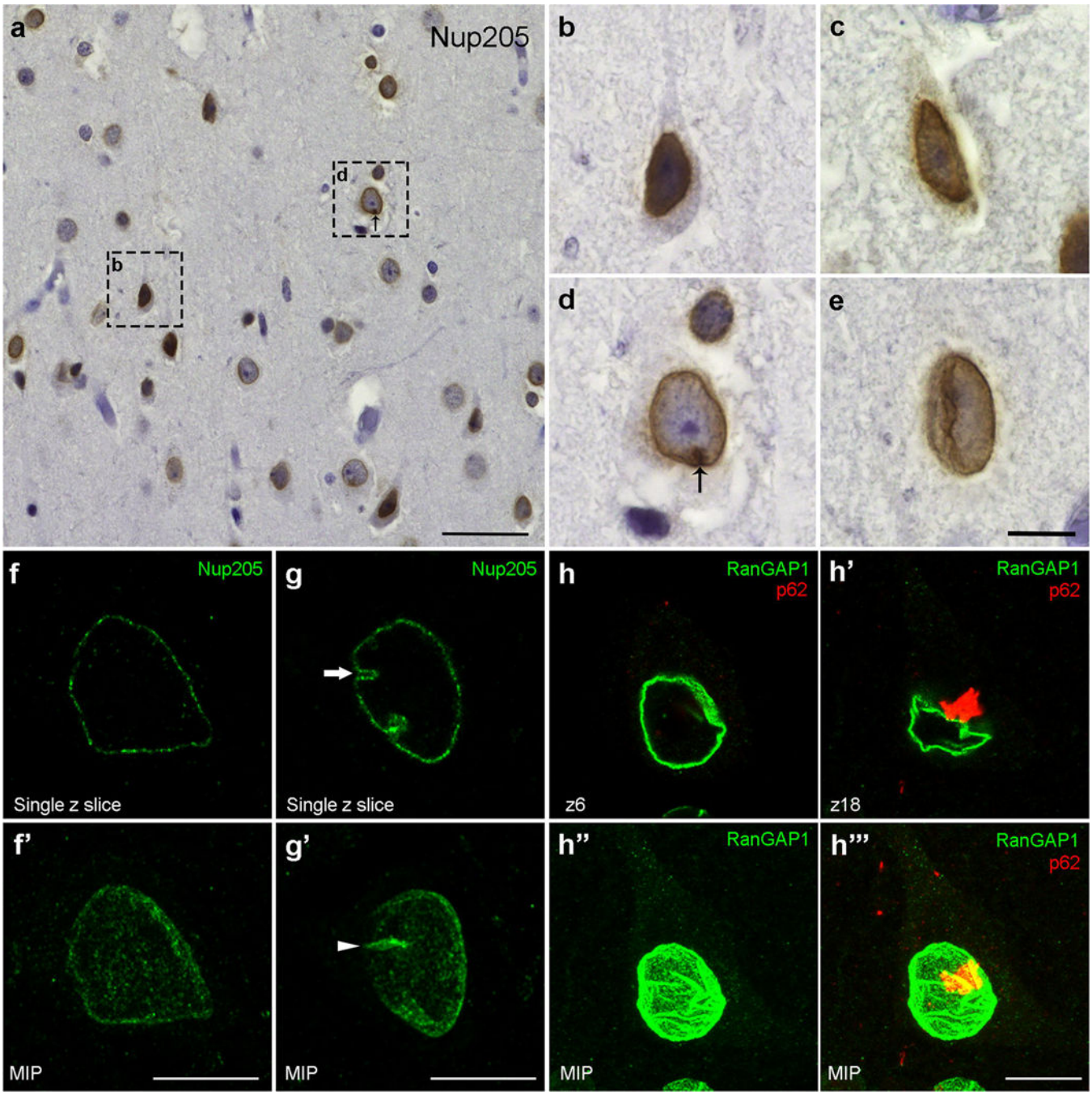

Fig. 4. Nucleocytoplasmic transport system: abnormalities in *C9orf72*-FTD/ALS?

(a-e) Brightfield images of Nup205 staining in anterior cingulate cortex in *C9orf72*-associated and sporadic FTLD-TDP, Type B, shows neurons with apparent diffuse nuclear staining **(a, b, d)** and folded nuclear membranes, which can appear as large, focal nuclear puncta (thin arrow) adjacent to the nuclear membrane **(d)** or as folds spanning the nucleus **(e)**. **(b)** and **(d)** are also shown inset in **(a)**. **(f-g)** Confocal imaging of Nup205 reveals punctate staining that outlines the nuclear membrane in a single z slice **(f, g)** but appears as diffuse nuclear staining on a maximum intensity projection **(f', g')** of the whole nucleus.

Folds of the nuclear membrane (arrow) evident on a single Z slice **(g)** can appear as an aggregation (arrowhead) on a maximum intensity projection **(g')**, possibly simulating the appearance on brightfield imaging. **(h)** Perinuclear RanGAP1 staining that appears correctly localized in single z slices (h, h') can also appear as diffuse nuclear staining on a maximum intensity projection **(h")**. p62-positive inclusions in *C9orf72* FTD are often adjacent to the nuclear membrane **(h')**. Scale bars represents 25 μm **(a)** and 10 μm **(b-h)**.

Table 1:

Quantitative studies of neuronal RNA foci in *C9orf72*-FTD/ALS

Study	N per clinical syndrome			FISH method	Brain regions and other tissue	SF (% cells)	ASF (% cells)	SF + ASF (% cells)
	FTD	ALS	FTD-ALS					
DeJesus-Hernandez et al. [27]	2	-	-	SF/ASF[a]	Frontal cortex	25	-	-
					Spinal cord AHC	25	-	-
Mizielinska et al. [94]	7	-	-	SF/ASF[a] or SF+ASF[b]	Anterior frontal cortex	37[a]	26[a]	14[b]
					Hippocampus-DG	25[a]	18[a]	7[b]
					Cerebellum-GL	21[a]	9[a]	3[b]
Gendron et al. [43]	-	-	4	SF/ASF[a]	Frontal cortex (layers 1–3)	~10	~8	-
Zu et al. [175]	3 unspecified			SF/ASF[a]	Frontal cortex	19	9	-
					Peripheral blood leukocytes	29	7	-
Cooper-Knock et al. [22]	-	3	-	SF/ASF[a]	Cerebellum-GL	39	-	-
					Spinal cord AHC	61	-	-
Cooper-Knock et al. [21]	1	4–6[c]	1	SF/ASF[a]	Cerebellum-PN	55	85	-
					Cerebellum-GL	56	0.75	-
					Spinal cord AHC	62	71	-
Vatsavayai et al. [155]	2	-	-	SF+ASF[b]	Hippocampus-DG	30	19	-
					Hippocampus-CA4	60	43	-
					aMCC	30	19	13
					sACC	39	9	12
					Ventral striatum	33	12	31
					Inferior temporal gyrus	33	17	11
					Hippocampus-DG	35	13	6
					Entorhinal cortex	30	18	10
					Precentral gyrus	28	24	29
					Postcentral gyrus	30	19	24
					mPULV	24	15	42
					Calcarine cortex	34	21	27
DeJesus-Hernandez et al. [26][d]	26	18	16	SF/ASF[a]	Middle frontal gyrus	32	16	-

Study	N per clinical syndrome			FISH method	Brain regions and other tissue	SF (% cells)	ASF (% cells)	SF + ASF (% cells)
	FTD	ALS	FTD-ALS					
					Cerebellum-PN	70	74	-
					Cerebellum-GL	23	1	-
Vatsavayai et al. (present data)	-	1	3	SF/ASF/p62[b]	Spinal cord AHC	29	73	23

[a] Foci proportions are based on staining for sense or antisense foci only;

[b] Foci proportion are based on staining for sense and antisense foci together;

[c] number of cases used varied from 4–6 per brain region;

[d] 3 AD subjects were also included in the study.

Abbreviations: aMCC: anterior midcingulate cortex; AHC: anterior horn cells of spinal cord; CA4: CA4 subfield of hippocampus; DG: dentate gyrus of hippocampus; GL: granular layer of cerebellum; mPULV: medial pulvinar thalamus; PN: Purkinje neurons of Cerebellum; and sACC: subgenual anterior cingulate cortex.

Table 2:

Human neuropathological studies of DPR pathology in *C9orf72*-FTD/ALS

Study	N per syndrome			Tissues/regions analyzed	DPR(s) studied	Study method	Major DPR-related findings
	FTD	MND	FTD-MND				
Mori *et al.* [97]	5	-	4	**Cerebral cortex:** frontal or temporal **Limbic/Subcortical:** Hipp	GA, GP, GR	semi-quant (NCI)	• Co-discovered presence of DPR NCIs • GA NCI more abundant than GP or GR • DPR NCI rarely co-occur with pTDP-43 NCI
Ash et al. [2]	14	9	7	**Cerebral cortex:** frontal, temporal, PreCG, ERC **Limbic/Subcortical:** Hipp-DG, CA3, basal ganglia, Thal, hypothalamus, Amyg **Brainstem:** SN and XII CNN **Cerebellum:** GL, ML, and PL **Spinal cord:** AH **Other:** heart, lung, spleen, liver, kidney, muscle, peripheral nerve, and testis	GP	semi-quant (NCI)	• Co-discovered presence of DPR NCIs • GP NCI observed across CNS regions • GP NCI in neurons but not astrocytes • Sertoli cells of testes showed GP positive NCI and NII, but absent in germ cells of the seminiferous tubules
Mackenzie *et al.* [82]	9	8	18	**Cerebral cortex:** PreCG, frontal, occipital **Limbic/Subcortical:** Hipp-DG, CA3/4, aSTR **Brainstem:** SN and XII CNN **Cerebellum:** GL, ML and PL **Spinal cord:** AH	GA	semi-quant (NCI, NII, and DN) vs. clinical syndrome, AAO, SxDur, ND	• GA NCI most abundant in cerebellum, neocortex, and Hipp • Regional GA NCI burden similar across syndromes • High GA in STR and SN with parkinsonism • No correlation between GA inclusions & ND
Mann *et al.* [87]	9	3	4	**Limbic/Subcortical:** Hipp-DG, CA4 **Cerebellum:** GL	GA, GP, GR, PA, PR	semi-quant (NCI) vs. FTLD-TDP subtypes, p62	• GA, GP, GR make up most p62+/TDP- NCIs • DPR NCI burden similar in TDP Types A, B
Gendron *et al.* [43]	3	3	3	**Limbic/Subcortical:** Hipp-DG, CA3 **Cerebellum:** GL	GP, PA, PR	semi-quant (NCI)	• Antisense DPR NCIs detected • GP much more abundant that PA and PR • SF/ASF and GP NCI rarely co-occur
Mori *et al.* [96]	6	2	3	**Cerebral cortex:** frontal, temporal, parietal, occipital, PreCG **Limbic/Subcortical:** Hipp-DG, CA1/4,Thal, CN, PUT, and claustrum **Brainstem:** SN, olive, and pontine nuclei **Cerebellum:** ML and GL **Spinal cord:** AH **Other:** Olfactory bulb	GR, PA, PR	descriptive and quant (NCI)	• Rostro-caudal gradient of GR NCI • Antisense DPR NCIs detected • Sense and antisense DPRs may co-aggregate • RAN translation extends beyond repeat into c-terminal region
Zu *et al.* [175]	2	3	3	**Cerebral cortex:** motor **Limbic/Subcortical:** Hipp **Spinal cord:** AH	GA, GP, GR, PA, PR	semi-quant (NCI)	• Sense and antisense GP NCI detected • DPR NCI seen in isolated cells or in high density clusters of neighboring cells • DPRs can have unique c-terminal fragments
Liu *et al.* [77]	5	8	4	**Cerebellum:** GL	GA, GP, GR	quant (NCI) vs. *C9orf72* promoter methylation	• *C9orf72* promoter hypermethylation correlates with fewer DPR NCI
Davidson *et al.* [25]	8	7	6	**Cerebral cortex:** frontal, temporal, cingulate, insular, parietal, PreCG, ERC, occipital	GA	semi-quant (NCI) vs. clinical syndrome,	• NCI most abundant in neocortex, hipp, and cerebellum • NCI show similar abundance across syndromes

Study	N per syndrome			Tissues/regions analyzed	DPR(s) studied	Study method	Major DPR-related findings
	FTD	MND	FTD-MND				
				Limbic/Subcortical: FuG, Hipp-DG, subiculum, CN, PUT, GP, Thal, amyg **Brainstem:** SN, LC, DRN, X and XII CNN **Cerebellum:** GL, PL, DN **Spinal cord:** AH of cervical and/or lumbar		AAO, SxDur, APOE ε4, TMEM106B	• Negative correlation between poly-GA NCI burden and age at onset
Cooper-Knock *et al.* [21]	1	4	1	**Cerebellum:** GL **Spinal cord:** AH	GA, PA	quant (NCI and NII)	• GA inclusions higher in granule cells whereas PA inclusions higher in AH cells
Schuldi *et al.* [124]	3	3	8	**Cerebral cortex:** SFG, PreCG, Calc, cing gyr, parietal, temporal, ERC **Limbic/Subcortical:** NAcc, amyg, Hipp-DG, CA½, and CA3/4; CN, PUT, GP, STN, Thal **Brainstem:** DRN, ION, LC, XII CNN, pontine nuclei, SN **Cerebellum:** GL, ML, and PL **Spinal cord:** AH and/or PH of cervical, lumbar, thoracic, and sacral	GA, GP, GR, PR	quant (5 regions based on NCI and NII) and semi-quant (36 regions based on NCI, NII, and DN) vs. clinical syndrome	• DPR inclusions most abundant in neocortex, Hipp, Thal • In cerebellar GL, GA more abundant in FTD vs. MND and FTD-MND • PR NCI/NII more abundant in CA3/4 in FTD vs. MND • DPR distribution not correlated with ND
Gomez-Deza *et al.* [49]	-	10	-	**Spinal cord:** AH	GA, GP, GR, PA, PR	quant and semi-quant (NCI and NII)	• DPR NCI/NII rare in lower motor neurons but TDP-43 NCI are abundant • DPR NCI/NII & TDP-43 NCI rarely co-occur
Gendron *et al.* [46]	24	12	19	**Cerebral cortex:** MFG, PreCG **Limbic/Subcortical:** Hip-DG, CA4, subiculum **Cerebellum:** cerebellum	GA, GP	quant immunoassay vs. clinical syndrome, AAO, SxDur, expansion size, *C9orf72* variants 1/3	• GP levels highest in the cerebellum • Cerebellar GP significantly lower in MND vs. FTD or FTD-MND • Cerebellar GA trended lower in MND vs. FTD or FTD-MND • Cerebellar GP associated with cognitive symptoms • Cerebellar GP and GA associated with *C9ORF72* variant 3 mRNA expression
Mackenzie *et al.* [83]	11	8	16	**Cerebral cortex:** premotor frontal cortex **Brainstem:** XII CNN **Cerebellum:** cerebellum **Spinal cord:** AH	GA, GP, GR, PA, PR	quant and semi-quant (NCI, NII, and DN) vs. clinical syndrome, AAO, SxDur, ND	• DPR abundance: GA > GP > GR > PA/PR • Overall no relationship between any DPR NCI/NII and syndrome or ND • In frontal cortex, GA DN correlated with ND • Strong correlation between insoluble DPR proteins and number of visible inclusions
Davidson *et al.* [24]	5	9	6	**Cerebral cortex:** frontal, temporal, occipital **Limbic/Subcortical:** Hipp-DG, CA3/4, VM Thal **Cerebellum:** GL and PL **Spinal cord:** cervical and/or lumbar	GA, GP, GR, PA, PR	semi-quant (NCI)	• GA, GP, and GR NCI abundant in Hipp dentate granule cells and cerebellum • NCIs rare in Purkinje and AH cells
Saberi *et al.* [119]	-	5	-	**Cerebral cortex:** frontal, PreCG, parietal, occipital, retrosplenial **Limbic/Subcortical:** Hipp **Cerebellum:** cerebellum **Spinal cord:** AH, PH **Other:** Olfactory bulb	GA, GP, GR, PA, PR	quant (NCI and DN) vs. clinically affected and unaffected brain regions	• DPR NCI widely distributed across brain • In PreCG, occasional GR DN observed and most of these co-localized with pTDP-43 • GR NCI more abundant in clinically affected vs. unaffected regions

Study	N per syndrome			Tissues/regions analyzed	DPR(s) studied	Study method	Major DPR-related findings
	FTD	MND	FTD-MND				
Sakae *et al.* [120]	13	13	14	**Cerebral cortex:** MFG, PreCG **Limbic/Subcortical:** Hipp-DG, CA4, CA2/3	GA, GP, GR	quant (NCI and DN) with IHC vs. clinical syndromes and ND	• More frontal GR NCI in FTD-MND vs. MND • More GR NCI in Hipp CA2/3 in FTD-MND vs. FTD • Frontal GR density correlated with ND

Abbreviations: aSTR: anterior striatum; ASF: antisense foci; APOE: Apolipoprotein E; Amyg: amygdala; AH: anterior horn of spinal cord; AAO: age at onset; Ctx: cortex; CNN: cranial nerve nuclei; CN: caudate nucleus; Calc: calcarine cortex; cing gyr: cingulate gyrus; DRN: dorsal raphe nucleus; DN: dentate nucleus of cerebellum; ERC: entorhinal cortex; FTLD: frontotemporal lobar degeneration; FTD: frontotemporal dementia; FuG: fusiform gyrus; GL: granular layer of cerebellum; GP: globus pallidus; GA: poly-GA; GP: poly-GP; GR: poly-GR; Hipp: hippocampus; Hipp-DG: dentate gyrus of hippocampus; Hipp-CA1: CA1 subfield of hippocampus; Hipp-CA2: CA2 subfield of hippocampus; Hipp-CA3: CA3 subfield of hippocampus; Hipp-CA4: CA4 subfield of hippocampus; HN: hypoglossal nucleus; ION: inferior olive nucleus; MFG: middle frontal gyrus; LC: locus caeruleus; ML: molecular layer of cerebellum; MND: motor neuron disease; N: number of subjects; NAcc: accumbens nucleus; ND: neurodegeneration; PUT: putamen; PreCG: precentral gyrus; PL: Purkinje layer of cerebellum; PA: poly-PA; PR: poly-PR; PH: posterior horn of spinal cord; quant: quantitative; semi-quant: semi quantitative; SF: sense foci; SFG: superior frontal gyrus; SxDur: symptom duration; SN: substantia nigra; STN: subthalamic nucleus; TDP-43: Transactive response DNA-binding protein of 43 kDa; TMEM106B: transmembrane protein 106B; and Thal: thalamus.

Table 3:

Systems anatomical principles to guide *C9orf72*-FTD/ALS neuropathological research

Relevant syndrome	Regions most often evaluated in human NP studies to date	Functional role of region	Involvement of region in syndrome	Recommended region*	Functional role of region	Involvement of region in syndrome
bvFTD	"Frontal cortex", usually middle frontal gyrus when specified	Executive	Variable, often late	ACC or AI, esp. Layer 5 VENs/ fork cells	Social-emotional	Universal, often early
bvFTD	Hippocampus, usually dentate gyrus or CA1	Memory	Variable	Amygdala, esp. central nucleus	Emotion	Universal, often early
bvFTD	"Cerebellum" usually granule or Purkinje cells from unspecified cerebellar lobule	Cannot determine w/o anatomical details	Variable, may be absent, usually late	Thalamus, esp. medial pulvinar nucleus **	Social-emotional, reward	Universal, may be earliest-affected region in C9-bvFTD
ALS	Primary motor cortex	Voluntary motor	Universal, often early	Primary motor cortex, esp. Layer 5 upper motor neurons	Voluntary motor	Universal, often early
ALS	Spinal cord anterior horn cells	Motor, muscle trophic support	Universal, often early	Spinal cord anterior horn cells or bulbar motor neurons	Motor, muscle trophic support	Universal, often early

* based on in vivo whole brain imaging, quantitative neuropathology, or established clinico-anatomical relationships.

** If medial pulvinar thalamus not available, ventral/anterior striatum makes a suitable alternative. Abbreviations: ACC: anterior cingulate cortex; AI: anterior insula; ALS: amyotrophic lateral sclerosis; bvFTD: behavioral variant frontotemporal dementia; NP: neuropathological; and VENs: von Economo neurons.