Dipeptide-repeat protein pathology in *C9ORF72* mutation cases: Clinico-pathological correlations

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Abstract

Hexanucleotide repeat expansion in *C9ORF72* is the most common genetic cause of frontotemporal dementia and motor neuron disease. Recently, unconventional non-ATG translation of the expanded hexanucleotide repeat, resulting in the production and aggregation of di-peptide repeat (DPR) proteins (poly-GA, -GR and GP), was identified as a potential pathomechanism of *C9ORF72* mutations. Besides accumulation of DPR proteins, the second neuropathological hallmark lesion in *C9ORF72* mutation cases is the accumulation of TDP-43.

In this study, we characterized novel monoclonal antibodies against poly-GA and performed a detailed analysis of the neuroanatomical distribution of DPR and TDP-43 pathology in a cohort of 35 cases with the *C9ORF72* mutation that included a broad spectrum of clinical phenotypes. We found the pattern of DPR pathology to be highly consistent among cases regardless of the phenotype with high DPR load in the cerebellum, all neocortical regions (frontal, motor cortex and occipital) and hippocampus, moderate pathology in subcortical areas and minimal pathology in lower motor neurons. No correlation between DPR pathology and the degree of neurodegeneration was observed, while a good association between TDP-43 pathology with clinical phenotype and degeneration in key anatomical regions was present. Our data confirm that the presence of DPR pathology is intimately related to *C9ORF72* mutations. The observed dissociation between DPR inclusion body load and neurodegeneration might suggest inclusion body formation as a potentially protective response to cope with soluble toxic DPR species. Moreover, our data imply that alterations due to the *C9ORF72* mutation resulting in TDP-43 accumulation and dys-metabolism as secondary downstream effects likely play a central role in the neurodegenerative process in *C9ORF72* pathogenesis.

Introduction

Frontotemporal dementia (FTD) and motor neuron disease (MND), with amyotrophic lateral sclerosis (ALS) as the most frequent form, are closely related clinical syndromes with overlapping molecular pathogenesis. Abnormal intracellular accumulation of the transactive response DNA binding protein with molecular weight 43 kD (TDP-43) is a characteristic neuropathological feature of the most common molecular subtype of FTD (frontotemporal lobar degeneration with TDP-43 pathology, FTLD-TDP) and the majority of ALS cases [10, 21, 29]. Recently, abnormal expansion of a GGGGCC hexanucleotide repeat in a non-coding region of the chromosome 9 open reading frame 72 gene (*C9ORF72*) was discovered to be the most common genetic abnormality in familial and sporadic FTD and MND, and the cause in most families where both, FTD and MND, are inherited in an autosomal dominant fashion [14, 17, 31]. The number of the hexanucleotide repeats in the normal population ranges from 2-24 [13, 14, 17, 31, 39], whereas up to several thousand repeats (range 700-4400 repeats) are described in the pathologically expanded allele as estimated by southern blot analysis [5, 13, 14]. The mechanism by which the mutation leads to neurodegeneration is uncertain; however, initial reports provided evidence for both loss-of-function and toxic-gain-of-function mechanisms, by demonstrating a reduction in *C9ORF72* mRNA levels and the presence of potentially toxic RNA foci, respectively [14, 17].

The neuropathology of cases carrying the *C9ORF72* mutation and of cases previously described as linked to chromosome 9 has consistently been reported to be characterized by TDP-43 immunoreactive inclusions in neurons and glia [7, 10, 12, 14, 17, 19, 23, 26, 33-35, 38]. Cases with clinical FTD are found to have FTLD-TDP which most often fits the type B pattern, although some cases having additional features of type A [19, 22, 26]. Cases with the mutation and clinical ALS show TDP-43 pathology indistinguishable from classical sporadic ALS, with TDP-43 inclusions in both upper and lower motor neurons [12, 35, 38]. The extent of pathological overlap among cases is even greater than the overlap in clinical features, with almost all *C9ORF72* mutation cases having at least some TDP-43 pathology in both the frontotemporal cortex and the pyramidal motor system, as well as a wide range of other neuroanatomical regions [19, 35].

In addition to TDP-43 pathology, a unique and consistent feature of cases with the *C9ORF72* mutation is the presence of inclusions that are immunoreactive for markers of the ubiquitin proteasome system (UPS; including ubiquitin, ubiquilins and p62), but negative for TDP-43, particularly in the cerebellar cortex, hippocampus and cerebral neocortex [1, 8, 12, 19, 23, 35, 38]. Recently, two independent studies demonstrated that this UPS-positive, TDP-43-negative pathology is the result of unconventional translation of the abnormally expanded GGGGCC repeat region [4, 24]. Translation of the sense transcript in the three alternate reading frames generates three different polypeptides, each composed of repeating units of two amino acids (dipeptide repeats, DPRs): glycine-alanine (GA), glycine-proline (GP) or glycine-arginine (GR). In each study, antibodies generated against these DPR proteins were shown to label TDP-43-negative inclusions in cases with the *C9ORF72* mutation but not in any control samples. A similar process of repeat associated non-ATG-initiated translation has been reported in other non-coding repeat expansion disorders, including spinocerebellar ataxia type 8, myotonic dystrophy type 1, and fragile X tremor ataxia syndrome, in disease-relevant tissues [11, 25, 36, 41], suggesting DPR production as another possible pathogenic mechanism of *C9ORF72* mutations.

In the present study, we generated novel monoclonal antibodies against poly-GA with the aim to characterize the neuroanatomical distribution and clinico-pathological association of DPR pathology in a large cohort of cases with the *C9ORF72* mutation that included a broad spectrum of clinical phenotypes.

Material and Methods

Generation of monoclonal anti-GA antibody

Since our previous study showed the most abundant staining of DPR pathology with a polyclonal antiserum against poly-GA [24], we decided to generate monoclonal antibodies against poly-GA for this study. 30 µg insoluble recombinant peptide with 10 GA repeats linked to polyethylene glycol (C-PEG-GA₁₀) were used to immunize CBL mice subcutaneously and intraperitoneally with a mixture of 5 nmol CpG 2006 oligonucleotide (Tib Molbiol, Berlin, Germany), 150 µl PBS and 150 µl incomplete Freund's adjuvance. After a six weeks interval, mice were boosted with 30 µg peptide in PBS. Hyperimmune spleen cells were fused with the

mouse myeloma cell line P3X63Ag8.653 using standard procedures. Supernatants were first screened in an enzyme-linked immunosorbent assay (ELISA) and selected supernatants tested by immunoblot and immunohistochemistry. The clone 5E9 of subclass IgG1 and clone 5F2 of subclass IgG2a were stably subcloned and used for further characterization.

Immunoblot

Specificity of mAb 5E9 and 5F2 was tested by SDS-PAGE and immunoblot. 250 ng of recombinant GST-GA₁₅, GST-GP₁₅, GST-GR₁₅, GST-AP₁₅ and GST-PR₁₅ proteins, generated as described [24] were separated by 12 % SDS-polyacrylamid gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Following transfer, membranes were blocked with 0.2% I-Block (Applied Biosystems) in TBS with 0.2% Triton X-100 for 1 h, incubated with primary monoclonal antibody 5E9 and 5F2 (dilution 1:50) overnight at 4 °C. Antibody binding was detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega) and signals were visualized by an HRP-based chemiluminescent reaction using the ECL reagents (GE healthcare) and exposed to X-ray films (SuperRX, Fujifilm).

Cases

35 cases with a *C9ORF72* mutation from the brain banks affiliated with the University of British Columbia, University of Munich, University of Tuebingen, and Amsterdam were included in the study. Consent for autopsy was obtained from the legal representative in accordance with local institutional review boards. Some of the cases had previously been included in the original study that identified *C9ORF72* as the disease gene [14], while the other cases were identified by routine genetic testing for *C9ORF72* repeat expansion of FTD and ALS cases in the respective brain banks using repeat-primed PCR as described [14, 31]. Clinical records from all cases were reviewed for age at onset, disease duration, family history, features of dementia, signs of MND and other neurologic-psychiatric symptoms.

Neurological control cases used to investigate specificity of anti-GA immunohistochemistry included FTLD-TDP with *GRN* mutations (n=2), sporadic FTLD-TDP (n=2), sporadic ALS-

TDP (n=4), FTLD-tau (n=2), Alzheimer's disease (n=2), Lewy body disease (n=2), FTLD-FUS (n=2), and normal control tissue from elderly patients with no history of neurological disease (n=2).

Histology and immunohistochemistry

Histological examination was performed on 5-8 μ m thick sections cut from formalin-fixed, paraffin embedded tissue of nine different CNS regions from *C9ORF72* mutation cases when available: primary motor cortex, frontal cortex, occipital cortex, striatum, hippocampus, midbrain, medulla, cerebellum, and spinal cord. Sections were stained with hematoxylin and eosin, and Luxol fast blue–periodic acid–Schiff, or used for immunohistochemistry.

In addition to the anti-poly-GA mAbs 5E9 and 5F2 used to demonstrate DPR pathology (tested dilution range for both 1:5-1:2000), other antibodies used in this study included rabbit polyclonal anti-TDP-43 (ProteinTech Group; dilution 1:2000), rat monoclonal anti-pTDP-43 clone 1D3 (own production [28], dilution 1:1000), polyclonal rabbit anti-ubiquitin (DAKO, dilution 1:500), and mouse monoclonal anti-p62 (Ick ligand, BD Biosciences, dilution 1:500).

Immunohistochemistry was performed using the Ventana BenchMark XT automated staining system (Ventana, Tuscon, AZ) with the AEC detection kit or iVIEW DAB detection kit, or the Leica BOND-MAX automated staining system (Leica) with Novocastra Bond Polymer Refine Detection Kit. For all antibodies heat-induced antigen retrieval with citrate buffer pH 6 was performed.

To ensure consistency of anti-poly-GA staining among different staining systems used at the three participating centers (Vancouver, Tubingen, Munich), selected sections were stained with different protocols showing comparable immunoreactivity. All sections and cases were stained with mAb 5E9, anti-p62 and anti-TDP-43. Selected cases were also stained with the mAb 5F2 which showed similar results to those with mAb 5E9.

Double-label immunofluorescence:

Double-label immunofluorescence was performed on selected cases using one of the anti-poly-GA antibodies and ubiquitin, p62, pTDP-43, NeuN (polyclonal, Millipore, dilution 1:200), GFAP (mouse monoclonal, DAKO, 1:400), Iba1 (rabbit polyclonal, Wako Chemicals, dilution

1:2000), and CNPase clone SMI91 (mouse monoclonal, Sternberger Monoclonals, dilution 1:500). The secondary antibodies were Alexa Fluor 594 or Alexa Fluor 488 conjugated anti-mouse (IgG (H+L), IgG1 and IgG2a) or anti-rabbit IgG (IgG (H+L); Invitrogen, 1:500) depending on the used primary antibodies. Hoechst 33342 was used for nuclear counterstaining. Sections were treated with Sudan black to reduce autofluorescence [32].

Semi-quantitative evaluation of pathology

The number of DPR, TDP-43, and p62-immunoreactive inclusions was scored in the different anatomical regions using a semiquantitative grading system, in which the overall number of immunoreactive inclusions (total score) as well as the number of neuronal cytoplasmic inclusions (NCI), neuronal intranuclear inclusions (NII) and dystrophic neurites (DN) were each rated as being absent (0), rare (1), occasional (2), moderate (3), or numerous (4). Inclusions were considered “rare” if only a few examples could be found in the entire region examined, “occasional” if they were relatively easy to find, but not present in every medium power microscopic field, “moderate” if at least a few examples were present in most microscopic fields, and “numerous” when many were present in every microscopic field.

Degeneration of brain regions was assessed on H&E and myelin-stained (corticospinal tract) sections and graded as absent (0), mild (1), moderate (2) and severe (3) based on the presence of spongiosis, neuronal loss, gliosis and demyelination in the cortical, subcortical, brainstem and spinal cord regions. For cerebellum sections were screened for cell loss in the granular cell layer, Purkinje cell loss, and thinning of the molecular layer.

Statistics

Statistical analysis was performed with the GraphPad Prism software (version 5.0).

Group comparison for age at onset and disease duration were analyzed by one-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* test. Nonparametric Kruskal–Wallis (ANOVA) followed by Dunn’s *post hoc* test or Mann-Whitney U test was used to assess differences in degeneration levels and inclusion scores among clinical groups for each anatomical region. Significance level was set at $p < 0.05$ (two-sided).

Correlation analysis was examined for age at onset, disease duration and levels of neurodegeneration with DPR and TDP-43 pathology in distinct brain regions using Spearman's rank correlation coefficient, with p values < 0.01 adjusted for multiple comparisons considered as significant.

Results:

Characterization of novel anti-GA monoclonal antibodies

Monoclonal antibodies were generated against aggregated synthetic peptide with 10 GA repeats. Hybridoma supernatants were pre-screened by ELISA and positive supernatants were tested by immunoblot. mAb 5E9 and 5F2 showed a selective and sensitive detection of recombinant GST-GA in immunoblot (Fig. 1a) and was used for further analysis.

Next, mAb 5E9 and 5F2 were tested by immunohistochemical analysis on cerebellum of selected cases to determine their applicability and sensitivity in this assay. Both antibodies robustly and specifically labeled pathologic inclusions in paraffin-embedded tissue of *C9ORF72* case including NCI, NII and DN (Fig 1b) using a wide dilution range from 1:5 – 1:500 with heat antigen retrieval being found to significantly enhance immunoreactivity. In contrast, no labeling was observed in the cerebellum of ALS-TDP and FTLD-TDP cases without a *C9ORF72* mutation (Fig. 1b) or in the characteristic pathological lesions of other neurodegenerative diseases such as Lewy bodies, neurofibrillary tangles and senile plaques (data not shown).

Double-labeling immunofluorescence of cerebellum and hippocampus of selected cases revealed a complete co-localization of poly-GA immunoreactivity with p62-positive inclusions (Fig. 1c and d). Furthermore, some NCI and DN were only labeled for poly-GA (Fig. 1c). Together, these data demonstrate the high sensitivity and specificity of the novel mAb 5E9 and 5F2 to detect *C9ORF72* mutation associated poly-GA DPR pathology.

Study Cohort

A total of 35 cases with a *C9ORF72* mutation were included in the study to investigate the neuroanatomical distribution pattern of DPR pathology in detail. Demographics and clinical features of the cases are summarized in Table 1. According to the available clinical

information, cases were grouped into those with a pure/predominant MND phenotype (MND group, n=8), those with a pure/predominant FTD phenotype (FTD group, n=9), and those with mixed FTD/MND (FTD/MND group, n=18). Significant differences were identified among the groups for disease onset (later onset in the MND group than in the FTD/MND and FTD group, $p=0.0262$, ANOVA, $p<0.05$, Newman-Keuls post hoc test) and for disease duration (longer disease duration in the FTD group than in the FTD/MND and MND group; $p=0.0165$, ANOVA, $p<0.05$, Newman-Keuls post hoc test).

Spectrum and distribution of DPR pathology in *C9ORF72* cases

Multiple CNS regions were analyzed for DPR pathology by immunohistochemistry with the mAb 5E9 and the presence and frequency of poly-GA-immunoreactive lesions (total amount as well as individual scores for NCI, NII and DN) was assessed semi-quantitatively for each region (Table 2).

Overall, mAb 5E9 staining was found to be robust and intense in all cases with no obvious influence of postmortem delay or duration of formalin fixation. A remarkably homogenous distribution pattern and amount of DPR pathology was observed in all *C9ORF72* mutation cases in the neocortical regions examined (including frontal, motor and occipital cortex), with moderate to numerous NCI in layers II-VI (Fig. 2a and b). The morphology of NCI varied from granular dot-like in small non-pyramidal neurons to ring and star-like inclusions in pyramidal neurons (Fig 2c and d). Sometimes neurons with diffuse cytoplasmic anti-GA staining (“pre-inclusions”) were observed (Fig. 2e). Interstitial white matter neurons were often found to contain NCI, while no inclusions in glial cells or axonal processes were seen in the white matter. NII and DN were a consistent but more variable finding. NII could be found in cells with and without NCI. DN were usually small and delicate (Fig. 2c) and large swollen neurites were rare.

In the hippocampus, moderate to numerous NCI and NII were detected in all cases in the dentate granule cells (Fig. 2f) and in the pyramidal layer, primarily the CA3/4 region (Fig. 2g and h). NCI in CA pyramidal neurons had a mainly star-like or ring-like appearance.

All *C9ORF72* cases showed abundant DPR pathology in the cerebellum. In addition to small granular, dot-like NCI and NII in the granule cells (Fig. 1b and 2j), NCI and NII were

consistently found in the molecular layer (Fig. 2i) and occasionally NCI were identified in Purkinje cells (Fig. 2j). A subset of cases revealed rare “pre-inclusions” with diffuse labeling of Purkinje cells (Fig. 2k) and neurons in the molecular layer. Neuritic pathology in the cerebellum was most abundant and consistent in the molecular layer (Fig. 2i) and more variable in the granular layer.

DPR pathology ranging from absent to abundant was observed in the striatum (Fig. 2l) and in the pigmented neurons of the substantia nigra pars compacta (Fig. 2m).

DPR pathology was absent or sparse in the hypoglossal motor nuclei and the anterior horn of the spinal cord, with a small number of cases in each clinical group showing single NCI (Fig. 2n). In addition, single NCI in the posterior horn of the spinal cord and in the cuneate nuclei were detected.

Many of the small dot like cytoplasmic inclusions in the cortical areas were observed in cells with small nuclei, which could either be small neurons or glial cells. To further address this, we performed double-label immunofluorescence for poly-GA and cell specific markers. Cytoplasmic and nuclear poly-GA inclusions were found in cells co-labeled with the neuronal marker NeuN, but there were no poly-GA positive inclusions in cells that labeled with the astrocyte marker GFAP, the microglial marker Iba1 or CNPase as oligodendroglial marker (Supplementary Fig. 1).

Relation of DPR and TDP-43 pathology

In order to investigate the relationship between DPR and TDP inclusion body formation we assessed TDP-43 pathology in each of the examined regions (Table 3). All cases with sufficient cortical pathology were classified as FTLD-TDP type B pathology (n=30), characterized by compact or diffuse granular NCI and some DN in superficial and deep cortical layers. A subset of cases (n=8) had additional features of FTLD-TDP type A with compact oval or crescentic NCI with abundant short DN, predominantly localized to layer II, and one had additional pathological features of type C with many long DN.

Double-label immunofluorescence for TDP-43 and poly-GA was performed in selected cases. Neurons in neocortical regions were often found to contain either TDP-43 or poly-GA inclusions (Fig. 3a). Notably, vulnerable neurons like Betz cells in cases with MND were

found to contain TDP-43 pathology, whereas DPR pathology was usually seen in small neurons (Fig. 3b). However, in the hippocampal dentate granule cells in cases with high TDP-43 load in this region, accumulation of both proteins in individual neurons was observed with poly-GA aggregates present in the center being surrounded by accumulated TDP-43 (Fig. 3c-e). No association between cytoplasmic TDP-43 accumulation and presence/absence of poly-GA NII pathology was observed and no poly-GA immunoreactivity was observed with oligodendroglial TDP-43 inclusions in the white matter.

Clinico-pathological correlations

To gain further insights into the pathomechanisms of the *C9ORF72* mutation we performed detailed statistical analysis to investigate the correlations among clinical phenotypes, regional neurodegeneration, DPR pathology and TDP-43 pathology.

First, we investigated group differences in the amount and anatomic distribution of pathologies among the three clinical groups. As shown in Table 2 and illustrated in the lesion profiles (Fig. 4) different patterns of neurodegeneration were present among the three clinical groups, as expected. Cases with clinically pure MND had significantly less degeneration in the frontal cortex compared to the FTD/MND ($p<0.05$, Dunn's post hoc test) and FTD groups ($p<0.001$, Dunn's post hoc test). In contrast, cases with clinically pure FTD had less degeneration in the anterior horn of the spinal cord compared to MND ($p<0.01$, Dunn's post hoc test) and FTD/MND cases ($p<0.05$, Dunn's post hoc test) and less degeneration of the hypoglossal nucleus compared to the FTD/MND group ($p<0.01$, Dunn's post hoc test). TDP-43 pathology profiles closely paralleled those of degeneration with significantly less TDP-43 pathology in the frontal cortex in the pure MND group than in the pure FTD group ($p<0.05$, Dunn's post hoc test) and more abundant TDP-43 pathology in the spinal cord in the MND group than in pure FTD cases ($p<0.05$, Dunn's post hoc test) (Table 3 and Fig. 4).

For DPR pathology, a strikingly similar pattern was observed among all cases with high scores in all neocortical regions, cerebellum and hippocampus, moderate numbers in striatum and midbrain and low scores in medulla and spinal cord (Table 2 and Fig. 4). As result, there were no significant differences among the three clinical groups in the amount of DPR pathology in any of the brain regions examined ($p>0.05$, Kruskal-Wallis ANOVA). Notably,

the highest levels of DPR pathology in the striatum and substantia nigra were observed in cases with additional clinical features of parkinsonism. A trend for higher levels of DPR pathology in these regions was observed among cases with extrapyramidal syndromes (Table 1, n=8 with brain regions available), however it did not reach statistical significance ($p=0.2371$ substantia nigra, $p=0.2365$ striatum, Mann-Whitney-U test).

We further investigated the relationship between the amount of DPR and TDP-43 pathology with the grade of neurodegeneration (Spearman's rank correlation coefficient, Table 4). There were strong positive correlations between the degree of degeneration and TDP-43 pathology in the frontal cortex ($\rho=0.62$, $p<0.0001$), primary motor cortex ($\rho=0.70$, $p<0.001$), spinal cord ($\rho=0.74$, $p<0.0001$) and hypoglossal nucleus ($\rho=0.75$, $p<0.0001$) and a moderate positive correlation in the substantia nigra ($\rho=0.58$, $p<0.001$). Moreover, a strong negative association was seen between the disease duration and the severity of TDP-43 pathology in the spinal cord ($\rho=-0.70$, $p<0.0001$) and hypoglossal nucleus ($\rho=-0.58$, $p<0.0005$).

No significant correlations were identified between the severity of DPR pathology and the degree of neurodegeneration in any of the examined regions (**Table 4**), the age at onset and disease duration.

Discussion

An abnormal hexanucleotide expansion in *C9ORF72* is the most common genetic cause of MND and FTD. However, the mechanisms of how this intronic mutation contributes to neurodegeneration is unclear, with three pathogenic mechanism proposed: loss of *C9ORF72* function, toxic-RNA gain of function and toxicity due to unconventional non-ATG translation products [6, 13, 30].

Here, we describe for the first time a detailed clinico-pathological correlative study of DPR and TDP-43 pathology in 35 *C9ORF72* mutation cases that provide new insights into the potential roles and links of DPR and TDP-43 aggregation in *C9ORF72* pathogenesis.

The novel anti-GA antibodies 5E9 and 5F2 generated in this study were characterized as powerful tools for sensitive and specific detection of DPR pathology. This was demonstrated by the robust and highly specific labeling of inclusions in *C9ORF72*

mutation cases, the absent immunoreactivity in healthy and neurologic controls, in line with previous studies using polyclonal antibodies [4, 24] and the high sensitivity of these antibodies to detect the TDP-43-negative, UPS-positive pathology characteristic for *C9ORF72* mutation cases [1, 8, 12, 19, 23, 35, 38] as demonstrated by double-labeling experiments showing complete co-localization with p62 immunoreactivity in selected anatomical regions, such as the cerebellum and CA3/4 region.

The demographics and clinical phenotypes of our studied cohort of 35 *C9ORF72* cases were representative of the wide clinical spectrum previously associated with the *C9ORF72* mutation [7, 9, 12, 15, 19, 23, 26, 33-35, 40]. About 50 % of our cases presented with features of mixed FTD and MND and about 25% with predominant FTD, or MND, respectively. Other clinical features that have been described in some patients with the *C9ORF72* mutation, such as extrapyramidal symptoms [7, 12, 15, 19, 26, 33] and psychiatric features [2, 7, 15, 16, 19, 33, 34], were also represented in our cohort.

The predominant types of DPR pathology were cytoplasmic and intranuclear inclusions restricted to neurons, as confirmed by double labeling experiments with NeuN as a specific neuronal marker. No inclusions were detected in cells labeled with an astrocyte specific marker, consistent with previous reports [4] and moreover inclusions were also not found in microglia and oligodendrocytes. The morphology, amount and anatomical distribution of DPR pathology was found to be strikingly homogenous among all cases with high numbers in the cerebellum, hippocampus and throughout the cerebral neocortex, more variable in the striatum and substantia nigra and rare or absent in the medulla and spinal cord, consistent with the distribution pattern described using anti-GP antibodies [4].

Aggregation of proteins into characteristic lesions and inclusion bodies is a common theme in neurodegenerative diseases; however, the actual pathomechanistic function of inclusion bodies is a hot topic that has been debated in the field of neurodegenerative diseases for decades [20, 37]. Based on the observations that there is sometimes only poor correlation between the neuropathological hallmark lesions seen with neurodegeneration or phenotype, with the most prominent examples perhaps being the rather poor association of amyloid beta load with cognitive impairments [27] and intranuclear inclusions in Huntington's disease [18], there is increasing evidence that insoluble aggregates *per se* might not be the

primary neurotoxic agents and that soluble precursor species to mature aggregates might be the causative pathogenic species [3, 20, 37]. In line with this, inclusion body formation might instead serve as a protective function of the cell to dispose the neurotoxic species.

Our data do not support a direct causative role for aggregated DPR proteins in neurotoxicity, as evidenced by the poor correlation between the anatomical distribution of DPR inclusion bodies and neurodegeneration (e.g. low DPR load in vulnerable motor neurons and high load in occipital cortex and cerebellum, regions usually not affected). However, our data are consistent with a model where soluble precursors to aggregated DPR proteins play a role in neurotoxicity, while the formation of visible DPR inclusion bodies is possibly protective. While studies such as this one using post-mortem tissue from patients with end stage disease can provide important insights into potentially relevant disease mechanisms, the further elucidation of probably neurotoxic soluble DPR species can only be addressed by *in vitro* and *in vivo* experimental studies in *C9ORF72* cell culture and animal models.

Another interpretation of our finding of a highly consistent distribution pattern of DPR pathology is that this might reflect the physiological pattern of *C9ORF72* expression. In support of this, initial studies [14, 31] demonstrated RNA levels of *C9ORF72* to be highest in cerebellum, a region heavily affected by DPR pathology. However, a detailed analysis of *C9ORF72* expression including analysis of the relative expression of the various *C9ORF72* transcripts, ideally on the cellular level, will be needed to further address this.

The other characteristic hallmark lesion in *C9ORF72* mutation cases is TDP-43 accumulation [7, 10, 12, 14, 17, 19, 23, 26, 33-35, 38] with so far one notable exception of a Belgian FTD patient who carried the *C9ORF72* mutation but lacked detectable TDP-43 pathology [24]. All *C9ORF72* mutation cases in our cohort of 35 cases showed TDP-43 immunoreactive inclusions in a range of neuroanatomical regions consistent with previous reports. There were significant differences among the clinical groups, with MND cases showing more TDP-43 pathology in lower motor neurons than cases with pure FTD (unfortunately, primary motor cortex was not available in FTD cases) and, patients with predominant FTD showing more TDP-43 pathology in the frontal cortex compared to the other groups. Together with the strong positive correlations between the amount of TDP-43 pathology and the degree of degeneration found in key anatomical regions such as frontal

cortex, motor cortex and spinal cord, our data imply that alterations caused by the *C9ORF72* mutation result in TDP-43 dys-metabolism and accumulation as a secondary downstream effect and that this likely plays a central role in the neurodegenerative process. The link between *C9ORF72* mutation and TDP-43 is so far completely unknown. Although the presence of DPR and TDP-43 co-accumulation in the same neurons was a rare event, the composition of these combined inclusions with a central core consisting of DPR proteins being surrounded by TDP-43 suggests that DPR pathology precedes TDP-43 accumulation and might therefore trigger TDP-43 accumulation, at least in a subset of neurons. Future studies using cellular and animals models are needed to further elucidate the link between DPR and TDP-43 aggregation, as well as other effects of the *C9ORF72* repeat expansion (such as reduced production of specific *C9ORF72* isoforms, the formation of RNA foci and the GGGGCC repeat length) on TDP-43 metabolism. Moreover, the identification of additional *C9ORF72* mutation cases with pure DPR pathology would provide import insights as to the role and relevance of DPR and TDP-43 pathology in disease pathogenesis.

In summary, our data confirm the presence of DPR pathology as a highly specific and sensitive marker for the *C9ORF72* repeat expansion mutation and extend the description of the morphology and anatomical distribution of DPR inclusions. Novel DPR antibodies such as ours will be powerful tools in the routine diagnosis of neurodegenerative diseases, in the evaluation of emerging *C9ORF72* cellular and animal models to dissect the complex mechanisms of the *C9ORF72* mutation and potential links between DPR proteins, RNA toxicity, *C9ORF72* haploinsufficiency and TDP-43 accumulation and possibly in the development of *in vivo* diagnostic tests.

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Figure 1:**Characterization of novel anti-poly-GA monoclonal antibodies mAb 5E9 and 5F2**

a Immunoblot (IB) analysis of recombinant GST and 15-mer GA, GP, GR, AP and PR fused to GST with the indicated primary antibodies, demonstrating a specific signal for GA with mAb 5E9 and 5F2.

b Immunohistochemistry of cerebellum from *C9ORF72* mutation case (C9+) showing strong immunoreactivity of neuronal cytoplasmic inclusions, neuronal intranuclear inclusions and dystrophic neurites with mAb 5E9 and 5F2 in the granule cell layer. In contrast, no staining is observed in FTLD-TDP cases without a *C9ORF72* mutation (C9-). Scale bar: 20 μ m.

c and **d** Double label immunofluorescence for p62 (green) and poly-GA (mAb 5F2, red) showing co-localization in the characteristic neuronal cytoplasmic and intranuclear inclusions in the granule cells in the cerebellum (**c**) and the pyramidal neurons in the CA4 sector of the hippocampus (**d**) of a *C9ORF72* mutation case. Note that some inclusions are only poly-GA positive (arrows in **c**). Nuclei stained with Hoechst (blue) in merged images. Scale bar: 50 μ m in **c** and 20 μ m in **d**.

Figure 2:**Spectrum of DPR pathology in *C9ORF72* cases**

Numerous poly-GA immunoreactive inclusions are present in all cortical layers, with accentuation in layer IV, irrespective of their vulnerability in MND and FTD, as shown here in the frontal cortex (**a**) and the occipital cortex (**b**). Higher magnifications demonstrate the numerous neuronal cytoplasmic inclusions (NCI) and few dystrophic neurites (DN) in the cerebral cortex (**c** and **d**). In addition to more compact NCI with a granular, star-like morphology (**d** and **e**) occasionally cortical neurons show diffuse staining of the perikaryal cytoplasm ("pre-inclusions") (**e**). Note the neuronal intranuclear inclusion (NII) in the cell with the compact NCI (**e**). Numerous NCI and NII are visible in the dentate granule cells (**f**) and the CA3/4 region of the hippocampus (**g** and **h**). Note the presence of NII, which can be found in cells with and without NCI (**h**). In the cerebellar molecular layer, abundant neuritic pathology is seen in addition to NCI (**i**). Occasionally, NCI are detectable in Purkinje cells (**j**), which rarely show a diffuse pre-inclusion staining (**k**). Inclusions in the striatum (**l**) and

pigmented neurons of the substantia nigra (**m**, arrow) are less abundant. NCI in motor neurons of the spinal cord and hypoglossal nucleus are rare and usually not more than a single inclusion in an entire section (**n**). Immunohistochemistry with mAb 5E9, developed with DAB. *Scale bar*: 125 μm (**a** and **b**), 100 μm (**g**), 50 μm (**c**), 25 μm (**d,f,h-j,l**), 12.5 μm (**e,k,m,n**).

Figure 3:

Double label immunofluorescence for poly-GA and TDP-43

Double label immunofluorescence for phosphorylated TDP-43 (green) and poly-GA (mAb 5E7, red) with nuclei stained with Hoechst (blue). **a** Numerous poly-GA and TDP-43 positive inclusions in the frontal cortex of a *C9ORF72* mutation case with clinical FTD with the majority of inclusions showing either TDP-43 or poly-GA immunoreactivity. Very rare neurons show co-accumulation of TDP-43 and poly-GA (arrow). **b** In the primary motor cortex of a *C9ORF72* mutation case with MND, TDP-43 pathology is observed in Betz cells, whereas DPR pathology is usually seen in smaller neurons. **c** Cases with numerous poly-GA and TDP-43 positive neuronal cytoplasmic inclusions in the dentate granule neurons show inclusions composed of either poly-GA or TDP-43 as well as combined inclusions within the same cells. When both proteins co-accumulate within the same cell, poly-GA is found in the center being surrounded by TDP-43 aggregates as shown in higher magnification in a dentate granule cell (**d**) and neuron in the CA4 region of the hippocampus (**e**). *Scale bar*: 50 μm (**a** and **c**), 25 μm (**b**) and 15 μm (**d** and **e**).

Figure 4

Lesion profiles of *C9ORF72* cases grouped by clinical phenotypes.

The graphs illustrate the severity of degeneration (red), as well as severity of total TDP-43 (blue) and DPR pathology (green) in the CNS regions examined based on the semi-quantitative assessment shown in Tables 2 and 3. Note the close correspondence in the patterns of TDP-43 pathology and neurodegeneration which vary according to the clinical phenotype (more frontal cortical involvement in cases with FTD and more hypoglossal nucleus and spinal cord involvement in cases with clinical MND). In contrast, DPR pathology

shows the same pattern in all disease groups with no correlation with the pattern of degeneration. CE cerebellum; FCtx, frontal cortex; MCtx, primary motor cortex; OCtx, occipital cortex; SC, anterior horn of spinal cord; SN, substantia nigra; STR, anterior striatum; XII, motor nucleus of hypoglossal nerve.

Supplementary Figure 1:

DPR inclusions are restricted to neurons

Double labeling immunofluorescence with cell type specific markers in green, poly-GA in red and merged images with Hoechst nuclei staining in blue. Poly-GA-positive cytoplasmic inclusions are localized to NeuN-positive neurons as shown in the frontal cortex of a *C9ORF72* mutation case (**a**). Poly-GA-positive inclusions are not found in GFAP-positive astrocytes (**b**), Iba1-positive microglia cells (**c**) or in CNPase-positive oligodendrocytes (**d**).

Scale bar 25 µm.

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Figure 1:

a

b

Figure 2

Figure 3

Figure 4:

Table 1: Summary of demographic and clinical data

Case No	FH	Age at onset [years] ^a	disease duration [years] ^b	Sex	Dementia	MND	EPS	Other clinical features
1	yes	74	2	F	-	ALS (B)	-	
2	no	57	2	F	-	ALS (L)	-	
3	yes	68	2	F	-	ALS (L)	-	
4	yes	60	1	M	-	PLS (L)	parkinsonism (PSP)	
5	yes	59	3	M	-	ALS (L)	-	
6	yes	63	3	F	-	ALS (B)	-	
7	yes	65	3	M	-	ALS (L)	-	
8	yes	60	3	F	-	ALS (B)	-	
MND group[#] N=8	87.5%	63.3± 5.6	2.4± 0.7	F:M 5:3				
9	yes	58	4	M	bvFTD	ALS (L)	tremor/ unsteady gait	
10	yes	66	5	M	bvFTD	dysarthria, dysphagia		
11	yes	67	9	M	PNFA	fasciculations		incontinence
12	yes	52	3	M	bvFTD/PNFA	ALS (B)		
13	yes	55	1	M	PNFA	ALS (L)		
14	yes	39	2	F	PNFA	ALS (L)	parkinsonism (CBS)	
15	yes	46	1	M	(bvFTD)	ALS (L)		
16	yes	56	3	F	bvFTD/PNFA	ALS (B)		
17	yes	53	3	M	bvFTD	ALS (B)		
18	yes	52	10	M	PNFA	hyperreflexia, choking	parkinsonism	
19	yes	35	8	F	bvFTD	dysphagia, spasticity	parkinsonism	ataxia, dystonia, incontinence
20	yes	72	3	M	bvFTD	ALS (L)		
21	yes	39	3	F	bvFTD	ALS (B)		
22	yes	58	3	F	bvFTD	weakness		depression, hallucinations, fluctuations,
23	no	46	0.4	F	(bvFTD)	ALS (L)		
24	no	60	5	M	(bvFTD)/PNFA	tetraparesis, dysphagia	parkinsonism (CBS)	
25	no	58	0.4	F	(bvFTD)	ALS (B)		
26	no	na	na	F	bvFTD	ALS (L)		
FTD/MND group[#] N=18	77.7%	53.7± 10.2	3.8± 2.9	F:M 8:10				
27	yes	62	7	M	bvFTD	-		
28	yes	55	18	M	bvFTD	-		
29	yes	58	8	M	bvFTD	-		
30	na	na	na	M	bvFTD	-	parkinsonism	
31	yes	53	3	M	bvFTD	-		
32	yes	49	2	F	bvFTD	-	gaze palsy	
33	no	47	11	M	bvFTD	-	mild rigor	hallucinations, schizophrenia
34	yes	35	6	M	bvFTD	-		
35	no	55	3	M	bvFTD	-		
FTD group[#] N=9	66.6%	51.7± 8.3	7.3± 5.3	F:M 1:8				

[#] Data for age at onset and disease duration are the mean ± standard deviation.

Statistical analysis for age at onset and disease duration: one-way ANOVA followed by Newman-Keuls post hoc test. ^a $p=0.0262$, $p<0.05$ MND versus FTD/MND and FTD; ^b $p=0.0165$, $p<0.05$ FTD versus FTD/MND and MND.

ALS, amyotrophic lateral sclerosis; B, bulbar onset; bvFTD, behavioural variant of frontotemporal dementia; (bvFTD), mild features of bvFTD; CBS, corticobasal syndrome; EPS, extrapyramidal symptoms; F, female; FH, family history; L, limb onset; M, male; MND, motor neuron disease; na, not available; PLS, primary lateral sclerosis; PNFA, progressive non-fluent aphasia; PSP, progressive supranuclear palsy;

Table 2: Semi-quantitative assessment of neurodegeneration and DPR pathology

Case No		Degeneration										poly-GA immunoreactive inclusions																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
												MCTx					FCTx				OCTx				HC DG				HC CA3/4				STR				SN				XII			SC			CE GL				CE PCL			CE ML																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
MND		MCTx ^a	FCTx	OCTx	STR	SN	XII ^b	SC ^c	CE	Total				NCI				NII				DN				Total				NCI				NII				DN				Total				NCI				NII				DN																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							

Highlighted columns depict those regions with significant differences among groups (Kruskal-Wallis ANOVA followed by Dunn's post hoc test). ^a $p=0.0013$; $p<0.001$ MND versus FTD and $p<0.05$

MND versus FTD/MND; ^b $p=0.0084$; $p<0.01$ FTD/MND versus FTD; ^c $p=0.0037$; $p<0.01$ MND versus FTD and $p<0.05$ FTD/MND versus FTD.

CE GL, cerebellar granular layer; CE ML cerebellar molecular layer, CE PCL, cerebellar Purkinje cell layer; DN, dystrophic neurites; FCtx, frontal cortex; FTD, frontotemporal dementia GL, granular layer; HC DG, hippocampus dentate gyrus; HC CA3/4, hippocampus CA3/4 region; MCtx, primary motor cortex; MND, motor neuron disease; NCI, neuronal cytoplasmic inclusions; NII, neuronal intranuclear inclusions; OCtx, occipital cortex; SC, anterior horn of spinal cord; SN, substantia nigra; STR, anterior striatum; XII, motor nucleus of hypoglossal nerve; -, not available;

Table 3: Semi-quantitative assessment of TDP-43 pathology

Case No		TDP-43 immunoreactive inclusions																																				
		MCtx				FCtx				OCtx				HC DG			HC CA3/4				STR				SN			XII				SC				CE		
		Total	NCI	NII	DN	Total ^a	NCI ^b	NII	DN	Total	NCI	NII	DN	Total	NCI	NII	DN	Total	NCI	NII	DN	Total	NCI	NII	DN	Total	NCI	NII	DN ^c	Total	NCI	NII	DN	Total	NCI ^d	NII	DN	Total
MND	FTLD-TDP subtype																																					
1	B	2	2	0	1	2	2	0	2	0	0	0	0	3	3	1	0	0	0	0	0	4	3	1	3	2	2	0	0	4	4	0	2	4	4	0	2	0
2	n.d.	2	1	0	1	1	0	0	1	-	-	-	-	0	0	0	0	0	0	0	1	1	0	1	2	2	0	1	4	3	0	2	4	4	0	2	0	
3	B	3	2	0	2	4	3	0	2	-	-	-	-	4	4	0	1	1	0	0	2	2	0	1	3	3	0	0	3	3	0	1	4	4	0	2	0	
4	B	4	2	0	4	4	2	0	3	-	-	-	-	4	4	0	1	1	0	1	3	2	0	2	1	1	0	1	0	0	0	1	1	1	0	1	0	
5	B	2	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	0	0	0	0	2	2	0	0	3	3	0	1	-
6	B	3	3	0	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	0	0	2	2	0	1	0	
7	n.d.	2	1	0	2	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	-	-	-	-	-	-	-	4	4	0	2	4	4	0	2	0	
8	n.d	3	3	0	2	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	3	3	0	0	0
mean		2.6	2.0	0.0	1.8	1.9	1.3	0.1	1.4	0.0	0.0	0.0	0.0	1.9	1.9	0.1	0.3	0.3	0.0	0.1	2.0	1.6	0.2	1.4	1.3	1.3	0.0	0.3	2.5	2.3	0.0	1.1	3.1	3.1	0.0	1.4	0.0	
FTD/MND																																						
9	A+B	0	0	0	0	3	2	0	3	-	-	-	-	1	1	1	0	0	0	0	3	3	0	2	2	2	0	1	4	4	0	2	3	3	0	2	0	
10	B+C	-	-	-	-	4	3	0	4	-	-	-	-	3	3	1	0	0	0	0	2	2	0	2	3	2	0	2	3	2	0	2	-	-	-	-	0	
11	n.d.	-	-	-	-	1	1	0	1	-	-	-	-	4	4	0	0	0	0	0	1	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	
12	B	-	-	-	-	3	3	0	2	-	-	-	-	1	1	0	0	0	0	0	4	2	0	4	2	2	0	1	3	3	0	1	4	4	0	1	0	
13	B	-	-	-	-	2	2	0	1	-	-	-	-	4	4	0	0	0	0	0	3	3	0	1	3	3	0	0	3	3	0	0	3	3	0	1	-	
14	B	4	3	0	4	3	3	0	2	0	0	0	0	4	4	0	0	0	0	0	4	3	0	2	3	3	0	1	3	3	0	1	3	3	0	2	0	
15	B	1	1	0	0	2	2	1	1	-	-	-	-	1	1	0	0	0	0	0	3	2	1	2	2	2	0	2	4	4	0	2	4	4	0	2	0	
16	B	2	1	0	2	4	3	0	2	0	0	0	0	3	3	0	0	0	0	0	3	3	0	2	3	3	1	1	3	3	0	1	3	3	0	1	0	
17	B	-	-	-	-	3	3	1	2	-	-	-	-	4	4	0	0	0	0	0	4	3	0	2	2	2	0	1	3	3	0	1	2	2	0	0	0	
18	A+B	-	-	-	-	4	3	1	4	-	-	-	-	4	4	0	0	0	0	0	4	4	1	4	3	3	1	2	1	0	0	1	1	1	0	1	0	
19	A+B	-	-	-	-	4	4	1	4	1	1	0	1	4	4	0	1	0	0	1	4	4	1	4	3	2	0	2	1	1	0	1	1	0	0	1	0	
20	B	2	2	0	0	1	1	0	0	0	0	0	0	3	3	0	2	2	0	0	-	-	-	-	-	-	-	-	3	3	0	1	-	-	-	-	0	
21	B	2	2	0	0	3	3	0	3	0	0	0	0	3	3	0	2	2	0	0	-	-	-	-	-	-	-	-	3	3	0	1	3	3	0	1	0	
22	B	3	3	0	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	1	1	0	0	3	3	0	0	3	3	0	0	0	
23	n.d.	3	3	0	3	1	1	0	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0	1	2	2	0	1	4	4	0	2	4	4	0	3	0	
24	B	3	2	1	3	3	2	0	3	1	0	1	0	2	2	1	2	1	0	2	3	3	0	3	3	2	0	3	0	0	0	0	1	0	0	1	0	
25	A+B	3	3	0	2	2	2	0	2	1	1	0	1	3	3	1	3	0	0	3	3	2	0	3	3	2	0	3	3	3	0	2	3	3	0	0	0	
26	B	2	2	0	2	3	3	0	2	1	1	0	1	3	3	0	1	1	0	0	4	4	0	4	3	3	0	2	4	4	0	2	3	3	0	2	0	
mean		2.3	2.0	0.1	1.5	2.6	2.3	0.2	2.0	0.5	0.4	0.1	0.3	2.7	2.7	0.2	0.6	0.3	0.0	0.3	3.0	2.5	0.2	2.3	2.4	2.1	0.1	1.4	2.7	2.5	0.0	1.1	2.6	2.4	0.0	1.1	0.0	
FTD																																						
27	A+B	-	-	-	-	4	3	1	4	0	0	0	0	4	4	0	1	1	1	1	4	4	2	4	3	2	0	2	1	1	0	1	1	0	0	1	0	
28	A+B	-	-	-	-	3	2	1	2	-	-	-	-	2	2	1	1	1	1	1	4	3	1	4	2	2	0	2	0	0	0	1	1	0	0	1	0	
29	B	-	-	-	-	3	3	0	2	-	-	-	-	4	4	0	0	0	0	0	1	1	0	1	2	2	0	1	2	2	0	1	2	2	0	1	0	
30	A+B	-	-	-	-	4	2	1	3	-	-	-	-	4	4	0	2	0	0	2	-	-	-	-	-	-	-	-	1	1	0	1	-	-	-	-	-	
31	B	-	-	-	-	3	3	0	1	-	-	-	-	4	4	0	0	0	0	0	2	2	0	1	3	2	0	2	3	3	0	1	3	3	0	0	0	
32	B	-	-	-	-	4	3	0	2	-	-	-	-	4	4	0	1	1	0	0	4	3	0	2	4	3	2	2	3	3	0	1	3	3	0	1	0	
33	A+B	0	0	0	0	4	3	2	2	0	0	0	0	3	3	0	1	1	0	0	3	3	1	2	2	2	0	2	0	0	0	0	0	0	0	0	0	
34	B	-	-	-	-	4	4	0	3	1	1	0	1	4	4	0	3	2	0	3	4	3	0	3	2	2	0	2	3	3	0	2	2	2	0	0	0	
35	B	-	-	-	-	3	2	0	3	0	0	0	0	3	3	0	3	0	0	3	3	3	0	3	3	3	0	2	-	-	-	-	-	-	-	-	0	
mean		/	/	/	/	3.6	2.8	0.6	2.4	0.3	0.3	0.0	0.3	3.6	3.6	0.1	1.3	0.7	0.2	1.1	3.1	2.8	0.5	2.5	2.6	2.3	0.3	1.9	1.6	1.6	0.0	1.0	1.7	1.4	0.0	0.6	0.0	

Highlighted columns depict those regions with significant differences among groups (Kruskal-Wallis ANOVA followed by Dunn's post hoc test).

^a $p=0.0328$, $p<0.05$ MND versus FTD; ^b $p=0.0233$, $p<0.05$ MND versus FTD/MND; $p<0.01$ MND versus FTD; ^c $p=0.0035$, $p<0.05$ MND versus FTD/MND; $p<0.01$ MND versus FTD; ^d $p=0.0483$, $p<0.05$ MND versus FTD. CE, cerebellum; DN, dystrophic neurites; FCtx, frontal cortex; FTD, frontotemporal dementia GL, granular layer; HC DG, hippocampus dentate gyrus; HC CA3/4, hippocampus CA3/4 region; MCtx, primary motor cortex; MND, motor neuron disease; NCI, neuronal cytoplasmic inclusions; NII, neuronal intranuclear inclusions; OCtx, occipital cortex; SC, anterior horn of spinal cord; SN, substantia nigra; STR, anterior striatum; XII, motor nucleus of hypoglossal nerve; -, not available; n.d. not determined if amount of cortical TDP-43 pathology was too mild for subtyping.

Table 4: Correlation of neurodegeneration with DPR and TDP-43 pathology

Degeneration	TDP pathology		DPR pathology	
	<i>rho</i>	[99% CI]	<i>rho</i>	[99% CI]
motor cortex	0.70*	[0.22-0.91]	0.12	[-0.48-0.64]
frontal cortex	0.62**	[0.24-0.83]	0.34	[-0.12-0.68]
occipital cortex	0.34	[-0.32-0.78]	0.27	[-0.72-0.43]
anterior striatum	0.43	[-0.06-0.75]	0.35	[-0.16-0.71]
substantia nigra	0.58*	[0.15-0.83]	-0.12	[-0.56-0.37]
hypoglossal nucleus	0.75**	[0.46-0.90]	0.03	[-0.42-0.47]
spinal cord	0.74**	[0.42-0.90]	0.03	[-0.44-0.48]
cerebellum				
granule cell layer	NA	NA	-0.31	[-0.67-0.17]
Purkinje cell layer	NA	NA	0.27	[-0.21-0.65]
molecular layer	NA	NA	-0.44	[-0.75-0.02]
Spearman's rank correlation coefficient <i>rho</i> , * <i>p</i> <0.001, ** <i>p</i> <0.0001 CI, confidence interval, NA, not applicable;				