

A 30-unit hexanucleotide repeat expansion in *C9orf72* induces pathological lesions with dipeptide-repeat proteins and RNA foci, but not TDP-43 inclusions and clinical disease

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Hexanucleotide repeat expansions (G_4C_2) in *C9orf72* (chromosome 9 open reading frame 72) are the most prevalent genetic cause of frontotemporal dementia and amyotrophic lateral sclerosis [4,8]. The normal repeat length is between two and ten units in 90% of the population [8] while expansions associated with disease, consist of several hundred or thousands units [2,4]. Expansions of between twenty and several hundred units are found in disease and healthy individuals, but the size of the smallest expansion unit that confers disease risk is unknown [2,10]. In disease, neuronal aggregates are formed, composed of i) sense and anti-sense RNA foci ii) dipeptide repeat proteins (DPRs), generated by an unconventional mechanism termed repeat-associated non-ATG (RAN) translation and separate TDP43 inclusions [9]. The minimum number of repeats required to generate pathology is unknown, but has important implications for understanding disease risk and pathogenesis.

We screened *C9orf72* repeat length from the brains of 86 cognitively normal cases from the Queen Square Brain Bank. Case 1 demonstrated an expanded pattern up to a 40-unit G_4C_2 while two control cases (cases 2 and 3, aged 75 and 86 respectively) demonstrated a 20-unit G_4C_2 (**Figure 1**). A sizing PCR of case 1 showed a range of 20-40 repeats with the most abundant peak at 30 repeats. The absence of a repeat larger than 40 units in case 1 was confirmed by Southern blotting (**figure 1**) [6].

Case 1 was cognitively normal with a history of poliomyelitis as a child leaving her with leg weakness. Her mobility was also affected by pain attributed to orthopaedic problems. She lived alone until she died aged 84 without any cognitive decline.

No neuropathological abnormalities were found in cases 2 and 3. Macroscopically case 1 appeared normal (brain weight 1360g). Microscopically there were 'age-related' changes with neurofibrillary tangle (Braak and Braak stage II) and $A\beta$ plaque pathologies (Thal phase 1). The hemispheric white matter showed several foci of recent ischaemic damage. No TDP43-positive lesions were present. There were sparse p62-positive inclusions containing all five DPRs, when compared with frontotemporal dementia cases with a large *C9orf72* repeat expansion (supplementary information). The DPRs were present in the hippocampus, cerebellum, frontal and temporal cortices (**figure 1**), but not observed in any other brain regions. Sense and anti-sense RNA foci were evident in the frontal cortex (**figure 1**) but were absent in the hippocampal formation and cerebellum. DPR inclusions and RNA foci were absent in both cases with a 20-unit G_4C_2 repeat, suggesting a critical unit repeat length, which may be required to initiate the formation of such abnormalities.

The mechanism of neurodegeneration in *C9orf72* expansion cases is unclear. The presence of motor dysfunction, defects in endocytosis and autophagy or axonopathy in different models [3,5]

indicates that loss of *C9orf72* function may play a role. Gain of function toxicity by DPRs and/or RNA foci is another possible mechanism; both DPRs and RNA foci were present in our case with a 30-unit G_4C_2 . All five DPRs being present in our case 1 suggests a threshold DPR length is required for toxicity. Pathological studies have also suggested the DPR inclusions may precede the TDP43 pathology [1,7]. However, the cases reported in these studies contained large *C9orf72* expansion repeats and were younger than case 1. Unlike in some other neurodegenerative diseases, at present no data support the notion that the DPR inclusions observed in our case 1 would be an 'age-related' phenomenon. Published observations together with those from our case 1 suggest that the formation of DPR inclusions may be the earliest event in *C9orf72* repeat expansion-related diseases [1,7]. It has been shown that wild-type *C9orf72* alleles (<20-unit G_4C_2) are stable between generations, whilst, small expansions (20–150-unit G_4C_2) are susceptible to unfaithful inheritance [2] or somatic instability [11]. A recent study reported a paternal ~70-unit G_4C_2 in *C9orf72* expanded and passed a large G_4C_2 expansion (~1,750-unit G_4C_2) on to four children [12], underpinning the significance of small expansions within the normal population, which could potentially be unstable and lead to larger expansions in future generations.

Our data show that *C9orf72* disease-specific DPRs produced from a 30-unit G_4C_2 formed characteristic p62-positive inclusions in a cognitively normal case without progressing to TDP43 pathology, indicating that the 30-unit G_4C_2 may not be long enough to trigger the entire disease cascade and that longer expansion repeats may be necessary to initiate downstream cellular events ultimately leading to TDP43 pathology, neurodegeneration and clinical disease.

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Figure Legend

Figure 1a-c: Repeat-primed PCR (rp-PCR) from cases 1-3: *C9orf72* rp-PCR carried out on DNA extracted from the cerebellum. Case 1 (a) demonstrating approximately 38-40 expansion repeats whilst cases 2 (b) and 3 (c) showed around 20 repeats. ***C9orf72* sizing PCR (d and e):** A normal control (d) with 2 and 8 repeats whilst Case 1 (e) demonstrated approximately 20-40 repeats. Note the constant non-specific peak at ~275 bp in both PCRs. The relative fluorescent unit y-axis scale has been zoomed-in to show the low-level peaks relating to the approximately 20-40 repeats in case 1. **Southern blot analysis of Case 1 (f).** Frontal cortex (lane 1) and cerebellum (lane 2) were analysed

using Southern blot analysis to size the *C9orf72* expansion repeat length. The results were compared to a blood sample from a *C9orf72* positive case (lane 3) and a known negative control (lane 4).

Pathological findings in Case 1 (g-t). p62 positive neuronal intranuclear inclusions (g) and neuronal cytoplasmic inclusions (h) were found in the cerebellum. Star-like neuronal cytoplasmic inclusions were found in the CA4 subregion of the hippocampus (i and j). The p62 positive star-like inclusions were also shown to contain the five DPRs; glycine-alanine (k), glycine-proline (l), and glycine-arginine (m) in the sense frames, and alanine-proline (n), and proline-arginine (o) in the anti-sense frames. Neuronal sense and anti-sense RNA foci were evident in the frontal cortex of Case 1. RNA fluorescent in-situ hybridisation for sense (red p-q) or anti-sense (green r-s) foci were combined with immunostaining for neurons with NeuN (green (q) or red (s)) and nuclear DNA staining with DAPI (blue). Quantification of the percentage of neurons containing sense or anti-sense RNA foci from RNA FISH in the frontal cortex (t). Scale bar in a represents 10µm in b-c and e-j and 50µm in d. In k-n scale bar represents 2 µm.

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