Neuropathological examination of sense and antisense RNA foci and c9RAN protein pathology in c9FTD/ALS

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Abstract

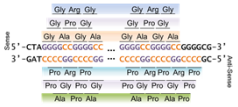
Introduction: Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are devastating neurodegenerative disorders with genetic, neuropathological, and clinical overlap. A hexanucleotide (GGGGCC) repeat expansion in *C9ORF72* is the major genetic cause of both diseases. The mechanisms by which this repeat expansion causes "c9FTD/ALS" are not definitively known, but RNA-mediated toxicity is a likely culprit. RNA transcripts of the expanded GGGGCC repeat form nuclear foci and also undergo repeat-associated non-ATG (RAN) translation resulting in the production of aggregation-prone proteins. The goals of this study were to examine whether antisense transcripts resulting from bidirectional transcription of the expanded repeat behave in a similar manner, and to examine the relationship between foci formation and RAN translation.

Methods: To evaluate RAN translation from sense and antisense transcripts of the expanded *C9ORF72* repeat, we generated novel rabbit polyclonal antibodies for the 5 potential c9RAN proteins: poly(GA), poly(GR), poly(GP), poly(PA), and poly(PR). These antibodies were used to analyze the presence of c9RAN proteins in brain tissue from FTD/ALS cases with or without the expanded *C9ORF72* repeat. To investigate foci formation from sense and antisense transcripts, RNA fluorescence *in situ* hybridization (FISH) was carried out on spinal cord, frontal cortex and cerebellar sections of c9FTD/ALS cases using probes to sense (GGGGCC) or antisense (CCCCGG) repeats. To examine the relationship between foci and RAN translation, tissue sections subjected to FISH were subsequently stained using a poly(GP) antibody followed by a fluorescent-labeled secondary antibody.

Results: Foci composed of sense or antisense transcripts are observed in the frontal cortex, spinal cord and cerebellum of c9FTD/ALS cases, and neuronal inclusions of poly(GP), poly(PA) and poly(PR) are present in various brain tissues in c9FTD/ALS, but not in other neurodegenerative diseases, including CAG repeat disorders. Although RNA foci and poly(GP) inclusions infrequently co-occur in the same cell, the brain region sampled (frontal cortex vs. cerebellum) and foci type (antisense vs. sense) both significantly affect the percentage of cells having both foci and inclusions, despite similar frequency of sense and antisense foci-bearing cells.

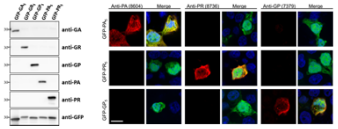
Discussion: That foci and poly(GP) inclusions are seldom observed in the same cells suggests that one event may preclude the other, and that they represent two distinct ways in which the *C9ORF72* repeat expansion may evoke neurotoxic effects.

Figure 1



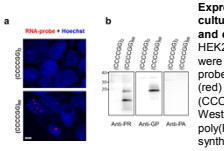
Schematic of the proteins generated by RAN translation of expanded GGGGCC and CCCCGG repeats in all possible reading frames.

Figure 2



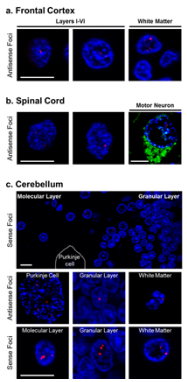
Antibody characterization for c9RAN proteins. (a) Western blot of lysates from HEK293T cells expressing enhanced GFP-tagged peptides with indicated antibodies. **(b)** Immunofluorescence staining of HEK293T cells expressing indicated enhanced GFP (green)-tagged peptides using anti-PA, anti-PR or anti-GP (red) antibodies. Nuclei are stained with Hoechst (blue). Scale bar=10 µm.

Figure 3



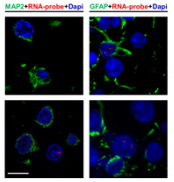
Expression of CCCCGG repeats in cultured cells leads to foci formation and expression of c9RAN proteins. (a) HEK293T cells expressing $(CCCCGG)_n$ were subjected to RNA-FISH using a probe against CCCCGG. Note the foci (red) in Hoechst-stained nuclei (blue) of $(CCCCGG)_{66}$-expressing cells. **(b)** Western blot of lysates shows that poly(PR) and poly(GP) proteins are synthesized in $(CCCCGG)_{66}$-expressing cells but not in cells expressing non-expanded $(CCCCGG)_2$. Scale bar=5 µm.

Figure 4



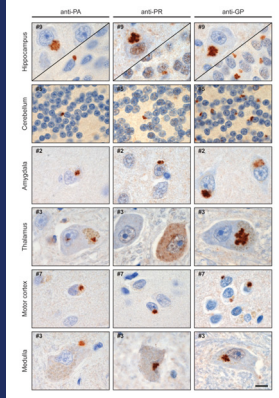
***C9ORF72* hexanucleotide repeat transcripts form nuclear RNA foci in frontal cortex, spinal cord and cerebellum in c9FTD/ALS. (a, b)** RNA-FISH of c9FTD/ALS frontal cortex and spinal cord tissue using a probe against the CCCCGG repeat transcripts shows RNA foci (red) in the nucleus (DAPI, blue) of cells. Note that foci are observed in motor neurons that stain positively for ChAT (green). **(c)** Cerebellar sections of c9FTD/ALS cases subjected to FISH using a probe against CCCCGG or GGGGCC transcripts. In most instances, foci-bearing cells were found in proximity to the Purkinje cell layer separating the molecular and granular layers. However, RNA foci were also observed in cells of the molecular layer, deep within the granular layer, in Purkinje cells, and in cells within the white matter. Scale bars=10 µm.

Figure 5



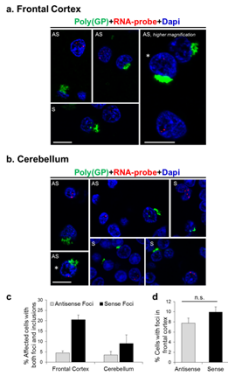
Nuclear RNA foci are present in both neurons and glia. RNA-FISH of c9FTD/ALS cerebellar tissue using a probe against the GGGGCC repeat was followed by immunofluorescence staining with the neuronal marker, MAP2, or the astrocytic marker, GFAP. Note that nuclear RNA foci are present in MAP2-positive and MAP2-negative cells, as well as GFAP-positive and GFAP-negative cells. Scale bar=10 µm.

Figure 6



Antisense c9RAN proteins in human postmortem tissue. Immunohistochemistry reveals poly(PA)-, poly(PR)-, and poly(GP)-reactive lesions throughout the CNS, including the hippocampus (endplate-CA3 on the top left, dentate fascia on the bottom right), cerebellum, amygdala, thalamus, motor cortex (layers 2-3), and medulla (inferior olivary nucleus). Lesions are often neuronal cytoplasmic inclusions (NCI) with a star-shaped morphology, but can also appear as dense NCI, small neuronal intranuclear inclusions, or diffuse neuronal "pre-inclusions". Anti-GP, which detects poly(GP) proteins that can be made from both sense and antisense transcripts of the *C9ORF72* expanded repeat, reveal greater pathologic burden compared to the anti-PA and PR antibodies. Scale bar=10 µm.

Figure 7



RNA foci and poly(GP) inclusions are seldom observed in the same cell. (a-b) RNA-FISH of c9FTD/ALS tissues using a probe against sense or antisense transcripts was followed by staining to detect poly(GP) inclusions. Though infrequent, both foci and poly(GP) inclusions can co-occur in the same cell (indicated by an asterisk). Scale bars=10 µm. AS = antisense foci, S= sense foci. **(c)** To determine the percentage of cells having both foci and poly(GP) inclusions, quantitative analysis was undertaken on four cases co-stained for poly(GP) inclusions and either sense or antisense foci. For each section, we examined 25 cells with inclusions and determined whether they had foci, and examined 25 cells with foci and determined whether they had inclusions. Data presented as mean ± SEM, n=4. The brain region sampled (frontal cortex vs. cerebellum, P=0.0292) and foci type (antisense vs. sense, P=0.0011) significantly affect the percentage of cells having foci and inclusions, as assessed by two-way ANOVA. **(d)** To determine the percentage of cells with antisense and sense foci, the number of cells with foci and the total number of cells were counted in twelve randomly selected, non-overlapping fields for each case. Data presented as mean ± SEM, n=4. n.s. = not significant by paired two-tailed t-test (P=0.1916).

Conclusion

Through the production of sense and antisense repeat RNA and five c9RAN proteins, the *C9ORF72* repeat expansion leads to the production of seven potentially toxic biomolecules. It is now of importance to determine if and how these biomolecules contribute to c9FTD/ALS pathogenesis, and whether the frequency or regional localization of RNA foci and c9RAN inclusions correlate with distinct clinical features.

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