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Supplemental data at Neurology.org/ng

ABSTRACT

Objective: We performed a genome-wide brain expression study to reveal the underpinnings of diseases linked to a repeat expansion in chromosome 9 open reading frame 72 (*C9ORF72*).

Methods: The genome-wide expression profile was investigated in brain tissue obtained from *C9ORF72* expansion carriers (n = 32), patients without this expansion (n = 30), and controls (n = 20). Using quantitative real-time PCR, findings were confirmed in our entire pathologic cohort of expansion carriers (n = 56) as well as nonexpansion carriers (n = 31) and controls (n = 20).

Results: Our findings were most profound in the cerebellum, where we identified 40 differentially expressed genes, when comparing expansion carriers to patients without this expansion, including 22 genes that have a homeobox (e.g., *HOX* genes) and/or are located within the *HOX* gene cluster (top hit: homeobox A5 [*HOXA5*]). In addition to the upregulation of multiple homeobox genes that play a vital role in neuronal development, we noticed an upregulation of transthyretin (*TTR*), an extracellular protein that is thought to be involved in neuroprotection. Pathway analysis aligned with these findings and revealed enrichment for gene ontology processes involved in (anatomic) development (e.g., organ morphogenesis). Additional analyses uncovered that *HOXA5* and *TTR* levels are associated with *C9ORF72* variant 2 levels as well as with intron-containing transcript levels, and thus, disease-related changes in those transcripts may have triggered the upregulation of *HOXA5* and *TTR*.

Conclusions: In conclusion, our identification of genes involved in developmental processes and neuroprotection sheds light on potential compensatory mechanisms influencing the occurrence, presentation, and/or progression of *C9ORF72*-related diseases. ***Neurol Genet* 2017;3:e161; doi: 10.1212/NXG.0000000000000161**

GLOSSARY

ALS = amyotrophic lateral sclerosis; **FTD** = frontotemporal dementia; **FTLD** = frontotemporal lobar degeneration; **IQR** = interquartile range; **MND** = motor neuron disease.

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are part of a disease continuum. Although ALS is the most common form of motor neuron disease (MND) and results in progressive muscle weakness, FTD is a frequent cause of dementia and is associated with changes in personality, behavior, and language. A hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9ORF72*) is a major genetic cause of both diseases.[1,2] Emerging evidence suggests that *C9ORF72*-related diseases are characterized by a loss of *C9ORF72* expression,[1] the formation of RNA foci with flawed RNA transcripts,[1] and the generation of dipeptide repeat proteins aberrantly translated from the repeat expansion,[3,4] with both RNA foci

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and dipeptide repeat proteins potentially contributing to disease by compromising nucleocytoplasmic transport.[5–7]

In our quest to increase our understanding of *C9ORF72*-related diseases, we assessed the genome-wide expression profile in brain tissue obtained from the Mayo Clinic Florida Brain Bank (n = 82). Of interest, in *C9ORF72* expansion carriers, we discovered an upregulation of genes involved in developmental processes and neuroprotection, particularly in the cerebellum, a region without substantial neuronal loss that demonstrates pathologic hallmarks of *C9ORF72*-related diseases,[3,8] and in which abnormalities associate with neuropathologic and clinical phenotypes.[9,10] Such findings may point toward mechanisms that could compensate for the harmful effects of *C9ORF72* repeat expansions.

METHODS **Participant selection.** From the Mayo Clinic Florida Brain Bank, participants were selected for our genome-wide expression study: patients with a pathologic diagnosis of frontotemporal lobar degeneration (FTLD) and/or MND who harbored *C9ORF72* repeat expansions (n = 32), patients with FTLD and/or MND without repeat expansions (n = 30), and controls without neurologic diseases (n = 20, table 1). To confirm the observed upregulation of homeobox A5 (*HOXA5*) and transthyretin (*TTR*), quantitative real-time PCR was performed, when expanding investigations to our entire pathologic cohort of *C9ORF72* expansion carriers for whom brain tissue was available (n = 56) as well as FTLD and/or MND patients without an expansion (n = 31) and controls without any neurologic disease (n = 20).[11] In this cohort, *C9ORF72* transcript levels, the length of the repeat expansion, and dipeptide repeat protein levels had already been determined.[10–12] To examine the cerebellar TTR protein, Western blots (n = 10) and immunohistochemistry (n = 13) were performed on a representative subset of samples (table e-1 at Neurology.org/ng). Next, an immunoassay was used to evaluate TTR protein levels in the CSF, studying 2 independent clinical cohorts obtained at either the Mayo Clinic (n = 67) or the University of Miami (n = 40, table e-1).

Standard protocol approvals, registrations, and patient consents. All participants agreed to participate in the study, and biological samples were obtained after informed consent with ethical committee approval from the respective institutions.

Methods and statistical analysis. To examine the genome-wide expression pattern, Whole-Genome DASL HT assays (Illumina, San Diego, CA) were used, which were processed by the Mayo Clinic Core Facility. Validation was performed with quantitative real-time PCR using TaqMan gene expression assays (Life Technologies, Carlsbad, CA). Western blotting was used to evaluate cerebellar TTR protein levels, complemented with immunohistochemistry to assess the presence of potential TTR protein aggregates. Meso Scale Discovery (MSD, Rockville, MD) electrochemiluminescence detection technology was used to establish a sandwich immunoassay for TTR. Cell culture experiments were then performed in an attempt to clarify underlying mechanisms. In U251 and HepG2 cells, a loss of *C9ORF72* expression was mimicked with small interfering RNAs (siRNAs, Dharmacon, Lafayette, CO), and in addition, the effect of full-length *C9ORF72* and the repeat expansion itself was examined by transfecting cells with expression vectors.[13] A detailed description of our methods and statistical analysis is provided in the supplemental data.

RESULTS **Upregulation of homeobox genes and *TTR* in *C9ORF72* expansion carriers.** We performed a genome-wide expression study in the cerebellum and frontal cortex to identify genes involved in *C9ORF72*-related diseases. First, we compared patients with or without a repeat expansion in *C9ORF72*. Although participants included in those groups are both affected by neurodegenerative diseases, this enabled us to find *C9ORF72*-specific differences. Second, we compared expansion carriers with controls without neurodegenerative diseases, allowing the detection of more general differences that could, theoretically, be due to the presence of a neurodegenerative disease.

In the cerebellum, when comparing expansion carriers to patients without expansions, we detected 40 differentially expressed genes (table e-2). Generation of a heat map of those genes revealed that expansion carriers generally cluster together (figure 1). Of interest, our list of differentially expressed genes contained 22 genes that have a homeobox (e.g., *HOX* genes) and/or are located within the *HOX* gene cluster (table e-2). In addition to the upregulation of multiple homeobox genes (top hit: *HOXA5*) that play a vital role in neuronal development,[14] we noticed a cerebellar upregulation of *TTR* (table e-2), an extracellular protein that is thought to be involved in neuroprotection.[15–19] We then compared expansion carriers with controls and

Table 1 Participant characteristics

Cohort/variable	C9Plus cohort (n = 32)	C9Minus cohort (n = 30)	Control cohort (n = 20)
Genome-wide expression			
Sex, male	20 (63)	12 (40)	7 (35)
Age at death, y	63.7 (58.4–71.7)	75.0 (64.0–81.8)	87.5 (81.8–93.0)
RIN cerebellum (value)	9.4 (9.2–9.6)	9.2 (8.7–9.4)	9.3 (8.5–9.4)
RIN frontal cortex (value)	9.0 (8.5–9.6)	9.1 (8.6–9.5)	8.9 (8.6–9.2)
Diagnosis			
FTLD	12 (38)	10 (33)	—
FTLD/MND	10 (31)	10 (33)	—
MND	10 (31)	10 (33)	—
Other	—	—	—

Abbreviations: FTLD = frontotemporal lobar degeneration; IQR = interquartile range; MND = motor neuron disease.
Data are sample median (IQR) or n (%). Information was obtained for patients with (C9Plus) and without (C9Minus) expansions in *C9ORF72*, as well as from controls. This study was performed in the cerebellum and frontal cortex.

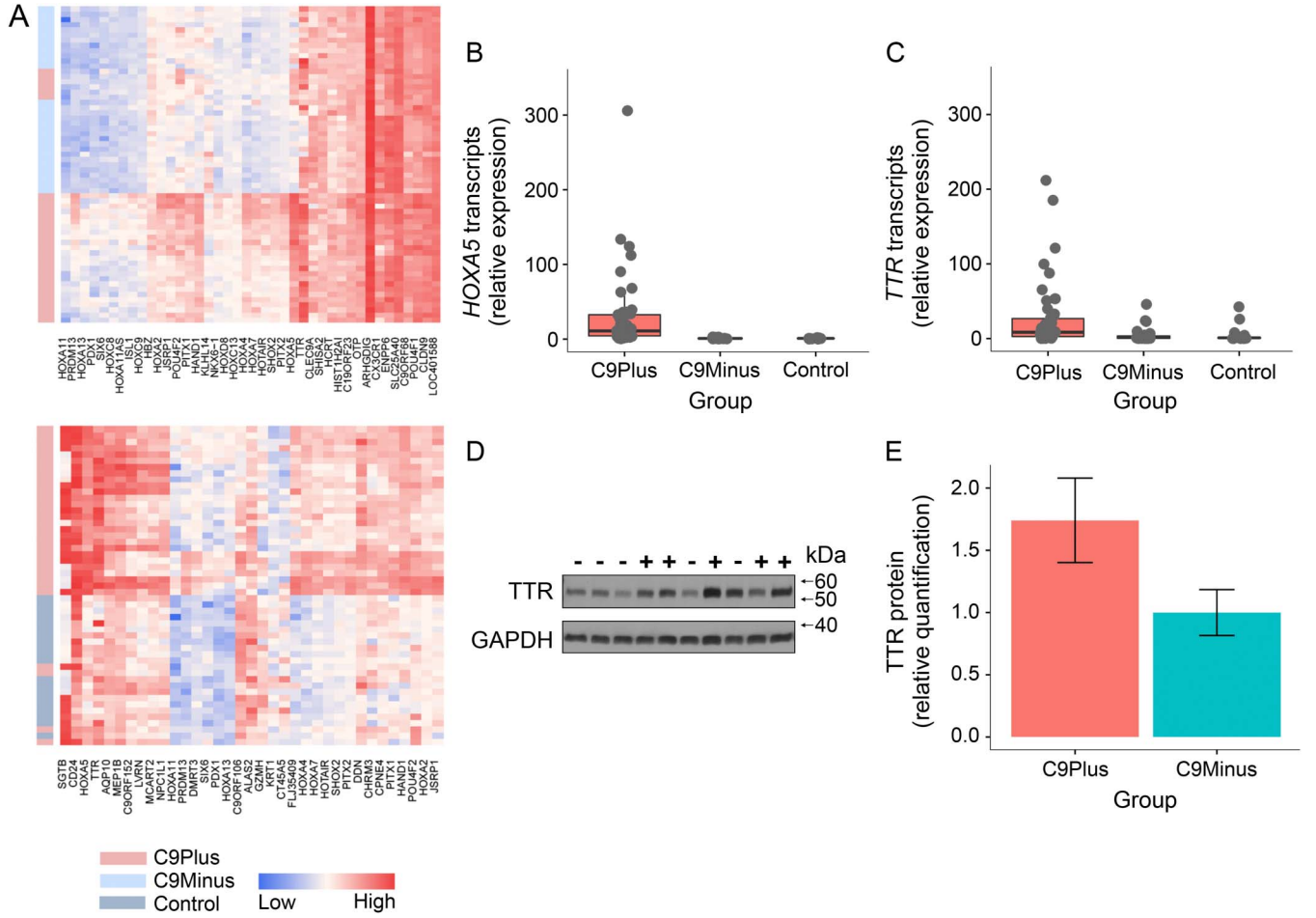
discovered 1,575 differentially expressed genes in the cerebellum (table e-2). Again, our heat map showed that expansion carriers tend to cluster together (figure 1). Of interest, our new list contained 37 of the 40 (93%) genes we identified previously (table e-2), including homeobox genes and *TTR*.

We also performed gene ontology analysis and observed an enrichment for pathways involved in the regulation of (anatomic) development, which was most profound when comparing expansion carriers with disease controls (e.g., organ morphogenesis, pattern specification process, regionalization, and skeletal system development, table e-3), but which was also seen when comparing expansion carriers with controls (table e-3).

In the frontal cortex, a comparison between patients with or without repeat expansions resulted in the detection of 3 differentially expressed genes: *HOXA5*, *C9ORF72*, and POU class 4 homeobox 2 (*POU4F2*; table e-2). We also compared expansion carriers with controls and revealed 679 differentially expressed genes, including *C9ORF72* and *TTR* (table e-2). Again, enrichment was observed for pathways involved in developmental processes (table e-3).

Associations of *C9ORF72* transcripts with *HOXA5* and *TTR* transcripts in our overall cohort. In previously published studies, we investigated the levels of known *C9ORF72* transcript variants (variant 1 [NM_145005.6], variant 2 [NM_018325.4], and variant 3 [NM_001256054.2])

Figure 1 **Expression of homeobox genes and transthyretin**



C9Plus = patients with *C9ORF72* repeat expansions; C9Minus = patients without *C9ORF72* repeat expansions; and control = controls without neurologic diseases. Heat map plots of intensity values of differentially expressed genes are displayed for the cerebellum, when comparing *C9ORF72* expansion carriers with patients without expansions (A, fold change above 1.2), and when comparing *C9ORF72* expansion carriers with controls (A, fold change above 2.5 [more stringent to allow visualization]). Rows (samples) and columns (genes) are grouped by hierarchical clustering using Manhattan distance measurements; low intensities are shown as blue, and high intensities are shown as red. In our expression cohort, cerebellar expression levels of homeobox A5 (*HOXA5*; B) and transthyretin (*TTR*; C) are increased in patients with *C9ORF72* repeat expansions as compared to patients without expansions or to controls. The median is represented by a solid line, and each box spans the 25th percentile to the 75th percentile (interquartile range). A Western blot is shown demonstrating higher cerebellar TTR protein levels in expansion carriers (+) than in patients without this expansion (−, D). Quantification of Western blot samples confirmed the cerebellar increase of TTR protein levels in patients with a repeat expansion as compared to patients without this expansion (E), which is displayed in a bar graph that represents the mean of the relative normalized TTR protein with the SEM, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control.

Table 2 Expression studies of *HOXA5* and *TTR* transcripts using TaqMan assays in the expression cohort

			C9Plus vs C9Minus cohort			C9Plus vs control cohort			C9Minus vs control cohort		
TaqMan	Group	p Value[a]	C9Plus	C9Minus	p Value	C9Plus	Control	p Value	C9Minus	Control	p Value
Cerebellum	*HOXA5*	1.24e-12	10.98 (4.43–33.01)	0.91 (0.55–1.30)	8.22e-10	10.98 (4.43–33.01)	1.00 (0.66–1.30)	5.41e-08	0.91 (0.55–1.30)	1.00 (0.66–1.30)	0.90
	TTR	3.01e-06	8.48 (2.69–26.86)	1.68 (0.27–3.55)	4.09e-05	8.48 (2.69–26.86)	1.00 (0.34–1.54)	7.69e-05	1.68 (0.27–3.55)	1.00 (0.34–1.54)	0.30
Frontal cortex	*TTR*	0.04	5.69 (1.88–15.42)	2.01 (0.35–21.18)	0.19	5.69 (1.88–15.42)	1.00 (0.43–6.92)	0.01	2.01 (0.35–21.18)	1.00 (0.43–6.92)	0.40

Abbreviation: IQR = interquartile range.
Data are sample median (IQR) or *p* value; *HOXA5* and *TTR* transcript levels are normalized to the geometric mean of endogenous control genes ribosomal protein, large, P0 (*RPLP0*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Information was obtained for patients with (C9Plus) and without (C9Minus) expansions in *C9ORF72*, as well as for controls. In the cerebellum, 2 tests were performed (*HOXA5* transcripts and *TTR* transcripts), and thus, *p* values below 0.025 were considered significant after Bonferroni correction; in the fontal cortex, the levels of *HOXA5* were too low for reliable detection using quantitative real-time PCR and only 1 test was performed (*TTR* transcripts), and thus, *p* values below 0.050 were considered significant.
[a] A Kruskal-Wallis rank-sum test was performed to determine whether expression levels differed between groups (cerebellum: $p < 0.025$ considered significant after Bonferroni correction, frontal cortex: $p < 0.050$ considered significant); when significant differences were detected, a Wilcoxon rank-sum test was used for pairwise comparisons ($p < 0.017$ considered significant after Bonferroni correction for 3 comparisons). Similar findings were obtained when normalizing to neuronal markers (not shown for simplicity).

as well as 2 intronic regions (1 upstream of the repeat expansion [intron 1a] and 1 downstream of the repeat expansion [intron 1b]).[11] In this study, we examined the same cohort to validate our findings related to *HOXA5* and *TTR*, which demonstrated that their cerebellar levels are indeed higher in *C9ORF72* expansion carriers than in (disease) controls (table 2, figure 1). In addition, in the frontal cortex, we showed that *TTR* levels are elevated in patients with a *C9ORF72* repeat expansion as compared to controls (table 2). Of note, we also performed a sensitivity analysis to assess whether our findings could have been biased by differences in age at death. It is important that similar findings were observed when restricting our analysis to a subset of participants with a comparable age at death (not shown). In addition, given the low levels of *TTR* and *HOXA5*, especially in (disease) controls, we also validated their upregulation in a subset of participants using other techniques, including digital molecular barcoding (not shown) and previously published RNA sequencing data (figure e-1).[20]

The vast amount of *C9ORF72* expression data available for this cohort then allowed us to determine whether the levels of *C9ORF72* transcripts were associated with the levels of *HOXA5* and *TTR*. In our overall cohort (expansion carriers, disease controls, and controls), lower cerebellar levels of *C9ORF72* transcript variant 2 were associated with higher cerebellar levels of both *HOXA5* ($r = -0.60$, $p = 3.21e\text{-}09$, Spearman test of correlation) and *TTR* ($r = -0.47$, $p = 2.21e\text{-}06$, Spearman test of correlation, table 3), which is not surprising given the fact that expansion carriers demonstrate decreased levels of *C9ORF72* transcript variant 2.[11] More excitingly, we noticed that higher cerebellar levels of intron-containing transcripts (both intron 1a and intron 1b) are associated with higher cerebellar levels of *HOXA5* transcripts (intron 1a: $r = 0.43$, $p = 6.47e\text{-}05$, intron 1b: $r = 0.36$, $p = 0.0008$, Spearman test of correlation, table 3). In the frontal cortex, lower *C9ORF72* variant 2 levels were also associated with higher *TTR* levels ($r = -0.28$, $p = 0.006$, Spearman test of correlation, table 3).

Associations of *C9ORF72* transcripts with *HOXA5* and *TTR* transcripts in expansion carriers. Because we were able to validate our findings related to *HOXA5* and *TTR* and detect significant associations with specific *C9ORF72* transcripts in our overall cohort, we then evaluated the presence of any potential associations within our cohort of *C9ORF72* expansion carriers. In the cerebellum, increased levels of total *C9ORF72* transcripts were associated with increased *HOXA5* transcripts ($r = 0.51$, $p = 8.86e\text{-}05$, Spearman test of correlation, table 4), most prominently in patients with a pathologic diagnosis of FTLD ($r = 0.65$,

Table 3 Associations of *HOXA5* and *TTR* transcripts with *C9ORF72* transcripts in the overall cohort

			Overall	
TaqMan	**Group**	**Association**	**Spearman *r* (95% CI)**	***p* Value**
Cerebellum	*HOXA5*	Total	−0.21 (−0.40 to 0.02)	0.07
		Variant 1	−0.19 (−0.39 to 0.03)	0.09
		Variant 2	−0.60 (−0.73 to −0.42)	3.21e-09
		Variant 3	0.06 (−0.14 to 0.27)	0.56
		Intron 1a	0.43 (0.23 to 0.60)	6.47e-05
		Intron 1b	0.36 (0.16 to 0.54)	0.0008
	TTR	Total	−0.19 (−0.39 to 0.02)	0.06
		Variant 1	−0.20 (−0.40 to 0.003)	0.05
		Variant 2	−0.47 (−0.62 to −0.28)	2.21e-06
		Variant 3	−0.03 (−0.24 to 0.18)	0.78
		Intron 1a	0.23 (0.03 to 0.42)	0.03
		Intron 1b	0.23 (0.04 to 0.41)	0.03
Frontal cortex	*TTR*	Total	−0.15 (−0.35 to 0.07)	0.16
		Variant 1	−0.06 (−0.25 to 0.14)	0.58
		Variant 2	−0.28 (−0.47 to −0.08)	0.006
		Variant 3	−0.17 (−0.35 to 0.03)	0.09
		Intron 1a	0.23 (0.02 to 0.42)	0.03
		Intron 1b	0.14 (−0.07 to 0.34)	0.19

Data are Spearman correlation coefficient *r* (95% confidence interval [CI]) or *p* value; *HOXA5* and *TTR* transcript levels are normalized to the geometric mean of endogenous control genes ribosomal protein, large, P0 (*RPLP0*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). In total, in our overall cohort (expansion carriers, disease controls, and controls), we examined 6 different associations (total *C9ORF72* transcripts, *C9ORF72* transcript variant 1, *C9ORF72* transcript variant 2, *C9ORF72* transcript variant 3, intron 1a–containing *C9ORF72* transcripts, and intron 1b–containing *C9ORF72* transcripts) for each outcome, and thus, *p* values below 0.0083 were considered significant after Bonferroni correction. A Spearman test of correlation was used ($p < 0.0083$ considered significant after Bonferroni correction). Similar findings were obtained when normalizing to neuronal markers (not shown for simplicity).

$p = 0.0009$, Spearman test of correlation). Of interest, we also noticed that elevated levels of intron-containing transcripts were associated with elevated levels of *HOXA5* or *TTR* (table 4). For *HOXA5*, this association (intron 1a: $r = 0.60$, $p = 1.61e\text{-}06$, intron 1b: $r = 0.54$, $p = 2.75e\text{-}05$, Spearman test of correlation, table 4) was driven by patients with a pathologic diagnosis of FTLD (intron 1a: $r = 0.72$, $p = 0.0001$, intron 1b: $r = 0.59$, $p = 0.003$, Spearman test of correlation). For *TTR*, however, the association (intron 1a: $r = 0.40$, $p = 0.003$, intron 1b: $r = 0.43$, $p = 0.001$, Spearman test of correlation, table 4) was most profound in patients with a pathologic diagnosis of MND (intron 1a: $r = 0.83$, $p = 0.0002$, intron 1b: $r = 0.88$, $p = 3.78e\text{-}05$, Spearman test of correlation).

Because we previously discovered associations between dipeptide repeat proteins and intron-containing transcripts,[11] we subsequently evaluated potential associations with poly(GP) and poly(GA) proteins, which can form abundant inclusions in the neocortical regions, hippocampus, thalamus, and cerebellum.[3,4,9,21–24] Although no significant associations were detected for *TTR*, we did observe an association for *HOXA5*: higher levels of dipeptide repeat proteins were associated with higher levels of *HOXA5* (poly[GP]: $r = 0.52$, $p = 0.0002$, poly[GA]: $r = 0.56$, $p = 3.89e\text{-}05$, Spearman test of correlation, table 4).

In the frontal cortex, we noted a trend between *C9ORF72* variant 2 and *TTR* ($r = -0.39$, $p = 0.004$, Spearman test of correlation), particularly in the subset of patients with a pathologic diagnosis of FTLD ($r = -0.62$, $p = 0.002$, Spearman test of correlation, table 4).

In both brain regions, we did not detect significant associations with other variables, such as expansion size (table 4), disease subgroup, sex, age at onset, age at death, or survival after onset (not shown).

Cerebellar changes in TTR transcripts are reflected by changes in protein levels. Given the fact that TTR is an extracellular protein, we determined whether changes in RNA levels were reflected by changes in protein levels, which could indicate that TTR may serve as a biomarker for *C9ORF72*-related diseases. Because our findings were most profound in the cerebellum, we extracted protein from this neuroanatomic region and performed Western blots. As expected, we detected a significant increase in cerebellar TTR protein levels in patients with a repeat expansion (mean 174% ± 34%) as compared to patients without a repeat expansion (mean 100% ± 18%, $p < 0.05$, 2-sample t test, figure 1). We also performed immunohistochemistry to examine whether an aggregated form of the TTR protein was present in the cerebellum because TTR protein aggregates have been reported in other diseases, such as familial amyloid polyneuropathy.[25] We observed diffuse cytoplasmic TTR staining in pyramidal neurons and Purkinje cells, and in the neuropil; however, no TTR deposits were detected similar to those seen in patients with TTR amyloidosis (not shown).

To further evaluate TTR as a potential biomarker, we determined its protein levels in the CSF. In our first cohort, the median TTR protein level in expansion carriers was 15.5 μg/mL (interquartile range [IQR] 13.7–17.6) and in the remaining participants 16.3 μg/mL (IQR 14.5–17.7), which was not significantly different ($p = 0.29$, Wilcoxon rank-sum test). Our second cohort revealed a median TTR protein level of 12.5 μg/mL in expansion carriers (IQR 11.0–12.6) and 12.3 μg/mL in other participants (IQR 11.8–14.4); again, this difference did not reach statistical significance ($p = 0.58$, Wilcoxon rank-sum

Table 4 Associations of *HOXA5* and *TTR* transcripts with *C9ORF72* transcripts, expansion size, and dipeptide repeat proteins in expansion carriers

			C9Plus cohort		FTLD cohort		FTLD/MND cohort		MND cohort	
TaqMan	**Group**	**Association**	**Spearman *r* (95% CI)**	***p* Value**	**Spearman *r* (95% CI)**	***p* Value**	**Spearman *r* (95% CI)**	***p* Value**	**Spearman *r* (95% CI)**	***p* Value**
Cerebellum	*HOXA5*	Total	0.51 (0.29 to 0.68)	8.86e-05	0.65 (0.32 to 0.83)	0.0009	0.20 (−0.40 to 0.65)	0.47	0.43 (−0.19 to 0.86)	0.13
		Variant 1	0.33 (0.08 to 0.54)	0.01	0.50 (0.11 to 0.78)	0.01	−0.12 (−0.68 to 0.44)	0.67	0.16 (−0.45 to 0.68)	0.59
		Variant 2	−0.13 (−0.40 to 0.15)	0.33	−0.07 (−0.51 to 0.41)	0.77	−0.19 (−0.68 to 0.36)	0.50	−0.08 (−0.67 to 0.50)	0.80
		Variant 3	0.38 (0.13 to 0.59)	0.005	0.35 (−0.12 to 0.69)	0.10	0.41 (−0.16 to 0.81)	0.12	0.22 (−0.42 to 0.77)	0.44
		Intron 1a	0.60 (0.37 to 0.77)	1.61e-06	0.72 (0.39 to 0.89)	0.0001	0.56 (−0.02 to 0.91)	0.03	0.41 (−0.21 to 0.82)	0.14
		Intron 1b	0.54 (0.31 to 0.71)	2.75e-05	0.59 (0.20 to 0.82)	0.003	0.65 (0.17 to 0.88)	0.009	0.42 (−0.13 to 0.80)	0.14
		C9ORF72 expansion size	−0.17 (−0.42 to 0.10)	0.24	−0.09 (−0.53 to 0.36)	0.68	−0.42 (−0.70 to 0.02)	0.12	−0.18 (−0.64 to 0.44)	0.55
		Poly(GP)	0.52 (0.26 to 0.72)	0.0002	0.32 (−0.12 to 0.71)	0.15	0.50 (−0.15 to 0.91)	0.08	0.45 (−0.09 to 0.84)	0.10
		Poly(GA)	0.56 (0.33-0.73)	3.89e-05	0.60 (0.20 to 0.85)	0.004	0.41 (−0.26 to 0.84)	0.17	0.09 (−0.59 to 0.62)	0.74
	TTR	Total	0.25 (−0.02 to 0.50)	0.07	0.44 (0.08 to 0.70)	0.03	−0.02 (−0.62 to 0.60)	0.95	0.35 (−0.23 to 0.83)	0.22
		Variant 1	0.07 (−0.23 to 0.36)	0.60	0.29 (−0.13 to 0.62)	0.18	−0.18 (−0.74 to 0.47)	0.53	0.03 (−0.52 to 0.61)	0.91
		Variant 2	−0.17 (−0.43 to 0.12)	0.21	−0.06 (−0.53 to 0.42)	0.77	−0.24 (−0.74 to 0.36)	0.40	−0.37 (−0.78 to 0.21)	0.19
		Variant 3	0.11 (−0.19 to 0.39)	0.43	0.10 (−0.34 to 0.52)	0.65	0.05 (−0.61 to 0.67)	0.85	0.24 (−0.36 to 0.75)	0.41
		Intron 1a	0.40 (0.15 to 0.59)	0.003	0.27 (−0.16 to 0.66)	0.22	0.28 (−0.31 to 0.73)	0.31	0.83 (0.54 to 0.96)	0.0002
		Intron 1b	0.43 (0.18 to 0.63)	0.001	0.37 (−0.04 to 0.67)	0.08	0.33 (−0.32 to 0.80)	0.23	0.88 (0.62 to 0.97)	3.78e-05
		C9ORF72 expansion size	0.01 (−0.29 to 0.30)	0.93	0.05 (−0.38 to 0.45)	0.83	0.12 (−0.57 to 0.66)	0.67	0.01 (−0.66 to 0.64)	0.96
		Poly(GP)	0.09 (−0.19 to 0.36)	0.55	−0.11 (−0.49 to 0.30)	0.62	0.19 (−0.43 to 0.66)	0.54	0.38 (−0.15 to 0.76)	0.17
		Poly(GA)	−0.02 (−0.29 to 0.26)	0.91	0.15 (−0.26 to 0.54)	0.50	0.00 (−0.60 to 0.63)	0.99	−0.002 (−0.53 to 0.57)	1.00
Frontal cortex	*TTR*	Total	−0.19 (−0.47 to 0.11)	0.17	−0.05 (−0.51 to 0.43)	0.84	−0.33 (−0.79 to 0.24)	0.26	−0.39 (−0.86 to 0.30)	0.19
		Variant 1	−0.03 (−0.32 to 0.26)	0.82	−0.09 (−0.40 to 0.56)	0.69	−0.12 (−0.58 to 0.40)	0.70	0.01 (−0.61 to 0.60)	0.96
		Variant 2	−0.39 (−0.59 to −0.14)	0.004	−0.62 (−0.80 to −0.31)	0.002	−0.51 (−0.90 to 0.07)	0.06	−0.11 (−0.68 to 0.53)	0.73
		Variant 3	−0.03 (−0.31 to 0.26)	0.81	0.32 (−0.10 to 0.67)	0.14	−0.33 (−0.79 to 0.30)	0.26	0.08 (−0.61 to 0.68)	0.80
		Intron 1a	0.18 (−0.08 to 0.41)	0.21	0.31 (−0.14 to 0.70)	0.16	0.14 (−0.39 to 0.58)	0.63	0.21 (−0.46 to 0.75)	0.49
		Intron 1b	−0.02 (−0.31 to 0.30)	0.88	−0.08 (−0.52 to 0.47)	0.72	−0.08 (−0.71 to 0.51)	0.79	0.22 (−0.54 to 0.80)	0.47
		C9ORF72 expansion size	0.02 (−0.28 to 0.30)	0.91	−0.25 (−0.62 to 0.19)	0.26	0.49 (−0.08 to 0.83)	0.07	−0.19 (−0.80 to 0.48)	0.55
		Poly(GP)	0.15 (−0.15 to 0.42)	0.32	0.40 (−0.10 to 0.71)	0.08	0.43 (−0.14 to 0.88)	0.12	0.05 (−0.63 to 0.65)	0.85

Abbreviations: FTLD = frontotemporal lobar degeneration; MND = motor neuron disease.
Data are Spearman correlation coefficient *r* (95% confidence interval [CI]) or *p* value; *HOXA5* and *TTR* transcript levels are normalized to the geometric mean of endogenous control genes ribosomal protein, large, P0 (*RPLP0*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). In total, within our cohort of *C9ORF72* expansion carriers, we examined 16 different associations (total *C9ORF72* transcripts, *C9ORF72* transcript variant 1, *C9ORF72* transcript variant 2, *C9ORF72* transcript variant 3, intron 1a–containing *C9ORF72* transcripts, intron 1b–containing *C9ORF72* transcripts, *C9ORF72* repeat length, poly[GP] levels, poly[GA] levels, disease subgroup, sex, age at onset, age at death, and survival after onset [using 3 different cutoff points]) for each outcome, and thus, *p* values below 0.0031 were considered significant after Bonferroni correction; in this table, only 9 of those 16 associations are displayed (total *C9ORF72* transcripts, *C9ORF72* transcript variant 1, *C9ORF72* transcript variant 2, *C9ORF72* transcript variant 3, intron 1a-containing *C9ORF72* transcripts, intron 1b-containing *C9ORF72* transcripts, *C9ORF72* repeat length, poly[GP] levels, and poly[GA] levels). A Spearman test of correlation was used ($p < 0.0031$ considered significant after Bonferroni correction). Similar findings were obtained when normalizing to neuronal markers (not shown for simplicity).

test). Of note, similar findings were obtained when adjusting for possible confounders, when removing outliers, and when restricting our analysis to specific (sub)groups (e.g., symptomatic participants).

Loss of *C9ORF72* expression increases *HOXA5* and *TTR* transcripts. Next, we performed cell culture experiments to determine which *C9ORF72*-related disease characteristics might drive the specific upregulation of *HOXA5* and *TTR*. Of interest, we observed an increase of *HOXA5* in response to a knock-down of total *C9ORF72* transcripts (mean 193% ± 3%, $p = 0.0002$, 2-sample t test). A comparable effect was seen on *TTR* (mean 129% ± 6%, $p = 0.01$, 2-sample t test) after knocking down total *C9ORF72* transcripts. Given our significant findings related to *C9ORF72* transcript variant 2, we then targeted variant 2. It is important that knocking down of variant 2 was sufficient to increase *HOXA5* levels (mean 274% ± 12%, $p = 0.005$, 2-sample t test). For *TTR*, targeting *C9ORF72* variant 2 also affected *TTR* levels (mean 293% ± 37%, $p = 0.03$, 2-sample t test). Overexpression of full-length *C9ORF72* or expression of 66 GGGGCC repeats, however, did not seem to affect *HOXA5* or *TTR* (not shown). Consequently, it seems that a loss of *C9ORF72* expression triggers an increase in *HOXA5* and *TTR*.

DISCUSSION We set out to increase our understanding of *C9ORF72*-related diseases and revealed elevated levels of multiple homeobox genes (e.g., *HOXA5*) and *TTR*. Of interest, our findings were most profound in the cerebellum, a region without substantial neuronal loss that demonstrates pathologic characteristics of diseases linked to a repeat expansion in *C9ORF72*.[3,8] In fact, in this neuroanatomic region, associations with neuropathologic and clinical features of the disease have already been reported. It has been shown, for instance, that dipeptide repeat protein levels are lower in pathologically diagnosed patients with MND as compared to patients with FTLD,[9,10] and that they are associated with the cognitive score of clinically diagnosed patients with ALS.[10] Moreover, an association between dipeptide repeat proteins and intron-containing transcripts has been described in the cerebellum, indicating that transcripts containing the entire first intron may serve as templates for repeat-associated non-ATG translation.[11] In addition, extensive alternative splicing and polyadenylation defects have been reported in the cerebellum of *C9ORF72* expansion carriers.[20] The fact that our findings were most prominent in the cerebellum emphasizes that this region may have been underappreciated and could play an important role in *C9ORF72*-related diseases.

The upregulation of multiple homeobox genes and *TTR* was further substantiated by the results of our gene ontology analysis that revealed enrichment for developmental processes. The observed enrichment for developmental processes is not surprising given the function of homeobox genes and TTR in neuronal development and neuroprotection.[14–19] Homeobox genes, for example, are involved in neuronal specification and target connectivity; they play a key role in the identity, organization, and peripheral connectivity of motor neuron subtypes.[14] TTR is important for the transportation of thyroid hormones (thyroxine [T4]) and retinol (vitamin A), and in addition, it is thought to participate in behavior, maintenance of normal cognitive processes during aging, neuropeptide processing, and nerve regeneration.[26] Of interest, homeobox genes and TTR are linked. For instance, an active metabolite of vitamin A (all-trans retinoic acid) functions in maintenance of many processes (e.g., brain function) and is essential for limb and organ development through homeobox gene–mediated mechanisms.[27]

Although the relative differences we observed in *HOXA5* and *TTR* are a reflection of small absolute differences, the fact that their levels are barely detectable in (disease) controls raises the possibility that their expression may have been (re)activated in the adult brain. Such a (re)activation could be specific to *C9ORF72*-related diseases and might be driven by certain mechanisms underlying those diseases. It is currently unknown where the elevated *HOXA5* and *TTR* levels are coming from; a change in cell-type composition, invading progenitor cells, and cell-autonomous effects need to be taken into consideration.

One of the pathologic hallmarks of *C9ORF72*-related diseases is a reduction in *C9ORF72* expression levels.[1] Because the most prominent decrease has been reported for *C9ORF72* variant 2 transcripts,[11] one could speculate that levels of this transcript might be associated with levels of *HOXA5* or *TTR*. Indeed, in our overall cohort, we demonstrated that lower levels of *C9ORF72* variant 2 transcripts were associated with higher levels of *HOXA5* and *TTR* transcripts. We also detected associations with intron-containing transcripts; in our expansion carriers, for instance, higher levels of intron-containing transcripts were associated with *HOXA5* transcript levels, especially in patients with a pathologic diagnosis of FTLD. In addition, *HOXA5* transcript levels were associated with dipeptide repeat proteins, which is in agreement with reports that describe associations between intron-containing transcripts and dipeptide repeat proteins.[11] For TTR, we also observed a correlation with intron-containing transcripts, but this association was most profound in patients with

a pathologic diagnosis of MND; no significant associations were observed with dipeptide repeat proteins. Thus, although some associations were shared between *HOXA5* and *TTR*, others differed.

We emphasize that in our study, as in any observational study, performing association analysis between 2 variables is not intended to provide information about possible mechanisms, but rather is intended to address the initial question whether 2 variables are related in any way. It is only after this initial question is addressed that further questions, such as mechanism, become relevant. To determine what may have driven an increase in *HOXA5* and *TTR*, we already performed cell culture experiments, demonstrating that lower levels of *C9ORF72* resulted in higher levels of *HOXA5* and *TTR*. These effects were observed when targeting either total *C9ORF72* transcripts or *C9ORF72* transcript variant 2; we cannot, however, exclude nonspecific effects on other transcript variants, and further studies are warranted. Additional studies could also help to learn more about mechanisms that link C9ORF72 to HOXA5 and TTR, particularly because little is known about the function, and interaction partners, of C9ORF72. Moreover, future studies could examine downstream targets and/or upstream regulators that might contribute to the observed differences.

It is important that TTR protein levels have been evaluated as a potential biomarker for ALS and FTD,[28–33] but findings were inconsistent, which could, in part, be explained by the genetic, pathologic, and clinical heterogeneity observed in those patients. Although our results seem to indicate that cerebellar TTR protein levels are elevated in *C9ORF72* expansion carriers, we could not detect significant differences in CSF TTR protein levels. The lack of a significant difference could be due to the presence of posttranslational modifications to the TTR protein that are undetectable using our immunoassay. Alternatively, it might be possible that the secretory pathway is affected, hampering the secretion of TTR into the CSF. Future experiments using mass spectrometry and immunoassays with different antibodies as well as experiments investigating the secretion of TTR (e.g., in cell culture models) should be used to test these hypotheses. In addition, future studies should examine whether TTR protein levels are associated with features of the disease (e.g., in the CSF or plasma) and whether they change over time, especially because one could postulate that a single time point in a clinical cohort may not reflect changes observed in a pathologic cohort (end-stage disease).

Thus, we discovered elevated levels of multiple homeobox genes and *TTR*, reported to be involved in developmental processes and neuroprotection, in brain tissue obtained from *C9ORF72* expansion carriers. Our findings may point to the presence of compensatory mechanisms aiming to mitigate the progression of *C9ORF72*-related diseases.

AUTHOR CONTRIBUTIONS

NiCole A. Finch, Matthew C. Baker, Tania F. Gendron, Kevin F. Bieniek, Mariely DeJesus-Hernandez, Patricia H. Brown, Jeannie Chew, Karen R. Jansen-West, Lillian M. Daughrity, Alexandra M. Nicholson, Melissa E. Murray, and Robert Bowser: acquisition of data, analysis or interpretation of data, and revising the manuscript for content, including writing of content. Xue Wang and Yan W. Asmann: analysis or interpretation of data and drafting the manuscript for content, including writing of content. Michael G. Heckman: analysis or interpretation of data, statistical analysis, and drafting the manuscript for content, including writing of content. Joanne Wuu: contribution of vital reagents/tools/patients and revising the manuscript for content, including writing of content. Keith A. Josephs, Joseph E. Parisi, David S. Knopman, Ronald C. Petersen, Leonard Petrucelli, Bradley F. Boeve, Neill R. Graff-Radford, Dennis W. Dickson, Michael Benatar, and Kevin B. Boylan: revising the manuscript for content, including writing of content, contribution of vital reagents/tools/patients, and obtaining funding. Rosa Rademakers: study concept or design, acquisition of data, analysis or interpretation of data, drafting the manuscript for content, including writing of content, revising the manuscript for content, including writing of content, study supervision or coordination, and obtaining funding. Marka van Blitterswijk: study concept or design, acquisition of data, analysis or interpretation of data, statistical analysis, drafting the manuscript for content, including writing of content, revising the manuscript for content, study supervision or coordination, and obtaining funding.

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DISCLOSURE

NiCole A. Finch and Xue Wang report no disclosures. Matthew C. Baker holds the following patents: US Patent No. 12/302,691 (Detecting and Treating Dementia [2008]) and US Patent No. 12/413,869 (Methods and Materials for Detecting and Treating Dementia [2009]). Michael G. Heckman has served on the editorial board of *Parkinsonism & Related Disorders*. Tania F. Gendron has received speaker honoraria from Johns Hopkins; holds a patent for Methods and materials for detecting poly (GP) proteins in tissues from C9ORF72 repeat expansion carriers; has received research support from NIH, the ALS Association, and the Muscular Dystrophy Association; and receives license fee payments for C9ORF72 repeat expansion constructs and viruses, and for antibodies against C9ORF72 dipeptide repeat proteins. Kevin F. Bieniek reports no disclosures. Joanne Wuu has received research support from the Muscular Dystrophy Association, the NIH, the Food and Drug Administration, Eli Lilly and Company, the University of Miami, Department of Neurology, the ALS Association, and the Department of Defense. Mariely DeJesus-Hernandez holds a patent on Methods to screen for the hexanucleotide repeat expansion in the *C9ORF72* gene. Patricia H. Brown, Jeannie

Chew, Karen R. Jansen-West, and Lillian M. Daughrity report no disclosures. Alexandra M. Nicholson has received research support from the Association for Frontotemporal Degeneration. Melissa E. Murray has served on the editorial boards of *BMC Neurology* and *Frontiers in Neurology* and has been a consultant for Avid Radiopharmaceuticals. Keith A. Josephs receives research support from the NIH, the Dana Foundation, and the Alzheimer's Association; and is an editorial board member for *Acta Neuropathologica* and *Journal of Neuropathology & Experimental Neurology*. Joseph E. Parisi has served on the Defense Health Board Health Care Delivery Subcommittee; has received publishing royalties from Oxford University Press; and has received research support from the NIH. David S. Knopman has served on the scientific advisory boards of the Bluefield Project, Lundbeck Pharmaceuticals, and DIAN study DSMB; served on a Data Safety Monitoring Board for Lundbeck Pharmaceuticals and the DIAN study; was an investigator in clinical trials sponsored by Lilly, TauRx, and Biogen Pharmaceuticals; has received travel funding/speaker honoraria from the Alzheimer Conference (Seoul, Korea) and the Behavioral Neurology Conference (Hyderabad, India); has served on the editorial board of *Neurology*; and has received research support from the NIH. Ronald C. Petersen has served on the scientific advisory boards of Pfizer, Janssen Alzheimer Immunotherapy, Elan Pharmaceuticals, Wyeth Pharmaceuticals, and GE Healthcare; has received publishing royalties from Oxford University Press; has been a consultant for Roche Incorporated, Merck, GeneTech, Biogen, and Eli Lilly; has received research support from the NIH; and has served on the National Advisory Council on Aging. 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Boeve has served as an investigator for clinical trials sponsored by GE Healthcare and FORUM Pharmaceuticals; receives royalties from the publication of a book entitled *Behavioral Neurology of Dementia* (Cambridge Medicine, 2009); serves on the Scientific Advisory Board of the Tau Consortium; has consulted for Isis Pharmaceuticals and Ionis Pharmaceuticals; and receives research support from the NIH, GE Healthcare, FORUM Pharmaceuticals, C2N Diagnostics, the Little Family Foundation, and the Mangurian Foundation. Neill R. Graff-Radford has served on the editorial board of *Alzheimer's Research & Therapy*; has received publishing royalties from *UpToDate*; has consulted for Cytox; and has received research support from TauRx, Lilly, Biogen, Axovant, and the NIH. Yan W. Asmann reports no disclosures relevant to this manuscript. Dennis W. Dickson has served on the editorial boards of *Acta Neuropathologica*, *Brain*, *Brain Pathology*, *Neurobiology of Aging*, *Annals of Neurology*, *Neuropathology*, *International Journal of Clinical and Experimental Pathology*, and *American Journal of Neurodegenerative Disease*; has received travel funding and speaker honoraria from Novartis; and has received research support from the NIH, the Society for PSP: Foundation for PSP/CBD and Related Disorders, and the Mangurian Foundation. Michael Benatar has served on the scientific advisory boards of Denali, Ra Pharmaceuticals, Alnylam, and Mitsubishi Tanabe; has served on the editorial board of *Journal Watch Neurology*; has been consultant for Congressionally Directed Medical Research Program (CDMRP) ALS Research Program Integration Panel; has received research funding from the Muscular Dystrophy Association, the ALS Association, the NIH, the Food and Drug Administration, the Department of Defense, Cytokinetics Inc., Alexion Pharmaceuticals, Kimmelman Estate, Eli Lilly and Company, Neuraltus, and the ALS Recovery Fund; and has had involvement in legal proceedings regarding Morris James LLP. Robert Bowser is a founder of Iron Horse Diagnostics, Inc. (a company focused on biomarkers related to ALS that holds patents on TTR as a biomarker for motor neuron diseases); has served on the scientific advisory boards of Denali Therapeutics and Above & Beyond, LLC; has served on the editorial boards of *Scientific Reports*, the *International Journal of Proteomics*, and the *American Journal of Neurodegenerative Disease*; holds patents for Biomarkers for the diagnosis and prognosis of ALS, Biomarkers to monitor drug treatment of ALS and other neuromuscular disease patients, and Biomarkers for detecting and treating joint related pain; has been a consultant for Cytonics, Inc. and Merck; has received research support from the NIH, ALS Association, and Target ALS; and holds stock/stock options for Iron Horse Diagnostics, Inc. Kevin B. Boylan receives research support from the NIH, ALS Association, Genentech, Cytokinetics Inc., the Mayo Foundation, Neuraltus Pharmaceuticals, GlaxoSmithKline, Avanir Pharmaceuticals, and Synapse Biomedical. 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Go to Neurology.org/ng for full disclosure forms.

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Abnormal expression of homeobox genes and transthyretin in *C9ORF72* expansion carriers

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