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Abnormal expression of homeobox genes and transthyretin in C9ORF72 expansion carriers

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Supplemental data
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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. Neurology 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. Emerging evidence suggests that C9ORF72-related diseases are characterized by a loss of C9ORF72 expression, the formation of RNA foci with flawed RNA transcripts, and the generation of dipeptide repeat proteins aberrantly translated from the repeat expansion, with both RNA foci

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and dipeptide repeat proteins potentially contributing to disease by compromising nucleocytoplasmic transport.\textsuperscript{3–7}

In our quest to increase our understanding of \textit{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 \textit{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 \textit{C9ORF72}-related diseases,\textsuperscript{3,8} and in which abnormalities associate with neuropathologic and clinical phenotypes.\textsuperscript{9,10} Such findings may point toward mechanisms that could compensate for the harmful effects of \textit{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 \textit{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 (\textit{HOXA5}) and transthyretin (\textit{TTR}), quantitative real-time PCR was performed, when expanding investigations to our entire pathologic cohort of \textit{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 (\(n = 20\)).\textsuperscript{11} In this cohort, \textit{C9ORF72} transcript levels, the length of the repeat expansion, and dipeptide repeat protein levels had already been determined.\textsuperscript{10–12} To examine the cerebellar \textit{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 \textit{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, Carlebad, CA). Western blotting was used to evaluate cerebellar \textit{TTR} protein levels, complemented with immunohistochemistry to assess the presence of potential \textit{TTR} protein aggregates. Meso Scale Discovery (MSD, Rockville, MD) electrochemiluminescence detection technology was used to establish a sandwich immunoassay for \textit{TTR}. Cell culture experiments were then performed in an attempt to clarify underlying mechanisms. In U251 and HepG2 cells, a loss of \textit{C9ORF72} expression was mimicked with small interfering RNAs (siRNAs, Dharmacon, Lafayette, CO), and in addition, the effect of full-length \textit{C9ORF72} and the repeat expansion itself was examined by transfecting cells with expression vectors.\textsuperscript{13} A detailed description of our methods and statistical analysis is provided in the supplemental data.

RESULTS Upregulation of homeobox genes and \textit{TTR} in \textit{C9ORF72} expansion carriers. We performed a genome-wide expression study in the cerebellum and frontal cortex to identify genes involved in \textit{C9ORF72}-related diseases. First, we compared patients with or without a repeat expansion in \textit{C9ORF72}. Although participants included in those groups are both affected by neurodegenerative diseases, this enabled us to find \textit{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., \textit{HOX} genes) and/or are located within the \textit{HOX} gene cluster (table e-2). In addition to the upregulation of multiple homeobox genes (top hit: \textit{HOXA5}) that play a vital role in neuronal development,\textsuperscript{14} we noticed a cerebellar upregulation of \textit{TTR} (table e-2), an extracellular protein that is thought to be involved in neuroprotection.\textsuperscript{15–19} We then compared expansion carriers with controls and

| Table 1 Participant characteristics |
|--------------------------|--------------------------|--------------------------|
| Cohort/variable          | \(C9\text{Plus}\) cohort (\(n = 32\)) | \(C9\text{Minus}\) 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 \(C9\text{Plus}\) and without \(C9\text{Minus}\) expansions in \textit{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 HOX5 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]).

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

![Expression of homeobox genes and transthyretin](image)

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.
### Expression studies of HOXA5 and TTR transcripts using TaqMan assays in the expression cohort

| Group            | HoXa5 C9Plus vs control cohort | HoXa5 C9Minus vs control cohort | C9Plus vs C9Minus | TTR C9Plus vs control cohort | TTR C9Minus vs control cohort | C9Plus vs C9Minus |
|------------------|--------------------------------|--------------------------------|-------------------|------------------------------|------------------------------|-------------------|
| Cerebellum       | 1.24e-12 (4.43–33.01)         | 0.91 (0.55–3.30)               | 1.00 (0.66–1.30)  | 5.41e-08 (8.59–26.86)        | 0.91 (0.55–3.30)               | 1.00 (0.66–1.30)  |
| Frontal cortex   | 0.04                           | 5.69 (1.88–15.42)              | 0.19               | 2.01 (0.35–21.18)            | 0.01                         | 0.01              |

Abbreviation: IQR = interquartile range.

Data were analyzed using Kruskal-Wallis rank-sum test and then pairwise comparisons were done using Bonferroni correction. 

As well as 2 intronic regions (1 upstream of the repeat expansion [intron 1a] and 1 downstream of the repeat expansion [intron 1b]). 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-09, Spearman test of correlation) and TTR (r = -0.47, p = 2.21e-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-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-05, Spearman test of correlation, table 4), most prominently in patients with a pathologic diagnosis of FTLD (r = 0.65,
transcript variant 1, ferroni correction. A Spearman test of correlation was used (significant after Bonferroni correction). Similar findings were obtained when normalizing to each outcome, and thus, controls, we examined 6 different associations (total HOXA5 and transcript variant 2, C9ORF72 transcripts). In total, in our overall cohort (expansion carriers, disease controls, and transcripts), 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. 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).

### 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. 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 test).

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

| TaqMan | Group | Association | Spearman r (95% CI) | p Value |
|--------|-------|-------------|---------------------|---------|
|        |       |             | Overall             |         |
| 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 |
| TTR    | Intron 1b | 0.36 (0.16 to 0.54) | 0.0008 |
|         |       | Total       | -0.19 (-0.39 to 0.02) | 0.06    |
|         |       | Variant 1   | -0.20 (-0.40 to 0.003) | 0.06    |
|         |       | 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-06, intron 1b: r = 0.54, p = 2.75e-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-05, Spearman test of correlation).

Because we previously discovered associations between dipeptide repeat proteins and intron-containing transcripts, 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. Although no significant associations were detected for TTR, we observed 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-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).
### Table 4

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

| TaqMan | Group | Association | C9Plus cohort | FTLD cohort | FTLD/MND cohort | MND cohort |
|--------|-------|-------------|---------------|-------------|----------------|------------|
|        |       |             | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |
|         |       | 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 |

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 (RPL0), 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, 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. 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, and that they are associated with the cognitive score of clinically diagnosed patients with ALS. 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. In addition, extensive alternative splicing and polyadenylation defects have been reported in the cerebellum of C9ORF72 expansion carriers. 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. 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. 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. 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.

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. Because the most prominent decrease has been reported for C9ORF72 variant 2 transcripts, 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. 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. Daughtry, 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. Keith A. Josephs, Joseph E. Parisi, David S. Knopman, Ronald C. Peterson, Leonard Perrucelli, Bradley F. Boeve, Nell L. Graff-Radford, Dennis W. Dickson, Michael Benatar, and Kevin B. Boyle: 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 Wu 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
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, Cytokinetix Inc., the Mayo Foundation, Neuraltus Pharmaceuticals, GlaxoSmithKline, Avanir Pharmaceuticals, and Synapse Biomedical. Rosa Rademakers receives research support from the NIH, the ALS Therapy Alliance, the Consortium for Frontotemporal Degen-
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Abnormal expression of homeobox genes and transthyretin in C9ORF72 expansion carriers
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