HTT exon 1 splice variants are non-allele specific and expressed at low levels in human brain

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ABSTRACT

As *HTT* mRNA-targeting therapeutics progress into clinical trials for Huntington's disease (HD), understanding the abundance of *HTT* isoforms in disease-relevant tissues is crucial. Previous studies in rodent models and human patients identified an *HTT* mRNA isoform, *HTT1a*, produced through alternative splicing within the first intron. *HTT1a* encodes an HTT exon 1 protein fragment which is prone to aggregation when it is derived from the expanded allele. However, short-read sequencing and PCR methods have been unable to accurately quantify *HTT1a* RNA and protein levels in HD patient brains.

To address this, we obtained fresh-frozen cortical samples and formalin-fixed paraffin-embedded (FFPE) caudoputamen samples from HD patients and healthy donors through the Netherlands Brain Bank. We then used next-generation sequencing, biochemistry, and histology to investigate *HTT1a* RNA and protein abundance. Advanced techniques, including long-read PacBio sequencing and digital PCR, revealed multiple *HTT1a* RNA isoforms in cortical samples, with one isoform significantly elevated in HD patients. These isoforms were transcribed at similar rates from both non-expanded (CAG <36) and mutant (CAG ≥36) alleles. *HTT1a* RNA levels were generally low (5–12% relative to full-length *HTT* mRNA) but reached up to 35% in severe HD cases (Vonsattel grade 4). HTT1a protein levels were also scarce (~5–11% of total mutant HTT protein) in cortical tissues, as detected by the newly developed 11G2 antibody targeting the Exon 1 neo-epitope. Immunohistological staining with the same antibody further showed no detectable HTT1a-derived protein in the caudate and putamen regions of HD brains.

These findings indicate that substantial *HTT1a* RNA expression may only occur during advanced neurodegeneration, with HTT1a protein remaining difficult to detect. Consequently, therapeutic strategies should prioritize targeting the more abundant full-length *HTT* mRNA or protein in early-stage HD.

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BACKGROUND

- HD is a rare neurodegenerative disorder caused by an autosomal dominant CAG repeat expansion (≥ 36) in the *HTT* gene which generates the mutant HTT protein (mHTT)[1]
- mHTT is believed to drive disease primarily through a toxic gain-of-function mechanism, which triggers nearly all cellular pathologies underlying disease development in medium spiny neurons in the striatum [2]
- Neuronal dysfunction and toxicity may be caused by the altered function of the full-length mHTT on its cellular targets and the accumulation of mHTT fragments in the cytoplasm and nucleus of medium spiny neurons [2-3]
- HTT lowering is considered a promising treatment for HD that aim to reduce the levels of mHTT protein[4]
- It is poorly understood whether toxicity is due to mHTT protein fragments generated from protease cleavage of the full-length protein and/or from alternative splicing that produces aberrant transcripts encoding solely for exon 1 protein fragment (HTT1a)[5-6]
- Understanding the abundance of HTT1a in human brain tissue can guide the design and development of HTT-lowering therapeutics

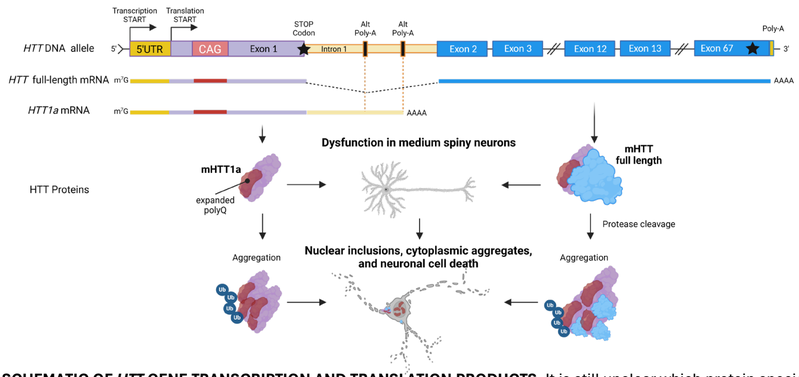

FIGURE 1. SCHEMATIC OF *HTT* GENE TRANSCRIPTION AND TRANSLATION PRODUCTS. It is still unclear which protein species (N-terminal cleaved or HTT1a derived) ultimately drives toxicity and cell death.

This study aims to advance the understanding of HTT1a abundance in human brain tissue and its relevance in driving HD pathology

EXPERIMENTAL APPROACH

Human Brain Samples								
	Case ID	Sample ID	Gender	CAG size 1	CAG size 2	Vonsattel Grade	Disease (yrs)	Age of Death	Cause of Death
CORTICAL FLASH-FROZEN SAMPLES	2009-063	HD 1	F	20	46	4	11	64	Pneumonia
	2021-063	HD 2	M	19	47	3	9	57	Pneumonia
	2013-076	HD 3	M	19	43	1	8	71	Euthanasia
	2019-070	HD 5	F	19	45	1	9	44	Euthanasia
	2020-001	HD 6	F	19	44	1	19	67	Euthanasia
	2021-050	HD 11	M	19	43	3	11	73	Infection
	2019-036	Ctrl 4	F	19	21	-	-	85	Cancer
	2020-054	Ctrl 7	F	20	27	-	-	81	Cancer
	2020-113	Ctrl 8	M	19	19	-	-	87	Cancer
	2020-071	Ctrl 9	F	12	20	-	-	79	NA
	2021-051	Ctrl 10	M	19	19	-	-	77	Cancer
	2021-119	Ctrl 12	M	17	20	-	-	73	NA
FFPE CAUDOPUTAMEN	2019-070	HD 16	F	NA	NA	2	9	44	Euthanasia
	2020-001	HD 6	F	19	44	1	19	67	Euthanasia
	2021-063	HD 18	M	NA	44	NA	9	57	Pneumonia
	1996-052	Ctrl 13	F	NA	NA	-	-	73	NA
	2001-011	Ctrl 14	F	NA	NA	-	-	46	NA
	2014-043	Ctrl 15	F	NA	NA	-	-	60	Cancer
	2021-051	Ctrl 17	M	NA	NA	-	-	77	Cancer

Crtl 12 sample excluded from analysis due to poor RIN; dPCR = digital PCR; HTRF = Homogeneous Time Resolved FRET

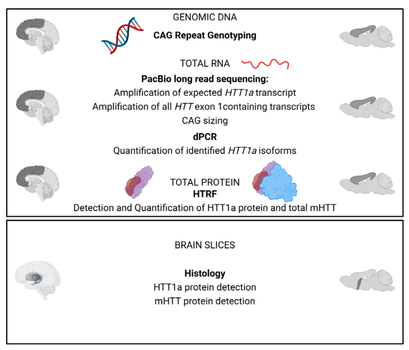

FIGURE 2. SCHEMATIC OF THE EXPERIMENTAL PIPELINE TO DETECT AND QUANTIFY HTT1a PROTEIN

RESULTS

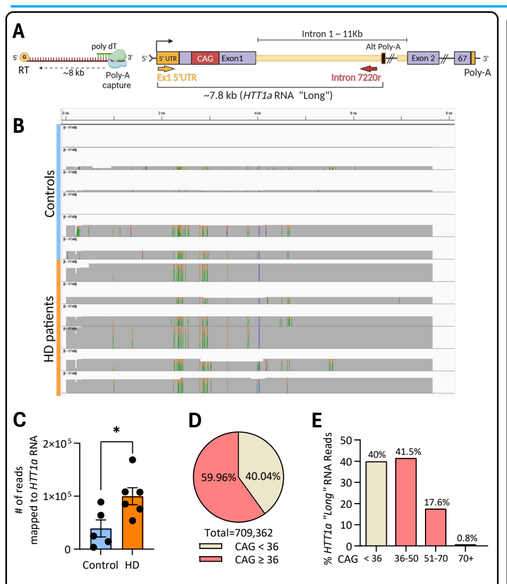

FIGURE 3. HIGHER EXPRESSION OF *HTT1a* "LONG" RNA IN HD PATIENTS IS NOT DRIVEN BY SOMATIC INSTABILITY, INDICATING THAT CAG LENGTH ALONE DOES NOT SOLELY DETERMINE *HTT1a* ALTERNATIVE SPLICING. A. *Left*. Overview of RT-PCR of total RNA from human cortical tissue by end-point PCR followed by PacBio long read sequencing. *Right*. Schematic of *HTT* pre-mRNA. PolyA site[7] for the "long" *HTT1a* isoform is positioned ~7.8kb downstream from the transcription start site (+1 nt, NM001388492.1). *Arrows* indicate primer sets used to generate the long RT-PCR amplicons. **B.** Alignment of Pacbio long read amplicon sequencing to the predicted *HTT1a* "Long" reference. **C.** Total Pacbio reads mapping to *HTT1a* RNA reference. Two tailed *t*-test, *p < 0.05. *Error bars* are SEM. **D.** Percentage of *HTT1a* reads from HD patients from the mutant and normal allele. Binomial test indicates significant difference between the expected 50% frequency p < 0.0001). **E.** Percentage of *HTT1a* RNA reads from HD patients with CAG sizes between the indicated ranges.

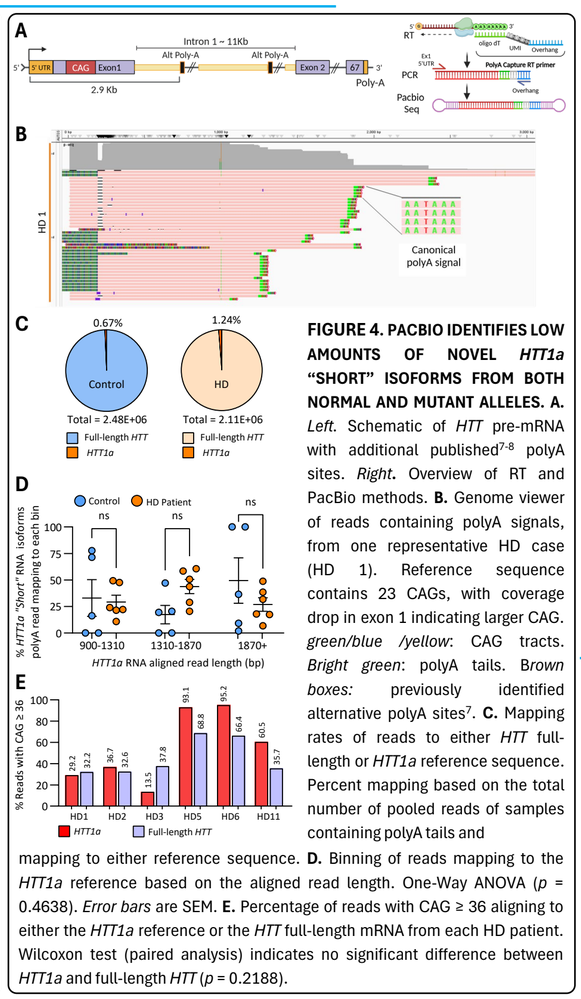

FIGURE 4. PACBIO IDENTIFIES LOW AMOUNTS OF NOVEL *HTT1a* "SHORT" ISOFORMS FROM BOTH NORMAL AND MUTANT ALLELES. A. *Left.* Schematic of *HTT* pre-mRNA with additional published[7-8] polyA sites. *Right.* Overview of RT and PacBio methods. **B.** Genome viewer of reads containing polyA signals, from one representative HD case (HD 1). Reference sequence contains 23 CAGs, with coverage drop in exon 1 indicating larger CAG. *green/blue /yellow*: CAG tracts. *Bright green*: polyA tails. *Brown boxes:* previously identified alternative polyA sites[7]. **C.** Mapping rates of reads to either *HTT* full-length or *HTT1a* reference sequence. Percent mapping based on the total number of pooled reads of samples containing polyA tails and mapping to either reference sequence. **D.** Binning of reads mapping to the *HTT1a* reference based on the aligned read length. One-Way ANOVA (p = 0.4638). *Error bars* are SEM. **E.** Percentage of reads with CAG ≥ 36 aligning to either the *HTT1a* reference or the *HTT* full-length mRNA from each HD patient. Wilcoxon test (paired analysis) indicates no significant difference between *HTT1a* and full-length *HTT* (p = 0.2188).

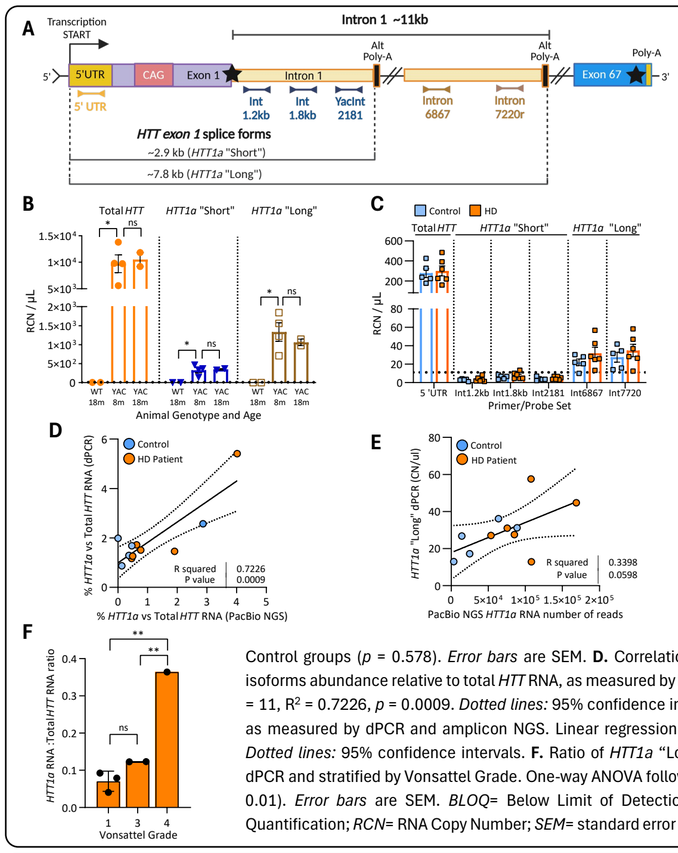

FIGURE 5. *HTT1a* "LONG" RNA ISOFORMS ARE MORE ABUNDANT THAN *HTT1a* "SHORT ISOFORMS, BUT OVERALL STILL EXPRESSED AT MUCH LOWER LEVELS THAN FULL-LENGTH *HTT* RNA. A. Schematic of *HTT* primer sets for dPCR analysis. **B.** Total *HTT*, "Short", and "Long" *HTT1a* RNA dPCR quantification in YAC128 cortical samples. WT samples were *BLOQ*. Two-way ANOVA analysis: significant differences between groups (p= 0.0169), primer sets (p=0.0012), and the interaction between group and primer set (p=0.017). Multiple comparisons evaluated with Tukey's post-hoc test (* p < 0.05). *Error bars* are SEM. **C.** *HTT1a* isoforms dPCR quantification in cortical human samples (n = 5-6 per group). *HTT1a* "Short" RNA isoforms were mostly *BLOQ*, while *HTT1a* "Long" RNA isoforms accounted for an average of ~8-10% of total *HTT* RNA in controls and ~13-14% in HD patients. *Dotted line: LLOQ*. Two-way ANOVA analysis: significant differences between primer sets (p < 0.0001); no significant difference between HD and Control groups (p = 0.578). *Error bars* are SEM. **D.** Correlation of the average of all *HTT1a* "Short" RNA isoforms abundance relative to total *HTT* RNA, as measured by NGS and dPCR. Linear regression analysis, n = 11, R^2 = 0.7226, p = 0.0009. *Dotted lines:* 95% confidence intervals. **E.** Correlation of *HTT1a* "Long" RNA as measured by dPCR and amplicon NGS. Linear regression analysis, n = 11, R^2 = 0.3398, p = 0.0598. *Dotted lines:* 95% confidence intervals. **F.** Ratio of *HTT1a* "Long" RNA to total *HTT* RNA as measured by dPCR and stratified by Vonsattel Grade. One-way ANOVA followed by Tukey's multiple comparisons (**p < 0.01). *Error bars* are SEM. *BLOQ*= Below Limit of Detection; *LLOQ* (11.22 RCN/µL)= Lower Limit of Quantification; *RCN*= RNA Copy Number; *SEM*= standard error of the mean.

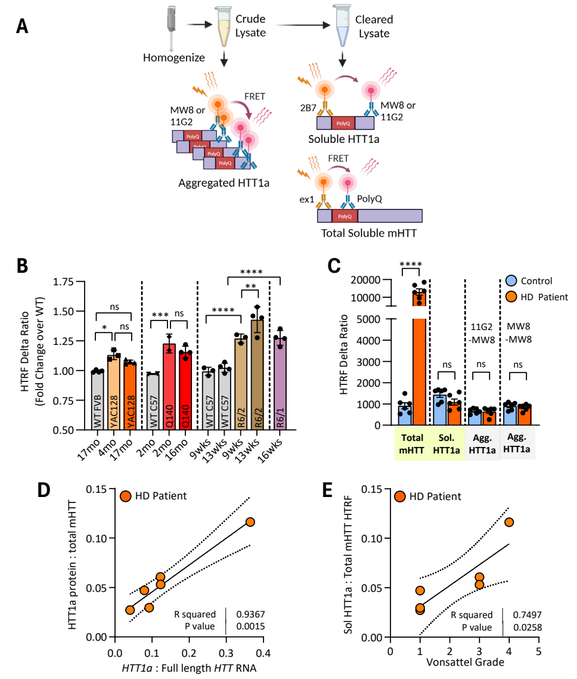

FIGURE 6. HTT1a PROTEIN ANALYSIS IS CONSISTENT WITH *HTT1a* RNA FINDINGS, INDICATING THAT HTT1a PROTEIN LEVELS ARE LOW IN HD PATIENTS BUT SLIGHTLY INCREASE IN MORE SEVERE PATHOLOGICAL STAGES. A. Overview of the HTRF method. Antibody pairs: anti-exon1 and anti-polyQ (total soluble mHTT); 2B7 and either 11G2 or MW8 (soluble (*Sol.*) HTT1a); both MW8 or both 11G2 (aggregated (*Agg.*) HTT1a). **B.** Test of the 2B7-11G2 soluble HTT1a protein assay on striatal lysates from various HD mouse lines at different ages and relative WT controls. Data represent the background subtracted HTRF delta ratio normalized to WT littermates. One-way ANOVA (p < 0.0001) followed by Sidak's multiple comparisions test between the indicated groups. *ns* = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. **C.** HTRF analysis of human cortical lysates from HD patients and controls. Two tailed *t*-tests were carried out for each assay. *ns* = not significant; ****p < 0.0001. **D.** Correlation between the ratio of HTT1a protein and full-length mHTT protein as measured by HTRF and *HTT1a* "long" abundance relative to total *HTT* (5' UTR primer set) as measured by dPCR. *Dotted lines*: 95% confidence intervals. **E.** Correlation between the ratio of HTT1a protein and full-length mHTT as measured by HTRF and Vonsattel pathology grade. *Dotted lines*: 95% confidence intervals.

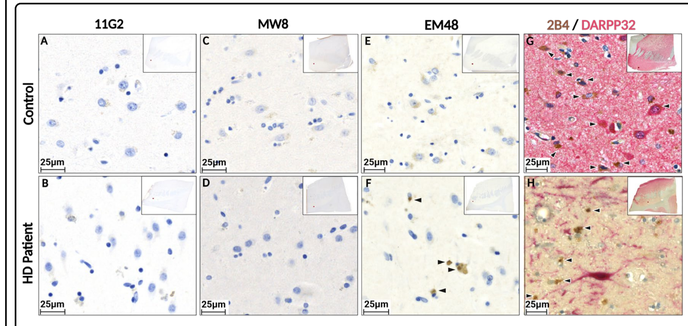

FIGURE 7. HTT1A DERIVED PROTEIN IS NOT READILY DETECTABLE IN STRIATAL SAMPLES, WHEREAS FULL-LENGTH AND OTHER N-TERMINAL FRAGMENTS ARE PRESENT. Representative image from control (#14) and HD patient (#6) putamen. 20X images. Counterstained blue with Hematoxylin. **A-B-C-D.** HTT1a protein detection with 11G2 or MW8 antibodies. **E-F.** mHTT specific detection with EM48 antibody (*arrow heads*) antibody. **G-H.** total HTT protein detection with 2B4 antibody (*brown, arrow heads*) and DARPP32 (*red*, medium spiny neuron marker).

CONCLUSIONS

- Use of sensitive assays and reagents that enhance detection and quantification of *HTT* transcript and protein isoforms confirmed that HTT1a is expressed only at low levels in adult-onset HD patients' cortical and striatal brain regions, as previously published
- Long-read sequencing results suggest that *HTT1a* transcripts can be produced from both the expanded and non-expanded *HTT* alleles in individuals with HD
- HTT1a RNA and protein are more abundant in patients with severe neuropathology but remain a minimal fraction of total HTT, suggesting they are not major contributors to HD pathology
- Since *HTT1a* transcripts make up only a small fraction of total *HTT* mRNAs and histology of terminal samples reveals HTT but not HTT1a aggregates, HTT-lowering therapies targeting transcripts downstream of exon 1 are expected to provide similar benefit to those targeting exon 1 directly.

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