Supporting Information

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SI Materials and Methods

Specificity Validation of pT3 Singulex Assay on HEK293T Cell Lysates by siRNA (Endogenous and Overexpressed FL-HTT). For real-time PCR, relative HTT mRNA expression was evaluated by standard calculation of threshold values (Ct) (Fig. S2*A*); for Singulex assays, the relative signal (%) was calculated for each readout using the signal in the linear range and fixing the Scrambled siRNA signal as reference (Fig. 3*B*).

Specificity Validation by T3A Mutants (Fig. 3*D*). HEK293T cell lysates were analyzed by MW1/2B7 and MW1/pT3 Singulex assays. The fold difference between EX1 and EX1 T3A mutants was calculated using the MW1/pT3 signal in the linear range and fixing the pT3 signal detected on the T3A mutant (HTT endogenous signal) as reference; paired *t*-test was performed (two-tailed; $^{*}P < 0.05$; $^{**}P < 0.01$) using GraphPad Prism software.

PolyQ Influence on T3 Phosphorylation in Knockin Mouse Cortex (Figs. 4 *B* and *C* and 5 *B*, *ii*), cerebellum (Fig. 5 *A*, *ii*), striatum (Fig. S6*B*), and iPSCs (Fig. 6*A*). Relative pT3 HTT levels normalized on total HTT levels are determined as the ratio between the fold increase obtained from the MW1/pT3 Singulex assay and the fold increase calculated on the MW1/2B7 (or 2166) readout. This ratio was obtained as follows: MW1/pT3 Singulex assay and MW1/2B7 (or 2166) Singulex assay were performed in parallel on the same samples. For each readout, a curve fitting (described by a four-parameter logistic curve fit, as shown in Figs. S3, S5, S6*A*, and S7) was calculated. For both MW1/pT3 and MW1/2B7 (or 2166) readouts, a fold increase among curves was assessed, which was determined by interpolating an arbitrary constant signal value within the linear range, above the limit of detection and shared among the curves of each readout (fixing as a reference one of the analyzed samples). The statistical significance was assessed using an unpaired *t*-test (two-tailed; $^{*}P < 0.05$ $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$) in which CTRL samples versus HD samples were compared, and using one-way ANOVA where more groups were compared.

PolyQ Influence on T3 Phosphorylation in PBMCs (Fig. 6*B*). The pT3-HTT and total-HTT concentrations were obtained through a back-calculation on the reference standard (pT3-HTT semisynthetic protein) by interpolation of the signals of MW1/pT3 (Fig. S8*A*) and MW1/2B7 (Fig. S8*B*) readouts for each sample (tested in duplicate and in serial dilution points). The relative pT3 HTT levels normalized with MW1/2B7 were obtained by calculating the ratio of the pT3 HTT on total-HTT concentration for each sample. Unpaired *t*-test was applied (two-tailed; $^{*}P < 0.05$).

Conformational TR-FRET Immunoassay on Semisynthetic Proteins (Fig. 7 *A* and *B*). The data analysis and statistics were performed as previously described (24) (one-way ANOVA test; $^{***}P < 0.005$).

Densitometry (Fig. 7*C*). The relative signal (%) was obtained by a densitometric analysis, performed using the ImageJ software (paired *t*-test, two-tailed: $^{*}P < 0.05$; $^{**}P < 0.01$).

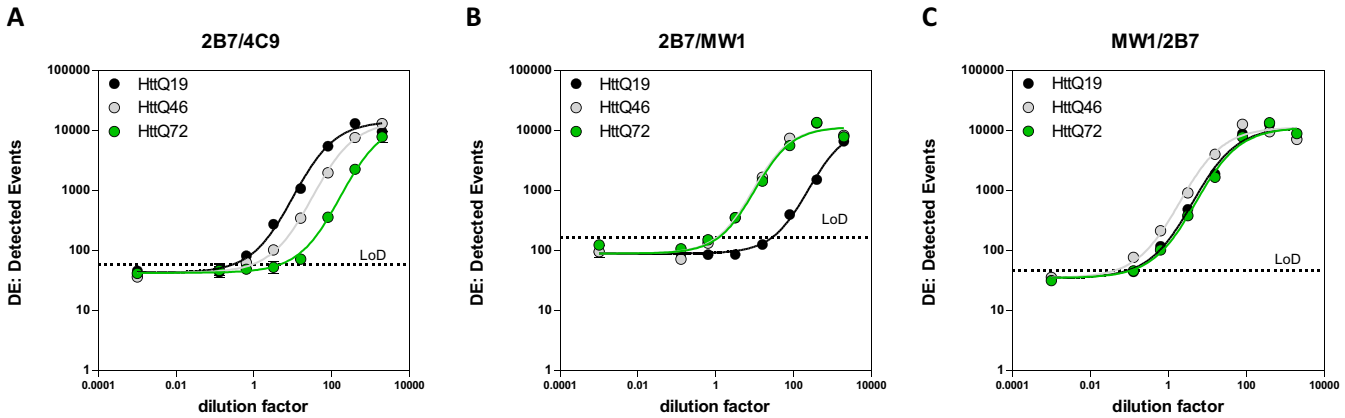

Fig. S1. Comparative analysis of different anti-HTT Ab combinations in the Singulex assay on recombinant HTT N548 proteins with different CAG repeats (Q19, Q46, Q72). The MW1/2B7 assay clearly detects WT HTT and is much less sensitive to (although not devoid of) variations in polyQ length than the combination 2B7/MW1. (*A*) Signal obtained using a polyQ-independent Singulex immunoassay, employing 2B7 (anti-N17 domain) used as a capture Ab and 4C9 (anti-polyP domain) used as a detection Ab. (*B*) Signal obtained on the same protein dilutions using a Singulex immunoassay employing 2B7 (anti-N17 domain) used as a capture Ab and MW1 (anti-polyQ domain) used as a detection Ab. (*C*) Signal obtained on the same protein dilutions with MW1 as a capture Ab in combination with 2B7 as a detection Ab.

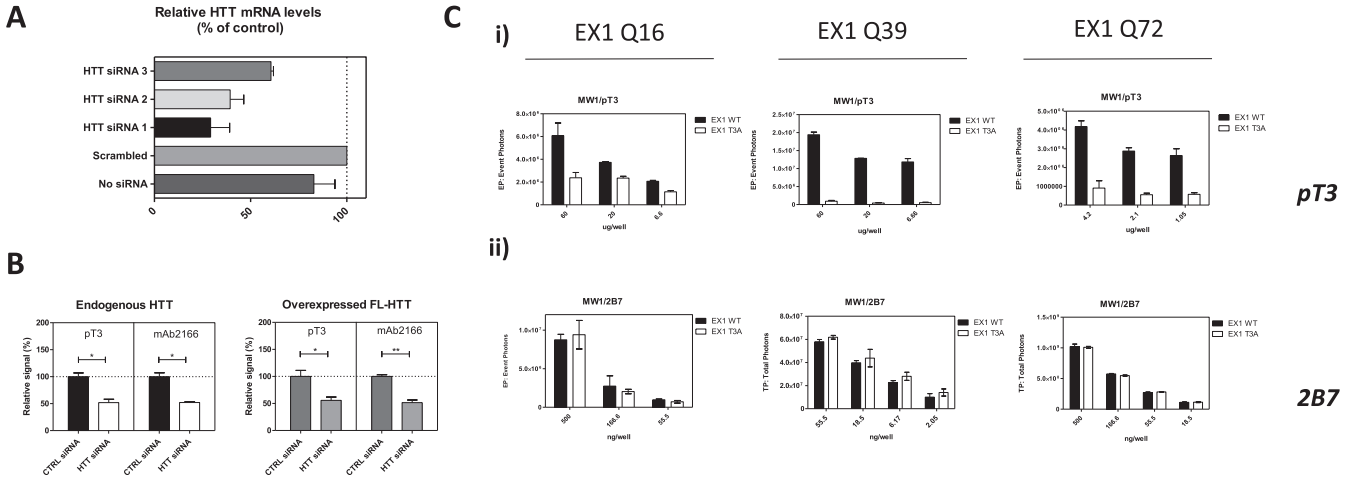

Fig. S2. Specificity evaluation of pT3-HTT detection by siRNA and phosphor-abrogative mutation in HEK293T cells. (*A*) Real-time PCR evaluation of HTT mRNA levels knockdown efficiency of three commercial siRNAs specific for *HTT* in HEK293T cells. Relative levels (to cells not transfected with siRNA) are shown, from which *HTT* siRNA1 was selected as the most effective. (*B*) Densitometric analysis of corresponding WB (Fig. 3*A*). Means and SD of three independent experiments, paired *t*-test (two-tailed; $*P < 0.05$; $**P < 0.01$). (*C*) Singulex analysis using MW1/pT3 (*i*) or MW1/2B7 (*ii*) showing the effects of T3A mutations on HTT pT3 or total HTT signals. Data demonstrate that the T3A mutation affects only the signal from the HTT pT3 Singulex immunoassay; results are from a representative experiment from a set of three independent experiments.

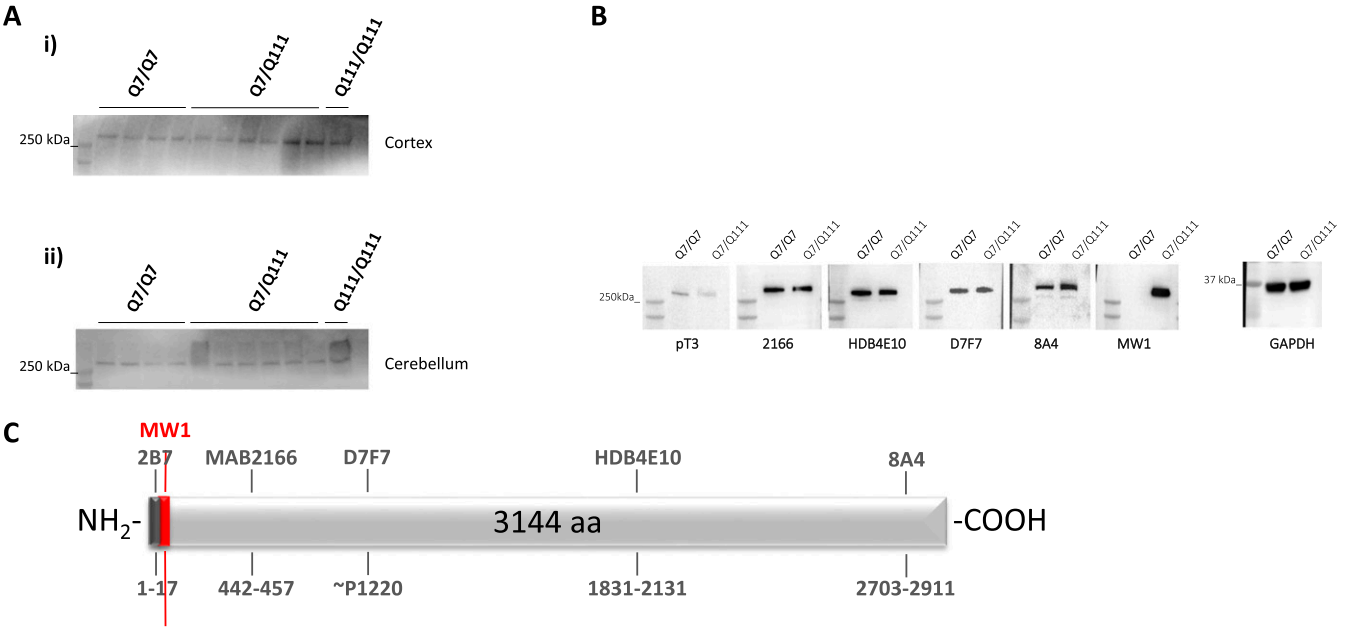

Fig. S3. Western immunoblots of CNS tissue samples from Q7/Q111 and Q111/Q111 knock-in HD mice probed with different anti-HTT antibodies. (*A*) Western immunoblot of (*i*) cortex or (*ii*) cerebellum homogenates from Hdh knock-in mouse (6 mo), analyzed using monoclonal antibody D7F7 for total HTT (epitope around P1220), confirming comparable expression of huntingtin protein in all samples as also detected by MAB2166 (Figs. 4*A* and 5). (*B*) pT3 levels and total HTT levels analyzed in homogenates from pooled whole brains of Hdh knock-in mice. pT3 levels are decreased in whole brains from mutant mice, despite comparable levels of HTT expression in WT and mutant HD mice as demonstrated by a variety of anti-HTT antibodies specific for different epitopes on the HTT protein. (*C*) Position of epitopes for different anti-HTT antibodies used in this study along the HTT protein.

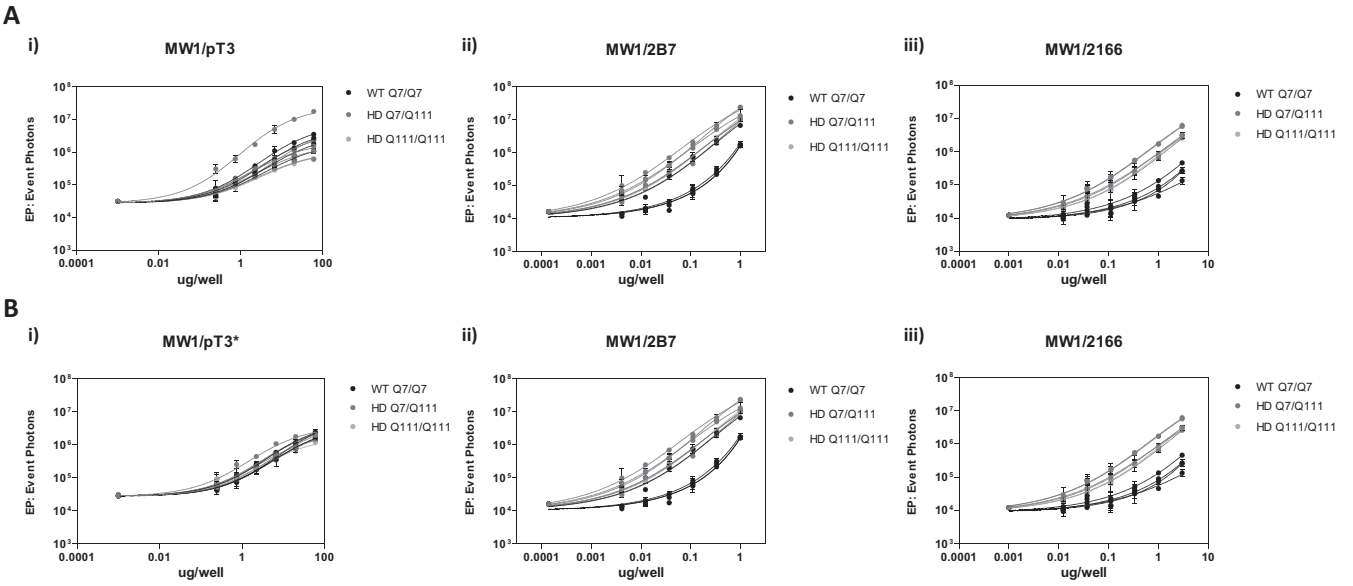

Fig. S4. Singulex assay analysis of brain cortex homogenates. (*A*) Serial dilutions of each brain cortex homogenate were subjected to Singulex immunoassay for HTT pT3 (MW1/pT3) (*i*) or for total HTT using two independent immunoassays [MW1/2B7 (*ii*) and MW1/2166 (*iii*)]. (*B*) Serial dilutions of brain cortex homogenates (same as in Fig. S3*A*) were subjected to Singulex immunoassay for HTT pT3 (MW1/pT3 with anti-pT3 Ab from ref. 39) (*i*) or for total HTT using two independent immunoassays [MW1/2B7 (*ii*) and MW1/2166 (*iii*)].

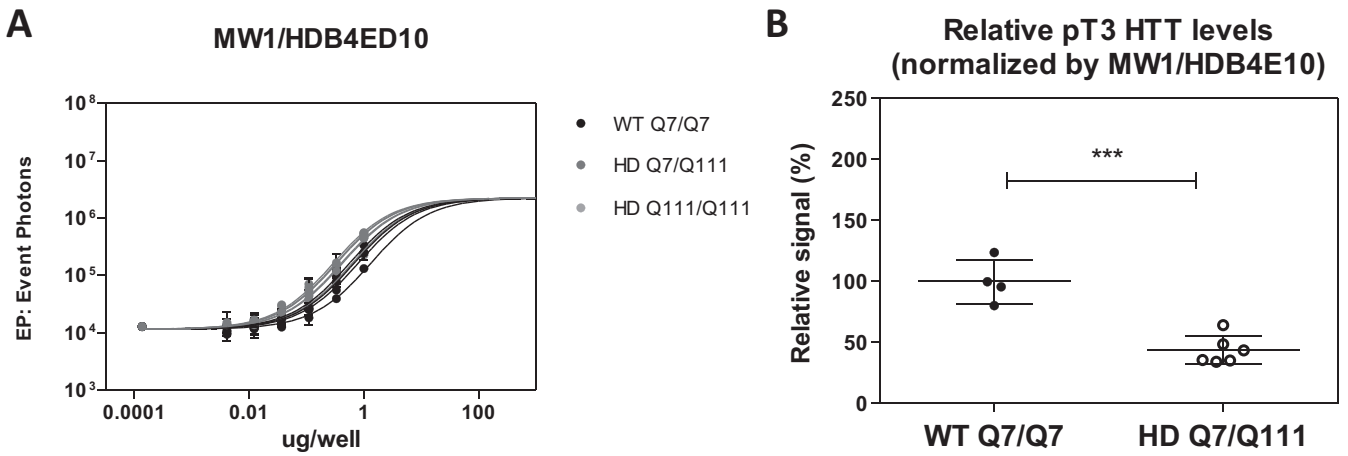

Fig. S5. Total HTT levels in mouse brain cortex homogenates using the MW1/HDB4E10 Singulex immunoassay. (*A*) Serial dilutions of brain cortex homogenates (same as in Fig. S4) were subjected to Singulex immunoassay for HTT using MW1 as a capture antibody and HDB4E10 as a detection antibody. (*B*) Relative HTT pT3 Singulex signal in brain cortex samples from WT (Q7/Q7) and heterozygous mutant (Q7/Q111) Hdh HD animals, normalized to total HTT measured with the MW1/HDB4E10 Singulex immunoassay, demonstrating the coherence of the effects of the HD mutation on normalized pT3 HTT levels in WT and mutant mouse brain cortex samples irrespective of the anti-HTT antibody employed for total HTT measurement. Means and SD of four Q7/Q7 samples and six Q7/Q111 samples, unpaired *t*-test (two-tailed; ***$P < 0.05$).

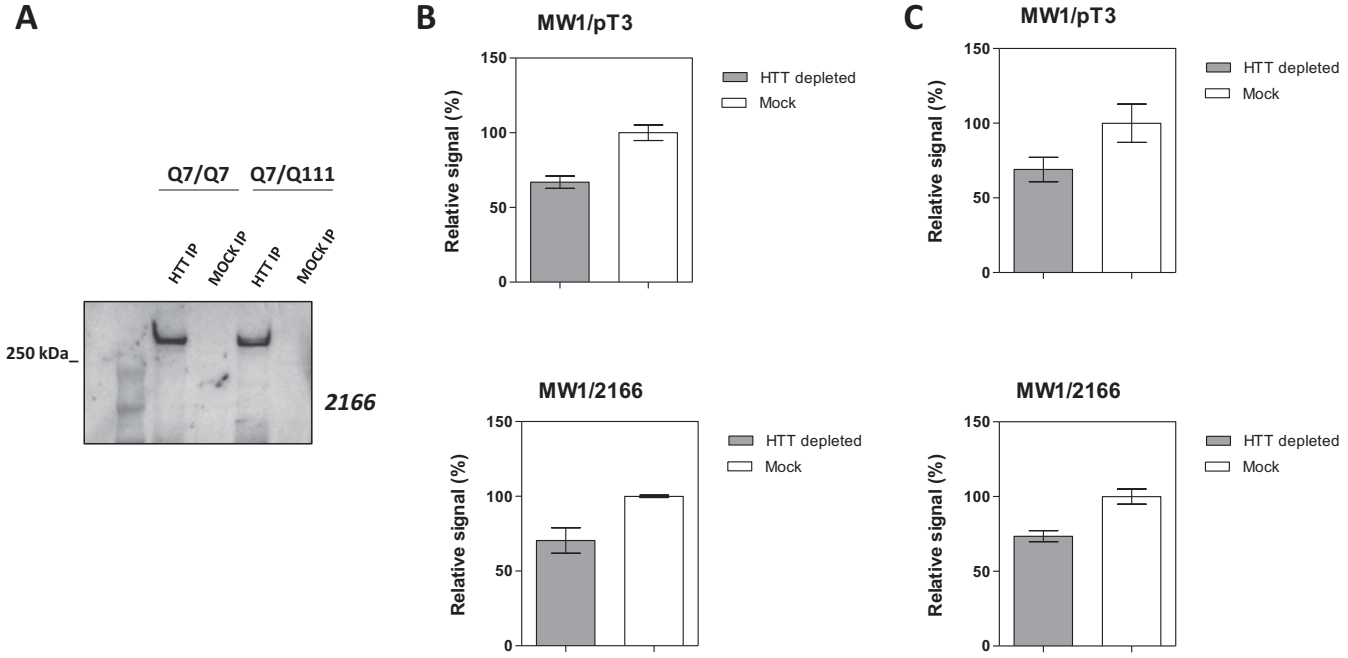

Fig. S6. Specificity of the HTT pT3 signal obtained in mouse brain cortex. (*A*) Western blotting of HTT protein immunoprecipitated from WT Hdh Q7/Q7 or heterozygous mutant Q7/Q111 mouse brain cortex using antibody 2B7 or an unrelated antibody, demonstrating successful (specific) immunoprecipitation of HTT protein from the samples. (*B*) Hdh Q7/Q7 mouse brain cortex immunodepleted samples were subjected to analysis using the MW1/pT3 or MW1/2166 Singulex immunoassays, demonstrating a specific decrease in both HTT pT3 and total HTT signal in HTT immunodepleted samples. (*C*) Same as *B*, but for Hdh Q7/Q111 mouse brain cortex.

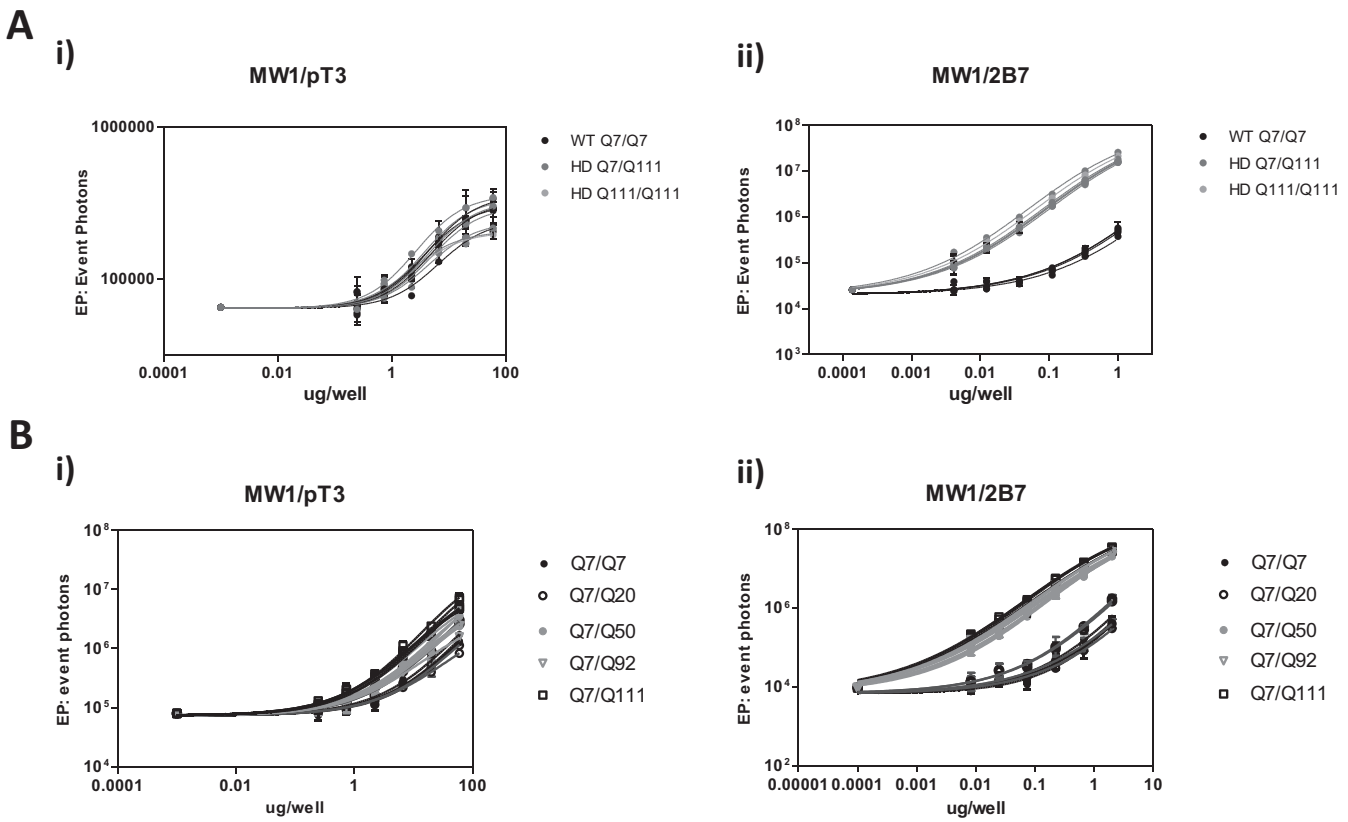

Fig. S7. Singulex assay analysis of brain cerebellum homogenates. (*A*) Serial dilutions of each brain cerebellum homogenate were subjected to a Singulex immunoassay for HTT pT3 (MW1/pT3) (*i*) or for total HTT using the MW1/2B7 Singulex immunoassay (*ii*). (*B*) Serial dilutions of brain cortex homogenates from Hdh mice (allelic series, Q7-Q111; Fig. 5*B*) were subjected to a Singulex immunoassay for HTT pT3 (MW1/pT3) (*i*) or for total HTT using the MW1/2B7 Singulex immunoassay (*ii*).

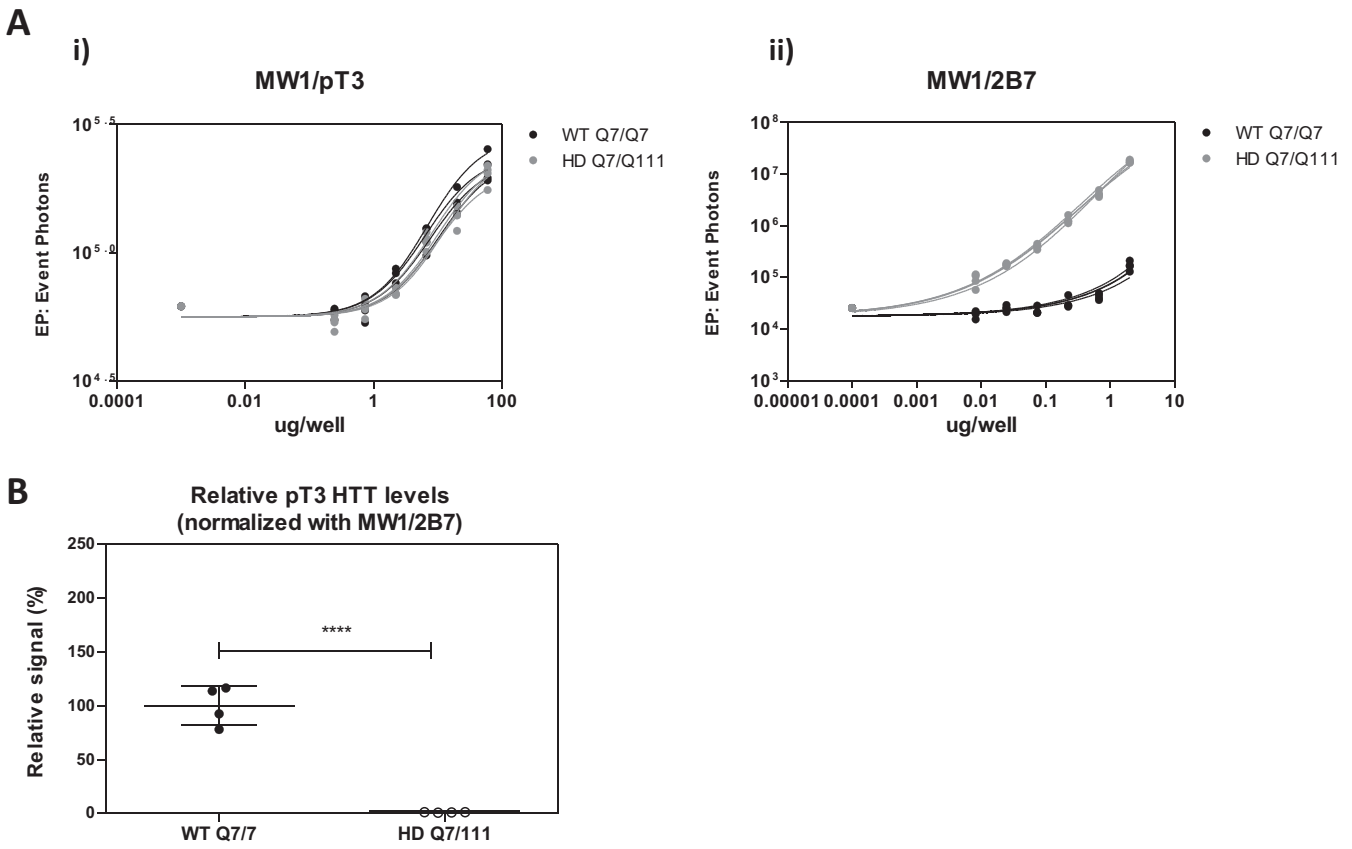

Fig. S8. Singulex assay analysis of brain striatum homogenates. (*A*) Serial dilutions of each brain striatum homogenate were subjected to Singulex immunoassay for HTT pT3 (MW1/pT3) (*i*) or for total HTT using the MW1/2B7 Singulex immunoassay (*ii*). (*B*) Relative HTT pT3 Singulex signal in brain striatum samples from WT (Q7/Q7) and heterozygous mutant (Q7/Q111) Hdh HD animals, normalized to total HTT measured with the MW1/2B7 Singulex immunoassay. Means and SD of four Q7/Q7 samples and four Q7/Q111 samples, unpaired *t*-test (two-tailed; ****$P < 0.001$).

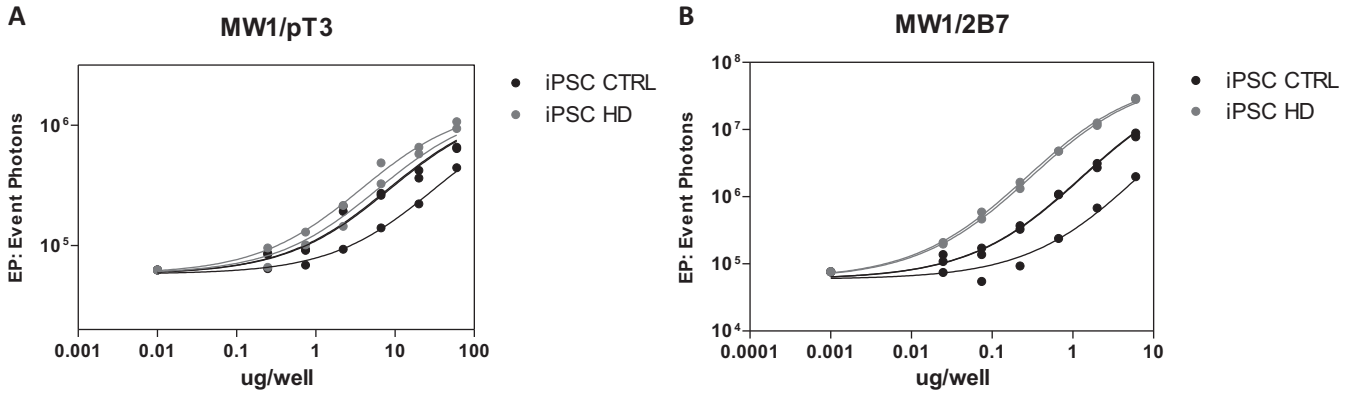

Fig. S9. Singulex assay analysis of IPSC-derived neuronal population protein lysate. Serial dilutions of each iPSC-derived neuronal population protein lysate were subjected to Singulex immunoassay for HTT pT3 (MW1/pT3) (*A*) or for total HTT using the MW1/2B7 Singulex immunoassay (*B*).

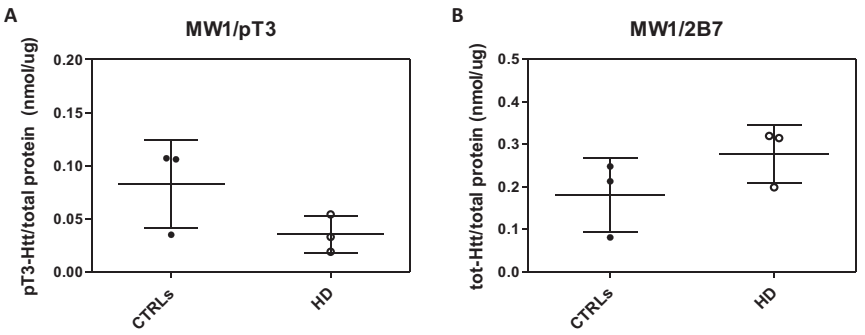

Fig. S10. Singulex assay analysis of lysates from peripheral (blood) PBMCs isolated from HD individuals and normal individuals. Back-calculated HTT pT3 protein concentration (*A*) or total HTT protein (*B*) in lysates from peripheral (blood) PBMCs isolated from HD individuals or from normal individuals, detected by Singulex assays (MW1/pT3 and MW1/2B7, respectively).