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Mutational analysis implicates the amyloid fibril as the toxic entity in Huntington's disease

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Abstract

In Huntington disease (HD), an expanded polyglutamine (polyQ > 37) sequence within huntingtin (htt) exon1 leads to enhanced disease risk. It has proved difficult, however, to determine whether the toxic form generated by polyQ expansion is a misfolded or avid-binding monomer, an α -helix-rich oligomer, or a β -sheet-rich amyloid fibril. Here we describe an engineered htt exon1 analog featuring a short polyQ sequence that nonetheless quickly forms amyloid fibrils and causes HD-like toxicity in rat neurons and *Drosophila*. Additional modifications within the polyQ segment produce htt exon1 analogs that populate only spherical oligomers and are non-toxic in cells and flies. Furthermore, in mixture with expanded-polyQ htt exon1, the latter analogs in vitro suppress amyloid formation and promote oligomer formation, and in vivo rescue neurons and flies expressing mhtt exon1 from dysfunction and death. Thus, in our experiments, while htt exon1 toxicity tracks with aggregation propensity, it does so in spite of the toxic construct's possessing polyQ tracts well below considered to be disease-associated. That is, aggregation propensity proves to be a more accurate surrogate for toxicity than is polyQ repeat length itself, strongly supporting a major toxic role for htt exon1 aggregation in HD. In addition, the results suggest that the aggregates that are most toxic in these model systems are amyloid-related. These engineered analogs are novel tools for mapping properties of polyQ self-assembly intermediates and products

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that should similarly be useful in the analysis of other expanded polyQ diseases. Small molecules with similar amyloid inhibitory properties might be developed into effective therapeutic agents.

Keywords

polyglutamine; β -hairpin; oligomers; primary neurons; *Drosophila*; thioflavin T; amyloid; huntintin exon 1; aggregation

Introduction

Huntington's disease (HD) is a progressive neurological disorder characterized by a CAG repeat expansion coding for polyglutamine (polyQ) in the first exon (htt-ex1) of the *huntingtin* gene (Bates et al., 2015). Normal human huntingtin varies between 10–35 glutamine repeats, while the disease threshold is at ~37–40 repeats. Therapies for directly intervening in disease progression at the protein level are lacking, in part because of our ignorance of the identities and nuances of the post-expression molecular events that trigger the initial cellular toxic events. At least three distinct hypotheses for a basic-of-function" molecular mechanism have articulated, positing that expanded polyQ tracks either (a) drive formation of a toxic conformation within the monomer ensemble (Peters-Libeu et al., 2012; Trotter et al., 1995), (b) enhance the interaction energy between the disordered monomer and a cellular target (Bennett et al., 2002), or (c) drive formation of a toxic self-associated or aggregated state (Davies et al., 1997).

Support for the aggregation hypothesis includes observation of large inclusions with fibrillary ultrastructure in post-mortem brains of patients and in animal and cell models (Bauerlein et al., 2017; Davies et al., 1997; DiFiglia et al., 1997) along with the demonstration that polyQ aggregates formed *in vitro* are toxic when taken up by cells in culture (Kar et al., 2014; Nekooki-Machida et al., 2009; Yang et al., 2002). In addition, the polyQ repeat length dependence of HD age of onset and severity (Bates et al., 2015), as well as amyloid aggregate burden and disease progression in cell and animal models (Lunkes and Mandel, 1998; Schilling et al., 1999), are mutually correlated with the repeat length dependence of polyQ amyloid formation kinetics *in vitro* (Chen et al., 2001; Scherzinger et al., 1997). These correlations lead directly to the attractive hypothesis that polyQ repeat expansion increases polyQ aggregation, which in turn causes disease. However, it could be argued that polyQ repeat expansion directs HD pathology *via* other, as yet uncharacterized, biophysical, biochemical and biological mechanisms, while aggregation in response to repeat expansion is an independent phenomenon and a red herring.

The self-association landscape of htt-ex1 is complex, featuring, at a minimum, monomers, dimers and tetramers (Sahoo et al., 2016), spherical oligomers (Poirier et al., 2002), isolated nanofibrils (Sahl et al., 2016; Sahoo et al., 2016), and clusters of mature fibrils (Scherzinger et al., 1997) and fibril-rich inclusions (Bauerlein et al., 2017; DiFiglia et al., 1997). Adding to the complexity are large overlaps in the time scales of interconversion between these forms. Thus, the near-simultaneous appearance of several self-associated states during a phased aggregation process complicates the interpretation of experiments seeking to correlate the timing of the appearance of specific misfolded forms with toxic events within

the cell (Sahoo et al., 2016). The implied low energy barriers to the interconversion of various forms also complicate attempts to isolate and test particular intermediates in cell and *in vitro* models.

The mechanistic and structural basis of htt-ex1 self-association and aggregation have been described (Fig. 1). In a polyQ repeat length dependent manner, the intrinsically disordered htt-ex1 monomer undergoes non-covalent self-association into dimers, tetramers, and higher non- β ("spherical") oligomers via concerted formation and packing α -helical forms of the htt-ex1 N-terminal segment, htt^{NT} (Arndt et al., 2015; Chen and Wolynes, 2017; Jayaraman et al., 2012a; Monsellier et al., 2015; Pandey et al., 2018; Sahoo et al., 2016; Thakur et al., 2009). This self-association dramatically enhances the local concentration of polyQ chains within oligomers, facilitating a second kinetic phase featuring polyQ-repeat length dependent formation of a ThT-positive amyloid core (Chen and Wolynes, 2017; Jayaraman et al., 2012a; Misra et al., 2016; Pandey et al., 2018; Thakur et al., 2009). The resulting fibrils are initially small and diffusible (Sahoo et al., 2016) but ultimately grow into long amyloid fibrils whose 3D structures consist of polyQ β -hairpins aligned into anti-parallel β -sheets (Hoop et al., 2016).

Armed with this knowledge of fundamental polyQ solution behavior (Wetzel, 2012) and the htt-ex1 self-association mechanism, in this paper we design, construct and validate *in vitro* a series of htt-ex1 analogs in which we alter both the polyQ-repeat length dependence of aggregation and the time scales for interconversion of aggregation intermediates. We then probe the toxicity of these forms in transfected primary rat neurons and in *Drosophila*. The results show that a htt-ex1 analog that rapidly aggregates to an amyloid endpoint in spite of its low polyQ length is toxic in neurons and flies. Furthermore, novel, amyloid-impaired htt-ex1 analogs that in isolation aggregate slowly to a non- β oligomer endpoint are - toxic completely. non Finally, the amyloid-impaired htt-ex1 analogs slow expanded polyQ htt-ex1 amyloid formation *in vitro* by prolonging the non- β -oligomer phase, and, *in vivo*, these inhibitory analogs protect neurons and flies from polyQ toxicity. Our results clarify the nature of the toxic species in HD, provide general tools for the similar analysis of other expanded polyQ diseases, and suggest ways to configure *in vitro* screens for small molecule inhibitors that should be more precisely focused on inhibiting formation of the HD toxic species.

Materials and methods

Materials.

Water (HPLC grade), acetonitrile (99.8% HPLC grade), formic acid, and HFIP (99.5%, spectrophotometric grade) were from Acros Organics. Trifluoroacetic acid (99.5%) was from Pierce and phosphate buffered saline from Invitrogen. All peptides were synthesized at the Small Scale Synthesis facility at the Keck Biotechnology Resource Laboratory of Yale University and supplied crude. All synthetic peptides were purified and disaggregated, immediately before use, as previously described (O'Nuallain et al., 2006).

Sedimentation Assay.

Following rigorous disaggregation (O’Nuallain et al., 2006), ultracentrifugation (100,000 rpm, 4 °C, 2 hrs), and passage through a 0.02 µm filter, samples were allowed to aggregate in PBS buffered water. At specific times, aliquots of the reaction were centrifuged (21,000 g, 4 °C, 30 mins), and the supernatant concentrations were measured using analytical HPLC based standard curves, as previously described (O’Nuallain et al., 2006). Nucleation analysis was carried out using the initial rates from the sedimentation assay, as previously described (Chen et al., 2002; Kar et al., 2011; O’Nuallain et al., 2006).

Fractional population analysis of aggregation reactions.

Previously we demonstrated that oligomers lacking measureable ThT fluorescence are populated early in the amyloid formation reaction of htt-ex1 peptides (Thakur et al., 2009) and that they correspond to structures held together by α -helical bundles of the htt^{NT} segments (Jayaraman et al., 2012a). Here we develop a mathematical approach to estimating the levels of these α -helical oligomers along the aggregation reaction coordinate by independently determining the weight fractions of monomers and amyloid fibrils at each time point. We analyzed aliquots of the reactions for residual monomer (actually, monomer/dimer/tetramer (Sahoo et al., 2016)) concentration using analytical size exclusion chromatography (SEC). For amyloid levels we used ThT fluorescence. At any time point, a knowledge of the total protein available (based on starting monomer amount) as well as the amounts of monomer and amyloid fibrils determined at that time point, allows straightforward calculation of the amount of ThT-negative aggregates (i.e., α -oligomers).

We carried out the SEC analysis on a GE Superdex 75 5/150 GL column, run on an Agilent 1200 single pump analytical HPLC system equipped with a UV-VIS detector, in PBS at 0.3 ml/min and integrated the monomer peak using system software. SEC time points were taken by first exposing an aliquot of the aggregation reaction mixture to low speed centrifugation (10 mins at 15,000g, carried out to protect the column) followed by injection of the supernatant. Monomer peaks were integrated and the peak areas converted to peptide mass according to a previously constructed standard curve for that peptide in a manner similar to that previously described for reverse phase HPLC analysis of reaction supernatants (O’Nuallain et al., 2006). Monomer weight concentrations at reaction time $t = n$ (i.e., $[m]_n$) were then calculated based on the standard curve readout and a knowledge of the dilutions used in the analysis. Analytical SEC was also used to determine the starting concentration of monomer, a quantity whose accuracy is critical to this analysis. Since for some htt-ex1 peptides aggregation (and, specifically, α -oligomer formation) is extremely fast once reactions are initiated, special measures had to be taken to determine this $t = 0$ value. To determine an accurate starting monomer concentration, we analyzed and averaged two replicate SEC runs on aliquots of the relatively stable pH2 (TFA-water) solution of htt-ex1 monomers just prior to adding PBS to initiate the aggregation reaction. The value obtained was then adjusted for the dilution that occurs on PBS addition to determine $[m]_{(start)}$, the concentration of monomer in the PBS reaction mixture at $t = 0$.

Determining $[m]$ at various stages allows determination of several important values. First, the weight fraction of monomers present at any reaction time point n , $Fm(n)$, is equal to

$[m]n / [m](start)$. Second, $[f](end)$, the concentration of amyloid fibrils at the end of the reaction, when ThT intensity is no longer changing with time, is equal to $[m](start) - [m](end)$. Third, $Ff(end)$, the weight fraction of fibrils in the homogeneous aggregated sample at the end of the reaction is equal to the concentration of fibrils at the end of the reaction divided by the total concentration of protein present, which should be the starting concentration of monomer; thus, $Ff(end) = [f](end) / [m](start)$.

We determined the weight concentration of amyloid fibrils (f) at any time using ThT fluorescence measurements and an analysis of the fluorescence intensities obtained. For each time point, the aggregation reaction aliquot was gently vortexed to mix the particles, then 40 μ L of the peptide suspension was added to a solution of ThT in double distilled H₂O to give a final volume of 400 μ L and final concentrations of ThT (15 μ M) and total peptide ($\sim$ 1 μ M). Raw ThT fluorescence (λ_{ex} 450 nm, λ_{em} 489 nm) was determined in a Horiba FluoroMax 4 instrument, with a 4 mm path length cuvette, a 5 nm excitation slit width, and a 2 nm emission slit width. All measurements were read for a minimum of one minute and averaged. The fluorescence intensity of a buffer blank of 15 μ M ThT in 400 μ L water was also determined, and this value was subtracted from the intensities of the values for reaction aliquots to generate the net fluorescence intensity, ThT_{net} , of the sample. These ThT values were interpreted according to the concept of aggregate-weight-normalized (AWN) ThT values, which shows that, under standard measurement conditions, homogeneous polymorphic amyloid fibrils or other β -aggregates made in a particular way yield reproducible weight-normalized ThT intensities that, at the same time, can differ substantially from the intensities of other polymorphic forms (Chemuru et al., 2016; Misra et al., 2016; Wetzel et al., 2018).

To complete the analysis it is first necessary to calculate the $AWN-ThT$ value for the sample being produced. This is simply the new ThT fluorescence intensity of the time point chosen to represent the end of the reaction, $ThT_{net}(end)$, divided by the weight fraction of fibrils at this end point, $Ff(end)$ (see above). This $AWN-ThT$ value can now be used in turn to calculate the weight fraction of amyloid fibrils at any time point n , $Ff(n)$, by dividing $ThT_{net}(n)$ by $AWN-ThT$.

Then, at any time point n , the weight fraction of α -oligomers, $Fo(n)$, is equal to “1 – $Fm(n) - Ff(n)$ ”. That is, at each time point, Ff is the weight fraction of the starting monomer that has been converted to fibril, Fm is the fraction of the starting weight of monomer that remains monomeric, and Fo is the weight fraction of starting monomer that has been converted into ThT-negative oligomers.

Electron Microscopy.

Transmission electron microscopy was conducted as described previously (Thakur et al., 2009) using a Tecnai T12 microscope (FEI) operating at 120 kV and 30,000 $\times$ magnification and equipped with an UltraScan 1000 CCD camera (Gatan).

Fourier Transform Infrared Spectroscopy.

Aggregates from end time points of each reaction were isolated and analyzed on an ABB Bomem FTIR instrument. A total of 400 scans were collected at room temperature with 4 cm^{-1} resolution. Residual buffer absorption was subtracted to yield second-derivative minima. PROTA software (Biotools, Inc.) was used to identify spectral components.

Circular Dichroism.

Far-UV CD measurements were performed on a JASCO J-810 spectropolarimeter using a 1-mm path length cuvette. CD samples were prepared in parallel to sedimentation assays (10–30 μM , PBS). CD spectra were analyzed using the CONTINLL program from the CDPro package.

Primary Neurons.

Rat primary cortical neurons were isolated from embryonic day 18 pups and nucleofected (Lonza) at plating (Jacob et al., 2005). As the tested htt toxicity model in neurons combines coexpression of a pathogenic htt-ex1 with an inhibitory htt-ex1 derivative, for all other neuronal transfections, transgene and total introduced DNA content were balanced by performing co-transfections with equivalent amounts of fluorescent protein vector (EGFP or mCherry). For viability assays, live neurons were imaged on a Nikon A1 Confocal Microscope (40x oil immersion lens at 2.0 zoom) at room temperature. Samples for confocal imaging were sequentially scanned with individual lasers (488, 561, and 640 nm) and appropriate emission band pass filters (500–550 and 575–625 nm) or long pass filter (650LP). 75 μL of fresh NucRed Dead 647 (ReadyProbes) in 1 mL HEPES-buffered imaging saline (135 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl_2 , 10 mM HEPES, 2.5 mM CaCl_2 , 11 mM glucose, pH 7.4) was added to neurons and incubated at 37 °C for 20 mins. Neurons were imaged for the following 45 minutes. Neurons with healthy, intact membranes reject the NucRed Dead 647 dye and are not stained, while dead or unhealthy neurons with compromised membrane integrity allow the NucRed Dead 647 to enter the nucleus and bind DNA, amplifying its signal. At least 50 neurons per condition were captured. For puncta/aggregate counting, neurons grown on coverslips were fixed in 4% paraformaldehyde and imaged using an Olympus Fluoroview confocal microscope (60x oil immersion lens) at room temperature. Random fields were scored blind (>100 cells per condition over 3–5 independent experiments) for the percentage of Map2 positive neurons also positive for EGFP or mCherry puncta. Images were analyzed using ImageJ software.

Drosophila.

The *UAS-attB-IVS-8/9* vector (GenBank accession #KU963520) vector was created for improved protein expression from the UAS-attB vector system (Bischof et al., 2007) by including *ATPalpha* intervening sequence 8/9 (*IVS 8/9*), using a previously published strategy (Pfeiffer et al., 2010). Wild type and mutant human *huntingtin exon1* were directionally cloned into the *UAS-attB-IVS-8/9* vector (GenBank accession #KU963520) resulting in the series of vectors used in these studies that express control (htt-ex1-Q25-EGFP #KU963524) or polyQ expanded (htt-ex1-Q46-EGFP #KU963525; htt-ex1-Q97-EGFP #KU963526) proteins, as well as engineered huntingtin analogs (htt-ex1- βHP -

mCherry #KU963521; htt-ex1-βHP-P2-mCherry #KU963522; htt-ex1-βHP-P1,2-mCherry #KU963523). Site-specific insertion was achieved using the *attP18* site on the X chromosome, balanced with *FMO* and flies homozygous for these *huntingtin* transgenes with *elav-Gal4* on the 2nd chromosome were generated. Upon eclosion, non-virgin female flies were selected and kept at 29 °C in 12:12 hour light:dark cycles, initially with 16 flies per vial on standard fly food. Media was changed every other day and the number of live and dead flies was counted. A minimum of 100 flies per genotype/condition were tested. Antibodies used to confirm expression of our DNA constructs were anti-GFP (D5.1) XP® Rabbit mAb (Cell Signaling #2956) and anti-mCherry mAb [1C51] (Ab Cam ab125096).

Behavioral Assay.

RING assay was performed at the indicated ages. Age-matched samples were transferred to fresh media-free vials and allowed 1 minute to acclimate. The flies were knocked down with gentle tapping and filmed. Height climbed after 8 seconds was recorded, with 5 vertical cm being the maximum. A minimum of 30 flies per condition was tested.

Reproducibility and Statistical Analysis.

All sedimentation assay results in Figure 2 contain error bars representing standard error of the mean as technical replicates of n 2; in some cases error bars are smaller than the data point symbols and so are not evident. Critical nucleus error is presented as a 95% confidence interval of the slope of the log-log plots. Primary neuron toxicity and puncta data error bars are standard deviation, analyzed using one-way ANOVA Tukey's multiple comparisons test. Resulting individual p values are indicated in the figure. Htt-ex1-βHP-EGFP + Cherry, htt-ex1-Q25-EGFP + Cherry, and htt-ex1-βHP-P2-mCherry + EGFP from the puncta assay in figure 6a and htt-ex1-Q25-EGFP + mCherry from the viability assay in Figure 6b used 4 independent primary neuronal cultures. All other conditions in the puncta and viability of Figures 6a,b used 5 independent primary neuronal cultures. 100 cells per condition were used to assess both puncta and viability. No statistical methods were used to predetermine sample sizes for neuronal cultures, but sizes were similar to those generally employed in the field. The data distribution of neuron puncta and viability was assumed to be normal. A Browne-Forsythe and Bartlett's test were used to test for similar variances between groups. Primary neuron colocalization was analyzed using a two-tailed 95% confidence interval Pearson's correlation between the EGFP (from htt-ex1-Q97-EGFP (6c-d)) and the mCherry signal (from untagged mCherry (6c) or htt-ex1-βHP-P2-mCherry (6d)). Fifteen 1-dimensional correlations from 15 cells over 5 independent cultures were imaged and analyzed blind to yield correlation ± standard deviation. All neuron imaging and data analysis was performed blind. Drosophila survival was statistically analyzed using Log-rank Mantel-Cox test of Kaplan-Meier survival curves, *** = $p < 0.0001$. Drosophila survival experiments were performed once (100 flies per condition). A sample size of 100 flies is sufficient for a 95% confidence interval at 80% power assuming a standard deviation of 10 days to detect a change of 4 days from the mean. Drosophila locomotor activity was analyzed using two-way ANOVA with Bonferroni's multiple comparisons test (*** = $p < 0.01$ and error bars represent standard error of the mean). Drosophila locomotor experiments involved an average of three independent RING tests involving at least 30 flies total (10 per biological replicate). A total of 30 flies per condition was sufficient to for a 95% confidence interval at

80% power assuming a standard deviation of 1 cm to detect a change of 1 cm from the mean. Animal studies were not performed blind or randomized. Locomotor assays were performed on 10 flies at a time in technical triplicate. No data were excluded as outliers. Animal studies were assumed to have normal distribution. Aggregation experiments were generally performed at least twice. All statistical data was analyzed using GraphPad Prism software.

Data availability.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Design and testing of polyQ-mutated huntingtin exon1 analogs.

Previously we used -hairpin β -enhancing mutations to develop frameworks for aggregation-enhanced (Kar et al., 2013) and aggregation-suppressed (Kar et al., 2017) simple peptides with short polyQ repeat lengths, such as AcWQ₁₁pGQ₁₁WTGK₂ (Table 1) with its 22 Gln residues. To move these frameworks into a htt-ex1 background expressible in cells however, required some structural adjustments. We designed a highly related analog β HP (Table 1) in which-terminal acetylthe groupN (a key component of the trpzip motif(Kier et al., 2010)) is replaced by GlyGly, and the central D-Pro residue is replaced by L- Pro. We found that although($\blacktriangle$)aggregates β HPless efficiently than AcWQ₁₁pGQ₁₁WTGK₂ ($\blacktriangledown$), it nonetheless aggregates faster than both a K₂Q₂₃K₂ ($\bullet$)with a similar polyQ length and a K₂Q₃₇K₂ ($\blacksquare$) with a pathological repeat length (Fig. 2a). Importantly, in contrast to the critical nucleus (n*) of 4 found for short polyQ peptides like K₂Q₂₃K₂ ($\bullet$) (Kar et al., 2011), β HP replicates the n* $\blacktriangle$; of 1 (Fig. Supplementary Fig. 1) previously found for AcWQ₁₁pGQ₁₁WTGK₂ ($\blacktriangledown$) (Kar et al., 2013) as well as for long repeat peptides like K₂Q₃₇K₂ ($\blacksquare$) (Kar et al., 2011). Mature β HP fibrils also resemble those of AcWQ₁₁pGQ₁₁WTGK₂ by transmission electron microscopy (TEM) (Fig. 3a) and Fourier transform infrared spectroscopy (FTIR) (Supplementary Fig. 2).

Previously we reported that mid-strand Pro insertions within conformationally constrained polyQ sequences produce peptides exhibiting greatly inhibited spontaneous aggregation (Kar et al., 2017; Thakur and Wetzel, 2002) along with a new ability to inhibit aggregation of other polyQ peptides in *trans* (Kar et al., 2017; Thakur et al., 2004). We report here that similarly mutated versions of the n β HP-P^{1,2} likewise show greatly diminished spontaneous aggregation (Fig. 2a, $\blacklozenge$) and, when mixed with K₂Q₃₇K₂, inhibit its amyloid formation in *trans* (Fig. 2c).

We next inserted this β HP motif into a-ex1chemicallybackground access (“htt-ex1^{P10}”; Table 1) previously shown to faithfully replicate full-length htt-ex1 (Sahoo et al., 2014) and found that htt-ex1^{P10}- β HP – with only 22 Gln residues – aggregates faster (Fig. 2d, $\blacktriangle$, t_{1/2} = 10 hrs) than htt-ex1^{P10}-Q23 ($\bullet$, t_{1/2} = 200 hrs) and at a rate in the same range as that of htt-ex1^{P10}-Q37 ($\blacksquare$, t_{1/2} = 15 hrs). In contrast, the same htt-ex1^{P10}- β HP framework now containing-strand Proresiduesmid in one (Fig. 2d, $\blacktriangledown$, $\blacklozenge$) or both (Fig. 2d, $\star$) of

β HP's 11 segments Q exhibits very poor spontaneous aggregation which does not progress beyond the oligomer stage (Fig. 3 e,f) under these conditions. Finally, these Pro-interrupted peptides also inhibit the aggregation of expanded polyQ molecules like htt-ex1^{P10}-Q37 (Fig. 2e). Thus, while 10 μ M htt-ex1^{P10}-Q37 aggregates with a $t_{1/2}$ of 14 hours (Fig. 2e, ■), incubation of this peptide with 20 μ M htt-ex1^{P10}- β HP-P1 (▼), htt-ex1^{P10}- β HP-P2 (▲) or htt-ex1^{P10}- β HP-P1,2 (●) extends the $t_{1/2}$ to 54, 125, and 125 hrs (Fig. 2e), respectively – up to nearly an order of magnitude delay. These inhibitors are also effective at equal and sub-stoichiometric ratios (Supplementary Fig. 3).

Htt-ex1^{P10}- β HP is unstructured as a monomer in aqueous solution (Supplemental Fig. 4a, $t = 0$) and only β -structure acquires as the amyloid assembly reaction proceeds (Supplementary Figs. 4 a,c). The structures of the oligomeric (Fig. 3d) and final amyloid (Fig. 3j) aggregates of htt-ex1^{P10}- β HP resemble those of htt-ex1^{P10}-Q37 (Fig. 3 c,i) and htt-ex1^{P10}-Q23 (Fig. 3 b,h) by TEM as well as by FTIR (Supplementary Fig. 2) and cross-seeding ability (Fig. 2f).

Mechanism of β HP enhancement of htt-ex1- β HP aggregation.

The mechanism for how β -hairpin enhancing mutations influence the aggregation of simple polyQ peptides has been described (Kar et al., 2013). But the amyloid nucleation mechanism of simple polyQ (■) (Kar et al., 2011) is fundamentally different from that of polyQ in htt-ex1 (Jayaraman et al., 2012a; Thakur et al., 2009) (Fig. 1). It is therefore of great interest as to how β HP mutations produce enhanced aggregation rates- when ex1 background. One possible mode might be dramatic enhancement of the “simple polyQ” nucleation mechanism (Fig. 1, step 1; kinetic competition with normally dominant htt^{NT}-mediated, exon1-type mechanism (Jayaraman et al., 2012b)). However, nucleation kinetics analysis clearly reveals an htt^{NT}-mediated, exon1-type mechanism is retained in the aggregation of htt-ex1^{P10}- β HP. Thus, -log plot of initial aggregation rate vs. initial concentration for htt-ex1^{P10}- β HP (Fig. 2b, ★) exhibits a shallow slope in a range that is characteristic of all htt-ex1 peptides examined previously (Jayaraman et al., 2012b; Thakur et al., 2009), such as htt-ex1^{P10}-Q37 (Fig. 2b, ◆), and is markedly different from those for simple polyQ peptides (Fig. 2b, ▲, ■, ●, ▼) that generate n^* values in the 1–4 range (Kar et al., 2011).

This result suggests that the β HP somehow enhance the htt^{NT}- mediated aggregation pathway (Fig. 1, step 2->5). In the context of this mechanism (Fig. 1), there appear to be three different ways in which a mutation might augment aggregation rates: (a) enhance non- β oligomer formation (Fig. 1, step 2), (b) enhance the efficiency of amyloid nucleation within these oligomers (Fig. 1, step 3), or (c) enhance the elongation rates of newly formed amyloid fibrils (Fig. 1, step 4). We investigated each of these possibilities.

Quantitative determination of non- β -oligomer levels is challenging. We developed a new procedure (Methods) using the concept of aggregate-weight-normalized ThT fluorescence (AWN-ThT) intensities (Wetzel et al., 2018). For the aggregation reaction of 7.2 μ M freshly-ex1^{P10}- β HP disaggregated in PBS at 37°C, for example, we monitored reaction time points for both ThT intensity and for the amount of low-molecular weight forms eluting in analytical size exclusion chromatography (SEC) (Fig. 4a). From the final ThT intensity and the final aggregate weight concentration we calculated the AWN-ThT intensity of htt-ex1 amyloid

fibrils, then used this value along with the ThT intensities of various time points to determine the weight fraction of amyloid (Ff) at each time point (Fig. 4b). The difference between these Ff values and the total aggregate weights at each time point yielded the weight fractions of ThT-insensitive, non- β oligomers, (Fig. 4b)F. The data show that, in htt-ex1^{P10}- β HP aggregation, a small- β amount-oligomers isofgeneratednon rapidly and transiently, while amyloid begins to develop later, just as the Fo value begins to decay (Fig. 4b). Importantly, TEM images at several time points (Fig. 4b) are consistent with these time courses, with no aggregates apparent at $t = 0$, homogeneous spherical oligomers at 1 hr, a mix of oligomers and fibrils at 4 hrs, and homogeneous fibrils at 24 hrs.

Similar analyses of the other htt-ex1 analogs were conducted (Fig. 5 a-d) and the log scale time dependence of each species – Fo, Ff, and Fm – plotted together in groups for straightforward comparison (Fig. 6). The requirement for 5 logs in the timescale illustrates the wide difference in reaction kinetics of aggregation between the various analogs, as introduced by our mutational engineering. The Fo time course (Fig. 6a) shows that htt-ex1^{P10}-Q37 (■) undergoes a rapid increase in Fo peaking at a weight fraction of ~ 0.25 at ~ 10 mins, followed by a rapid decay, consistent with a burst of amyloid production (Ff > 0.05) within the first hour of incubation (Fig. 6b). In contrast, htt-ex1^{P10}-Q23 (▲) exhibits a maximum Fo value of only ~ 0.07 , which peaks significantly later – about 2 hrs (Fig. 6a); this retarded aggregation rate and extent is consistent with the previously reported polyQ repeat length dependence of non- β oligomer formation (Pandey et al., 2018; Sahoo et al., 2016). In comparison, the Fo of htt-ex1^{P10}- β HP (●) rises to a maximum of ~ 0.14 at ~ 35 mins (Fig. 6a), consistent with the onset of appreciable (Ff > 0.05) amyloid formation by 5 hrs (Fig. 6b). These data show that both the initial rates of non- β oligomer formation, and the maximal fractiona oligomers formed, vary in the order Q37 $>$ β HP $>$ Q23 (Fig. 6a). This suggests that the more rapid onset of amyloid fibril formation by htt-ex1^{P10}- β HP compared with similar- poly length htt-ex1^{P10}-Q23 (Fig. 6b) may be partly due to the more rapid and extensive (but transient) formation of non- β oligomers by-ex1the^{P10}- β HPhtptide (Fig. 1, step 2)

Next, we tested the capacity of the β HP sequence to increase the nucleation within non- β oligomers. We incubated htt-ex1^{P10}-Q23 the presence or absence of a very small portion of htt-ex1^{P10}- β HP. As shown in Figure 2g, addit only 1% by weight of monomeric β HP to 10 μ M monomeric-ex1^{P10}-Q23reduceshtt the time to 50% completion of the amyloid formation reaction by a factor of ~ 3 , from 160 hrs for htt-ex1^{P10}-Q23 (●) alone to 50 hrs for the mixture (■). The large nucleation rate increase bestowed by a trace amount of htt-ex1^{P10}- β HP implies a substantially enhanced ability of htt-ex1^{P10}- β HP co-assembled within non- β oligomers to initiate amyloid formation in the htt-ex1^{P10}-Q23 population in this experiment (Fig. 1, step 3)

Finally, to look for changes in the efficiency of monomer recruitment into growing fibrils (Fig. 1, step 4), we compared the abilities of equal concentrations of htt-ex1^{P10}-Q23 monomers and htt-ex1^{P10}- β HP monomers to undergo elongation-ex1^{P10}seeded- by Q23 fibrils. We found that under these conditions the initial rate of seeded elongation of htt-ex1^{P10}- β HP monomers is about 30 times faster than the23 corresponding monomers (Fig. 2h).

Taken together, these studies suggest a complex, multi-factorial mechanistic basis for the ability of the β HP sequence in htt-ex1 to accelerate spontaneous amyloid growth compared with the Q23 sequence. β HP appears to independently bo rate and extent of formation-helix-containing, of non the- β α oligomeric intermediates within which amyloid nucleation occurs (Fig. 6a), (b) the efficiency of formation of amyloid nuclei within these oligomers (Fig. 2g), and (c) the efficiency of elongation of amyloid nuclei and fibrils emerging from these oligomers (Fig. 2h). These quantitative assessments are in line with our previous qualitative observations that polyQ-expansion appears to independently enhance oligomer formation and amyloid nucleation by htt-ex1 (Sahoo et al., 2016; Thakur et al., 2009). Recent, independent studies confirm those previous observations (Pandey et al., 2018). Although it has not been directly demonstrated, we presume the enhanced nucleation and elongation abilities of htt-ex1^{P10}- β HP are both due to the greater tendency of the β HP poly β -hairpin conformation (Kar et al., 2013).

Mechanisms of *cis* and *trans* inhibition of amyloid formation by Pro insertions into polyQ tracks of htt-ex1- β HP's.

All three htt-ex1- β HP peptides with inserted- m strand Pro residues are highly impaired at spontaneous amyloid formation (Fig. 2d). hen incubated at 14–20 μ M, these peptides exhibit little or no aggregation until after 200 hrs (Fig. 2d). After that point, the two peptides with only a single interrupted Q11 track begin to aggregate, while the doubly interrupted htt-ex1 peptide continues to resist the formation of sedimentable aggregates. The failure to make amyloid is not because of a failure to make oligomers (Fig. 3 e,f); rather, it is because amyloid nucleation and growth is substantially inhibited. Thus, htt-ex1^{P10}- β HP-P² incubated at 7.9 μ M forms about 20%-oligomera α at 1,000 hrs (Fig. 6a), but no ThT-positive fibrils are formed in the same time frame (Fig. 6b). Incubation of this peptide for 24 hrs change in its signature random coil CD spectrum (Supplemental Fig. 4b), consistent With an absence of- amyloid. β sheets

The lack of efficient amyloid nucleation in these Pro-interrupted mutants is probably related to previous observations with similarly mutated simple polyQ peptides that efficient amyloid nucleation requires the potential to make β -hairpins containing two unbroken polyQ strands (Kar et al., 2017; Thakur et al., 2004). A single unbroken Q11 sequence is expected to be extremely inefficient at making a nucleation competent β -hairpin (Kar et al., 2011; Landrum and Wetzel, 2014). At the same time, α -helical packing interactions between the httNT segments in isolated peptides (Jayaraman et al., 2012a; Mishra et al., 2012) or in htt-ex1 are sufficient to support oligomer formation. The result is that these htt-ex1 peptides can make α -oligomers, albeit slowly, but not fibrils, consistent with previous observations on related peptides (Mishra et al., 2012). Given the similar polyQ contents of htt-ex1P10- β HP-P2 and htt-ex1P10- β HP and the demonstrated polyQ-repeat length dependence of α -oligomer formation (Pandey et al., 2018; Sahoo et al., 2016; Thakur et al., 2009) (Fig. 6a), the basis for the relative sluggishness of α -oligomer formation by htt-ex1P10- β HP-P2 is unclear.

All three htt-ex1 peptides containing the β HP-P¹, β HP-P², and - β HP^{1,2} sequences are also capable of inhibiting the aggregation of expanded polyQ htt-ex1 (Fig. 2e). Differences in the shapes of the inhibition kinetics curves suggest that htt-ex1-P¹ (▼) and htt-ex1-P^{1,2} (●)

specifically delay amyloid nucleation, while htt-ex-P² (▲) suppresses elongation. Short, simple and polyQ peptides combining β -hairpin mutations mid-strand Pro insertions have also been shown to inhibit spontaneous aggregation of other simple polyQ peptides in *trans* (Kar et al., 2017), but such peptides – probably due to the lack of an htt^{NT} segment – are not effective against htt-ex1 aggregation (Fig. 2i).

Htt-ex1 fragments consisting of only the htt^{NT} segment are modestly effective inhibitors of htt-ex1 aggregation, apparently acting by co-assembling -oligomers into α and thus reducing the local concentration of disordered polyQ chains and suppressing nucleation (Mishra et al., 2012). Aggregation-incompetent htt-ex1 analogs like htt-ex1^{P10}- β HP-P² are expected to provide superior nucleation inhibition through the dual operation of (a) this general dilution effect plus (b) the addition of an on-board polyQ aggregation inhibitor attached to the htt^{NT} (Mishra et al., 2012). In fact, while a 1:1 mixture of htt-ex1-Q37 and htt-ex1- β HP-P² leads to a substantial burst and prolonged duration of ThT-negative-oligomers α (Fig. 6a) containing both peptides (Supplementary Fig. 3c), nucleation of amyloid is greatly delayed (Fig. 6b). The important role of the htt^{NT} segment in inhibition of htt-ex1 aggregation is indicated by the superior inhibition of htt-ex1-Q37 aggregation by htt-ex1- β HP-P² compared with-Pa²peptide β HP lacking the htt^{NT} domain (Fig. 2i).

Interestingly, their differing kinetics profiles (Fig. 2e) suggest that while htt-ex1^{P10}- β HP-P^{1,2} (●) and htt-ex1^{P10}- β HP-P¹ (▼) delay the nucleation phase of htt-ex1^{P10}-Q37, htt-ex1^{P10}- β HP-P² (▲) has a lesser effect on nucleation and primarily slows the elongation phase. These differences are consistent with an especially important role of the N-terminal residues of the polyQ stretch in htt-ex1 in nucleation of amyloid structure. Our model for the role of the htt^{NT} segment in the enhanced aggregation of htt-ex1 peptides is that it drives the clustering of polyQ sequences to a high local concentration, and this would be expected to be particularly so in the htt^{NT}-proximal polyQ segments. Htt^{NT} may also play a role in ordering the polyQ chains to facilitate nucleation; indirect evidence for this is the involvement of the C-terminal residues of htt^{NT} in β -sheet amyloid structure ((Sivanandam et al., 2011)). Thus, the presence of Pro inserts in the htt^{NT}-proximal polyQ segments of these htt-ex1 derived inhibitors would be expected to be particularly effective in suppressing nucleation, whereas the presence of an unbroken polyQ would be expected to be less disruptive of nucleation.

The aggregation behaviors of the structure and mechanism-based enhancers and inhibitors of htt-ex1 amyloid formation described above suggest potential diagnostic value in cell and animal models expressing related molecules. In particular, will expression of htt-ex1- β HP in cells and animals be benign (as - expected range Gln content) or pathogenic (as suggested by its high aggregation propensity)? If aggregation is implicated, will toxicity trend with oligomer or amyloid content?

Aggregation and toxicity profiles of Htt-ex1- β HP derivatives in rat primary cortical neurons.

We constructed DNA vectors encoding analogs consisting of htt-ex1 fused at their C-termini to a fluorescent protein (Table 1). Previous studies showed that C-terminal fluorescent protein modifications do not alter the basic aggregation properties of htt-ex1 (Sahoo et al.,

2016; Sahoo et al., 2014). These htt-ex1 analogs were co-transfected with either a second htt-ex1 analog or a control vector into rat primary cortical neurons (Jacob et al., 2005). At day *in vitro* 14 (DIV14), neurons were fixed, stained for neuronal somatodendritic marker Map2, imaged *via* confocal microscopy, and scored blind (Methods) for aggregate/puncta formation (Fig. 7a). In addition, cytotoxicity was measured with confocal live imaging of a NucRed 647 Dead assay at DIV14 (Supplementary Fig. 6) and DIV21 (Fig. 7b). As anticipated, expression of negative control htt-ex1-Q25-EGFP / mCherry resulted in few neurons with visible puncta (Fig. 7a, ◆, $4.1 \pm 3.1\%$) and excellent cell viability (Fig. 7b, ◆, $84.4 \pm 4.0\%$ viable at DIV21). Conversely, as expected for an HD neuron model (King et al., 2008), a high percentage of neurons with the expanded polyQ repeat control (htt-ex1-Q97-EGFP / mCherry) showed visible puncta (Fig. 7a, ●, $84.0 \pm 9.5\%$) and significantly fewer viable cells (Fig. 7b, ●, $45.7 \pm 4.2\%$ viable at DIV21).

Dramatically, neurons expressing the β HP construct htt-ex1- β HP(-EGFP / mCherry) exhibited results unlike those from the htt-ex1-Q25 cells and more typical of expanded polyQ repeat length cells, with $39.3 \pm 4.2\%$ neurons exhibiting puncta (Fig. 7a, ▲) and only $47.1 \pm 7.8\%$ of neurons remaining viable at DIV21 (Fig. 7b, ▲). Thus, despite containing only 22 glutamines, htt-ex1- β HP shows aggregation-associated cytotoxicity comparable to an htt-ex1 featuring a very long Q97 repeat normally associated with highly aggressive juvenile HD (Bates et al., 2015).

In sharp contrast, we found that htt-ex1- β HP-P²-mCh / EGFP neurons exhibit low levels of visible puncta (Fig. 7a, ●, $6.4 \pm 2.1\%$) and low cytotoxicity (Fig. 7b, ●, $74.8 \pm 9.2\%$ viable at DIV21), comparable to the htt-ex1-Q25 construct. These cellular data are especially intriguing, given our *in vitro* data suggesting that the mid-strand Pro insertions in htt-ex1 are very effective at slowing overall aggregation and blocking amyloid formation. If the residual aggregates seen in the htt-ex1- β HP-P² cell model are ThT-negative oligomers, as seen *in vitro* (Fig. 6a; Fig. 3e), the data suggest that non-amyloid oligomers are most likely relatively benign. It is also possible, however, that the lack of both visible aggregates and significant toxicity in these cells is attributable to very slow formation of any type of aggregate, as observed *in vitro* (Fig. 5b; Fig. 6). Such an interpretation is consistent with the toxic aggregate hypothesis, but does not strongly implicate any particular aggregate in that toxicity.

However, consistent with the *in vitro* results (Fig. 2e), we also found that htt-ex1- β HP-P²-mCh is a good inhibitor of expanded polyQ htt-ex1 aggregation in cells. We observed that neurons expressing htt-ex1-Q97-EGFP plus htt-ex1- β HP-P²-mCh exhibit significantly fewer ($63.4 \pm 6.3\%$) neurons with puncta (Fig. 7a, ■) compared with neurons expressing the highly aggregation prone Q97 protein alone (Fig. 7a, ●, $84.0 \pm 9.5\%$). In addition, the htt-ex1- β HP-P² inhibitor effectively rescues cells from htt-ex1-Q97 toxicity at DIV21, exhibiting $69.3 \pm 7.1\%$ viable cells (Fig. 7b, ■) compared with neurons expressing Htt-ex1-Q97-EGFP alone (Fig. 7a, ●, $45.7 \pm 4.2\%$ viable). Thus, the results of these co-expression experiments, coupled with our *in vitro* studies (Fig. 6), speak against non- β oligomers as the toxic species and-ex1 amyloid

To probe the cellular mechanism of inhibition, we carried out confocal microscopy analysis of neurons co-expressing htt-ex1-Q97-EGFP and htt-ex1- β HP-P²-mCh, which revealed line intensity profiles of the bright EGFP puncta from htt-ex1-Q97-EGFP that superimpose well (Fig. 7d, Pearson's coefficient = from the inhibitor. In a control of neurons co-expressing htt-ex1-Q97-EGFP and isolated mCherry protein, there is no correlation (Fig. 7c, Pearson's coefficient = 0.17) results are consistent with a direct role of the htt-ex1- β HP-P² peptide (which when expressed alone in cells forms few puncta (Fig. 7a, ●)) in slowing aggregation of htt-ex1-Q97. The intensity of the htt-ex1- β HP-P²-mCh component of these inclusions suggests substantial co-incorporation of the inhibitor in the cellular aggregates, as implied by *in vitro* results suggesting co-aggregation of target protein and inhibitor (Supplementary Fig. 3c).

In *Drosophila*, Htt-ex1- β HP-EGFP shortens lifespans and generates behavioral defects, while mid-strand Pro mutants are non-toxic and rescue htt-ex1-Q97-EGFP induced toxicity.

While cellular models are valuable for studying the molecular basis of disease, many symptoms of HD are known to be derived from changes in connectivity and communication between different neuronal populations (McColgan et al., 2015). These aspects of pathology are recapitulated in *Drosophila* models of HD, which are well characterized, mimic the main pathophysiology of HD in humans, and have robust endpoints for quantitative and qualitative outcomes (Jackson et al., 1998; Weiss et al., 2012). Using site-directed gene insertion, we generated a series of homozygous female flies engineered for expression of fluorescently labeled htt-ex1 analogs and drove transgenic *UAS-attB-Htt-ex1* expression pan-neuronally using the *elav*-Gal4 promoter (Weiss et al., 2012). After confirming protein expression of the transgenes (Supplementary Fig. 7) we analyzed age-dependent behavior and lifespans.

To test locomotor function, female flies were screened using a Rapid Iterative Negative Geotaxis (RING) assay, which challenges flies to climb a maximum of 5 vertical cm in 8 seconds. Both a wild type *attR* control (which contains no added *htt* gene) and *htt-ex1-Q25-EGFP* flies showed a pattern of decline in RING scores typical for normal aging (Fig. 8a). *Htt-ex1-Q46-EGFP* flies, featuring a polyQ repeat length in the same range as most HD patients, exhibit an intermediate decrease in RING performance with age. *Htt-ex1-Q97-EGFP* flies, featuring a polyQ repeat length comparable to very aggressive juvenile HD in humans, show a statistically significant drop in performance at all ages tested. The same trend was observed for longevity, with *htt-ex1-Q25-EGFP* flies exhibiting normal longevity (median survival 68 days) comparable to that of *attR* control flies, while *htt-ex1-Q46-EGFP* flies have moderate, statistically significant lifespan defects (median 52 days) and *htt-ex1-Q97-EGFP* flies show greatly reduced longevity (median 36 days) (Fig. 8b). Thus, consistent with HD genetics and previous reports (Jackson et al., 1998; Weiss et al., 2012), HD-associated pathology correlates with polyQ repeat length in our *Drosophila* model.

In contrast, however, to this well-established notion of the overriding importance of polyQ repeat length in HD pathology, *htt-ex1- β HP-mCh* exhibits significant age-dependent locomotor defects (Fig. 8a) and shortened lifespan (median 56 days, Fig. 8b) despite its short polyQ repeat length. In the RING assay, *Htt-ex1- β HP-mCh* severity is midway between *htt-*

ex1-Q46-EGFP and *htt-ex1-Q97-EGFP*, while *Htt-ex1-βHP-mCh* exhibits activity comparable to that of *htt-ex1-Q97-EGFP* in the longevity assay. Thus, as in the primary neuron experiments, pathology is less associated with absolute polyQ content than it is with intrinsic amyloidogenicity. Conversely, both the *htt-ex1-βHP-P²-mCh* and *htt-ex1-βHP-P^{1,2}-mCh* flies have uncompromised locomotion (Fig. 8a) and lifespan (Fig. 8b). Thus, while *htt-ex1-βHP-mCh* generates an HD phenotype, Pro-interrupted *htt-ex1-βHP* analogs of very similar Gln content are well tolerated.

To confirm the inhibitory effects of the mid-strand proline versions of *htt-ex1-βHP* observed *in vitro* (Fig. 2e) and in primary neurons (Fig. 7 a,b), we generated *Drosophila* co-expression models. Four expression-balanced lines were constructed, each containing one copy of *htt-ex1-Q97-EGFP* plus a second copy of either *htt-ex1-Q97-EGFP*, *htt-ex1-Q25-EGFP*, *htt-ex1-βHP-P²-mCh*, or *htt-ex1-βHP-P^{1,2}-mCh*. The lifespan of the *htt^{Q97}/htt^{Q25}* flies was indistinguishable from that of the *htt^{Q97}/htt^{Q97}* flies (Fig. 8d), with both exhibiting reduced longevity compared to control flies. Likewise, we found that the *htt^{Q97}/htt^{Q25}* flies exhibit an age-dependent decline in locomotor function nearly as severe as that of the *htt^{Q97}/htt^{Q97}* flies (Fig. 8c). In sharp contrast, flies expressing a combination of *htt-ex1-Q97* and either of the two mid-strand Pro versions of *htt-ex1-βHP* exhibit normal locomotion and improved longevity. The age-dependent locomotion for the *htt^{Q97}/htt^{P2}* and *htt^{Q97}/htt^{P1,2}* flies (Fig. 8c) is comparable to those of control flies (Fig. 7a) and dramatically improved from *htt^{Q97}/htt^{Q25}* and *htt^{Q97}/htt^{Q97}* flies. Longevity of *htt^{Q97}/htt^{P2}* (median 52 days) and *htt^{Q97}/htt^{P1,2}* flies (median 54 days) was significantly improved compared to *htt^{Q97}/htt^{Q97}* (median 36 days) or *htt^{Q97}/htt^{Q25}* (median 36 days) (Fig. 8d). This is equivalent to a ~60% rescue with respect to the normal median longevity of ~68 days. Thus, co-expression of mid-strand Pro mutants of *htt-ex1-βHP* with highly expanded polyQ *htt-ex1*, associated with a prolongation of the non-β oligomer phase (Fig. 6a) and inhibition of amyloid nucleation and/or elongation (Fig. 2e; Fig. 6b), dramatically abrogates the toxic effects of the mutant *htt-ex1*, continuing the theme of HD-related toxicity being associated with amyloid fibrils and not ThT-negative α-oligomers.

Discussion

Studies into the toxic species of *htt-ex1* often give ambiguous results, due to a multitude of interconvertible physical states that develop from disordered monomers with overlapping time scales (Sahoo et al., 2016; Wetzel and Mishra, 2014). Here we construct and test a series of short-polyQ *htt-ex1* analogs that are mutationally constrained to energetically favor or disfavor particular aggregation states and thus greatly alter the time scales of their formation. Despite its low Gln content, *htt-ex1-βHP* transits from disordered monomer to amyloid fibril at a rapid rate comparable to expanded polyQ *htt-ex1* molecules. In contrast, *htt-ex1* analogs containing-P¹, the βHP βHP-P², and -βHP^{1,2} motifs in isolation are only capable of self-assembly into non-β oligomers, while in co-mixture with expanded polyQ *htt-ex1* they are effective inhibitors, extending the α-oligomer phase and delaying amyloid formation. The availability of these well-characterized proteins in forms that can be expressed in biological systems provides a unique opportunity to assess the relative toxic contributions of various *htt-ex1* physical states, in a way that has not been possible using *htt-ex1* with normal polyQ sequences.

The linkage between disease age-of-onset and polyQ expansion beyond a specific repeat length threshold is the defining genetic feature of HD (Bates et al., 2015). Some proposals for the HD toxic mechanism posit polyQ expansion-dependent enhancement of particular htt-ex1 physical properties not related to its aggregation (Owens et al., 2015; Truant et al., 2008). Other mechanisms focus on aggregated states, reflecting the well-documented, consistent parallel between repeat length dependence of disease risk and the repeat length dependence of aggregation propensity (Chen et al., 2001; Scherzinger et al., 1997). By demonstrating that highly aggregation-prone htt-ex1-βHP is quite toxic to cells and flies despite content, our experiments show that polyQ aggregation propensity is actually a more robust predictor of HD-related pathology than is polyQ repeat length. Stated differently, polyQ repeat length appears to be an excellent predictor of HD risk only because it happens to be a predictor of the propensity of biological polyQ chains to form toxic aggregates.

The behavior of our constrained mutants *in vitro* and *in vivo* also allows us to infer with some confidence which aggregated state is most likely to be the toxic species. We found *in vitro* that, like expanded polyQ htt-ex1 and unlike normal length htt-ex1, htt-ex1-βHP yields a burst-helical, spherical of aggregates which then swiftly decay as amyloid fibrils accumulate. Neurons making htt-ex1-Q⁹⁷ or htt-ex1-βHP exhibit enhanced levels of both puncta and cytotoxicity, and similar toxicity results were obtained in flies. These data thus support the aggregation hypothesis, but they do not provide compelling clues to which aggregates are the toxic species, since *in vitro* the rates of formation-oligomers of and αamyloid fibrils are increased roughly in parallel (Figs. 4–6). At the same time, the oligomer hypothesis of toxicity is clearly not supported by the Pro-interrupted htt-ex1-βHP constructs. Thus, -βHP-P²htt incubated *in vitro* by itself leads only to ever increasing amounts of oligomers, and expressed in neurons and flies is non-toxic. Much more dramatically, incubation of this protein with an expanded polyQ htt-ex1 *in vitro* leads to a prolonged and amplified burst of non-β oligomers and a corresponding delay in amyloid growth expressed in neurons and flies exhibits reduced toxicity. We therefore conclude that htt-ex1 -oligomers are relatively benign while its amyloid is relatively toxic.

Our data provide no support for hypotheses-rich promoting “mi monomers as the toxic species-likees. and βHP-²βHP-like sequences probably possess similarly low (< 1%) levels of hairpin stable at equilibrium β (Kar et al., 2017), yet in the htt-ex1-β context one is quite toxic-toxic and. In the any case, other it is not clear non that htt-ex1 molecules containing expanded polyQ sequences even exist in appreciable amounts as monomers in the cell, since experiments with htt-ex1 molecules possessing precisely correct N-termini exhibit tetramers and no monomers even at very low concentrations (Sahoo et al., 2016).

The molecular mechanisms by which htt-ex1-βHP enhances amyloid nucleation and growth are likely related to the parallel effects of simple polyQ expansion on htt-ex1 physical behavior. We show here that 22 Gln htt-ex1-βHP forms amyloid much more readily than htt-ex1-Q23 through a trifecta - of oligomer enhanced formation, α enhanced nucleation within those oligomers, and enhanced elongation of nuclei and fibrils. This is consistent with and extends previous observations that expansion of polyQ in htt-ex1 enhances both spherical oligomer generation and nucleation of amyloid (Pandey et al., 2018; Sahoo et al., 2016; Thakur et al., 2009).

The biological evaluation of our structure and mechanism-based htt-ex1 analogs implicates polyQ amyloid -fibrils, oligomers and conformational states of monomers, as the major toxic entity in HD. This conclusion is consistent with the recent report of the prion-like spread of toxic htt aggregates (Pearce et al., 2015), and is also supported by previous studies showing that polyQ or htt-ex1 amyloid fibrils inserted into mammalian cells are toxic (Kar et al., 2014; Nekooki-Machida et al., 2009; Yang et al., 2002). It remains to be determined what form of htt-ex1 amyloid is most toxic to cells, and its mechanism of toxicity. Although Arrasante et al. (Arrasate et al., 2004) found large, htt-ex1 inclusions to be protective, more recent results suggest possible toxicity of amyloid-rich inclusions particularly associated with membrane disruption (Bauerlein et al., 2017; Liu et al., 2015). At the same time, it is now also clear that difficult-to-detect htt-ex1 nanofibrils can form very early in the aggregation process and are probably already present in the cell coincident with the first evidence of cytotoxicity (Sahoo et al., 2016). Small clusters of htt-ex1 fibrils have also been shown to co-exist with inclusions at later stages of aggregation (Sahl et al., 2016). Some amyloid polymorphs may also be more toxic than others (Lin et al., 2017; Nekooki-Machida et al., 2009). Given the generally very high efficiency of fibril elongation even at low monomer concentrations, the potential toxic role of small amyloid fibrils should revive previously postulated cytotoxicity mechanisms proposing recruitment of other polyQ proteins into inactive cellular aggregates (Kar et al., 2014; McCampbell and Fischbeck, 2001; Perez et al., 1998).

There are at least nine other expanded polyQ repeat diseases differing in repeat-length thresholds, host proteins, and neurological profiles (Adegbuyiro et al., 2017; Bates and Benn, 2002; Storey, 2014), and the polyQ analogs exploited here should be helpful in identifying the toxic species in many of these diseases. Polypeptides related to the described inhibitory htt-ex1 mutants, or small molecules discovered by focusing screening assays specifically on reducing htt-ex1 amyloid formation, may provide new amyloid-limiting approaches to HD therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We crafted htt exon1 analogs mutated in polyQ to yield novel self-assembly profiles
- In neurons and flies, a short polyQ form that rapidly makes amyloid is highly toxic
- A slowly aggregating form that doesn't assemble beyond oligomers is non-toxic
- Co-expressed with mhtt-ex1, this form inhibits both amyloid formation and toxicity
- The results implicate the amyloid fibril as the toxic species in Huntington disease

Figure 1.

Mechanisms of polyQ aggregation. Htt-ex1 monomers (1) composed of three intrinsically disordered segments (htt^{NT}, green; polyQ, orange; proline rich domain, black) can engage two distinct pathways of amyloid nucleation (Jayaraman et al., 2012b). In one pathway, the polyQ component engages directly in assembling a critical nucleus (8) whose elongation (9) initiates an ongoing amyloid formation reaction. In the other pathway, monomers undergo a concentration-dependent self-association (2) *via* α -helical bundle formation involving the htt^{NT} segments. The high local polyQ concentration in these oligomers stimulates polyQ amyloid nucleation (3) to generate amyloid fibrils (4) that grow by monomer addition (5). Initial presence of htt^{NT} peptides with or without added elongation-impaired polyQ segments leads to assembly of mixed α -oligomers (6) with reduced capacity to nucleate amyloid. Some htt-ex1 analogs with Pro-interrupted polyQ tracts can also inhibit htt-ex1 elongation by adding to fibrils while disallowing further additions (7).

Figure 2.

Aggregation properties of polyglutamine proteins. (a) Time-dependent loss of simple polyQ monomeric peptides from solution on incubation in PBS at 37 °C. (b) log-log plots of initial aggregation rates vs. initial concentration for a series of polyQ peptides. Slopes and corresponding calculated n^* values are: $\text{K}_2\text{Q}_{23}\text{K}_2$ (slope = 5.9 ± 0.5 ; $n^* = 4$); $\text{K}_2\text{Q}_{37}\text{K}_2$ (slope = 3.0 ± 0.2 ; $n^* = 1$); βHP (slope = 0.3 ± 0.1 ; $n^* = 1$); $\text{AcWQ}_{11}\text{PGQ}_{11}\text{WTGK}_2$ (slope = 3.0 ± 0.3 ; $n^* = 1$); $\text{htt-ex1}^{\text{P10}}\text{-}\beta\text{HP}$ (slope = 0.2 ± 0.1 ; $n^* = 1$); $\text{htt-ex1}^{\text{P10}}\text{-Q}_{37}$ (slope = 0.9 ± 0.3). The dashed lines represent a 95% confidence interval for each fitted line. Data for $\text{K}_2\text{Q}_{23}\text{K}_2$ and $\text{K}_2\text{Q}_{37}\text{K}_2$ are from reference (Kar et al., 2011) and for $\text{AcWQ}_{11}\text{PGQ}_{11}\text{WTGK}_2$ from reference (Kar et al., 2013). Underlying data for βHP are in Supplementary Fig. 1; (c) Inhibition of $\text{K}_2\text{Q}_{37}\text{K}_2$ aggregation by $\beta\text{HP-P}^{1,2}$ showing βHP delay in the aggregation curve of $\text{K}_2\text{Q}_{37}\text{K}_2$ without detectable co-aggregation of the $\beta\text{HP-P}^{1,2}$ peptide; (d) Time-dependent aggregation of htt-ex1 peptide analogs on incubation in PBS at 37 °C., determined as loss of monomers from solution; (e) Time-dependent loss of monomeric $\text{htt-ex1}^{\text{P10}}\text{-Q}_{37}$ in the presence or absence of different mid-strand Pro mutants of $\text{htt-ex1}^{\text{P10}}\text{-}\beta\text{HP}$; (f) Time-dependent loss of monomeric $\text{htt-ex1}^{\text{P10}}\text{-Q}_{23}$ peptide from solution in the presence or absence of various polyQ amyloid fibril seeds. (g) Effect of an added 1% of monomeric $\text{htt-ex1}^{\text{P10}}\text{-}\beta\text{HP}$ on aggregation kinetics of $\text{htt-ex1}^{\text{P10}}\text{-Q}_{23}$ (nucleation test); (h) Relative ability of $\text{htt-ex1-}\beta\text{HP}$ and $\text{htt-ex1}^{\text{P10}}\text{-Q}_{23}$ monomers to respond to $\text{htt-ex1}^{\text{P10}}\text{-Q}_{23}$ fibril seeds

(elongation test); (i) Role of an attached htt^{NT} segment in the ability of β HP-P² peptides to inhibit htt^{ex1}^{P10}-Q37 aggregation.

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Figure 3.

TEM images of various physical states of polyQ peptides (scale bar = 100 nm; all images at same magnification): htt-ex1^{P10}-Q23 oligomers; β HP fibrils; (c) (b) htt-ex1^{P10}-Q37 oligomers; (d) htt-ex1^{P10}- β HP oligomers; (e) htt-ex1^{P10}- β HP-P² oligomers; htt-ex1^{P10}- β HP-P^{1,2} oligomers; (g) empty grid (PBS buffer); (h) htt-ex1^{P10}-Q23 fibrils; htt-ex1^{P10}-Q37 fibrils; (j) htt-ex1^{P10}- β HP fibrils; (k) fibrils from htt-ex1^{P10}-Q37 plus htt-ex1^{P10}- β HP-P²; (l) fibrils from htt-ex1^{P10}-Q37 plus htt-ex1^{P10}- β HP-P^{1,2}.

Figure 4.

Aggregation kinetics-ex1^{P10}-βHPof 7in.3 PBSμM hatt 37 °C. (a) Raw d of ThT readings and monomer concentration as assessed by SEC. (b) Component analysis of the raw data (Methods) showing weight fractions-oligomers, of mono and amyloid fibrils at different time points. (c) Electron micrographs obtained directly from time points indicated (hrs); scale bar = 100 nm.

Figure 5. Component analysis aggregation kinetics of other htt-ex1^{P10} peptides under identical conditions to Fig. 4 -reactionex1^{P10}-Q23; (b)(a) 7.97-ex1.μM8^{P10}μM-htt P²; (c) 7.7-ex1μM^{P10}-httQ37; (d) 7.3-ex1μM^{P10}-httQ37plus 7.5 -μMex1^{P10}htt-P². TEM micrographs are shown at identical magnification, with scale bar = 100 nm.

Figure 6.

Aligned time courses (log scale) for weight fraction plots for Fo (a), Ff (b), and Fm (c) using data derived from Fig. 4b and Figs. 5 a-d. All plots were fit to log(gaussian), with the exceptions of: (a) Fo plot for htt-ex1^{P10}-Q37 plus 7.5 -μM htt ex1^{P10}-P2, which is manually fit to two-component log(gaussian); (b) Fm plots, which are fit by a two-phase disassociation; (c) Ff plots, which are fit by sigmoidal. Supporting ThT and SEC profiles are in Supplementary Fig. 5.

Figure 7.

Effect of htt-ex1 analog expression on rat cortical neurons. (a) Percentages of Map2 positive neurons that also have visible puncta, scored blind, representing at least 100 neurons per condition over 4–5 independent cultures. An example image is in Supplementary Fig. 6c. (b) Primary rat cortical neuron viability was determined using a NucRed 647 Dead assay (sample image, DIV14, Supplementary Fig. 6b) in live neurons at DIV21, 50 neurons, 4–5 independent cultures. NucRed647 Dead assay data from DIV14 are in Supplementary Fig. 6a. Statistics for panel a and b data were by a one-way Tukey's multiple comparisons ANOVA. (c,d) Primary rat cortic httex1-Q97-EGFP (green) and either (c) mCherry (red) or (d) htt-ex1-βHP-P²-mCh were fixed and stained for Map2 (blue). Fluorescence intensity line traces along the straight line paths indicate no obvious co-localization between the intense scattered green puncta signal and the diffuse mCherry signal in c, but significant co-localization in d. For each panel, sets of similar randomly generated one-dimensional line intensity profiles were taken from a total of 15 cells over 3 cultures yielding coefficient of 0.17 (c) or 0.86 (d).

Figure 8.

Effect of *htt-ex1* analog expression in *Drosophila*. (a,c) Flies were challenged to RING locomotor assays as they aged. At least 30 flies and three technical repeats at 10 flies per vial were used per condition. (a) Flies expressing either *htt-ex1-Q97-EGFP*, *htt-ex1-Q46-EGFP*, or *htt-ex1-βHP-mCh* exhibited impaired locomotion compared to the *AttR/AttR* negative control at the last time point tested (Day 45) (two-way ANOVA, Bonferroni's multiple comparisons test, ***p < 0.001). (c) Co with either *htt-ex1-βHP-P2-mCh* or *htt-ex1-βHP-P1.2-EGFP* improved locomotion compared with flies expressing *htt-ex1-Q97* alone (two-way ANOVA, Bonferroni's multiple comparisons test, ***p < 0.05). Error bars are standard error of the mean. (b,d) Survival curves of female flies expressing a total of two copies of various huntingtin constructs, as shown. Flies were aged at 29 °C immediately after eclosion and dead flies were counted and removed every two days. A log-rank Mantel-Cox comparison of survival curves was used to determine statistical significance versus *htt-ex1-Q25-EGFP* flies (*** p < 0.0001) (b) and *htt-ex1-Q97-EGFP/htt-ex1-Q25-EGFP* flies (*** p < 0.001) (d).

Table 1.Protein sequences described in this study^a.

Peptide Name	Amino Acid Sequence
htt-ex1 (htt exon1)	MATLEKLMKAFESLKSF-----Q _N -----PRD
K ₂ Q _N K ₂	KK-----Q _N -----KK
AcWQ ₁₁ PGQ ₁₁ WTGK ₂	-----AcW-Q ₁₁ PGQ ₁₁ -WTG-----KK
βHP	KK-----GGW-Q ₁₁ PGQ ₁₁ -WTG-----KK
βHP-P ¹	KK-----GGW-Q ₅ PQ ₅ PGQ ₁₁ -WTG-----KK
βHP-P ²	KK-----GGW-Q ₁₁ PGQ ₅ PQ ₅ -WTG-----KK
βHP-P ^{1,2}	KK-----GGW-Q ₅ PQ ₅ PGQ ₅ PQ ₅ -WTG-----KK
htt-ex1 ^{P10} -Q _N ^b	MATLEKLMKAFESLKSF-----Q _N -----P ₁₀ KK
htt-ex1 ^{P10} -βHP ^c	MATLEKLMKAFESLKSF-----GGW-Q ₁₁ PGQ ₁₁ -WTG-----P ₁₀ KK
htt-ex1 ^{P10} -βHP-P ¹	MATLEKLMKAFESLKSF---GGW-Q ₅ PQ ₅ PGQ ₁₁ -WTG-----P ₁₀ KK
htt-ex1 ^{P10} -βHP-P ²	MATLEKLMKAFESLKSF---GGW-Q ₁₁ PGQ ₅ PQ ₅ -WTG-----P ₁₀ KK
htt-ex1 ^{P10} -βHP-P ^{1,2}	MATLEKLMKAFESLKSF---GGW-Q ₅ PQ ₅ PGQ ₅ PQ ₅ -WTG---P ₁₀ KK
htt-ex1-Q _N -EGFP ^b	MATLEKLMKAFESLKSF-----Q _N -----PRD-EGFP
htt-ex1-βHP-mCh	MATLEKLMKAFESLKSF-----GGW-Q ₁₁ PGQ ₁₁ -WTG-----PRD-mCh
htt-ex1-βHP-P ² -mCh	MATLEKLMKAFESLKSF---GGW-Q ₁₁ PGQ ₅ PQ ₅ -WTG-----PRD-mCh
htt-ex1-βHP-P ^{1,2} -mCh	MATLEKLMKAFESLKSF---GGW-Q ₅ PQ ₅ PGQ ₅ PQ ₅ -WTG---PRD-mCh

^aPRD = PPPPPPP PPPQLPQPPP QAQPLLQPQ PPPPPPPPP GPAVAEEPLH RP; Ac = acetyl group; p = D-Pro; EGFP = enhanced green fluorescent protein; mCh = mCherry fluorescent protein. AcW.....WTG and GGW.....WTG are “trpzip” motifs k β-hairpin structure into some short peptide sequences (Kier et al., 2010).

^bFor the P10 analogs of htt-ex1, N is either 23 or 37. For full-length htt-ex1, it is either 25 or 97.

^cThe βHP motif is considered to 24, since the Pro analog-Gly residues of replace Q Gln residues.