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Prefibrillar huntingtin oligomers isolated from HD brain potently seed amyloid formation

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ABSTRACT

Many neurodegenerative diseases are associated with deposits of aggregated protein in the brain. The molecular pathways through which soluble proteins misfold to form amyloids and large protein aggregates often include diverse oligomeric species, only some of which progress to the amyloid state. Here we show that prefibrillar huntingtin (HTT) oligomers, isolated from Huntington's disease (HD) affected human brain samples or mouse models, stimulate polyglutamine amyloid formation. Fibrillar HTT oligomers have been shown to be unstable under denaturing conditions and appear not to lead to amyloid formation. Here we show that prefibrillar HTT oligomers are remarkably stable and are potent seeds of polyglutamine amyloid formation. Therefore, our findings help to dissect the complex molecular pathway of HTT misfolding.

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1. Introduction

Huntington's disease (HD) is an autosomal-dominant, neurodegenerative disorder caused by an expansion of CAG repeats in the huntingtin (HTT) gene [1]. An invariably fatal disorder, HD is characterized by involuntary movement, behavioral abnormalities, and progressive decline in cognitive function. One of the salient features of HD brain pathology is the presence of neuronal intranuclear and perinuclear inclusions, which are primarily composed of an N-terminal proteolytic fragment of the huntingtin (HTT) protein [2]. Transgenic mouse models have been developed to emulate the pathogenesis of HD, thus enabling investigation of the molecular species associated with neurodegeneration resulting from expression of disease-related HTT and HTT fragments [3].

The roles of protein misfolding and inclusion formation in HD pathogenesis have been controversial: some studies provide evidence for the protein misfolding process that leads to inclusions as a central event in pathogenesis [2,4–7], while others suggest that large inclusions are neuroprotective [8,9]. Heterogeneous oligomeric intermediates form along the protein misfolding pathway [10] and have been associated with toxicity [11–16]. For Aβ, a peptide found in amyloid plaques in the brains of patients with Alzheimer's disease, two different oligomeric conformations have been isolated, both of which seed the propagation of their own oligomeric strains [17,18]. In another study, prefibrillar Aβ oligomers were shown to act as seeds for amyloid formation [19]. Which oligomeric conformations of HTT are on the amyloid formation pathway is less well defined.

We recently developed and applied a seeding assay to detect the presence of misfolded HTT in biological samples [20]. This assay relied upon the propensity of pathological protein conformations to accelerate the conversion of monomeric proteins into amyloid, a process which can be monitored using the fluorescent dye Thioflavin T. Similar assays have been developed for the detection of prions [21,22] and Aβ [24,46]. When applied to *drosophila* models of HD, seeding potency correlated well with toxicity [25]. Here, we demonstrate that prefibrillar HTT oligomers isolated from two HD mouse models (Table 1) and human HD brain homogenates by immunopurification with the oligomer-specific antibody A11, seed polyglutamine amyloid formation. These HTT oligomeric species have strong seeding potency compared to the seeding activity of HTT conformations immunopurified using a panel of antibodies recognizing different epitopes within the HTT protein (see Table 2).

Author contributions: O.A.M. and S.G. collected and interpreted the data, and wrote the paper. D.W.C. designed the study, analyzed the data, and wrote the paper. All authors discussed the results and commented on the manuscript.

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Table 1
Mouse models used in this study.

Mouse model	PolyQ length	Protein context	References
R6/2	>180	Exon1 product	[34]
YAC128	128	Full HTT product	[43]

2. Results

We purified misfolded HTT from crude brain homogenates using magnetic bead-assisted immunopurification to test for amyloid seeding activity (Fig. 1A). Magnetic beads labeled with the MW8 antibody [26] were incubated with clarified brain homogenates of end-stage R6/2 mice (or age matched WT controls), captured with a magnet, and rinsed. The beads were incubated with 6 M GdnHCl to elute HTT, and the spent magnetic beads were removed with a magnet. Misfolded HTT has been shown to be stable in these denaturing conditions [27]. The eluent was incubated with a solution of monomeric $K_2Q_{44}K_2$ peptide and amyloid formation kinetics were monitored with Thioflavin T (ThT) fluorescence. The solution containing eluent from beads which had been incubated with R6/2 brain homogenate formed amyloid more rapidly than that with control eluent (Fig. 1B). To simplify kinetic data analysis throughout this report, we quantified ThT fluorescence at a time point just after the control samples began to spontaneously form amyloid, which we called $t_{1.2}$ (defined in Methods and indicated by the dashed line in Fig. 1B). At this time, ThT fluorescence was significantly ($P < 0.001$) higher for samples containing R6/2 HTT extracts compared to control (Fig. 1B).

Incubation of R6/2 brain homogenates with beads labeled with 9E10 antibody, which is specific for c-myc and does not recognize HTT, resulted in no increase in ThT intensity at $t_{1.2}$ when added to amyloid formation reactions (Fig. 1C). Similarly, elimination of the primary antibody in the immunopurification protocol led to a loss of signal in the seeding assay (Fig. 1C). The direct addition of the magnetic beads containing HTT immunopurified from R6/2 brain tissue also accelerated amyloid formation (SI Fig. 1), however the discrimination between positive and negative samples was not as pronounced as that observed following elution with GdnHCl, and thus elution was used for all subsequent experiments.

We applied the same immunopurification and seeding technique using A11, an antibody that is specific for oligomeric protein species and does not bind to monomeric or amyloid protein conformations [28]. Brain homogenates from three different sources (end stage R6/2, YAC128, and human HD) or appropriate negative control sources were incubated with A11-labeled beads, and the eluent was added to amyloid formation reactions. The ThT fluorescence signal at $t_{1.2}$ was significantly higher for eluent derived from disease brain compared to wild-type mouse and normal (non-HD) human control brain samples (Fig. 2A, $P < 0.001$, $N = 3$ brains of each type).

The limit of detection for misfolded HTT in the R6/2 model was determined by gradually reducing the amount of brain homogenate used per experiment while keeping the quantity of A11 antibody the same (Fig. 2B). Seeding activity of A11-purified HTT oligomers was observed with as little as 0.02 mg of R6/2 brain tissue equivalent. Use of the immunopurification method described here with the seeding assay did not significantly alter the limit of detection compared to the original protocol, which relied on sucrose gradient purification method [20,36].

Immunogold-EM was used to confirm that A11 immunopurifies oligomeric species from brain homogenate. The eluent from A11 labeled beads incubated with brain homogenates from R6/2 mice, YAC128 mice, and human HD samples had small EM48-positive aggregates in the size range of 20–70 nm (Fig. 2C). The characteristics of oligomers observed were comparable to those reported by others for HTT oligomers [30–33]. Eluents from control brain homogenate samples had only monomeric immunoreactivity, as identified by the lack of clustering gold particles, while eluents immunopurified with the MW8 antibody recognizing HTT contained large aggregates (Fig. 2C).

Table 2
Primary antibodies used to immunopurify HTT from HD brain homogenates.

Antibody	Epitope	References
A11	Protein oligomers	[28]
MW8	AEEPLHRP after polyglutamine stretch	[26]
3B5H10	Polyglutamine	[14]
mEM48	PPGPAVA after polyglutamine stretch	[44]
H7540	N-terminal residues 3–16 of human HTT	Sigma–Aldrich

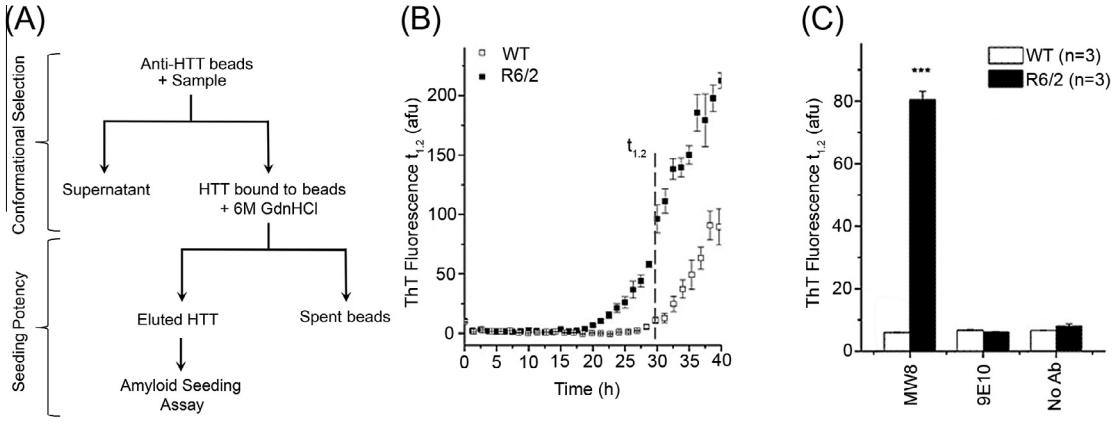

Fig. 1. Immunopurified HTT from R6/2 mouse brain tissue accelerates conversion of polyglutamine peptides into amyloid. (A) Schematic of the experimental approach used for conformational selection of HTT protein and determination of seeding activity. In brief, clarified brain homogenate was incubated with magnetic beads coated with HTT-specific antibodies, followed by separation of the magnetic beads from the tissue supernatant in a magnetic field. The purified HTT protein was then tested for amyloid seeding activity after elution from the antibodies in 6 M GdnHCl. (B) Amyloid formation kinetics were measured in a buffered solution of $K_2Q_{44}K_2$ peptide containing eluted HTT from R6/2 and wild type control brain homogenate immunopurified with MW8. Accelerated amyloid formation was observed by ThT fluorescence for R6/2 brain samples compared to control samples. Accelerated conversion of polyglutamine into amyloid was quantified using the measurement observed just after the end of the lag phase for the control sample ($t_{1.2}$ indicated on graph). (C) No significant increase in ThT fluorescence was observed when a negative control antibody (9E10), or no antibody at all (No Ab), was used in the immunopurification step. Error bars denote standard error ($N = 4$ independent measurements for each sample; $^{***}P < 0.001$).

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