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·Original Article·

Soluble N-terminal fragment of mutant Huntingtin protein impairs mitochondrial axonal transport in cultured hippocampal neurons

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ABSTRACT

Huntington's disease (HD) is an autosomal dominant, progressive, neurodegenerative disorder caused by an unstable expansion of CAG repeats (>35 repeats) within exon 1 of the interesting transcript 15 (*IT15*) gene. This gene encodes a protein called Huntingtin (Htt), and mutation of the gene results in a polyglutamine (polyQ) near the N-terminus of Htt. The N-terminal fragments of mutant Htt (mHtt), which tend to aggregate, are sufficient to cause HD. Whether these aggregates are causal or protective for HD remains hotly debated. Dysfunctional mitochondrial axonal transport is associated with HD. It remains unknown whether the soluble or aggregated form of mHtt is the primary cause of the impaired mitochondrial axonal transport in HD pathology. Here, we investigated the impact of soluble and aggregated N-terminal fragments of mHtt on mitochondrial axonal transport in cultured hippocampal neurons. We found that the N-terminal fragment of mHtt formed aggregates in almost half of the transfected neurons. Overexpression of the N-terminal fragment of mHtt decreased the velocity of mitochondrial axonal transport and mitochondrial mobility in neurons regardless of whether aggregates were formed. However, the impairment of mitochondrial axonal transport in neurons expressing the soluble and aggregated N-terminal fragments of mHtt did not differ. Our findings indicate that both the soluble and aggregated N-terminal fragments of mHtt impair mitochondrial axonal transport in cultured hippocampal neurons. We predict that dysfunction of mitochondrial axonal transport is an early-stage event in the progression of HD, even before mHtt aggregates are formed.

Keywords: Huntington; mitochondria; axonal transport; hippocampal neurons

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant, progressive, neurodegenerative disorder characterized by chorea, cognitive decline, and psychiatric symptoms[1]. Currently, there is no effective treatment to prevent its progression[2]. The cause of HD is an unstable expansion of CAG repeats (>35 repeats) within exon 1 of the interesting transcript 15 (*IT15*) gene. There is an inverse correlation between the age of onset and the number of CAG repeats; more CAG repeats are associated with an earlier age of disease onset. Normal *IT15* gene CAG repeats range from 6 to 35. This gene encodes a protein called Huntingtin (Htt), and mutation of the gene can result in a polyglutamine (polyQ) near its N-terminus[3]. Mutant Htt (mHtt) is cleaved to form N-terminal fragments containing the polyQ, which are toxic to neurons[4, 5]. Preventing the formation of N-terminal fragments by inhibiting mHtt cleavage reduces

the toxicity in cultured striatal neurons[6]. Exon 1 of the *IT15* gene containing extended CAG repeats induces a typical neurological phenotype in transgenic mice[7]. These data suggest that the N-terminal fragments of mHtt are sufficient to cause HD. In addition, it is believed that polyQ tends to aggregate. mHtt forms intracellular aggregates in nuclei and neurites[8]. Whether these aggregates are causal or protective for HD remains hotly debated[9]. Some evidence suggests that aggregate formation plays a causative role in the pathology of HD[10], while others have suggested that aggregate formation is beneficial, in that aggregates sequester the toxic soluble (non-aggregate-forming) mHtt into less toxic aggregates[11].

Under physiological conditions, the mitochondria in neurons move anterograde toward the synapse and retrograde toward the cell body along the axons[12]. Dysfunctional mitochondrial axonal transport is associated with neurodegenerative diseases[13]. It has been demonstrated that mHtt aggregates impair mitochondrial trafficking in cultured cortical neurons[14]. Recent studies have also shown that soluble mHtt impairs mitochondrial trafficking in cultured primary striatal neurons[15]. It remains unclear which form of mHtt (aggregated or soluble) impairs mitochondrial transport more severely.

The hippocampus is important for cognitive processes. Atrophy of the hippocampus contributes to the cognitive difficulties in both pre-symptomatic and symptomatic HD patients[16, 17]. Evidence that mHtt impairs mitochondrial trafficking in hippocampal neurons is still lacking. Thus, we investigated mitochondrial axonal transport in primary hippocampal neurons expressing N-terminal fragments of mHtt. We compared the effects of soluble and aggregated mHtt to identify their exact roles in this process.

MATERIALS AND METHODS

DNA Constructs

The vector for mitochondria-targeted DsRed2 (pDsRed2-Mito) was from Clontech (Mountain View, CA). This construct contains mitochondria-targeted DsRed fluorescent protein *via* the targeting peptide of cytochrome c oxidase. As described previously, we generated cDNA constructs encoding green fluorescent protein (GFP)-tagged N-terminal fragments of wild-type (19Q) or mutant (70Q) Htt in the eukaryotic expression vectors GFP-Htt and GFP-mHtt, respectively[18]. A similar fragment can be produced by proteolytic cleavage of full-length mHtt in HD[4] and is sufficient to cause HD[7].

Cell Culture

Primary cultured hippocampal neurons were obtained from Sprague-Dawley rats on embryonic day 17, as described previously[19]. Cultures were maintained in basal Eagle's medium supplemented with B27 and incubated in a humidified environment at 37°C and 5% CO_2. Half of the medium was changed every 3 days. Hippocampal neurons were transfected on day 7 *in vitro* using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, as described previously[20].

Imaging and Aggregate Formation

Twenty-four hours after GFP-Htt, GFP-mHtt, or GFP transfection, the cultured hippocampal neurons were placed in extracellular solution in a temperature-controlled chamber at 37°C and imaged with a Fluoview500 laser confocal microscope (Olympus, Tokyo, Japan) using a 60× water objective. The percentages of cells containing aggregates were determined by counting the cells containing visible aggregates and the total number of cells emitting green fluorescence. All experiments were carried out in triplicate. In each experiment, 20 fields of view were randomly selected and analyzed.

Imaging and Mitochondrial Transport Analysis

Twenty-four hours after co-transfection with pDsRed2 and GFP-Htt, GFP-mHtt, or GFP, the cultured hippocampal neurons were placed in extracellular solution in a temperature-controlled chamber at 37°C and imaged with the Fluoview500 laser confocal microscope using a 60× water objective. Typical axons, which were identified based on morphological characteristics such as uniform thickness and lack of branching, were selected for mitochondrial axonal transport measurements. Imaging was performed at a rate of one frame every 5 s for 8 min in total. Individual mitochondria were tracked manually using Image J software (National Institutes of Health, Bethesda, MD). The velocity of mitochondria was calculated by dividing the total distance traveled by the total time of movement, and the velocities were imported into Excel for further analysis. Stationary mitochondria were identified as those with no displacement throughout the observation period. We

assessed 159 mitochondria from eight control neurons co-transfected with pDsRed2-Mito and GFP, 153 mitochondria from eight neurons co-transfected with pDsRed2-Mito and GFP-Htt, 160 mitochondria from eight neurons co-transfected with pDsRed2-Mito and GFP-mHtt without aggregate formation, and 148 mitochondria from eight neurons co-transfected with pDsRed2-Mito and GFP-mHtt with aggregate formation. Time-space plots were constructed using the "kymograph" plugins in Image J[21]. The plots were constructed by converting a time-lapse movie along an axon into a spatial position *versus* time image, displaying the distance travelled on the x-axis and the time recorded on the y-axis.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0. Data are presented as mean ± SEM. Significant differences between groups were identified using one-way analysis of variance with a post-hoc Tukey's test.

RESULTS

The N-terminal Fragment of mHtt Aggregates in Cultured Hippocampal Neurons

In neurons transfected with GFP, the entire cell was diffusely labeled with green fluorescence 24 h after transfection. The N-terminal fragment of Htt was diffusely expressed in the cytoplasm without aggregation. In contrast, N-terminal fragments of mHtt formed aggregates throughout the cell, in the nucleus, cytoplasm, dendrites, and axons (Fig. 1), in 44 ± 2.87% of transfected neurons.

Effects of Soluble and Aggregated mHtt on the Velocity of Mitochondrial Axonal Transport

We quantified the velocity of mitochondrial axonal transport in the transfected hippocampal neurons that expressed GFP-Htt, GFP-mHtt, or GFP and pDsRed2-Mito. While the N-terminal fragments of mHtt formed aggregates in some neurons, this did not occur in others that expressed what we considered to be soluble mHtt. Therefore, we studied the influence of mHtt on transport under different circumstances: with or without aggregate formation (in this case, soluble mHtt was the major type of mHtt in the cells) (Fig. 2). We found that Htt expression did not alter the velocity of mitochondrial movement compared with control GFP-transfected neurons (anterograde, Htt 0.30 ± 0.03 *versus* control 0.28 ± 0.03 μm/s; retrograde, Htt 0.28 ± 0.03 *vs* control 0.28 ± 0.02 μm/s). However, compared with control, aggregated mHtt (anterograde, 0.11 ± 0.01 *vs* 0.28 ± 0.03 μm/s; retrograde, 0.12 ± 0.01 *vs* 0.28 ± 0.02 μm/s) and soluble mHtt (anterograde, 0.16 ± 0.02 *vs* 0.28 ± 0.03 μm/s; retrograde, 0.14 ± 0.02 *vs* 0.28 ± 0.02 μm/s) greatly

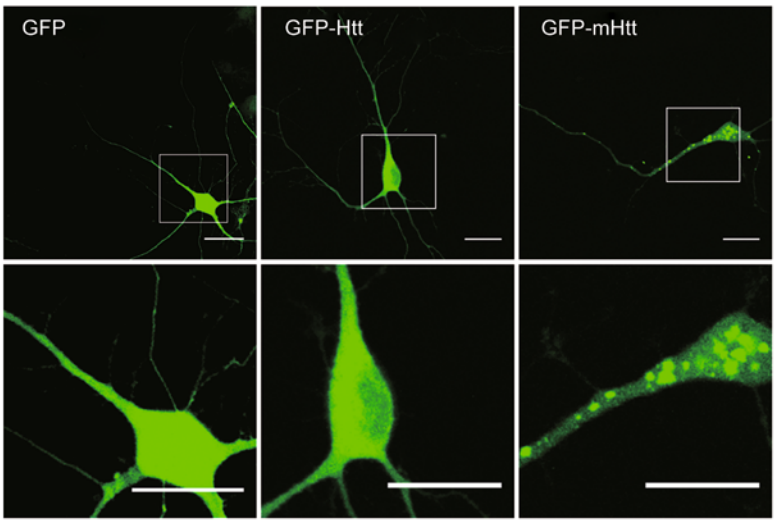

Fig. 1. The N-terminal fragment of mHtt formed aggregates in hippocampal neurons 24 h after transfection. Left panel, GFP was diffusely expressed throughout the cell. Middle panel, the N-terminal fragment of Htt was diffusely expressed in the cytoplasm. Right panel, the N-terminal fragments of mHtt formed aggregates throughout the cell. Scale bars, 20 μm.

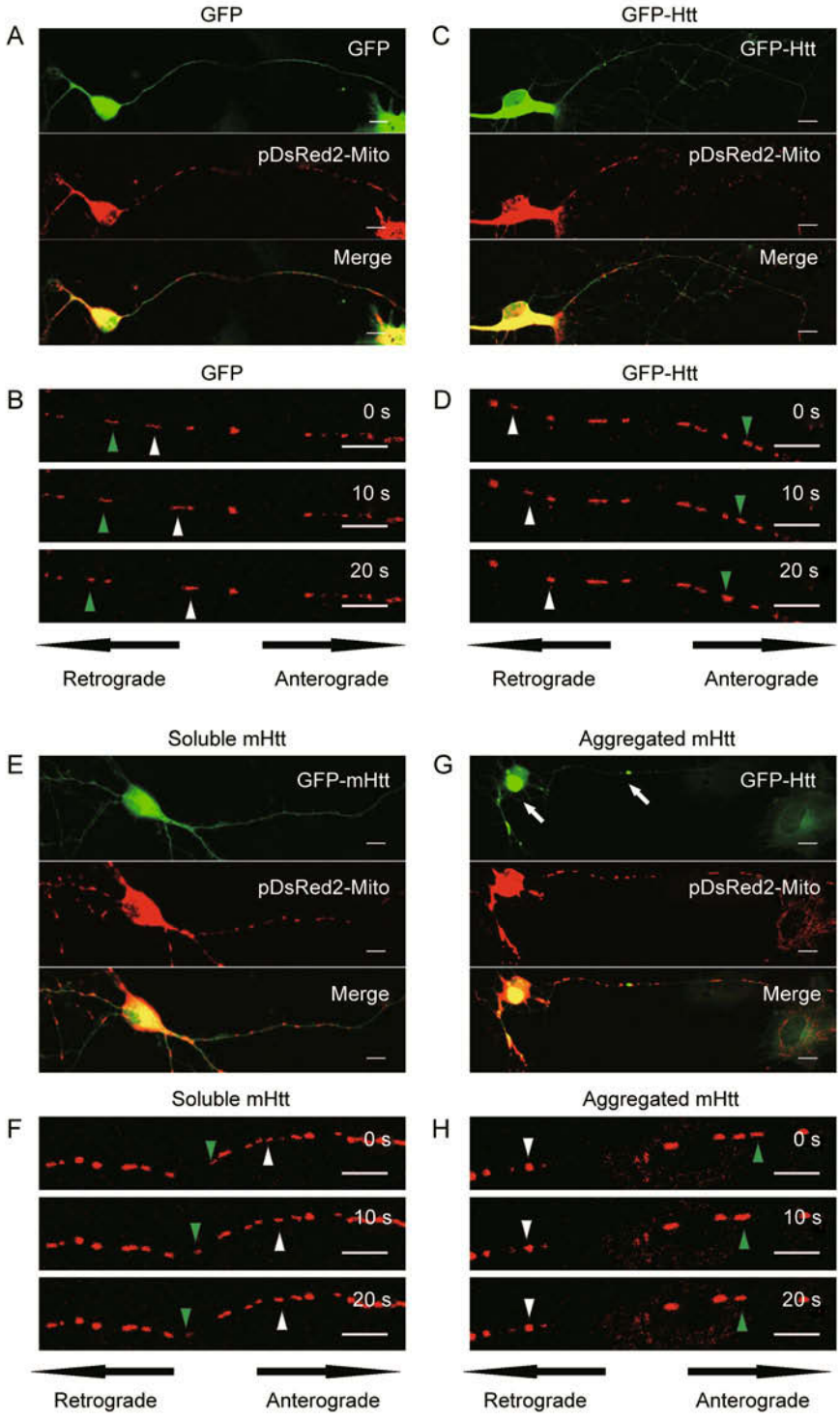

Fig. 2. Soluble and aggregated mHtt impaired mitochondrial axonal transport in hippocampal neurons. A: Neurons co-transfected with GFP and pDsRed2-Mito. C: Neurons co-transfected with GFP-Htt and pDsRed2-Mito. E, G: Presence or absence of aggregate formation in neurons co-transfected with GFP-mHtt and pDsRed2-Mito. White arrows indicate aggregates. B, D, F, H: Examples of moving mitochondria from transfected neurons. White triangles indicate mitochondria migrating in an anterograde direction along the axon. Green triangles indicate mitochondria migrating in a retrograde direction along the axon. Scale bars, 5 μm.

reduced the velocity. There were no significant differences in the velocity of anterograde and retrograde mitochondrial movement between neurons expressing aggregated mHtt and soluble mHtt (Fig. 3). These data suggested that the soluble and aggregated forms of mHtt both impaired mitochondrial axonal transport in cultured hippocampal neurons. The aggregated mHtt did not impair transport more severely than the soluble form.

Effects of Soluble and Aggregated mHtt on Mitochondrial Mobility

We quantified mitochondrial mobility as the percentage of stationary mitochondria in the co-transfected hippocampal neurons that expressed GFP-Htt, GFP-mHtt, or GFP, and pDsRed2-Mito. Htt did not alter the percentage of stationary mitochondria compared with control GFP-transfected neurons (68.02 ± 2.15% *vs* 65.29 ± 5.42%). Soluble mHtt (78.27 ± 1.78% *vs* 65.29 ± 5.42%) and aggregated mHtt (78.98 ± 1.85% *vs* 65.29 ± 5.42%) increased the percentage of stationary mitochondria compared with the control. There were no significant differences in the percentage of stationary mitochondria between neurons expressing soluble and aggregated mHtt (78.27 ± 1.78% *vs* 78.98 ± 1.85%; Fig. 4). Consistent with the data on mitochondrial velocity, soluble and aggregated mHtt both decreased the mobility. The impairment of mitochondrial mobility in neurons expressing soluble and aggregated mHtt showed no difference.

We also visualized the movement of mitochondria in axons with time-space plots[21], a useful tool for analyzing axonal transport[22], in which moving mitochondria are represented as diagonal lines. The gradient of a line corresponds to the velocity and stationary mitochondria are visible as vertical lines. Using this technique, we observed that mitochondria were present in both moving and stationary clusters in the axons. Compared with control GFP-transfected neurons, soluble and aggregated mHtt both diminished mitochondrial movement (Fig. 5).

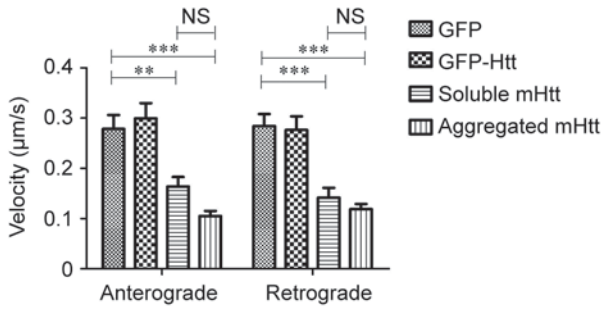

Fig. 3. Soluble and aggregated mHtt reduced the velocity of mitochondrial movement along the axons of cultured hippocampal neurons. Htt did not alter the velocity of anterograde and retrograde mitochondrial movement compared with control GFP-transfected neurons. The velocity of anterograde and retrograde mitochondrial movement was reduced in neurons expressing soluble mHtt or aggregated mHtt compared with the control. There was no significant difference in the velocity between neurons expressing soluble and aggregated mHtt (NS, not significant; **P <0.01; ***P <0.001).

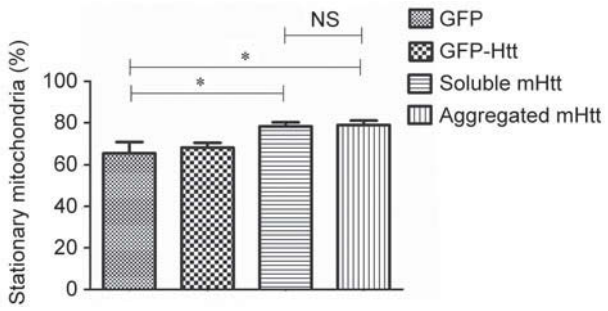

Fig. 4. Mobility of mitochondria in cultured hippocampal neurons. Htt did not alter the percentage of stationary mitochondria compared with control GFP-transfected neurons. Soluble or aggregated mHtt increased the percentage of stationary mitochondria compared with the control. There was no significant difference in the percentage of stationary mitochondria between neurons expressing soluble and aggregated mHtt (NS, not significant; *P <0.05).

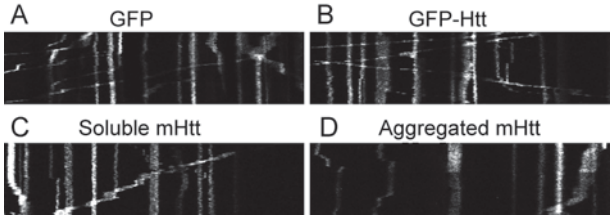

Fig. 5. Representative time-space plots of mitochondrial transport in transfected neurons. A: Mitochondrial movement through a GFP-transfected axon. B: Mitochondrial movement through an axon transfected with pDsRed2-Mito and GFP-Htt. C, D: Mitochondrial movement through axons transfected with pDsRed2-Mito and GFP-mHtt expressing soluble (C) and aggregated (D) mHtt.

DISCUSSION

Intracellular aggregates of mHtt are the hallmark of HD[9]. In the present study, the N-terminal fragment of mHtt formed aggregates in the nucleus, perinuclear region, dendrites and axons in 44 ± 2.87% of transfected hippocampal neurons, consistent with the classical feature of HD. The N-terminal fragment of mHtt did not form aggregates in the remaining neurons and was considered soluble mHtt. This allowed us to compare the severity of mitochondrial axonal transport impairment caused by soluble and aggregated mHtt.

Impairment of mitochondrial axonal transport is associated with the pathophysiology of HD[23, 24]. Several mechanisms for the impairment of transport by mHtt have been proposed, such as direct physical blockage by mHtt aggregates or abnormal transport-related protein interactions with soluble mHtt[25]. It has been reported that mHtt aggregates impair mitochondrial trafficking in cultured cortical neurons[14]. In addition, a recent report suggested that soluble mHtt impairs mitochondrial axonal transport in primary striatal neurons[15]. However, whether the soluble or aggregated form of mHtt is the primary cause of the impaired transport in HD remains unknown. Further research is needed to investigate the mechanisms by which aggregated and soluble mHtt impair mitochondrial axonal transport during the pathogenesis of HD. We compared the effects of soluble and aggregated mHtt directly in cultured hippocampal neurons, and found that mHtt decreased both the transport velocity and mitochondrial mobility regardless of whether it formed aggregates. However, this impairment did not differ between neurons expressing soluble and those expressing aggregated mHtt. Indeed, mHtt affects many aspects of the cell, such as protein clearance, mitochondrial dysfunction, N-methyl-*D*-aspartate receptor-mediated excitotoxicity, oxidative stress, and gene transcription[26]. Mitochondrial axonal transport might be impaired as a secondary consequence of reduced cell viability. In our experiments, we could not establish that mitochondrial traffic dysfunction is the first step in the pathogenesis induced by mHtt. Our studies suggested that the transport deficits were caused by soluble mHtt independent of aggregate formation, supporting the hypothesis that soluble mHtt directly impairs mitochondrial axonal transport in neurons[15]. The transport deficit could be an early-stage event in HD progression, even before mHtt aggregates are formed. Aggregated mHtt did not impair mitochondrial axonal transport more severely than soluble mHtt, suggesting that blockage by aggregated mHtt does not play a major role in the transport deficit in HD. On the other hand, mHtt aggregates did not play a protective role in our experiments either. As aggregated mHtt did not reduce the impairment of mitochondrial axonal transport, the exact role of aggregates of mHtt remains unclarified. The mechanism by which soluble mHtt disrupts transport may be associated with the altered interaction between axonal transport-related proteins (such as dynein and kinesin)[12] and soluble mHtt. This molecular mechanism requires further study, and the development of molecules that improve mitochondrial axonal transport in HD would be worthwhile.

Previous findings have shown that mHtt disrupts the axonal transport of mitochondria in striatal and cortical neurons[14, 27]. There was little evidence for impairment of mitochondrial axonal transport by mHtt in hippocampal neurons, although neuron loss has been reported in the hippocampus of HD patients[17]. Here, we demonstrated that the N-terminal fragment of mHtt affected mitochondrial transport in cultured hippocampal neurons. We suggest that this impairment is potentially the molecular mechanism that mediates hippocampal neuron loss in HD.

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