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Aggregate formation and the impairment of long-term synaptic facilitation by ectopic expression of mutant huntingtin in *Aplysia* neurons

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Abstract

Huntington's disease (HD) is caused by an expansion of a polyglutamine (polyQ) tract within huntingtin (htt) protein. To examine the cytotoxic effects of polyQ-expanded htt, we overexpressed an enhanced green fluorescent protein (EGFP)-tagged N-terminal fragment of htt with 150 glutamine residues (Nhtt150Q-EGFP) in *Aplysia* neurons. A combined confocal and electron microscopic study showed that *Aplysia* neurons expressing Nhtt150Q-EGFP displayed numerous abnormal aggregates (diameter 0.5–5 μm) of filamentous structures, which were formed rapidly (approximately 2 h) but which were sustained for at least 18 days in the cytoplasm. Furthermore, the overexpression of Nhtt150Q-EGFP in sensory cells impaired 5-hydroxytryptamine (5-HT)-induced long-term synaptic facilitation in sensori-motor synapses without affecting basal synaptic strength or short-term facilitation. This study demonstrates the stability of polyQ-based aggregates and their specific effects on long-term synaptic plasticity.

Keywords: aggregation, *Aplysia*, huntingtin, polyglutamine, synaptic facilitation.

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Huntington's disease (HD) is an inherited neurological disorder that causes progressive degeneration of the cognitive and motor functions (Harper 1991). The HD gene mutation is an expansion of a CAG repeat, a trinucleotide motif that encodes a polyglutamine (polyQ) stretch within the mature protein. Moreover, it is known that repeat expansion of greater than approximately 35–40 repeats causes neurodegenerative disease (Evert *et al*. 2000; Paulson 2000). Increasing evidence indicates that the expanded glutamine repeats cause small protein fragments to accumulate and aggregate in the nuclei or cytoplasm of cells (Davies *et al*. 1997; Becher *et al*. 1998; Gutekunst *et al*. 1999). Neuronal loss in HD appears to be initiated by the production of N-terminal fragments of polyQ-expanded huntingtin (htt) and cellular aggregation (DiFiglia *et al*. 1997). Moreover, abnormal behavior and aggregate formation have been observed in transgenic mice expressing htt with an expanded polyQ stretch (Brouillet *et al*. 1999). The association between HD and aggregation has led to the idea that such aggregates are toxic and play an important role in the pathology of HD. However, the dominant mechanism by which polyQ expansion causes neurodegenerative disease is unknown. An understanding of the pathogenic mechanism of HD will have a wide impact on the biology of neurodegenerative processes.

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Abbreviations used: CBP, CREB binding protein; CRE, cyclic AMP response element; CREB, CRE binding protein; DRPLA, dentatorubral-pallidoluysian atrophy; EGFP, enhanced green fluorescent protein; EM, electron microscopy; EPSP, excitatory post-synaptic potential; HD, Huntington's disease; htt, huntingtin; 5-HT, 5-hydroxytryptamine; MAP kinase, mitogen-activated protein kinase; Nhtt, N-terminal fragment of huntingtin; PBS, phosphate-buffered saline; PC-12, pheochromocytoma-12; PI, propidium iodide; PKA, protein kinase A; polyQ, polyglutamine; TBP, TATA binding protein.

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In order to understand the mechanism of polyQ-expanded htt pathogenesis, we overexpressed polyQ-expanded htt fused to enhanced green fluorescent protein (EGFP) in *Aplysia* neurons, which provide a useful model for the study of synaptic plasticity. Accordingly, we found that overexpressed polyQ-expanded htt caused abnormal aggregation and impaired synaptic plasticity in an *Aplysia* cell model.

Materials and methods

Subcloning, microinjection, and fluorescence microscopy

N-terminal fragment of htt with 150 glutamine residues (Nhtt150Q-EGFP) and Nhtt16Q-EGFP have been described previously (Wang *et al.* 1999; Jana *et al.* 2001). Each construct contains 1–90 amino acids of truncated N-terminal fragment and different polyQ length (16Q and 150Q), fused to N-terminus of EGFP. Each form of Nhtt150Q-EGFP or Nhtt16Q-EGFP was subcloned into *Hind*III/*Xba*I-digested pNEXδ (Kaang *et al.* 1993) to create pNEXδ-Nhtt150Q-EGFP and pNEXδ-Nhtt16Q-EGFP, respectively. Nhtt150Q-EGFP was subcloned into *Pst*I/*Xba*I-digested pNEX2 or pNEX3 (Kaang 1996) to create pNEX2-Nhtt150Q-EGFP or pNEX3-Nhtt150Q-EGFP, respectively. To examine the effect of overexpressed polyQ-expanded htt in *Aplysia* neurons, cultured pedal cells or sensory cells were microinjected with a solution containing pNEXδ-Nhtt150Q-EGFP, pNEX2-Nhtt150Q-EGFP, pNEX3-Nhtt150Q-EGFP or pNEXδ-Nhtt16Q-EGFP (1 mg/mL; Kaang 1996). Microinjection into *Aplysia* neurons was performed using air pressure, as described previously (Kaang *et al.* 1992). To determine the time course of the aggregation process, fluorescent images of the cells were obtained every 15 min from the same visual field using a time-lapse fluorescence confocal laser-microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, CA, USA), 20–24 h after transfection. To quantify the total number of aggregates in a cell, aggregates with a diameter of more than 0.5 μm were counted. The area occupied by these aggregates was calculated by summing the areas of representative circles using Laserpix software (Image-Pro Plus version 4.0, Bio-Rad).

Immunocytochemistry

Immunocytochemistry was carried out as described in Martin *et al.* (1997). Cultured cells were fixed 18 days after transfection. The primary antihuntingtin antibody (mAb 5374, Chemicon International, Temecula, CA, USA) and the secondary Cy3-conjugated anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used at dilution ratios of 1 : 50 and 1 : 100, respectively. Fluorescent images were obtained through a confocal laser-scanning microscope (Radiance 2000, Bio-Rad).

Propidium iodide staining

Fixed sensory neurons were propidium iodide [PI; Sigma Chemical Co., St Louis, MO, USA; 20 μg/mL in phosphate-buffered saline (PBS)] stained in dissociated cultures for 5 min at room temperature in the dark, as previously described by Lim *et al.* (2002). After the staining solution had been removed by washing, the shapes of nuclei were observed under a confocal microscope (Radiance 2000, Bio-Rad).

Electron microscopy

Aplysia neurons were grown on poly-L-lysine-coated film coverslips (Aclar film, Pelco International, Redding, CA, USA) and Nhtt150Q-EGFP or Nhtt16Q-EGFP expression was induced for 3 days. For fixation, cultured neurons were rinsed three times with artificial seawater, fixed in a mixture of 1% paraformaldehyde, 2.5% glutaraldehyde, 10% dimethylsulfoxide, 10% sucrose, and 0.05% $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4) for 1 h, bathed in 2% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer for 16–20 h at room temperature, and treated with 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Finally, the samples were dehydrated with ethanol and embedded in Spurr's resin. Serial sections were cut using an ultramicrotome (Reichert-Jung, Leica, Depew, NY, USA), stained with uranyl acetate and lead citrate, and examined using a JEOL electron microscope (1200 EX-II; JEOL, Peabody, MA, USA).

Cell cultures, electrophysiology, and the induction of long-term facilitation by 5-hydroxytryptamine

Culture dishes and medium were prepared as previously described (Montarolo *et al.* 1986; Schacher and Proshansky 1986). Pedal neurons were isolated from the pedal ganglia of adult animals, and incubated for 2–3 days at 18°C. Sensory cells isolated from the pleural ganglia of adult animals (100–150 g) were co-cultured with the motor neuron LFS obtained from the abdominal ganglia of adult animals, as described previously (Montarolo *et al.* 1986). The co-culture was maintained for 3–4 days at 18°C to allow time for the formation and stabilization of synaptic connections. The motor cell was then impaled intracellularly with a glass microelectrode filled with 2 M K–acetate, 0.5 M KCl, 10 mM K–HEPES (10–15 MΩ), and the membrane potential was held at −30 mV below its resting value. The excitatory post-synaptic potential (EPSP) was evoked in LFS by stimulating the sensory neurons with a brief depolarizing stimulus using an extracellular electrode. To examine basal synaptic transmission, the EPSP was measured before and 48 h after microinjection. To investigate the effect of polyQ-expanded htt on synaptic plasticity, the initial EPSP value was measured 48 h after microinjection. The cultures then received one pulse of 5-hydroxytryptamine (5-HT) to induce short-term facilitation or five pulses of 5-HT for 5 min at 15-min intervals to induce long-term facilitation. The amount of synaptic facilitation was calculated as the percentage change in EPSP amplitude recorded after the 5-HT treatment, versus its initial value before treatment. All data are presented as the mean percentage change ± SEM in EPSP amplitude.

Results

Aggregates formed by polyQ-expanded huntingtin

Many groups have reported that aggregates are formed by polyQ repeats of polyQ-expanded htt (Cooper *et al.* 1998; Li and Li 1998; Lunkes and Mandel 1998). To determine whether aggregates could be formed in *Aplysia* neurons, we overexpressed by microinjection EGFP-tagged truncated N-terminal fragments of htt with 150 or 16 glutamine repeats (pNEXδ-Nhtt150Q-EGFP, pNEXδ-Nhtt16Q-EGFP; Wang *et al.* 1999) in *Aplysia* sensory or pedal neurons.

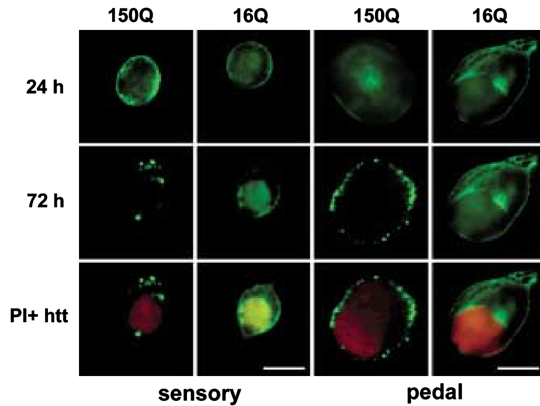

Fig. 1 Ectopic expression of polyQ-expanded htt in *Aplysia* cultured neurons. Cultured *Aplysia* sensory neurons or pedal neurons were injected with Nhtt150Q-EGFP or Nhtt16Q-EGFP. The confocal images were taken of cell bodies 24 h and 72 h after DNA microinjection. The fluorescent images show that aggregates appear in Nhtt150Q-EGFP-positive cells 72 h after microinjection. Cells were stained with PI 72 h after microinjection. In the merged images, aggregates (green image) mainly appear in the cytoplasmic region of Nhtt150Q-EGFP-positive cells. However, Nhtt16Q-EGFP is diffusely expressed in the nucleus and the cytoplasm. Scale bar, 50 μm.

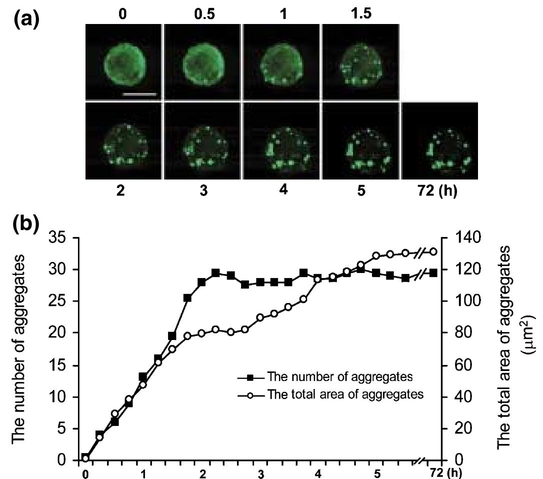

Fig. 2 The aggregation process of polyQ-expanded htt in *Aplysia* cultured neurons. The aggregates in Nhtt150Q-EGFP-positive cells were monitored every 15 min using time-lapse confocal microscopy 20–24 h after transfection. The number of aggregates rapidly increased but saturated at 2 h after the first aggregates appeared, whereupon the aggregates grew in size at 5 h. (a) A representative set of confocal images showing the time course of the aggregate formation. This typical cell shows that most of the aggregates were rapidly formed within 2 h of the first aggregate appearing. Scale bar, 50 μm. (b) The histogram shows the averaged total area and the number of aggregates. Data show the mean values of two representative cells.

Initially, 24 h after microinjection, Nhtt150Q-EGFP or Nhtt16Q-EGFP was found to be diffusely expressed in the cell bodies. However, 48–72 h after transfection, the majority of the sensory cells (80%, $n = 40/50$) or of the pedal cells (79.4%, $n = 27/35$) expressing Nhtt150Q-EGFP formed abnormal aggregates, which were found mainly in the cell body and a few axons (Figs 1 and 3). Nuclear staining by PI showed that the aggregates were mainly localized in the cytoplasm (Fig. 1). In contrast, *Aplysia* neurons expressing Nhtt16Q-EGFP did not show aggregation over this time course or even longer (up to 10 days) and, furthermore, neither did lower expression levels of Nhtt150Q-EGFP driven by pNEX2-Nhtt150Q-EGFP (Kaang 1996). Taken together, these data indicate that the aggregation process requires a certain length and expression level of polyQ repeats.

The aggregation process of polyQ-expanded htt

To further examine the aggregation of polyQ-expanded htt, we monitored the fluorescent aggregates in cells that expressed Nhtt150Q-EGFP using time-lapse confocal microscopy (Fig. 2a). Nhtt150Q-EGFP was diffusely distributed over the entire cell body area until small aggregate cores began to appear some 20–24 h after transfection. Interestingly, the size and the number of aggregates rapidly increased during the 2-h period immediately following the initial appearance of the small aggregate cores in the cytoplasm (Figs 2a and b). As diffusely distributed Nhtt150Q-EGFP gradually decreased in the nucleus and cytoplasm, aggregate formation by polyQ repeats increased in the cytoplasmic region approximately 5 h after small aggregate cores first appeared (Fig. 2b). The size of aggregates was variable (0.5–5 μm), and some of the smaller aggregates (< 1 μm) seemed to move locally during the aggregation process.

We then monitored time-dependent changes in the distribution of aggregates, for 18 days after transfection, and found that large-sized aggregates (> approximately 3 μm) did not move. Figure 3 shows that the overall location of larger aggregates (> approximately 3 μm) in the cell did not change significantly during the 2- to 18-day period after transfection, although the locations of small aggregates did change. Therefore, the larger aggregates are not easily degraded once formed, at least during the observed time frame. Next, we examined if Nhtt150Q-EGFP fusion proteins were intact in the aggregates. As shown in Fig. 3(b), GFP aggregates were stained with a monoclonal antibody (mAb 5374) against the N-terminal of htt 18 days after transfection. Taken together, these results suggest that Nhtt150Q-EGFP fusion protein is intact and that larger aggregates might form a stable structure.

Ultrastructure of polyQ-expanded htt

The ultrastructure of the fluorescent cellular aggregates in cells expressing Nhtt150Q-EGFP was examined by electron microscopy (EM). PolyQ aggregates were identified at low magnification (×1000) by using the co-ordinates of fluorescent aggregates obtained from a confocal micrograph (data

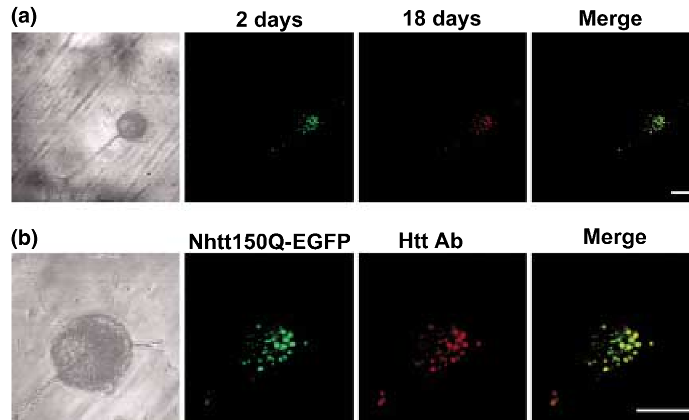

Fig. 3 Time course of aggregate distribution in a cultured *Aplysia* neuron. (a) The pictures were taken 2 and 18 days after transfection into a sensory neuron. Fluorescence images show the distribution of aggregates within the neuron. The green image represents aggregates at 2 days and the red image shows aggregates 18 days after transfection. This merged image shows that the distribution of most of the large-sized aggregates (> approximately 3 μm) was unchanged 18 days after transfection. Scale bar, 50 μm. (b) The cultures were fixed 18 days after transfection and were stained with an antibody against the N-terminal of huntingtin (mAb 5374) and Cy3-conjugated anti-mouse antibody. The GFP fluorescent aggregates were also stained with mAb 5374. Scale bar, 50 μm.

not shown). Aggregates were usually electron-dense spherical structures (Fig. 4a). Diameter were diverse from approximately 0.5–5 μm, and relatively large aggregates with diameters > 3 μm had a low-density center (Fig. 4a). At a high magnification (×30 000), the EM data showed that aggregates contained electron-dense, filamentous components (Fig. 4b), which consisted of small, circular units of diameter approximatley 10 nm (Fig. 4c). Some of the aggregates were found to be composed of multiple-aggregates, which seemed to have agglomerated with time, as observed under the confocal microscope (Fig. 4d). Interestingly, lysosomes were frequently observed near aggregates, and some lysosomes created indentations of aggregates (Fig. 4e). Large lysosomes were found in some neurons expressing Nhtt-150Q-EGFP (data not shown).

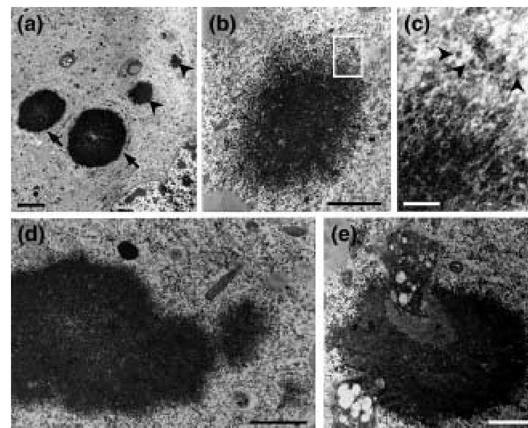

Fig. 4 Ultrastructure of polyQ-aggregates. These electron micrographs were taken from *Aplysia* neurons expressing Nhtt150Q-EGFP for 3 days. (a) Aggregates (arrows and arrowheads) are usually distributed in the cytoplasm and large aggregates with a diameter of > approximately 3 μm have a low-density center. Arrows indicate aggregates with a low-density core. Scale bar, 2 μm. (b) A magnification of a single aggregate shows a typical filamentous structure. Scale bar, 400 nm. (c) A higher magnification (×30 000) reveals that the aggregate is composed of circular units of diameter approximately 10 nm. Arrowheads indicate each circular aggregate unit. Scale bar, 100 nm. (d) A large aggregate is composed of several fused aggregates. Scale bar, 1 μm. (e) Indentation of an aggregate by a lysosome. Scale bar, 600 nm.

Cell type-specific morphological changes due to polyQ-expanded htt in *Aplysia* neurons

We investigated if any gross morphological changes occurred in sensory or pedal cells overexpressing pNEXδ-Nhtt150Q-EGFP or pNEXδ-Nhtt16Q-EGFP, and found that pedal cells with abnormal Nhtt150Q-EGFP aggregates showed reduced neurite length and number (85.7%, $n = 30/35$ polyQ-positive cells) 72 h after transfection (Fig. 5a). Lower expression levels of Nhtt150Q-EGFP driven by pNEX2, which was shown to be unable to produce aggregates, was not associated with any neurite reduction, even up to 10 days after transfection (data not shown). These results indicate that cellular toxicity, resulting in, for example, neurite reduction, may be somewhat associated with the formation of polyQ aggregates.

In contrast with the effect of mutant htt on neurite reduction in pedal cells, we did not observe the neurite reduction in sensory cells containing abnormal aggregates of Nhtt150Q-EGFP ($n = 0/40$; Fig. 5b). These different effects of mutant htt on neurite reduction in pedal and sensory cells may stem from the expression level discrepancies of mutant htt in the different cell types. The lack of neurite reduction

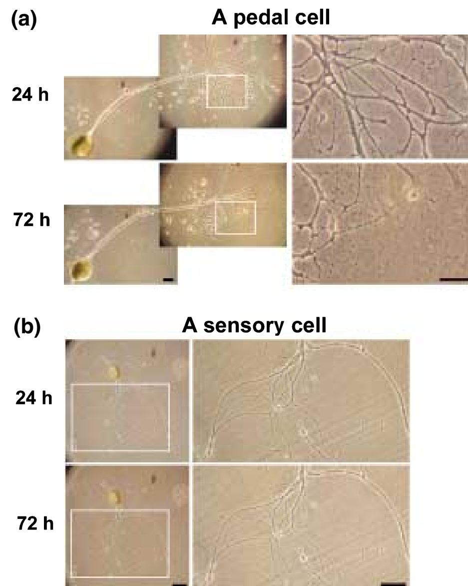

Fig. 5 The effect of polyQ-expanded htt on morphological change in *Aplysia* neurons. Phase-contrast images show that the length and the number of neurites in Nhtt150Q-EGFP-positive pedal cells (a) was reduced 72 h after transfection, but not in Nhtt150Q-EGFP-positive sensory cells (b). Insets in the left panels are magnified in the right panels. Scale bar, 50 μm.

could be due to a lower expression level in small sensory neurons than in relatively large pedal neurons. To test this possibility, we used pNEX3 expression vector, which is known to express target genes more than pNEXδ (Kaang 1996). We found that sensory cells microinjected with pNEX3-Nhtt150Q-EGFP also had abnormal aggregates, but did not show any neurite reduction 10 days after transfection (data not shown). These results show that the reason for the lack of neurite reduction in sensory cells is not simply due to the expression levels of Nhtt150Q-EGFP. We also observed that the number and length of neurite were not reduced in cells expressing Nhtt16Q-EGFP, irrespective of cell type (data not shown). These results suggest the possibility that abnormal aggregates induced by polyQ-expanded htt are able to induce cytological dysfunction, such as neurite reduction in a cell-type specific manner.

Effects of polyQ-expanded htt on *Aplysia* sensory to motor synapses

We asked whether polyQ-expanded htt can affect synaptic function. To test this idea, we overexpressed Nhtt150Q-EGFP or Nhtt16Q-EGFP for 2 days in *Aplysia* sensory neurons that had been synaptically connected to a motor neuron in culture.

First, we examined the effect of polyQ-expanded htt on basal-synaptic transmission in these cultures. The overexpression of Nhtt150Q-EGFP and subsequent aggregate formation in the sensory neurons had no significant effect on basal synaptic strength (% change, −22.8 ± 9.0%, $n = 10$), which was comparable to the synaptic strength of control cells (− 13.1 ± 2.7%, $n = 7$) or Nhtt16Q-EGFP positive cells (− 10.5 ± 3.3%, $n = 6$; data not shown).

Secondly, the sensori-motor co-culture was exposed to one pulse (5 min) of 10 μM 5-HT, which normally produces a short-term synaptic facilitation 48 h after transfection with a polyQ DNA construct. Compared with Nhtt16Q-EGFP positive cells or polyQ-negative control cells (97.9 ± 30.2%, $n = 7$ and 97.7 ± 21.7%, $n = 6$, respectively), the over-expression of Nhtt150Q-EGFP did not impair the short-term synaptic facilitation induced by one pulse of 5-HT (90.2 ± 16.6%, $n = 6$; Fig. 6a). We also measured the membrane excitability, another form of short-term facilitation, induced by one pulse of 5-HT in Nhtt150Q-EGFP-positive cells. Membrane excitability was found to be normally enhanced by 5-HT treatment in these cells (data not shown).

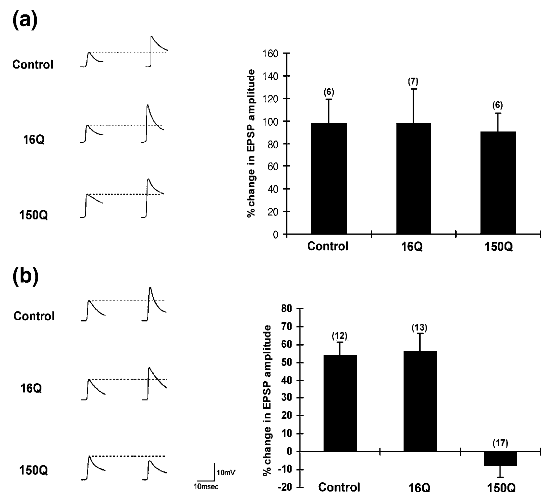

Fig. 6 The effect of polyQ-expanded htt on synaptic plasticity in *Aplysia* sensori-to-motor synapses. (a, left panel) Examples of EPSPs recorded in motor neuron LFS after stimulation of sensory neurons before and after one pulse of 5-HT (10 μM) treatment for 5 min. The control failed to show cDNA expression after DNA microinjection. Nhtt150Q-EGFP-positive (150Q) or Nhtt16Q-EGFP-positive (16Q) cells induced short-term synaptic facilitation after 5-HT treatment. (a, right panel) The height of each bar corresponds to the mean percentage change ± SEM of EPSP amplitude as tested 5 min after 5-HT (10 μM) treatment. Numbers in parentheses represent the numbers of sensory to motor synaptic connections tested. (b, left panel) Examples of EPSPs recorded in motor neuron LFS before and 24 h after five pulses of 5-HT (10 μM) treatment. Overexpressed Nhtt150Q-EGFP, but not Nhtt16Q-EGFP, blocked 5-HT-induced long-term synaptic facilitation. (b, right panel) The height of each bar corresponds to the mean percentage change ± SEM of EPSP amplitude 24 h after 5-HT (10 μM) treatment. One-way analysis of variance and Duncan's multiple range test were used to determine the significance of the effect of Nhtt150Q-EGFP on synaptic facilitation ($F = 20.4$, d.f. = 2, $p < 0.0001$).

Finally, the sensorimotor co-culture was exposed to five pulses of 5-HT to induce long-term synaptic facilitation, which is known to require new protein, mRNA synthesis, and structural changes. Nhtt150Q-EGFP-positive cells with the abnormal aggregates did not produce an increase in the amplitude of synaptic potential 24 h after 5-HT treatment ($-7.5 \pm 6.6\%$, $n = 17$). On the other hand, Nhtt16Q-EGFP-positive cells and polyQ-negative control cells showed a normal increase in synaptic potential amplitude ($56.1 \pm 9.9\%$, $n = 13$ and $53.9 \pm 7.4\%$, $n = 12$, respectively) 24 h after 5-HT treatment (Fig. 6b). To examine the effect of lower expression of mutant htt on synaptic facilitation, we overexpressed pNEX2-Nhtt150Q-EGFP in sensory cells of the sensory to motor synapse. Nhtt150Q-EGFP-positive cells did not show abnormal aggregates but showed a normal increase in synaptic potential amplitude ($53.8 \pm 12.5\%$, $n = 7$) 24 h after 5-HT treatment. These results show that aggregates formed by polyQ-expanded htt (Nhtt150Q, but not Nhtt16Q) impaired 5-HT-induced long-term synaptic facilitation, but did not impair the short-term facilitation or basal synaptic function in *Aplysia* sensory to motor synapses in a polyQ length-dependent manner.

Discussion

In this study, we examined the cytological effects of polyQ-expanded htt by overexpressing Nhtt150Q-EGFP or Nhtt16Q-EGFP in *Aplysia* neurons. Previous studies have reported that aggregate formation correlates well with the length of polyQ (Paulson *et al.* 1997; Hazeki *et al.* 1999; de Almeida *et al.* 2002). Our results confirm this and show that polyQ-expanded htt induces aggregation in a time- and polyQ length-dependent manner. These results are also similar to those of previous reports that N-terminal truncations with expanded repeats formed intracellular aggregates (Cooper *et al.* 1998; Li and Li 1998; Lunkes and Mandel 1998). These studies found that aggregates appeared in both the nucleus and the cytoplasm in *in vivo* and in *in vitro* models of polyQ diseases, and that aggregates were detected in some axonal processes in some mammalian cells (Davies *et al.* 1997; Sapp *et al.* 1999; Murphy *et al.* 2000; Steffan *et al.* 2000; Parker *et al.* 2001).

In our experiment, we observed aggregates mainly in the cytoplasm and in some neurites of Nhtt150Q-EGFP-positive cells, though the free form of Nhtt150Q-EGFP seemed to be localized evenly in the nucleus and cytoplasm before the aggregation process began. We did not observe aggregate formation in the nucleus even 18 days after transfection, and therefore, cannot rule out the possibility of nuclear aggregate formation after a longer incubation. Indeed, in mice or patients, aggregates develop over a period of weeks or years, respectively, and some neuroblastoma cells have shown that long-term (6–12 days after transfection) expression of mutant htt reduced cytoplasmic aggregates but increased intranuclear aggregates (Lunkes and Mandel 1998). The cytoplasmic localization of aggregates in our study could be also explained by another possibility, namely that the cell phenotype may influence the intracellular region where aggregates are formed. For example, dentatorubral-pallidoluysian atrophy (DRPLA) is a neurodegenerative disease caused by polyQ, and it was shown that in COS-7 cells the expression of truncated DRPLA protein with an expanded polyQ stretch formed aggregates mainly in the cytoplasm, whereas polyQ-induced aggregates in differentiated pheochromocytoma-12 (PC-12) cells were formed in the nucleus (Igarashi *et al.* 1998; Sato *et al.* 1999). Recently, Waelter *et al.* (2001) also showed that, in human 293 cells, htt with a polyQ repeat (> 51 glutamines) formed aggresome-like cytoplasmic inclusions.

Our results also show that the process of aggregation proceeds very rapidly after small aggregate cores appeared in the cytoplasm and that it then takes only an hour to form a substantial number of aggregates. These data are consistent with a previous report by Hazeki *et al.* (1999) that in COS-7 cells, the N-terminal fragment of htt with 77Q aggregates were rapidly formed within 40 min. Our result shows that neuronal and non-neuronal cell types may share a common aggregation process.

We also found that the distribution pattern of larger aggregates did not change appreciably once they were formed. Similarly, the immobilization of htt and ataxin-3 aggregates was recently demonstrated in a live-cell photobleaching study (Chai *et al.* 2002).

Our results also show that the sizes of aggregates did not reduce with time, at least, during our observations of up to approximately 18 days, and that aggregates composed of GFP fusion protein seemed to be fairly intact, implying that larger aggregates may be resistant to the degradation process. Similarly, Jana *et al.* (2001) and Waelter *et al.* (2001) reported that the formation of insoluble fibrillar structures by polyQ-expanded stretch interfered with proteasomal activity. Moreover, recent reports have suggested that hydrogen bonding by polar-zipper formation and covalent bonding by transglutaminase-catalyzed cross-linking are involved in the process of aggregation, i.e. aggregates are stabilized by hydrogen bonding and covalent bonding (Perutz *et al.* 1994; Hoffner and Djian 2002). These structural properties enable aggregates to be stable for long periods. However, the exact mechanisms underlying the stability and immobility of aggregates require further study.

Many groups have shown intranuclear aggregates in HD patients or in HD transgenic mice, by EM (Davies *et al.* 1997; DiFiglia *et al.* 1997). Our EM study shows that cytoplasmic aggregates are spherical in shape (0.5–5 μm) and contain fibrillar components. Electron-dense filamentous materials in *Aplysia* cells expressing 150Q are similar to the HD exon 1 fibrils that were previously shown *in vitro* and in a HD transgenic model (Davies *et al.* 1997; Scherzinger

et al. 1997). This ultrastructure is also consistent with that of an inclusion body, as previously reported by Waelter *et al.* (2001). Interestingly, our EM study also shows that lysosomes concentrate near aggregates. Some neurons expressing Nhtt-150Q-EGFP contained large, abnormally developed lysosomes. Recent studies have also reported that lysosomal components and proteasomal machinery are involved in the degradation of aggregated proteins (Kegel *et al.* 2000; Ravikumar *et al.* 2002), and our EM data support the possibility that lysosomes may be involved in the degradation of polyQ aggregates.

Our results also show that Nhtt150Q-EGFP, but not Nhtt16Q-EGFP, reduces the length and the number of neurites of large neurons in the pedal ganglion, but not of sensory neurons in the pleural ganglion. It has been reported that PC-12 cells overexpressing htt exon1 containing 150 polyQ had defective neurite outgrowth after treatment with neurotrophic factors in a polyQ length-dependent manner (Li *et al.* 1999). Li *et al.* (2000a) also showed that mutant htt induced selective neuronal loss in striatal neurons despite its widespread expression. In our study, it seems that neurite reduction due to aggregate formation is cell-specific, because sensory neurons (approximately 50 μm in diameter), which are smaller than pedal neurons (approximately 200 μm in diameter), did not show defective neurite morphology. This cell type-specific neurite reduction did not seem to be simply related to the expression level of polyQ aggregates in different cell types. Neurite reduction in pedal cells overexpressing 150Q may reflect the altered gene expression of molecules or proteins that are important for maintaining normal neuronal processes. Li *et al.* (1999) demonstrated that mutant htt induced multiple cellular defects by interfering with multiple gene expressions, including those that are necessary for neurite outgrowth. Recently, several researchers have reported that mutant htt alters the expressions of genes involved in normal cellular functions using the microarray technique (Luthi-Carter *et al.* 2000; Sipione *et al.* 2002). In our study, aggregates by polyQ htt may alter gene expression involved in the outgrowth or maintenance of neurites in pedal cells. Alternatively, polyQ aggregates also may sequester many proteins important for neurite maintenance. For example, polyQ aggregates are known to sequester the cytoskeletal proteins (HIP-1; Wanker *et al.* 1997) or HAP-1 (Li *et al.* 2000b) involved in neurite extension. It will be interesting to compare the gene expression and protein profiles of pedal and sensory cells overexpressing polyQ-expanded htt in order to investigate the cell type-specific effects of mutant huntingtin in *Aplysia* neurons.

Previous, post-mortem studies have suggested that the first symptoms (both motor and cognitive) appear in the absence of clear neuronal cell loss and that impaired cognition, such as learning and memory, was likely to be caused by cellular dysfunction (Vonsattel *et al.* 1985). Usdin *et al.* (1999) showed that in mice carrying the HD mutation, induction of long-term potentiation was significantly reduced. Furthermore, a recent study reported that HD transgenic mice (R6/2) show marked alteration in hippocampal synaptic plasticity and impaired spatial cognitive performance in the Morris water maze (Murphy *et al.* 2000). It was also shown that basal synaptic transmission appeared to be normal in spite of impaired synaptic plasticity (Usdin *et al.* 1999; Murphy *et al.* 2000). Our study also shows that the aggregates formed by polyQ-expanded htt protein impair 5-HT-induced long-term synaptic plasticity without affecting short-term facilitation or basal synaptic transmission. PolyQ aggregates may interrupt the induction pathway or the suppression pathway of long-term facilitation.

In *Aplysia*, cyclic AMP response element binding proteins (CREBs), transcription factors, are activated by protein kinase A (PKA) and mitogen-activated protein (MAP) kinase in the nucleus of sensory neurons by repeated pulses of 5-HT. This phosphorylation results in the activation of the cyclic AMP-inducible genes required for long-term synaptic facilitation (Kaang *et al.* 1993). Recently, it was reported that polyQ-expanded htt caused decreased cyclic AMP response element (CRE)-mediated transcription (Nucifora *et al.* 2001; Wyttenbach *et al.* 2001). It is known that htt protein interacts with transcription factors or with the transcriptional apparatus, such as with CREB binding protein (CBP), TATA-binding protein (TBP), and that such interactions mediate alterations in transcription (Steffan *et al.* 2000). In our studies, it is possible that overexpressed polyQ-expanded htt causes abnormal interactions with the transcription factors such as C/EBP (Alberini *et al.* 1994; Lee *et al.* 2001) or CBP (Guan *et al.* 2002), or transcription apparatus required for long-term synaptic facilitation, resulting in impaired synaptic plasticity in *Aplysia* sensory-motor synapses. Though it seems unlikely that cytosolic polyQ aggregates can sequester nuclear transcription factors, Nucifora *et al.* (2001) showed that cytoplasmic aggregates could sequester transcription factors, such as CBP, by redistributing them to cytoplasmic aggregates.

It has been reported that regulated proteolysis by the ubiquitin–proteasome pathway has a critical function in memory storage (Abel and Kandel 1998; Milner *et al.* 1998; Chain *et al.* 1999). Hegde *et al.* (1997) showed that the induction of Ap-uch (ubiquitin C-terminal hydrolase in *Aplysia*) resulted in the upregulation of the ubiquitin–proteasome pathway and the subsequent degradation of specific proteins, such as the regulatory subunits of PKA, which inhibit long-term facilitation. Chain *et al.* (1999) also showed that ubiquitin-mediated proteolysis is essential for consolidating the long-term facilitation of *Aplysia* sensory-to-motor synapses. Recently, it has been proposed that intracellular aggregates impair the proteasome–ubiquitin pathway (Jana *et al.* 2001; Sherman and Goldberg 2001; Sakahira *et al.* 2002). The 19S regulatory complex, a component of proteosomes, is known to be trapped to the aggregates by polyQ repeats and that as a result proteasomal

activity is reduced. Failure to eliminate the Ub (ubiquitin)-protein deposits might result from a malfunction of the ubiquitin/ATP-dependent pathway or from structural changes in the protein substrates, rendering them inaccessible to proteolysis. In the present study, our cytoplasmic aggregates may have impaired the proteasome–ubiquitin pathway required to degrade suppressor proteins. Moreover, the accumulation of suppressor proteins may block long-term facilitation.

Long-term facilitation is also accompanied by structural changes in sensory to motor synapses. Thus, it is possible that some factors such as cell adhesion molecules or actin involved in structural changes may interact with polyQ-expanded htt and disrupt the structural modifications required for long-term synaptic facilitation. However, all these possibilities need to be further examined. In the *Aplysia* sensory to motor synapse, neither short-term facilitation nor basal synaptic function was impaired by ectopic expression of polyQ-expanded htt, indicating that synaptic transmission and the activation of signal pathway by 5-HT treatment are normal in polyQ-expanded htt-positive sensory cells. From these results, we may conclude that the impairment of synaptic facilitation by the polyQ aggregates is specific for 5-HT-induced long-term facilitation that requires gene transcription, new protein synthesis, and structural changes.

In summary, polyQ-expanded htt induces abnormal aggregates and causes cellular dysfunction, such as impaired synaptic plasticity in an *Aplysia* cell model. Therefore, the *Aplysia* giant cell model provides a valuable approach to the study of the cellular and molecular mechanisms of HD pathology by which polyQ-expanded htt induces cellular defects.

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