Aged monkey brains reveal the role of ubiquitin-conjugating enzyme UBE2N in the synaptosomal accumulation of mutant huntingtin

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Abstract

Although misfolded proteins are ubiquitinated and cleared by the proteasome, they can accumulate in synapses in aged neurons to promote synaptic dysfunction in a variety of neurodegenerative diseases, including Huntington’s disease (HD), which is caused by polyglutamine expansion in huntingtin. The mechanism behind this aging-related phenomenon is unknown and has been difficult to investigate using animals with short life spans. With brain tissues from longer-lived rhesus monkeys of different ages, we found that aging reduces ubiquitin-proteasomal activity and also increases the level of ubiquitin-conjugating enzyme UBE2N (Ubc13) in synaptosomes. Synaptosomal fractions from aged monkey brain increase in vitro ubiquitinated huntingtin, whereas depletion of UBE2N markedly reduces this increase. Overexpressing UBE2N increases the aggregation of mutant huntingtin, and reducing UBE2N attenuates huntingtin aggregation in cellular and mouse models of HD. Our studies suggest that increased UBE2N plays a critical role in the synaptosomal accumulation of mutant huntingtin with age.

Introduction

Protein misfolding causes a variety of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington’s disease (HD). All these diseases share several common pathological features, including the age-dependent accumulation of misfolded proteins in affected brain regions. In HD, the disease protein, huntingtin (htt), carries an expanded polyglutamine (polyQ, >37Q) domain that causes htt to accumulate in the nucleus, as well as in neuronal processes and terminals (1–3). The accumulation of mutant htt in synapses is found to alter synaptic transmitter release in HD mouse models (4–7). In addition, targeting mutant htt to the presynaptic terminal in transgenic mice also leads to neurological symptoms and early death (8), and reducing synaptic mutant htt levels alleviates neurological symptoms (7), indicating a critical role for mutant htt in synapses and synaptic dysfunction are commonly seen in a variety of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s, at early disease stages (9–12). All these findings underscore our need to understand how misfolded proteins accumulate in the synapses if we are to unravel the pathogenesis of neurodegenerative diseases. The age-dependent accumulation of misfolded proteins is thought to be due to an impaired cellular capacity to clear...
these misfolded proteins (7,13–17). Two major routes for removing misfolded proteins are the ubiquitin-proteasome system (UPS) and autophagy (14,18–21). To remove misfolded proteins via the UPS, first the proteins need to be ubiquitinated, and then targeted to the proteasomes for degradation. This process involves the concerted action of three enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and a number of ubiquitin-ligating (E3) enzymes (22), which confer substrate specificity by facilitating the interaction between a particular substrate and an E2 (23). UPS activity is found to decline with age in different tissue types (24–28), and also in different subcellular regions (7). Since misfolded proteins selectively accumulate in synapses, there must be other determinants that contribute to this selective accumulation. Knowing the mechanism for this selectivity would help us to understand the pathogenesis of age-dependent neurodegenerative diseases.

Our current understanding of disease pathogenesis has been based largely on studies of small animal models, especially mouse models expressing misfolded proteins. Although these mouse models have yielded valuable information for important pathological and mechanistic events in neurodegenerative diseases, species differences and short life spans have also limited the usefulness of small animal models to recapitulate some important pathological changes seen in patient brains. For example, most transgenic mouse models do not mimic the striking neurodegenerative phenomena in different neurodegenerative diseases, such as AD, PD and HD (29,30). Transgenic models of large animals that are closer to humans appear to be more sensitive to misfolded proteins and show more robust pathological changes than transgenic mouse models. For example, transgenic HD monkeys die earlier and display more severe axonal degeneration than mouse models expressing the same short mutant htt fragment (31,32). The differences seen in primate and rodent models indicate clearly that species differences can determine the nature of neuropathology, underscoring the importance of studying pathogenesis in non-human primates (33).

In the current study, we used rhesus monkey brain tissues to investigate how mutant htt accumulates in the brain. Because synaptic dysfunction is the common pathological feature in many different neurodegenerative diseases, we focused on synaptosomal fractions of monkey brains at different ages. Our studies revealed that the level of ubiquitin-conjugating enzyme UBE2N (Ubc13) in synaptosomes increases with aging and that its expression levels influence the accumulation of mutant htt in neuronal cells. Our findings have uncovered a new target for the selective accumulation of misfolded proteins in neuronal terminals.

Results

Age-dependent decrease in UPS activity in monkey brain

The fact that misfolded proteins accumulate in the aged brain led us to examine UPS activity in the brain tissues of monkeys at different ages. We deeply anesthetized rhesus monkeys at 2, 8 and 22 years of age, and then immediately isolated tissues from different brain regions and peripheral organs. Such freshly isolated tissues allowed us to biochemically analyze primate brain and peripheral tissues without the influence of postmortem-related protein degradation. We compared chymotryptsin-like and postglutamyl-like activities in the monkey brain cortex, striatum, liver and muscle tissues. These proteasomal activities are higher in liver than in brain and muscle tissues (Fig. 1A and B). Importantly, proteasomal activities in the brain (striatum and cortex) tissues significantly decline with age, but are not significantly altered in liver and muscle tissues (Fig. 1A and B).

The decreased proteasomal activity we saw could lead to the accumulation of ubiquitinated proteins, so we examined the ubiquitination of proteins in these tissues via western blotting with
anti-ubiquitin. The results indeed showed that some ubiquitinated proteins are more abundant in the brain cortex and striatum than in the liver and muscle. Importantly, aging apparently increases the levels of ubiquitinated proteins (Fig. 1C and D). The differential accumulation of ubiquitinated proteins in monkey tissues is similar to what we recently found in mouse tissues (34). Thus, using freshly isolated monkey tissues, we found that proteasome activity in primate brain tissues is more vulnerable to aging than in the peripheral tissues.

Increased UBE2N expression in synaptosomes of aged monkey brain tissues

Several neurodegenerative diseases, as among them AD, PD and HD, are characterized by the accumulation of misfolded proteins in synapses and synaptic dysfunction (7,35–37). To investigate whether aging alters the levels of proteins that are involved in protein degradation in synapses, we used monkey brain striatum to isolate the subcellular fraction P2 that is enriched in synaptosomes (Supplementary Material, Fig. S1). Isolated synaptosomes from monkeys at three different ages were subjected to quantitative proteomics analysis in which samples were differentially labeled with four-plex isobaric tag for relative and absolute quantitation (iTRAQ) reagents (114–117), and then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)-based multidimensional protein identification technology (2D-LC-MS/MS-iTRAQ) (Fig. 2A, Supplementary Material, Fig. S2). This assay allows simultaneous analysis of up to four samples with minimal variations between LC-MS runs. The iTRAQ assay revealed increases in 300 proteins and decreases in 150 proteins from ages 2-8 years in monkey synaptosomes, whereas ~50 and 75 proteins showed upregulation and downregulation, respectively, from 8 to 22 years (Fig. 2B). These results suggest that more proteins in the synapse show altered expression from juvenile (2 years) to adult (8 years) ages. The proteins identified are differentially enriched for several functional groups via Fisher’s exact test analysis. We next categorized all proteins in 17 functional synaptic protein groups (Fig. 2C, Supplementary Material, Fig. S3). More mitochondrial proteins are upregulated from 2 to 8 years than from 8 years to 22 years. Interestingly, there is concomitant down- and up-regulation of several proteins for ‘Protein synthesis, folding and degradation’ from 2 to 8 years, whereas greater downregulation is seen from 8 to 22 years. This result is consistent with the age-dependent decline in clearance of misfolded proteins in neurodegenerative diseases. Of these proteins, UBE2N was increased in adult monkey brains. The protein-protein interactions network determined by STRING analysis also revealed that UBE2N is associated with the proteasome 26S subunit ATPase and non-ATPase in the network (Fig. 2D). Taken together, our data show an age-dependent dysregulation of distinct synaptic proteins in monkey brains.

Since UBE2N is important for targeting misfolded proteins for degradation, we focused on the analysis of UBE2N and its effect on the accumulation of misfolded proteins. To verify the age-dependent altered level of UBE2N seen in the iTRAQ assay, we performed western blotting of monkey brain samples. UBE2N was increased in synaptosomes from the striatum and cortex of 8- and 22-year-old monkeys compared with 2-year-old monkey (Fig. 3A and B). However, UBE2K, another E2 conjugating enzyme, was unchanged, suggesting a selective increase in UBE2N in adult monkey brains. There is a slight decrease in UBE2K and UBE2N in 22-year-old monkey brain compared with 8-year-old monkey brain, which may be due to a loss of synapses in very old animals. Furthermore, we examined P2 fractions from liver and muscle, which were isolated using similar conditions for isolating synaptosomes, and found that UBE2N was not increased in these peripheral tissues with age (Fig. 3C). All these results support the selective upregulation of UBE2N in synaptosomes in monkey brains with age and the relevance of this change to selective neuropathology in age-dependent neurodegenerative diseases. Since the age of 8 years in monkey is equivalent to adult humans, the increased UBE2N at 8 years of age implies that synaptic UBE2N is upregulated in adult primate monkey brains.

Synaptosomal fractions from aged monkey brains promote mutant htt accumulation

We thought it was important to explore whether misfolded proteins are more likely to accumulate in the synapse from aged monkeys. We used mutant htt for this investigation, as htt misfolding is mediated by polyQ expansion in its N-terminal region. Because of the early death of previously established transgenic HD monkeys (32), we did not have monkey brain tissues that express mutant htt. We therefore used an in vitro assay, which we developed recently (34), to examine the effect of synaptosomes from monkeys at different ages on htt accumulation. In this assay, transfected exon1-97Q htt from HEK293 cells was precipitated by anti-htt (Supplementary Material, Fig. S4A). The precipitated htt was then incubated with synaptosomal fractions isolated from the cortex of monkeys at 2, 8 and 22 years of age (Fig. 4A). We assumed that the in vitro incubation of synaptosomes from monkeys at different ages under the same conditions would reveal age-related factors that may alter htt stability. Indeed, we saw that the synaptosomal fractions from older monkeys caused more exon1-97Q htt to accumulate in the incubation (Fig. 4B, left panel). To verify that this assay does reflect the ubiquitin-proteosomal degradation event, we added the proteasome inhibitor MG132 to the incubation and saw that inhibiting the UPS eliminated the age-dependent difference in htt accumulation (Fig. 4B, right panel), which is consistent with the age-dependent decrease in UPS activity in aged monkey brain (Fig. 1).

Next, we were interested to know whether there is age-related ubiquitination in monkey brain synaptosomes, so we examined htt ubiquitination after incubating htt with synaptosomal fractions from monkeys of different ages. As expected, synaptosomal fractions from older monkeys caused more extensive ubiquitination of htt, suggesting there are increased amounts of ubiquitinated htt in the aged synaptosomes (Fig. 4C). To verify that this increased ubiquitination is related to the presence of UBE2N, we used anti-UBE2N to deplete UBE2N in synaptosomal fractions via immunoprecipitation. This depletion markedly reduced the ubiquitination of mutant htt (Fig. 4C), suggesting that the age-dependent increase in htt ubiquitination requires UBE2N.

We already know that UBE2N mediates K63 ubiquitination of proteins (38–40), and our recent study shows that N-terminal htt is differentially ubiquitinated by ubiquitin with mutations at different lysine residues (41). Using the same approach to immunoprecipitate N-terminal htt that was transfected with ubiquitin or its mutant forms (K48, K48R, K63, K63R), we indeed confirmed that exon1-97Q htt is ubiquitinated via K63 to a greater extent than via K48 (Supplementary Material, Fig. S4B). Importantly, when UBE2N was coexpressed with exon1-97Q htt, it dramatically promoted K63 ubiquitination of htt compared with the absence of transfected UBE2N (Fig. 4D). Taken together, these results suggest that increased levels of UBE2N can promote the accumulation and aggregation of mutant htt.
Alter the accumulation of mutant htt by UBE2N expression

The age-dependent accumulation of misfolded proteins is determined by multiple factors, including decreased proteasome activity and increased production of ubiquitinated proteins that tend to be more stable and form aggregates. To provide direct evidence for the causative role of UBE2N in the accumulation of misfolded proteins in cells, we used two siRNAs to inhibit the expression of endogenous UBE2N in cultured HEK293 and PC12 cells (Supplementary Material, Fig. S5A). The results showed...
that decreasing UBE2N reduces the levels of aggregated mutant htt in both HEK293 cells and PC12 cells (Fig. 5A), though there is no significant effect of UBE2N siRNA on the level of normal N-terminal exon1 htt with 20Q. Immunofluorescent staining also showed a decrease in the density of htt aggregates formed by mutant htt in PC12 cells (Fig. 5B) and HEK293 cells (Supplementary Material, Fig. S5B) by UBE2N siRNA. This inhibitory effect was verified by quantifying the number of htt aggregates in transfected HEK293 cells (Fig. 5C).

If inhibiting UBE2N can reduce htt accumulation and aggregation, overexpressing UBE2N may lead to the opposite effect. To test this idea, we generated a plasmid that can overexpress UBE2N and RFP (Supplementary Material, Fig. S6A) and transfected it to HEK293 cells that express either exon1-97Q htt or exon1-120Q (42). The results showed that overexpression of UBE2N increased the level of both mutant htt proteins (Fig. 5D and E). Since UBE2N plasmid also expressed RFP, we could evaluate htt accumulation in RFP-positive cultured cells. Our examination revealed that RFP-positive cells (HEK293 and PC12 cells) do show htt aggregates compared with those without RFP expression (Supplementary Material, Fig. S6B).

To provide in vivo evidence for the regulatory effects of UBE2N, we generated AAV vector that expresses GFP or human UBE2N and lentiviral vector that expresses UBE2N-shRNA or its scramble shRNA with GFP, which allows us to identify cells with inhibition of UBE2N via shRNA (Fig. 6A). After verifying the effects of these viral vectors to alter UBE2N expression in transfected HEK293 cells (Fig. 6B), we used these viral vectors to examine their effects

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**Figure 3.** Age-dependent change of UBE2N in the synaptosomes of monkey brain at different ages. (A) Western blotting with anti-UBE2N or anti-UBE2K revealing the age-dependent increase of UBE2N, but not UBE2K, in the synaptosome of monkey striatum. The isolated synaptosome contains γ-tubulin, which serves as a loading control. Quantitative results of the ratios of UBE2N or UBE2K to γ-tubulin are beneath the blot. In A and B, *P < 0.05; **P < 0.01 (n = 3). (C) UBE2N in the P2 fractions of liver and muscle of monkeys at 2, 8, and 22 years of age is analyzed via western blotting. (D) Quantitative results in (C) of the ratios of UBE2N to γ-tubulin. The P2 fractions were isolated using conditions similar to isolating synaptosomes from monkey brains. There is no increase in UBE2N in the P2 fractions in the liver and muscle from old monkeys.
Figure 4. In vitro assay of htt degradation with synaptosomal fractions from aged monkey cortex. (A) Schematic of in vitro assay of htt degradation. Transfected htt in HEK293 cells was immunoprecipitated and then incubated for 2 or 4 h with synaptosomes isolated from monkeys at different ages. The incubation was then analyzed via EM48 western blotting to detect the degradation of transfected htt. (B) Western blot analysis of transfected htt that has been incubated with brain synaptosomes isolated from monkeys at different ages (left panel). The proteasome inhibitor MG132 was also added in the incubation (right panel). Quantitative assessment of the ratio of transfected htt to control is presented beneath the blots. Control is the incubation for 2 h without brain lysates. * P < 0.05 (n = 3). Note that inhibiting the UPS by MG132 eliminates differences caused by aged synaptosomes. (C) Immunoprecipitated htt from transfected cells was incubated with monkey synaptosomes and analyzed via western blotting with anti-ubiquitin. There is increased ubiquitination of transfected htt when incubated with synaptosomes from aged monkey cortex. The synaptosomes were also immunoprecipitated with anti-UBE2N overnight to deplete UBE2N, and this depletion markedly reduces htt ubiquitination. The relative levels of ubiquitinated htt (ratio to the precipitated htt) are shown below the Western blots. * P < 0.05. (D) N-terminal htt (Exon1-97Q htt) was transfected with HA-ubiquitin (K63 or K63R) and/or without UBE2N. Mutant htt was then immunoprecipitated and probed with anti-HA for ubiquitinated htt. Note that UBE2N promotes K63-mediated htt ubiquitination. Control is non-transfected cells.
Figure 5. Altering UBE2N expression influences the formation of htt aggregation in cultured cells. (A) Western blotting with mEM48 showing that the aggregated htt in the stacking gel decreased when co-transfected with UBE2N siRNA in HEK293 and PC12 cells. Note that normal N-terminal htt (Exon1-20Q htt) did not show altered expression by UBE2N siRNA. (B) Immunofluorescent staining of PC12 cells transfected with Exon1-120Q htt showing that UBE2N siRNA decreased the formation of htt aggregation. (C) The relative density (mean ± SE) of htt aggregates per image (20X) was counted (n = 5 images per group) * P < 0.05. (D) Western blotting with mEM48 revealing that overexpression of UBE2N via UBE2N-P2A-RFP transfection increases aggregated mutant htt (Exon1-97Q or Exon1-120Q htt) in the stacking gel (SG). (E) Quantitative analysis of the intensity (ratio to tubulin) of aggregated htt on western blot is presented in the right panel. * P < 0.05.
on the accumulation and aggregation of mutant htt (N212-120Q), which was expressed via our previously established lentiviral vector (34). We injected the viral vectors into the mouse striatum and 3 weeks later used immunofluorescent staining to confirm the expression of transgenic proteins (Supplementary Material, Fig. S7) and to evaluate htt aggregation and accumulation. Compared with the control viral expression of GFP in the right striatum, expression of viral UBE2N significantly increased the number of aggregates in the left striatum in the same animal (Fig. 6C), which was also verified by quantitative assessment of the density of htt aggregates (Fig. 6D).

To validate the effect of UBE2N on the expression of full-length mutant htt at the endogenous level, we injected lentiviral vector (UBE2N-shRNA-GFP) into the right striatum of 11-month-old HD 140Q KI mice and lentiviral GFP alone into the left striatum in the same animal. Four weeks later, we examined htt aggregation via htt immunofluorescent staining. As expected, HD KI mice at 11 months of age showed abundant htt aggregates in both the nuclei and neuropil, which still existed in the region injected with lentiviral GFP. However, there was a marked reduction of nuclear and neuropil aggregates in the viral UBE2N-shRNA-injected area (Fig. 7A). Thus, inhibiting UBE2N can also attenuate htt accumulation and aggregation in the brain expressing full-length mutant htt. Based on these findings and the age-dependent up-regulation of UBE2N, we proposed a model for the role of UBE2N. In this model, age-dependent decrease of UPS activity may lead more mutant htt to be ubiquitinated via UBE2N in the synaptosomes. The K63-mediated ubiquitination will contribute to the accumulation of mutant htt in the synaptosomes in aged brain and related neuropathology (Fig. 7B). Thus, reducing UBE2N in aged neurons could help decrease the synaptic accumulation of mutant htt and perhaps other misfolded proteins, as well.

**Discussion**

Age-dependent neurodegenerative diseases, including AD, PD and HD show increased accumulation of misfolded proteins in neuronal cells with age, resulting in the formation of inclusions in the brain (1,13,43-46). Because of its associated polyQ expansion that causes protein misfolding, HD makes an ideal model to investigate how aging can cause the accumulation of misfolded proteins. Clearance of misfolded proteins by the UPS involves sequential reactions mediated by multiple proteins. Ubiquitin is covalently attached to substrate lysines in a three-
enzyme cascade catalyzed by E1, E2 and E3 enzymes (47). In this cascade, E1 activating enzymes initiate ubiquitination, E2 enzymes conjugate ubiquitin to substrates and E3 ligases specify targeting the right E2-conjugated substrates to the proteasome for degradation. Using monkey brain tissues with a focus on the accumulation of mutant htt, our findings suggest that an age-dependent increase in the UBE2-conjugated enzyme UBE2N plays an important role in the accumulation of misfolded proteins in synaptosomes.

Evidence that supports an important role for UBE2N in the age-dependent accumulation of misfolded proteins comes from the following findings in our studies. Proteomics and western blotting revealed that the level of UBE2N is increased in synaptosomes from rhesus monkeys at the age of 8 years, which is equivalent to the age of 28 years in humans; this is sustained until 22 years of age, which corresponds to the age of 77–80 years in humans. Since we know aging can reduce the activity of UPS, the increase in UBE2N in adult monkey brain is likely to contribute to the late onset of symptoms in diseases caused by misfolded proteins. Consistently, old monkey brain synaptosomal fractions can promote the accumulation of mutant htt in vitro, and this accumulation can be attenuated by removing...
UBE2N via immunoprecipitation. Overexpression of UBE2N in cultured cells can also increase the accumulation of mutant htt, and more importantly, reducing UBE2N in the brain of HD KI mice decreases the density of htt aggregates. These findings support a causal role for UBE2N in the accumulation of misfolded proteins in aged brains.

The above role of UBE2N is also supported by its unique function. UBE2N is the only E2 conjugating enzyme that specifically mediates the elongation of K63-linked polyubiquitin chains (48–50). Consistent with its broad modulation of K63-linked polyubiquitination in the subcellular localization and function of a variety of proteins, UBE2N is found to regulate the functions of a number of important proteins, such as P53 (51,52), the NF-kappaB pathway (53), IkappaB kinase (IKK) (54) and the Rap80/Abraaxas/Brcal/Srcc36 complex in response to DNA damage (55). K63 ubiquitination can also lead to the accumulation of ubiquinated proteins when the clearance of these proteins by the UPS is impaired (14,44,56). Indeed, in aged monkey brains, we also saw a decline in UPS activity in the same manner as rodent brains (51).

UBE2N also appears to regulate the progression of Huntington’s disease. UBE2N in the synapses also contributes to the accumulation of K63-ubiquitinated mutant htt in nerve terminals if K63-ubiquitinated htt is degraded by the proteasome. Since we did not find a global increase of UBE2N in the brain lysates, the age-dependent increase of UBE2N in the synaptosomal fraction in aged monkey brains suggests that this increase is specific for the accumulation of misfolded proteins in the nerve terminals.

Because UBE2N can conjugate ubiquitin to various proteins, we would expect increased UBE2N to occur under different pathological conditions. Indeed, UBE2N has more than threefold upregulation in metastases in the lungs (61) and is upregulated in the striatum and hippocampus in methamphetamine-induced neurotoxicity in different regions of rat brain (62). Moreover, proteasome inhibition promotes Parkin–UBE2N interaction and lysine 63-linked ubiquitination (39), and UBE2N upregulation is thought to contribute to parkin-mediated mitophagy, as knockdown prevents specifically K63-linked ubiquitination at mitochondrial sites (63). Also, alpha-synuclein knockdown exacerbates glutamate neurotoxicity and stimulates the expression of UBE2N (64).

While E3 ligases are involved in substrate selection, E2 conjugating enzymes are the main determinants for selection of lysine to construct ubiquitin chains, which thereby directly control the cellular fate of substrates. In humans, there are 35 active E2 enzymes versus 16 to 35 E2 family members in other species. These differences also underscore the importance of studying the E2 enzymes in primates if we are to understand how misfolded proteins accumulate or are degraded in primate brains. Using the monkey brain tissue and iTRAQ proteomics approaches, we identified UBE2N as an important target in the accumulation of misfolded proteins in the synaptosomes of adult brains. A key implication of this age-dependent increase of UBE2N is that it may also underlie the age-dependent accumulation of misfolded proteins in the synapses in other neurodegenerative diseases. By investigating the age-related accumulation of aggregated htt, our findings offer a new mechanistic insight into the age-dependent accumulation of misfolded proteins in the synapse, as well as an additional therapeutic target for HD treatment.

### Materials and Methods

#### Animals

Mouse experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. Full-length htt CAG140Q (i.e., HD KI) mice were provided by Michael Levine (University of California, Los Angeles, CA) and maintained at the Emory University Animal facility in accordance with the IACUC guidelines. Rhesus monkeys were maintained at Kunming Biomed International, Kunming, China, and their experimental procedures were approved by the Institutional Animal Care and Use Committee of Kunming Biomed International, Kunming, China. Male rhesus monkeys at the ages of 2, 8 and 22 years (n = 3 each group) were deeply anesthetized with a lethal dose of sodium pentobarbital, and their brains and peripheral tissues were rapidly isolated and stored in liquid nitrogen.

#### Quantitative protein identification using LC-MS

The brain striatum of monkeys at 2, 8 and 22 years of age (n = 3 each age) were used to isolate synaptosomal fractions. The fractions of the same amounts of proteins from the monkeys at the same age were pooled. We treated the fractions (100 μg protein for each sample) with 10 mM DTT at 37°C for 4 h, and then 55 mM iodoacetamide at room temperature for 1 h in the dark. The alkylated lysates were then digested with trypsin (1:50 [w:w]) at 37°C overnight, resulting in tryptic peptides that were collected and labeled with iTRAQ® Reagents (AB Sciex Inc., MA, USA) according to the manufacturer’s manual. The labeled samples were mixed together in equal ratio amounts and were then desalted and fractionated with C18-strong cation exchange (SCX) stop-and-go-extraction tips (StageTips) into six fractions. The separated peptides were desalted again with C18 StageTips and concentrated with a SpeedVac.

The peptides were analyzed by a TripleTOF 5600 mass spectrometer (AB SCIEX) coupled online to an Eksigent nanoLC Ultra in Information Dependent Mode. Tandem mass spectra were recorded in high-sensitivity mode (resolution > 15,000) with rolling collision energy on and iTRAQ reagent collision energy adjustment on. Peptide and protein identification and quantification were performed with the ProteinPilot 4.5 software (AB SCIEX) using the Paragon database search algorithm. The UniProt proteome sequences for Macaca mulatta (including canonical and isoforms) were used for database searching, and the mass tolerance was set to 0.05 Da. The false discovery rate (FDR) analysis was performed using the software PSPEP integrated with the ProteinPilot.

#### Proteasome activity assay

To determine proteasome activity, all the tissue samples were adjusted to 0.5 mg/ml total protein by dilution with homogenization buffer. All assays were done in triplicate. Chymotrypsin-like activity of 20S-beta-5 was determined using the substrate Suc-LLVY-aminomethylcoumarin (AMC) (40 μM; Bimol), and post-glutamyl activity of 20S-beta-1 was determined using the substrate Z-LLL-AMC (400 μM; Bimol). Equal amounts (10 μg) of the extracts were incubated with corresponding substrates in 100 μl proteasome activity assay buffer (0.05 M Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM ATP and 1 mM DTT) for 30–60 min at 37°C. The reactions were stopped by adding 0.8 ml of cold water and placing the reaction mixtures on ice for at least 10 min. The free AMC fluorescence was quantified using the CytoFluor multi-well...
plate reader (FLUOstar; BMG LABTECH) with excitation and emission wavelengths at 380 and 460 nm, respectively. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methyl-coumarin (AMC) solution (40 mM), normalized by the protein concentrations and expressed as nmol/min/mg protein.

**In vitro ubiquitination assay**

HEK293 cells stably expressing transfected full-length htt were grown to confluence in a 10 cm plate. Cells were washed in the plate, then lysed in cold assay buffer (25 mM Tris–HCl, pH 7.6, 10 mM MgCl₂, 100 µg/ml purified rabbit creatine kinase, 50 mM phosphocreatine, 1 mM ATP). Wild-type monkey cortex tissues of different ages were rapidly collected and homogenized at 1 g/ml in cold assay buffer using 20 strokes of a glass dounce hand homogenizer. Both cell lysates and tissues were centrifuged 500 × g for 5 min to pellet unbroken tissues and membranes. The supernatant was collected and stored in ice while protein concentrations were determined using a BCA assay (Thermo Scientific). Both tissue and cell lysates were prepared at 170 µg protein/500 µl assay buffer, and the appropriate number of 200 µl aliquots were prepared. The samples containing both cell and tissue lysates were mixed (200 µl cell lysates + 200 µl tissue lysates). The control samples contained equal volumes of tissue or cell lysates and assay buffer. The samples were incubated at 37°C with 300 RPM shaking for 0, 8, or 16 h. The reaction was stopped by the addition of protease inhibitors, PMFS, MG132 and protein loading dye.

**Fractionation of brain tissues**

The brains of monkeys at 2-, 8- or 22-years of age were dissected to isolate the cortex, striatum and other brain regions. The tissues were homogenized for 25 strokes in ice-cold homogenization buffer (0.32 M sucrose, 15 mm Tris–HCl pH 8.0, 60 mm KCl, 15 mm NaCl, 5 mm EDTA, 1 mm EGTA, 0.02% NaN₃ and 2 mM ATP) containing protease inhibitor (Roche) and 100 µM PMSF. Twenty-five percent of lysates were stored as total lysate sample. Nuclei and cellular debris were pelleted (P1) at 800 × g for 5 min. The supernatant (S1) was transferred to a new tube and spun at 20,000 × g for 30 min at 4°C to obtain a mitochondria- and synaptosome-enriched pellet (P2) and the supernatant (S2). The supernatant (S2) was then used for the soluble cytoplasmic fraction. Crude nuclear pellets were washed four times with ice-cold homogenization buffer to remove cytoplasmic contaminants. The P2 fraction was resuspended in an equal amount of homogenization buffer (0.32 M sucrose) and layered on two-step sucrose gradients consisting of 12 ml each of 0.8 and 1.2 M sucrose. The gradients were centrifuged at 9100 g for 2 h in a swinging bucket rotor, SW28, using ultracentrifuge. Three fractions were collected corresponding to material at the 0.32–0.8 M sucrose interface (myelin enriched fraction, LP1), at the 0.8–1.2 M sucrose interface (synaptosome-enriched fraction, LP2), and the below 1.2 M sucrose interface (mitochondria-enriched fraction, LP3). The 0.8 M sucrose layer and most of the loose pellet containing the synaptosomes were collected and resuspended in 0.32 M sucrose/4 mm HEPES and protease inhibitors and centrifuged at 9100 g again (2 h) to pellet the synaptosome-enriched fraction, which was separated from the mitochondrial fraction. Resuspended pellets in PBS were saved as aliquots (10 µg/µl) at −80°C. The same amounts of proteins of each fraction were resolved in Tris–glycine gel (Invitrogen) and subjected to western blotting.

**Stereotaxic injection**

Adult WT and HD 140Q KI mice at the age of 11 months (n = 2 each group) were anesthetized by i.p. injection of 2.5% Avertin, and their heads were placed in a Kopf stereotaxic frame (Model 1900) equipped with a digital manipulator, a UMP3–1 Ultra pump, a 10 µl Hamilton microsyringe (Hamilton Co., Reno, NV, USA), and a 33 G needle was inserted through a 1 mm drilled hole on the scalp. Injections were made at the following stereotaxic coordinates: 0.5 mm anterior to bregma; 2.0 mm lateral to the midline (left or right side); 3.3 mm ventral to the dura, with bregma set at zero. The microinjections were carried out at a rate of 0.20 µl/min. The microsyringe was left in place for an additional 10 min before and after each injection. A total of 2.5 × 10¹⁵ vg virus (AAV-UBE2N or AAV-GFP as control virus) was stereotaxically injected into the right striatum of mice. N212-12Q-htt lentivirus (described before) (34) was injected bilaterally. Two weeks after injections, animals were killed, and the striatum was isolated and subjected to immunofluorescent staining to examine the expression of GFP or hUBE2N. For each animal, multiple brain sections (>6) were used for quantification of htt aggregates per image (×40).

**UBE2N knockdown**

The cells were transiently transfected with UBE2N siRNA (Gene Pham Co. sequence GCCUUAUGCGGCAUAAUAt or CCGAU- GAUCAUGACAAAt) or control siRNA (scrambled sequence) using RNAi Max transfection reagent (Invitrogen) according to the manufacturer’s protocol. At 48 h following transfection, cells were harvested and lysed in ice-cold 0.5% Triton X in 1× PBS solution lysis buffer with protease inhibitor mixture (Roche) and 100 µM PMSF on ice. Following this, the lysates were spun at 16,873 g for 15 min, and protein concentrations were determined using a BCA assay (Thermo Scientific). Protein lysates were resolved in Tris–glycine gel (Invitrogen) and blotted to a PVDF membrane (Hybond ECL, GE Health Care/Amersham Biosciences). The blots were immunoblotted using anti-UBE2N and anti-γ-tubulin (loading control). The western blots were developed using the ECL Prime Chemiluminescence kit (GE Health Care/Amersham Biosciences). The lentivirus vectors of shRNA-UBE2N with the target sequence of CCAGAUGAUCAUGACAAAtt and shRNA-Control with the scrambled sequence were packaged and purified by Gene Chem Co. (Shanghai, China). Titers of shRNA-UBE2N or control virus were confirmed by qPCR.

**Statistical analyses**

Statistical significance was assessed using the two-tailed Student’s t-test whenever two groups were compared. Three monkeys at each age group provided tissues for western blotting, and three or more western blot experiments were often repeated for each money tissue. When analyzing multiple groups, we used one-way ANOVA to determine statistical significance. Data are presented as mean ± SEM. Calculations were performed with GraphPad Prism software (GraphPad Software, Inc.). A P-value <0.05 was considered statistically significant.

**Supplemental Material**

Supplementary Material is available at HMG online.

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