Deficiency of Ube3a in Huntington’s disease mice brain increases aggregate load and accelerates disease pathology

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Abstract

Huntington’s disease (HD) is an inherited neurodegenerative disorder caused by abnormal expansion of CAG repeats in the gene encoding huntingtin. Mutant huntingtin undergoes proteolytic processing and its N-terminal fragment containing polyglutamine repeat accumulates as inclusion not only in nucleus but also in cytoplasm and neuronal processes. Here we demonstrate that removal of ubiquitin ligase Ube3a selectively from HD mice brain resulted in accelerated disease phenotype and shorter lifespan in comparison with HD mice. The deficiency of Ube3a in HD mice brain also caused significant increase in global aggregates load and these aggregates were less ubiquitinated when compared with age-matched HD mice. These Ube3a-maternal deficient HD mice also showed drastic reduction of DARPP-32, a dopamine regulated phospho protein in their striatum. These results emphasize the crucial role of Ube3a in the progression of HD and its immense potential as therapeutic target.
INTRODUCTION

Huntington’s disease (HD) is an autosomal-dominant progressive neurodegenerative disorder, which is characterized by motor, cognitive and psychiatric disturbances (1-3). HD is caused by CAG repeat expansion in the gene encoding for protein huntingtin (4). The disease falls under the category of polyglutamine (polyQ) disorders that includes spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and several spinocerebellar ataxias (SCAs)(1, 5-8). Presence of intra-nuclear inclusions of mutant huntingtin and selective neurodegeneration of specific brain regions are the characteristic pathological features of HD (9-12). Full-length mutant huntingtin cleaved by specific proteases and its N-terminal region containing polyQ repeats rapidly misfolded and accumulated as inclusion bodies (10, 13-15). These inclusions sequester essential component of chaperones and ubiquitin-proteasome system (UPS) contributing to impaired cellular protein quality control system and eventually neurodegeneration (16-22).

Multiple approaches have been pursued to understand and regulate the progression of HD. One such strategy has been to study the role of UPS and autophagy in the clearance of mutant huntingtin and pathogenesis of HD(21, 23-25). This is based on the fact that mutant huntingtin and its aggregates are ubiquitinated, a pre-requisite step for a misfolded protein to be cleared not only by proteasome but also through autophagy(26, 27). In fact, multiple evidences now indicate that mutant huntingtin and its aggregates can be degraded by proteasome and autophagic machinery(20, 21, 28). Ubiquitin ligase (E3 enzyme) is the crucial component of UPS because of its substrate recognition property and existence as large diversities(26). There is a growing body of evidence suggesting their ability to slow down polyQ-induced disease pathogenesis. CHIP, a chaperone-dependent E3 ubiquitin ligase, has been shown to ubiquitinate and degrade polyQ proteins and reduce its associated toxicity in mice and a zebra fish model of HD(29, 30). Its over-expression has also been shown to have
beneficial effects on SBMA phenotype by promoting degradation of mutant androgen receptor and improving mice behaviour(31). Over-expression of ER-associated ubiquitin ligase Hrd1 in cellular model degrades polyQ protein and prevents cell death(32). Partial suppression of E3 ligase parkin has been demonstrated to affect motor behaviour and increased death of striatal neurons in HD model mice, while its over-expression has been shown to reduce aggregation and cytotoxicity of mutant ataxin-3 fragment(33, 34). Silencing of cullin1 and skp1 has been shown to increase aggregation propensity in fly models of HD and SCA3(35).

Recently we have demonstrated function of Ube3a (also known as E6-AP) as a protein quality control ubiquitin ligase and its involvement in clearance of misfolded polyQ proteins(36, 37). Ube3a has also been seen to recruit to aggregates and its soluble levels depleted in several brain regions of R6/2 mice model of HD, indicating its possible involvement in disease progression(38). Its deficiency has previously been shown to intensify SCA1 pathalogy by specifically degenerating Purkinje cells layer with surprising concomitant reduction of aggregates (39). Although the level of Ube3a is reduced globally and its role is suggested in HD, there is no direct evidence to implicate its involvement in HD pathogenesis(38, 40). Furthermore, how its deficiency decrease aggregates in Purkinje cells is not clear. Ube3a gene is paternally imprinted in the brain particularly in neurons (41-43) and loss of function of maternally inherited Ube3a causes Angelman syndrome (AS), a neurodevelopmental disorder characterised by cognitive and motor deficits (44, 45). Ube3a-maternal deficient mice (model mice for AS) also recapitulate many essential features of AS including learning and memory impairment along with motor and other behavioural abnormalities (46, 47). In the present study, we used the feature of preferential expression of maternal Ube3a gene to generate Ube3a-maternal deficient HD mice (that will not express Ube3a specifically in the brain) in order to better understand the role of Ube3a in HD.
progression. These Ube3a-maternal deficient HD mice demonstrates an overall accelerated disease phenotype, evidenced by higher neuronal aggregates load, worsening of motor behavioural performance and finally increased mortality rate in comparison with HD.

RESULTS

Absence of Ube3a in HD mice brain results in reduced body weight and shorter lifespan

Ube3a has been shown to promote the clearance of mutant huntingtin, recruits to mutant huntingtin aggregates and its level is decreased in the brain of symptomatic R6/2 HD mice (36-38). To understand the precise role of Ube3a in the clearance of mutant huntingtin and disease progression, we generated brain Ube3a-deficient R6/2 HD mice by crossing male R6/2 HD with Ube3a-maternal deficient heterozygous female (AS model mice). It is important to note that Ube3a-maternal deficient heterozygous mice will not express Ube3a gene in their neurons because paternally inherited Ube3a gene is imprinted in the neuron(41-43). Mice thus generated were genotyped and categorized as wild type, Ube3a-maternal deficient, HD and Ube3a-maternal deficient HD (Fig.1A). Immunoblot analysis of IC2 (detects extended polyQ tract) and Ube3a in the cortical lysates further confirmed the specified genotypes (Fig.1A, bottom panel). Interestingly, we noticed lifespan of Ube3a-maternal deficient HD mice were reduced to almost half in comparison with HD mice (Fig.1B). While HD mice survived up to 100-115 days, double-mutants started dying as early as 40 days and survived not longer than 60-65 days of age. Ube3a-maternal deficient mice survived normally similar like wild type. All four genotypes appeared to be phenotypically similar till the age of 4 weeks. From the age of 5-6 weeks, HD and Ube3a-maternal deficient HD mice showed decrease in body weight compared to wild type and Ube3a-maternal deficient mice (Fig.1C). Double mutant mice exhibited significant differences in body weight from 6 weeks onwards compared to HD.
Loss of Ube3a in HD mice brain accelerates behavioral abnormalities

We next compared the disease progression between HD and Ube3a-maternal deficient HD mice. All four age-matched genotypes obtained were tested for a battery of motor tests and aspects of locomotion from 4 weeks of age. Gait abnormalities were tested by analyzing footprint pattern, while mice walked on a narrow straight trail. HD and Ube3a-maternal deficient HD mice began to display unevenly spaced, non-uniform gait with statistically shorter stride length from 6 weeks onwards (Fig. 2A). However, at 7 weeks of age, Ube3a-maternal deficient HD mice exhibited significantly shorter stride length compared to HD (Fig. 2A). The stride length of AS mice was not statistically different from the wild type group up to 7 weeks of age. Motor co-ordination was assessed by allowing these mice to perform on rotating rod at a constant speed of 10 rpm. At 4 weeks of age, Ube3a-maternal deficient HD mice showed significant difference in rotarod performance compared to HD and became worse over time (Fig. 2B). HD mice were performing equally well like wild type and AS mice at 4 weeks of age, but their performance starts deteriorating from 5-6 weeks onwards. The rotarod performance of AS mice was very similar to wild type in this experimental setup, however, significant difference can be observed at higher speed of roratod. When suspended from tail, Ube3a-maternal deficient HD mice mostly clasped their hind and forelimbs firmly at 5 weeks of age with increasing severity over time (Fig. 2C). HD mice at 5-7 weeks of age showed only intermittent hind limbs clasping, while AS mice in these age did not exhibit any sign of clasping. It is important to note that AS mice has been shown to exhibit hind limbs clasping at 7-8 months old age (48). The proportion of Ube3a-maternal deficient HD mice that showed clasping was always higher than that of HD mice during 5-7 weeks of age (Fig. 2D). To further compare fine motor co-ordination and balance, mice were assessed on their ability to traverse a narrow beam. Up to 7 weeks of age, all genotypes were able to traverse the beam with equal ease except Ube3a-maternal deficient
HD mice, which took much longer time to traverse the beam and made frequent hind-limb slips (Fig.2E and F). These double mutant mice also manifested increasing incidence of seizure particularly during advanced stage of disease progression (6-8 weeks of age). During behavioral studies, many double mutant mice (about 20-30% at 7 weeks of age) died because of seizures and this could be due to handling. Therefore mice used for survival studies were not allowed to perform behavioral tests. AS or HD mice at this age did not exhibit seizures during behavioral studies.

**Reduced brain weight, accelerated striatal atrophy accompanied by altered histopathology upon loss of Ube3a in HD mice**

Reduced striatal volume due to severe atrophy and reduced total brain weight is the characteristic feature of HD neuropathology. In R6/2 model of HD mice, decrease in brain weight and striatal volume is clearly observed from 8-10 weeks onwards. After sacrificing the animals at 8 weeks of age, total brain weights were measured and found to be significantly reduced in Ube3a-maternal deficient HD mice in comparison with HD and other mice group (Fig.3A). Although AS and HD mice showed reduced brain weight compared to wild type control it was not statistically significant. To determine the extent of striatal atrophy, stereological evaluation of striatal volume using Nissl-stained sections of 8 weeks old animals was done. Striatal volume of Ube3a-maternal deficient HD mice was found to be significantly lower than that of HD and other mice group (Fig.3B and C). HD mice at this age also exhibited reduced striatal volume compared to wild type or AS mice but the decrease were not statistically significant, which could be because of early stage of detecting these alterations.

Although, average brain weight of Ube3a-maternal deficient HD mice was much lower than HD mice, there was no detectable loss of neurons, changes in cell body
morphology or apoptotic cell death in different brain areas of these mice (Supplementary Fig.S1). We did not observe any sign of microgliosis in the striatum and cortex of Ube3a-maternal deficient HD brain as checked by immunostaining of Iba1, a microglial marker (Supplementary Fig.S2A). We also checked for induction of various cytokines by cytokine bead array (CBA) and found no gross change in principle cytokines between HD and Ube3a-maternal deficient HD mice striatum (Supplementary Fig.S2B). However, cortical dendritic projections of Ube3a-maternal deficient HD mice brain appeared to be abnormally shorter compared to same age group (8 weeks) HD mice (Fig.3D). This was confirmed by using different neuronal markers, which stain neurofilaments (detected by SMI32), cytoskeleton components (detected by β-III tubulin or Tuj-1), microtubules (detected by microtubule-associated protein 2 or MAP2) and neuronal nuclei (detected by NeuN). The cortical dendritic projections of AS mice brain were also slightly shorter when compared to wild type or HD mice (Supplementary Fig.S3).

**Increased load of huntingtin aggregates upon loss of Ube3a in HD mice brain**

We next compared distribution and frequency of mutant huntingtin aggregates in age-matched HD versus Ube3a-maternal deficient HD mice using huntingtin-specific antibody. This huntingtin antibody (Santa Cruz, SC-8767) was generated against N-terminal region of human huntingtin and detects both human and mouse huntingtin. At 8 weeks of age, Ube3a-maternal deficient HD mice brain showed profuse aggregation across all brain regions (Fig.4 and Supplementary Fig.S4). HD mice brain at the same age exhibited comparatively reduced number of aggregates but strong diffused expression of mutant huntingtin. When these huntingtin-positive aggregates were double-labeled with ubiquitin antibody, most huntingtin-positive aggregates were also strongly stained with ubiquitin in HD but not in Ube3a-maternal deficient HD mice brain. Many huntingtin-positive aggregates in Ube3a-maternal
deficient HD brain were devoid of or very weakly stained with ubiquitin (indicated by arrow in Fig.4 and also shown in zoomed images). Immunostaining pattern of either huntingtin or ubiquitin in AS mice brain was very similar to wild type (Supplementary Fig.S5).

Approximate numbers of huntingtin-positive aggregates were then counted across different brain regions (motor cortex, striatum, hippocampus and Purkinje cells layer of cerebellum). Frequency of huntingtin-positive aggregates was found to be significantly higher across all regions in Ube3a-maternal deficient HD mice brain compared to HD (Fig. 5A). We next compared the ratios of huntingtin and ubiquitin-positive aggregates in different brain regions of 8 and 12 weeks old HD and 8 weeks old Ube3a-maternal deficient HD mice brain after double immunofluorescence staining of brain sections using huntingtin and ubiquitin antibodies. The ratio of ubiquitin to huntingtin-positive aggregates was nearly similar in various brain regions of HD mice at different ages. In contrary, the ratio was significantly reduced in case of Ube3a-maternal deficient HD mice brain (Fig.5B).

Approximately, 95% of huntingtin aggregates were positive for ubiquitin in cortical, hippocampal and striatal region of HD mice that was reduced to about 70% in Ube3a-maternal deficient HD mice. Although, Purkinje cells in Ube3a-maternal deficient HD mice did not show significant difference in the ratio of ubiquitin to huntingtin aggregates, the aggregates in these cells were less intensely labeled with ubiquitin compared to huntingtin. These findings strongly imply significant contribution of Ube3a in the ubiquitination of mutant huntingtin.

Severe depletion of DARPP-32 levels in the striatum of Ube3a-maternal deficient HD mice brain

Selective degeneration of striatal medium spiny neurons (MSNs) is another major pathological feature of HD. Since we did not find any gross neuronal cell loss in striatum of
We first analyzed total DARPP-32 level (a dopamine-regulated phospho protein marker for MSN activity) since its altered expression is reported in HD mice brain(49). As expected, DARPP-32 staining was reduced in striatal sections obtained from HD mice in comparison with wild type or Ube3a-maternal deficient brain at 8 weeks of age. The reduction was much more dramatic in the striatum of Ube3a-maternal deficient HD mice when compared to HD (Fig.6A). The same was re-confirmed through immunoblot analysis of DARPP-32 (Fig.6B and C). Interestingly, we also observed a significant reduction of DARPP-32 in Ube3a-maternal deficient mice striatum, indicating that the drastic reduction of this protein in Ube3a-maternal deficient HD striatum could be due to additive effect. In contrast, levels of GAD65/67 (general marker for GABAergic neurons) were indistinguishable among all genotypes confirming specific defect in dopaminergic-signaling of MSNs (Fig.6D). Figure 6E showed the significant reduction of Ube3a protein in the cortex and striatum of HD mice compared to wild type control at 8 weeks of age. Similar reduction of Ube3a was also observed in the hippocampus of HD mice (Supplementary Fig.S6).

**Discussion**

In this work we have highlighted ubiquitin ligase Ube3a as an important mediator of HD progression. By creating a HD model that selectively lacked Ube3a in the brain, we report severe acceleration of disease phenotype contributing to early death of these mice. Removal of Ube3a from the HD mice brain also resulted significant global increase in aggregates load in comparison with HD mice.

The findings presented here are in agreement with some of our earlier observations that indicate Ube3a function as cellular protein quality control ubiquitin ligase and promote clearance of misfolded polyQ proteins including mutant huntingtin(36, 37). Ube3a also recruits to mutant huntingtin aggregates and its soluble level was found to be progressively
decreased in HD mice brain indicating further the critical role of Ube3a in disease progression(38). Here we have also found that the reduction in endogenous Ube3a in HD mice can be seen as early as 8 weeks of age. Ube3a dysfunction is linked with AS (41, 44, 45) and its mice model exhibits many crucial features of AS including cognitive, motor and other behavioural abnormalities(46, 47). Thus loss of function of Ube3a in HD mice could not only increase the global aggregate load and associated pathology but also might be directly connected with progressive cognitive and motor abnormalities observed in HD.

An earlier studies have linked lack of Ube3a to increased SCA1 pathology along with a surprising reduction in ataxin-1 aggregates in Purkinje cells, opposing the role of aggregates in the disease pathology (39). We instead demonstrated a significant increase in aggregates frequency along with an overall increased HD pathology and therefore could favour the idea of the role of aggregates in disease pathogenesis(50-52). The discrepancy in aggregation profile between two polyQ disease models is somewhat perplexing and needs further investigation. The biochemical nature of huntingtin and ataxin-1, their size and length of polyQ repeats, their affinity towards Ube3a as well as in the cell they are expressed could be the determining factor for the discrepancies. The increased disease severity in Ube3a-deficient HD or SCA1 models could be due to some targets of Ube3a that is also affected in response to polyQ protein or their aggregates. For example, p53 has been shown as a target of Ube3a (46) and its levels also up-regulated in HD mice brain(53). DARPP-32 could be another potential common target leading severe dysfunction of striatal MSN function and this abnormality could be very specific to HD.

Interestingly, we also observed a significant reduction in the ubiquitination of huntingtin aggregates in Ube3a-maternal deficient HD brains compared to HD. Ubiquitination of mutant huntingtin or their aggregates could target them for degradation through proteasome as well as by autophagy(27). Decrease in ubiquitination therefore could
affect the clearance as has been observed in these double mutant mice. Notably, in the CHIP-deficient HD mice, ubiquitination of huntingtin aggregates was unaffected, although there was an increase in aggregates load(30). Reduction of ubiquitination in absence of Ube3a (not in CHIP) suggests Ube3a can be a major ubiquitin ligase targeting mutant huntingtin.

The phenotypic traits of 6-8 weeks old Ube3a-deficient HD mice were observed to be very similar to 12-14 week old HD mice, indicating an accelerated disease progression. We saw increased mortality rate, decreased total body and brain weight in these mice. This was accompanied by severe atrophy of the striatum and enlarged ventricles. To our surprise, we did not observe any overt neuronal death in these areas but their dendritic projections were degenerated. Also the expression of DARPP-32, which is essential to maintain dopamine signalling in MSN of striatum(54), was found to be severely reduced in double-mutant mice. Its level is known to be reduced even in pre-symptomatic R6/2 HD mice and has been linked to striatal dysfunction(49). We found an even further reduction of DARPP-32 in Ube3a-maternal deficient HD mice suggesting accelerated MSN dysfunction. Our study also indicates that Ube3a could be an important regulator of DARPP-32 and MSN function as the level of Ube3a was progressively decreased along with DARPP-32 in the striatum of HD mice. In fact, dysfunction of nigrostriatal dopaminergic system has been reported in AS mice(48).

Behavioural performance tasks, which require motor learning and coordination are tightly controlled by cortico-striatal circuitry were also impaired to a level much below age-matched control HD mice. Cortical dendritic projections appeared shorter and fragmented in Ube3a-maternal deficient HD mice without any obvious neurodegenerartion. This stunting in Ube3a-deficient HD mice might have aggravated due to lack of Ube3a. Because Ube3a is required for the specification of the apical dendrites and dendrite polarization and Ube3a-maternal deficient mice exhibit reduced dendritic arborisation (55, 56). Overall, this severe
pathology in \textit{Ube3a}-maternal deficient HD mice can be attributed to additive effect of accumulation of Ube3a substrates and toxicity of polyQ proteins or their aggregates.

Altogether, our studies provide strong evidence that Ube3a could play a crucial role in the progression of HD and therefore might be a potential therapeutic target to slow down the disease progression. Recently, topoisomerase inhibitors have been shown to unsilence the dormant paternal Ube3a in the brain\cite{57}. Restoration of Ube3a functions in the brain, by the use of such drugs, might provide promising reversal of the HD pathology.

\section*{MATERIALS AND METHODS}

\subsection*{Materials}

Goat polyclonal anti-huntingtin and rabbit polyclonal anti-Ube3a were purchased from Santa Cruz Biotechnology, rabbit polyclonal anti-ubiquitin was from DAKO, mouse monoclonal anti-SMI32 was from Covance, rabbit polyclonal anti-\(\beta\)III-tubulin (Tuj-1) and anti-\(\beta\)-actin were from Sigma, rabbit polyclonal anti-MAP2 was from Cell signalling and mouse monoclonal NeuN, 1C2 and rabbit polyclonal anti-GAD were purchased from Millipore, Rabbit polyclonal anti-DARPP-32 was from Abcam. Donkey anti-goat, anti-rabbit or anti-mouse alexa fluor (AF)-594 and donkey anti-rabbit or anti-mouse AF-488 were purchased from Molecular Probes. AP- and HRP-conjugated anti-mouse, anti-rabbit IgG, ABC kit, ImmPACT Novared Peroxidase Substrate and vectashield mounting media with DAPI were purchased from Vector Laboratories. PCR reagents were obtained from TaKaRa Biomedical and Tunel assay kit was from Promega.

\subsection*{Animals}

Ovarian-transplanted hemizygous female mice carrying the HD exon 1 gene with \(\sim\)150 CAG repeats (strain B6CBA-Tg (HDexon1) 62Gpb/3J) was purchased from The Jackson Laboratory and crossed with B6CBAF1/J males. Genotyping was carried out using PCR as described previously\cite{58}. Heterozygous \textit{Ube3a} mice were also obtained from the Jackson
Laboratory (strain 129-\textit{Ube3atm1Alb/J}) and maintained in the C57BL/6 background. Genotyping was carried out as described\cite{46}. HD transgenic males were crossed with maternally \textit{Ube3a}-deficient females, to obtain \textit{Ube3a}-intact HD and \textit{Ube3a}-deficient HD mice along with \textit{Ube3a}-deficient and \textit{Ube3a} intact control mice. These mixed background mice were used in all experiments. Animals had free access to a pelleted diet and water \textit{ad libitum}. Mice were either sacrificed by cervical dislocation and brain parts were stored at -80°C or anesthetised and transcardially perfused with PBS containing 4\% PFA (w/v). All animal experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee of the National Brain Research Centre.

**Survival study**

Mice were assessed for viability from age of 4 weeks twice daily. Mice used for this study did not undergo behavioural assessment, to rule any affect of them on death of animals. Survival data was analysed by Kaplan-Meier survival analysis and multiple curves were compared by log rank test.

**Behavioural tests**

**Gait analysis**

For foot-print gait analysis, the fore and hind paws of the mice were dipped in blue and red nontoxic paints respectively. Mice were placed at an open end of a wooden tunnel (40 cm×5 cm) lined with paper. The mice were trained for 3 days to walk through the tunnel and then tested for three trials per week. Two to four steps from the middle portion of each run were measured for hind-stride length and hind-base width.

**Rotarod test**

Mice were placed on the rotarod (Scientific Instruments, New Delhi) and were taught to stay on the rod which was rotating with a constant speed of 5 rpm. Mice that would fall were repeatedly placed back on the rod until they were able to stay on the rotarod for at least 30
sec. Mice were trained for 3 days and then tested for 5 trials per week (each trial comprised of 3 sessions of 60 sec) at a constant rod speed of 10 rpm. Sessions of all 5 trials in each week were averaged to calculate the overall time for each mouse with a maximum possible score of 60 sec. Data were expressed as % total time in rotarod.

**Clasping behaviour**

Mice were suspended with their tails for 60 sec for 2 trials per session and the proportion of mice that clasped by firmly bringing their all four limbs together was evaluated. Clasping scores were allotted to each mouse in every session as, 0 if no clasping is observed, 1 if abnormal extension of the hind limbs was noticed, 2 if mouse is starting to clasp, and 3 if clasping is strongly established. All tests were performed in a blinded fashion.

**Beam walk test**

Mice were allowed to walk and cross an 80 cm long and 20 mm wide beam which was at a height of 50 cm from ground. Mice were trained for 3 days and during that time all mice learned to cross the beam and reach dark box kept on the other end. Mice were tested for three trials per day. Total amount of time taken and number of slips made while crossing the beam by each mouse was calculated from the video recordings made during experiments.

**Measurement of striatal volume**

Mice were perfused with 4% paraformaldehyde and brains were carefully removed. Coronal cryosections (20 μm) were performed and then processed for Nissl staining. The stereological volume of striatum was calculated in arbitrary sections of a systematic set (e.g. every 5th section with the first section selected randomly among first five sections) from the front of the striatum to levels slightly posterior to the level of the crossing of the anterior commissure(59). The perimeter of the striatum was traced in each of the sections using 1x
Nissl stained images in the NeuroLucida software. Subsequently, total striatal volume was calculated as described (60).

**Immunostaining and aggregates counting**

Paraformaldehyde fixed brains were processed for cryo-sectioning to obtain 20 µm thick sections. Sections were then processed for immunostaining using reagents from Vector Laboratories as described earlier (61). Briefly, after antigen retrieval of 45 min at 70°C, sections were blocked and probed using various primary antibodies. Huntingtin, ubiquitin, and DARPP-32 antibodies were used at 1:1000, 1:200 and 1:200 dilutions respectively. Biotinylated secondary antibodies were used at a dilution of 1:500 and signal was enhanced using ABC kit and developed using ImmPACT Novared Peroxidase Substrate. Stained sections were imaged using a Leica DM RXA2 microscope. For immunofluorescence staining, secondary antibodies conjugated either with AF-594 and AF-488 was used at 1:2000 dilutions and the sections were counterstained by DAPI. Primary antibodies against SMI32, Tuj, MAP2, NeuN and huntingtin were used at 1:1000 dilutions and ubiquitin was at 1:200 dilutions. Total numbers of huntingtin and ubiquitin positive aggregates were counted in multiple random sections of 40X magnification (0.200 x 0.150 mm area) from different regions of the brain.

**Immunoblotting**

Mice brain parts were dissected and homogenised in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and complete protease inhibitor cocktail) and were kept on ice for 30 mins. Lysed protein samples were centrifuged at 15,000 x g for 10 minutes and supernatant was used for immunoblotting after protein estimations as previously described (62). Primary antibodies Ube3a, DARPP-32 and GAD were used at 1:1000 dilutions, 1C2 1:5000 dilutions and β-actin at 1:5000 dilutions.
Cytokine bead array and Tunel staining

Cytokine bead array (CBA, mouse inflammation kit, BD Biosciences) was used to quantitatively measure the inflammatory cytokines expression in the striatal samples obtained from all 4 genotyped mice at 8 weeks old age. The assay was performed according to the manufacturer instruction using FACS calibur (BD Biosciences) and data was analyzed using CBA software. Tunel assay was done using Tunel assay kit as per manufacturer instruction.

Statistical analysis

Statistical analysis was performed using SigmaStat software. Values were expressed as mean ± SEM. Datas were analyzed by using one or two-way ANOVA followed by Holm-Sidak post-hoc test. Log rank test was used to analyze survival data. In some experiments, two-tailed Student’s t test was used for inter group comparison. P<0.05 was considered statistical significant.

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Conflict of Interest statement: None
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FIGURE LEGENDS

Figure 1. *Ube3a*-maternal deficient HD mice show shorter life-span and reduced body weight compared to their HD counterpart. A) Generation of *Ube3a*-maternal deficient HD mice \{HD/Ube3a(m-/p+)\} by crossing *Ube3a*-maternal deficient females \{Ube3a(m-/p+)\} with HD transgenic males. Genotypes were confirmed by PCR using HD and *Ube3a* gene specific primers. Bottom panel shows immunoblot analysis of Ube3a and 1C2 to re-confirm the expression of Ube3a and HD transgene respectively. Cortex samples from 8 weeks old mice were used for immunoblot analysis. Note the absence of Ube3a expression in maternally *Ube3a*-null wild-type \{wild/Ube3a(m-/p+)\} or HD mouse \{HD/Ube3a(m-/p+)\} and presence of HD transgene as detected by 1C2 in HD and HD/Ube3a(m-/p+) mice. B) Survival study of the indicated genotypes (n = 10 for each genotype). C) Comparison of the body weight showing significant gradual decrease in total body weight of *Ube3a*-deficient HD mice when compared to HD mice. Values are mean ± SEM with 8 animals per group. *P* < 0.05 compared to HD/Ube3a(m+/p+) group. Data were analysed by two-way ANOVA followed by Holm-Sidak post-hoc test.

Figure 2. Loss of Ube3a in HD mice brain accelerates motor abnormalities. *Ube3a*-maternal deficient HD mice exhibits significant reduction in the motor performance and coordination as evaluated from stride length of foot print gait analysis (A), rotarod (B) clasping (C,D) and beam-walk test (E,F). All these tests were conducted in a blinded manner. Behavioural data obtained from each animal (through multiple sessions and/or multiple trials) at each time point were then made average with the total number of animals in each group. Data represented as mean ± SEM with 8 animals in each group. *P* < 0.05 in compared to HD/Ube3a(m+/p+) when analysed by two-way ANOVA followed by Holm-Sidak post-hoc test.
Figure 3. Reduced brain weight, accelerated striatal atrophy and altered histopathology in Ube3a-maternal deficient HD mice. A) Significant reduction in total brain weight of Ube3a-maternal deficient HD mice in comparison with age-matched (8 weeks) HD and other mice groups. B,C) Ube3a-maternal deficient HD mice exhibited increased dilation of lateral ventricles (B) and decreased striatal volume (C) compared to other mice group at 8 weeks of age seen upon stereological evaluation of Nissl-stained sections of cerebrum as described in Methods. Scale bar, 500 µm. Five mice were used in each group. *P< 0.01 in comparison with all three other groups when calculated by two-way ANOVA followed by Holm-Sidak post-hoc test. Arrow indicates the dilation of lateral ventricle of Ube3a-maternal deficient HD mice. D) Altered histopathology of Ube3a-maternal deficient HD mice brain. Representative images of motor cortex of 8 weeks old HD and Ube3a-deficient HD mice brain, showing truncated dendritic projections upon loss of Ube3a in HD mice brain as seen by SMI32, Tuj-1 or β-III tubulin and MAP2 immunofluorescence staining. In the bottom panel, sections were subjected to immunofluorescence staining using NeuN antibody showing no differences in cell numbers between the two genotypes. AF-594-conjugated secondary antibody was used to detect SMI32, Tuj-1 and MAP2 (red) while AF-488-conjugated secondary antibody was used to label NeuN (green). Brain sections obtained from 3 different mice (one section from each mouse) in each group was evaluated for above mentioned staining. Scale bar, 20 µm.

Figure 4. Ube3a-maternal deficient HD mice brain exhibits higher frequency of huntingtin positive aggregates. Representative images of huntingtin and ubiquitin double immunofluorescence staining of 8 weeks old HD and Ube3a-maternal deficient HD mice brain of different regions. A) Striatum and cerebellum. B) Cortex and hippocampus. AF-488-conjugated secondary antibody was used to label ubiquitin (green) and AF594--conjugated secondary antibody was used to detect huntingtin (red). Note the increase in huntingtin-
positive aggregates number in every region of Ube3a-maternal deficient HD brain and some huntingtin-positive aggregates showed reduced or no ubiquitination (indicated by arrow).

Inset shows 3X magnified images around an arrow marked by white area. Five mice in each group were analysed and 3 sections from each mouse were stained for immunostaining. Section from different mice were placed on the same slide and processed for immunostaining. Scale bar, 20 µm.

Figure 5. Increased aggregation but reduced ubiquitination upon loss of Ube3a in HD mice brain. A) Approximate number of huntingtin-positive nuclear aggregates in different brain regions of HD and Ube3a-maternal deficient HD mice. Huntingtin-positive nuclear aggregates were counted in 40X immunohistochemically stained images (0.200 x 0.150 mm area) obtained from 3-5 different field in each region. Two brain sections of particular region from each mouse were analysed for aggregate counting and there was 5 animals in each group. Average aggregate numbers of specific brain region in each animal was first calculated followed by average value for 5 animals and statistical analysis. *P<0.01 in comparison with HD/Ube3a(m+/p+) mice. Values were analysed by two-way ANOVA followed by Holm-Sidak post-hoc test. B) Reduced ubiquitination of huntingtin aggregates in the absence of Ube3a. Brain sections obtained from HD and Ube3a-maternal deficient HD mice were placed in the same slides and processed for double-immunofluorescence staining for huntingtin and ubiquitin as in Fig.4. Aggregates stained by both huntingtin and ubiquitin with almost equal intensity were counted. Total number of huntingtin and ubiquitin positive aggregates in different areas of each section was counted and the ratios were made for each mouse. Ratios obtained from 4 individual mice were made average and subjected to statistical analysis. While almost all aggregates were ubiquitinated in 8 and 12 weeks old HD mice, the ratio of ubiquitin to huntingtin-positive aggregates was significantly reduced in Ube3a-maternal deficient HD brain sections. Values are mean ± SEM of 4 different mice in each
group. *P<0.01 in comparison with HD/Ube3a(m+/p+) mice data when calculated by two-way ANOVA followed by Holm-Sidak post-hoc test.

**Figure 6.** Severe reduction of striatal DARPP-32 levels in *Ube3a*-maternal deficient HD mice. A) Representative images of DARPP-32 immunostaining of the striatas of 8 weeks old animals of specified genotypes indicated in the figure. Brain sections obtained from 4 different groups were put on the same slides and processed for immunostaining. Three mice in each group were analysed for DARPP-32 staining. Striatum of HD and *Ube3a*-deficient HD mice showed reduced DARPP-32 staining in comparison to wild-type and *Ube3a*-maternal deficient mice. Scale bar, 500 µm. B) Brain lysate (cortex and striatum) obtained from different genotypes were subjected to immunoblotting to check levels of DARPP-32 and GAD 65/67 and Ube3a. C) The band intensity of DARPP-32 normalized to β-actin was quantified and plotted from 3 different mice striatal and cortical samples in each group. *P<0.01 in comparison with WT/Ube3a(m+/p+) mice and **P<0.05 compared to HD/Ube3a(m+/p+). Data were analysed by two-way ANOVA followed by Holm-Sidak post-hoc test. D and E) Quantification of band intensities of GAD 65/67 (D) and Ube3a (E) normalized to β-actin from 3 different mice in each group. *P<0.01 in comparison with WT/Ube3a(m+/p+) mice.
A: Stride length (cm) vs Age of mice (weeks)

B: Time in rotarod (% of total time) vs Age (weeks)

C: Clasping score vs Age of mice (weeks)

D: Proportion of mice that clasp vs Age of mice (weeks)

E: Time (sec) vs Age of mice (weeks)

F: Number of slips vs Age of mice (weeks)