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Compensatory changes in the ubiquitin–proteasome system, brain-derived neurotrophic factor and mitochondrial complex II/III in YAC72 and R6/2 transgenic mice partially model Huntington's disease patients

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Intraneuronal protein aggregates of the mutated huntingtin in Huntington's disease (HD) brains suggest an overload and/or dysfunction of the ubiquitin–proteasome system (UPS). There is a general inhibition of the UPS in many brain regions (cerebellum, cortex, substantia nigra and caudate-putamen) and skin fibroblasts from HD patients. In the current experiment, the widely used mutant huntingtin-exon 1 CAG repeat HD transgenic mice model (R6/2) (with 144 CAG repeat and exon 1) during late-stage pathology, had increases in proteasome activity in the striatum. However, this discrepancy with HD patient tissue was not apparent in the mutant CAG repeat huntingtin full-length HD (YAC72) transgenic mouse model during post-symptomatic and late-stage pathology, which then also showed UPS inhibition similar to HD patients' brains. In both types of HD model mice, we determined biochemical changes, including expression of brain-derived neurotrophic factor (BDNF) and mitochondrial complex II/III (MCII/III) activities related to HD pathology. We found increases of both BDNF expression, and MCII/III activities in YAC72 transgenic mice, and no change of BDNF expression in R6/2 mice. Our data show that extreme CAG repeat lengths in R6/2 mice is paradoxically associated with increased proteasome activity, probably as a cellular compensatory biochemical change in response to the underlying mutation. Changes in HD patients for UPS function, BDNF expression and MCII/III activity are only partially modeled in R6/2 and YAC72 mice, with the latter at 16 months of age being most congruent with the human disease.

INTRODUCTION

Huntington's disease (HD) is an autosomal progressive movement disorder, which is caused by long polyglutamine repeats (>36 CAG) in the exon 1 of huntingtin gene (IT15). The progressive pathology of HD includes selective degeneration and loss of medium-sized spiny neurons with nuclear inclusion bodies in the caudate-putamen and cortical area of the patients' brains. The selective vulnerability and abnormal protein aggregates in specific cells may be explained by dysfunction of multiple factors, such as cellular protein degradation, trophic factor regulation, mitochondrial energy metabolism and *N*-methyl-D-aspartic acid (NMDA) receptor excitotoxicity (1).

One of the explanations of the failure of abnormal protein degradation is the downregulation of the ubiquitin–proteasome system (UPS) (2,3). UPS is a cellular process for the non-lysosomal protein degradation of abnormal, misfolded or oxidized proteins. We previously reported that proteasome activities are generally inhibited in several brain regions and skin fibroblasts of HD patients (4), and recently demonstrated that enhancing UPS function by proteasome activator can improve cell survival against glutamate toxicity in the cell

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culture model of HD (5). Downregulation of brain-derived neurotrophic factor (BDNF) levels also explained the progressive HD pathology in HD patients and HD model mice (4,6–8). Previous studies reported that mitochondrial complex II/III (MCII/III) activity is downregulated in the caudate-putamen area of HD patients' brain (4,9). Striatal cells expressing mutant huntingtin showed significant impairment of mitochondrial enzyme activities and ATP production (10). Benchoua *et al*. (11) suggested that N-terminal fragment of mutated huntingtin affects the defects of MCII/III activities in the Htt171 striatal cell model.

There are several animal models for HD, widely used in the study of potential pathological mechanisms of HD. The transgenic mice R6/2 contain a mutant N-terminus segment of the huntingtin gene with an approximately 144 CAG expansion in exon 1. In R6/2 transgenic mice, symptoms begin around 5–7 weeks as subtle neurological dysfunctions, which become more prominent at 8–9 weeks and progressively worse by 14 weeks. Nuclear inclusions appear in striatal neurons at 4–5 weeks, although selective neuronal death is not seen until 14 weeks. The loss of mGluR2 receptors that is responsible for regulating glutamate release in corticostriatal terminals has been demonstrated in the R6/2 mice. Recently, it was reported that dopamine release is compromised in R6/2 mice models of HD (12). In 14–16 weeks old R6/2 mice, muscle mitochondria showed low stability against Ca^{2+} (13). YAC72 transgenic mice are developed with yeast artificial chromosome with N-terminal CAG repeat for the full size huntingtin and all its regulatory elements with 72 CAG repeats. The pathological phenotype including nuclear inclusions develops more gradually than in the R6/2 (14–16). There are yet other HD animal models such as knock-in HD model mice series (for example, HdhQ111 mice), which developed a progressive neuropathological phenotype with specificity for striatal neurons (17). Tet/HD94 conditional HD model showed conditional expression of mutant huntingtin and HD pathology and was used for the RNA interference studies against mutant huntingtin (18,19). Therefore, each HD model appears to represent specifically interesting pathological features, and all are thus valuable in the study of specific biochemical abnormalities and cellular responses relevant to understanding pathogenesis. It is not clear if biochemical changes in patients' postmortem samples are also reflected in HD animal models. Consequently, it is of interest to determine whether these biochemical dysfunctions are also shown in HD transgenic models. In this study, we determined the regulation of proposed HD pathological factors in two major HD transgenic mice: R6/2 and YAC72, used in research to find therapeutics for HD.

RESULTS

Previously, we demonstrated that the UPS is downregulated in several regions of HD patients' brain and skin fibroblasts (4). In HD patients, we also found decreased BDNF protein levels in most brain regions and downregulation of MCII/III in the striatum and cerebellum, while ubiquitin levels were increased only in the striatum. Since there are several transgenic mouse models available, we wanted to experimentally compare these factors in two major but different HD transgenic mice, R6/2 and YAC72, with real HD pathology and biochemistry (see Introduction).

UPS is upregulated in R6/2 transgenic mice

We first tested the movement behaviors of R6/2 (with 180 CAG repeats in Huntington-exon 1 region) transgenic mice every week from 6 weeks of age to 12 weeks of age to create a baseline and progression of their disease. R6/2 mice showed reduced total locomotor counts in open-field tests and an increased frequency to fall in rotor-rod tests compared with wild-type littermate control mice (Fig. 1A). This motor malfunction in R6/2 mice worsened with age and these mice had very low levels of locomotor activity by 12 weeks of age. Post-mortem R6/2 mice brains (at 13 weeks of age) showed prominent ubiquitin-positive nuclear inclusions in various brain regions including striatum, cortex and hippocampus (Fig. 1B). The striatum region of R6/2 mice clearly had the highest density of inclusion body formation. Unexpectedly, compared with the clinical data (4), R6/2 mice (13 weeks of age) showed significant increase of proteasome activities including chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities (PGPH) in the frontal cortex (FC), striatum (STR) and cerebellum (CB). Similar findings were seen in parallel groups of R6/2 mice at 9 weeks of age with pathological symptoms (data not shown), but not at 6 weeks of age (Fig. 1C).

UPS is downregulated in YAC72 HD transgenic mice at 16 months of age

We determined proteasome activities in various brain regions of YAC72 HD transgenic mice (at 16 months of age, chronic phase). As in HD patients' brains and skin fibroblasts (4), YAC72 mice showed decrease of proteasome activities including chymotrypsin-like and PGPH activities in several brain regions including FC, STR and CB at 16 months of age (chronic stage of disease) (Fig. 2). However, this decrease of proteasome activities were not detected in the brain regions of YAC72 mice at younger age (8 months; Fig. 2). These data suggest that progressive and age-dependent decrease of UPS in YAC72 transgenic mice (but not in R6/2 mice) mimic those in HD patients.

Regulation of BDNF protein expression levels in YAC72 and R6/2 transgenic mice

We and others previously determined that BDNF protein expression is downregulated in HD patients' brain regions including striatum (4). To study whether the HD transgenic model mice shows similar abnormalities in the neurotrophic regulation, we determined BDNF protein expression levels in the striatum of post-mortem brains from YAC72 and R6/2 transgenic mice using BDNF enzyme-linked immunosorbent assay (ELISA). In contrast to HD patients, R6/2 mice did not show significant changes of BDNF protein expression levels in the striatum compared with wild-type littermate control mice (Fig. 3A). In both wild-type and R6/2 mice, BDNF protein levels decreased by age (6–13 weeks)

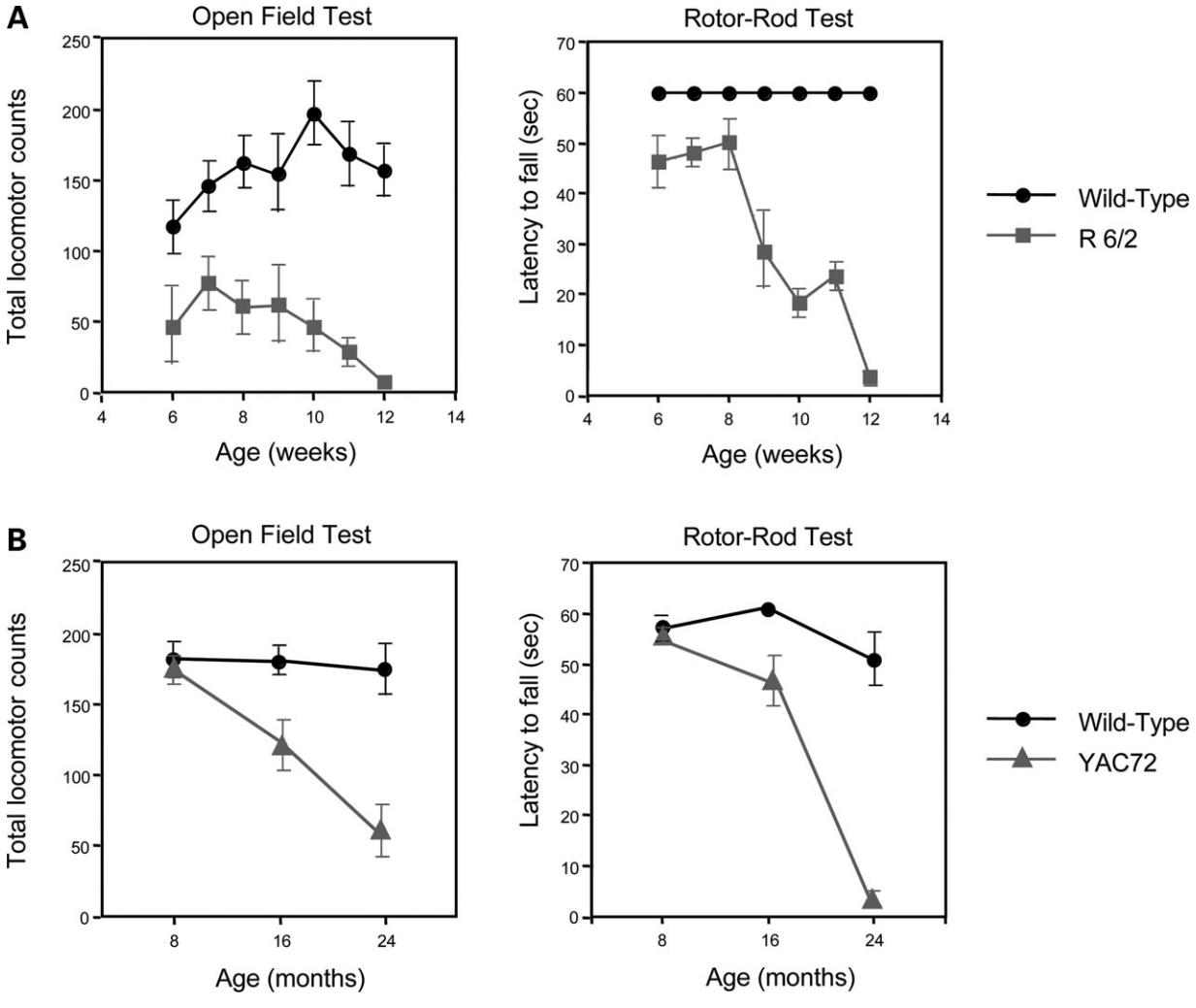

Figure 1. Behavioral assessment of R6/2 Huntington disease (HD) transgenic mice (**A**) and YAC72 transgenic mice (**B**) in open-field and rotor-rod tests. R6/2 mice showed significantly reduced total locomotor counts in open-field test and latency to fall in rotor-rod compared with wild-type littermate control mice. With age, the R6/2 mice locomotor counts and latency to fall worsened and were non-functional by 12 weeks of age and were sacrificed for biochemical analysis. YAC72 mice also showed significant reduction in their total locomotor counts and latency to fall in open-field and rotor-rod test at 16 months and 24 months of age ($n = 8$ for each group). (**C**) Proteasome activities in the brain regions of R6/2 HD transgenic mice at 6 and 13 weeks of age. We determined chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities in frontal cortex (FC), striatum (STR), cerebellum (CB) of R6/2 transgenic mice and wild-type littermate control mice. Compared with wild-type, R6/2 transgenic mice showed significantly higher proteasome activities at 13 weeks of age in all the three brain regions tested ($^*P < 0.05$, $n = 8$ for each group).

(Fig. 3A). Unexpectedly, YAC72 mice showed slightly higher BDNF expression levels in FC, STR and CB compared with wild-type littermate control mice at 16 months of age.

Regulation of MCII/III activities in YAC72 HD transgenic mice

Previous studies of R6/2 transgenic mice showed no significant changes of MCII/III enzyme activities but some reduction of complex IV at 12 weeks of age (20). R6/2 mice did not show significant changes of MCII/III enzyme activities by 13 weeks of age. YAC72 HD transgenic mice at 16 months of age (chronic stage) showed increased MCII/III enzyme activities in the FC and STR (Fig. 4).

DISCUSSION

Progressive pathology in HD models

There are several transgenic animal models for HD including R6/2 and YAC72 mice (14,21). Although each model is valuable in understanding pathological aspects, it is of interest to study the specific biochemical abnormalities and progressions seen in these model animals. Here, we have investigated these two transgenic HD mice with a focus on the progressive changes in behavior, UPS, BDNF and MCII/III. The decreased UPS function seen in patients was better modeled in YAC72 transgenic mice, by 16 months of age. Other measures, such as metabolic activity (MCII/III) and BDNF levels showed compensatory changes (upregulation), which may reflect similar changes in HD at very early stages of cellular pathology. Further down we discuss these findings and interpretations.

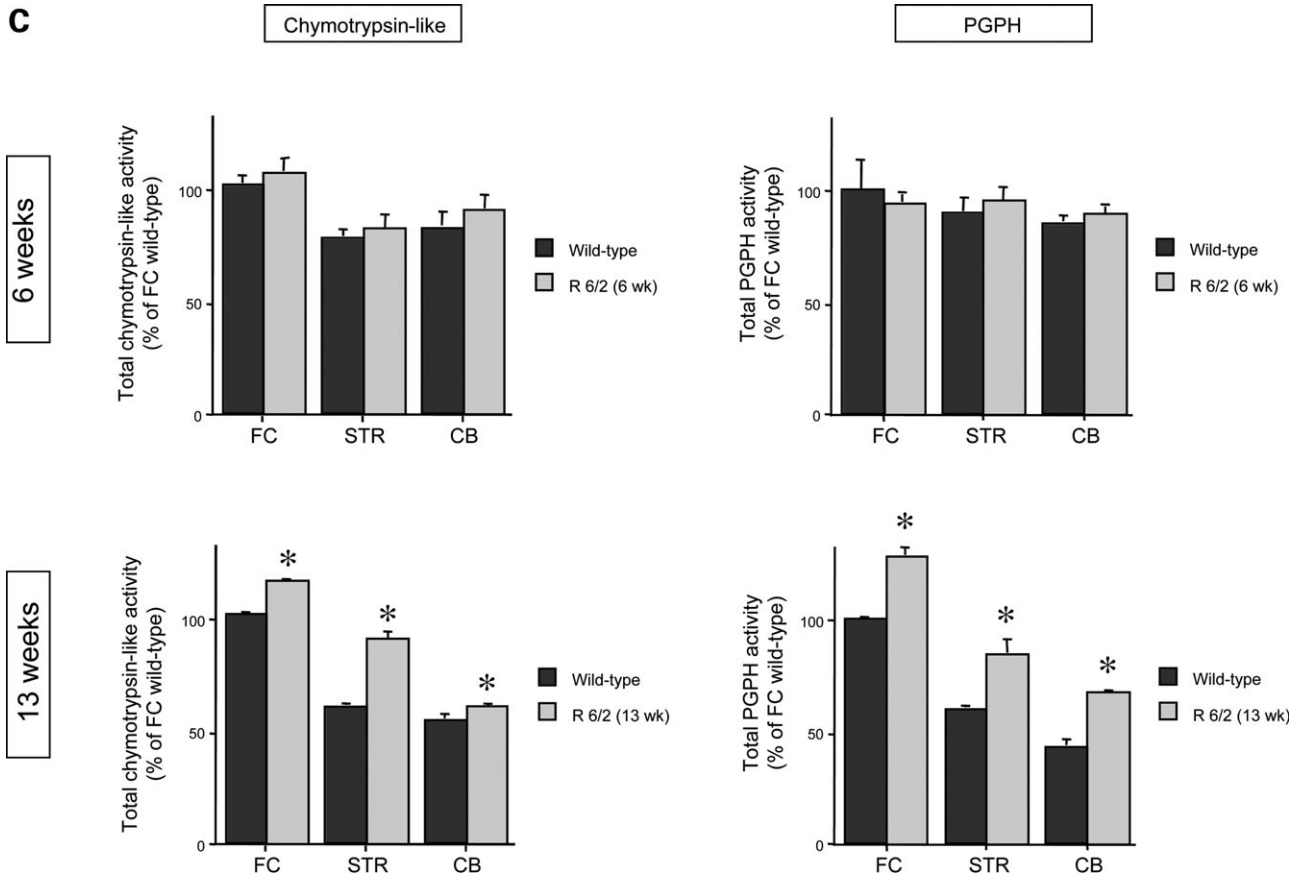

Figure 1. continued.

Our data indicate that specific cellular responses to the mutant protein *in vivo* can model the disease, and intriguingly may also model adaptive and protective responses by the affected cells. Given that patient material is not available for such longitudinal studies, these results provide unique insights to compensatory mechanisms of cells affected by the mutant huntingtin.

Mutant huntingtin downregulates UPS function in YAC72 transgenic mice but not in R6/2 transgenic mice

As reported in previous studies, dysfunctional protein degradation has been used to explain the generation of protein aggregates in the HD pathology (3,22). Proteasome and autophagy pathway are major ways to clear protein aggregates with mutant huntingtin expression (2,23). There are some primary data about the effects of mutant huntingtin on proteasome activities. For example, using *in vitro* cell culture systems, the huntingtin filaments, which was isolated from Tet/HD94 mouse model inhibited 26S proteasome activity (19). In previous studies, we determined that the function of UPS, non-lysosomal protein degradation system, is generally decreased in the several brain regions and skin fibroblasts of HD patients (4). Proteasome activity was also reduced in HD model striatal neurons containing mutant huntingtin (105 CAG repeats), and it could be improved by lentiviral gene transfer of proteasome activator, PA28γ suggesting a potential therapeutic strategy through proteasome activation (5). However, the proteasome activity in the striatum of one case of a 6-year-old boy with extremely high CAG repeats (180 CAG repeats) showed two times higher proteasome activity than in normal subjects (4), possibly as compensatory response to the accumulation of the toxic proteins. In the present study, YAC72 HD model mice (with 72 CAG repeats) at 16 months of age showed decreased proteasome activities in the striatum as seen in HD patients, but R6/2 transgenic mice (with 144 CAG repeats) showed paradoxical increases of proteasome activities in the striatum. Therefore, it is possible that extreme CAG repeats in HD patients and R6/2 mice might cause increased proteasome activities in the striatum, prior to the time when full degenerative changes are apparent in most of the cells.

The effects of mutant huntingtin on BDNF function

BDNF is anterogradely transported to the striatum from the cortex suggesting functional interaction between these two brain regions (24,25). Several previous studies emphasized the dysfunction of BDNF in HD and potential neuroprotection strategies by BDNF for the improvement of HD pathology (4,7). In HD patients, BDNF protein levels were decreased in several brain regions including frontal cortex, striatum, substantia nigra and cerebellum (4,7). Zuccato *et al*. determined that the mRNA levels of BDNF were downregulated by disease progression in the cerebral cortex of R6/2 mice (6, 8 and 12 weeks). Cortical and striatal BDNF protein levels

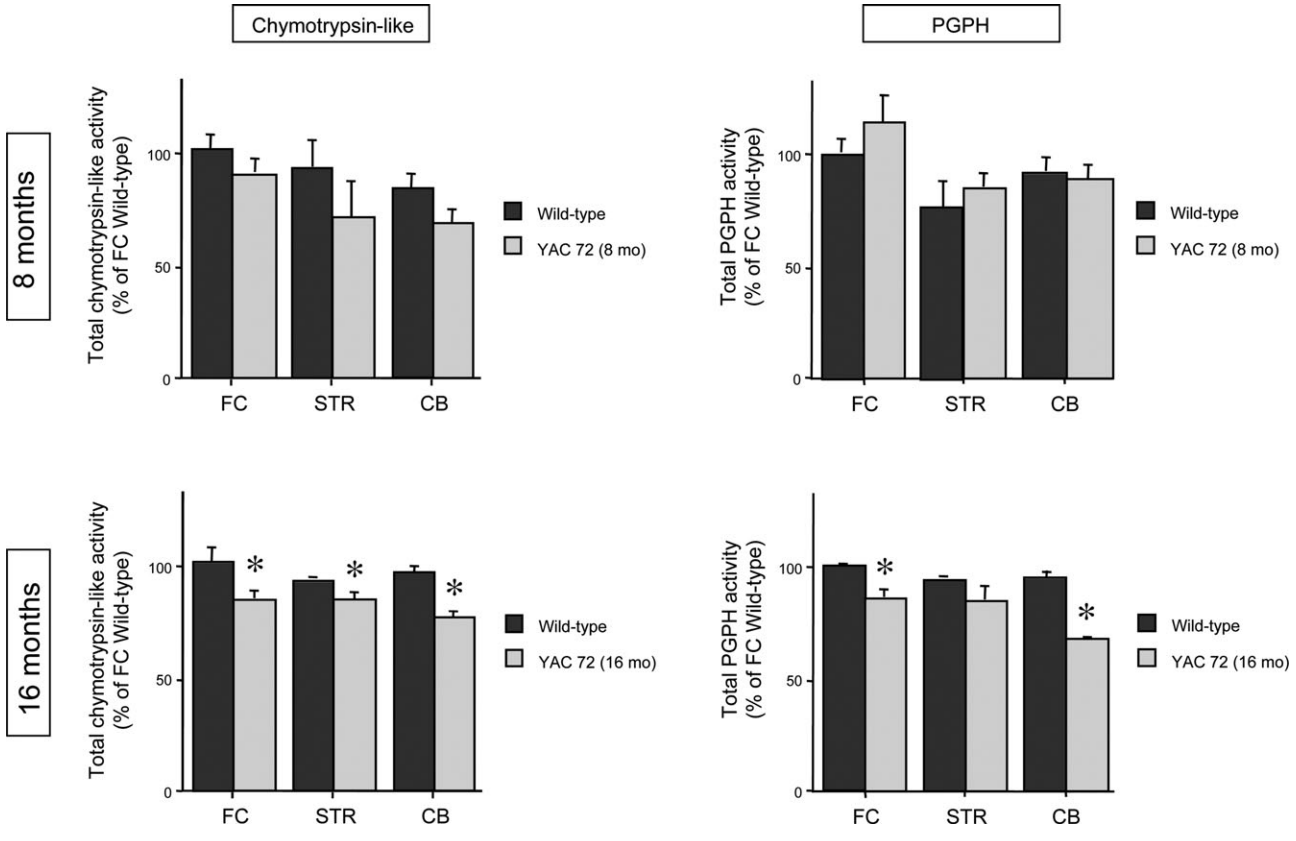

Figure 2. Proteasome activities in the brain regions of YAC72 Huntington disease (HD) transgenic mice at 8 and 16 months of age ($n = 8$ for each group). Generally the proteasome activities including the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities were slightly decreased in frontal cortex (FC), striatum (STR), cerebellum (CB) of YAC72 HD transgenic mice at 16 months of age compared with littermate control mice ($^*P < 0.05$). However, there were only tendency of decreased chymotrypsin activity in YAC72 transgenic mice at 8 months of age.

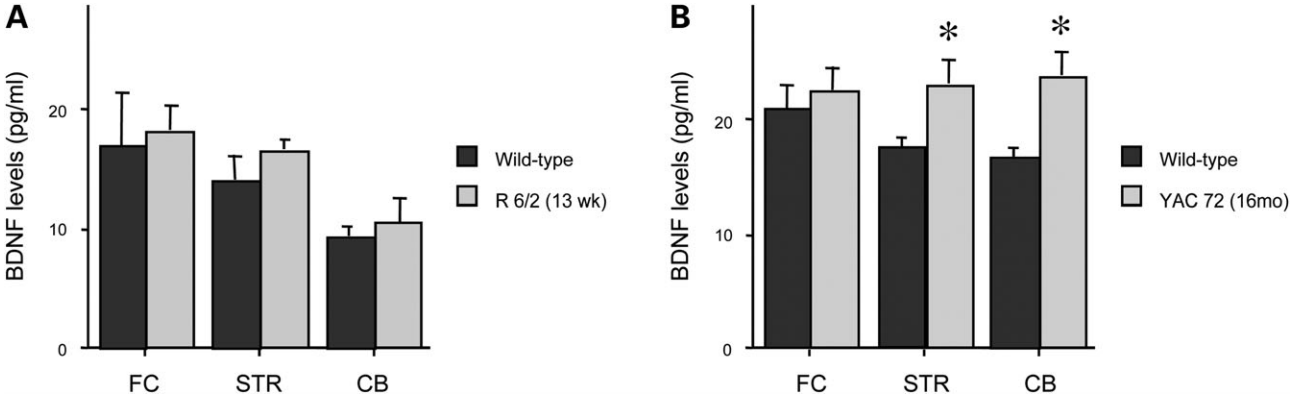

Figure 3. Striatal brain-derived neurotrophic factor (BDNF) protein expression levels in Huntington disease (HD) transgenic mice. (**A**) BDNF protein expression levels were determined using ELISA from the striatum of R6/2 transgenic mice and wild-type control at three different ages (6, 9 and 13 weeks). BDNF expression levels were not significantly altered in R6/2 HD transgenic mice compared with wild-type littermate control mice at all three age points we tested. BDNF protein expression levels were decreased by age in both wild-type littermate control and R6/2 transgenic mice ($\S P < 0.05$ in comparison with the wild-type mice at 6 weeks of age, $\#P < 0.05$ in comparison with the R6/2 HD transgenic mice at 6 weeks of age). (**B**) BDNF protein expression levels in YAC72 HD transgenic mice at 16 months of age. YAC72 HD transgenic mice showed significantly higher BDNF protein expression levels in striatum and cerebellum ($^*P < 0.05$).

were reduced in the N171-82Q HD mice (26). The protein levels of BDNF detected by ELISA were reduced in the hippocampus and striatum of R6/1 HD mice at 5 months of age (27). Depending on the length and levels of expression of the CAG repeats, BDNF levels were also reduced in cultured HD cell model (6).

However, unexpectedly, BDNF protein levels, which were detected by ELISA did not show significant changes in R6/2

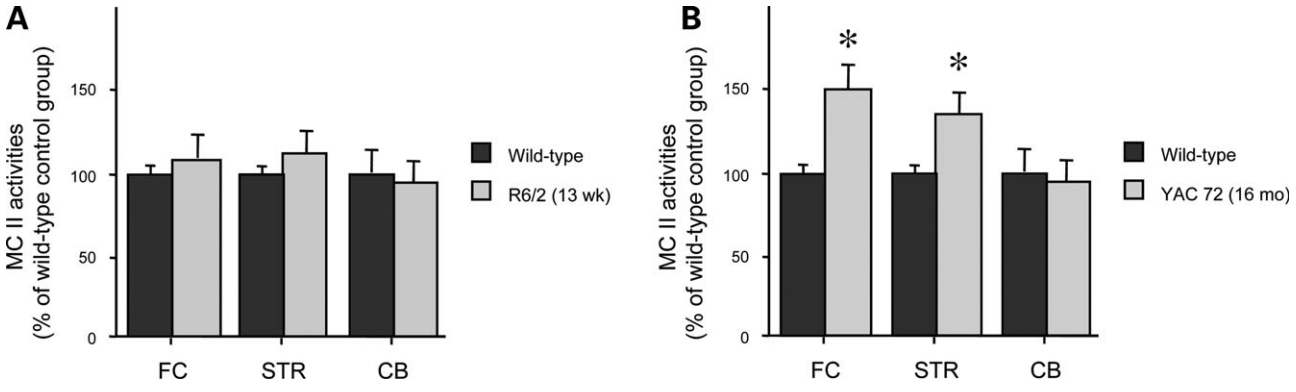

Figure 4. Mitochondrial complex II/III (MCII/III) enzyme activities in R6/2 (**A**) and YAC72 (**B**) HD transgenic mice. MCII/III activity levels were significantly higher in YAC72 HD transgenic mice compared with wild-type control mice ($^*P < 0.05$).

mice at 6, 9 and 13 weeks of age (end-stage), and significant increases were seen in the striatum and cerebellum, but not frontal cortex, of YAC72 mice at 16 months of age in the present study (Fig. 3). Interestingly, recent studies suggest that protein trafficking of BDNF is reduced by mutant huntingtin in HD cell models (28). The expression of tyrosine kinase receptor (TrkB), BDNF receptor, was specifically reduced in transgenic exon-1 (R6/1) and full-length knock-in HD model mice (29). The absence of anterograde cortical BDNF in BDNF knock-out mice caused striatal dendrite deficits followed by neuronal loss (30). In addition, huntingtin and BDNF double-mutant mice showed lower activity in response to amphetamine indicating a dopaminergic neuronal dysfunction (25). From these data we suggest that BDNF transcription level is downregulated by mutant huntingtin, but does not alter the BDNF protein levels in the striatum of R6/2 mice at 13 weeks of age. In that case, anterogradely transported BDNF from cortical cells to striatum, would accumulate at or in the receptor striatal cells of YAC72 mice, possibly by reduced degradation and/or altered level of BDNF uptake given the reduced expression of TrkB (29). Similar growth factor level fluctuations in the hippocampus have been discussed in the context of Alzheimer's disease (31–33).

Effects of mutant huntingtin on MCII/III function

Dysfunction of energy metabolism is known to be associated with the HD pathology (34). HD can be induced by the mitochondrial toxin, 3-nitropropionic acid (3-NP; complex II inhibitor) in animal model systems (35–37) indicating mitochondrial dysfunction in the HD pathology. These 3-NP HD model mice in older age showed a greater susceptibility to striatal neurotoxicity (38) suggesting age-dependent metabolic impairment in HD. Interestingly, this 3-NP HD model showed decrease of the threshold for NMDA-mediated glutamate toxicity (38–40). Mitochondrial dysfunctions including reduced MCII/III activity in HD patients at grade II–IV has been reported (4,9). Tabrizi *et al*. reported no significant changes on the MCII/III enzyme activities, but significant reduction of complex IV in R6/2 mice at 12 weeks of age (end-stage). In this study, we did not detect significant changes in MCII activity in R6/2 mice at 13 weeks of age (end-stage), but it was paradoxically increased in YAC72 mice at 16 months of age (chronic stage).

Recent studies demonstrate that the expression of full-length mutant huntingtin decreases mitochondrial-dependent handling (41) and reduces the stability of muscle mitochondria against Ca^{2+} in R6/2 mice (13). Interestingly, in the R6/2 mice at 12–13 weeks and YAC128 mice at 12 months exhibit increased mitochondrial Ca^{2+} loading capacity of forebrain mitochondria during steady Ca^{2+} infusion, indicating reduced bioenergetic metabolism (42). Using a cell respirometer, these authors also found high *in situ* respiration yield of mitochondria of cultured striatal neurons from HD mice, in the appropriate cellular environment. These data suggest early signs of mitochondrial dysfunction in HD model mice (42). The transcriptional expression of peroxisome proliferator-activated receptor gamma coactivator-1 alpha, which is a transcriptional coactivator that regulates several metabolic processes including mitochondrial biogenesis and respiration, was inhibited by mutant huntingtin expression suggesting downregulation of transcriptional control related to biogenesis in the vulnerable cells by mutant huntingtin (43).

Taken together, our data suggest a compensatory increase of functional activities of MCII/III activity in the targeted striatal cells in HD pathology, as a response to the huntingtin mutation and toxicity. In the case of R6/2 mice, which do not show striatal cell loss as seen in HD patients, the increase of mitochondrial activity may reflect such a compensation.

Multiple factors involved in HD pathology and their correlations

Based on the current experiments, we suggest that the generalized UPS inhibition, the downregulation of BDNF and MCII/III seen in HD patients are modeled in mice only under realistic mutant protein and age conditions (Fig. 5). Although we cannot explain all the mechanisms for the direct interaction among multiple factors in the pathological mechanisms of HD, these multiple risk factors can be considered as potentially synergistic in the selective dysfunction and/or loss of striatal and cortical neurons in HD. First, proteasome function and mitochondrial energy metabolism may be linked. For example, altered proteasomal function owing to the expression of polyglutamine-expanded truncated N-terminal huntingtin-

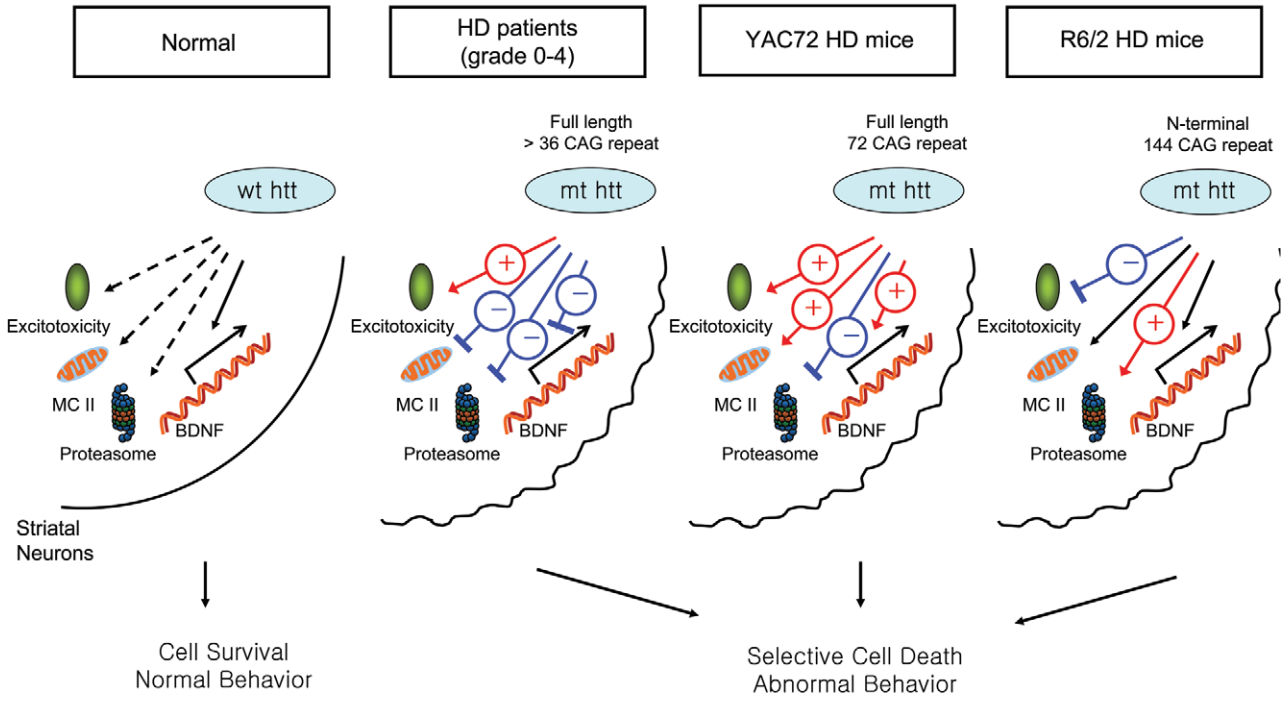

Figure 5. Schematic diagram of multiple risk factors involved in the progressive Huntington disease (HD) pathology in patients and model mice; proteasome, brain-derived neurotrophic factor, MCII/III.

induced apoptosis by caspase activation through mitochondrial cytochrome *c* release (44). Secondly, mitochondrial function is related to neurotrophic regulation such as BDNF function. For instance, protective factors against 3-NP, MCII inhibitor, significantly increase cortical BDNF levels, but not striatal BDNF levels in N171-82Q mice (26). These data suggest that mitochondrial dysfunction can also alter cortical regulation of BDNF levels. Thirdly, BDNF regulation is potentially associated with proteasome function. Sommerfeld *et al*. (45) determined that BDNF-induced reduction of TrkB receptors was blocked by proteasome inhibitor in cerebellar granule cells (45). These data indicate some of the potential interactions between BDNF, TrkB receptor, mitochondria and proteasome systems (45). These multiple risk factors might be affected by non-neuronal supporting cells, in the pathological progress of HD and other neurodegenerative diseases (46). Several studies have shown the potential involvement of glial cells and/or inflammatory cells in the neurodegenerative processes (47–51). However, there is no significant finding about differences of cell population in the striatum of these HD animal models.

From all of these studies, we conclude that there are combinations and summations of multiple risk factors leading to the progressive neuropathology by age in HD patients. The age of onset and severity of progressive HD pathology in patients is also correlated with genetic and cellular components such as higher CAG repeat in mutant huntingtin and possibly excitotoxicity (1,40). Consequently, overall proteasome activity, BDNF and mitochondrial function (MCII/III activity) would progressively decrease by age, increasing CAG repeat length and disease stage. As we show here, in different transgenic HD animal models, the progression may include cellular compensatory changes at both presymptomatic and symptomatic stages. Therefore, different HD animal models also represent different compensatory responses as well as specific key risk factors at a given disease stage. Notably, the animal models do not represent all aspects of HD-like cellular pathology. Conversely, the compensatory changes likely represent attempts by individual cells to overcome the effects of the pathogenic proteins, and can therefore provide ideas for therapeutic intervention and molecular targets.

MATERIALS AND METHODS

Behavioral assessment

The behavioral testing was carried out for HD model R6/2 mice at three different age groups (6, 9 and 13 weeks) in the rotor-rod test and open-field test as previously described (52,53). For rotor-rod test (San Diego Instrument, San Diego, CA, USA), mice were subsequently tested with three trials in 1 day each being 2 h apart. Fixed-speed rotarod testing was completed at a speed of 24 rpm to a maximum time of 60 s. The latency to fall was recorded for each mouse (52). Open-field test was performed using an automated open-field system (San Diego Instrument). The activity of mice in the open-field trials was assessed in the dark during the light cycle and was measured automatically as the number of photobeam breaks during each trial as previously described (52).

Sample preparation

The dissected brain tissue samples were homogenized in cell lysis buffer (4) and then sonicated, After centrifugation at

14 000*g* for 30 min at 4°C, supernatants were collected for the protein quantification. After protein levels were quantified using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), samples containing equal amounts of total protein were used for the proteasome assay, BDNF ELISA, MCII activity determination.

Determination of proteasomal function

Proteasome function was determined by continuously measuring the fluorescence of 7-amido-4-methylcoumarin (AMC) (excitation 380 nm, emission 460 nm) generated from peptide–AMC-linked substrates. Reactions were conducted with a final volume of 200 μl containing 50 mM Tris–HCl buffer (pH 7.5) and 1 mM ethylene diammine tetraacetic acid (EDTA). After adding samples to the reaction mixtures, reactions were initiated by adding the following substrates: Suc-Leu-Leu-Val-Try-AMC (65 μM) for chymotrypsin activity and Z-Leu-Leu-Glu-AMC (75 μM) for PGPH-like activity. Reactions were followed for 240 min at 25°C and enzymatic activities determined at linear rates and expressed as fluorescence units (FU/min/mg) of protein.

Immunohistochemistry

Brains of R6/2 transgenic mice were cryoprotected and cut into 40 μm sections using a freezing microtome. Immunoperoxidase staining was performed as previously described using primary antibodies raised against ubiquitin (Dako, Carpinteria, CA, USA; 1:2000) and incubated with the tissue sections for 72 h at 4°C (4).

Western blots

Samples containing equal amounts of total protein were analyzed in Western blot using a secondary horseradish peroxidase-linked anti-mouse IgG antibody (Jackson Laboratories, Bar Harbor, ME, USA; 1:6000) and the following primary antibodies: monoclonal anti-proteasome β-subunit (Calbiochem, La Jolla, CA, USA; 1:5000), monoclonal anti-ubiquitin (Dako; 1:2000), anti-PA28 α subunit (Calbiochem; 1:2500), anti-PA700 (Calbiochem; 1:2500). Quantification of the immunoreactive bands in western blot was performed using quantitative densitometry. The results were confirmed by duplicate measurements of the same sample.

BDNF ELISA

BDNF expression levels were determined using BDNF Emax TM Immunoassay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. From the samples BDNF levels were calculated from standard curve, which was created with serial dilutions of known BDNF concentrations. The results were confirmed by triplicate measurements of the same sample.

Detection of MCII/III enzyme activity

Mitochondria samples from brain tissues were prepared using established mitochondria fractionation methods (4,54). In brief, all the brain tissue samples were homogenized in 100 μl of H-buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes, 0.5% bovine serum albumin; pH 7.2). After centrifugation at 1400*g* for 5 min at 4°C, the supernatant were centrifuged at 7900*g* for 15 min. The pellets were resuspended in H-buffer and then sonicated for 10 s before enzyme assays. The enzyme assay for MCII activities were performed as previously described (9) in the following reaction conditions; 40 mM K_2HPO_4 (pH 7.4), 20 mM succinate, 50 μM cytochrome *c*, 0.5 mM EDTA and 1.5 mM KCN at room temperature. Enzyme activity was detected at 550 nm and normalized for protein concentration and reaction time (in nmol/min/mg) before statistical analysis.

Statistical analysis

All statistical analyses were carried out using JMP (version 3.1.6, SAS Institute, Cary, NC, USA). Data were objectively compared between different groups at different stages of disease using unpaired Student's *t*-test and two-way analysis of variance followed by Turkey–Kramer *post hoc* analysis. Differences between groups were considered statistically significant when $P < 0.05$. Regression analyses were performed with linear-fit for two independent variables with correction for multiple comparisons (statistical criterion was $P < 0.05$).

Conflict of Interest statement. None declared.

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