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Brain-derived neurotrophic factor over-expression in the forebrain ameliorates Huntington's disease phenotypes in mice

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Abstract

Huntington's disease (HD), a dominantly inherited neurodegenerative disorder characterized by relatively selective degeneration of striatal neurons, is caused by an expanded polyglutamine tract of the huntingtin (htt) protein. The htt mutation reduces levels of brain-derived neurotrophic factor (BDNF) in the striatum, likely by inhibiting cortical BDNF gene expression and anterograde transport of BDNF from cortex to striatum. However, roles of the BDNF reduction in HD pathogenesis have not been established conclusively. We reasoned that increasing striatal BDNF through over-expression would slow progression of the disease if BDNF reduction plays a pivotal role in HD pathogenesis. We employed a *Bdnf* transgene driven by the promoter for the alpha subunit of Ca^{2+}/calmodulin-dependent kinase II to over-express BDNF in the forebrain of R6/1 mice which express a fragment of mutant htt with a 116-glutamine tract. The *Bdnf* transgene increased BDNF levels and TrkB signaling activity in the striatum, ameliorated motor dysfunction, and reversed brain weight loss in R6/1 mice. Furthermore, it normalized DARPP-32 expression of the 32 kDa dopamine and cAMP-regulated phosphoprotein, increased the number of enkephalin-containing boutons, and reduced formation of neuronal intranuclear inclusions in the striatum of R6/1 mice. These results demonstrate crucial roles of reduced striatal BDNF in HD pathogenesis and suggest potential therapeutic values of BDNF to HD.

Keywords: brain atrophy, brain-derived neurotrophic factor, gene expression, Huntington's disease, motor coordination, neuronal intranuclear inclusions.

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Huntington's disease (HD), a dominantly inherited neurodegenerative disorder characterized by abnormalities of movement and cognition along with changes in psychiatric symptoms, is caused by expansion of a polyglutamine tract at the N-terminus of huntingtin (htt). The clinical signs and symptoms result from relatively selective degeneration of striatal neurons (Vonsattel and DiFiglia 1998). It remains unknown how the mutation in ubiquitously expressed htt leads to relatively selective striatal degeneration. Discovery of neuronal intranuclear inclusions containing mutant htt in HD patients and mouse models led to suggestions that these protein aggregates might cause neuronal death (Davies *et al.* 1997; DiFiglia *et al.* 1997). However, studies in mice and cultured neurons indicate that formation of nuclear inclusions does not correlate with neuronal death, suggesting that soluble mutant htt could be toxic to neurons (Saudou *et al.* 1998; Kim *et al.* 1999). As many proteins containing polyglutamine tracts function as transcription factors (Alba and Guigo 2004), one possible harmful effect of soluble mutant htt is disruption of transcription (Sugars and Rubinsztein 2003).

Recent studies suggest that loss of beneficial activities of normal htt might also contribute to HD pathogenesis. Htt is important for neuronal survival in the CNS (Dragatsis *et al.* 2000). Moreover, in cortical neurons normal htt has been shown to induce BDNF gene expression, while mutant htt has been shown to suppress BDNF gene expression (Zuccato *et al.* 2001). As striatal neurons express TrkB but very little BDNF and BDNF in the striatum arrives by anterograde transport from cell bodies primarily in the cerebral cortex and

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Abbreviations used: BDNF, brain-derived neurotrophic factor; BTg, *Bdnf* transgene; Ca^{2+}/CaMKIIα, calmodulin-dependent kinase II; DARPP-32, 32 kDa dopamine and cAMP-regulated phosphoprotein; HD, Huntington's disease; htt, huntingtin; TBS, Tris buffered saline; WT, wild type.

secondarily in the substantia nigra (Altar *et al.* 1997; Conner *et al.* 1997), inhibition of BDNF gene expression in the cerebral cortex by the htt mutation should reduce striatal BDNF. Loss of normal htt further reduces striatal BDNF by decreasing BDNF axonal transport (Gauthier *et al.* 2004). Consistent with these observations, levels of striatal BDNF are reduced in HD patients and mice (Ferrer *et al.* 2000; Spires *et al.* 2004). Furthermore, intrastriatal injection of BDNF-expressing adenovirus or grafting of BDNF-expressing cells protects striatal neurons from loss in excitotoxic rat models of HD (Bemelmans *et al.* 1999; Perez-Navarro *et al.* 2000), and progression of disease phenotypes in R6/1 HD mice is accelerated in *bdnf* +/− mice (Canals *et al.* 2004). However, as BDNF is only one of many proteins affected in HD (Cha *et al.* 1999; Luthi-Carter *et al.* 2000; Zuccato *et al.* 2001) and as deficiency in BDNF-mediated signaling alone is sufficient to cause dendritic abnormalities and neuronal loss in the cerebral cortex and striatum (Xu *et al.* 2000; Baquet *et al.* 2004), it remains unclear whether the reduction in striatal BDNF seen in HD is such a crucial part of disease pathogenesis that restoration of striatal BDNF would normalize many aspects of HD pathology. Here we report that BDNF over-expression in the forebrain significantly ameliorates many disease phenotypes in R6/1 mice.

Materials and methods

Animals

R6/1 mice were obtained from the Jackson Laboratory (Bar Harbour, ME, USA) via recovery of frozen embryos. They were on the genetic background of B6CBA. Generation of *Bdnf* transgenic (BTg) mice under the control of the Ca^{2+}/calmodulin-dependent kinase II (*CaMKIIα*) promoter was reported previously (Huang *et al.* 1999b). BTg mice were maintained on the genetic background of C57BL/6. R6/1 mice were crossed to BTg mice to produce wild type (WT), BTg, R6/1, and BTg;R6/1 mice. Each mouse was marked with an ear tag. We used PCRs to identify the two transgenes. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

Antibodies

Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (BDNF, 1 : 1000), Cell Signaling Technology (Danvers, MA, USA) (DARPP-32, 1 : 1000 for western blots and 1 : 500 for immunohistochemistry; Akt, 1 : 1000; phospho-Akt, 1 : 1000), Chemicon International (Temecula, CA, USA) (EM48, 1 : 500), and Sigma (St. Louis, CA, USA) (α-tubulin, 1 : 7500). Antibodies to TrkB (1 : 1000) and phospho-TrkB (1 : 1000) were kindly provided by Dr. Louis Reichardt (University of California, San Francisco, CA, USA) and Dr. Moses Chao (New York University, New York, NY, USA), respectively.

In situ hybridization

In situ hybridization was performed as described previously (Xu *et al.* 2003). In brief, mouse brains were dissected and frozen immediately in an isopentane-dry ice bath. BDNF *in situ* hybridization was performed on cryostat coronal sections at 10 μm using ^{35}S–labeled antisense cRNA probes complementary to the coding region of the mouse BDNF cDNA. After hybridization and washes, sections were exposed to Beta–Max Hyperfilm (Amersham, Piscataway, NJ, USA). Images from three sections separated by 100 μm were scanned at 600 dpi for each mouse, and the optical density of *in situ* signals in the striatum and the cortical area dorsal to the striatum was determined using Image J (NIH, Bethesda, MD, USA). After subtraction from the background, the mean optical density of a brain region was used for comparisons.

Western blotting

Dissected cortical and striatal tissues were homogenized in lysis buffer (80 mmol/L Tris–Cl, 2% sodium dodecyl sulfate, 10% glycerol, pH 6.8) and centrifuged at 14 300 *g* for 30 min. Protein concentrations of extracts (supernatants) were measured using the D_c protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Extracts (25 μg) in SDS loading buffer were denatured for 5 min at 100°C, separated on an sodium dodecyl sulfate-polyacrylamide gel, and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk in TBST (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.5% Tween-20) for 1 h and then incubated with primary antibodies overnight at 4°C. After two washes with TBST, the membrane was incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) for 1 h at 25°C. The membrane was then washed three times with TBST, and proteins were visualized using the chemiluminescence plus system (Amersham Biosciences). The membrane was then stripped and blotted with an antibody to α-tubulin. To quantify western blots, X-ray films were scanned into digital images and analyzed with the NIH image J software. Intensity of each band was normalized to the level of α-tubulin on the same lane.

Rotarod test

Motor coordination was tested using a Rotarod apparatus (UGO Basile, Italy). The observer was unaware of the genotype of each mouse during tests. Mice were trained for three consecutive days and each day had three trials, each with a 1-h interval. A mouse was placed on a rotating rod which accelerated from 4 to 40 rpm in 5 min. The time the mouse stayed on the rotating rod was recorded. If the mouse still stayed on the rotating rod after 5 min, a score of 300 s was recorded. The best score on the third day was used for comparisons among genotypes.

Immunohistochemistry

Mice were anaesthetized with avertin and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde sequentially. Their brains were removed from the skull, post-fixed in 4% paraformaldehyde overnight, and soaked in 30% sucrose. Coronal brain sections (50 μm) were obtained with a sliding microtome, rinsed once with Tris buffered saline (TBS; 10 mmol/L Tris–HCl, 150 mmol/L sodium chloride, pH 7.5), and incubated with 10% methanol-3% hydrogen peroxide in TBS to quench endogenous peroxidase. After incubating with a blocking buffer (0.4% Triton X-100, 2.5% bovine serum albumin, and 10% horse serum in TBS) for 1 h, the sections were incubated with a primary antibody diluted in

the blocking buffer overnight at 25°C. After three washes in the blocking buffer, the sections were incubated with an appropriate biotinylated secondary antibody followed by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) according to the instructions of the manufacturer. Sections were developed in 0.05% 3-3′-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 0.1 mol/L Tris–HCl (pH 7.5), mounted onto slides, dehydrated, and coverslipped with dibutyl phthalate-xylene mixture.

Cortical volume, striatal volume, and ventricle size

Perfused brains were cut coronally into 50 μm sections throughout the striatum. Every 12th coronal brain section was stained with cresyl violet. Stereo Investigator software and Neuroexplorer software (MicroBrightField Inc, Williston, VT, USA) were used to trace areas of interest and calculate volume, respectively. Cortical volume was estimated in the region with the largest percentage of striatal volume (bregma 1.18–0.22 mm), as described by Slow *et al.* (2003). Size of the lateral ventricle and dorsal 3rd ventricle was estimated on all sections with the striatum.

Huntingtin aggregates

Color digital images of htt aggregates were obtained with a 100× objective lens from the dorsal lateral striatum and converted to gray-scale images using the Image J software. The threshold of the gray-scale images were then adjusted until staining of soluble htt disappeared. The same parameters were used to take and adjust all images. We used the particle analysis function of Image J to measure the number and size of htt aggregates larger than 0.1 μm^2. Four to five images from each mouse were analyzed.

Statistical analysis

All data are expressed as mean ± SEM. Results on protein levels, rotarod tests, weights, volumes, aggregate number, and aggregate size were compared using an unpaired Student's *t*-test.

Results

BDNF over-expression in the forebrain increases striatal BDNF in R6/1 mice

We employed a BTg under the control of the promoter for the alpha subunit of CaMKIIα to over-express BDNF in the forebrain. In agreement with the previous observation (Huang *et al.* 1999b), *in situ* hybridization showed that the transgene led to a ~3-fold increase in levels of BDNF mRNA in the cerebral cortex (Fig. 1a and b). The BTg was also expressed in the striatum where the activity of the endogenous *Bdnf* gene is very low (Fig. 1a).

In order to determine whether the BTg was able to increase levels of striatal BDNF protein in HD mice, we crossed BTg mice to R6/1 mice to generate WT, BTg, R6/1, and BTg;R6/1 mice. R6/1 mice express exon 1 of the human htt with a tract of 116 glutamine in the brain as well as in the peripheral tissues (Mangiarini *et al.* 1996). We prepared protein extracts from striatal and cortical tissues dissected from the four genotypes of mice at 6 months of age and examined levels of mature BDNF with western blots. In the cerebral cortex, the BDNF level in R6/1 mice was similar to that in WT mice (Fig. 1c and d). The BTg increased the cortical BDNF level by more than 76% in both BTg mice and BTg;R6/1 mice (Fig. 1c and d). In contrast, the level of mature BDNF in the striatum was dramatically reduced in R6/1 mice, compared to WT mice (Fig. 1e). Transgenic BDNF over-expression greatly increased levels of striatal BDNF in BTg and BTg;R6/1 mice (Fig. 1e and f). Levels of striatal BDNF protein in R6/1 mice were only 28% of that in WT mice ($p < 0.01$), while those in BTg mice and BTg;R6/1 mice were increased by 80% ($p < 0.05$) and 57% ($p < 0.05$), respectively, in comparison with WT mice (Fig. 1f). Importantly, the striatal BDNF level in BTg;R6/1 mice was nearly 5 fold higher than that in R6/1 mice (Fig. 1f, $p < 0.001$). There was not a significant difference in striatal BDNF levels between BTg mice and BTg;R6/1 mice (Fig. 1f, $p = 0.44$), suggesting that the htt N-terminal fragment with expanded polyglutamine does not significantly interfere with expression of the BTg and that over-expressed BDNF in the cerebral cortex is still transported to the striatum in R6/1 mice. These results demonstrate that the BTg can be used to increase striatal BDNF supply in HD mice.

Activation of the TrkB receptor in R6/1 and BTg mice

To investigate whether TrkB activation is altered in R6/1 mice and whether BDNF over-expression in the forebrain enhances TrkB activation in the striatum, we first examined striatal levels of the TrkB receptor in the four genotypes of mice at 6 months of age. There are two isoforms of TrkB receptors: the full-length TrkB receptor tyrosine kinase and the truncated TrkB receptor that lacks the tyrosine kinase domain (Klein *et al.* 1990). Western blot analyses with an antibody against the TrkB extracellular domain revealed that levels of either isoform of TrkB receptors were not altered in R6/1 mice or by BDNF over-expression (Fig. 2a and b). We then examined TrkB activation with an antibody specific to the activated TrkB receptor in which the tyrosine residue at the docking site for phospholipase C-gamma1 is phosphorylated. Levels of the activated TrkB receptor were reduced by 49% in R6/1 mice and normalized in BTg;R6/1 mice (Fig. 2c and d). This is consistent with the observed alterations in striatal BDNF levels in these two genotypes of mice (Fig. 1e and f). To further confirm that TrkB signaling is diminished in R6/1 mice and enhanced in BTg;R6/1 mice, we investigated activation of Akt, one of the downstream targets of activated TrkB receptors in the striatum. Although the amount of total Akt protein in striatal extracts was only slightly lower in R6/1 mice than in WT mice (Fig. 2c and e), levels of phospho-Akt (Ser473) were dramatically reduced in R6/1 mice, compared to WT mice (Fig. 2c and f). Furthermore, striatal levels of phospho-Akt (Ser473) in BTg mice and BTg;R6/1 mice were nearly twice as high as those in WT mice (Fig. 2c and f), which correlates well with striatal levels of BDNF in these mice (Fig. 1f). These

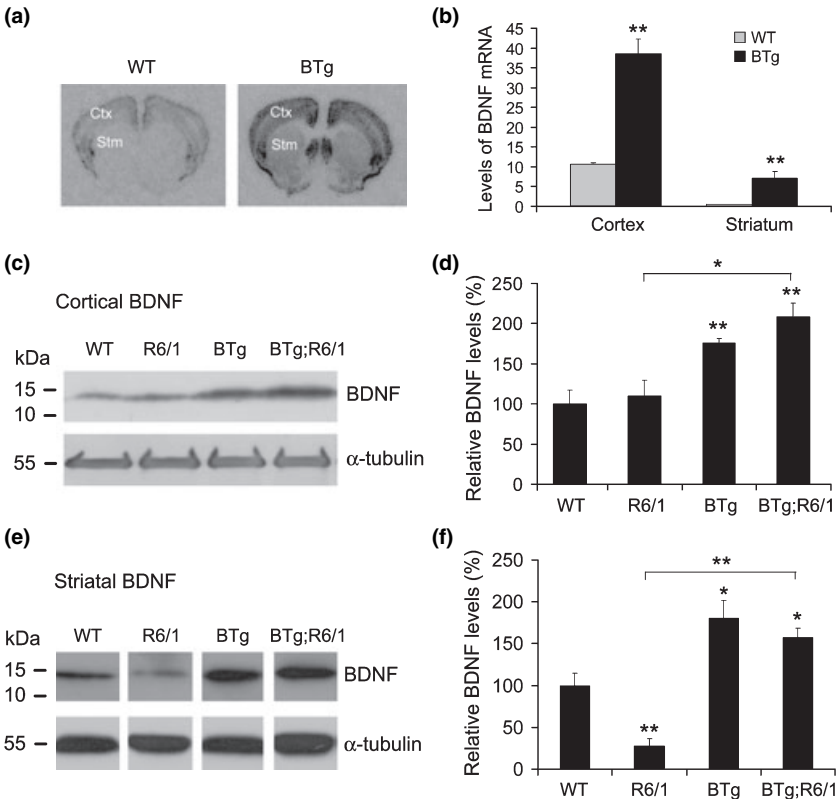

Fig. 1 Cortical and striatal levels of brain-derived neurotrophic factor (BDNF) protein in R6/1 and *Bdnf* transgenic (BTg) mice. (a) Representative brain sections of BDNF *in situ* hybridization show that levels of BDNF mRNA are increased in the cerebral cortex (Ctx) and striatum (Stm) of BTg mice. The *in situ* probe was ^{35}S-labeled antisense RNA complementary to the coding region of BDNF transcripts. (b) The optical density of BDNF *in situ* signals was measured with Image J software on three sections for each mouse. Four adult mice were used for each genotype. (c) Representative western blots show that levels of mature BDNF protein in the cerebral cortex. (d) Relative levels of cortical BDNF were determined from four WT mice, four R6/1 mice, four BTg mice, and three BTg;R6/1 mice. (e) Representative western blots show that levels of mature BDNF protein in the striatum are reduced in R6/1 mice and elevated in BTg mice and BTg;R6/1 double transgenic mice. (f) Relative levels of striatal BDNF were determined from four WT mice, five R6/1 mice, four BTg mice, and three BTg;R6/1 mice. Student's *t* test: $*p < 0.05$; $**p < 0.01$.

results indicate that R6/1 mice have diminished TrkB signaling in the striatum because of a reduced striatal BDNF level, which can be reversed by BDNF over-expression in the forebrain.

BDNF over-expression ameliorates disease phenotypes in R6/1 mice

R6/1 mice display several HD-related phenotypes, including poor motor coordination, brain atrophy, and loss of body weight (Mangiarini *et al.* 1996; Spires *et al.* 2004). We performed rotarod tests to monitor motor coordination when mice were at 2, 3, 4, 5, and 6 months of age. WT mice and BTg mice performed similarly well on the test and stayed on the rotating rod for the maximum amount of allowed time in most trials (Fig. 3a). R6/1 mice stayed on an accelerating rotating rod for a significantly shorter time, compared to WT mice (Fig. 3a, $p < 0.01$ at all time points). Although double transgenic mice (BTg;R6/1) did not perform as well as WT mice (Fig. 3a, $p = 0.017$ at 3 months of age and $p < 0.01$ at other time points), they performed significantly better on the test than R6/1 mice at 4 months of age and afterward (Fig. 3a, $p = 0.019$ at 4 months of age and $p < 0.01$ at 5 and 6 months of age). These results indicate that over-expression of BDNF in the forebrain slows progression of motor dysfunction in R6/1 mice.

Male and female R6/1 mice displayed differential progression in motor dysfunction. Both male and female R6/1 mice stayed on an accelerating rotating rod for a significantly shorter time than sex-matched WT mice (Fig. 3b, $p < 0.01$ at 3 months of age and later time points). The motor dysfunction progressed faster in male R6/1 mice than in female R6/1 mice, so that male R6/1 mice performed significantly worse on rotarod tests than female R6/1 mice at 5 months of age (Fig. 3b, $p = 0.011$). Despite of the accelerated disease progression, BDNF over-expression still dramatically improved motor coordination in male R6/1 mice (Fig. 3c, $p < 0.01$ between male R6/1 mice and male BTg;R6/1 mice at ages of 4, 5, and 6 months).

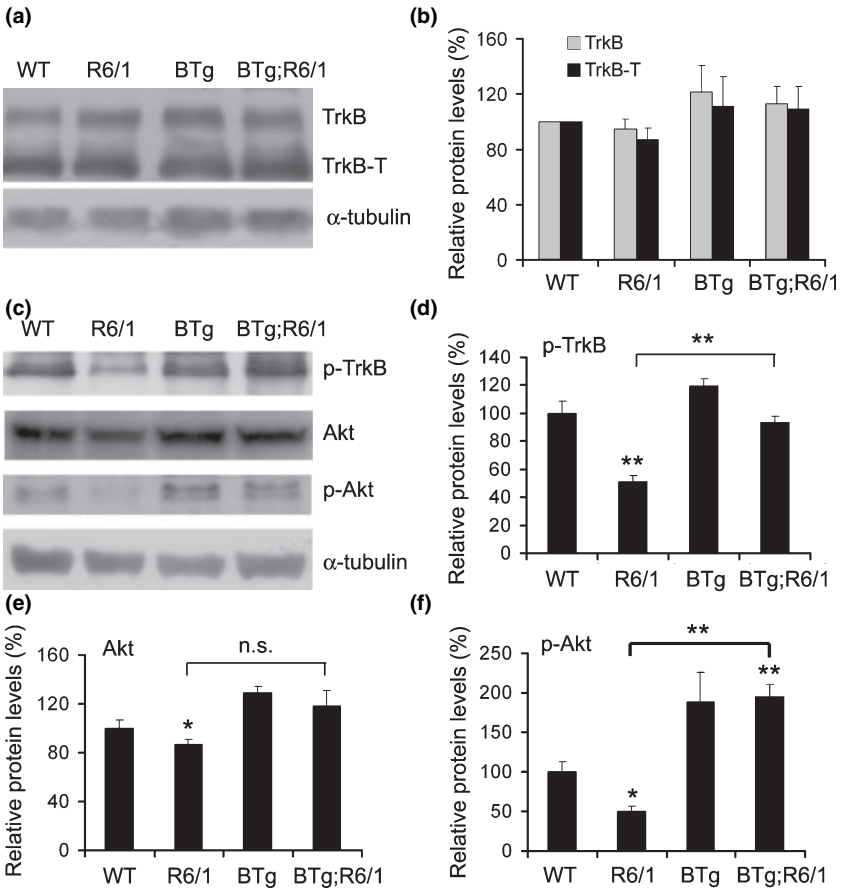

Fig. 2 Expression and activation of the TrkB receptor in R6/1 and *Bdnf* transgenic (BTg) mice. (a) Representative western blots show expression of the full-length TrkB receptor (TrkB) and the truncated TrkB receptor (TrkB-T) in the striatum. (b) Levels of TrkB and TrkB-T are similar in striata of WT ($n = 4$), R6/1 ($n = 4$), BTg ($n = 4$), and BTg;R6/1 ($n = 3$) mice. The graph represents the average of three independent experiments. (c) Representative western blots show levels of activated TrkB (*p*-TrkB), Akt, and activated Akt (*p*-Akt) in the striatum. (d–f) Striatal levels of *p*-TrkB, Akt, and *p*-Akt in WT ($n = 4$), R6/1 ($n = 3$), BTg ($n = 4$), and BTg;R6/1 ($n = 3$) mice. Student's *t* test: $^{*}p < 0.05$; $^{**}p < 0.01$.

Body weights were measured for some mice at 6 months of age. BTg mice had a similar body weight to WT mice for both genders (Fig. 3e and f). Although both male and female R6/1 mice had significantly lower body weights than their sex-matched WT littermates, the reduction of body weight was more severe in male R6/1 mice (Fig. 3e and f, 40% in male vs. 29% in female), which is consistent with the gender difference in motor dysfunction in R6/1 mice (Fig. 3b). BDNF over-expression had little effect on the body weight of male R6/1 mice ($p = 0.478$), but significantly increased the body weight of female R6/1 mice ($p = 0.027$).

We perfused the four genotypes of mice at 6 months of age, and dissected and weighed their brains. Brain weight of R6/1 mice was 18% lighter than that of WT mice (Fig. 3d, $p < 0.001$). Brain weight of BTg;R6/1 mice was significantly higher than that of R6/1 mice and similar to that of WT and BTg mice (Fig. 3d). Thus, BDNF over-expression in the forebrain completely reverses the loss of brain weight in R6/1 mice.

To determine if the change in brain weight reflects an alteration in brain size, we sectioned fixed brains, performed Nissl staining, and measured volumes of the cerebral cortex, striatum, and ventricles. An examination of comparable coronal sections suggests that R6/1 mice have smaller brains (Fig. 4a). Using the Nissl-stained coronal sections, we employed Stereo Investigator software to measure the striatal volume, cortical volume, and size of the lateral and dorsal third ventricles. In agreement with the previous observation (Canals *et al.* 2004), cortical and striatal volumes were significantly smaller by 12% and 18% in R6/1 mice than in WT mice, respectively (Fig. 4b and c). Importantly, BDNF over-expression in the forebrain normalized both cortical volume and striatal volume in R6/1 mice (Fig. 4b and c). BDNF over-expression also significantly increased the striatal volume in BTg mice by 13% (Fig. 4c), which is consistent with the observation that BTg mice have significantly smaller ventricles (Fig. 4d). There was not a significant difference in ventricle size among WT, R6/1, and BTg;R6/1 mice (Fig. 4d). Taken together, these results demonstrate that BDNF over-expression under the control of the *CaMKIIα* promoter at least partially reverses many aspects of disease phenotypes in R6/1 mice.

Effect of BDNF over-expression on levels of DARPP-32 and enkephalin in the R6/1 striatum

DARPP-32 is a 32 kDa dopamine- and cAMP-regulated phosphoprotein important for dopamine neurotransmission (Svenningsson *et al.* 2004), while enkephalin serves as a peptide neurotransmitter for striatal neurons at the origin of the indirect pathway (Kawaguchi 1997). Expression of

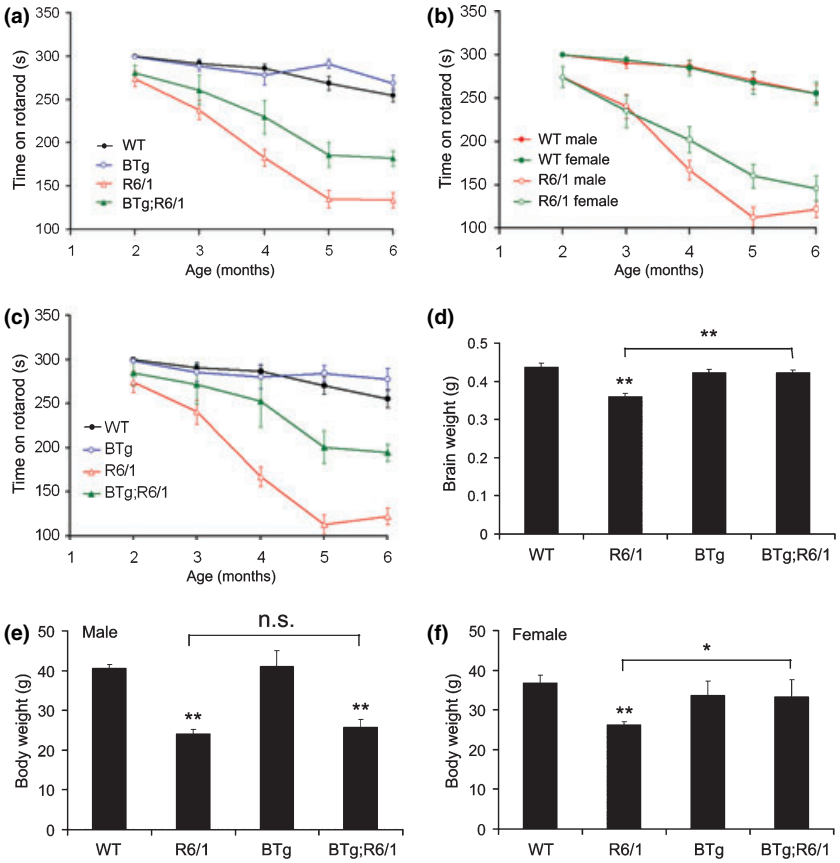

Fig. 3 Brain-derived neurotrophic factor over-expression improves motor coordination and reverses loss of brain weight in R6/1 mice. (a) Performance on rotarod tests. Not every mouse was tested at each time point. The mouse number at each time point was as follows: 30 (2 months), 31 (3 months), 30 (4 months), 30 (5 months), and 24 (6 months) for WT mice; 31, 32, 32, 34, and 24 for R6/1 mice; 13, 16, 15, 21, and 16 for *Bdnf* transgenic (BTg) mice; 12, 12, 12, 13, and 11 for BTg;R6/1 mice. (b) Differential rotarod performance in male and female R6/1 mice. The mouse number at each time point was as follows: 18 (2 months), 18 (3 months), 18 (4 months), 18 (5 months), and 12 (6 months) for male WT mice; 12, 13, 12, 12, and 12 for female WT mice; 18, 18, 17, 18, and 12 for male R6/1 mice; 13, 14, 15, 16, and 11 for female R6/1 mice. (c) Performance of male mice on rotarod tests. The mouse number at each time point was as follows: 18 (2 months), 18 (3 months), 18 (4 months), 18 (5 months), and 12 (6 months) for male WT mice; 18, 18, 17, 18, and 12 for male R6/1 mice; 8, 10, 9, 11, and 7 for male BTg mice; 4, 6, 6, 8, and 7 for male BTg;R6/1 mice. (d) Brain weights of WT (six mice), R6/1 (eight mice), BTg (four mice) and BTg;R6/1 (four mice) at 6 months of age. (e) Body weight of male WT (10 mice), R6/1 (nine mice), BTg (six mice) and BTg;R6/1 (six mice) at 6 months of age. (f) Body weight of female WT (ten mice), R6/1 (nine mice), BTg (five mice) and BTg;R6/1 (four mice) at 6 months of age. Student's *t* test: $^{*}p < 0.05$; $^{**}p < 0.01$.

DARPP-32 and enkephalin in the striatum is severely impaired in HD mice (Luthi-Carter *et al.* 2000; Spires *et al.* 2004) and may be regulated by BDNF (Ivkovic and Ehrlich 1999; Canals *et al.* 2004). To determine if BDNF over-expression slows disease progression of R6/1 mice in part by normalizing expression of DARPP-32 and enkephalin, we examined striatal levels of these two polypeptides in the four genotypes of mice at 6 months of age using western blots and immunohistochemistry.

Immunohistochemistry showed that the striatal level of DARPP-32 was diminished in R6/1 mice, as reported (Spires *et al.* 2004), and that BDNF over-expression reversed the reduction (Fig. 5a–d). To quantify the change we performed western blots with striatal protein extracts prepared from mice at 6 months of age. As shown in Fig. 5e and f, the level of DARPP-32 in R6/1 mice was only 21% of that in WT mice. Although the BTg did not significantly alter the level of DARPP-32 on the WT genetic background, it increased the striatal level of DARPP-32 in BTg;R6/1 mice by ~5 fold (Fig. 5e and f). This result suggests that down-regulation of DARPP-32 in the striatum of R6/1 mice likely results from BDNF deficiencies.

As expected, enkephalin immunoreactivity in the striatum was reduced in R6/1 mice as compared to WT mice (Fig. 6a and b). Introduction of the BTg to either WT or R6/1 mice did not appear to significantly increase striatal enkephalin immunoreactivity (Fig. 6c and d). However, BDNF over-expression in the forebrain did increase the number of

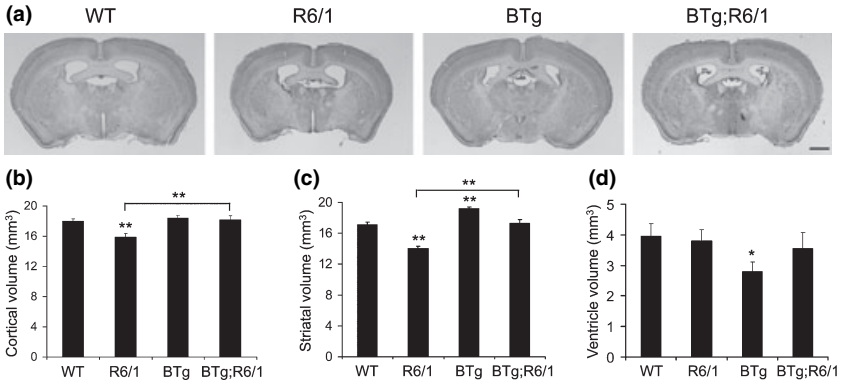

Fig. 4 Brain-derived neurotrophic factor over-expression in the forebrain increases volumes of the cerebral cortex and striatum in R6/1 mice. (a) Representative coronal brain sections of four genotypes of mice. Note that R6/1 mice have a smaller brain. Scale bar, 1 mm. (b) Cortical volumes of WT ($n = 7$), R6/1 ($n = 6$), BTg ($n = 7$), and BTg;R6/1 ($n = 5$) mice at 6 months of age. (c) Striatal volumes of WT ($n = 8$), R6/1 ($n = 6$), BTg ($n = 5$), and BTg;R6/1 ($n = 5$) mice at 6 months of age. (d) Ventricle volumes of WT ($n = 9$), R6/1 ($n = 10$), BTg ($n = 7$), and BTg;R6/1 ($n = 5$) mice at 6 months of age. The volume of the lateral ventricle and dorsal 3rd ventricle was calculated on coronal sections containing the striatum. Student's *t* test: $^{*}p < 0.05$; $^{**}p < 0.01$.

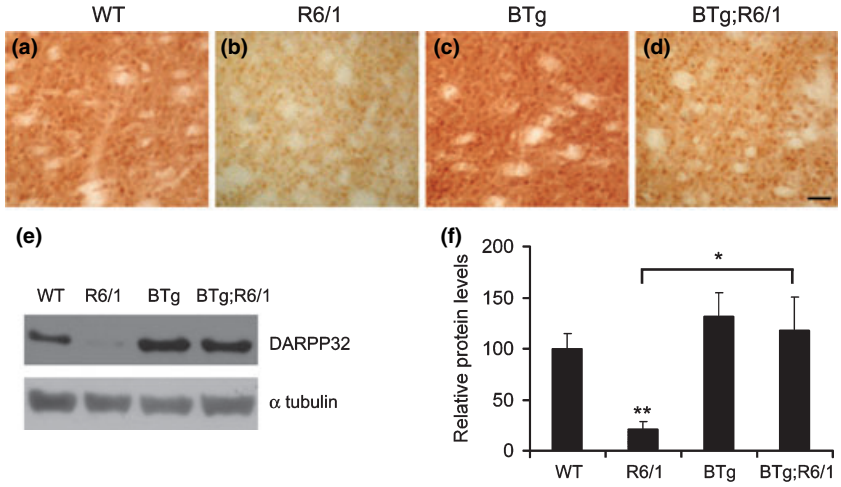

Fig. 5 Brain-derived neurotrophic factor over-expression normalizes levels of DARPP-32 in the striatum of R6/1 mice. (a–d) Immunohistochemistry shows striatal expression of DARPP-32 in four genotypes of mice. Scale bar, 50 μm. (e) Representative blots show levels of striatal DARPP-32 in four genotypes of mice. (f) Levels of DARPP-32 protein were determined in the striatal extracts from WT ($n = 4$), R6/1 ($n = 3$), BTg ($n = 4$), and BTg;R6/1 ($n = 3$) mice. Student's *t* test: $^{*}p < 0.05$ and $^{**}p < 0.01$.

enkephalinergic boutons in the R6/1 striatum. These boutons are the pre-synaptic terminals of collaterals from enkephalinergic medium-sized spiny neurons to innervate other striatal neurons (Kawaguchi 1997). Very few enkephalin-immunoreactive boutons were observed in the striatum of R6/1 mice and, if present, they were small, compared to those in WT mice (Fig. 6e and f). BDNF over-expression increased the number of enkephalin-immunoreactive boutons in the striatum of BTg;R6/1 mice (Fig. 6f and h), although it had a minimal effect on these boutons in the striatum of BTg mice (Fig. 6e and g). However, BDNF over-expression does not completely reverses the deficit in enkephalin-immunoreactive boutons as these boutons were still fewer and smaller in BTg;R6/1 mice than in WT mice (Fig. 6e and h). This result indicates that an elevated BDNF level in the striatum partially rescues alterations in enkephalin synthesis and/or formation of enkephalinergic synapses on striatal neurons in R6/1 mice.

Reduced formation of htt aggregates in double transgenic mice

To examine the effect of BDNF over-expression on formation of htt aggregates, we analyzed brain samples from R6/1 and BTg;R6/1 mice at 6 months of age with EM48 antibodies against polyglutamine tracts (DiFiglia *et al.* 1997). Striata of R6/1 mice showed extensive EM48-positive aggregates (Fig. 7a). Fewer and smaller htt aggregates were observed in striata of BTg;R6/1 mice (Fig. 7a). To quantify the difference between htt aggregates in the two genotypes of mice, we measured the density and size of the aggregates in the striatum. The average size of htt aggregates and the number of the aggregates in a unit of striatal area were significantly reduced by 35% and 31% respectively in BTg;R6/1 mice compared to R6/1 mice (Fig. 7b and c). A further analysis based on the cross area of an aggregate showed that the reductions resulted from the presence of fewer large htt aggregates in BTg;R6/1 mice (Fig. 7d). Thus,

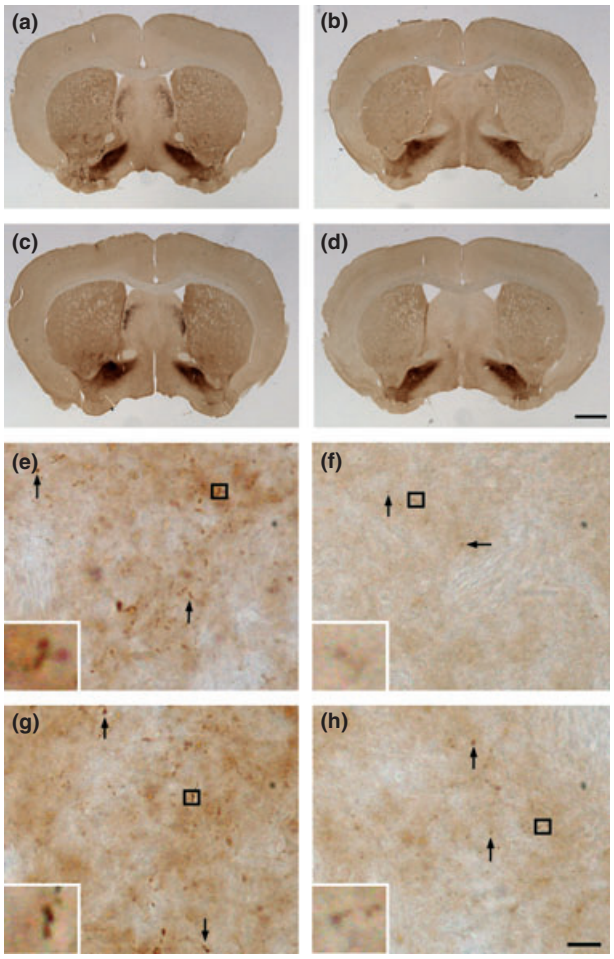

Fig. 6 Effect of brain-derived neurotrophic factor over-expression on enkephalergic medium-sized spiny neurons. (a–d) Immunohistochemistry shows that striatal levels of enkephalin in R6/1 mice (b) are reduced in comparison with those in WT mice (a). Brain-derived neurotrophic factor over-expression does not appear to alter significantly striatal levels of enkephalin on either the wild-type (c) or the R6/1 (d) genetic background. Scale bar, 1 mm. (e–h) High-magnification images show that the number and size of striatal enkephalin-containing boutons (arrows) in four genotypes of mice. Inserts are enlarged images of the boxed areas to show representative boutons. Scale bar, 10 μm.

an elevation in striatal BDNF reduces formation of neuronal intranuclear htt aggregates in R6/1 mice.

Discussion

It has been shown that intrastriatal injection BDNF-expressing adenovirus or grafting of BDNF-expressing cells protects striatal neurons from loss in excitotoxic rat models of HD (Bemelmans *et al.* 1999; Perez-Navarro *et al.* 2000). However these types of studies have several limitations in evaluating the role of BDNF deficiency in HD pathogenesis and the value of BDNF or its mimetics as HD therapeutic agents. First, the excitotoxic models do not allow for examination of motor dysfunction or alterations in gene expression related to HD. Second, it is unclear if the excitotoxic rat model is physiologically relevant to HD as R6/1 and R6/2 mice were observed to be resistant to intrastriatal injections of the NMDA-receptor agonist quinolinic acid (Hansson *et al.* 1999). Third, it is difficult to perform similar studies in transgenic HD mice, as these mice show no or only a small loss of striatal neurons (Rubinsztein 2002). Finally, it is not feasible to use viral injection or cell grafting to examine the effect of BDNF on a whole brain region. Here we examined roles of BDNF deficiency in HD pathogenesis through transgenic over-expression of BDNF in the R6/1 forebrain. In this system, the BTg likely increases levels of striatal BDNF by over-expressing BDNF in the striatum and cerebral cortex. Our results provide three lines of evidence to support a crucial role of BDNF deficiency in HD pathogenesis. First, striatal BDNF levels were sharply reduced in R6/1 mice and increased by BDNF over-expression in the forebrain. Consistent with this observation, our results show that TrkB signaling in the striatum is decreased in R6/1 mice and enhanced in BTg;R6/1 mice. Second, BDNF over-expression ameliorated motor dysfunction and normalized brain weight in R6/1 mice. Third, BDNF over-expression normalized expression of DARPP-32. These observations suggest that BDNF or its mimetics should have therapeutic values to HD.

Previous studies obtained conflicting results with regard to levels of striatal BDNF in R6/1 mice. Spires *et al.* observed significantly reduced levels of mature BDNF protein in the R6/1 striatum but not in the cerebral cortex by using western blots (Spires *et al.* 2004). However, two other studies reported no changes in levels of striatal BDNF in R6/1 mice by using ELISA assays (Canals *et al.* 2004; Pang *et al.* 2006). The discrepancy likely results from the different detection methods employed in these studies. If BDNF antibodies used in ELISA assays also bind to other proteins, the non-specific binding will mask a reduction in BDNF which is expressed at low levels. Furthermore, ELISA assays do not distinguish mature BDNF from pro-BDNF. In light of these considerations, western blots offer some advantages over ELISA assays. We were able to identify a specific band for mature BDNF on the basis of its molecular weight. In agreement with the observation made by Spires *et al.*, we found that levels of mature BDNF protein were dramatically reduced in the striatum but not in the cerebral cortex in R6/1 mice at 6 months of age. This observation supports the notion that both production of BDNF in the cortex and its anterograde transport from cortex to striatum are impaired in R6/1 mice.

The activity of the BDNF-to-TrkB pathway can be further diminished if levels of the TrkB receptor are also reduced in the R6/1 striatum. In fact, one recent study reported that

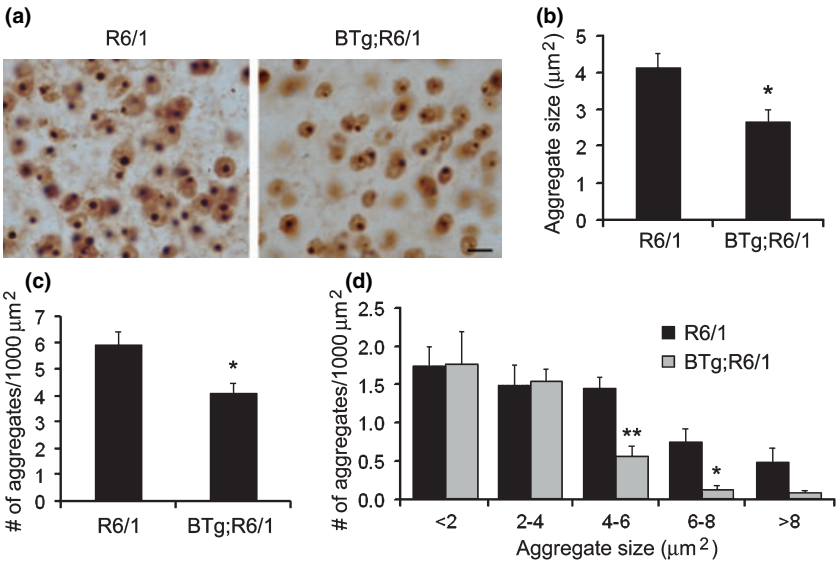

Fig. 7 Formation of neuronal intranuclear inclusion is reduced in BTg;R6/1 mice. (a) Immunohistochemistry revealed huntingtin (htt) aggregates in striata of R6/1 and BTg;R6/1 mice. Scale bar, 10 μm. (b) The average size of htt aggregates was smaller in BTg;R6/1 mice than in R6/1 mice. (c) The number of htt aggregates on one area unit was reduced in BTg;R6/1 mice in comparison with R6/1 mice. (d) BTg;R6/1 mice had fewer large htt aggregates than R6/1 mice. Five R6/1 mice and four BTg;R6/1 mice at 6 months of age were used for the analysis of neuronal intranuclear inclusions. Student's *t* test: $^{*}p < 0.05$ and $^{**}p < 0.01$.

levels of striatal TrkB are reduced in HD mice (Gines *et al.* 2006). We revisited this issue by using highly specific antibodies raised against the TrkB extracellular domain (Huang *et al.* 1999a; Paredes *et al.* 2004). We did not observe a change in levels of either the full-length TrkB receptor or the truncated receptor in the striatum of R6/1 mice at 6 months of age. Consistent with the results on levels of striatal BDNF and TrkB, we found that levels of activated TrkB and Akt were reduced by ~50% in the R6/1 striatum. Therefore, our results suggest that reduced TrkB signaling in the R6/1 striatum results from decreased BDNF availability.

It has been reported that the htt mutation reduces axonal transport of BDNF in cultured neurons (Gauthier *et al.* 2004). However, it is unlikely that the htt mutation will completely block BDNF axonal transport because a significant amount of striatal BDNF is still present in HD mouse models and in human HD patients (Gauthier *et al.* 2004; Spires *et al.* 2004). Therefore, over-expression of BDNF in the cerebral cortex should be able to increase striatal levels of BDNF in HD mice. Our data on levels of BDNF in the striatum and cortex support this argument. First, levels of cortical and striatal BDNF were not significantly different between BTg mice and BTg;R6/1 mice. Second, levels of striatal BDNF in BTg;R6/1 mice were significantly higher than those in WT mice, although levels of BDNF mRNA produced from the BTg in the striatum were not as high as those in WT cortex. Therefore, both locally expressed BDNF and anterogradely transported BDNF contribute to elevated levels of striatal BDNF in BTg;R6/1 mice. However, increased cortical BDNF levels and decreased striatal BDNF levels in BTg;R6/1 mice in comparison with BTg mice, despite statistically insignificant, do suggest that mutant htt inhibits BDNF anterograde transport.

How might BDNF over-expression in the forebrain normalize brain weight in R6/1 mice? The cerebral cortex, hippocampus, and striatum are atrophic in HD patients as well as in R6/1 mice (Vonsattel *et al.* 1985; Mangiarini *et al.* 1996). The BTg should increase BDNF levels in these brain regions (Huang *et al.* 1999b) and thus might protect these regions from atrophy in R6/1 mice. Indeed, introduction of the BTg into R6/1 mice normalizes cortical and striatal volumes. BDNF has been shown to be essential for dendritic growth and/or maintenance and long-term survival of neurons in the cortex and striatum (Xu *et al.* 2000; Baquet *et al.* 2004). As loss of striatal medium-sized spiny neurons in R6/1 mice is not significant (Mangiarini *et al.* 1996), BDNF over-expression likely restores size and weight of the brain by overcoming detrimental effects of the mutant htt N-terminal fragment on dendritic growth and maintenance.

Brain-derived neurotrophic factor over-expression in the forebrain significantly, but not fully, reverses motor dysfunction in R6/1 mice, even though it normalizes brain weight, striatal volume, and expression of some genes. This could be because of the fact that R6/1 mice develop early onset and severe motor dysfunction in association with pathology in peripheral tissues such as skeletal muscle (Mangiarini *et al.* 1996). It is unlikely that BDNF over-expression in the forebrain of R6/1 mice would ameliorate dysfunction of their skeletal muscle and therefore be able to completely restore rotarod performance.

Brain-derived neurotrophic factor over-expression significantly increases body weight in female R6/1 mice but not in male R6/1 mice. One possibility for this differential effect is that BDNF over-expression disturbs the balance of TrkB signaling in different subsets of hypothalamic neurons, which leads to more weight gain in females because female mice develop more severe obesity when TrkB signaling is

deficient (Xu *et al.* 2003). However, this does not appear likely as BTg mice had similar body weights to WT mice. A more plausible possibility is that loss of body weight in male R6/1 mice is too severe to be overcome by BDNF over-expression in the brain.

The number and size of neuronal intranuclear inclusions are reduced in BTg;R6/1 mice, compared to R6/1 mice. This is the first demonstration that signaling cascades activated by receptor tyrosine kinases regulate aggregation of mutant htt. This is a somewhat surprising finding considering the previous observation that deletion of one copy of the *Bdnf* gene does not affect htt inclusions in R6/1 mice (Canals *et al.* 2004). One explanation for the apparent discrepancy is that TrkB signaling has already been so reduced in R6/1 mice that a further reduction in levels of striatal BDNF will not increase formation of htt aggregates anymore. A reduction in formation of htt aggregates could result from either decreased levels of the mutant htt or reduced propensity of soluble mutant htt to form aggregates. The latter mechanism should increase levels of soluble mutant htt in neurons. However, EM48 immunoreactivity of soluble mutant htt appeared to be lower in BTg;R6/1 mice than in R6/1 mice (Fig. 7a). Therefore, it is likely that increased TrkB signaling somehow reduces levels of the mutant htt in neurons. It is an intriguing open question whether TrkB signaling decreases cellular content of the mutant htt by stimulating protein degradation pathways, by keeping striatal neurons healthier so that they are more efficient in clearing misfolded proteins, or by other mechanisms. As the BTg completely restored brain weight in BTg;R6/1 mice while many intranuclear inclusions were still formed in these mice, BDNF over-expression should provide direct beneficial effects on gene expression and dendritic morphology independent of its potential role in degradation of mutant htt.

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