

Lack of Huntingtin-Associated Protein-1 Causes Neuronal Death Resembling Hypothalamic Degeneration in Huntington's Disease

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Huntington's disease (HD) is caused by a polyglutamine expansion in the disease protein huntingtin. The polyglutamine expansion causes huntingtin to interact abnormally with a number of proteins. However, it is unclear whether, and how, huntingtin-associated proteins are involved in the neurodegeneration in HD. Here, we show that huntingtin-associated protein-1 (HAP1), which is involved in intracellular trafficking of epidermal growth factor receptor (EGFR), is highly expressed in the hypothalamus. Mice lacking HAP1 die after birth because of depressed feeding activity. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining and electron microscopic examination revealed the degeneration in hypothalamic regions that control feeding behavior. Hypothalamic degeneration was also observed in HD transgenic mice that have a significant loss of body weight. Inhibition of HAP1 expression decreases EGFR signaling and cell viability, whereas overexpression of HAP1 enhances this signaling activity and inhibits mutant huntingtin-mediated cytotoxicity. These results suggest that the effect of mutant huntingtin on HAP1 and EGFR signaling may contribute to the hypothalamic neurodegeneration and loss of body weight in HD.

Key words: hypothalamus; degeneration; EGFR; Huntington; polyglutamine; huntingtin

Introduction

Huntington's disease (HD) is characterized by progressive neurodegeneration that primarily occurs in the striatum and, as the disease progresses, extends to other brain regions including the hypothalamus (Vonsattel et al., 1985; Kremer et al., 1990, 1991). The marked degeneration (up to 90%) of the lateral hypothalamic neurons is found in HD patients in late stages of the disease (Kremer et al., 1990, 1991). These patients often show a loss of body weight (Sanberg et al., 1981; Pratley et al., 2000). HD is caused by a polyglutamine (polyQ) expansion in N-terminal huntingtin (Htt). Expression of N-terminal mutant Htt in mice results in neurological symptoms and body weight loss, followed by early death. However, obvious neurodegeneration was not found at the light microscopic level (Davies et al., 1997; Schilling et al., 1999).

Like other polyQ proteins, mutant Htt forms inclusions or aggregates (Zoghbi and Orr, 2000). Whereas the role of polyQ aggregates remains elusive, it is widely accepted that N-terminal mutant Htt has a gain of function by abnormally interacting with other proteins. A number of proteins have been found to bind N-terminal Htt, and their binding is altered by polyQ expansion (Cattaneo et al., 2001). However, little is known about the patho-

logical relevance of the interactions between Htt and its interacting proteins.

Among the identified Htt-interacting proteins, Htt-associated protein-1 (HAP1) is an interesting candidate for HD neuropathology. First, it is enriched in the brain (Li et al., 1995, 1996; Gutekunst et al., 1998; Page et al., 1998; Dragatsis et al., 2000), and its binding to Htt is enhanced by polyQ expansion (Li et al., 1995, 1998a). Second, HAP1 seems to be involved in neuronal vesicular transport through its interactions with microtubule-based transporters and vesicles (Block-Galarza et al., 1997; Engelder et al., 1997; Li et al., 1998b). Recently, HAP1 was found to be involved in the endosomal trafficking of epidermal growth factor receptor (EGFR) (Li, 2002b). EGFR is important for neuronal survival (Kornblum et al., 1998; Sibilio et al., 1998). Consistent with these important roles, mice lacking HAP1 die after birth (Chan et al., 2002), although the pathological basis of their death is not clear.

Here, we report that mice lacking HAP1 develop neuronal degeneration in the hypothalamus. We also observed degeneration in the hypothalamus in HD transgenic mice. Mutant Htt binds HAP1 and decreases its expression, leading to decreased EGFR signaling and increased cytotoxicity. These studies indicate an important role for HAP1 in hypothalamic function and suggest that the dysfunction of HAP1 and defective EGFR signaling contribute to neuronal degeneration in HD.

Materials and Methods

Antibodies and reagents used. Rabbit anti-HAP1 (Li et al., 1998a), rabbit anti-Htt (EM48) (Li et al., 1998b), and mouse anti-Htt (mEM48) (Li et

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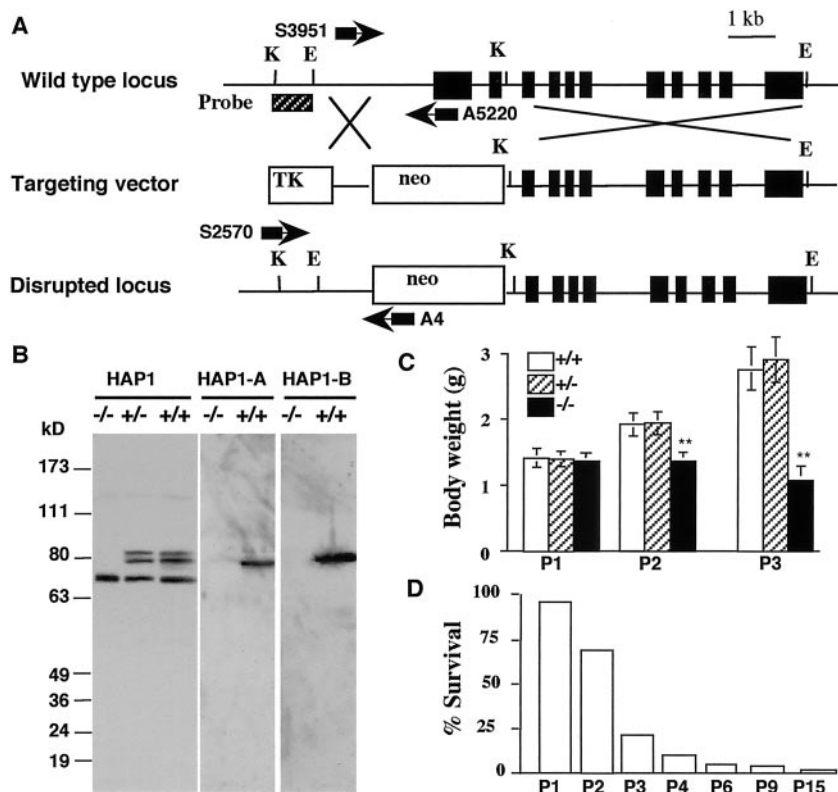


Figure 1. Targeted disruption of the mouse HAP1 gene. *A*, DNA structures of the wild-type allele, targeted vector, and mutant allele. The targeted vector contains neomycin (neo) and TK markers flanked by 1.6 and 5.8 kb of the mouse HAP1 gene. The first two exons (black boxes) of the HAP1 gene are deleted in the mutant allele. The probe used for Southern blot analysis is indicated, and the primers (arrows) for PCR genotyping are also indicated. K, *KpnI*; E, *EcoRI*. *B*, Western blot analysis of brain cortex extracts of wild-type, HAP1 (+/+), and HAP1 (-/-) mice. The blots were probed with antibodies against HAP1, HAP1-A, or HAP1-B. *C*, Pups from HAP1 (+/+), HAP1 (+/-), and HAP1 (-/-) mice were weighed at postnatal days 1, 2, and 3. The data (mean \pm SEM) were obtained from 54–204 pups for each group. ** $p < 0.01$ compared with wild-type mice. *D*, The percentage of surviving HAP1 (-/-) pups after birth. The majority of HAP1 (-/-) pups died at days 3–4 after birth.

al., 2002a) were described previously. Guinea pig anti-HAP1 (EM78) was produced by Covance (Denver, PA) using the same antigen as for rabbit anti-HAP1 (Li et al., 1995). Other antibodies used were mouse monoclonal anti-EGFR (Sigma, St. Louis, MO), anti- γ -tubulin (Sigma), anti-extracellular signal-regulated kinase (ERK) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated ERK, anti-protein kinase B (AKT), and anti-pAKT (Cell Signaling Technology, Inc., Beverly, MA). AG1478 was obtained from Calbiochem (La Jolla, CA), and other reagents from Sigma.

Homologous recombination of the HAP1 gene. Mouse genomic clones covering all 11 exons of the mouse HAP1 gene (Nasir et al., 1998) were isolated from a 129 SVJ mouse genomic DNA library (Stratagene, La Jolla, CA). A 1,575-bp fragment between *EcoRI* (2,705) and *BamHI* (4,269) was inserted 5' of a neomycin (NeoR) and thymidine kinase (TK) cassette in the pLoxpneo vector. Another 5,792-bp fragment between *KpnI* (7,126) and *EcoRI* (13,008) was connected 3' of the NeoR cassette. Approximately 4% of neoR/TK-selected TC-1 embryonic stem cell clones (185) were correctly targeted. Three of these were microinjected into C57BL/6 blastocysts, implanted into the uterus of pseudopregnant Swiss Webster foster mothers, and developed to term (Deng et al., 1994). Male chimeras were mated with National Institutes of Health black Swiss females (Taconic, Germantown, NY). No sex bias was observed in the offspring, and the expected Mendelian ratio (1:2:1) was observed among wild-type, heterozygous, and homozygous mutant mice. PCR genotyping of HAP1 knock-out mice used the following primers: S2570 (5'-GTGGCGCTGAGTCTG-GATTGAC-3') and A4 (5'-TACCCTACCCGGTAGAATTCG-3') for the knock-out allele; S3951 (5'-TTTGGAGGTCTGGTCTCGCTCTG-3') and A5220 (5'-CGTCTCCATCTTAGTGCCTTCAC-3') for the wild-type HAP1 allele.

Analysis of HD mice. N171–82Q mice [B6C3F1-TgN (HD82Gln)81Dbo], which express the first 171 amino acids of Htt with an additional 82-glutamine repeat (Schilling et al., 1999), and R6/2 mice [B6CBA-TgN (HDexon1)62], which express the first exon of Htt (67 amino acids) with an additional 115- to 150-glutamine repeat (Davies et al., 1997), were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Emory Animal Facility. Light and electron microscopy were performed as described previously (Li et al., 2000b). Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was performed using a kit obtained from Promega (Madison, WI) and following the manufacturer's instructions. For Western blots, the brain extracts (50–100 μ g of protein) were loaded onto each lane of the SDS gel, and the immunoreactive products were visualized with ECL kits (Amersham Inc). Immunoprecipitation was performed as described (Li et al., 2002a) using whole-brain extracts from R6/2 mice at 4 weeks of age and guinea pig anti-HAP1.

Analysis of transfected cells. PC12 cell lines stably expressing exon1 Htt containing 20- or 150-glutamine repeats were obtained from our previous study (Li et al., 1999). An anti-HAP1 construct in the PRK vector that contains the reverse sequences from the N-terminal region of HAP1 (amino acids 1–296) was cotransfected with pCDN3 (5:1) into PC12 cells. Stably transfected PC12 cells were selected with G418 (500 ng/ml) and analyzed by Western blots to verify the decreased expression of HAP1. Of 12 cell lines isolated, we focused on the A2, A3, and A6 lines, which showed a significant decrease in HAP1 expression. Transient transfection of HEK293 cells and assays of cell viability and caspase-3 activity were described previously (Li et al., 1999, 2002a). For EGF treatment, cells were cultured in serum-free medium overnight before EGF (100 ng/ml) was added for 5 or 45 min.

Primary neuronal culture and transfection. Hypothalamic cells were cultured and maintained as described previously (Perez-Martinez et al., 2001) using hypothalami from embryonic days 14–16 rats. Cells were cultured in DMEM supplemented with 10% FBS, 5 μ M cytosine arabinoside (Sigma), and 50% glial-conditioned medium. Neurons that had been cultured for 2–4 d were transfected with HAP1-A, HAP1-B, and Htt cDNAs using CaPO₄ transfection. After a 36- to 48-hr transfection, cells were analyzed. For immunofluorescent double labeling, we used mEM48 and rabbit HAP1 antibodies. We performed three to five transfection experiments for each group, examined 139–187 transfected neurons for each group, and counted neurons that showed nuclear DNA fragmentation.

Statistics. Data were analyzed using an unpaired *t* test, with $p < 0.05$ indicating statistical significance.

Results

Targeted deletion of the mouse HAP1 gene leads to postnatal death

We used a targeted gene disruption approach to eliminate the first 131 amino acids of HAP1 (Fig. 1A). Deletion of the expression of HAP1 in HAP1 (-/-) mouse brain was confirmed by Western blotting with three antibodies: anti-HAP1, anti-HAP1-A, and anti-HAP1-B (Fig. 1B). HAP1-A and HAP1-B, which have different C-terminal sequences (Li et al., 1995) and were represented by the two top bands on the blot, were both eliminated in homozygous mutant mice (Fig. 1B).

The striking gross phenotype of HAP1 (-/-) mice was that

they were unable to gain body weight after birth (Fig. 1C). Similar to HAP1 mutant mice that were generated by deleting exon 1 of the HAP1 gene (Chan et al., 2002), HAP1 ($-/-$) pups had very little milk in their stomachs, suggesting a feeding defect that may retard growth and cause postnatal death at days 3–4. Some female mice could produce newborn mutant mice that lived longer. However, no HAP1($-/-$) mice survived >15 d (Fig. 1D). In those HAP1($-/-$) mice that lived longer than 9 d, we did not observe abnormalities in movement, tactile response, motor control, skin, bones, or organs. HAP1(+/-) heterozygous mice showed a similar expression level of HAP1 to wild-type mice and lived as long as wild-type mice with no obvious behavioral or body weight abnormalities. We, therefore, focused our studies on HAP1($-/-$) mice to compare them with age-matched wild-type littermates.

Degeneration in the hypothalamus of HAP1($-/-$) mice

We observed that in wild-type mice, HAP1 is highly expressed in the hypothalamus (Fig. 2A), including the arcuate nucleus (ARC), the paraventricular nuclei (PVN), the lateral hypothalamus (LH), and the ventromedial hypothalamic nucleus (VMN) (data not shown). The ARC receives peripheral hormone signals, such as leptin and insulin, and projects to the LH, VMN, and PVN to regulate central feeding behavior (Elmqvist et al., 1998). The expression of HAP1 in the hypothalamus and the feeding phenotype of HAP1($-/-$) mice are virtually the same as those of HAP1 knock-out mice reported by Chan et al. (2002). Despite the small size of the brain and body of HAP1($-/-$) mice, the previous study using creyl violet or hematoxylin and eosin staining did not reveal obvious neuropathology or abnormal gross morphology in these mice (Chan et al., 2002). Our examination of the gross morphology did not reveal any significant neuropathology in the brains of HAP1($-/-$) mice either. We then used TUNEL labeling and electron microscopy to examine whether there is any abnormality in HAP1($-/-$) mouse brains. TUNEL labeling clearly revealed positive cells in the hypothalamus of HAP1($-/-$) mice. At postnatal day 1 (P1), most TUNEL-positive cells were observed in the PVN and the LH (Fig. 2B). Very few or no TUNEL-positive cells were found in the ARC. At P3, TUNEL-labeled cells were more widely distributed in various regions of the hypothalamus, including the VMN (Fig. 2D), which also controls eating, food intake, and energy metabolism (Meguid et al., 2000). The distribution of TUNEL-positive neurons is well correlated with the high level of HAP1 in the hypothalamic nuclei.

Several other lines of evidence also suggest the presence of specific hypothalamic degeneration in HAP1($-/-$) mice. First, there were few or no TUNEL-positive cells in the hypothalamus of wild-type mouse brain at P1 (Fig. 2C). For example, quantitative analysis revealed a significant increase in TUNEL-positive cells in the HAP1($-/-$) PVN (113 per image; 100 \times) as compared with the PVN of wild-type pups (11 per image). Second, other brain regions such as the cortex, striatum (Fig. 2E), cerebellum, and hippocampus (data not shown) in HAP1($-/-$) mice had much fewer TUNEL-positive cells or showed no difference from those in wild-type control mice. Thus, deletion of HAP1 preferentially kills neurons that normally express a high level of HAP1 in the hypothalamus in mouse pups.

Ultrastructural evidence for the degeneration of hypothalamic neurons in HAP1($-/-$) mice

Electron microscopy revealed that hypothalamic neurons at P1 contained degenerating mitochondria that were swollen and had

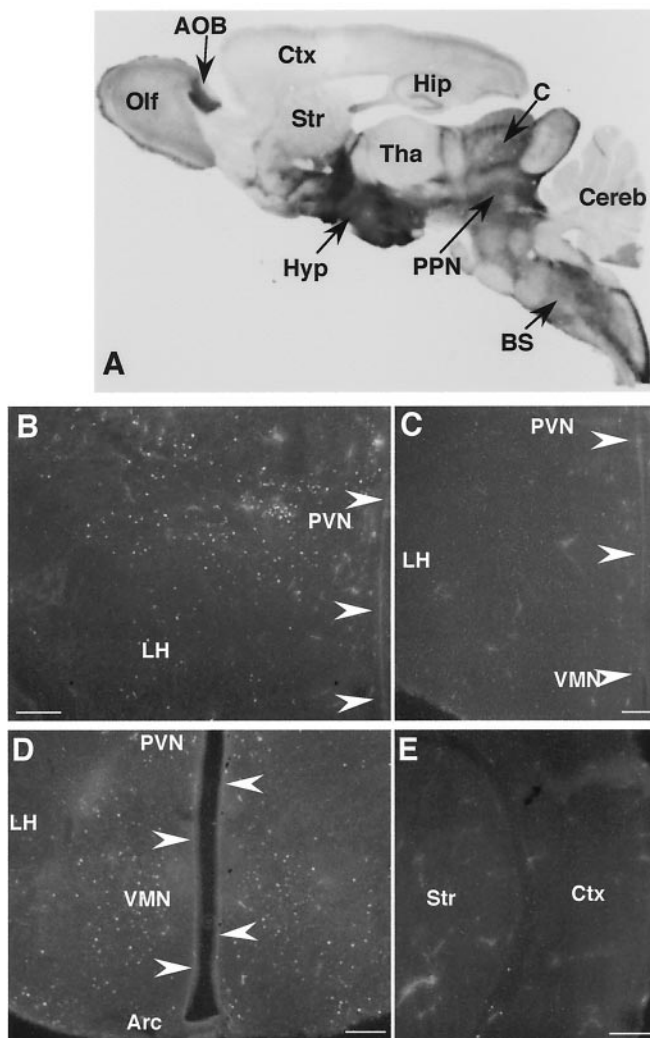


Figure 2. TUNEL staining of mouse brain. *A*, HAP1-B immunostaining of a sagittal section of adult mouse brain shows abundant expression of HAP1 in the hypothalamus (Hyp). HAP1 is also highly expressed in the accessory olfactory bulb (AOB), superior and inferior colliculi (C), pedunculo-pontine nucleus (PPN), and brain stem (BS). Olf, Olfactory bulb; Ctx, cerebral cortex; Str, striatum; Hip, hippocampus; Tha, thalamus; Cereb, cerebellum. *B–D*, Elimination of HAP1 results in TUNEL-positive cells in the hypothalamus. At P1, TUNEL-positive cells are seen in the PVN and the LH in HAP1($-/-$) mice (*B*) but not in HAP1($+/+$) mice (*C*). At P3, more TUNEL-positive cells are seen in the hypothalamic areas including the VMN, whereas the ARC shows fewer TUNEL-positive cells in HAP1($-/-$) mice (*D*). *E*, In the cortex of the same HAP1($-/-$) mouse, no or very few TUNEL-positive cells are seen. The third ventricle is indicated by arrowheads. Scale bar, 100 μ m.

no intact membrane structure (Fig. 3A). These abnormal morphological changes were not found in the hypothalamus of wild-type pups at P1 (Fig. 3B) or in the cortex of the same mutant pup (Fig. 3C). Some HAP1($-/-$) neurons in the hypothalamus showed condensed and fragmented nuclei that constituted apoptotic bodies (Fig. 3D). This apoptotic profile was more prominent at P3–P4, as evidenced by large and multiple apoptotic bodies that were surrounded by many swollen mitochondria, large vacuoles, and lysosome-like structures (Fig. 3E). A late stage apoptotic neuron is shown in Figure 3F, in which an apoptotic body is engulfed by a glial cell. We did not find such degenerating neurons in the striatum or cortex of the same HAP1($-/-$) mouse. Thus, electron microscopy confirms the selective degeneration of hypothalamic neurons in HAP1($-/-$) mice.

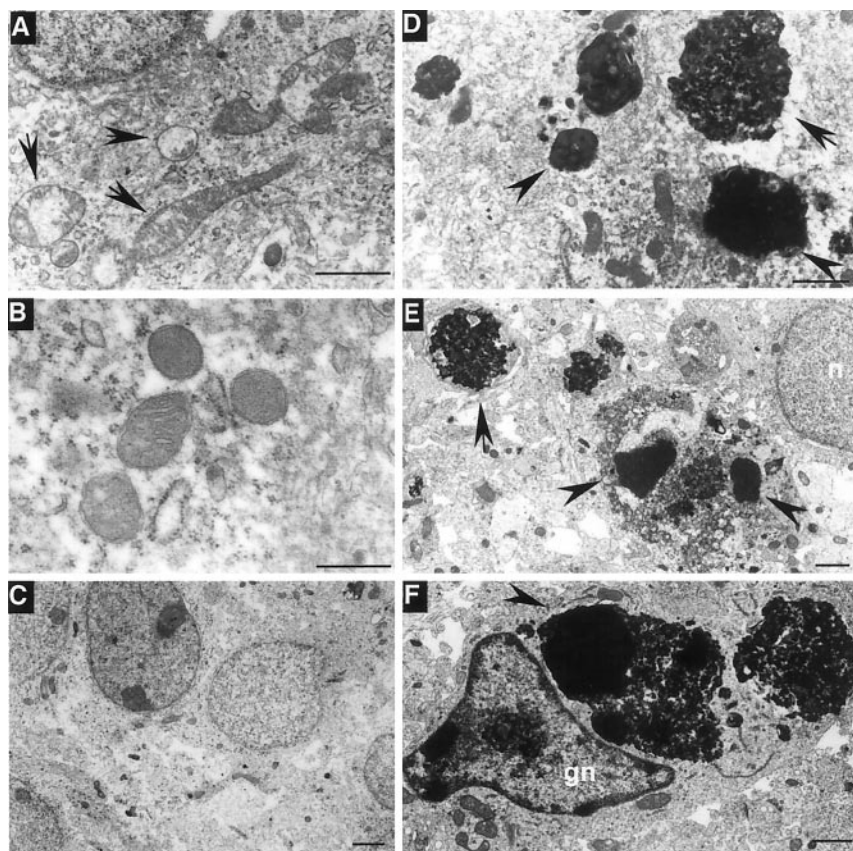


Figure 3. Electron microscopic examination of the brain of HAP1(−/−) mice. *A–D*, Ultrastructural morphology of HAP1(−/−) or HAP1(+/+) mice at P1. *A*, Electron microscopy reveals swollen mitochondria with disrupted structures of internal membranes (arrows) in the LH of a HAP1(−/−) mouse. *B*, Mitochondria in the hypothalamus of a HAP1(+/+) mouse pup at P1 have normal morphology. *C*, The cortex of a HAP1(−/−) mouse at P1 reveals no apoptotic cells. *D*, In the hypothalamus of the same HAP1(−/−) pup, fragmented and condensed nuclei form apoptotic bodies (arrowheads). *E, F*, Apoptotic profiles of the hypothalamic neurons of a HAP1(−/−) mouse at P3 showing nuclear pyknosis, chromatin fragmentation, and darkening structures (arrows). Apoptotic bodies (arrowheads) are surrounded by degenerated mitochondria and lysosome-like structures (*E*). A normal nucleus (*n*) is present near apoptotic cells (*E*). A large apoptotic body (arrowhead) is being engulfed by a glial cell (*gn*), indicating a late stage of apoptosis (*F*). Scale bars: *A, B*, 0.5 μm; *C–F*, 1 μm.

Degeneration of hypothalamic neurons in HD transgenic mice

Because wasting and body weight loss are typical symptoms both for HD patients (Sanberg et al., 1981; Pratley et al., 2000) and HD transgenic mice (Davies et al., 1997; Schilling et al., 1999), we examined whether there is also neuronal degeneration in the hypothalamus of N171–82Q mice that express the first 171 amino acids with a 82–glutamine repeat (Q). These mice have early death occurring at 5–6 months and display a striking reduction in body weight resulting from the inability to gain weight beginning 2 months after birth (Schilling et al., 1999). Previous studies using hematoxylin/eosin and silver stains did not reveal obvious neuronal loss in these mice (Schilling et al., 1999). Using electron microscopy, we observed many degenerated neurons in the hypothalamus, as evidenced by the disrupted structures of mitochondria, condensed and dark nuclei, and some dark, electron-dense cytoplasmic structures (Fig. 4*A, B*). To examine the distribution of mutant Htt, we also performed EM48 immunogold labeling, which showed that transgenic Htt was diffuse or scattered around the degenerated organelles (Fig. 4*C*). Vacuolar structures were often recruited into these degenerated organelles. In the nuclei of neurons that have not shown apoptotic bodies, mutant Htt formed intranuclear inclusions (Fig. 4*D*). Degener-

ated neurons in the HD mice were present in various hypothalamic areas, including the LH, VMN, and PVN. However, other brain regions, including the cortex and striatum, also had degeneration (Yu et al., 2003), perhaps because the transgenic mutant Htt is widely expressed in brain. Wild-type mice at 5 months of age did not show such neurodegeneration (data not shown).

Interaction of HAP1 with Htt and decreased expression of HAP1 and EGFR in HD mice

The abnormal interaction between mutant Htt and HAP1 may affect the function of HAP1. To provide evidence for their interaction *in vivo*, we immunoprecipitated HAP1 from R6/2 HD mouse brain, which expresses mutant Htt at a higher level than does N171–82Q. EM48, which reacts strongly with mutant Htt but very weakly with endogenous rodent Htt (Li et al., 2000b), detected the soluble and aggregated forms of mutant Htt. Both forms were precipitated by HAP1 antibody but not by preimmune serum (Fig. 5*A*). Probing the same blot with anti-HAP1 confirmed that Htt precipitation was specific to the presence of HAP1.

Intranuclear mutant Htt affects the transcriptional level of HAP1 in PC12 cells (Li et al., 1999). We performed Western blot analysis and also observed a reduced expression of HAP1 in N171–82Q mouse hypothalamus (Fig. 5*B*). This decrease was apparently correlated with the presence of mutant Htt. Immunohistochemistry also showed a decrease in HAP1 staining of the LH and VMN in HD mice, as compared

with that in wild-type mice (Fig. 5*C*). This decrease was evidenced by the reduced cytosolic HAP1 staining and fewer stigmoid bodies, which are a cytoplasmic structure of unknown function and are intensively labeled by anti-HAP1 antibody (Gutkunst et al., 1998). Other brain regions that express HAP1 at a relatively lower level did not show a significant difference in HAP1 staining between wild-type and HD mice (data not shown).

Because HAP1 overexpression reduces EGFR degradation in transfected cells (Li et al., 2002b), we wanted to examine the expression of EGFR in HAP1(−/−) mouse brain. Because the postnatal hypothalamus is too small to use for Western blotting, we examined the expression of EGFR in the cortex of HAP1(−/−) pups. The result showed a significant decrease in the expression of EGFR (Fig. 5*D*). Analysis of the expression of EGFR in N171–82Q mice at 4 months of age also showed a similar decrease in EGFR expression in the hypothalamus (Fig. 5*D*).

Decreased expression of HAP1 and overexpression of mutant Htt inhibit EGFR signaling

The decreased expression of EGFR in HD brains prompted us to use cell models to examine whether EGFR signaling pathways are impaired by decreased HAP1 expression or increased expression

of mutant Htt. We generated PC12 cell lines that stably express exon1 Htt containing 150Q or 20Q (Li et al., 1999). Stably transfected PC12 cells that express antisense HAP1 RNA were also established. Three cell lines (A2, A3, and A6) showed a significant decrease in the expression of HAP1 (Fig. 6A). The A2, A3, and A6 lines all had reduced neurite outgrowth in response to NGF (Fig. 6B). This result is consistent with our previous observation that overexpression of HAP1 can promote neurite outgrowth (Li et al., 2000a). We then focused on A2 cells and examined EGFR signaling, which triggers numerous downstream signaling pathways mainly through phospholipase C- γ , Ras, and phosphatidylinositol 3-kinase (PI3-kinase) to activate protein kinase C, ERK1/2 cascade, and protein kinase B (AKT), respectively (Wells, 1999). Compared with nontransfected PC12 cells, A2 cells showed much less phosphorylation of ERK and AKT after EGF stimulation (Fig. 6C). This decrease was apparently associated with the reduced expression of EGFR. A similar decrease in EGFR, phosphorylated ERK, and AKT was also observed in 150Q cells, but not in 20Q cells, suggesting that defective EGFR signaling pathways were presented in both A2 and 150Q cells.

The impaired EGFR signaling could confer increased sensitivity of A2 and 150Q cells to EGFR signaling inhibitors. We, therefore, treated these cells with AG1478, the specific EGFR tyrosine kinase inhibitor, and wortmannin, an inhibitor of PI3-kinases. A2 and 150Q cells were more sensitive than wild-type PC12 cells to the cytotoxic effect of these inhibitors and, thus, displayed a greater decrease in their viability. The 150Q cells had the greatest reduction of viability in response to the highest dose of wortmannin (100 nM) (Fig. 6D), perhaps because 150Q activates multiple pathological pathways. Decreased viability of 20Q cells was seen with 100 nM wortmannin, suggesting that N-terminal Htt, when overexpressed, could also interfere with cellular function to some extent. The similar decrease in the viability of A2 and 150Q cells supports the idea that mutant Htt affects EGFR signaling activity in the same manner as the dysfunction of HAP1.

Protection of HAP1 against Htt toxicity

If mutant Htt affects the function of HAP1, overexpression of HAP1 may reduce Htt-mediated toxicity. We used transfected HEK293 cells to test this hypothesis. As reported previously (Li et al., 1999), we also observed that overexpression of HAP1 reduced the degradation of EGFR induced by EGF. This prevention, however, was inhibited by coexpression of N-terminal Htt (1–208 amino acids) containing 120Q (N208–120Q), but not by the same N-terminal huntingtin with a normal repeat (N208–23Q) (Fig. 7A). Consistently, the phosphorylation of ERK was enhanced by HAP1 overexpression and decreased by coexpression of N208–120Q. We then examined whether overexpression of HAP1 could protect against Htt toxicity in response to wortmannin, which promotes EGFR degradation and kills neurons by a caspase-dependent mechanism (Chen et al., 1999; Oriike et al.,

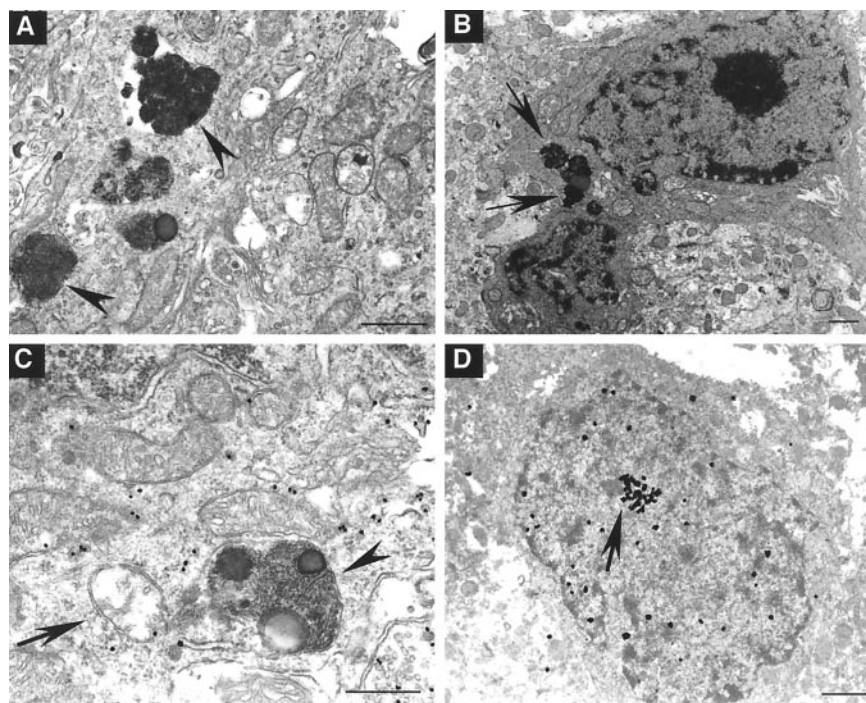


Figure 4. Hypothalamic degeneration in HD mice. Electron microscopic examination of the LH of HD mice at 4 months of age. *A*, Several condensed and darkening apoptotic bodies (arrowheads) are evident. Some swollen mitochondria are also seen. *B*, A condensed nucleus with dark chromatin is shown in a degenerating neuron. The nuclear membrane is not intact, and several cytoplasmic condensed structures (arrows) are also seen. *C*, *D*, EM48 immunogold labeling of hypothalamic neurons in HD mice. Immunogold particles are diffusely distributed in a neuron in which degenerating mitochondria (arrow) and lysosomal structures (arrowhead) are also present (*C*). Mutant Htt also forms aggregates (arrow) in a nucleus that is undergoing chromosomal condensation (*D*). Scale bars: *A*, *C*, 0.4 μ m; *B*, *D*, 0.8 μ m.

2001). We measured caspase-3 activity, because this assay is sensitive to huntingtin toxicity in cultured cells. Wortmannin significantly increased caspase-3 activity in HEK293 cells transfected with N208–120Q. This increase, however, was inhibited by coexpression of HAP1 (Fig. 7B).

It would be interesting to see whether HAP1 also protected hypothalamic neurons from Htt toxicity. We transfected primary hypothalamic neurons with HAP1 and the same mutant Htt as described previously. Transfected HAP1-A formed small cytoplasmic inclusions resembling stigmoid bodies, but had no effect on cell viability (Fig. 7C). Expression of mutant Htt, however, resulted in the formation of large aggregates in the cytoplasm and neurites. Nuclear DNA fragmentation (Fig. 7C) and neuritic degeneration (data not shown) were often seen in mutant Htt-transfected cells. When HAP1 (HAP1-A or HAP1-B) was cotransfected with mutant Htt into cultured neurons, both transfected HAP1 and Htt were diffusely distributed in the cell body and neurites. Some Htt aggregates in the neurites also contained HAP1 immunoreactive product, suggesting a colocalization of mutant Htt and HAP1 (Fig. 7D). Because the majority of HAP1 was diffuse in the transfected cells, most HAP1 was likely to bind to mutant Htt that had not formed microscopic aggregates. Importantly, neurons cotransfected with HAP1 often showed normal cellular morphology without obvious DNA fragmentation. We observed that 36.3% of mutant Htt-transfected cells showed nuclear DNA fragmentation, which is higher than that of normal Htt-transfected cells (10.1%) or HAP1-transfected cells (6.9%). When mutant Htt was cotransfected with HAP1, this percentage was reduced to 19.9% (Fig. 7E). These results also demonstrated

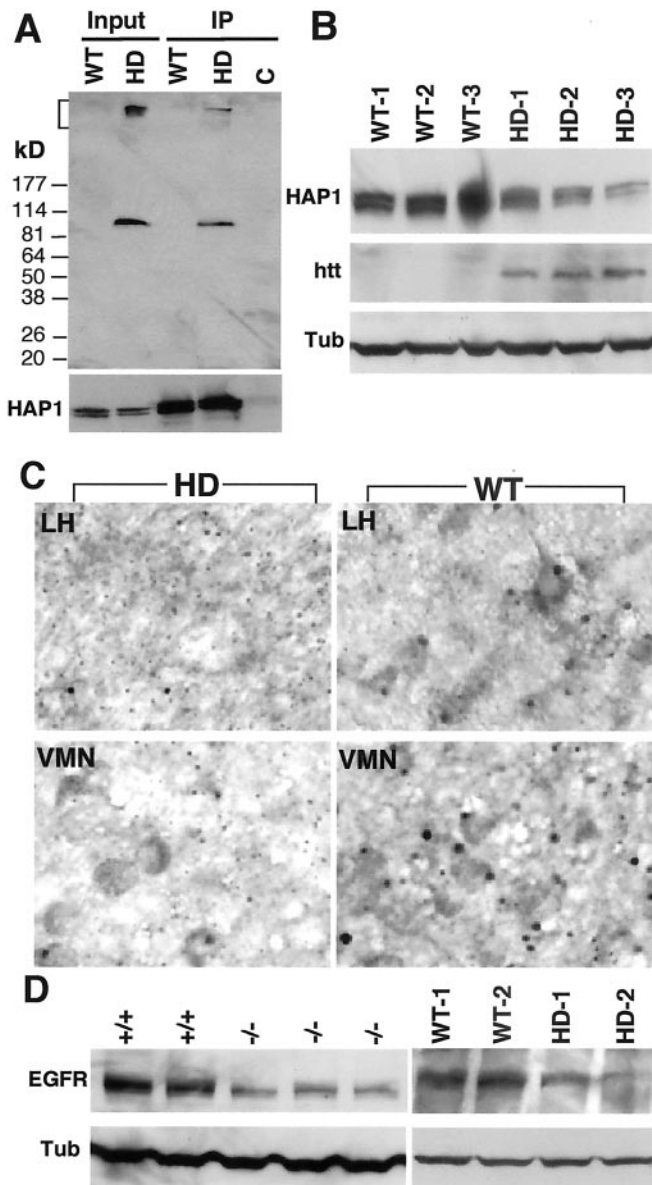


Figure 5. Expression of HAP1 and EGFR in HD transgenic mice. *A*, Immunoprecipitation of HAP1 and Htt from brains of wild-type mice (WT) and R6/2 mice (HD) at 4 weeks of age. Anti-HAP1 or the preimmune serum (lane C) was used for immunoprecipitation, and the precipitates were blotted and probed with mEM48. The bottom panel is the same blot reprobed with anti-HAP1 antibody. Input, 20% of tissue lysates. The bracket indicates the stacking gel in which aggregated Htt is retained. *B*, Western blot analysis of the expression of HAP1, transgenic huntingtin (htt), and tubulin shows that the expression of HAP1 is lower in the hypothalamic tissue from three different N171–82Q mice (HD-1, -2, -3) than in that from wild-type mice (WT-1, -2, -3). *C*, HAP1 immunocytochemistry of the LH and VMN of N171–82Q and wild-type (WT) mice at 4 months of age. *D*, Western blot analysis of brain cortical tissues from HAP1(+/+) and HAP1(-/-) pups at P1 and the hypothalamic tissues from two wild-type mice (WT) or N171–82Q mice (HD) at 4 months of age. The blots were probed with antibodies to EGFR and tubulin (Tub).

that coexpression of HAP1 can inhibit the cell death caused by mutant Htt.

Discussion

Eating behavior after birth is largely dependent on the activation of NPY signaling pathways in hypothalamic neurons by hunger signals, such as those caused by active depletion of body fat stores and/or reduced leptin/insulin signaling to the brain (Kalra et al.,

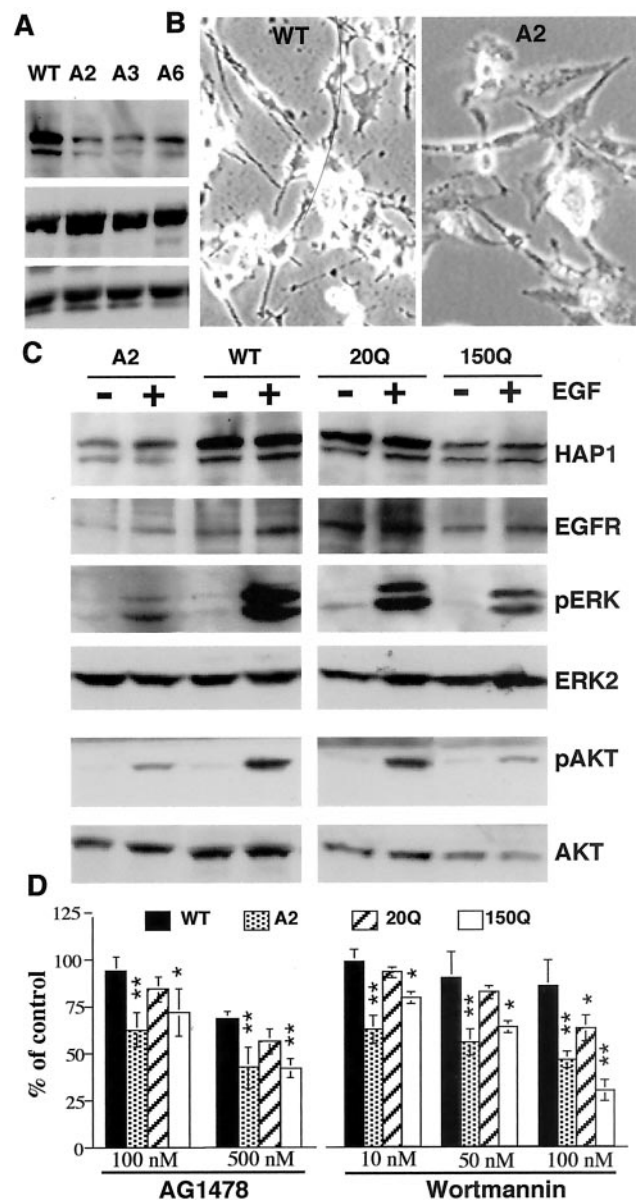


Figure 6. Decreased EGFR signaling activity in PC12 cells expressing antisense HAP1 or mutant Htt. *A*, Western blot analysis of PC12 cell lines (A2, A3, and A6) that were stably transfected with antisense HAP1 construct. WT, Wild-type PC12 cells. The expression of HAP1 (top), dynactin p150 (middle), and tubulin (bottom) is shown. *B*, Neurite outgrowth of PC12 cells (wild-type and A2 cell line) after NGF (100 ng/ml) treatment for 48 hr. *C*, Expression of HAP1, EGFR, phosphorylated ERK (pERK), ERK2, phosphorylated AKT (pAKT), and AKT in wild-type cells, A2 cells, and PC12 cells stably expressing exon1 Htt with a 150-glutamine repeat (150Q) or 20-glutamine repeat (20Q) after EGF (100 ng/ml) treatment for 5 min. *D*, The viability of wild-type, A2, 20Q, and 150Q PC12 cells after treatment with AG1478 and wortmannin for 5 and 8 hr. The control is untreated cells for each group. Data are expressed as mean \pm SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$ compared with WT.

1999; Schwartz et al., 2000). The ARC is a major site for transducing afferent input from circulating leptin and insulin into a neuronal response (Elmqvist et al., 1998; Schwartz et al., 2000). Other hypothalamic areas, including the PVN and the LH, are richly supplied by axons from NPY/agouti-related peptide and pro-opiomelanocortin and cocaine and amphetamine-regulated transcript neurons in the ARC (Elmqvist et al., 1998; Kalra et al., 1999). The abundant expression of HAP1 in various hypothalamic regions is consistent with its pivotal role for the function of

hypothalamic neurons. The lack of HAP1 causes hypothalamic neurons to die and may lead these neurons unable to respond to hunger signals.

EGFR, when translocated in the endosome, is also functional and activates the major signaling pathways, leading to cell proliferation and survival (Wang et al., 2002). Deletion of EGFR results in postnatal neurodegeneration and death in mice (Kornblum et al., 1998; Sibilio et al., 1998). HAP1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is involved in endosome-to-lysosome trafficking of EGFR, and overexpressed HAP1 protects against EGFR degradation (Li et al., 2002b). The decreased expression of HAP1 could cause a rapid degradation of EGFR in certain types of neurons and, thus, decrease its signaling activity for neuronal survival, which may contribute to the neurodegeneration in HAP1(−/−) mice. Also, HAP1 seems to possess multiple functions, such as binding to dynactin P150, which is involved in microtubule-based transport (Engelender et al., 1997; Li et al., 1998b) and neurite outgrowth (Block-Galarza et al., 1997; Li et al., 2000a). These functions could also be impaired in the absence of HAP1. Because HAP1 is highly expressed in the hypothalamic regions that control feeding behavior, deletion of HAP1 may preferentially affect neuronal function and cause degeneration in these hypothalamic regions, leading to a defect in feeding behavior. As a result, HAP1(−/−) mice die earlier than EGFR(−/−) mice, preventing us from investigating whether HAP1 deletion also causes neurodegeneration in other brain regions in older neonatal or adult mice. The role of HAP1 in other brain regions can be better investigated in future studies with a conditional HAP1 knock-out mouse model.

The function of HAP1 for hypothalamic neuronal survival and EGFR signaling may be affected by mutant Htt. Indeed, similar hypothalamic degeneration was found in HD transgenic mice that show wasting and body weight loss. Weight loss in HD patients (Sanberg et al., 1981; Pratley et al., 2000) is consistent with altered release of the growth hormones from the hypothalamus (Hayden et al., 1977) and severe neuronal degeneration in the hypothalamus of HD patients (Kremer et al., 1990, 1991). Several other lines of evidence also suggest that dysfunction of HAP1 and defective EGFR signaling occur in HD brains. First, immunoprecipitation showed that mutant Htt abnormally interacted with HAP1 in HD brain. Second, consistent with our previous findings that HAP1 expression was reduced in HD patient brains and HD cells (Li et al., 1998a, 1999), the expression of HAP1 was decreased in HD mouse brains. The decreased expression of HAP1 could be because of its abnormal interaction with mutant Htt in the cytoplasm and the inhibitory effect of mutant Htt in the nucleus on gene transcription. We

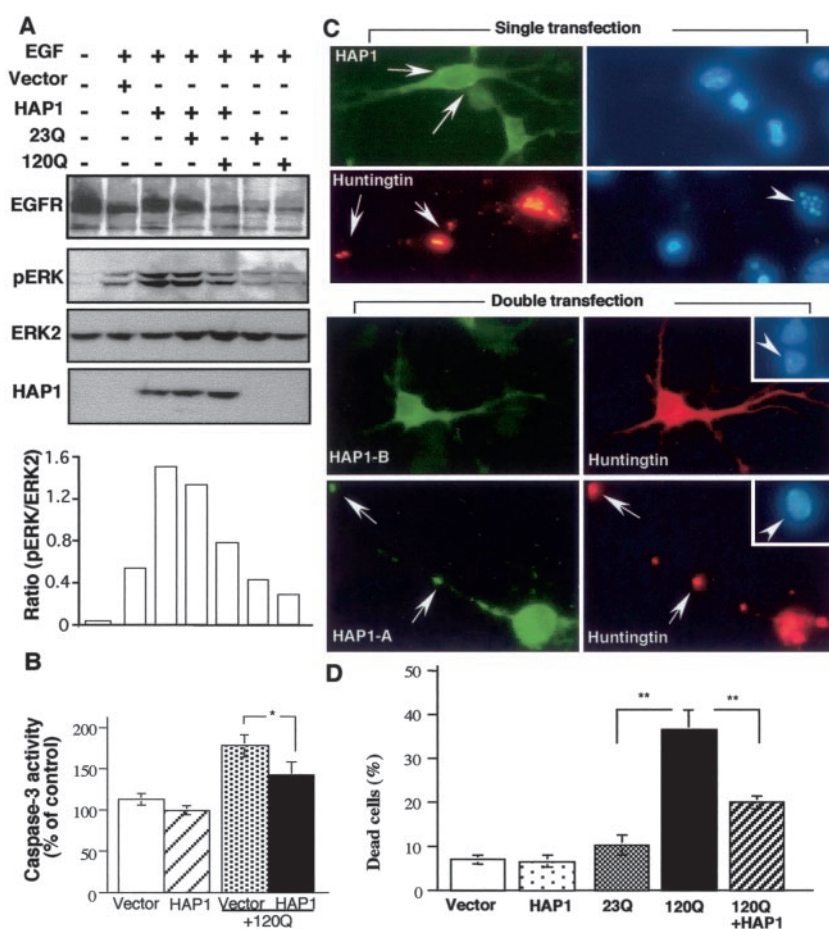


Figure 7. Protection of HAP1 against Htt toxicity. *A*, Transfection of HEK293 cells with HAP1-B, N-terminal Htt (1–208 amino acids) containing 23 or 120 glutamines (N208–23Q or –120Q), or HAP1-B with Htt. Transfected cells were treated with EGF (100 ng/ml) for 45 min. The expression of EGFR, phosphoralted ERK (pERK), ERK2, and HAP1 was analyzed by Western blotting. The levels of pERK were quantified and expressed as a ratio (pERK/ERK) for each sample. *B*, HEK293 cells transfected with HAP1-B, N208–120Q alone, N208–120Q with vector, or HAP1 were treated with wortmannin (500 nM) for 4 hr, followed by caspase-3 activity assay. Control is untreated cells. Data are expressed as mean \pm SD ($n = 4$), and $*p < 0.05$. *C*, Transfection of primary hypothalamic neurons with HAP1-A (top) or mutant Htt (bottom). Cytoplasmic HAP1 and Htt inclusions are indicated by arrows. Hoechst DNA staining (right) shows nuclear DNA fragmentation (arrowhead) in Htt-transfected neurons. Coexpression of HAP1-B (top) or HAP1-A (bottom) with Htt resulted in diffuse HAP1 (green) and Htt (red) and a colocalization of some HAP1 with Htt aggregates (arrows). Note that doubly transfected cells show intact nuclei (arrowheads in insets). *D*, The percentage of transfected cells showing nuclear DNA fragmentation. Cultured neurons were singly transfected with vector, HAP1-B (HAP1), N208–23Q, or N208–120Q and HAP1-B together (120Q + HAP1). Data are mean \pm SE of three to five transfections. $**p < 0.01$ compared with 120Q transfection.

found that the promoter of the mouse HAP1 gene contains Sp1-binding sites (data not shown), which could be affected by nuclear Htt that binds Sp1 (Dunah et al., 2002; Li et al., 2002a). Third, the increased degradation or reduced expression of EGFR was found in the brains of HAP1 knock-out and HD mice. Consistent with the *in vivo* evidence, both mutant Htt and decreased HAP1 expression caused a similar defect in EGFR signaling pathways, making cultured cells more susceptible to EGFR signaling inhibitors. Similarly, previous studies have shown that in PC12 cells mutant Htt interferes with cellular signaling mediated by EGFR (Song et al., 2002) and EGF protects against Htt toxicity (Li et al., 1999).

Although the decreased EGFR signaling activity is associated with the decreased expression of HAP1, the loss of HAP1 may also cause other cell type-specific dysfunction that contributes to neurodegeneration in the hypothalamic regions. This may explain why in HAP1(−/−) cortical tissues we did not observe

obvious neurodegeneration, even the expression of EGFR was also decreased. Similarly, mutant Htt may also cause cells to die via different pathological pathways, such as promoting NMDA toxicity (Zeron et al., 2002), activating caspases (Gervais et al., 2002; Sanchez et al., 2003), and affecting neurotrophic factor production (Zuccato et al., 2001). Different types of neurodegeneration in HD may depend on cellular context and the abnormal interactions of mutant Htt with various proteins including Hip1, which is involved in clathrin-mediated endocytosis (Metzler et al., 2001; Rao et al., 2001), therefore, leading to a variety of neurological symptoms. For example, degeneration or dysfunction of neurons in the striatum and cortex observed in different transgenic HD mouse models (Reddy et al., 1998; Hodgson et al., 1999) could be associated with motor and behavioral abnormalities, whereas degeneration in the hypothalamus may lead to wasting and body weight loss. Consistent with this idea, environmental enrichment of R6/2 mice slows the progression of neurological symptoms but not the loss of body weight (Hockly et al., 2002). Our recent studies show that huntingtin context specifies the types of neurodegeneration (Yu et al., 2003), suggesting that the interactions of various N-terminal huntingtin fragments with other proteins are involved in the different types of neurodegeneration in HD.

An interesting question is why hypothalamic degeneration occurs in the late stages of HD. N-terminal mutant Htt may accumulate in aged neurons when their capability to remove toxic protein products is reduced. The increased concentration of N-terminal mutant Htt fragments is then able to cause abnormal protein interactions and altered gene expression to affect the function of Htt-interacting proteins. In support of this idea, overexpressed mutant N-terminal Htt rapidly killed the primary cultured neurons, and its toxicity could be reduced by coexpression of HAP1. Also, it remains to be investigated whether the reduced expression or partial dysfunction of HAP1 in hypothalamic neurons in adult mice leads to the feeding and wasting phenotypes similar to those of HD. Conditional HAP1 knock-out mouse models would allow one to address this issue and to investigate whether and how HAP1 is involved in regulating food intake and energy balance in adult animals. Identification of the pivotal role of HAP1 in hypothalamic neurons and its involvement in EGFR signaling pathways may help find therapeutic targets to slow the progressive neurodegeneration in HD.

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