Huntingtin-associated Protein-1 Interacts with Pro-brain-derived Neurotrophic Factor and Mediates Its Transport and Release*\[S\]

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Brain-derived neurotrophic factor (BDNF) plays a pivotal role in brain development and synaptic plasticity. It is synthesized as a precursor (pro-BDNF), sorted into the secretory pathway, transported along dendrites and axons, and released in an activity-dependent manner. Mutant Huntington with expanded polyglutamine (polyQ) and the V66M polymorphism of BDNF reduce the dendritic distribution and axonal transport of BDNF. However, the mechanism underlying this defective transport remains unclear. Here, we report that Huntingtin-associated protein-1 (HAP1) interacts with the prodomain of BDNF and that the interaction was reduced in the presence of polyQ-expanded Huntingtin and BDNF V66M. Consistently, there was reduced coimmunoprecipitation of pro-BDNF with HAP1 in the brain homogenate of Huntington disease. Pro-BDNF distribution in the neuronal processes and its accumulation in the proximal and distal segments of crushed sciatic nerve and the distribution in the neuronal processes and its accumulation in the brain homogenate of Huntington disease. Pro-BDNF partition in axonal transport and activity-dependent release of pro-BDNF by interacting with the BDNF prodomain. Accordingly, the decreased interaction between HAP1 and pro-BDNF in Huntington disease may reduce the release and transport of BDNF.

Neurotrophins play important roles in the proliferation, differentiation, and survival of neurons during development and in the maintenance of normal functions of the mature nervous system by activating their respective tyrosine kinase receptors TrkA, TrkB, and TrkC and the common receptor p75NTR (1–6). Neurotrophins are synthesized as precursors (proneurotrophins), which are either cleaved intracellularly by furin (2, 7, 8) and released as mature forms (9), or cleaved extracellularly by several proteases, including prohormone convertases, tissue-activated plasminogen/plasmin, MMP-3, and MMP-7 (8, 10, 11). Recently, it has been shown that unprocessed, the nerve growth factor precursor and the brain-derived neurotrophic factor precursor (pro-BDNF) bind both Sortilin and p75NTR with a high affinity and preferentially activate p75NTR, leading to apoptosis (12–15).

Although the retrograde neurotrophic hypothesis is well recognized, accumulating evidence indicates that neurotrophins such as BDNF and neurotrophin-3 are also trafficked anterogradely within dendrites and axons, released in an activity-dependent manner, and uptaken by second- or third-order target neurons (16–19). The anterogradely transported and released BDNF regulates neuronal survival, differentiation, dendritic morphology, and synaptic plasticity (17, 20–22). Both Sortilin and carboxypeptidase E play important roles in post-translational Golgi sorting of BDNF to the regulated secretory pathway and activity-dependent release by interacting with the prodomain (23) and the mature domain, respectively (24). Recently, we showed that pro-BDNF, like mature BDNF, is also transported anterogradely and retrogradely within axons of sensory neurons (25). However, how pro-BDNF and mature BDNF are transported within dendrites and axons remain to be investigated. A single nucleotide polymorphism in the BDNF gene (BDNFmet) at codon 66 in the prodomain results in the reduction of BDNF transport and activity-dependent secretion (26). The mutation was associated with reduction of hippocampal volume, impairment of episodic learning (27, 28), and a number of neurological disorders (29–31).

The polyglutamine (polyQ) expansion in Huntington (Htt) and knocking down of Htt-associated protein-1 (HAP1) and p150Glued can reduce BDNF transport and lead to the degeneration of striatal neurons (26, 32). Htt is a scaffold protein predominantly found in the cytoplasm where it associates with various vesicular structures and molecular motors to form a cargo complex and may play a role in intracellular trafficking (32, 33). The expanded (>37) glutamine stretch repeat in the Htt N terminus causes abnormally assembled protein complexes. HAP1 is distributed throughout the brain and spinal cord and is involved in axonal transport of BDNF (34). However, the mechanism for this transport remains to be investigated. It is also unclear how mutant Htt interferes with the transport of BDNF. Furthermore, whether mutant Htt interferes with the transport of mature BDNF or pro-BDNF remains

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to be investigated (32). Here, we demonstrate that HAP1 associates with the prodomain of BDNF and is required for the intracellular trafficking, axonal transport, and activity-dependent secretion of pro-BDNF. Moreover, polyQ-expanded Htt intracellular trafficking, axonal transport, and activity-dependent secretion of pro-BDNF. Moreover, polyQ-expanded Htt

EXPERIMENTAL PROCEDURES

Animals and Human Brain Tissue—All procedures involving animals were approved by the Animal Welfare Committee of Flinders University and undertaken according to the guidelines of the National Health and Medical Research Council of Australia. HAP1 knock-out mice were generated previously (35). All animals were kept under standardized barrier breeding conditions (12-h light/12-h dark cycle) with free access to water and food. Brain samples from HD cases (2 males, age 75 and 73) and control cases (2 males, age 68 and 73) were obtained from the Flinders University Brain Bank and approved by Flinders Human Ethics Committee. The CAG repeat length was identified using the following primer: CTACGAGTCCCTCAAGTC-CTTCCAGC and GACGCGACGGCGGCTGCTG. PCR genotyping of HAP1 knock-out mice were carried out as described (35).

Preparation of Pro-BDNF (Full Length), the Prodomain of BDNF (Prodomain Fragment 1–130), and V66M Prodomain BDNF Recombinant Proteins—For preparation of pET100/D-TOPO pro-BDNF, pET100/D-TOPO prodomain, and pET100/D-TOPO-V66M prodomain, the cDNA fragments of rat pro-BDNF (749 bp), the prodomain (423 bp), and the V66M prodomain (423 bp) were amplified by *Pfu* Turbo DNA polymerase (Stratagene) from AApro-BDNF (amino acids 129 and 130, RR to AA point mutation to generate pro-BDNF furin-resistant recombinant protein) or V66M proBDNF-EGFP constructs (gifts from Dr. Masami Kojima, Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, Ikeda, Osaka, Japan) by the following PCR primers (GeneWorks Pty Ltd): prodomain (forward) 5′-CACCACCATCTTTTCCTAC-TATG, and prodomain (reverse) 5′-CTAGCGCCGAAACCTT-CATAAG; pro-BDNF (reverse) 5′-CTACCTTCCCCTTT-TAATGGT-3′. Pro-BDNF fragment, wild type, and V66M prodomains were amplified by using corresponding primers. The amplified fragments were subcloned in-frame into pET100/D-TOPO by following the instruction manual of Champion pET Directional TOPO expression kit (Invitrogen), and the sequences of the final constructs were verified by DNA sequencing.

The plasmids pET100/D-TOPO-AApro-BDNF, pET100/D-TOPO-prodomain BDNF, and pET100/D-TOPO-V66M prodomain were transformed into *Escherichia coli* BL21, and the proteins were expressed according to the protocols provided by the manufacturer (Invitrogen). Briefly, after overnight culture in isopropyl β-d-thiogalactopyranoside, the bacteria were harvested, and the pellet was resuspended in binding buffer, lysed, and sonicated. Inclusion bodies were then collected, washed, and solubilized in 8 M urea solution. The proteins were purified using nickel column chromatography. The final protein concentration was assayed using BCA™ protein assay kit (Pierce). For the assay of full-length pro-BDNF, supernatant lysate was used without urea such that naturally refolded protein was affinity-purified on the nickel column. The mature BDNF recombinant protein expressed from *E. coli* was obtained from PeproTech (Rocky Hill, NJ). All recombinant proteins were characterized by SDS-PAGE with Coomassie Blue staining and Western blot analysis. An example of the prodomain SDS gel is shown in supplemental Fig. S1. Mature BDNF was a gift from Regeneron.

Preparation of BDNF-binding Proteins from the Rat Brain Homogenate, Two-dimensional DIGE, Two-dimensional SDS-PAGE, and Mass Spectrometry Analysis—Rat brains samples were homogenized in RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris, 1 mM EDTA) containing protease inhibitors (protease inhibitor mixture, Roche Applied Science), and the crude homogenate was centrifuged for 20 min at 5,000 × g. The supernatant was added to the columns of the prodomain and mature BDNF, which were immobilized on CNBr-activated Sepharose™ 4B beads based on the manufacturer’s instructions (Amersham Biosciences). The columns were washed using gravity flow with washing buffer A (56 mM NaH2PO4, 144 mM Na2PO4, 2 mM NaCl, pH 7.2, plus 1% Tween 20) and then with washing buffer B (56 mM NaH2PO4, 144 mM Na2PO4, 1 mM NaCl, pH 7.2) to remove the detergent (Twen 20). The elution buffer (buffer C, 0.1 M glycine-HCl, pH 2.5) was then added to the columns. The elution fractions were collected, and the pH was adjusted immediately to 7.2 with 1 M Tris base. The proteins were dialyzed using distilled water at 4 °C. Each protein preparation was cleaned by using PlusOne two-dimensional clean-up kit (GE Healthcare) and quantified by using PlusOne Two-dimensional Quan Kit (GE Healthcare).

For sample labeling, 50 μg of protein fraction from each column was labeled with 400 pmol of cyanine dyes, Cy3 for the prodomain-binding proteins, and Cy5 for the mature BDNF-binding proteins according to a standard protocol. The labeled samples were mixed to allow the gel to contain 50 μg each of Cy3- and Cy5-labeled samples, respectively. For the first dimension separation, the labeling mixture was applied to Immobiline™ DryStrips (13 cm, pH 3–11 linear) by cup loading with a total running time of 55 kV·h of isoelectric focusing. The second dimension was carried out with 10% SDS-polyacrylamide gels, and gel images were subsequently acquired at the recommended wavelengths by using a Typhoon™ Variable Mode imager (GE Healthcare).

For identification of the separated proteins, 100 μg of prodomain-binding proteins were separated in two-dimensional SDS-PAGE as above. After the two-dimensional electrophoresis, the gel was stained with SYPRO Ruby. The spots on the gel were analyzed using a PE Biosystems MALDI-TOF Voyager-DE STR mass spectrometer. For the data base searches, the peptides were selected in the mass range of 750–900 kDa and evaluated using the ExPasy server.

Pulldown Assay, Subcellular Fractionation, and Immunoprecipitation of Human Brain Samples—Five hundred ng of recombinant protein (pro-BDNF, the prodomain, mature BDNF) were incubated with 4 μg of HAP1-GST proteins together with glutathione-Sepharose beads at 4 °C for 2 h without or
with the presence of an equal quantity of PC12 cell lysate transfected with Htt-23Q or Htt-103Q (gift from E. S. Schweitzer, Departments of Physiological Science and Neurology, Brain Research Institute, UCLA) or with the same quantity of normal or HD human brain lysates. The beads were washed three times and boiled in loading buffer to release the bound proteins. Resolved by SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) and detected by immunoblotting using rabbit anti-HAP1 antibody (gift from M. DiFiglia) and rabbit anti-prodomain (25, 36).

Subcellular fractions of wild type and HAP1−/− mice cortex were prepared essentially as described (37, 38). The SDS-polyacrylamide gels were loaded with equal amounts of proteins from each fraction (65 μg per lane) to conduct Western blotting using sheep anti-HAP1 (raised against recombinant HAP1) and rabbit anti-pro-BDNF.

For immunoprecipitation experiments, human brains were lysed in RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris, 1 mM EDTA) containing protease inhibitors (protease inhibitor mixture, Roche Applied Science), and the crude homogenate was centrifuged for 20 min at 5,000 × g. The supernatants were pre-cleared by incubation with an excess of protein A + G-agarose. The protein lysates (1 mg) were incubated with 2 μg of HAP1 antibody immobilized in 25 μl of protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. The immunoprecipitation products and exogenous prodomain and mature BDNF as positive controls were detected by Western blotting using rabbit anti-prodomain antibody (36), mouse anti-mature BDNF, rabbit anti-sortilin (Abcam), and or a sheep anti-HAP1 antibody.

Sciatic Nerve Crush—All the experiments were done on postnatal day 1. When the mouse was motionless on ice, an incision 0.5 cm long was made in the left thigh, and the left sciatic nerve was exposed and crushed at the mid-thigh level for 10 s with a pair of fine forceps. The skin was closed, and animals were kept on a warm blanket for recovery. After recovery, the neonates were returned to their mother. Six hours after sciatic nerve crush, puppies were anesthetized on ice again and killed by perfusion through the heart with 200 ml of 0.1% heparin. The thoracic cavity was opened, the heart was removed, and the sciatic nerve was exposed and crushed at the mid-thigh level for 20 min at 4 °C.

Sciatic Nerve Crush—All the experiments were done on postnatal day 1. When the mouse was motionless on ice, an incision 0.5 cm long was made in the left thigh, and the left sciatic nerve was exposed and crushed at the mid-thigh level for 10 s with a pair of fine forceps. The skin was closed, and animals were kept on a warm blanket for recovery. After recovery, the neonates were returned to their mother. Six hours after sciatic nerve crush, puppies were anesthetized on ice again and killed by perfusion through the heart with 4% paraformaldehyde. The sciatic nerve was dissected and processed for immunohistochemistry.

Pro-BDNF Immunohistochemistry—Antibodies to pro-BDNF were generated by immunization of rabbits with synthetic peptide, corresponding to the 14 amino acids of the prodomain sequence of pro-BDNF (MTILFLTMVISYFG), which were conjugated to keyhole limpet hemocyanin. This antibody recognizes endogenous pro-BDNF, recombinant pro-BDNF, and recombinant prodomain. Its immunoreaction specificity was verified after affinity purification by reaction with the immunizing antigen and characterized by Western blotting and immunohistochemistry with the negative staining of brain sections of BDNF−/− mice as reported (36). The immunohistochemistry for pro-BDNF on sciatic nerve and cultured neurons was performed as described previously (25, 36).

ELISA—Constitutive and regulated secretion of pro-BDNF from cultured cortical neurons was examined as described (23). The pro-BDNF protein concentrations in the respective medium samples were determined using the pro-BDNF ELISA system with the recombinant prodomain as a standard.

RESULTS

HAP1 Associates with Pro-BDNF, the Prodomain, but Not Mature BDNF—To investigate mechanisms underlying the axonal transport of BDNF, we attempted to identify the proteins that interact with BDNF. To do this, we first generated recombinant prodomain and mature BDNF (Fig. 1A) for affinity purification of interacting proteins from the rat brain. Proteins bound to the prodomain and mature BDNF were labeled by cyanine dyes Cy3 and Cy5, respectively, and resolved by two-dimensional DIGE. The results showed that the proteins interacting with mature BDNF from the rat brain are obviously different from those interacting with the prodomain (Fig. 1B), as most proteins detected from the two sets of samples are not colocalized and displayed different colors.

To further identify which proteins interact with the prodomain, the protein samples isolated from the prodomain affinity column were subjected to two-dimensional SDS-PAGE, and the protein spots were determined by MALDI-TOF Voyager-DE STR mass spectrometer and data base search. One of these proteins was HAP1. To confirm the result, the prodomain-binding proteins were analyzed by Western blotting using anti-HAP1 rabbit antibody. Western blot confirmed that HAP1 was present in the protein fraction isolated from the prodomain column (Fig. 1C).

To strengthen the finding that HAP1 associates with pro-BDNF and to test whether HAP1 directly interacts with BDNF fragments, we performed GST pulldown experiments. The pulldown assay showed that the HAP1 fragment (280–445 amino acids) could bind both the prodomain and full-length pro-BDNF but not mature BDNF (Fig. 1D). Quantitative analysis showed 56% pro-BDNF and 84% prodomain were pulled down by HAP1-GST (Fig. 1E).

Mutant Huntingtin Decreases the Association of HAP1 with Pro-BDNF—Growing evidence has shown that mutant Htt affects the axonal transport of BDNF (32, 34, 39, 40), but the
BDNF or HAP1 and prodomain in the presence of cell lysates of wild type Htt (Fig. 2). This finding is in agreement with the transfected PC12 cell lysates, although less protein amount from the brain lysates than from PC12 cells was added in the incubation mixture.

To further confirm the interaction between HAP1 and pro-BDNF, we immunoprecipitated HAP1-binding proteins from normal human brain (28 CAGs and 32 CAGs) or Huntington disease human brain (94 CAGs and 82 CAGs heterozygous HD) by the pulldown experiments. The result showed that the pull-down of pro-BDNF and the prodomain in the presence of HD human brain cell lysates was significantly decreased compared with the normal human brain cell lysates (Fig. 2C). The quantitative data showed the pulldown of pro-BDNF in the presence of HD brain lysate was decreased to 23.6% and the prodomain to 28.7% of normal brain lysates (Fig. 2D). This finding is in agreement with the transfected PC12 cell lysates, although less protein amount from the brain lysates than from PC12 cells was added in the incubation mixture.

Pro-BDNF, HAP1, and Sortilin Are Associated in the Human Brain, and the Association Is Reduced in Huntington Disease Brain—To further confirm the interaction between HAP1 and pro-BDNF, we immunoprecipitated HAP1-binding proteins from human brain homogenates with an antibody to HAP1. We also tested whether the association is altered in the brain homogenate from HD. As shown in Fig. 3, there are several species of pro-BDNF detected in the HAP1-immunoprecipitated sample at molecular masses of 32 and 64 kDa of normal human brain. The bands did not appear when the membrane was probed with the antibodies pre-absorbed with recombinant pro-BDNF protein. The amount of pro-BDNF in the complex immunoprecipitated from the HD brain was significantly reduced (Fig. 3, A and B). Quantification results showed that amount of pro-BDNF immunoprecipitated from HD brain was 66.9% (32-kDa band) and 51.7% (64-kDa band) of the normal brain, when probed with rabbit anti pro-BDNF, and was 59.3% (32 kDa) and 51.7% (64 kDa) of the normal brain, when probed with rabbit anti pro-BDNF, and was 59.3% (32 kDa) and 51.7% (64 kDa) of the normal brain, when probed with rabbit anti pro-BDNF, and was 59.3% (32 kDa) and 51.7% (64-kDa band) of the normal brain, when probed with rabbit anti pro-BDNF, and was 59.3% (32 kDa) and 51.7% (64-kDa band) of the normal brain, when probed with rabbit anti pro-BDNF, and was 59.3% (32 kDa) and 51.7% (64-kDa band) of the normal brain.
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51.6% (64 kDa) of normal brain when probed with monoclonal anti-mature BDNF antibody (Fig. 3B). For positive controls, we also loaded the prodomain and mature BDNF, respectively. As shown in Fig. 3A, the pro-BDNF antibody, which against the prodomain, did not recognize mature BDNF, and the mature BDNF antibody did not recognize the prodomain. However, both antibodies recognized the endogenous full-length pro-BDNF. The multiple bands in Fig. 3A, lane 1, are likely dimers and oligomers of the prodomain. The SDS gel showed that the fresh purified prodomain was a single band with 95% purity and formed oligomers in 1 week at 4 °C (supplemental Fig. S1), but Western blot analysis confirmed that the purified prodomain monomer forms dimers and oligomers with the time (1 week) at 4 °C in water (supplemental Fig. S1, A and B). We detected minor bands (Fig. 3A, lanes 3 and 4) with the higher molecular weight detected by the pro-BDNF antibody but not by the mature BDNF antibody. These bands disappeared when the antibody was pre-incubated with an excessive amount of recombinant pro-BDNF.

It is well known that HAP1 is associated with vesicles as demonstrated by electron microscopic studies and by sucrose gradient fractionations (44, 45). HAP1 may play roles in trafficking of pro-BDNF by interacting with sortilin, which also interacts with the prodomain of BDNF (23). To test the possibility, we probed the HAP1 immunoprecipitated complex from the

human brain with a sortilin antibody. We found that sortilin was present in the HAP1-associated complex from the human brain (Fig. 3A). Interestingly, less sortilin (73%) was immunoprecipitated from the brain of HD than from the control (Fig. 3B).

Effects of the V66M Mutation on the Interaction between HAP1 and the BDNF Prodomain—20–30% of humans have an allele of the V66M polymorphism in the prodomain of the BDNF gene (46). The polymorphism is known to cause the reduction of axonal BDNF transport and activity-dependent release, leading to a reduction in the volume of the hippocampus and development of various mental disorders (23, 47, 48). The mechanism underlying the reduction of BDNF transport is not known. Here, we examined whether the V66M mutation affects the interaction between HAP1 and the prodomain. As shown in Fig. 4, A and B, compared with the WT prodomain, the amount of V66M prodomain pulled down by HAP1 was significantly reduced (84 versus 60%). The reduction in the interaction between HAP1 and V66M prodomain was further reduced by incubation in the presence of expanded polyQ Htt in PC12 cell lysates (46 versus 19%) or in HD brain lysates (28.7 versus 13%). These results indicate that the V66M mutation in the prodomain can reduce the interaction with HAP1, which may cause the reduction in pro-BDNF transport and release.

Colocalization of Pro-BDNF and HAP1 in Transfected Cells and Neurons—To see whether HAP1 is colocalized with pro-BDNF, we carried out three different experiments (Fig. 5). First, we cotransfected two different sets of plasmids conjugated with different fluorescent markers in PC12 cells. Through Live Cell
The interactions between HAP1 and pro-BDNF were examined in transfected PC12 cells. HAP1-A (green fluorescence) was highly colocalized with pro-BDNF (red fluorescence) (Fig. 5A). The colocalization was quantitatively analyzed using a Leica SP5 confocal microscope. The data presented as mean ± S.E. (n = 20). C, double labeling of pro-BDNF and HAP1 in cultured cortical neurons from wild type and HAP1−/− mice. Pro-BDNF is stained in red and HAP1 in green. No HAP1 was stained in HAP1−/− neurons. Pro-BDNF and HAP1 are highly colocalized in the cytoplasm and neurites in wild type neurons. In HAP1−/− neurons, pro-BDNF is only present in soma. Phase contrast images showed cell bodies and neurites of wild type and HAP1−/− neurons.

In vivo experiments in sciatic nerve, as BDNF is synthesized and transported in nerve terminals from the cell body to the neuronal process. The in vitro results were further confirmed by our in vivo experiments in sciatic nerve, as BDNF is synthesized and transported in nerve terminals from the cell body to the neuronal process.
with immunized peptide or in brain sections of BDNF knockout mice (16, 25).

HAP1 Plays a Critical Role in Activity-dependent Secretion of Pro-BDNF—To assess whether HAP1 affects regulated and constitutive secretory pathways, we performed two series of experiments on the constitutive and regulated secretion in primary cultured cortical neurons from P1 neonatal wild type (wt) and HAP1<sup>−/−</sup> mouse cortex. A subcellular fractionation of sucrose gradient from P1 neonatal wild type (wt) and HAP1<sup>−/−</sup> mouse cortex were detected by Western blot using sheep anti-pro-BDNF and rabbit anti-HAP1. Equal amounts of protein (65 μg) were loaded in each lane. B, pro-BDNF immunohistochemistry of primary cultured cortical neurons from P1 neonatal wt mouse. C, higher magnification view of the field highlighted in B. D, pro-BDNF immunohistochemistry of primary cultured cortical neurons from P1 neonatal HAP1<sup>−/−</sup> mouse (scale bar, 15 μm). E, higher magnification view of the field highlighted in D. F, photomicrograph of a crushed sciatic nerve section stained for pro-BDNF from a P1 neonatal wt mouse. G, photomicrograph of a crushed sciatic nerve section stained for pro-BDNF from a P1 neonatal HAP1<sup>−/−</sup> mouse. H, quantitative data on the relative staining of pro-BDNF in the sciatic nerve. Y axis stands for the relative amount quantified by the ImageJ program. The data are presented as mean ± S.E., n = 3. **, p < 0.01, Student’s t test, scale bar, 15 μm. I, high magnification of micrograph of the proximal segment of crushed nerve stained for pro-BDNF from a P1 neonatal wt mouse; scale bar, 60 μm. J, high magnification of micrograph of the proximal segment of crushed nerve stained for pro-BDNF from a P1 neonatal HAP1<sup>−/−</sup> mouse.

HAP1 Directly Binds to Pro-BDNF—Defects in BDNF transport and secretion may underlie a wide range of nervous system diseases, such as epilepsy, Rett syndrome, neurodegenerative diseases, and neuropsychiatric diseases (32, 51–55). However, how BDNF-containing vesicles are transported is not known. Substantial evidence suggests that the anterograde transport of BDNF is dependent on the prodomain sequence. Sortilin interacts with the prodomain of BDNF fused with mature nerve growth factor (BDNF) and sorts pro-BDNF into the regulated pathway (23). Nerve growth factor is normally released constitutively from dendrites of neurons (56–58), but nerve growth factor chimeric molecule (the prodomain of BDNF fused with mature nerve growth factor) can be sorted into the regulated pathway and released in an activity-dependent manner (23). The neuronal

![Figure 6. HAP1 is required for the transport of pro-BDNF. A, subcellular fractions of sucrose gradient from P1 neonatal wild type (wt) and HAP1<sup>−/−</sup> mouse cortex were detected by Western blot using sheep anti-pro-BDNF and rabbit anti-HAP1. Equal amounts of protein (65 μg) were loaded in each lane. B, pro-BDNF immunohistochemistry of primary cultured cortical neurons from P1 neonatal wt mouse. C, higher magnification view of the field highlighted in B. D, pro-BDNF immunohistochemistry of primary cultured cortical neurons from P1 neonatal HAP1<sup>−/−</sup> mouse (scale bar, 15 μm). E, higher magnification view of the field highlighted in D. F, photomicrograph of a crushed sciatic nerve section stained for pro-BDNF from a P1 neonatal wt mouse. G, photomicrograph of a crushed sciatic nerve section stained for pro-BDNF from a P1 neonatal HAP1<sup>−/−</sup> mouse. H, quantitative data on the relative staining of pro-BDNF in the sciatic nerve. Y axis stands for the relative amount quantified by the ImageJ program. The data are presented as mean ± S.E., n = 3. **, p < 0.01, Student’s t test, scale bar, 15 μm. I, high magnification of micrograph of the proximal segment of crushed nerve stained for pro-BDNF from a P1 neonatal wt mouse; scale bar, 60 μm. J, high magnification of micrograph of the proximal segment of crushed nerve stained for pro-BDNF from a P1 neonatal HAP1<sup>−/−</sup> mouse.]

DISCUSSION

BDNF is sorted into the regulated secretory pathway, anterogradely transported to presynaptic nerve terminals, released in an activity-dependent manner, and plays a critical role in synaptic plasticity (49, 50). The activity-dependent secretion of BDNF is critical for various forms of synaptic plasticity and the long term regulation of synaptic structure and function. In this study, we have provided mechanistic insight into how BDNF is transported anterogradely along axons.
transport of BDNF is likely mediated by its interacting proteins. In this study, the DIGE experiments and Western blot results demonstrated that HAP1 associates with the prodomain. Further interaction studies using GST pulldown assays with recombinant proteins confirmed that the prodomain associates with a HAP1 fragment (amino acids 280–445).

**Mutant Htt Affects the Association of HAP1 with Pro-BDNF**—A large body of evidence suggests that mutant Htt abnormally interacts with HAP1. One of the pathophysiological mechanisms underlying neuronal death in HD is that mutant Htt down-regulates the expression and transport of BDNF. Several studies have already indicated that wild type Htt is an anti-apoptotic protein that could enhance intracellular transport of BDNF, but mutant Htt causes retardation of BDNF transport along microtubules (32, 59) and impairs the post-Golgi trafficking of WT BDNF but not V66M BDNF (60). However, how mutant Htt impairs the axonal transport and post-Golgi trafficking of BDNF is not known. We showed that mutant Htt significantly reduced the association of HAP1 with pro-BDNF (Fig. 2), suggesting that mutant Htt may disrupt pro-BDNF transport by interfering with the interaction between the prodomain and its cargo-carrying molecule HAP1. Further supporting this notion is the evidence that HAP1 in the brain of HD patients binds less pro-BDNF than that in the normal human brain (Fig. 3). It should be pointed out that mutant htt also affects the transcription of BDNF (40). Thus, the reduction in BDNF pulldown from HD brain could also be due to reduced expression of pro-BDNF.

**V66M Polymorphism Reduces Its Association with HAP1**—In human, 20–30% individuals carry the mutation of a valine to methionine substitution at codon 66 in the prodomain (46). The previous studies showed that the V66M BDNF polymorphism may associate with several neurodegenerative diseases (30, 61). The V66M mutation leads to the reduction in activity-dependent secretion and impairs the intracellular trafficking of BDNF without affecting the constitutive release of BDNF (26, 61). This mutation in human also causes the reduction in hippocampal volume (28) and affects hippocampal processing of episodic learning and memory (27). The phenomenon suggests that the impairment of activity-dependent secretion of BDNF is responsible for dysfunction in hippocampal plasticity and for a number of human psychological disorders (62). However, how the mutation results in reduction in activity-dependent secretion and transport is not clear. In this study, we showed that the mutation form of the prodomain reacts with HAP1 with less efficiency compared with the wild type form. Our data suggest that the mutation may reduce its association with HAP1. We also showed that the combination of V66M and mutant Htt further reduces the association of the prodomain with HAP1.

BDNF intracellular distribution can also be affected by its mRNA localization and trafficking. The BDNF mRNA transcript with long 3′-untranslated region tends to go to dendrites, whereas the transcript with short 3′-untranslated region is restricted to the soma (63). A recent study shows that the dendritic targeting of the long 3′-untranslated region transcript is regulated by Translin, an RNA-binding protein implicated in mRNA trafficking, and the G196A polymorphism, which results in V66M substitution and impairs the interaction between translin and BDNF oligonucleotides (64). Whether HAP1 also regulates BDNF mRNA dendritic targeting remains to be determined.

**HAP1 May Play a Role in Intraneuronal Trafficking, Axonal Transport, and Activity-dependent Release of BDNF**—HAP1 is an Htt-associated protein and interacts with the dynactin subunit, p150Glued (65), which is the accessory protein of dynein motor (66–68), and the kinesin light chain (69), which is a subunit of the kinesin motor (68, 70). As it is known that the dynein motor carries cargos retrogradely and the kinesin motor carries cargos anterogradely along axons (68), HAP1 may be the BDNF cargo-carrying molecule for these motors. Several lines of evidence support the idea that HAP1 is a BDNF cargo-carrying molecule. Cotransfection studies showed that HAP1 is highly colocalized with the prodomain of BDNF. Endogenous pro-BDNF is also highly colocalized with HAP1 in cultured neurons. Biochemical analysis of brain homogenate from wild type mice showed that BDNF is present in all precipitated components with different spin forces and is associated with the fraction of vesicles in which HAP1 is also present. However, in
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HAP1 knock-out mice, the normal distribution pattern of BDNF is altered (Fig. 6), and BDNF is not detectable in the vesicle fraction (LP2). Also, BDNF is not in the pellet of moderate speed centrifugation (P2) but was increased in P1, S1, and P3 fractions in the absence of HAP1. Immunohistochemical data showed that pro-BDNF was present in the neuritic processes (dendrites and axons) of most cortical neurons from wild type mice but not in those of neurons from HAP1 mutant mice. The data suggest that BDNF-containing vesicles fail to reach dendrites and axons when HAP1 is absent.

In support of the above idea, the transport of endogenous pro-BDNF in the sciatic nerve in HAP1 knock-out mice is abolished. Sciatic nerve is an ideal model to examine BDNF transport, as the BDNF is highly expressed in primary sensory neurons and transported anterogradely and retrogradely within the sciatic nerve (16, 25). Our data showed that the inactivation of the HAP1 gene completely blocks the transport of endogenous pro-BDNF in the sciatic nerve and in the processes of cortical neurons. These data indicate that HAP1 may be essential for the transport of pro-BDNF. Interestingly, both anterograde and retrograde transports are abolished in these animals, suggesting that HAP1 may not only be essential for carrying BDNF forward by the kinesin motor through interacting with its prodomain but also important for carrying the receptor-mediated internalized cargos of pro-BDNF to the cell body.

Previous studies showed that HAP1 is associated with secretory vesicles (44) and plays a role in protein trafficking and vesicular transport (34). It is likely that HAP1 is present both inside and outside vesicles. Biochemical and electron microscopic study of adult mouse basal forebrain and striatum showed that HAP1 is preferentially enriched in membrane fractions and localized to membrane-bound organelles, including large endosomes, tubulovesicular structures, and budding vesicles in neurons. HAP1 was also strongly associated with large dense organelles and within vesicles in dendrites (44, 45). Pro-BDNFs may directly interact with HAP1 within the vesicles and/or indirectly interact with pro-BDNF via sortilin, which is the type I transmembrane receptor for pro-BDNF (13, 15) and also interacts with pro-BDNF within vesicles (23). We provided evidence by HAP1 immunoprecipitation from the human brain that HAP1 is associated with sortilin, and the association was reduced in the HD brain (Fig. 3).

Consistent with the axonal transport data, the activity-dependent release of pro-BDNF is largely abolished in HAP1−/− mice, whereas the constitutive release is not affected. Our data suggest that the activity-dependent release but not the constitutive release of pro-BDNF may require the function of HAP1. As BDNF is critical for the survival and differentiation of many groups of neurons in the brain, the impairment of axonal transport and activity-dependent release of BDNF in HAP1−/− mice may underlie the death of neurons in the hypothalamus in these animals (35, 71) and explain the function of HAP1-A in neurite outgrowth (72, 73).

In summary, our findings suggest a new mechanism underly- ing the intraneuronal trafficking, axonal transport, and release of pro-BDNF via association with HAP1. Deletion of HAP1 diminishes the intraneuronal trafficking, axonal transport, and activity-dependent release of pro-BDNF. We also showed that mutant Het and V66M polymorphism reduce the association of HAP1 with pro-BDNF. Thus, we conclude that HAP1 may play a role in the transport and release of pro-BDNF and that the reduced association of HAP1 with the prodomain may underlie the decreased transport and release of BDNF in HD and V66M polymorphism.

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