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Mutant Huntingtin Impairs BDNF Release from Astrocytes by Disrupting Conversion of Rab3a-GTP into Rab3a-GDP

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Brain-derived neurotrophic factor (BDNF) is essential for neuronal differentiation and survival. We know that BDNF levels decline in the brains of patients with Huntington’s disease (HD), a neurodegenerative disease caused by the expression of mutant huntingtin protein (mHtt), and furthermore that administration of BDNF in HD mice is protective against HD neuropathology. BDNF is produced in neurons, but astrocytes are also an important source of BDNF in the brain. Nonetheless, whether mHtt affects astrocytic BDNF in the HD brain remains unknown. Here we investigated astrocytes from HD140Q knock-in mice and uncovered evidence that mHtt decreases BDNF secretion from astrocytes, which is mediated by exocytosis in astrocytes. Our results demonstrate that mHtt associates with Rab3a, a small GTPase localized on membranes of dense-core vesicles, and prevents GTP-Rab3a from binding to Rab3-GAP1, disrupting the conversion of GTP-Rab3a into GDP-Rab3a and thus impairing the docking of BDNF vesicles on plasma membranes of astrocytes. Importantly, overexpression of Rab3a rescues impaired BDNF vesicle docking and secretion from HD astrocytes. Moreover, ATP release and the number of ATP-containing dense-core vesicles docking are decreased in HD astrocytes, suggesting that the exocytosis of dense-core vesicles is impaired by mHtt in HD astrocytes. Further, Rab3a overexpression reduces reactive astrocytes in the striatum of HD140Q knock-in mice. Our results indicate that compromised exocytosis of BDNF in HD astrocytes contributes to the decreased BDNF levels in HD brains and underscores the importance of improving glial function in the treatment of HD.

Key words: BDNF; exocytosis; glial cell; Huntington’s disease; Rab3

Significance Statement
Huntington’s disease (HD) is an inherited neurodegenerative disorder that affects one in every 10,000 Americans. To date, there is no effective treatment for HD, in part because the pathogenic mechanism driving the disease is not fully understood. The dysfunction of astrocytes is known to contribute to the pathogenesis of HD. One important role of astrocytes is to synthesize and release brain-derived neurotrophic factor (BDNF), which is vital for neuronal survival, development, and function. We found that mutant huntingtin protein (mHtt) at the endogenous level decreases BDNF secretion from astrocytes by disrupting the conversion of GTP-Rab3a into GDP-Rab3a and that overexpressing Rab3a can rescue this deficient BDNF release and early neuropathology in HD knock-in mouse brain. Our study suggests that astrocytic Rab3a is a potential therapeutic target for HD treatment.
(Zuccato et al., 2001) and its axonal transport from cortical neu-
rons to the striatum (Gauthier et al., 2004), implicating neuronal
BDNF dysfunction in HD pathogenesis. Astrocytes are also an
important source of BDNF (Miyamoto et al., 2015). BDNF is
released from astrocytes via exocytosis to support the neighbor-
ning neurons and regulate their function (Wu et al., 2008; Parpura
and Zorec, 2010; Quessevre et al., 2013). However, whether
mHtt affects astrocytic BDNF production or secretion in the HD
brain remains unknown. A previous study found that overex-
pressing mHtt in cultured astrocytes decreased BDNF levels by
sequestering BDNF transcriptional factors into mHtt aggregates
(Wang et al., 2012). Considering that very few mHtt aggregates
are formed in astrocytes in the HD brains that express mHtt at the
endogenous level (Shin et al., 2005), we wanted to investigate whether
mHtt at the endogenous level can affect BDNF production
or secretion in astrocytes.

We used both full-length HD140Q knock-in (KI) and GFAP-
Htt transgenic (TG) mouse models to investigate the effect of
mHtt on astrocytic BDNF. In these mouse models, full-length
mHtt with 140Q is expressed at the endogenous level in knock-in
mice (Menalled et al., 2003), and N-terminal Htt (1–208 aa) with
160Q is expressed at levels similar to endogenous Htt (Bradford
et al., 2009). Using primary astrocytes and brain-slice cultures, we
found that secreted BDNF from HD astrocytes is reduced, and
that this reduction stems from impaired docking of BDNF vesicles
attributable to an abnormal association between mHtt and
Rab3a in HD astrocytes. More importantly, overexpression of
Rab3a improves docking of BDNF-containing vesicles and BDNF
release from HD astrocytes. Moreover, overexpression of Rab3a
ameliorated reactive astrocytes in the striatum of full-length
HD140Q knock-in mice, which is an early HD pathology. Our
findings suggest a new mechanism behind the reduced BDNF
levels in HD brains and indicate the importance of improving the
function of astrocytes in the treatment of HD.

Materials and Methods

Animals. KI mice were kindly provided by Dr. Michael Levine of the
University of California, Los Angeles (Hickey et al., 2008). TG mice with
160Q were generated previously by our laboratory (Bradford et al.,
2009). Mice were maintained at the Emory University animal facility.
Both male and female pups from these mice were used for primary cul-
tures. Male adult mice of different ages were used for viral injection and
brain-slice preparation. This study was performed in strict accordance
with the recommendations in the Guide for the Care and Use of Labora-
tory Animals of the National Institutes of Health. The protocol was ap-
proved by the Committee on the Ethics of Animal Experiments of Emory
University (permit number 2002557).

Antibodies and reagents. Antibodies used were anti-expanded polyQ
(1C2; Millipore, MAB1574), anti-Htt (mEM48), anti-Rab3a (BD Biosci-
ences, 610379), anti-GFP-Rab3a (NewEast Biosciences, 26920), anti-
Rab3-GAP (Proteintech, 21663-1-AP), anti-V5 (Life Technologies,
46-0705), anti-TrkB (Cell Signaling Technology, 80E3), anti-phospho-
TrkB (Epitomics, 2149-1), anti-BDNF (Santa Cruz Biotechnology,
sc-546), anti-actin (Sigma-Aldrich, A5060), anti-GFAP (Millipore,
MAB360), and anti-NeuN (Millipore, ABN78). Secondary antibodies
were HRP-labeled donkey anti-mouse, donkey anti-rabbit, donkey anti-
mouse Alexa Fluor 488 or 594, and donkey anti-rabbit Alexa Fluor 488 or
594 from Jackson ImmunoResearch. Proteinase inhibitor mixture, phor-
bol 12-myristate 13-acetate (PMA), ATP Bioluminescent Assay Kit, and
quinacrine dihydrochloride were purchased from Sigma-Aldrich.
Rab3-V5 adenovirus was purchased from SignaGen Laboratories
(SL174810). The Glutamate Assay Kit was purchased from BioVision.

Primary cell cultures. Both male and female brains of postnatal (day
1–3) murine pups were used for culturing cortical astrocytes. Following
dissection, the cortex was subjected to 0.3 mg/ml papain digestion. Cell
suspension was filtered through 70 µm nylon cell strainers ( Fisher Sci-
cientific). Microglia and oligodendrocytes were removed from cultures at
DIV 14 by shaking. Remaining cells were detached with 0.25% trypsin
and plated for the following experiments. For cortical neuron cultures, cortical neurons were prepared from both male and female postnatal day
0 murine pups. The cortex was digested with 0.3 mg/ml papain. Cell
suspension was filtered through 40 µm nylon cell strainers ( Fisher Sci-
cientific) to remove debris. Neurons were plated at a density of 1 × 10^5
on poly-a-lysine-coated six-well plates and cultured in Neurobasal-A me-
sium supplemented with B27 and glutamine (Invitrogen).

Preparation of astrocyte-conditioned medium. Primary astrocytes (DIV
25) were cultured at a density of 2 × 10^5 in six-well plates and treated
with 28 µm KCl for 30 min at 37°C. The culture medium was collected for
measuring released BDNF via ELISA.

ELISA. Levels of BDNF were determined by a BDNF ImmunoAssay
System (Promega) following the manufacturer’s protocol. Briefly, sam-
ple s or standards (100 µl) were added to 96-well plates, incubated at 4°C
overnight, and washed extensively, followed by a 2 h incubation with the
anti-BDNF antibody and a 1 h incubation with anti-IgY HRp conjugate
plus substrate for signal development. The absorbance was recorded at
450 nm on a plate reader (Microplate Reader, BioTek). The amount of
BDNF in each sample was calculated based on the standard curve pre-
pared in the same experiment.

Western blotting. Primary cultures or brain tissues were homogenized
in ice-cold NP-40 buffer containing a protease inhibitor mixture
(Thermo Fisher Scientific) and 100 µM PMSF. Samples were boiled for 5
min in SDS/β-mercaptoethanol protein loading dye and run on 4–12%
Tris-glycine gels purchased from Invitrogen (catalog #EC60385).
Pro-
teins were transferred to a nitrocellulose membrane in Tris-glycine buf-
fer. After blocking, blots were probed with Rab3a or other primary
antibodies overnight. The Western blots were developed using the ECL
Prime Chemiluminescence kit (GE Healthcare).

Gluotamate measurement. Glutamate release from astrocytes was deter-
mined by a Glutamate Assay Kit (BioVision) following the manufacturer’s
protocol. Briefly, each culture medium was diluted in the assay buffer. Sam-
ple s or standards (50 µl) were added to 96-well plates. Reaction Mix (100 µl)
was added to each well containing the glutamate standard and test samples.
Reactions were incubated for 30 min at 37°C and protected from light. Mea-
sured OD was at 450 nm in a microplate reader (BioTek). The amount of
glutamate in each sample was calculated based on the standard curve pre-
pared in the same experiment.

qRT-PCR. Total RNA was isolated from WT, TG, and KI astrocytes.
Reverse transcription reactions were performed with 1.5 µg of total RNA
using the Superscript III First-Strand Synthesis System (Invitrogen). One
microliter of cDNA was combined with 10 µl SYBR Select Master Mix
(Applied Biosystems) and 1 µl of each primer in a 20 µl reaction. The
reaction was performed in a thermal cycler (Eppendorf, RealPlex
Mastercycler).

Knockdown assay. Rab3a siRNA duplexes and negative control siRNA
were purchased from OriGene (SR402766). The following combination of
oligonucleotides was used to target the Rab3a gene: CGACUAUAUGU
CAAGAUCCUGATC, GGAGUCAUUUAAUGCAGUGCAGGAC, CG
CACGUCUUGACUAAAGAAGAT.

Sterotaxic injection of virus. Male adult mice were used for Rab3-V5
adenoviral and control GFP adenoviral injections (TG, n = 4; WT, n = 4;
KI, n = 4). Heads of the animals were placed and fixed in a stereotaxic
frame (David Kopf Instruments, Model 1900) equipped with a digital
manipulator and a UMP3–1 Ultra pump. The mice were kept deeply
anesthetized as assessed by monitoring pinch withdrawal and respiration
rate. Animals were injected in the striatum (0.6 mm anterior to bregma,
2.0 mm lateral to the midline, 3.5 mm ventral to dura). The injections
were performed at a rate of 0.2 µl/ml. The needle was left in place for 10
min after each injection to minimize the upward flow of viral solution
after raising the needle. Rab3a-V5 was allowed to be expressed for 21–30
d in vivo before slicing the brains.

Preparation of brain slices. Brains from male mice were immersed in
chilled artificial CSF (ACSF), and then were cut with the vibratome into
250 µm coronal slices containing striatum (WT, n = 4; TG, n = 4;
N171-82Q, n = 4). To stimulate BDNF release from the brain slices, 56 mM KCl was added to the ACSF.

Rab3 GTPase activity assay. GST-Rab3a was loaded with 20 μCi of [γ-32P]-GTP (5000 Ci/mmol; GE Healthcare Pharmacia Biotech) in 40 μl of loading buffer (20 mM Tris, pH 7.6, 5 mM EDTA, and 0.1 mM DTT) for 15 min at room temperature. The loading reaction was stopped by adding 2 μl of 0.4 M MgCl₂. Five microliters of preloaded GTPase was diluted in assay buffer (20 mM Tris, pH 7.6, 1 mM GTP, 1 mg ml⁻¹ BSA, and 0.1 mM DTT) to yield a final volume of 50 μl. The total amount of [γ-32P]-GTP-loaded GTPase was measured by removing 5 μl of the loading sample (t₀) into 1 ml of ice-cold dilution buffer (50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM MgCl₂). WT or KI astrocyte lysates without Rab3-GAP1 were then added to the assay and incubated with [γ-32P]-GTP (5000 Ci/mmol; GE Healthcare Pharmacia Biotech) in 40 μl of sample was precleared with protein A agarose beads three times. Proteins from the immunoprecipitates and inputs were immunoprecipitated with 1C2 and anti-V5 antibodies, respectively, for 1 ha at 4°C. Ice-cold lysis buffer was used to wash the immunoprecipitates for 1 h at 4°C. Ice-cold lysis buffer was used to wash beads three times. Protein concentrations were measured with BCA assay (Thermo Fisher Scientific). A total of 500 μg of samples were precleared with protein A agarose beads (Sigma-Aldrich), and huntingtin and Rab3a-V5 proteins were immunoprecipitated with IC2 and anti-V5 antibodies, respectively, at 4°C overnight. Protein A agarose beads were added to capture the immunoprecipitates for 1 h at 4°C. Ice-cold lysis buffer was used to wash beads three times. Proteins from the immunoprecipitates and inputs were subjected to Western blotting.

Immunoprecipitation. Cells were harvested and lysed in ice-cold 0.5% Triton X-100/PBS solution with protease inhibitor mixture and 100 μl PMSF on ice. The lysates were centrifuged at 16,000 × g for 15 min. Protein concentrations were measured with BCA assay (Thermo Fisher Scientific). A total of 300 μg of samples were precleared with protein A agarose beads (Sigma-Aldrich), and huntingtin and Rab3a-V5 proteins were immunoprecipitated with IC2 and anti-V5 antibodies, respectively, at 4°C overnight. Protein A agarose beads were added to capture the immunoprecipitates for 1 h at 4°C. Ice-cold lysis buffer was used to wash beads three times. Proteins from the immunoprecipitates and inputs were subjected to Western blotting.

Immunofluorescence staining. Cultured astrocytes were fixed with 4% paraformaldehyde for 8–10 min. Mouse brains were sliced at 10 μm thickness with a cryostat at −20°C, mounted onto gelatin-coated slides, and fixed with 4% paraformaldehyde for 10 min. Fixed samples were blocked with 3% BSA/0.2% Triton X-100 for 30 min at room temperature. Following incubation of fixed samples with primary antibodies at 4°C overnight and washes, fluorconjugated secondary antibodies and Hoechst nuclear dye were added to the samples for staining. Images were acquired with an Imager Z1 microscope. Quantitative analysis of GFAP levels was performed using ImageJ. For each condition, 3 independent experiments were performed, and values were expressed as mean ± SEM. Statistical analysis was performed using Student’s *t* test, **p < 0.01, ***p < 0.001; ns, not significant. 

Figure 1. BDNF secretion from HD astrocytes is reduced. A, ELISA assay showed that cultured primary astrocytes from TG (Student’s *t* test, n = 6 independent experiments, p = 1.6381E-05) and KI (Student’s *t* test, n = 4 independent experiments, p = 0.0208) mice release less BDNF than astrocytes from WT mice. ELISA results also indicated that BDNF secretion is decreased from brain slices of TG mice compared with WT mice (Student’s *t* test, n = 3 independent experiments, p = 0.0449). However, BDNF secretion is unchanged from brain slices of N171-82Q mice compared with WT mice (Student’s *t* test, n = 3 independent experiments, p = 0.1489). B, WT neurons were treated with ACM containing released BDNF from WT or KI astrocytes, and then analyzed via Western blotting to examine p-TrkB levels. p-TrkB is decreased in KI ACM-treated neurons, confirming decreased BDNF in KI ACM. C, Glutamate measurement assay showing no significant reduction in glutamate release in cultured astrocytes from TG mice (Student’s *t* test, n = 5 independent experiments, p = 0.1027) or KI mice (Student’s *t* test, n = 6 independent experiments, p = 0.1647) compared with WT astrocytes. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.
staining was performed using the method in our previous studies (Yang et al., 2015). Briefly, ImageJ software was used to measure GFAP immunostaining intensity. Colored images obtained with a 63×1100×3 objective were converted to eight-bit black-and-white images. The “Threshold” function was used to adjust the background to highlight GFAP-specific staining. The same threshold was applied to all images analyzed. Finally, the “Measure” function was used to quantify GFAP staining intensity in each image. Each group had 7–10 images per section and eight sections per group were examined.

**ATP assay.** ATP levels in astrocyte culture medium were analyzed by an ATP bioluminescence assay kit (Sigma-Aldrich) and a luminometer (BioTek) according to the manufacturer’s instructions. Briefly, WT, KI, and Rab3a-overexpressed KI astrocytes were treated with PMA for 15 min at 37°C. Each culture medium was harvested for ATP assay. Each sample was run in duplicate and assayed within 5–10 min of collection.

**Statistical analyses.** Statistical analyses were performed with unpaired two-tailed Student’s t tests. Results are expressed as means ± SEM. P value <0.05 was considered significant. Statistical significance level was set as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

mHtt at endogenous levels impairs BDNF secretion from astrocytes

To examine whether BDNF secretion from astrocytes is affected by mHtt in KI mice, we measured BDNF secretion by cultured astrocytes from WT, KI, and TG mice. TG mice were used because the expression of N-terminal mHtt under the control of the GFAP promoter in these mice is astrocyte-specific (Bradford et al., 2009). Using ELISA to directly detect BDNF in the cell culture medium following 28 mM KCl stimulation, we found that primary astrocytes from either TG (n = 6 independent experiments, p = 1.6381E-05) or KI mice (n = 4 independent experiments, p = 0.4087) compared with WT astrocytes. Western blotting revealing similar mature mBDNF and precursor of BDNF (pro-BDNF) levels in cultured astrocytes from TG (C) and WT mice. Quantifying ratios of pro-BDNF or mBDNF to actin in TG astrocytes (Student’s t test; pro-BDNF, n = 8 independent experiments, p = 0.6983; mBDNF, n = 5 independent experiments, p = 0.6349) ns, Not significant.

Figure 2. Transcription or translation of BDNF is not changed in the HD astrocytes. A, B, qRT-PCR results revealing no significant reduction in BDNF mRNA levels in cultured astrocytes from TG mice (A, Student’s t test, n = 5 independent experiments; BDNF transcripts generated by different promoters are indicated: BDNF promoter I, p = 0.5844; BDNF promoter II, p = 0.3669; BDNF promoter III, p = 0.5765; BDNF promoter IV, p = 0.67; BDNF promoter pan, p = 0.2406) or KI mice (B, Student’s t test, n ≥ 5 independent experiments; BDNF promoter I, p = 0.6688; BDNF promoter II, p = 0.2283; BDNF promoter III, p = 0.6892; BDNF promoter IV, p = 0.0809; BDNF promoter pan, p = 0.7733) compared with WT astrocytes. C, D, Western blotting revealing similar mature mBDNF and precursor of BDNF (pro-BDNF) levels in cultured astrocytes from TG (C) and WT mice. Quantifying ratios of pro-BDNF or mBDNF to actin in TG astrocytes (Student’s t test; pro-BDNF, n = 8 independent experiments, p = 0.6983; mBDNF, n = 5 independent experiments, p = 0.6349). ns, Not significant.
brain slices from TG mice released less BDNF than slices from WT mice (Fig. 1A; n = 3 independent experiments, p = 0.0449), which also suggests that the expression of mHtt in astrocytes affects BDNF secretion. Nevertheless, BDNF release from brain slices of N171-82Q mice that express N-terminal mHtt primarily in neurons is unchanged compared with WT brain slices, further indicating that mHtt selectively impairs BDNF secretion from astrocytes (Fig. 1A; n = 3 independent experiments, p = 0.1489).

To show the functional consequences of reduced BDNF release, we treated WT neurons with either WT or KI astrocyte-conditioned medium (ACM) for 10 min to induce phosphorylation of TrkB, as binding of BDNF to TrkB receptors leads to TrkB phosphorylation (p-TrkB; Duman and Voleti, 2012), so the extent of p-TrkB reflects BDNF levels in the ACM. Consistently, we found that neurons treated with KI ACM showed reduced p-TrkB levels (p-TrkB/actin) compared with those treated with WT ACM (Fig. 1B; n = 4 independent experiments, p = 0.0157). This result further supports our observation that BDNF levels are reduced in KI ACM compared with WT ACM.

To verify that mHtt specifically impairs dense-core vesicle release in astrocytes, we also measured glutamate release from astrocytes. This glutamate is stored in small synaptic-like clear-core vesicles. The glutamate measurement assay showed no significantly decreased levels of glutamate in TG astrocyte culture medium (Student’s t test, n = 5 independent experiments, p = 0.1027) or in KI astrocyte culture medium (Student’s t test, n = 6 independent experiments, p = 0.3647) compared with WT astrocyte culture medium (Fig. 1C). Collectively, our results indicate that mHtt at endogenous levels impairs BDNF secretion from astrocytes.

mHtt does not affect the generation of BDNF in HD astrocytes
To explore the mechanism behind the decreased BDNF secretion from HD astrocytes, we first examined BDNF production in cultured astrocytes, as previous studies suggested that overexpressing mHtt influenced BDNF transcription and translation in astrocytes (Wang et al., 2012). We performed a qRT-PCR assay using BDNF-specific primers and found that levels of different BDNF mRNA isoforms are not significantly changed in TG or KI astrocytes compared with WT astrocytes (Fig. 2A, B). Moreover, using Western blotting and BDNF-specific antibody, we found no significant changes in either mature BDNF (mBDNF) or precursor of BDNF in cultured astrocytes from TG or KI mice (Fig. 2C, D). These results suggest that mHtt at the endogenous level does not affect BDNF mRNA or protein levels.

mHtt binds to Rab3a in astrocytes
Since BDNF synthesis was unchanged, we reasoned that the decreased BDNF protein secretion might be due to decreased exo-
cytosis of BDNF vesicles. Rab3 is a small GTPase comprising four isoforms (Rab3a–Rab3d; Takai et al., 1996). Rab3a, the isoform highly enriched in the brain, is localized on membranes of secretory vesicles, including synaptic vesicles and dense-core vesicles, and mediates the exocytosis of vesicles (Tsuboi and Fukuda, 2006). Although Rab3a is expressed in astrocytes (Maienschein et al., 1999), its function in astrocytes remains elusive. We used Rab3a siRNA to knock down Rab3a expression in cultured WT astrocytes and found that knocking down Rab3a results in the defective release of BDNF and ATP, another cargo in dense-core vesicles, from astrocytes (Fig. 3A, B; BDNF release, Student’s t test, n = 3 independent experiments, p = 0.0492; ATP release, Student’s t test, n = 4 independent experiments, p = 0.0041) and in the corpus callosum of KI mice (C, Student’s t test, n = 4 independent experiments, p = 0.0106). D, Rab3-GAP1 levels did not change in KI astrocytes. E, GTPase activity of purified GST-Rab3a was not affected by binding to mHtt (Student’s t test, n = 4 independent experiments; 30 min, p = 0.4807; 60 min, p = 0.9353; 90 min, p = 0.4071). *p < 0.05, **p < 0.01; ns, not significant.

Figure 4. Reduced association between Rab3-GAP1 and Rab3a by mHtt results in increased GTP-Rab3a in HD astrocytes. A, GTP-Rab3a protein levels were increased in cultured KI astrocytes (Student’s t test, n = 3 independent experiments, p = 0.0492). B, C, Association between GTP-Rab3a with Rab3-GAP1 is decreased in both cultured KI astrocytes (C, Student’s t test, n = 4 independent experiments, p = 0.0106). D, Rab3-GAP1 levels did not change in KI astrocytes. E, GTPase activity of purified GST-Rab3a was not affected by binding to mHtt (Student’s t test, n = 4 independent experiments; 30 min, p = 0.4807; 60 min, p = 0.9353; 90 min, p = 0.4071). *p < 0.05, **p < 0.01; ns, not significant.

mHtt binds Rab3-GAP1 to prevent its association with Rab3-GAP in astrocytes

The ability to associate/dissociate GTP and GDP is essential for the function of Rab3a in dense-core vesicle docking (van Weering et al., 2007). Given the increased binding of mHtt to GTP-Rab3a, we wanted to know whether mHtt disrupts the conversion of GTP-Rab3a into GDP-Rab3a (GTP/GDP-Rab3a exchange), which could result in altered levels of GTP-Rab3a. Using Western blotting with a GTP-Rab3a-specific antibody, we found that GTP-Rab3a protein levels were indeed increased in KI astrocytes (Fig. 4A), supporting the idea that mHtt inhibits the conversion of GTP-Rab3a. As Rab3-GAP binds Rab3-GAP, the catalytic subunit of Rab3-GAP, indeed, we found that the association between GTP-Rab3a and Rab3-GAP1 is significantly reduced in astrocytes.
from KI mice compared with WT astrocytes (Fig. 4B; C n = 4 independent experiments, p = 0.0041). In addition, we observed the same result in protein lysates from the KI corpus callosum, a brain region enriched in glial cells (Fig. 4B; C n = 4 independent experiments, p = 0.0106). Western blotting using a Rab3-GAP1-specific antibody showed that there is no difference in Rab3-GAP1 levels between WT and KI astrocytes, ruling out the possibility that the increased GTP-Rab3a is caused by a reduction in Rab3-GAP1 levels between WT and KI astrocytes, thereby ruling out the possibility that mHtt in KI lysates does not directly affect the GTPase activity of Rab3a but disrupts GTP/GDP-Rab3a exchange by interfering with the association between GTP-Rab3a and Rab3-GAP1.

Overexpression of Rab3a in HD astrocytes rescued deficits of BDNF secretion

If mHtt binds Rab3a to inhibit GTP/GDP-Rab3a exchange, overexpression of Rab3a should antagonize this inhibition and the related decrease in BDNF release. We therefore generated adenoviral vector to overexpress Rab3a-V5 in cultured astrocytes from KI mice. Immunofluorescent staining and Western blotting of these cells verified the expression of Rab3a-V5 (Fig. 5A, B). Using stereotaxic techniques, we injected Rab3a-V5 adenovirus into the striatum of TG mice and confirmed the expression of Rab3a-V5 via Western blotting (Fig. 5C). It is important to see whether overexpressed Rab3a could increase BDNF release in HD astrocytes, so we isolated mouse brain slices 30 d after stereotaxic injection. TG mouse brain slices containing the striatum area were prepared and incubated in ACSF, immediately followed by stimulation with 56 mM KCl for 30 min to trigger BDNF release, and the ACSF in the culture dish was harvested and used for ELISA (Fig. 5D). Similar to cultured KI astrocytes (n = 3 independent experiments, p = 0.0056), the BDNF secretion from TG brain slices is also increased after viral Rab3a-V5 injection (n = 3 independent experiments, p = 0.0464) compared with control virus-infected TG mice (Fig. 5E). Moreover, this increased BDNF release is further supported by the increased levels of p-TrkB found in cultured WT neurons incubated with ACM from Rab3a-overexpressing KI astrocytes (Fig. 5F; n = 5 independent experiments, p = 0.0292). These results demonstrated that defective BDNF secretion from HD astrocytes could be rescued by overexpressing Rab3a.

Overexpression of Rab3a rescued the defective release of ATP from HD astrocytes

Since BDNF is not the only cargo in dense-core vesicles, we also examined whether mHtt also impairs the release of ATP, which is stored in dense-core vesicles. Quinacrine often serves as a fluorescein marker for intracellular ATP-enriched vesicles. After PMA treatment, quinacrine staining was performed by incubating living cultured astrocytes with quinacrine for 15 min at room temperature.
ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test; WT vs KI: WT, n = 12 cells; KI, n = 14 cells, p = 0.0204; KI vs KI + Rab3a-V5: KI, n = 14 cells; KI + Rab3a-V5, n = 15 cells, p = 0.0068). C, Bioluminescence assay showed that ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test, n = 5 independent experiments, WT vs KI, p = 0.00006; KI vs KI + Rab3a-V5, p = 0.0125). **p < 0.01, ***p < 0.001. Scale bar, 10 μm.

**Figure 6.** Overexpression of Rab3a rescues the deficient release of ATP from HD astrocytes. A, Quinacrine staining was performed by incubating living cultured WT, KI, and Rab3a-overexpressed KI astrocytes for 15 min after treatment of PMA. B, The quantitative results showed that ATP levels are higher in KI astrocytes than in WT astrocytes after stimulation, and overexpression of Rab3a reduces ATP levels in astrocytes compared with KI astrocytes (Student's t test; WT vs KI: WT, n = 12 cells; KI, n = 14 cells, p = 0.0204; KI vs KI + Rab3a-V5: KI, n = 14 cells; KI + Rab3a-V5, n = 15 cells, p = 0.0068). C, Bioluminescence assay showed that ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test, n = 5 independent experiments, WT vs KI, p = 0.00006; KI vs KI + Rab3a-V5, p = 0.0125). **p < 0.01, ***p < 0.001. Scale bar, 10 μm.

**Overexpression of Rab3a rescues the deficient release of ATP from HD astrocytes.** WT and KI astrocytes were transfected with the adenoviral Rab3a and GFP injection sides (Fig. 6C). We also examined NeuN-positive neuronal cells and mutant Htt aggregates, but did not see differences between adenoviral Rab3a-V5 and GFP adenovirus injection sides (Fig. 6C,D). Thus, overexpression of Rab3a could selectively decrease reactive astrocytes in the HD KI mouse model. Based on these findings, we propose that mHtt binds Rab3a to affect its GTP/GDP exchange and impairs the docking of dense-core vesicles in HD astrocytes.

**Discussion**

Decreased BDNF levels in HD brains are believed to play a critical role in HD pathogenesis (Zuccato and Cattaneo, 2007). While previous studies have focused on the effect of mHtt on neuronal BDNF, our findings demonstrate that mHtt in astrocytes can also affect BDNF release, yielding new insights into the decreased BDNF levels in the HD brain.

Given the fact that >70% of cells in the striatum are astrocytes, the role of astrocytes in the striatum is vitally important. Astrocytes have a wide range of functions to support neuronal cells, including the reuptake of neurotransmitters and release of ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test; WT vs KI: WT, n = 12 cells; KI, n = 14 cells, p = 0.0204; KI vs KI + Rab3a-V5: KI, n = 14 cells; KI + Rab3a-V5, n = 15 cells, p = 0.0068). C, Bioluminescence assay showed that ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test, n = 5 independent experiments, WT vs KI, p = 0.00006; KI vs KI + Rab3a-V5, p = 0.0125). **p < 0.01, ***p < 0.001. Scale bar, 10 μm.

**Figure 6.** Overexpression of Rab3a rescues the deficient release of ATP from HD astrocytes. A, Quinacrine staining was performed by incubating living cultured WT, KI, and Rab3a-overexpressed KI astrocytes for 15 min after treatment of PMA. B, The quantitative results showed that ATP levels are higher in KI astrocytes than in WT astrocytes after stimulation, and overexpression of Rab3a reduces ATP levels in astrocytes compared with KI astrocytes (Student's t test; WT vs KI: WT, n = 12 cells; KI, n = 14 cells, p = 0.0204; KI vs KI + Rab3a-V5: KI, n = 14 cells; KI + Rab3a-V5, n = 15 cells, p = 0.0068). C, Bioluminescence assay showed that ATP secretion was rescued from cultured KI astrocytes infected with Rab3a-V5 adenovirus (Student's t test, n = 5 independent experiments, WT vs KI, p = 0.00006; KI vs KI + Rab3a-V5, p = 0.0125). **p < 0.01, ***p < 0.001. Scale bar, 10 μm.

**Overexpression of Rab3a rescues the deficient release of ATP from HD astrocytes.** WT and KI astrocytes were transfected with the adenoviral Rab3a and GFP injection sides (Fig. 6C). We also examined NeuN-positive neuronal cells and mutant Htt aggregates, but did not see differences between adenoviral Rab3a-V5 and GFP adenovirus injection sides (Fig. 6C,D). Thus, overexpression of Rab3a could selectively decrease reactive astrocytes in the HD KI mouse model. Based on these findings, we propose that mHtt binds Rab3a to affect its GTP/GDP exchange and impairs the docking of dense-core vesicles in HD astrocytes, resulting in decreased release of BDNF (Fig. 9).

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growth factors. mHtt was found to reduce the transcription of an important neurotransmitter transporter, glutamate transporter (GLT-1; Bradford et al., 2009) in astrocytes, which may contribute to the increased glutamate levels and excitotoxic neuronal death in the brains of HD mice (Espey et al., 1998; Liévens et al., 2001; Behrens et al., 2002; Hassel et al., 2008; Estrada-Sánchez et al., 2009; Bradford et al., 2010; Wolfram-Aduan et al., 2014). Our findings indicate that mHtt can also affect the secretion of neurotrophic factors of astrocytes. Astrocytic secretion of neurotrophic factors in supporting neuronal function and survival is a well-studied subject (Quesseveur et al., 2013). BDNF is reported to play an important role in neuronal development and survival, and decreased BDNF levels in neurodegenerative diseases, including HD, Parkinson’s disease, and Alzheimer’s disease, have been proposed as a major player in neuronal death (Howells et al., 2000; Lee et al., 2005; Zuccato and Cattaneo, 2007). Elucidating the mechanism behind the decreased BDNF in these diseases could help us find ways to restore BDNF levels in diseased brains, providing new therapeutic targets for HD, as well as other neurodegenerative diseases.

In the present study, using primary cultures and mouse brain slice models from HD mice, we found that mHtt at the endogenous level was able to compromise BDNF secretion from HD astrocytes but not from HD neurons. This idea is supported by the fact that brain slices from the N171-82Q mouse model, in which N-terminal Htt with 82Q is primarily expressed in neuronal cells (Schilling et al., 1999), do not show defective BDNF release. Additionally, cultured astrocytes and brain slices from TG mice that express mHtt selectively in astrocytes show defective release of BDNF. This selectivity is likely due to the specific binding of mHtt to Rab3a in astrocytes, but not in neurons. Although it remains to be investigated how mHtt selectively binds Rab3a in astrocytes, we can speculate that astrocyte-specific molecules, proteins, and post-translational modifications may contribute to this selective binding, given that mHtt can associate with a large number of proteins and its function is regulated by complex post-translational modifications. Our studies also indicate that N-terminal mHtt fragments can affect the secretion of BDNF. This is because immunoprecipitation results show that N-terminal mHtt binds Rab3a and that TG mice expressing N-terminal mHtt show defective BDNF release from their astrocytes.

On the other hand, we found no changes in BDNF mRNA and protein levels in cultured astrocytes from astrocyte-specific transgenic HD or HD knock-in mice. This finding differs from an earlier report that showed that BDNF mRNA in cultured astrocytes was decreased by overexpressed N-terminal mHtt via adenoviral infection (Wang et al., 2012). This discrepancy may be due to different expression levels of mHtt. In our studies, we used astrocytes that express endogenous full-length mHtt or N-terminal mHtt at a level similar to endogenous Htt.

Rab3 undergoes functional conversion between active and inactive forms, which is essential for vesicle docking and exocytosis in cells (Darchen and Goud, 2000; Schlüter et al., 2004; Tsuibo and Fukuda, 2006; van Weering et al., 2007). It has been reported that Rab3 is located on the membrane of synaptic vesicles and dense-core vesicles regulating vesicle exocytosis in neurons; however, the location and function of Rab3 in astrocytes remains unknown. Although electron microscopy studies have shown that astrocytes contain both dense-core vesicles and synaptic-like clear-core vesicles, whether the exocytosis of these vesicles is mediated by different mechanisms is currently unknown (Parpura and Zorec, 2010). Despite this, we know that BDNF is packed in dense-core vesicles in astrocytes in a similar manner as in neurons, which requires GTP/GDP-Rab3a exchange (Parpura and Zorec, 2010). We found that the association of mHtt with Rab3a perturbs this exchange. First, we detected an increased level of GTP-Rab3a in KI astrocytes, which may be the result of the impaired GTP/GDP-Rab3a exchange. Second, our results demonstrate that the binding of mHtt to GTP-Rab3a inhibits the association of GTP-Rab3a with Rab3-GAP1. Previous studies showed that Rab27 and Rab3 sequentially regulate dense-core vesicle exocytosis in human sperm cells (Bustos et al., 2012). However, we did not find the association of mHtt with Rab27 by
Figure 8. Overexpression of Rab3a reduces reactive astrocytes in the striatum of HD140Q KI mice. A. Five-month-old WT mice were injected with Rab3a-V5 adenovirus into the left side of the striatum. After 21 d, immunofluorescent staining showed that Rab3a-V5 preferentially infected astrocytes in the striatum. B. Stereotaxic injection of 9-month-old HD140Q KI mice. C. Low-magnification (10×) micrographs showing NeuN and GFAP staining in adenoviral GFP and Rab3a-V5-injected striatum. High-magnification (63×) micrographs showing GFAP (red) or mutant Htt aggregates (arrows, red) staining in the merged images in which the nuclei are stained by Hoechst (blue). Scale bars, 10 μm. D. Quantitative analysis of the GFAP immunofluorescent density showing GFAP staining is significantly decreased in the Rab3a-V5-injection side compared with GFP-injection side (Student’s t test, randomly selected 7–10 images per section, n = 8 sections per group, p = 7.51767E-13). The percentage of NeuN-positive cells and the number of mutant Htt aggregates per image are unchanged (Student’s t test, n = 600 cells per group, NeuN-positive cells, p = 0.1016; aggregates number, p = 0.1511). ***p < 0.001; ns, not significant.
immunoprecipitation (data not shown). These results suggest that the disruption of the GTP/GDP-Rab3a exchange causes the deficient release of BDNF from HD astrocytes by binding mHtt to Rab3a. More importantly, we found that overexpressing Rab3a significantly improved vesicle docking and BDNF release from HD astrocytes.

Previous studies also showed that overexpression of Rab3a increases vesicle docking (Martelli et al., 2000), and conversely, disruption of GTP/GDP-Rab3 exchange inhibits vesicle docking (van Weering et al., 2007). Using TIRFM, we found a reduced number of BDNF-containing docking vesicles underneath the plasma membranes of KI astrocytes, which was increased through Rab3a overexpression. This result also supports the notion that disrupting conversion of Rab3-GTP to Rab3-GDP suppresses vesicle docking. Because BDNF is not a unique cargo for dense-core vesicles (Parpura and Zorec, 2010), we examined ATP release and the docking of ATP-containing dense-core vesicles. Consistently, we found defective ATP secretion and less docking of ATP-containing dense-core vesicles, which are also rescued by Rab3a overexpression. Therefore, our results indicate that mHtt impairs the docking of dense-core vesicles by intervening in the conversion of GTP/GDP-Rab3a in astrocytes. The defective docking of dense-core vesicles in astrocytes could impair the release of BDNF from HD astrocytes in which mHtt is expressed at endogenous levels. Our findings also suggest that mHtt could affect other functions of astrocytes or the secretion of different molecules via a similar mechanism. Furthermore, our finding that Rab3a overexpression can rescue the defective docking, increase BDNF secretion, and ameliorate reactive astrocytes in vivo suggests that improving the exocytosis function of astrocytes could be beneficial to the treatment of HD.

Substances like BDNF released from astrocytic dense-core vesicles are well known to play broad roles in neuronal survival and excitability, as well as astrocyte–neuron communication. Our findings provide new insight into the decreased secretion of BDNF from HD astrocytes in which mHtt is expressed at endogenous levels. Our findings also suggest that mHtt could affect other functions of astrocytes or the secretion of different molecules via a similar mechanism. Furthermore, our finding that Rab3a overexpression can rescue the defective docking, increase BDNF secretion, and ameliorate reactive astrocytes in vivo suggests that improving the exocytosis function of astrocytes could be beneficial to the treatment of HD.

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