A Role for Huntington Disease Protein in Dendritic RNA Granules*§

Regulated transport and local translation of mRNA in neurons are critical for modulating synaptic strength, maintaining proper neural circuitry, and establishing long term memory. Neuronal RNA granules are ribonucleoprotein particles that serve to transport mRNA along microtubules and control local protein synthesis in response to synaptic activity. Studies suggest that neuronal RNA granules share similar structures and functions with somatic P-bodies. We recently reported that the Huntington disease protein huntingtin (Htt) associates with Argonaute (Ago) and localizes to cytoplasmic P-bodies, which serve as sites of mRNA storage, degradation, and small RNA-mediated gene silencing. Here we report that wild-type Htt associates with Ago2 and components of neuronal granules and co-traffics with mRNA in dendrites. Htt was found to co-localize with RNA containing the 3′-untranslated region sequence of known dendritically targeted mRNAs. Knockdown of Htt in neurons caused altered localization of mRNA. When tethered to a reporter construct, Htt down-regulated reporter gene expression in a manner dependent on Ago2, suggesting that Htt may function to repress translation of mRNAs during transport in neuronal granules.

Transport and local translation of mRNAs in neurons play key roles in modulating synaptic strength and maintaining proper neural circuitry (1, 2). Neuronal RNA granules serve as sites of transport and translational repression of dendritic mRNAs. Several types of cytoplasmic RNA granules in neurons have been described that contain distinct as well as shared components (3). Processing (P)-bodies are dynamic assemblies of RNA and proteins found in the cytoplasm of somatic cells (4). In response to stress, P-bodies form to store mRNAs targeted for degradation or translational control. It was recently reported that neuronal ribonucleoprotein particles in Drosophila that contain RNA-binding proteins Staufen and fragile X mental retardation protein are related to somatic P-bodies in structure and function (5). Further, P-body-like structures have been described in mammalian dendritic neurons that are heterogeneous in composition and respond to neuronal activity (6, 7). Somatic P-bodies also serve as sites of microRNA (miRNA)-mediated translational repression. Given the similarities between neuronal ribonucleoprotein particles and somatic P-bodies, it has been proposed that miRNAs may help to maintain silencing of mRNAs during transport by neuronal RNA granules.

We recently reported that Huntington disease (HD) protein huntingtin (Htt) associates with Argonaute proteins, localizes to P-bodies, and contributes to post-transcriptional gene silencing (8). HD is a dominantly inherited late-onset progressive neurodegenerative disorder caused by an expansion of CAG trinucleotide repeats, resulting in a long tract of polyglutamines in the N terminus of Htt whose one or more normal functions remain unclear. Mouse models of HD have provided evidence for a disease mechanism that involves a gain of function of the mutant HD protein (9–12). Many studies have focused on deciphering the pathogenic mechanisms involving mutant Htt; however, new studies point to a role for wild-type Htt in the disease process (13). Determining the molecular function of wild-type Htt may prove critical to understanding HD pathogenesis and the eventual development of effective treatment strategies.

Htt has been reported to function in retrograde transport of vesicles in neurons; it is required for transport in axons at rates...
consistent with microtubule-associated vesicles (14, 15). Huntingtin-associated protein 1 (HAP1) was identified in a yeast two-hybrid screen using the N-terminal 230 amino acids of Htt as bait (16). HAP1 interacts with p150Glued, a subunit of the vesicular motor protein complex dynein, and HAP1 may act as a link between the motor proteins and their cargo (14). Htt has been proposed to affect vesicle mobility or cargo binding affinity. Overexpression and knockdown assays showed that Htt plays an important role in controlling brain-derived neurotrophic factor trafficking (17); however, trafficking of Htt itself has not been reported. In this study we present findings that implication Htt in RNA transport and local translation through neuronal RNA granules.

**EXPERIMENTAL PROCEDURES**

**Dissection and Culture of Neurons**—Rat neurons were isolated and cultured as previously described (18, 19). All rats were maintained under veterinary supervision at the New York University School of Medicine Animal Care Facility in accordance with the guidelines established by the National Institutes of Health (NIH) for the care of laboratory animals, and all procedures were approved by the Institutional Animal Care and Use Committee. To prepare brain slices for immunostaining and FISH, wild-type Wistar rats (7 days old) were perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde under deep anesthesia induced by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (25 mg/kg). Brains were extracted from the skull and fixed with 4% paraformaldehyde overnight at 4 °C. The hippocampus was then dissected out, and 100-μm Vibratome sections were prepared with a Vibratome Series 1000 Classic (Vibratome Co., St. Louis, MO) and transferred into 24-well plates filled with PBS.

All dilutions and washes (3 × 30 min) between stages were performed in PBS. Vibratome sections were washed for 20 min with PBS, blocked with 5% goat serum (Sigma) for 2 h, and incubated overnight with mouse α-Htt and rabbit α-Dcp1 antibody (in solution containing 5% goat serum) at 4 °C. Sections were then incubated overnight with Alexa 488-conjugated goat α-mouse IgG (BD Pharmingen) and Alexa 555-conjugated goat α-rabbit IgG (BD Pharmingen) at 4 °C. After immunolabeling, sections were transferred to a Lab-Tek 2-well chamber cover glass (Nalge Nunc International) for confocal microscopy.

**Confocal Microscopy/Indirect Immunofluorescence**—Endogenous proteins were examined in rat hippocampal neurons (DIV 14) grown on poly-D-lysine-coated glass coverslips (Glaswabenfabrik Karl Hecht KG, Sondheim, Germany). Cells were fixed at room temperature with 4% (w/v) paraformaldehyde in PBS (similar results were obtained with −20 °C methanol for 3 min), permeabilized with 0.2% Triton X-100 for 20 min, followed by three PBS washes. Cells were subsequently blocked for 1 h at room temperature in 10% FBS plus 0.25% saponin (Sigma) in PBS. Primary antibodies were diluted in blocking buffer and probed overnight at 4 °C. Cells were washed three times with 7% fish gelatin plus 0.025% saponin in PBS for 5 min each. Conjugated goat α-mouse Alexa 488, goat α-rabbit Alexa 555, and goat α-chicken Alexa 633 (Molecular Probes) were diluted 1:250 in the blocking buffer and incubated for 1 h at 37 °C. Cells were washed in PBS three times for 5 min and treated at room temperature for 15 min with Hoechst stain and washed three times with PBS before mounting with Dako or Vectashield H1400 (Vector Laboratories). Confocal imaging was performed using an LSM META510 confocal scanning laser system on an Axiovert 200M microscope (Carl Zeiss). The instrument settings are detailed in **supplemental Table 1.** Images were acquired with a Plan-Apochromat 100×/1.3 oil-immersion objective lens. Brightness and contrast of images were adjusted before export to Photoshop for further processing.

**Htt Trafficking Movie**—Cortical neurons were grown on glass bottom dishes (WillcoWells) and transfected at DIV 5–6 with an RFP-Htt590-25Q expression plasmid using Lipo-fectamine 2000 (Invitrogen). The following day, the medium was changed to phenol red-free Neurobasal medium (Invitrogen) supplemented with 30 mm HEPES-NaOH, pH 7.4. Transfected cells were imaged on an Olympus IX71 inverted microscope driven by IPLab software (BD Biosciences) equipped with a 60× PlanApoN objective (numerical aperture 1.42), a Hamamatsu EM-CCD camera, and a heated stage maintained at 37 °C. Images were taken every 5 s. All movies were exported and processed using IPLab (BD Biosciences) and ImageJ (NIH) software. Co-trafficking data were acquired on a Zeiss LSM710 with heated chamber and humidity control unit at 37 °C and 5% CO₂. Movie files were compressed with Virtual Dub (Version 1.9.0.0) and then saved as AVI documents. Cinepak Codec by Radius was used for the compression.

**Antibodies Used in Microscopy Experiments and Western Blotting**—The following antibodies were used: α-Htt (Millipore 2166 and 5492 antibodies gave same results, immunofluorescence (IF)-dilution: 1:250, Western blot (WB)-dilution 1:1,000), rabbit polyclonal α-Staufen1 (Millipore AB5781, IF-dilution 1:250, WB-dilution 1:500), rabbit polyclonal α-Staufen (gift of Susana de Lucas (National Centre for Biotechnology, Madrid, Spain), 1:400), chicken polyclonal α-MAP2 (Covance PCK-554P, IF-dilution 1:10,000), α-FLAG (Sigma, M2 monoclonal, IF-dilution 1:100), rabbit polyclonal α-DDX6 (Bethyl A300-460A, IF-dilution 1:500), rabbit polyclonal α-Dcp1a (gift of J. Lykke-Andersen (University of Colorado, Boulder), IF-dilution 1:200), α-rpS6 (Cell Signaling 2217, IF-dilution 1:500), rabbit polyclonal α-GFP (gift of E. Ziff (NYU School of Medicine, New York, NY), IF-dilution 1:1,000), rabbit polyclonal α-elf4E (Cell Signaling 9742, WB-dilution 1:1,000), anti-β-tubulin (MMS-410P, WB-dilution 1:1,000), α-Ago2 (Cell Signaling 2897, WB-dilution 1:1,000), α-Ago2 (gift of Ramin Shiekhattar (The Wistar Institute, PA), IF-dilution 1:1,000), α-HA (12CA5, WB-dilution 1:1,000), and GAPDH (Millipore MAB374, WB-dilution 1:300).

**RNA/Htt Dendritic Branch Point Localization Assays**—Rat hippocampal neurons were cultured (DIV 14) as described above. Live neurons were incubated with 500 nM SYTO® RNASelect (Invitrogen) for 20 min at 37 °C in culture medium as recommended by the manufacturer. The untreated cells were fixed with −20 °C methanol for 3 min and handled as described in the indirect immunofluorescence section. For the treated neurons (DNase & RNase), after SYTO® RNASelect incubation, the cells were permeabilized and incubated with PBS containing RQ1 RNase-Free DNase (2 μl of 1 unit/μl, Promega, Madison, WI) or RNase (1–2 μl of 10 mg/ml, Sigma) for.
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30 min at room temperature, then fixed with −20 °C methanol for 3 min. Subsequently, the fixed neurons were blocked and immunostained as described above. Dendritic branch points were identified by visualization of MAP2 staining.

**Plasmids**—mRFP-Htt590-25Q was made by inserting Htt590-25Q fragment from pOZ-N-Htt590-25Q (8) into pmRFP-C1 (Clontech). mRFP-Htt480-17Q was a gift of Florian Then (MGH) (20). HA-Ago2 and AN-HA-Ago2 have been described (21). HA-Htt590-25Q and AN-HA-Htt590-25Q were made by subcloning Htt590-25Q fragment from pOZ-N-Htt590-25Q into pCGN plasmid (22) and pAN-HA plasmid (21), respectively. FLAG–HttFL–25Q was constructed by moving the C-terminal Htt sequence from full-length Htt cDNA into pOZ-N-Htt590-25Q. mRFP–hStau1 was made by subcloning hStau1 into pmRFP-C1.

GFP–hStau1, NLS–MS2–Venus, and IP3R1 3’UTR–MS2–Venus (23) were gifts of Hiroko Bannai (RIKEN). pAN22-mEGFP3-M9 and pmRFP–4boxB–β-actin–zipcode (24) were gifts of Jan Ellenberg (EMBL).

**Neuronal Transfections**—Rat cortical neurons (DIV 5–10) were typically transfected with 2 μg of plasmid DNA, 1 μl of Lipofectamine 2000 (Invitrogen) with Opti-MEM (Invitrogen) per 24 wells as recommended by the manufacturer. The neuronal cultures were fed 12–24 h prior to transfection. Thirty minutes before transfection, 50% of the culture medium was removed and later used to replace the medium after a 45-min incubation with the transfection mixture. Neurons were fixed and analyzed 24–36 h after transfection.

**Glycerol Gradients**—1% Nonidet P-40 lysates were prepared from rat cortical neurons (DIV 14), and 1 mg of total lysate was analyzed on 10–40% glycerol gradients essentially as described (25). Twenty-five fractions (~200 μl each) were collected, and the pellet was resuspended in sample buffer. The even-numbered fractions were precipitated with trichloroacetic acid and analyzed by Bradford assay to determine the protein concentration, and the odd-numbered fractions were analyzed by qRT-PCR reactions with gene-specific primers. qRT-PCR reactions were performed in 20-μl reactions with 1 μl each of primer (100 μM), 10 μl of SYBR green reagent (2×, USB) on a LightCycler (Roche Applied Science). To ensure the identity of the product, the primers were designed to span an intron, and control reactions were performed in parallel without reverse transcriptase.

**RT-PCR Primers**—The primers used were as follows: mBetaCatFWD, 5′-GAAGCGGCTTTTCAGCTGAGC-3′; mBetaCatREV, 5′-TCAGCAGCCCACTCAACTGCG-3′; mIP3R1-FWD, 5′-AGGCTTTCCAAACACGAGACAT-3′; mIP3R1-REV, 5′-TGCTGATCTCCGCATAC-3′; mMAP2-FWD, 5′-GATCAACGG-3′; mMAP2-REV, 5′-CGGAGATGATGACCCTTTTG-3′; mPGAM1-FWD, 5′-TACCCCCCTTCTACAGGCACA-3′; and mPGAM1-REV, 5′-GGGAGAGTCTGTTTCAGTAA-3′.

**Lentiviral shRNA Constructs**—Lentiviral shRNA plasmids were constructed, and viral particles were prepared essentially as described (26). To ensure similar multiplicity of infection between viral preparations, we examined GFP expression in 293T cells after infection with serially diluted viruses. The Htt shRNA-1 sequence (rat/mouse) was designed (17): 5′-GATCCCCGGAACCTCAGCCACAAGTTCAAGAGAC-3′ (top) and 5′-AGCTTTTCAAAGAAGAATCTCAGGACCAAAAGATTCTCTTGAGGAGGT-3′ (bottom). The Htt shRNA-2 sequence (rat) was: 5′-GATCCCCGGAACCTCAGCCACAAGTTCAAGAGAC-3′ (top) and 5′-AGCTTTTCAAAGAAGAATCTCAGGACCAAAAGATTCTCTTGAGGAGGT-3′ (bottom). The SCB (scrambled) shRNA sequence was: 5′-GATCCCCCACAAGCTAGCTACCTGCTGTAGAGATTGAGGC-3′ (top) and 5′-AGCTTTTCAAAGAAGAATCTCAGGACCAAAAGATTCTCTTGAGGAGGT-3′ (bottom). The Ago2 shRNA sequence (rat/mouse) was designed (27): 5′-GATCCCCACAAACCCGTTTTTATGGTTGAGGAGGTCTGCAGTCTGTAAG-3′ (top) and 5′-AGCTTTTCAAAGAAGAATCTCAGGACCAAAAGATTCTCTTGAGGAGGT-3′ (bottom). Cortical neurons were infected at DIV 3 and cultured for 5–7 days.

**Image Quantification**—To quantify endogenous protein co-localization (Table 1), 30 μm of dendrite (~10 μm from cell body) was cropped and analyzed essentially as described (5, 28). First, we examined the green channel and counted Htt-positive granules. Next, we examined the red channel and counted DCP1 (or Staufen, DX6, and Ago2) granules, and lastly we counted yellow granules in the merged image. In order for a granule to be scored positive for co-localization, at least 30% of the red/green signal was required to overlap in the merged image; thus adjacent localization without significant overlap was not considered as co-localized. MAP2 staining was used to identify dendrites.

For quantitative comparison of Htt/AN-GFP versus Htt/RFP (Fig. 4D), RGB images were handled and processed with the
RESULTS

Huntingtin Is Present at Dendritic RNA Granules—Using antibodies to establish markers of neuronal ribonucleoprotein particles, we investigated if Htt localizes to dendritic RNA granules or dendritic P-body-like structures, which were recently shown to be structurally and functionally related to somatic P-bodies (PBs) (5–7). We examined rat hippocampal neurons triple-stained with Htt, Staufen (NG marker), and MAP2 antibodies to determine if Htt is present in mammalian dendritic RNA granules. The monoclonal antibody 2166 against Htt (Millipore) was used; the antibody is specific to Htt as demonstrated by the loss of staining in Htt−/− ES cells (29) compared with wild type (supplemental Fig. 1). Htt co-localized with many Staufen granules present in dendrites (Fig. 1A). To directly examine if these Htt dendritic granules are related to PBs—bodies we immunostained additional neurons with antibodies to DCP1 (a PB marker), DDX6 (also known as p54/RCK, PB resident, and Ago-interacting protein), and Ago2. Most dendritic Htt granules contained DCP1, DDX6, and Ago2 (also shown in Ref. 8) suggesting that these granules are structurally related to somatic PBs (Fig. 1B). To quantitatively assess Htt co-localization with these markers we examined multiple neurons and found that Htt dendritic granules often contained Htt dendritic granules containing DCP1, DDX6, and Ago2. The intensity of mRNA, Htt, and Ago2 was normalized to the area of GFP staining, which constituted the size of dendrites. For Htt, DyLight 649-conjugated goat α-rabbit IgG was used. The secondary antibody for mouse α-GFP (Abnova) was Alexa 488-conjugated goat α-mouse IgG. To detect Ago2, DyLight 484-conjugated goat α-rabbit IgG was used.

To quantify the FISH signal, 25-μm-long image segments of dendrites were selected and copied to ImageJ (National Institutes of Health). The intensities of protein/mRNA and the area of GFP staining were measured by using the “Measure” function in ImageJ. For comparison, the intensity of mRNA, Htt, and Ago2 was normalized to the area of GFP staining, which represented the size of dendrites.

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To investigate if Htt is present in neuronal granules in vivo, we examined immunostained rat hippocampal brain slices and found Htt to be coincident with DCP1 in many granules in the rat brain (supplemental Fig. 2). Next, arsenite treatment, which induces PBs and stress granules (SGs) (31), was used to test the effect on Htt and Staufen granule formation. The size and distribution of Htt did not markedly change after arsenite treatment (supplemental Fig. 3). Some Staufen granules significantly increased in size, consistent with previous reports that Staufen can be transferred from NGs to SGs in response to stress (Ref. 32 and references therein). This suggests that Htt granules are not highly sensitive to arsenite, but a subset of Staufen granules shows sensitivity similar to SGs. Thus, Htt granules likely represent distinct structures from SGs, consistent with our previous result obtained in somatic cells (8).

To confirm the localization of Htt to dendritic neuronal RNA granules, we transfected neurons with FLAG-HttFL (full-length)-25Q and GFP-Staufen1 and stained with α-FLAG and rabbit α-GFP was DyLight 488-conjugated goat α-rabbit IgG. For Htt, DyLight 649-conjugated goat α-mouse IgG was used. The secondary antibody for mouse α-GFP (Abnova) was Alexa 488-conjugated goat α-mouse IgG. To detect Ago2, DyLight 484-conjugated goat α-rabbit IgG was used.

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We found that overexpressed Htt localized to dendritic RNA granules based on co-localization with GFP-Staufen1 and MAP2 staining (Fig. 1C). Further, the N-terminal 480 amino acids of Htt containing 17 glutamines fused to RFP was sufficient to co-localize with GFP-Staufen1, GFP-DCP1a, and GFP-Ago2, but not GFP alone (data not shown). Taken together these results provide compelling evidence that the majority of dendritic Htt granules contain DCP1 and Staufen; thus they are structurally related to PBs and NGs.

**Huntingtin Interacts with Dendritic mRNAs**—Huntingtin has been observed at neuritic branch points but details regarding the function of these structures or the identity of additional components of these Htt structures have yet to be reported (33). Staufen is an RNA-binding protein involved in transport of neuronal mRNAs. Immunostaining of neurons showed co-localization of endogenous Htt with Staufen in large structures at or near dendritic branch points (supplemental Fig. 4). To test if RNA is present with Htt at these branch points, we incubated live hippocampal neurons with the cell-permeable RNA-specific dye SYTO RNASelect, and then fixed and immunostained the cells with Htt and MAP2 antibodies and examined them by microscopy. We found Htt near these neuritic branch point structures to co-localize with RNA (Fig. 2A). To verify that the signal from SYTO RNASelect was specific for RNA, we incubated the fixed cells with RNase or DNase and imaged the cells after immunostaining. Importantly, treatment with RNase was sufficient to abolish the SYTO RNASelect signal, while DNase treatment had no observable effect (Fig. 2A). Additionally, we found Htt present with Staufen and RNA in common granules near branch points by immunostaining and FISH with oligo(dT) as probe to detect polyadenylated mRNAs (Fig. 2B).

We next examined live neurons transfected with RFP-Htt480-17Q and treated with SYTO RNASelect. Many Htt-containing structures possessed abundant quantities of RNA and showed localized movements at or near branch points (Fig. 2, C and D, and supplemental Movie S1). RNA is known to transport at broad distribution of speeds; our observation falls

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within the reported distribution. We conclude that RNA is present with Htt near dendritic branch points. The observation that RNA is present at branch points in granules is supported by many previous reports (Ref. 34 and references therein).

To confirm biochemically that Htt is present in NGs together with known PB residents, we fractionated mouse brain extracts by glycerol gradient sedimentation and analyzed the distribution of Htt, Ago2, Staufen, eIF4E, and a ribosomal protein rpS6 by Western blotting (Fig. 3A). The results demonstrated that Htt was recovered predominantly in fractions 14–18 while Ago2 sedimented between fractions 10–18. Staufen was found in two populations: the majority in the pellet fraction and a minor in fractions 6–18, with an increased abundance in fractions 12–18. eIF4E was distributed only in the top half of the gradient (fractions 2–10) and rpS6 was found only in the pellet fraction. The observation that Staufen fractionates in two distributions, one heavy pool containing ribosomes and a minor light pool, is consistent with a previous report from rat brain (35). Fractions 14–18 are enriched for Htt, Ago2, and Staufen, suggesting these proteins migrate in a complex of ~1 MDa that lacks ribosomes and translation initiation factors (Fig. 3A). We also immunoprecipitated endogenous Htt from unfractionated mouse brain extract and found endogenous Htt to associate with Ago2 (Fig. 3B) as already shown in somatic cells (8).

To investigate the possibility that Htt associates with dendritic mRNAs we performed quantitative reverse transcription-PCR analysis of the same Htt immunoprecipitates from mouse brain. We chose to examine the well characterized dendritic mRNAs IP3R1, CaMKII, and MAP2 in addition to β-catenin, which has only been detected previously in dendrites after RNA amplification (36). For negative control mRNAs restricted to the cell body, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate mutase. Htt immunoprecipitates showed significant enrichment (relative to IgG control) for all dendritic mRNAs assayed, with 20-fold enrichment for IP3R1, 16-fold for CaMKII, 10-fold for β-catenin, and 6-fold for MAP2 mRNA compared with GAPDH mRNA (Fig. 3C). The biochemical results suggest that Htt may be associated with a variety of mRNAs in neurons.

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Huntingtin Traffics in Primary Neurons—Several mechanisms have been proposed for how Htt contributes to intracellular trafficking; however, direct evidence that Htt traffics in live cells has yet to be reported (37). Toward this end, we prepared two Htt N-terminal constructs, 480–17Q and 590–25Q within the reported distribution. We conclude that RNA is present with Htt near dendritic branch points. The observation that RNA is present at branch points in granules is supported by many previous reports (Ref. 34 and references therein).

To confirm biochemically that Htt is present in NGs together with known PB residents, we fractionated mouse brain extracts by glycerol gradient sedimentation and analyzed the distribution of Htt, Ago2, Staufen, eIF4E, and a ribosomal protein rpS6 10-fold for β-catenin, and 6-fold for MAP2 mRNA compared with GAPDH mRNA (Fig. 3C). The biochemical results suggest that Htt may be associated with a variety of mRNAs in neurons.
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Fused to mRFP, and performed live videomicroscopy of transfected cortical neurons with each Htt construct. We found that Htt480-17Q and Htt590-25Q traffic in rectilinear trajectories in both dendrites and axons (supplemental Movies S2–S5 and data not shown). The two Htt constructs showed similar distribution and trafficking rates; therefore, we performed detailed analysis of the Htt590-25Q movements, because the construct contained more Htt sequence (Table 2).

We determined the average speed of dendritic Htt granules to be 0.09 μm/s (±0.11) and observed both anterograde and retrograde transport, consistent with previous reports that Htt is associated with both plus-end and minus-end-directed microtubule motor proteins (37). This speed is similar to the average speed reported for GFP-Staufen (0.11 μm/s) in mammalian neurons (6, 38). Analysis of Htt trafficking revealed that 27.7% of Htt granules trafficked in axons and 24.1% in dendrites, similar to previous observations made for DCP1, which revealed 26.9% of the granules trafficked (6). Taken together with the co-trafficking data shown for Htt480-17Q with SYTO RNASelect (Fig. 2, C and D, and supplemental Movie 1), this is to our knowledge the first report that Htt traffics in mammalian neurons.

Huntingtin Is Present in Dendritic Granules with the 3′UTR of IP3R1 and Actin mRNA—Our biochemical analysis revealed that Htt immunoprecipitates were significantly enriched with the IP3R1 mRNA suggesting Htt associates (either directly or indirectly) with this dendritic mRNA (Fig. 3C). To address this further, we used the MS2-fluorescent reporter RNA localization system to test if Htt can be detected in dendritic granules

![Graph](image_url)

**FIGURE 3.** Huntingtin co-purifies with Ago2 and dendritic mRNA. A, 1% Nonidet P-40 lysates from rat cortical neurons (DIV 14) were separated on 10 – 40% glycerol gradients. Even-numbered fractions were analyzed by immunoblotting with α-Htt, α-Ago2, α-Staufen, α-eIF4E, and α-rpS6 antibodies. Odd-numbered fractions were used to determine total protein per fraction, which is graphed above (scale of 1 = 50 μg). In (input) represents 5% of protein (50 μg) analyzed in the gradient. B, the soluble S1 fraction from mouse brain (P10) was immunoprecipitated with α-Htt or control IgG antibodies and probed with α-Htt and α-Ago2 antibodies. The percent recovery of Ago2 was determined by the LI-COR software and indicated below the IP lanes. C, the immunoprecipitated material was analyzed by quantitative RT-PCR. The assayed mRNAs are indicated along the x axis and are grouped based on their subcellular distribution. Relative enrichment was determined by dividing the Htt signal (IP/input) by the IgG signal (IP/input) and graphed relative to GAPDH (n = 3 independent experiments). Error bars indicate standard deviations. t test: equal variance (homoscedastic), two-tailed distribution, all compared relative to GAPDH. IP3R1 0.004, CaMKII 0.05, α-catenin 0.02, and MAP2 0.05.

**TABLE 2**

| Huntingtin traffics in live cortical neurons |
|--------------------------------------------|
| Cortical neurons (DIV 5–6) were transfected with mRFP Htt590-25Q and imaged every 5 s (see supplemental Movies S2–S5). |

| mRF-Htt590-25Q |
|----------------|
| Axon           |
| Total structures in axons | 36 |
| Anterograde    | 0  |
| Retrograde     | 10 |
| Speed (μm/s ± S.D.) | 0.13 ± 0.17 |
| Oscillating/stationary* | 369 single movements |
| % stopping of moving structures | 74.2 |

| Dendrite       |
| Total structures in dendrites | 54 |
| Anterograde    | 3  |
| Retrograde     | 0  |
| Bidirectional  | 10 |
| Speed (μm/s ± S.D.) | 0.09 ± 0.11 |
| Oscillating/stationary* | 845 single movements |
| % stopping of moving structures | 85.9 |

*Particles that do not move at least three steps (four frames) in a row in the same direction. Particles that move one or two steps, stop, move another step, stop, are scored as oscillating.
that contain the IP₃R1–3’UTR. This system has previously been used to investigate the role of SYNCRIP, an RNA-interacting protein and resident of RNA granules, in dendritic transport of IP₃R1 mRNA (23). Neurons were transfected with mRFP-Htt480-17Q or mRFP-Staufen1, NLS-MS2-Venus, and an RNA localization reporter plasmid expressing the IP₃R1–3’UTR fused to the binding sequence (MS2bs) of the bacteriophage MS2 protein. mRFP-Htt480-17Q and mRFP-Staufen1 showed significant co-localization with IP₃R1–3’UTR (detected by NLS-MS2-Venus) in cortical dendrites (Fig. 4A). As a negative control we omitted the IP₃R1–3’UTR plasmid and found the Venus signal restricted mainly to the nucleus presumably via the NLS (Fig. 4B). This result suggests that dendric co-localization of NLS-MS2-Venus with Htt480-17Q or Staufen1 is dependent on the IP₃R1–3’UTR mRNA. Additionally, when we transfected cells with mRFP without Htt, the RFP and Venus signals did not overlap in dendrites, suggesting the observed co-localization was dependent on Htt (data not shown).

To determine if endogenous Htt is present in dendritic RNA granules, a second fluorescent reporter RNA localization system was used. Cortical neurons were transfected with two plasmids, the first plasmid containing the RFP coding sequence termed was used. Cortical neurons were transfected with two plasmids expressing the IP₃R1–3’UTR fused to the binding sequence (MS2bs) of the bacteriophage MS2 protein. mRFP-Htt480-17Q and mRFP-Staufen1 showed significant co-localization with IP₃R1–3’UTR (detected by NLS-MS2-Venus) in cortical dendrites (Fig. 4A). As a negative control we omitted the IP₃R1–3’UTR plasmid and found the Venus signal restricted mainly to the nucleus presumably via the NLS (Fig. 4B). This result suggests that dendric co-localization of NLS-MS2-Venus with Htt480-17Q or Staufen1 is dependent on the IP₃R1–3’UTR mRNA. Additionally, when we transfected cells with mRFP without Htt, the RFP and Venus signals did not overlap in dendrites, suggesting the observed co-localization was dependent on Htt (data not shown).

To determine if endogenous Htt is present in dendritic RNA granules, a second fluorescent reporter RNA localization system was used. Cortical neurons were transfected with two plasmids, the first plasmid containing the RFP coding sequence linked to the β-actin-3’UTR-zipcode element plus four copies of boxB element, and the second plasmid expressing GFP fused to AN, the N protein of bacteriophage λ that binds to boxB and facilitates visualization of boxB-containing mRNA. Indeed, transfected neurons stained with Htt antibodies revealed significant co-localization with the β-actin-3’UTR-zipcode element reporter, a previously characterized dendritically targeted mRNA (39) (Fig. 4C, top two panels). Htt co-localized more often (~7-fold) with the β-actin-3’UTR-zipcode reporter RNA (detected by GFP fluorescence) than with the protein product of that reporter (RFP fluorescence) (Fig. 4, C and D). As a negative control, we omitted the β-actin-3’UTR-zipcode reporter and found the GFP signal restricted to the nucleus (data not shown). Together, these assays revealed that Htt is found in RNA granules with two known dendritically localized mRNAs.

Tethered Huntingtin Represses Reporter Gene Expression—Many proteins present at PBs and NGs function in post-transcriptional control of gene expression either by repressing translation or triggering RNA decay (4). Neuronal RNA transport and translational repression are linked processes, which are co-regulated in the context of large RNA/protein granules via various trans-acting proteins that include MOV10 (40) and Argonaute. Ago may play a role in the repression of transported RNA, which becomes de-repressed in response to synaptic activity permitting local translation at target sites (6, 41–43). Therefore, we tested the repressive effect of targeting Htt to an mRNA through a heterologous RNA-protein tether in the 3’UTR (44). We first transfected murine neuroblastoma cells (N2a) with Htt590-25Q or Ago2 alone, or each protein fused to the tethering peptide AN derived from the N protein of bacteriophage λ. Additionally, cells were transfected with a control firefly luciferase reporter and a Renilla luciferase reporter with ten copies of boxB elements (bound by AN) in the 3’UTR and assayed for luciferase activity. Cells transfected with the reporter containing ten boxB elements and the tethered AN–Htt plasmid showed ~3-fold reduction in reporter activity when compared with cells transfected with the un-tethered Htt plasmid (Fig. 5A, black bars), despite the lower cellular concentration of the tethered protein (Fig. 5B). Tethered AN-Ago2, a positive control for the assay (21), was somewhat more potent as a repressor, reducing reporter activity by ~9-fold. Both tethered Ago2 and Htt had only minor effects on the reporter lacking boxB elements (Fig. 5A, white bars). This analysis revealed that, like Ago2, Htt down-regulates gene expression when tethered to mRNA.

To determine if Htt-mediated repression is dependent on endogenous Ago2, we performed a similar experiment in N2a cells first transfected with siRNA to Ago2, or GFP (as a control). Consistent with our previous report that Htt interacts with Ago2 and plays a role in gene silencing (8), we found that tethered Htt-mediated reporter gene silencing was abolished in cells depleted of endogenous Ago2 by siRNA (Fig. 5C, compare lanes 7 and 8 with 11 and 12). These results suggest that Htt can function through Ago2 to repress gene expression.

Knockdown of Huntingtin Perturbs RNA Localization—To assess the effects of Htt on dendritic mRNA, we carried out experiments to deplete endogenous Htt. To this end we infected developing rat cortical neurons with lentiviruses that expressed two different Htt shRNAs. To ensure that these shRNAs were effective in reducing Htt protein levels and that viral infection alone did not affect Htt protein levels, we analyzed cell extracts by Western blotting of neurons infected with lentivirus-expressing GFP, a non-silencing shRNA, or two independent Htt shRNAs (Fig. 6A). The shRNA constructs also expressed GFP. The results revealed specific and potent knockdown of Htt from both viral constructs (98 and 70%) and no effect on Htt levels from control viruses.

Neurons were infected with lentivirus expressing shRNA corresponding to a scrambled sequence of Htt (SCB) or two independent shRNAs against Htt. We performed FISH with oligo(dT) as probe to examine the distribution of mRNA, and immunofluorescence to detect GFP and endogenous Htt (Fig. 6B). Upon knockdown of Htt (~4-fold), we found concomitant loss of punctate RNA staining, suggesting that Htt is required for discrete localization of mRNAs in primary neurons. The depletion of Htt also caused loss of granular Ago2 staining. This is consistent with our previous result showing that loss of Htt caused disassembly of PBs in non-neuronal cells, implicating a role for Htt in assembly/maintenance of PBs (8). By contrast, knockdown of Ago2 had little effect on Htt staining, but significantly altered the distribution of the mRNA signal (Fig. 6C). Thus, Htt may function through Ago2 to modulate RNA localization in neurons.

DISCUSSION

In this study we have demonstrated that endogenous Htt can associate with components of neuronal granules to participate in the transport of mRNA in dendrites. Htt was found to co-localize with RNA containing specific 3’UTR with known dendritic targeting sequence. Our findings also suggest that Htt is involved in repression of mRNA.

Role of Huntingtin in Trafficking—The first connection between Htt and trafficking was made through its interaction with huntingtin-associated protein 1, which is associated
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with the plus-end-directed microtubule motor protein kinesin, and the p150Glued dynactin that functions with the minus-end-directed microtubule motor dynein (15, 17, 45, 46). Additional evidence indicated that Htt interacts directly with dynactin (47). Based on recent findings, several models have been proposed that place Htt as a regulator of intracellular trafficking of vesicles and organelles along microtubules (reviewed in Ref. 37). Our study proposes an additional function to wild-type Htt, namely the ability to associate with dendritic mRNAs and influence RNA trafficking.

High throughput analysis of subcellularly localized mRNAs has led to the categorization of hundreds of mRNAs present in dendrites. A recent investigation has confirmed that more than 300 mRNAs are present in mammalian axons (48). Computational analysis of these mRNAs, however, has failed to uncover a common primary sequence or secondary cis-acting element required for dendritic targeting (36). This analysis and many others (see Refs. 7, 49) suggest there is no consensus sequence that marks mRNA for transport but rather they are selected through various independent mechanisms likely governed by complex secondary RNA structures present within the 3'UTRs (50). Further, all currently available data suggest that various mechanisms ensure temporally and spatially regulated translation of transported mRNAs within neurons.

Functional Relationship between Huntingtin and Ago2 in Neuronal Cells—We previously reported that Htt associates with Ago2 in somatic PBs and contributes to small RNA-mediated gene silencing (8). Recent

FIGURE 4. Huntingtin is present at dendritic RNA granules that contain IP₃, R1 and β-actin 3' UTR mRNAs. A, cortical neurons (DIV 5–6) were transfected with NLS-M52-Venus, IP₃, R1–3' UTR-M52bs reporter, and mRFP-Htt480-17Q or mRFP-Stauflen1 plasmids and imaged for RFP and Venus. As shown in the schematic, NLS (red circle)-M52-Venus served as a readout for IP₃, R1–3' UTR mRNA. The neurons were subsequently stained with α-MAP2 antibody to visualize dendrites. Scale bar, 10 μm. *, neuron enlarged in the lower panel. B, cortical neurons were transfected with mRFP-Htt480-17Q and NLS-M52-Venus but without the IP₃, R1–3' UTR-M52bs reporter. Scale bar, 10 μm. C, cortical neurons were transfected with α-GFP-NLS and 4xboxB-β-actin-zipcode plasmids and probed with α-Htt and α-GFP antibodies. In this experiment, mRNA containing the β-actin-zipcode sequence was detected by GFP, and its protein product was detected by RFP (see schematic, NLS in red circle). Scale bar, 10 μm. D, quantification of pixel-based co-localization from C (6 cells, 12 dendrites). *** p value (paired t test, two-tailed distribution) 2.29 x 10⁻⁶. Error bars indicate standard deviations.

FIGURE 5. A tethered function for huntingtin. A, N2a cells were transfected with HA-Ago2, NHA-Ago2, HA-Htt590-25Q, or NHA-Htt590-25Q and a Renilla luciferase reporter plasmid (RL) or without the element (white bars). Firefly luciferase reporter plasmid served as a normalization control. The ratio of Renilla to firefly luciferase activity is graphed as the percentage of expression relative to the cells transfected with un tethered plasmid (e.g. Ago2 and Htt590-25Q) set to 100%. Error bars indicate standard deviations. The data are representative of at least three independent experiments. B, Western blot analysis of lysates from cells transfected with the indicated plasmids. The murine N2a cells were first transfected with no siRNA (lanes 1–4), siRNA to murine (m)Ago2 (lanes 5–8), and siRNA to human (h)Ago2 (lanes 9–12). After 48 h, the cells were transfected with the indicated plasmid, a Renilla luciferase reporter plasmid with 10xboxB elements, and a firefly luciferase reporter plasmid (normalization control). The ratio of Renilla to firefly luciferase activity is graphed as for panel A. Error bars indicate standard deviations. The data are representative of at least three independent experiments. Western blots below the graph show expression levels of the transfected HA-tagged tethered and untethered constructs (all constructs are HA-tagged), endogenous mAgo2 (indicated by arrow) and transfected human Ago2 (not subject to siRNA knockdown), and GAPDH as a loading control. * NS, nonspecific protein band.

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FIGURE 6. Knockdown of Htt perturbs RNA localization. A, Western blot analysis of protein extracts from cortical neurons infected with lentivirus expressing GFP, non-relevant shRNA, shRNA-Htt-1, or shRNA-Htt-2 probed with α- Htt and α-β-tubulin antibodies. B, neurons infected with lentivirus-expressing scrambled (SCB) shRNA, or indicated shRNA against Htt were probed with oligo(dT) (FISH), α-Htt, α-GFP, and α-Ago2 antibodies. Proximal region is at the left. Scale bar, 5 μm. The table shows relative intensity of mRNA (measured by oligo(dT) signal), Htt, and Ago2 normalized to the area of GFP staining, which represents the size of dendrites. C, neurons infected with lentivirus-expressing scrambled (SCB) shRNA, or shRNA against Ago2 were probed with oligo(dT) (FISH), α-Ago2, α-GFP, and α-Htt antibodies. Scale bar, 5 μm.

function in dendritic transport and local translation of critical mRNAs that permit expression of survival factors such as neurotrophins at target sites. We speculate that mutant Htt performs similar functions as wild type; however, the expanded polyQ sequence may alter the composition/dynamics of NGs over time, which could eventually lead to the disease phenotype. Identification of specific mRNAs associated with Htt-containing neuronal granules will help to elucidate the mechanisms that contribute to progressive neurodegeneration in HD.

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studies from several laboratories have strengthened the connection between miRNA-based regulation and PTGS in NGs (30). Here we report that Htt co-localizes with Ago2 in dendritic granules, and knockdown of Htt in dendrites results in altered Ago2 distribution. We also found Ago2 required for Htt-mediated reporter gene silencing in neuronal cells. Together, these data provide support for a functional relationship between Htt and Ago2 in neurons. NGs that contain Htt may be enriched for Ago proteins and miRNA-targeted mRNAs. Whether Htt-Ago2 complex associates with a specific subset of miRNA/mRNAs remains to be determined.

Translational Repression and RNA Transport Are Linked Processes—mRNA transport and translational regulation are linked processes in neurons; this ensures protein production in spatial-temporal regions of neurites at high fidelity (3). We previously demonstrated that Htt is physically and functionally linked to the post-transcriptional gene-silencing (PTGS) pathway. Growing evidence suggests that Htt plays a critical role in cytoskeletal-dependent transport processes (37). Our results indicate that Htt may function in RNA transport and in translational control within dendrites and axons. Htt may serve as a structural bridge linking the transport machinery to the translational repression/PTGS machinery within neuronal granules. A recent study from several laboratories has shown that Htt is linked to the post-transcriptional gene-silencing (PTGS) machinery.
