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Aggregate-centered redistribution of proteins by mutant huntingtin

Leigh Anne Swayne, Janice E.A. Braun *

Hotchkiss Brain Institute, Department of Physiology and Biophysics, University of Calgary, Calgary, Alta., Canada T2N 4N1

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Abstract

Huntingtin is a widely expressed 350-kDa cytosolic multidomain of unknown function. Aberrant expansion of the polyglutamine tract located in the N-terminal region of huntingtin results in Huntington's disease. The presence of insoluble huntingtin inclusions in the brains of patients is one of the hallmarks of Huntington's disease. Experimentally, both full-length huntingtin and N-terminal fragments of huntingtin with expanded polyglutamine tracts trigger aggregate formation. Here, we report that upon the formation of huntingtin aggregates; endogenous cytosolic huntingtin, Hsc70/Hsp70 (heat shock protein and cognate protein of 70 kDa) and syntaxin 1A become aggregate-centered. This redistribution suggests that these proteins are eventually depleted and become unavailable for normal cellular function. These results indicate that the cellular targeting of several key proteins are altered in the presence of mutant huntingtin and suggest that aggregate depletion of these proteins may underlie, in part, the sequence of disease progression.

Keywords: Huntingtin; Huntingtin aggregates; Hsc70/Hsp70; Syntaxin

Huntingtin is a 350-kDa multidomain protein that is targeted by specific proteases including calpains [7,8,14] and caspases [9,10,18,32–34]. The resulting huntingtin fragments have been proposed to represent a functionally active species; however, the precise cellular function of either huntingtin or the huntingtin fragments remains to be established. The cellular trafficking of huntingtin is influenced by palmitoylation [35]. Deletion of the huntingtin gene results in embryonic lethality in mice [6,21,36]; while, huntingtin deletion in mature mice by conditional mutagenesis causes severe brain damage [5], indicating that huntingtin is essential. In humans, aberrant expansion of a polyglutamine tract located in the N-terminal region of huntingtin results in Huntington's disease, a neurodegenerative disorder characterized by progressive psychiatric, cognitive, motor dysfunction, and early death [12]. The severity of Huntington's disease depends on the length of the glutamine repeats. In unaffected individuals the polyglutamine tract contains between 6 and 39 repeats compared with 36–250 repeats in patients with Huntington's disease. Selective neurodegeneration is characteristic of Huntington's disease despite the ubiquitous nature of huntingtin expression. The initial target of cell degeneration in Huntington's disease is the striatal medium spiny GABAergic neuron [30], with neuronal loss also observed in the globus pallidus, cortex, hippocampus, thalamus, and cerebellum. The cellular role of huntingtin and the molecular basis for the selective vulnerability of neurons in Huntington's disease are currently the subject of intense investigation.

While the cascade of events that leads to the neurodegeneration found in Huntington's disease is not yet established, high levels of huntingtin proteolysis, the formation of huntingtin inclusion bodies, and abnormally high intracellular Ca^{2+} levels are observed in a wide variety of Huntington's disease models. We have recently shown that huntingtin with expanded polyglutamine repeats (i.e. mutant huntingtin) but not native huntingtin blocks the activity of the cysteine string protein chaperone system on G protein signaling cascades [19]. In addition, we have found that both expanded and nonexpanded huntingtin associate with the N-type Ca^{2+} channel and enhance

* Corresponding author. Fax: +1 403 283 8731.
E-mail address: braunj@ucalgary.ca (J.E.A. Braun).

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Ca^{2+} influx by removing inhibition of N-type Ca^{2+} channels [27].

Here, we have examined cellular protein localization in the presence of huntingtin with an expanded polyglutamine tract. We have found that a dramatic relocalization of native-(i.e. nonexpanded) huntingtin and Hsc70 occurs in the presence of huntingtin aggregates. Furthermore, the distribution of the membrane protein syntaxin 1A was extended to form rings around the huntingtin inclusions. Thus, aggregate-centered redistribution of proteins is an important consideration of current working models of Huntington's disease pathology.

Methods

Hippocampal neuron culture preparation and immunofluorescence microscopy. Dissociated hippocampal neurons were prepared from P0 mice as previously described [27]. Briefly, the tissue was digested for 30 min at 37 °C in a papain solution (20 U/ml, Worthington; 50 μM EDTA and Ca^{2+} chloride/L-cystenine (150 /100 mM)) which was prepared in cell plating media (Basal Medium Eagle (BME, Invitrogen), 0.3% glucose, 5% fetal bovine serum (FBS, Hyclone), 0.5 mM L-glutamine (Sigma), 10 mM Hepes–NaOH, pH 7.35, 2% B27 (Invitrogen), 15 mM NaPyruvate (Invitrogen), and 100 μg/ml penicillin–streptomycin (Invitrogen). After digestion the hippocampal tissue was washed and triturated to single cells. DNA constructs were electroporated into the cells. The tissue suspension was seeded ($\sim 5 \times 10^5$ cells/cm^2) onto coverslips pre-treated with poly-D-lysine (PDL; 70 kDa, 10 μg/ml, Sigma), and laminin (1 μg/ml, Sigma) in 24-well plates and maintained in a humidified atmosphere of 5% carbon dioxide 95% air at 37 °C.

After 2 days the neural cultures were rinsed in PBS, then fixed in 4% paraformaldehyde for 30 min to overnight at 4 °C. After washing, cells were incubated in the blocking solution (2% or 5% goat serum, 2% BSA, 0.1% Triton X-100 in PBS, and pH 7.4) for 1 h at 4 °C and then in the primary antibodies (diluted in the blocking solution) overnight at 4 °C. Neurons were washed several times in blocking solution in PBS before incubating the coverslips in the appropriate sheep secondary antibodies labeled with indocarbocyanine (Cy-3) (Sigma, or Jackson Immunoresearch Laboratories) for 1 h at room temperature [1]. Images were acquired with a Zeiss 510 Meta Laser scanning confocal microscope. Microscope composite assembly was performed with Adobe Photoshop 7.0.1.

HEK cell immunoflorescence microscopy and Huntingtin siRNA. Transient transfection of HEK 293 cells (50–80% confluency) was carried out in 35 mm tissue culture dishes using the lipofection technique. Briefly, 6–8 μl of Lipofectamine (Gibco/BRL) was mixed together with ~1.5 μg plasmid cDNA in 1 ml of serum-free culture medium (Dulbecco's modified Eagle's medium supplemented with L-glutamine and 4.5 g l^{-1} D-glucose) and placed on cells for 4–6 h in a humidified incubator containing 5 % CO_2 at 37 °C. DNA-containing medium was then aspirated and replaced with serum-containing medium. The following day, cells were detached from the dish by treatment with 0.05% (w/v) trypsin/0.5 mM EDTA and replated onto sterile glass coverslips.

HEK cells were plated onto sterile glass coverslips coated with poly-L-lysine and transfected by the Ca^{2+} phosphate method as previously described [15,20]. Two days post-transfection, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, washed and, permeabilized by incubation for 5 min in PBS containing 0.2% Triton X-100. Permeabilized cells were washed, blocked in PBS supplemented with 3% BSA, 5% normal Donkey serum, and 0.05% Tween 20 solution for 30 min, and incubated with the indicated primary antibody (diluted in blocking solution) overnight at 4 °C. Subsequent to several washes in blocking solution PBS, the cells were incubated with Cy3-conjugated secondary antibody (Jackson Immunoresearch Laboratories) diluted in blocking solution. Following several PBS washes, the coverslips were mounted on microscope slides with a drop of 50% glycerol in PBS. Images were captured with Olympus IX-70 wide-field fluorescence microscope. Development of immunomicrograph composites was performed with Adobe Photoshop 7.0.1.

Annealed double-stranded siRNA oligonucleotides targeted to huntingtin were purchased from Quiagen. The target sequence consisted of 5′-AACATTGTACATCCAAATTCC-3′ (huntingtin mRNA base 13439–13459 based on the sequence from Genbank accession # NM_002111) and the oligonucleotide sequences were 5′- (CAUUGUACAUCCAAAUUAA)d(TT)-3′ and 5′-r(UUAAUUUGGAUGUACAAUG)d(TT)-3′. The number of HEK cells seeded prior to transfection was optimized to 1×10^5 cells per well of a 24-well plates. The cells were seeded 24 h prior to addition of siRNA and incubated under normal growth conditions (37 °C and 5% CO_2). The ratio of μg of siRNA to μl of RNAifect transfection reagent was 1:9. On the day of transfection, 1 μg of huntingtin siRNA was diluted in Buffer EC-R and 9 μl were mixed by vortexing and incubated for 10 min at room temperature to allow formation of transfection complexes which were added dropwise onto the cells. The medium was exchanged 6 h post-transfection and gene silencing was monitored after 48 h.

Results and discussion

The cellular role(s) of huntingtin is currently unknown. We have previously shown a role for huntingtin N-terminal fragments in the uncoupling of syntaxin 1A, a SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) from N-type Ca^{2+} channels, a channel key in coupling synaptic vesicle exocytosis with Ca^{2+} influx [27]. We found that both normal and mutant huntingtin associate with the N-type Ca^{2+} channel. Does huntingtin-uncoupling of Ca^{2+} influx to exocytosis contribute to the neurodegeneration observed in Huntington disease? It is quite possible that dysregulation of cellular Ca^{2+} levels is the basis of Huntington's disease. For example, resting Ca^{2+} levels are increased by almost twofold in CA1 pyramidal neurons in a Huntington's disease mouse model expressing full-length expanded mutant huntingtin [11]. Basal Ca^{2+} levels are increased in mutant huntingtin-transfected striatal medium spiny neurons [28]. Mutant huntingtin has also been implicated in dysfunctional mitochondrial Ca^{2+} buffering [23]. In the presence of mutant huntingtin, the *N*-methyl-D-aspartate receptor subtype 2B (NR2B) is sensitized to glutamate, which leads to enhanced Ca^{2+} influx following receptor stimulation [3,37,38]. Mutant huntingtin increases the sensitivity of the inositol 1,4,5-triphosphate (IP_3) receptor to IP_3, which leads to enhanced Ca^{2+} release following mGluR1/5 activation [28]. Determination of the precise role of Ca^{2+} in the neurodegenerative sequence of events that underlie Huntington's disease will undoubtedly reveal if Ca^{2+} channel blockers might serve as effective therapies against Huntington's disease. Here, we show that in addition to the Ca^{2+} dysregulation, mutant huntingtin aggregation, the normal redistribution of cytosolic, and membrane proteins are altered in the presence of huntingtin aggregates. These data suggest that an aggregate-centered distribution of essential cellular proteins may, in part, underlie the pathogenesis of Huntington's disease.

The hallmark of Huntington's disease is the presence of insoluble huntingtin inclusions in the brains of affected

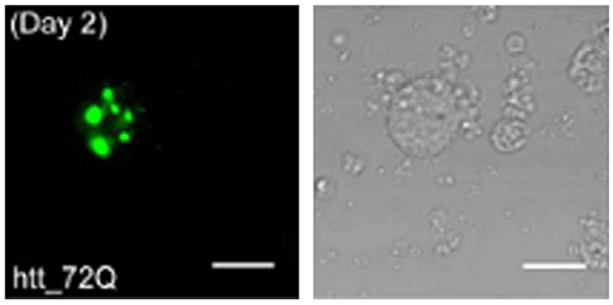

Fig. 1. Transient Expression of huntingtin with expanded polyglutamine repeats is toxic to hippocampal neurons. Confocal image of aggregates of EGFP-tagged huntingtin with a 72 polyglutamine stretch in hippocampal neurons (left panel) and brightfield of the same cells (right panel). Image was taken after two days in vitro. Scale bars = 10 μm.

individuals. It is controversial whether these inclusions are the disease causing agent(s). We have previously shown that native huntingtin as well as transiently expressed EGFP-tagged full-length huntingtin with a 17, 72 or 138 glutamine stretch and transiently expressed EGFP-tagged N-terminal huntingtin (i.e. exon1) with 25 or 72 polyglutamines are initially widely distributed in hippocampal neurons [27]. Huntingtin with 72 or 138 glutamines eventually form aggregates and is neurotoxic. Fig. 1 shows that expression of EGFP-tagged full-length huntingtin with 72 polyglutamines may cause excessive cell damage as early as 2 days after transfection. No neurons survive past 7 days (data not shown). These findings are consistent with the neural pathology observed in various Huntington disease models [16,25,29]. Due to this neurotoxicity of transiently expressed huntingtin with an expanded glutamine tract in hippocampal neurons, we chose to examine the effect of transiently expressed EGFP-tagged huntingtin with 72 polyglutamine repeats on the distribution of cellular proteins in human endothelial kidney (HEK) cells. Compared to hippocampal neurons, HEK cells have a greater survival rate (results not shown) after transient expression of huntingtin with expanded polyglutamine repeats and typically produce very large huntingtin inclusion bodies (Fig. 2) as previously shown [26,27].

Fig. 2A and B demonstrate that native full-length huntingtin is diffusely expressed throughout HEK cells as evaluated with anti-huntingtin monoclonal 2166 (Chemicon). Immunolocalization of huntingtin was specific as demonstrated by the reduction in huntingtin expression by small interfering RNA (huntingtin siRNA) (immunofluorescence middle panel and bright field right panel. Western blot analysis confirmed the huntingtin siRNA-reduction observed by immunolocalization (Fig. 2A). We then examined the distribution of native huntingtin after transient transfection of EGFP-tagged N-terminal huntingtin (i.e. exon 1) with 72 polyglutamine repeats. As shown in Fig. 2C, mutant huntingtin forms large aggregates and

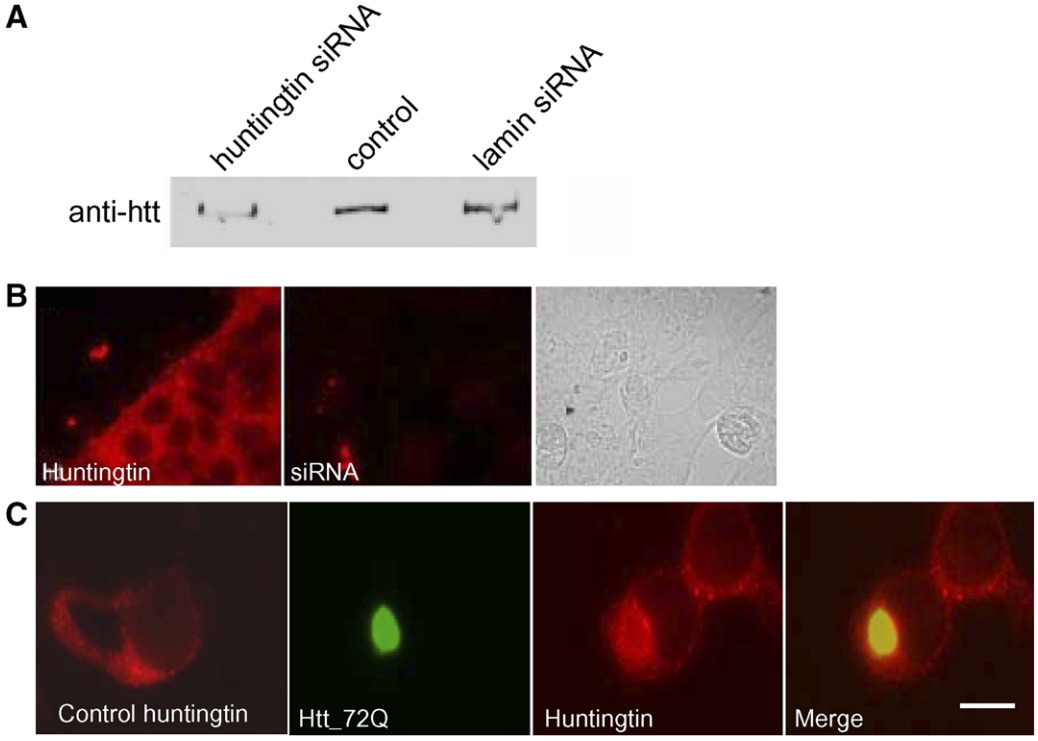

Fig. 2. Huntingtin aggregates trigger the redistribution of native huntingtin. (A) Western blot probed with anti-huntingtin (mAb2166, Chemicon) of HEK cell homogenates from huntingtin siRNA, control and lamin A/C siRNA-treated cells. (B) Huntingtin immunofluorescence (mAb 2166, Chemicon) in control cells (left panel), and with huntingtin siRNA (middle panel). Brightfield image of huntingtin siRNA-treated cells (same cells as in middle panel). (C) Confocal image of huntingtin distribution in the absence (left panel) and presence (right three panels Htt_72Q) of aggregates of EGFP-tagged huntingtin with a 72 polyglutaime stretch in HEK cells. Scale bars = 10 μm.

the widespread localization of huntingtin in HEK cells became huntingtin-aggregate-centered in the presence of EGFP-tagged N-terminal huntingtin (i.e. exon 1) with 72 polyglutamine repeats. These results suggest that the cellular function of native huntingtin is compromised in the presence of huntingtin with polyglutamine expansion.

Next, we examined the distribution of native Hsc70/Hsp70 after transient transfection of N-terminal huntingtin (i.e. exon 1) with 72 polyglutamine repeats. The Hsp70/Hsc70 (70-kDa heat shock protein and cognate protein) is an ancient and ubiquitous chaperone family that regulates the conformation of diverse substrate proteins [2,4,31]. The ATPase activity of Hsc70/Hsp70 s is harnessed for conformational work on specific proteins (e.g. recycling of clathrin coats by auxilin). Substrates of Hsc70 do not share homology but typically demonstrate significant conformational flexibility. Our previous work has revealed links between CSPα and heterotrimeric GTP binding protein (G protein) signal transduction pathways [17,19,20,22]. Anti-Hsp70 monoclonal (Sigma) which rec-

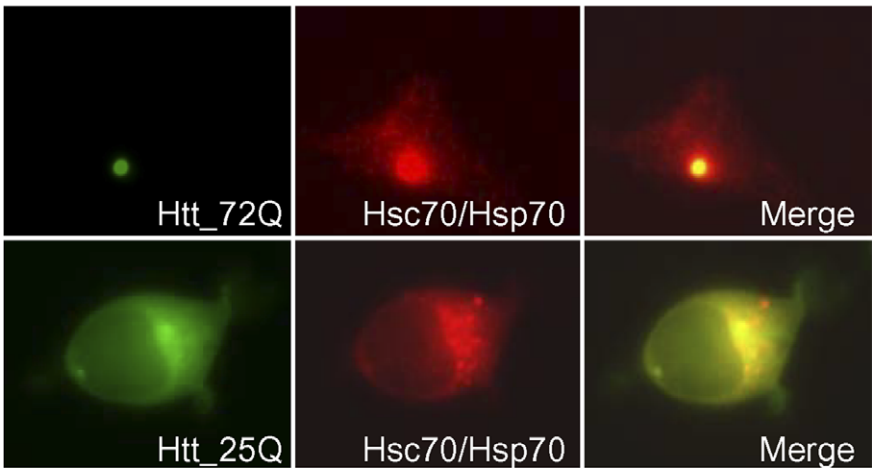

Fig. 3. Huntingtin aggregates sequester native Hsc70/Hsp70. Confocal image of hsc70/hsp70 (monoclonal Sigma) distribution in the presence of transiently expressed EGFP-tagged huntingtin with either a 72 (top panel Htt_72Q) or 25 polyglutamine (bottom panel Htt_25Q) stretch in HEK cells.

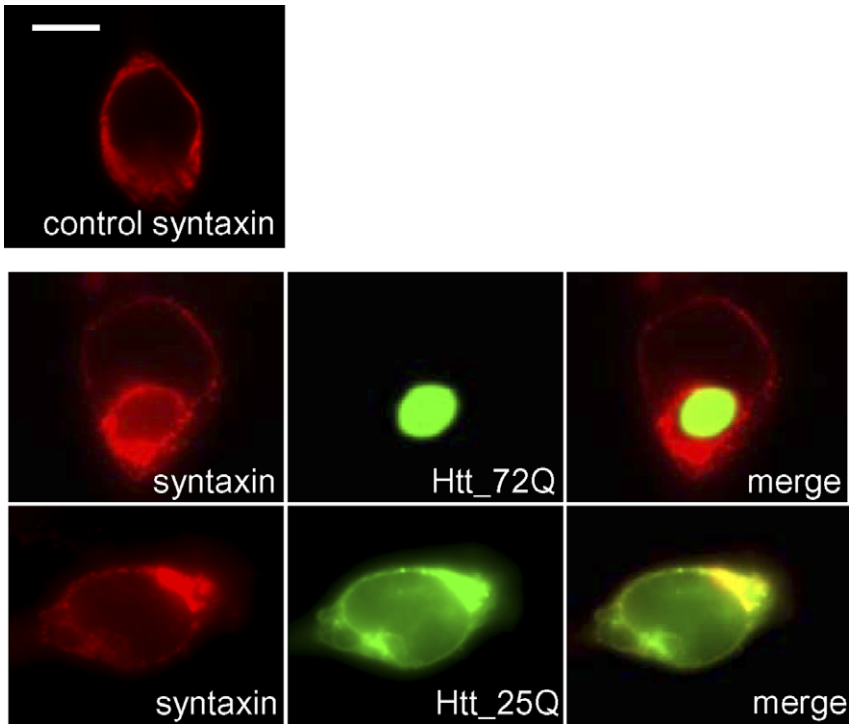

Fig. 4. Huntingtin aggregates trigger the redistribution of transiently expressed syntaxin 1A. Confocal image of the distribution of transiently expressed syntaxin 1A (Signal monoclonal) in the absence of huntingtin expression (top panel) or the presence of EGFP-tagged huntingtin with either a 72 (middle pane Htt_72Q) or 25 (bottom panel Htt_25Q) polyglutamine stretch in HEK cells. Scale bar = 10 μm.

ognizes both Hsp70 and Hsc70 [17] was used to immunolocalize Hsc70. Fig. 3 shows that Hsc70/Hsp70 was found to have widespread distribution in HEK cells transiently transfected with EGFP-N-terminal huntingtin (i.e. exon 1) with 25 polyglutamine repeats. In contrast, Hsc70/Hsp70 was found to have an aggregate-centered distribution in HEK cells transiently transfected with EGFP-N-terminal huntingtin with 72 polyglutamine repeats. These results indicate that mutant huntingtin with expanded polyglutamine repeats sequester Hsc70, eventually depleting Hsc70 availability. Exhaustion of molecular chaperone would leave the native targets of chaperones vulnerable to misfolding and result in loss of function.

Finally, we examined the distribution of transiently expressed syntaxin 1A coexpressed with either huntingtin with 72 polyglutamine repeats or 25 polyglutamine repeats. Syntaxin 1a is a member of the SNARE superfamily which drives membrane fusion [13,24]. We have previously shown that syntaxin 1A competes with huntingtin for association with N-type Ca^{2+} channels [26,27]. Fig. 4 shows that syntaxin 1A demonstrates primarily plasma membrane distributions in the presence or absence of transiently expressed EGFP N-terminal huntingtin with either 25 or 72 polyglutamine repeats. However, in addition, syntaxin 1A also showed an aggregate-centered ring-like distribution in the presence of transiently transfected EGFP-huntingtin exon 1 with 72 polyglutamines. Taken together our results indicate that huntingtin with expanded polyglutamines triggers incorrect targeting and possibly function of a number of key proteins in neurotransmission.

In summary, in Huntington's disease the polyglutamine tract of huntingtin is expanded beyond threshold inducing a conformational change that triggers a cascade of pathogenic events that remains to be characterized. Disruption of Ca^{2+} signaling pathways, the formation of insoluble inclusions and the depletion of chaperones have been implicated in Huntington's disease progression but the precise sequence of events remain to be identified. Our previous findings indicate that both (1) signaling mechanisms [19,27] and (2) chaperone activity [19] are compromised in Huntington's disease. Here, we show that the cellular distribution of essential proteins is altered in an aggregate-centered manner in Huntington's disease. The re-distribution of proteins and events that trigger re-distribution are important considerations vis a vis current working models of Huntington disease pathology.

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