A huntingtin-mediated fast stress response halting endosomal trafficking is defective in Huntington’s disease

Siddharth Nath¹, Lise N. Munsie² and Ray Truant¹,*

¹Department of Biochemistry and Biomedical Sciences, Michael G. DeGroote School of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, ON, Canada L8N 3Z5 and ²Centre for Applied Neurogenetics, University of British Columbia, Vancouver, BC, Canada V6T 2B5

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Cellular stress is a normal part of the aging process and is especially relevant in neurodegenerative disease. Canonical stress responses, such as the heat shock response, activate following exposure to stress and restore proteostasis through the action of isomerases and chaperones within the cytosol. Through live-cell imaging, we demonstrate involvement of the Huntington’s disease (HD) protein, huntingtin, in a rapid cell stress response that lies temporally upstream of canonical stress responses. This response is characterized by the formation of distinct cytosolic puncta and reversible localization of huntingtin to early endosomes. The formation of these puncta, which we have termed huntingtin stress bodies (HSBs), is associated with arrest of early-to-recycling and early-to-late endosomal trafficking. The critical domains for this response have been mapped to two regions of huntingtin flanking the polyglutamine tract, and we observe polyglutamine-expanded huntingtin-expressing cells to be defective in their ability to recover from this stress response. We propose that HSB formation rapidly diverts high ATP use from vesicular trafficking during stress, thus mobilizing canonical stress responses without relying on increased energy metabolism, and that restoration from this response is defective in HD.

INTRODUCTION

Huntingtin is a 350-kDa, 3144-residue protein involved in a variety of cellular functions including transcriptional regulation, mitotic spindle orientation and vesicular trafficking through energy-dependent molecular machinery (1–8). Initially identified as the protein product of the HTT gene implicated in Huntington’s disease (HD) (9,10), huntingtin is essential for development (4), and lowered huntingtin protein levels have been shown to sensitize cells to stress through specific restructuring of the endoplasmic reticulum (ER) (11). Structurally, huntingtin is composed largely of helix-turn-helix HEAT repeat motifs, characteristic of scaffolding proteins (12). Additionally, huntingtin has an amino-terminal alpha-helical domain, termed N17, that has been shown to modulate the protein’s intracellular localization, toxicity and function (13,14), as well as tethering huntingtin directly to the ER outer membrane and ER-derived vesicles. In HD, a CAG triplet repeat expansion in excess of 37 in the first exon of the coding region of the HTT gene leads to a polyglutamine tract expansion in huntingtin and, consequently, pathology.

We have previously established that huntingtin is involved in the cell stress response, as it translocates from the ER to the nucleus in a stress-dependent manner (14). More recently, we have also demonstrated that this localization can be modulated by a family of small molecules called kinase inhibitors, which may hold therapeutic benefit in HD (15). Upon induction of cell stress, huntingtin can also localize to nuclear cofilin–actin rods, similar to those seen in the cell cytosol in Alzheimer’s disease (16,17). Cofilin–actin rods function to transiently halt actin remodeling and thus increase available ATP during stress (18). Mutant huntingtin is defective in its ability to participate in this response, forming persistent rods that are unable to recapitulate actin dynamics (16). Moreover, an aberrant ATP/ADP ratio has been found in HD mouse and cell culture models, as well as HD brains (19). HD models have also been

*To whom correspondence should be addressed at: 4N54 Health Sciences Centre, McMaster University, 1200 Main Street West, Hamilton, ON, Canada L8N 3Z5. Email: truantr@mcmaster.ca

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noted to have chronic ER stress via the unfolded protein response (UPR) (20).

Here, we build on our previous work and further investigate the temporal role of huntingtin in the cell stress response. By visualizing huntingtin in live cells under heat shock stress, we observe a very rapid, dynamic cell stress response involving reversible accumulation of the protein at early endosomes. This localization is characterized by the formation of distinct cytosolic puncta, which we have termed huntingtin stress bodies (HSBs), and is associated with an arrest in early-to-late and early-to-recycling endosome fusion. In the context of HD, we demonstrate that cells expressing mutant huntingtin display a persistent HSB phenotype, requiring a longer time period to recover from this stress response when compared with cells expressing wild-type huntingtin.

We hypothesize that huntingtin has a critical function as a very early stress response protein, acting to arrest the energy-intensive process of endosomal trafficking, in addition to actin remodeling, to free pools of ATP for use within the cell during stress. Huntingtin stress body formation may therefore provide the critical energy required immediately for canonical stress responses, without relying on increased metabolism to produce ATP.

RESULTS

Huntingtin is involved in a rapid cell stress response

To observe huntingtin biology under stress conditions, we performed immunofluorescence on wild-type (STHdh<sup>Q7/Q7</sup>) mouse striatal-derived cells and cells derived from a knock-in mouse model of HD (STHdh<sup>Q111/Q111</sup>). Cells were either kept at 33°C (steady state) or challenged with heat shock stress for 10 min at 42.5°C. Cells kept at steady state showed diffuse staining of huntingtin (Fig. 1Aa and c), whereas stressed cells formed distinct cytosolic puncta (Fig. 1Ab and d), which we have termed huntingtin stress bodies (HSBs).

The dynamics of HSB formation were analyzed in STHdh<sup>Q7/Q7</sup> cells transfected with a construct encoding the first 586 residues of huntingtin with a wild-type polyglutamine tract length of 17 repeats, fused at its carboxyl-terminus to eYFP (1–586 Q17-eYFP). Live-cell imaging revealed that HSB formation was rapid, occurring within 30 s of induction of stress, and that HSBs are initially dynamic, but stationary following exposure to varying degrees of heat shock stress. The formation of nuclear HSF1 granules in response to stress was used as an indicator of activation of the HSR (23). As shown in Figure 1E, HSB formation was observed following only 10 min of heat shock stress, whereas the formation of HSF1 granules required that cells be challenged with at least 45 min of heat shock stress. This therefore suggests that HSB formation occurs upstream of the HSR as it precedes HSF1 activation.

Huntingtin stress bodies are distinct from stress granules and processing bodies

To determine whether HSBs are distinct from other canonical stress structures, such as processing bodies and stress granules, we co-transfected STHdh<sup>Q7/Q7</sup> cells with 1–586 Q17-eYFP, a construct encoding DCP1A, a decapping enzyme found in processing bodies, fused to mRFP (DCP1A-mRFP), and a construct encoding TIA1, a protein involved in stress granule formation, fused to CFP (TIA1-CFP) (24). Following challenge with 1 h of heat shock stress, we did not observe any co-localization between HSBs and DCP1A, or HSBs and TIA1 (Supplementary Material, Fig. S2C), indicating that these structures are distinct from HSBs.

N17 and huntingtin 81–171 are required for HSB formation

We next sought to determine which domain(s) of huntingtin are required for HSB formation. We transfected STHdh<sup>Q7/Q7</sup> cells with constructs encoding different fragments of huntingtin fused to a carboxyl-terminal eYFP fluorophore and, following challenge with heat shock stress, quantified the number of cells with puncta. As smaller huntingtin fragments are prone to aggregation, we performed live-cell imaging on cells expressing the various constructs, in parallel, to conclusively determine whether the puncta being observed were HSBs or huntingtin aggregates. We noted that the 1–171 and 1–586 fragments (and larger) formed HSBs most efficiently, with 1–117 aggregates. We noted that the 1–171 and 1–586 fragments (and larger) formed HSBs most efficiently, with 1–117 forming HSBs at a lower frequency (Fig. 2A and B, and Supplementary Material, Videos S1, S4, and S5). Cells expressing the N17 domain alone or 1–81 (huntingtin exon 1) did not form HSBs (Fig. 2A and B, Supplementary Material, Videos S2 and S3). As cells transfected with a huntingtin fragment smaller than 1–586 displayed punctate protein localization in the absence of stress, all subsequent work was performed using the 1–586 fragment.

To further delineate the role of different huntingtin domains in HSB formation, we generated constructs encoding deletions
of N17 and other critical regions, in the 1–586 context, fused at their carboxyl-terminus to eYFP. We then transfected these constructs into STHdh<sup>Q7/Q7</sup> cells and observed whether cells formed HSBs in response to heat shock stress. We noted that deletion of N17 (△2–13) completely abrogated HSB formation, as did deletion of exon 1 (Fig. 2C and D). Deletion of the poly-proline region flanking the polyglutamine tract (△polyP), however, did not affect HSB formation efficiency (Fig. 2C and D).

N17 structure influences HSB formation
We have previously shown that the N17 domain of huntingtin is a highly structured amphipathic alpha-helix that functions as a membrane-association domain (14) and a CRM1-dependent nuclear export signal (25). Furthermore, we have also demonstrated that the phosphorylation status of two serine residues (S13 and S16) within this region can modulate huntingtin localization through induction of structural changes (15). N17 has also been shown by solution and solid-state NMR to associate directly with the outer ER lipid leaflet (26). To build on our previous work (14,15) and results indicating that N17 is required for HSB formation (Fig. 2C and D), we further examined its role in this stress response. We generated constructs encoding various N17 mutations in the 1–586 context fused at their carboxyl-terminus to eYFP, transfected them into STHdh<sup>Q7/Q7</sup> cells and quantified the number of cells that formed puncta in response to stress. We observed that cells expressing the methionine-to-proline (M8P) mutant construct, which results

Figure 1. Huntingtin is involved in a rapid cell stress response. (A) Immunofluorescence of STHdh<sup>Q7/Q7</sup> and STHdh<sup>Q111/Q111</sup> cells at steady state (33°C) and following challenge with 10 min of heat shock at 42.5°C using anti-huntingtin mAb2166. (B) Imaging of fixed STHdh<sup>Q7/Q7</sup> cells transfected with 1–586 Q17-eYFP at steady state, and following challenge with 10 min of heat shock at 42.5°C, 120 min of cold shock at 4°C, 1 h of ATP depletion and 1 h of treatment with 400 μM H<sub>2</sub>O<sub>2</sub>. (C) Representative live-cell images of STHdh<sup>Q7/Q7</sup> cells transfected with 1–586 Q17-eYFP undergoing challenge with heat-shock stress. (D) Imaging of fixed primary cortical mouse neurons transfected with 1–586 Q17-eYFP following challenge with 10 min of heat shock at 42.5°C. (E) Immunofluorescence of HeLa cells transfected with 1–586 Q17-eYFP at steady state (33°C) and following challenge with varying amounts of heat shock stress using an anti-HSF1 antibody. (A–D) transfected protein and (E–H) endogenous HSF1. Secondary antibody was Cy5. All scale bars: 10 μm.
Figure 2. Huntingtin 1–171 is necessary for formation of HSBs. (A) Quantification of ability of different huntingtin fragments to form HSBs. STHdhQ7/Q7 cells transfected with indicated fragments fused C-terminally to eYFP were fixed after 10 min of heat shock, or at steady state, and 20 images were taken at 40 × magnification. The number of cells with puncta was counted. Experiments were performed in triplicate. \( n \approx 100 \) cells per triplicate, total \( n \approx 300 \) cells. Bars represent the mean, and error bars indicate standard deviation. (B) Representative images of graphical data presented in B. (C) Quantification of N17, exon 1 and poly-proline region deletion on HSB formation was performed as described in A. \( n \approx 100 \) cells per triplicate, total \( n \approx 300 \) cells. (D) Representative images of graphical data presented in D. (E) Quantification of ability of different N17 mutants in the 1–586 context to form HSBs. STHdhQ7/Q7 cells transfected with indicated constructs, fused C-terminally to eYFP, were fixed following challenge with 10 min of heat shock stress. Twenty images were captured at 40 × magnification and the number of cells with puncta counted. Experiments were performed in triplicate. \( n \approx 100 \) cells per triplicate, total \( n \approx 300 \) cells. Bars represent the mean and error bars indicate standard deviation. A student’s \( t \)-test was performed to test for statistical significance. *\( P \)-value < 0.0001. All scale bars: 10 \( \mu \)m.
in disruption of the secondary structure of the domain (14), formed HSBs significantly less frequently than cells transfected with the wild-type construct (Fig. 2E). Cells expressing the aspartic acid-to-alanine (E5A) mutant construct, which increases huntingtin’s affinity for membranes (14), displayed an opposite effect, with 100% of cells forming puncta in response to stress across all trials (Fig. 2E). Simulating post-translational modification of S13 and S16, or T3 through phospho-mimetic (S13E S16E, T3D, respectively) or phospho-resistant (S13A S16A, T3A, respectively) mutations did not significantly affect puncta formation (Fig. 2E). Similarly, a leucine-to-alanine (L4A) mutation, which decreases the ability of huntingtin to associate with membranes (14), did not significantly affect HSB formation but did reduce HSB formation efficiency (Fig. 2E). To further investigate the potential for N17 phosphorylation in modulating HSB formation, we treated STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP with either BMS 345 541 or DMAT, compounds we have previously shown to increase and decrease N17 phosphorylation, respectively (15) for 12 h prior to induction of stress, and observed no effect on HSB formation dynamics (Supplementary Material, Fig. S2A). Thus, we conclude that N17 secondary structure is critical to HSB formation, whereas phosphorylation is not.

Huntingtin localizes to early endosomes in response to cell stress

Motivated by data demonstrating that modulating huntingtin’s affinity for membranes via N17 structure can affect HSB formation, and literature suggesting that huntingtin is closely involved in intracellular vesicular trafficking (5–8), we sought to examine whether HSBs co-localize with endosomes. STHdhQ7/Q7 cells expressing huntingtin 1–586 Q17-eYFP following challenge with heat shock stress were immunostained for early (Rab5C), late (Rab7) and recycling (Rab11) endosomal markers (27). Imaging revealed that huntingtin co-localizes primarily with Rab5C following stress and does not co-localize with Rab7 or Rab11 (Fig. 3A).

We next determined whether other proteins involved in vesicular trafficking are present at HSBs. Through immunofluorescence, we assayed STHdhQ7/Q7 cells transfected with huntingtin 1–586 Q17-eYFP, following HSB formation, for the presence of clathrin, dynamin1, huntingtin-associated protein 1 (HAP1) and, through co-expression, huntingtin-associated protein 40 (HAP40) (Fig. 3B). Clathrin, a protein responsible for forming meshwork-like coats on inbound vesicles (28), was not found to co-localize with HSBs (Fig. 3B). Similarly, HAP1, which is involved in trafficking of late endosomes (29), did not co-localize with HSBs (Fig. 3B). In contrast, we noted that dynamin1, which functions to sever vesicles at the plasma membrane and aids in vesicular trafficking (30), and HAP40, a protein postulated to be a Rab5 effector (31), both co-localized with HSBs (Fig. 3B).

HSB formation results in arrest of early-to-recycling and early-to-late endosome fusion

As HSB formation requires huntingtin to localize to early endosomes, and we have previously shown huntingtin to inhibit high-energy processes in response to stress (16), we postulated that

Figure 3. Huntingtin localizes to early endosomes upon induction of cell stress. (A) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP following challenge with 10 min of heat shock stress using antibodies against early (Rab5C), late (Rab7) and recycling (Rab11) endosomal markers. Secondary antibodies were Cy5. (B) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP following heat shock using antibodies against dynamin1, clathrin and HAP1 (A–I). Secondary antibodies for clathrin and dynamin1 were Cy5, and Alexa594 was used for HAP1. Co-expression of 1–586 Q17-eYFP with mCh-6G-HAP40 in STHdhQ7/Q7 cells following 10 min of heat shock is shown in J–L. Co-localization is shown in white. R-values are representative of the PCC averaged over 10 cells in images that were not deconvolved. All representative images are post-deconvolution. All scale bars: 10 μm.
HSBs function to arrest energy-dependent vesicular trafficking processes.

Early endosomes serve as a sorting center for inbound cargo, branching into the recycling and late endosomal pathways, which subsequently lead to the plasma membrane, and lysosome, respectively (27). To analyze trafficking along the early-to-recycling pathway, we incubated STHdhQ7/Q7 cells transfected with huntingtin 1–586 Q17-eYFP with fluorescently labeled transferrin, a plasma glycoprotein that traffics from early endosomes to recycling endosomes (32–34). Following treatment with transferrin, cells were either kept at steady state or challenged with heat shock stress. Subsequently, we performed immunofluorescence against either Rab5C or Rab11 to visualize the early and recycling endosomal compartments, respectively. In cells kept at steady state, transferrin trafficked normally into recycling endosomes—as shown by extensive localization to the Rab11-positive compartment (Fig. 4A). In cells challenged with heat-shock stress, however, we observed a block in transferrin trafficking, with co-localization being observed between HSBs and Rab5C (Fig. 4B), but not transferrin and Rab11. This suggests that HSB formation resulted in sequestration of transferrin in the Rab5C-positive, early endosome compartment, preventing its progression into the Rab11-positive, recycling endosome compartment.

To analyze the effects of HSB formation on early-to-late endosome fusion, we replicated our trafficking assays using epidermal growth factor (EGF), which traffics from early endosome fusion, we replicated our trafficking assays using epidermal growth factor (EGF), which traffics from early endosome fusion, we replicated our trafficking assays using epidermal growth factor (EGF), which traffics from early endosome fusion. We incubated STHdhQ7/Q7 cells transfected with huntingtin 1–586 Q17-eYFP with fluorescently labeled transferrin, a plasma glycoprotein that traffics from early endosomes to recycling endosomes (32–34). Following treatment with transferrin, cells were either kept at steady state or challenged with heat shock stress. Subsequently, we performed immunofluorescence against either Rab5C or Rab11 to visualize the early and recycling endosomal compartments, respectively. In cells kept at steady state, transferrin trafficked normally into recycling endosomes—as shown by extensive localization to the Rab11-positive compartment (Fig. 4A). In cells challenged with heat shock stress, however, we observed a block in transferrin trafficking, with co-localization being observed between HSBs and Rab5C (Fig. 4B), but not transferrin and Rab11. This suggests that HSB formation resulted in sequestration of transferrin in the Rab5C-positive, early endosome compartment, preventing its progression into the Rab11-positive, recycling endosome compartment.

Mutant huntingtin can form HSBs and arrest endocytic trafficking

Next, we determined whether polyglutamine-expanded, mutant huntingtin could form HSBs and arrest endosomal trafficking in a similar manner. To determine whether mutant huntingtin differs from wild-type huntingtin in its ability to form HSBs, we transfected STHdhQ7/Q7 cells with constructs encoding huntingtin 1–586 in the context of different polyglutamine tract lengths, fused at their carboxyl-terminus to eYFP. We then quantified the number of cells with puncta following heat shock stress. Image analysis revealed no difference in HSB formation frequency across cells transfected with a wild-type huntingtin fragment (Q3 and Q17) when compared with cells transfected with polyglutamine-expanded huntingtin (Q138) (Supplementary Material, Fig. S2B). Following this, we replicated both our transferrin and EGF trafficking assays in STHdhQ7/Q7 cells, and in some cases, cells did not recover from stress, instead forming large aggregates prior to cell death (Supplementary Material, Video S6). To determine whether mutant huntingtin expression alters the ability of cells to recover from HSB formation, we replicated our live-cell imaging with STHdhQ7/Q7 cells transfected with huntingtin 1–586 Q138-eYFP. We observed that recovery from stress took longer in mutant huntingtin-expressing cells, and in some cases, cells did not recover from stress, instead forming large aggregates prior to cell death (Supplementary Material, Video S6). To quantify this difference, we transfected STHdhQ7/Q7 cells with either wild-type (1–586 Q17-eYFP) or mutant (1–586 Q17-eYFP) huntingtin and allowed them to recover for either 3 h or 24 h at 33°C, post-heat-shock stress. We subsequently quantified the number of cells with punctuation and noted that at the 3-h mark, almost all of the cells expressing wild-type huntingtin had recovered, showing diffuse huntingtin localization, whereas most of the cells expressing mutant huntingtin had punctate protein localization (Fig. 6A and B). No significant differences were observed in cells expressing wild-type versus mutant huntingtin at the 24-h mark, with both groups of cells having diffuse huntingtin localization. This therefore suggests that mutant huntingtin expression results in defective recovery from HSB formation.

DISCUSSION

The cell stress response is traditionally viewed as a long-term event involving the action of various chaperones and isomerases (36,37). These proteins correct the structure of peptides denatured as a result of stress and facilitate clearance and sequestration of toxic species (38). While all of these processes require ATP (39), the source of this energy remains unclear. Increased transcription and available energy is noted in ER stress responses, but for some stresses, the ER must respond very quickly or the cell will die, leaving minimal time for canonical signals to boost ATP production. It is thus likely that this ATP is sourced from early stress responses, which function to modulate or arrest high-energy activities within the cell and rapidly increase available ATP, without relying on the time required for upregulation of cellular metabolism.

Here, we present one such early stress response involving huntingtin protein. We demonstrate that huntingtin rapidly localizes to early endosomes in response to stress, forming distinct puncta that we have termed HSBs. HSB formation occurs within 30 s of a stress event, whereas canonical stress responses, such as the
Figure 4. HSB formation is associated with arrest of early-to-recycling and early-to-late endosome fusion. (A) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP and treated with Alexa594 transferrin at steady state using antibodies against Rab5C (A–D) and Rab11 (E–H). (B) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP and treated with Alexa594 transferrin following challenge with 10 min of heat shock using antibodies against Rab5C (A–E) and Rab11 (F–J). (C) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP and treated with TexasRed-EGF at steady state using antibodies against Rab5C (A–D) and Rab7 (E–H). (D) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q17-eYFP and treated with TexasRed-EGF following challenge with 10 min of heat shock using antibodies against Rab5C (A–E) and Rab7 (F–J). Secondary antibodies were Cy5. Three-channel co-localization is shown in white, and co-localization between green and blue channels is shown in aquamarine. All scale bars: 10 μm.
Figure 5. Mutant huntingtin HSB formation is associated with arrest of early-to-recycling and early-to-late endosome fusion. (A) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q138-eYFP and treated with Alexa594 transferrin at steady state using antibodies against Rab5C (A–D) and Rab11 (E–H). (B) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q138-eYFP and treated with Alexa594 transferrin following challenge with 10 min of heat shock using antibodies against Rab5C (A–E) and Rab11 (F–J). (C) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q138-eYFP and treated with TexasRed-EGF at steady state using antibodies against Rab5C (A–D) and Rab7 (E–H). (D) Immunofluorescence of STHdhQ7/Q7 cells transfected with 1–586 Q138-eYFP and treated with TexasRed-EGF following challenge with 10 min of heat shock using antibodies against Rab5C (A–E) and Rab7 (F–J). Secondary antibodies were Cy5. Three-channel co-localization is shown in white, and co-localization between green and blue channels is shown in aquamarine. All scale bars: 10 μm.
HSR and the UPR, and processes involved in translational arrest, such as stress granules and processing bodies, require minutes to engage and even longer to exert their effects (23,24,40). The HSR, for instance, is initiated by the heat shock protein-promoting transcription factor, HSF1, which activates as late as 45 min following stress (23), and signal transduction in the UPR may take even longer (40). HSBs are positive for dynamin1, Rab5C and HAP40, a Rab5 effector (31), but not clathrin or HAP1. This is consistent with our marker data, which show that huntingtin localizes exclusively to early endosomes during stress, and not late, nor recycling endosomes. The absence of clathrin from HSBs can be attributed to the nature of clathrin-mediated endocytosis, whereby the clathrin coat is lost prior to maturation of an early endosome.

The formation of HSBs is associated with arrest of early-to-recycling and early-to-late endosomal trafficking. We postulate that this occurs in order to rectify an imbalance in cellular bioenergetics. Endosomal trafficking requires a large pool of energy as the Rab proteins that denote different endosomal compartments function as small, membrane-bound, Ras family monomeric GTPases (27). As HSB formation is associated with an arrest in endosomal trafficking, it is likely that this stress response substantially increases the levels of ATP available to the cell during stress. We propose that this energy is subsequently used by the cell to fuel longer-term stress pathways, such as the HSR, which lie temporally downstream of HSB formation. This is congruent with our previous work demonstrating that huntingtin is required for nuclear cofilin–actin rod formation (16), a stress response that arrests actin treadmilling in order to increase available ATP during stress (18).

Cell stress is also of great importance in the context of neurodegeneration. As the brain ages, metabolic stresses, such as reactive oxygen species, increase owing to decreased mitochondrial efficiency (41), thereby placing emphasis on stress response pathways. Medium spiny neurons, the neuronal population most prominently affected in HD, are especially susceptible to these stresses as they require optimal cytoskeletal and vesicular trafficking dynamics (42). We demonstrate that mutant huntingtin expression results in a defective recovery process following HSB formation, with cells expressing mutant protein displaying a persistent HSB phenotype. This suggests that in HD, cells may not be able to relieve this huntingtin-mediated stress.
response, resulting in prolonged arrest of endocytic trafficking, neuronal dysfunction and, eventually, cell death. This is congruent with previous studies that show that aberrant vesicular trafficking in the context of HD can lead to improper transport of nutrients, such as brain-derived neurotrophic factor, leading to insufficient nourishment, and cell death (8,43). This hypothesis is also consistent with the presence of huntingtin on moving vesicles and direct scaffolding of GAPDH for the production of ATP by glycolysis for fast axonal transport (7). Therefore, a defective HSB response may explain the late age-onset and specific neurodegeneration observed in HD.

Huntingtin 1–171 is required for optimal HSB formation, whereas huntingtin 1–117 is able to form HSBs in limited cases. This suggests that residues located within 117–171 of the protein play a key role in HSB formation dynamics. It has been shown that residues 128–138 of huntingtin can promote aggregation (44). Hence, it is plausible that the absence of this region may limit the protein’s ability to nucleate to form HSBs.

HSB formation dynamics were also observed to be independent of microtubules and the actin cytoskeleton, which were disrupted using nocodazole and latrunculin A, respectively (Supplementary Material, Fig. S3). As HSB formation is associated with an arrest in endosomal trafficking, it may simply be that huntingtin is sequestering vesicles from the cytoskeletal machinery involved in transport, thereby eliminating the need for optimal cytoskeletal dynamics.

The requirement of N17 for HSB formation further underscores criticality of this domain in modulating huntingtin function. Structurally, N17 is an amphipathic alpha-helix that is capable of inserting into membranes, normally tethering huntingtin by direct lipid association to the outer leaflet of the ER (26,45,46). Our data indicate that disrupting the domain’s secondary structure reduces the cell’s ability to form HSBs in response to stress. This suggests that the membrane-association properties of N17 are critical to HSB formation as an intact structure is required for interaction with biological membranes. The requirement of distal regions of huntingtin, in addition to N17, for HSB formation is also congruent with our recent work highlighting the spatial orientation of N17 in relation to downstream regions of the protein, and the potential for intramolecular communication (47).

In summary, we have uncovered a novel, very early cell stress response mediated by huntingtin. We propose that this response functions to arrest endocytic trafficking in response to stress in order to increase available ATP for use by downstream stress pathways (Fig. 6C). Future work will be directed at investigating the potential role of huntingtin at other high-energy sites within the cell to understand the exact mechanism of normal huntingtin function in cell stress.

MATERIALS AND METHODS

Plasmid construction and molecular cloning

Plasmids encoding the N17, 1–81 Q17, 1–117 Q17, 1–171 Q17, 1–586 Q3, 1–586 Q17, 1–586 Q138, 1–586 Q17 Δ2–13, 1–586 M8P Q17, 1–586 S13A S16A Q17, 1–586 S13E S16E Q17 and 1–586 Q17 ΔpolyP fragments of huntingtin, all fused to eYFP at their C-terminus, as well as the mCh-6G-HAP40 construct, were cloned as described previously (14–16,48). Site-directed mutagenesis was performed using inverse PCR and subsequent gel purification using the Qiagen QIAEX II kit (as per manufacturer’s instructions), phosphorylation and ligation. All constructs were sent to Mobix Lab at McMaster University for sequencing analysis prior to use in experiments.

Tissue culture and transfection

STHdhQ7/Q7 STHdhQ111/Q111 cells (a kind gift from M. E. MacDonald, Massachusetts General Hospital) derived from the murine striatum were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS; Sigma) at 33°C with 5% CO2 in an air-jacketed incubator and were kept under clonal temperature selection using Geneticin G418 (Life Technologies) as described previously (49). HEK 293 cells (ATCC) were cultured in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS at 37°C with 5% CO2 in an air-jacketed incubator. HeLa cells (a kind gift from Jon Draper, McMaster Stem Cell and Cancer Research Institute) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS; Sigma) at 37°C with 5% CO2 in an air-jacketed incubator. Twenty-four hours prior to transfection, cells were split from 10-cm dishes of 75–80% confluence and seeded into 35-mm glass-bottom tissue culture dishes (Sarstedt) or Delta T Dishes (Biotopes, Inc.) using Trypsin–EDTA Solution (0.05% trypsin, 0.02% EDTA; Life Technologies). Seeded cells were transfected with 3 μg of plasmid DNA using 4 μl of TurboFect Transfection Reagent (Fermentas). Human wild-type (GM02149) and HD (GM01061) fibroblasts were obtained from Coriell Cell Repositories and cultured in Minimum Essential Medium (MEM; Sigma) supplemented with 15% FBS (Sigma) and 2 mM L-Glutamine (Sigma) at 37°C with 5% CO2 in an air-jacketed incubator. Cells were split into Delta T Dishes (Biotopes, Inc.) or 35-mm glass-bottom tissue culture dishes (Sarstedt) as described earlier. Three micrograms of plasmid DNA was nucleofected into cells using a 4D Nucleofector Type-X Electroporator (Lonza). Primary cortical neurons cultured from E16 embryonic mouse brains (a kind gift from Karun Singh, McMaster Stem Cell and Cancer Research Institute) were grown in Neurobasal media (Life Technologies), supplemented with B27, P15 and Q at 37°C with 5% CO2 in an air-jacketed incubator. Cells were cultured directly into 35-mm glass-bottom tissue culture dishes (Sarstedt) and were transfected with 3 μg of plasmid DNA using Lipofectamine LTX (Life Technologies).

Differentiation of striatal progenitor cells

STHdhQ7/Q7 cells were seeded into 35-mm glass-bottom tissue culture dishes and transfected with constructs of interest as described earlier. Twelve hours following transfection, medium was replaced with serum-free DMEM (Life Technologies) supplemented with a differentiation cocktail [0.65 mM 1-Dopamine (Sigma), 50 μM Forskolin (Sigma), 10 ng/μl alpha-Fibroblast Growth Factor (α-FGF; Sigma) and 250 μM 3-isobutyyl-1-methylxanthine (IBMX; Sigma)]. Cells were left at the growth conditions described earlier for 30 h to allow for differentiation to occur.
Heat shock stress challenge and cell fixation

Cells were cultured in 35-mm glass-bottom tissue culture dishes and transfected as described earlier. Following a 24-h expression period, dishes were wrapped with parafilm and placed in a pre-warmed water bath at 42.5°C for 10 min to heat shock cells. Cells were fixed immediately following heat shock using 4% paraformaldehyde (PFA; Sigma) in phosphate-buffered saline (PBS) for 20 min at room temperature. Cells were left in PBS for imaging.

ATP depletion, cold shock and reactive oxygen stress induction

ATP depletion was induced by incubating cells in pyruvate-free DMEM (Life Technologies) supplemented with 0.25 mM NaN₃ (Sigma) and 0.15 mM 2-deoxyglucose (Sigma) at 33°C for 60 min to disrupt glycolysis. Cold shock stress was induced by placing cells at 4°C for 120 min. Reactive oxygen stress was induced by incubating cells with 400 μM H₂O₂ (Sigma) in serum-free DMEM (Life Technologies) at 33°C for 60 min. Prior to induction of stress, cells were cultured and transfected as described previously and were permitted to express protein for 24 h.

Immunofluorescence

Paraformaldehyde/Triton X-100 method

Cells were fixed in 4% PFA (Sigma) in PBS for 20 min at room temperature and subsequently washed three times with PBS in 1-min intervals. Cells were then permeabilized with 0.5% Triton X-100 (BioShop) and 2% FBS (Sigma) in PBS for 15 min at 4°C. Following permeabilization, cells were blocked for 2 h in 30-min intervals with 2% FBS (Sigma) in PBS. Primary antibody anti-clathrin (mouse monoclonal, Abcam 2731, 1/50), anti-dynamin1 (mouse monoclonal, Abcam 13251, 1/50), anti-huntingtin-associated protein 1 (HAP1; goat polyclonal, Santa Cruz Biotechnology N18, 1/50), anti-huntingtin (mouse monoclonal, Millipore mAb2166, 1/100), anti-Rab11 (mouse monoclonal, Millipore 05–583/clone 47, 1/50), anti-Rab5C (rabbit polyclonal, Abcam 74 854, 1/50), anti-Rab7 (rabbit polyclonal, Abcam 74 906, 1/50), anti-HSF1 (rat monoclonal, Abcam 61 382, 1/100) and anti-β-tubulin (mouse monoclonal, University of Iowa Developmental Studies Hybridoma Bank E7, 1/100) were applied in antibody dilution solution [1% FBS (Sigma), 0.02% Tween-20 (Sigma) in PBS] and incubated overnight at 4°C. Following overnight incubation, the primary antibody was aspirated and cells were blocked for 30 min in blocker solution [2% FBS (Sigma) in PBS] in 10-min intervals. Cells were probed with secondary antibodies (Molecular Probes/Life Technologies) conjugated to either Alexa594 (1/500) or Cy5 (1/350) dye for 1 h at room temperature in antibody dilution solution [1% FBS (Sigma), 0.02% Tween-20 (Sigma) in PBS]. Following 1 h, the secondary antibody was aspirated, and cells were washed for 40 min with PBS at room temperature in 10-min intervals. Cells were left in PBS for imaging.

Methanol method

Cells were fixed-permeabilized in ice-cold methanol for 12 min at −20°C. Following fixation-permeabilization, cells were washed in PBS for 20 min at room temperature in 10-min intervals. Immunofluorescence was then performed as described earlier.

Transferrin uptake assay

Cells were cultured in 35-mm glass-bottom tissue culture dishes and transfected as described earlier. Following a 24-h expression period, cells were placed in serum-free DMEM (Life Technologies) for 60 min at the growth conditions described previously to remove any existing transferrin. Following this, medium was replaced with serum-free medium supplemented with 50 μg/ml of human transferrin conjugated to Alexa594 dye (Molecular Probes/Life Technologies). Cells were then either immediately heat shocked or left at steady state in the growth conditions described previously. Following transferrin uptake and/or stress, cells were fixed and imaged immediately or prepared for immunofluorescence analysis as described earlier.

EGF uptake assay

Cells were cultured in 35-mm glass-bottom tissue culture dishes and transfected as described earlier. Following a 24-h expression period, cells were cultured in serum-free DMEM (Life Technologies) for 60 min at the growth conditions described earlier to allow for degradation of existing EGF. Following this, medium was replaced with a solution of 3 μg/ml EGF labeled with TexasRed dye (Life Technologies), and 0.1% BSA (New England Biolabs) in serum-free DMEM. Cells were then either immediately heat shocked or left at steady state in the growth conditions described previously. Following EGF uptake and/or stress, cells were fixed and imaged immediately or prepared for immunofluorescence analysis as described earlier.

HSB recovery assay

Cells were cultured in 35-mm glass-bottom tissue culture dishes and transfected as described previously. Following a 24-h expression period, cells were heat shocked as described earlier and placed back in the incubator at 33°C. Cells were permitted to recover from stress for either 3 h or 24 h and were subsequently fixed as described earlier.

Nocodazole and latrunculin-A treatment

Cells were cultured in 35-mm glass-bottom tissue culture dishes and transfected as described previously. Following a 12-h expression period, cells were treated with 50 ng/ml, 75 ng/ml or 100 ng/ml nocodazole (Sigma) diluted in culture medium, for 12 h. Cells treated with latrunculin A were allowed to express protein for 20 h prior to being treated with 100 nM, 500 nM or 1 μM latrunculin A (Sigma) diluted in culture medium, for 1 h. Following treatments, cells were challenged with heat-shock stress, fixed and imaged as described earleir.

Microscopy

Following a 24-h expression period, cells transfected with protein(s) of interest were imaged live. Imaging was done using a Nikon Eclipse Ti inverted widefield epifluorescence microscope using either a 60× oil immersion N.A. 1.4 plan apochromat
objective or a 100 × oil immersion N.A. 1.4 plan apochromat objective (Nikon, Japan). Images were acquired using a Hamamatsu ORCA Flash 4.0 digital camera (Hamamatsu Photonics, Japan). NIS Elements Advanced Research 4.1 was used for microscope controlling and image acquisition. Live-cell videos were recorded with 10-s intervals between frames with acquisition of the first frame beginning prior to induction of heat shock at 42.5°C via a heated stage and heated dish lid (Bioptechs, Inc.). Live-cell imaging of recovery from HSB formation was performed by placing cells in a temperature-controlled microscope chamber set at 33°C. Frames were captured using a 40 X air N.A. 0.6 plan fluor objective every 30 s for 3 h with acquisition of the first frame immediately following challenge with heat shock stress.

Fixed cells were imaged using either a 40 × air N.A. 0.6 plan fluor, or 60 × oil immersion N.A. 1.4 plan apochromat objective (Nikon, Japan) as described earlier. Live cells were imaged in growth medium, whereas fixed cells were imaged in PBS. A Spectra LED lamp served as the light source for microscopy, attenuated with ND2 or ND4 filters as necessary. Filter sets and dichroic filters were supplied by Sutter Instruments. Qualitative images were generated by obtaining a multichannel Z-stack and performing blind 3D deconvolution using algorithms from AutoQuant (Media Cybernetics/Roper Industries, Inc., Rockville, MD, USA) within NIS Elements 4.1. All images were captured in a 16-bit non-compressed tagged-image format (TIF/tif) and converted to JPEGs or bitmaps (BMP) in ImageJ64 (National Institutes of Health) prior to figure preparation. Scale bars represent the diameter of the nucleus (~10 µm). Figures were prepared in Adobe Illustrator CS6 and Corel Draw X6. Live-cell imaging videos were captured in the Nikon proprietary .nd2 format and were converted to .avi using the NIS Elements Advanced Research 4.1 software.

Statistics and quantification

Quantitative imaging was performed as described earlier for fixed cells. Twenty images were taken per condition at 40 × magnification and the number of cells with puncta counted. All quantitative experiments were performed in triplicate. Where applicable, a student’s t-test was performed to determine statistical significance between conditions. Error bars on all graphs represent the standard deviation, and the bars represent the mean. Co-localization was assessed qualitatively through observation of white signal in overlays of green/magenta and red/green/blue images, and quantitatively by determining the Pearson’s Correlation Coefficient (PCC) and Mander’s Overlap values in NIS Elements 4.1 software. Graphing and statistical analysis was performed in GraphPad Prism 6 for Mac OS (GraphPad Software, Inc.)

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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