Dominant phenotypes produced by the HD mutation in STHdhQ111 striatal cells

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Lengthening a glutamine tract in huntingtin confers a dominant attribute that initiates degeneration of striatal neurons in Huntington’s disease (HD). To identify pathways that are candidates for the mutant protein’s abnormal function, we compared striatal cell lines established from wild-type and HdhQ111 knock-in embryos. Alternate versions of full-length huntingtin, distinguished by epitope accessibility, were localized to different sets of nuclear and perinuclear organelles involved in RNA biogenesis and membrane trafficking. However, mutant STHdhQ111 cells also exhibited additional forms of the full-length mutant protein and displayed dominant phenotypes that did not mirror phenotypes caused by either huntingtin deficiency or excess. These phenotypes indicate a disruption of striatal cell homeostasis by the mutant protein, via a mechanism that is separate from its normal activity. They also support specific stress pathways, including elevated p53, endoplasmic reticulum stress response and hypoxia, as potential players in HD.

INTRODUCTION

Huntington’s disease (HD) is a dominantly inherited disorder that is characterized by the progressive loss of neurons in the striatum (1). The HD mutation is an expanded CAG repeat tract that lengthens a polyglutamine segment in huntingtin to ~37 or more residues (2). This novel protein is required for normal nuclear organelles and perinuclear membrane compartments (3) and has been implicated in iron homeostasis (3), RNA biogenesis (3–7) and membrane trafficking (3,4,8–11).

The polyglutamine defect initiates the pathogenic process via a mechanism that conforms to genetic criteria that have been determined by genotype–phenotype studies in HD patients (12,13). These critical parameters include specificity for striatal neurons, increased severity with polyglutamine length and dominance over the wild-type protein.

Two mutant huntingtin phenotypes that fulfill these HD genetic criteria have been identified in model systems, strongly implicating the underlying process in each case in the human disorder. The promotion of mutant amino-terminal fragment into amyloid has implicated an abnormal property of the expanded polyglutamine tract (14–16). This property may, however, act either from the full-length mutant protein or, after cleavage, via an amino-terminal fragment (17,18). The other phenotype is the nuclear ‘mis-localization’ of the full-length mutant protein in HdhQ92 and HdhQ111 striatal neurons, which precedes the later formation of truncated product (19). This phenotype, produced by insertion of the HD mutation into the mouse HD locus, has revealed alternate forms of full-length huntingtin, distinguished by epitope accessibility, and has implicated a mechanism that involves the full-length mutant protein (19).

Moreover, the dominant phenotypes produced by the mutant protein in HdhQ111 striatal neurons may provide clues to the disrupted pathway that elicits eventual toxicity. One possibility is a perturbation that ensues from increases or decreases in an essential huntingtin activity, predicting a phenotype that entails abnormal organelles that require the protein’s function (3). This is because huntingtin excess and deficiency both produce a similar impact on the protein’s normal cellular pathway (3). Alternatively, the critical disruption may be elicited independently of any coincident change in the mutant protein’s normal activity, predicting phenotypes in homozygous and heterozygous mutant striatal cells that do not resemble altered huntingtin function.

Consequently, we have sought clues to the mutant protein’s abnormal pathway by assessing the dominant phenotypes exhibited by striatal cell lines established from mutant HdhQ111 knock-in compared with wild-type embryos. Our findings demonstrate alternate forms of huntingtin with properties that support roles in the response to hypoxia, in RNA biogenesis and in membrane trafficking. However, mutant cells display dominant phenotypes that imply toxicity due to a novel property

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of the mutant protein that elicits stress-response pathways to be explored in HD pathogenesis.

RESULTS

A set of clonal striatal cell lines from \( \text{Hdh}^{Q111} \) and wild-type littermates

To identify candidates for the mutant protein’s abnormal pathways in striatal cells, we established neuronal progenitor cell lines from E14 striatal primordia of \( \text{Hdh}^{Q111} \) knock-in (19) and wild-type littermate embryos using \( \text{tsA58 SV40} \) large T antigen (20). Multiple clonal lines of nestin-positive cells from the wild-type \( (\text{STHdh}^{+}/\text{Hdh}^+) \) and the heterozygous \( (\text{STHdh}^{Q111}/\text{Hdh}^+) \) mutant embryos were established. In contrast, only two clones from the mutant homozygote embryos were successfully ‘passaged’. These \( \text{STHdh}^{Q111}/\text{Hdh}^{Q111} \) exhibited longer doubling times at 33°C than did the wild-type clones (35 versus 26 h), suggesting that the mutant protein may impair the establishment pathway. However, heterozygous and homozygous mutant cells, like their wild-type counterparts, were conditionally ‘immortal’, as growth at 39°C resulted in the loss of \( \text{SV40 tsA58} \) protein and the cessation of proliferation (data not shown).

To test the differentiative capacity of the striatal cell lines, the growth medium was supplemented with a ‘dopamine cocktail’ (20,21). As shown in Figure 1a for a wild-type clone, all of the cells in the culture were MAP-2-positive and elaborated long processes. These characteristics of post-mitotic neuronal-like cells confirmed that the cell lines in each case were established from neuronal striatal progenitor cells. Notably, genotype-specific differences were not found, indicating that the mutant protein does not overtly affect the cellular pathways that mediate neuronal differentiation.

Normal and mutant huntingtin are expressed in the cultured striatal cells

Normal and mutant huntingtin were assessed by immunoblot analyses of wild-type and mutant striatal cell lysates with the reagent monoclonal antibody (mAb) 2166. The results, presented in Figure 1b, revealed the appropriate genotype-specific bands of the endogenous normal and/or mutant protein with 111 glutamines, which migrates more slowly. These bands were detected in the supernatant, rather than the pellet fraction, revealing similar partitioning of the normal and the mutant protein. However, the mutant band was always less intense than the normal band in the heterozygote lysates, although this ratio varied between experiments, indicating an abnormal property of the mutant protein that has been noted previously in HD patient cells (22) and \( \text{Hdh} \) knock-in brain (19).

To track the long polyglutamine segment of the mutant protein, the blot was probed with the polyglutamine mAb 1F8 (Fig. 1b), which revealed only a full-length mutant band in the soluble fraction. In this format, mAb 1F8 did not detect the short glutamine tract of the normal protein as expected (15,23). In some experiments, smears of 1F8 reactivity at approximately the mobility of the full-length mutant protein were detected, but reproducible low molecular weight bands were not evident (data not shown). Consistent with the unaltered half-life of the mutant protein in HD patient lymphoblastoid cells (22), these results do not support greatly altered turnover of the mutant protein. Rather they suggest that the long polyglutamine tract may confer differential ‘extractability’ or ‘detectability’ in the immunoblot format, although the basis of this abnormal behavior of the mutant protein remains unexplained.

Alternate forms of huntingtin in the nucleus and in the cytoplasm

The distribution of the full-length normal protein in striatal cells was investigated by staining the \( \text{STHdh}^{+}/\text{Hdh}^+ \) progenitor cells with a panel of five specific antibody reagents that detect amino-terminal (AP194, AP229, EM48), downstream (HP1) and internal (HF1) epitopes. As shown in Figure 2, the confocal images revealed overlapping yet reagent-specific patterns of reactivity that varied from cell to cell. A comparison of these patterns revealed alternate forms of the protein. An amino-terminal-accessible form that was detected by AP194, AP229 and EM48, but with inaccessible HP1 and HF1 internal epitopes, was prominent in the nucleus and also in cytoplasmic dots. In contrast, HP1 and HF1 revealed an alternate form of the protein with internal-accessible epitopes, but with amino-
terminal-inaccessible epitopes. This form was distributed to the nucleus and in the cytoplasm was found around the nucleus and in disperse dots. The distinct locations suggested that each version of the protein could associate with a different set of dynamic organelles.

To determine whether post-mitotic neuron-like cells exhibit the alternate versions of huntingtin, differentiated ST\textit{Hdh}\textsuperscript{+/Hdh}\textsuperscript{+} cells were stained with the antibody panel. The results indicated a similar subcellular distribution of reagent epitopes as was found for the striatal progenitor cells (Fig. 2). However, the rounded nuclei of the differentiated cells were densely stained and the amino-terminal-accessible version of the protein, for example detected by AP194, was more evident in the cytoplasm of the long projections.

Alternate versions of huntingtin colocalize with different sets of organelles

To determine the locations of the alternate versions of huntingtin, the wild-type striatal progenitor cells were costained for organelle-marker proteins. The results for the amino-terminal-accessible version, detected with AP194 or AP229 and markers of nuclear organelle, are shown in Figure 3A. This form of the protein exhibited extensive overlap with both ANA125-positive pre-mRNA splicing-speckles and the NuMA-reactive nuclear matrix. In contrast, the alternate internal-accessible version, detected with HF1, colocalized with fibrillarin-positive nucleoli, although it also co-stained with some ANA125 splicing-speckles (data not shown). As shown in Figure 3, co-staining revealed that this version of the protein was also localized in the vicinity of Concanavalin A (ConA) stain in the rough endoplasmic reticulum (ER) and with the GM130-reactive Golgi membrane. In some cells, the HF1 (or HP1) signals also displayed partial overlap with transferrin receptor-positive perinuclear foci, although the HF1- (or HP1-) reactive dots in the cellular processes were distinct from transferrin receptor-reactive vesicles. Thus, amino-terminal-accessible protein was located to splicing-speckles in the nucleus and the cytoplasm. In contrast, the alternate internal-accessible form was nucleolar and in the cytoplasm was found primarily in the proximity of membrane organelles near the nucleus.

Mislocalization of the mutant protein

To determine whether the polyglutamine expansion was associated with altered subcellular distribution of the mutant protein, homozygous mutant ST\textit{Hdh}\textsuperscript{Q111/Hdh}\textsuperscript{Q111} cells were stained with the antibody panel. The results for both the progenitor cells and the differentiated neuronal-like cells are shown in Figure 4A. In general, the pattern of amino-terminal and internal reagent signals was similar to that observed in the corresponding wild-type cells, indicating that the mutant protein also exhibits alternate amino-terminal-accessible (internal-inaccessible) and internal-accessible (amino-terminal-inaccessible) forms. Furthermore, co-staining experiments revealed that each version of the mutant protein was detected in the same set of nuclear and cytoplasmic organelles as its normal counterpart in wild-type cells (data not shown).

However, the staining patterns also revealed notable differences that are evident from the images in Figure 4A. The amino-terminal-accessible form, detected by AP194 and AP229, was unevenly distributed in the nucleus and was not detected in the processes of differentiated cells. Furthermore, the internal-accessible HF1- (and HP1-) reactive form of the mutant protein exhibited abnormal nuclear and perinuclear patches. These findings strongly suggested that a proportion of each version of the full-length mutant protein was ‘mislocalized’ due to the lengthened polyglutamine tract.

Nuclear location entails IF8-negative polyglutamine segments

Therefore, we assessed the polyglutamine tract of the full-length protein by co-staining the striatal cells with HF1 and the
polyglutamine mAb 1F8 (Fig. 4B). For wild-type cells, the bright 1F8 signal colocalized perfectly with the perinuclear HF1 stain, indicating that the short glutamine tract of the normal protein exhibited detectable 1F8 epitope in this format. In contrast, in the nucleus of the same cells the HF1-reactive nucleoli and speckles were 1F8 negative, revealing a difference in the polyglutamine tract. Furthermore, mAb 1F8 also failed to detect the speckle locations of the amino-terminal-accessible form of the protein prominent in the nucleus. Thus, 1F8 reveals a different property of the protein’s polyglutamine segment in the cytoplasm versus the nucleus, implying compartment-specific structural properties, modifications or interactors.

Abnormal forms of the mutant protein

Homozygous mutant STHdhQ111/HdhQ111 cells were co-stained with HF1 and mAb 1F8 to assess the expanded polyglutamine segment of the mutant protein (Fig. 4B). In general, these cells exhibited a pattern of HF1–1F8 colocalization similar to that found in the wild-type cells. The cytoplasmic versions of the mutant protein displayed 1F8-reactive expanded polyglutamine tracts, whereas the nuclear forms exhibited 1F8-negative polyglutamine segments, implying the same nuclear/cytoplasmic difference displayed by the shorter tract in the normal protein.

However, a proportion of the HF1-reactive protein in and around the nucleus exhibited reciprocally ‘switched’ 1F8 reactivities. For example, 1F8-reactive dots were embedded in the generally 1F8-negative HF1-stained nucleoli and large 1F8-negative sectors were found within the perinuclear HF1 signal, which typically was 1F8 reactive. Moreover, 1F8-reactive ‘worms’ in the nucleus suggested conglomerated splicing-speckles with abnormal expanded polyglutamine tracts. Thus, some proportion of the mutant protein in the nucleus and in the cytoplasm exhibited abnormal polyglutamine segments that may reflect aberrant modifications, binding partners or conformations.

Dominant phenotypes disclose a ‘stress’ response to mutant huntingtin

Although the growth properties were obviously different, we compared the homozygous mutant cells and the wild-type striatal cells to identify potential dominant phenotypes that may involve the protein’s normal function. This survey included the perinuclear membrane organelles and the iron pathway, which require huntingtin (3). The abnormal phenotypes that were specific to the mutant striatal cells can be grouped into three classes: (i) an altered establishment pathway due to elevated p53 levels; (ii) an enlarged ER compartment; and (iii) heightened activity of the iron pathway. Assessment of these phenotypes in heterozygous STHdhQ111/Hdh+ striatal cell lines revealed that the mechanism was dominant over the wild-type protein, although the phenotype in most cases was less severe than in the homozygous mutant cells. This finding may indicate a proposed protective effect of the wild-type protein (24) or may reflect the incomplete saturation of the mechanism by a single dose’s worth of the mutant protein.
Elevated levels of p53 protein

Consistent with the longer generation times of the mutant cells, the results of propidium iodide flow cytometry (FACS) (Fig. 5a) revealed that STdh0/111/Hdh0/111 and STdh0/111/Hdh+ cells exhibited twice the DNA content of STdh+/Hdh+ cells, although the size of the cells was similar for all genotypes. Increased ploidy is a hallmark of a blocked cellular p53–SV40 large T antigen establishment pathway (25), prompting an assessment of levels of these proteins by immunoblot analyses (Fig. 5b). Densitometry of the band intensities detected in these experiments demonstrated that, when normalized to spectrin signal, the homozygous mutant extract exhibited ~2-fold more SV40 tsA58 but >6-fold more p53 than the wild-type extract. As elevated p53 protein must be
overcome by high levels of SV40 large T antigen for ‘immortalization’ (26), this finding is consistent with our inability to establish more than two lines of ST\textsubscript{HdhQ111}/ST\textsubscript{HdhQ111} cells from the primordia of homozygous mutant embryos. Therefore, increased p53 levels in the mutant striatal cells may reflect an augmentation of stress pathways that are normally activated when cells are placed in tissue culture (26).

Abnormal ER response

Two of the dominant phenotypes were first identified by confocal images of the stained striatal cells, which indicated abnormal expansions of select perinuclear secretory organelles. As shown in Figure 6a, the mutant cells exhibited excessive ConA-reactive ER membrane. This was especially evident in cells that had been treated with the membrane fusion inhibitor, Brefeldin A. In contrast, the GM130-reactive Golgi membrane in these untreated or Brefeldin A-treated mutant cells was similar to wild-type, implying that the trafficking between the ER and Golgi apparatus may not be greatly affected. This relatively selective impact on the amount of ER membrane implies an ER-stress response, which may involve abnormal forms of the mutant protein that were detected in the nucleus and in the cytoplasm of the mutant striatal cells.

Heightened basal activity of the iron pathway

The images in Figure 6b illustrated the intense perinuclear transferrin receptor staining exhibited by the mutant cells, whether untreated or treated with Brefeldin A, which indicated that the recycling endosome compartment was enlarged and also suggested that transferrin receptor levels may be elevated. This was confirmed by immunoblot analyses (Fig. 6c), which revealed a more intense transferrin receptor band in the mutant cell extracts. When normalized to spectrin, the band was ~5-fold increased in heterozygous cell lysates compared with the wild-type level. However, whereas the confocal images indicated the most dramatic increase in the homozygous cells, these extracts typically exhibited only an ~2-fold elevation above the wild-type levels by immunoblot. This reproducible finding remains to be explained, although it may suggest an interaction of transferrin receptor with the mutant protein, which is also difficult to detect in this format.

Furthermore, the activity of the iron pathway in the mutant cells was dramatically elevated. The results of the uptake of FITC-tagged transferrin from the growth medium are shown in Figure 7a. Increased uptake compared with wild-type cells was evidenced by intense cytoplasmic and perinuclear FITC signal. Despite the already elevated activity of this pathway in mutant cells, treatment with deferoxamine mesylate led to increased transferrin uptake and transport for these cells and for the wild-type cells, indicating that the mutant protein does not alter the proper modulation of the iron pathway. These results imply that the heightened basal level of this pathway that was observed in the mutant striatal cells could reflect a normal response to hypoxia that has been elicited by the mutant protein.

Increased levels of the normal and the mutant protein in response to hypoxia

Huntingtin is an iron-response protein that has been implicated in cellular homeostasis (3), suggesting that the levels of huntingtin may be increased in the hypoxic mutant striatal cells. This possibility was assessed by immunoblot analyses of lysates from cells of all genotypes, without and with deferoxamine mesylate treatment. As shown in Figure 7b, increased levels of the normal protein were disclosed by the more intense mAb 2166-reactive bands in the treated compared with the untreated
Figure 6. Dominant high transferrin receptor reveals upregulated iron pathway. (a) Confocal images of ConA (white) ‘stain’ in wild-type (+/+), homozygous (III/III) and heterozygous (III/+), mutant striatal cells, reveal abnormal ER blobs in mutant cells, more evident after Brelefeldin A (BFA) treatment. (Insets) GM130-reactive Golgi in untreated and BFA-treated cells (asterisk) are relatively similar in all genotypes. (b) Confocal images of transferrin receptor stained homozygous (III/III) and heterozygous (III+/+) mutant cells reveal exaggerated perinuclear recycling compartment foci compared with wild-type (+/+), cells, also apparent after BFA treatment (higher magnification). (c) Immunoblot analysis of transferrin receptor levels in proteins extracted from STHdh+/Hdh+/ (+/+), STHdhQ111+/Hdh+/ (III/+), STHdhQ111+/HdhQ111 (III/III) cells. Transferrin receptor TnR bands, with differing mobility due to glycosylation, in the soluble (s) and pellet (p) fraction, are intensely reactive in mutant striatal cell extracts, indicating upregulation. This effect is most apparent in heterozygous cell extracts, as recovery from homozygous mutant cells is variable. Fodrin (spectrin) bands reveal comparable protein in each lane.
extracts for both the wild-type and the heterozygous mutant cells. Densitometry of the band intensities, normalized to the spectrin signal, indicated an ~2-fold increase in the wild-type and ~5-fold increase in the mutant heterozygous cell extract, indicating that the levels of the normal protein are modulated in striatal cells in response to hypoxia. Furthermore, despite the difficulty in detecting the mutant protein in this format, the band intensities of mutant huntingtin in the treated versus the untreated heterozygous and homozygous mutant cell lysates was reproducibly increased by ~3-fold, demonstrating iron regulation of the mutant allele.

These findings strongly suggested that the levels of huntingtin in the untreated mutant striatal cells might be elevated, as they exhibited a heightened basal activity of the iron pathway compared with the naïve wild-type cells. Although the differential ‘recovery’ of the mutant protein precludes an accurate comparison between the naïve wild-type and the mutant cell extracts, densitometry revealed that the ratio of the
band of normal protein to the spectrin band in wild-type cell extracts is typically ~1:1 (n = 2). In contrast, for the same exposures the ratio of the normal protein band to the spectrin band in the heterozygous mutant cell lysates was ~3:1 (n = 2). These ratios disclose upregulation of huntingtin in the untreated mutant striatal cells, implying that the basal levels of the mutant protein may also be elevated perhaps setting up a harmful cycle that might further stimulate stress-response pathways.

**Sensitivity of the mutant striatal cells to hypoxic stress**

During the course of the FITC–transferrin uptake experiments we discovered that exposure to deferoxamine mesylate followed by repeated changes of growth medium to remove excess FITC–ligand was associated with toxicity. The confocal images in Figure 7c demonstrated that wild-type striatal cells were refractory to the procedure. In contrast, the majority of the homozygous mutant cells and a proportion of the STHdhQ111/Hdh* cells had retracted their processes and were pyknotic or had already detached from the culture dish. Thus, additional hypoxia was harmful to the mutant striatal cells, implicating the already stimulated p53, ER and hypoxia stress-response pathways in this ultimately deleterious consequence of the mutant protein.

**DISCUSSION**

Mutant huntingtin provokes a biochemical disruption that initiates the eventual demise of striatal neurons in HD. This intriguing dominant mechanism may, but need not, involve some aspect of the protein’s normal activity. Consequently we have assessed dominant phenotypes in mutant ST HdhQ111 striatal cells to determine whether they resemble phenotypes caused by altered huntingtin function. Our results indicate that, although it shares properties of the normal protein that may be involved in neuronal selectivity, the full-length mutant protein may elicit eventual striatal cell toxicity via a mechanism that is separate from its normal activity.

The alternate forms of the full-length protein that were distinguished by their differing epitope accessibility support previous reports of distinct versions of the full-length protein in medium spiny neurons in the mouse striatum (19). These forms were localized to a different set of organelles in the nucleus and in the cytoplasm. Our data also demonstrated that nuclear and cytoplasmic forms of the protein were distinguished by different ‘availability’ of the polyglutamine segment. These alternate versions may reflect distinct modifications, binding partners and/or conformational properties. They are also likely to involve huntingtin’s HEAT protein interaction domains (27), found in nuclear–cytoplasmic proteins such as the importins (28,29), which may provide a scaffold for the organization of components of functional complexes.

An amino-terminal-accessible form of the full-length protein localized to nuclear sites that are addresses of huntingtin’s spliceosome, transcription factor and polyadenylation complex partners (3). This version may therefore fulfill the huntingtin requirement for normal compartmentalization of transcription factor partners (3), supporting a proposed role in RNA biogenesis (3–7). In contrast, the internal-accessible form of the protein was found in the vicinity of perinuclear membranes, consistent with huntingtin’s vesicle interactors (4,8–11), but was also associated with nucleoli. These organelles all require huntingtin, implicating this version of the protein in normal perinuclear membrane function, in a pathway that is linked with the nucleolus (3).

Although the mutant protein shared this spectrum of alternate forms, in support of the fulfillment of its intrinsic activities (30), the mutant striatal cells also displayed abnormal versions that were ‘mislocalized’. Detected in the nucleus and in the cytoplasm, these abnormal forms of the full-length mutant protein exhibited aberrant polyglutamine segment ‘accessibility’. This strongly suggests aberrant modifications, complexes and/or conformations of the full-length mutant protein, which are promoted by the expanded polyglutamine property. This dominant phenotype mirrors the early ‘mislocalization’ of the full-length mutant protein in the HdhQ111 knock-in striatum, which conforms to HD genetic criteria (19). The formation of abnormal nuclear and cytoplasmic forms of the full-length mutant protein may, therefore, be an early event in the pathogenic process in man.

The dominant mutant striatal cell phenotypes did not mirror the effects of altered huntingtin function, which yields diminished nucleoli and perinuclear secretory organelles. Instead, the abnormal phenotypes pointed to potentially harmful metabolic stress. This finding argues for an aberrant property of the full-length mutant protein that has the capacity to disrupt striatal cell homeostasis via a mechanism that is separate from its normal activity. This may involve, for example, an abnormal interaction with a protein whose function is essential to striatal neurons.

Several candidates for the disrupted cellular pathway are supported by these stress responses. An ‘ER unfolded protein response’ (31,32), implying elevated chaperones and proteasome subunits that mitigate the effects of mutant polyglutamine protein (33–35), implicates abnormal protein folding and degradation (36,37). p53-mediated homeostasis pathways (38), which may include a response to DNA damage, are consistent with aberrant gene transcription (39–41). The hypoxic response suggests disruption of pathways involved in energy metabolism that may also be awry in HD (42).

Importantly, our findings indicate that the mutant protein, like its normal counterpart, is upregulated in response to hypoxia. Striatal neurons are exquisitely sensitive to oxidative stress, suggesting a cycle of mutant protein upregulation that may hasten the harmful effect of the polyglutamine property. Thus, two aspects of the mutant protein are suggested: an abnormal ‘toxic’ activity that is separate from its intrinsic activity and its role as a hypoxia response protein, which may contribute the selective vulnerability of striatal neurons to this ultimately deleterious insult.

**MATERIALS AND METHODS**

**Establishment of E14 striatal precursor cell lines, cell culture and differentiation**

Striatal primordia were dissected from genotyped E14 littermates of a timed pregnant HdhQ111/Hdh* intercross, in ice-cold calcium- and magnesium-free Hanks’ balanced salt solution (HBBS; Gibco BRL, Gaithersburg, MD), supplemented with 3.9 g/l HEPES. Dissociated cells at ~2–5 × 10⁴ cells/cm² were
plated in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Gaithersburg, MD) plus 10% fetal calf serum (FCS; Imperial, UK). Infection with a defective retrovirus transducing the tsA58/U19 large T antigen, selection of G418 resistant colonies at the permissive temperature 33°C and ascertainment of nestin-positive colonies by immunostaining was as described (20). The genotype of resultant cell lines was confirmed by PCR analysis to determine HD CAG repeat size (18). All experiments were carried out with two independent cell lines for each genotype.

Striatal cells were grown at 33°C in 10% FCS in DMEM. Occasional cells, of all genotypes, exhibit microvilli and cytoplasmic vacuoles that are not correlated with mutant huntingtin. Neuronal differentiation was induced by incubating for 12 h in serum-free DMEM with α-FGF (10 ng/ml) (Promega, Madison, WI), IBMX (240 μM), TPA (20 μM), forskolin (48.6 μM) and dopamine (5 μM) (Sigma, St Louis, MO) (20,21).

**Protein extracts and immunoblot analysis**

Protein extracts were prepared from phosphate-buffered saline (PBS) washed cell pellets by lysis at 4°C in RIPA buffer (50 mM Tris–HCl pH 8, 1% NP40, 12 mM deoxycholic acid sodium salt, 0.1% SDS) with protease inhibitors. The supernatant was collected after centrifugation (25 min at 10 000 g), whereas the pellet in PBS was sonicated. For immunoblots 60 μg of total protein, boiled in SDS sample buffer, was loaded on a 6% SDS–polyacrylamide gel and blotted to nylon membrane. Protein concentration was by modified Bradford assay (BioRad, Hercules, CA). Blots were probed with primary antibody and specific signal was detected with appropriate secondary reagents by enhanced chemiluminescence.

**Immunostaining, nuclear matrix preparation and confocal microscopy**

For immunostaining PBS-washed cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, blocked in 1% bovine serum albumin in PBS and were then incubated with primary antibody (90 min), washed in PBS and exposed to secondary antibody (60 min). Similar results were obtained with methanol fixation. Fluorescein-labeled transferrin (Molecular Probes, Eugene, OR) uptake was performed over 30 min (43), in some cases 4 μM deferoxamine mesylate was added to growth medium 24 h earlier (Sigma). Brefeldin A (Calbiochem, La Jolla, CA) treatment (5 μM) was for 30 min. Nuclear matrices were prepared by serial extraction as described in Kern-matrix buffer (44). Secondary antibodies were Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and Oregon Green-conjugated goat anti-mouse. Confocal analyses was on a BioRad MRC-1024 laser confocal microscope using a 40× objective lens. Digitized images were saved as separate files for each channel and merged using Adobe Photoshop.

**Primary antibodies**

The specific huntingtin reagents were as follows: EM48 (amino acids 1–256) (45), AP194 (amino acids 1–17), AP229 (amino acids 55–66), HP1 (amino acids 80–113), HFI1 (amino acids 1981–2580) (14,23), mAb 2166 (amino acids 181–810) (Chemicon International, Temecula, CA) and mAb 1F8 (3,35).

Other reagents were as follows: MAP-2 (Boehringer Mannheim, Indianapolis, IN), nestin (Developmental Studies Hybridoma Bank, University of Iowa, IA), NuMA (Transduction Laboratories, San Diego, CA), ANA125 (a gift of Dr M. Bedford, Harvard Medical School, Boston, MA), fibrilarin 72B9 (a gift of Dr R. Terns, University of Georgia, Athens, GA), FITC–ConA (Molecular Probes), GM130 (Transduction Laboratories), transferrin receptor (Biosource International, Camarillo, CA) and SV40 large T antigen (Pab 416), p53 (Pab 421) (46) (gifts from Dr E. Harlow, Massachusetts General Hospital, Charlestown, MA).

**FACS analysis for DNA content**

DNA content was measured by flow cytometry of cells stained with propidium iodide (Calbiochem) and the data analyzed with DNA analysis CellQuest software. Briefly, after trypsinization cells were filtered through a 37 μm mesh, centrifuged at 90 g for 10 min and resuspended in HEPES–HBSS. Counted cells were fixed for 15 min in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 3 min at 4°C and resuspended in RNAse A (180 U/ml) for 40 min at 37°C. DNA staining was on 1–2×10⁶ cells in 1 ml of propidium iodide (50 μg/ml propidium iodide/ PBS/3% PEG-8000).

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