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Multiple Phenotypes in Huntington Disease Mouse Neural Stem Cells

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Abstract

Neural stem (NS) cells are a limitless resource, and thus superior to primary neurons for drug discovery provided they exhibit appropriate disease phenotypes. Here we established NS cells for cellular studies of Huntington's disease (HD). HD is a heritable neurodegenerative disease caused by a mutation resulting in an increased number of glutamines (Q) within a polyglutamine tract in Huntingtin (Htt). NS cells were isolated from embryonic wild-type (Htt^{7Q/7Q}) and "knock-in" HD (Htt^{140Q/140Q}) mice expressing full-length endogenous normal or mutant Htt. NS cells were also developed from mouse embryonic stem cells that were devoid of Htt (Htt^{-/-}), or knock-in cells containing human exon1 with an N-terminal FLAG epitope tag and with 7Q or 140Q inserted into one of the mouse alleles (Htt^{F7Q/7Q} and Htt^{F140Q/7Q}). Compared to Htt^{7Q/7Q} NS cells, HD Htt^{140Q/140Q} NS cells showed significantly reduced levels of cholesterol, increased levels of reactive oxygen species (ROS), and impaired motility. The heterozygous Htt^{F140Q/7Q} NS cells had increased ROS and decreased motility compared to Htt^{F7Q/7Q}. These phenotypes of HD NS cells replicate those seen in HD patients or in primary cell or in vivo models of HD. Huntingtin "knock-out" NS cells (Htt^{-/-}) also had impaired motility, but in contrast to HD cells had increased cholesterol. In addition, Htt^{140Q/140Q} NS cells had higher phospho-AKT/AKT ratios than Htt^{7Q/7Q} NS cells in resting conditions and after BDNF stimulation, suggesting mutant htt affects AKT dependent growth factor signaling. Upon differentiation, the Htt^{7Q/7Q} and Htt^{140Q/140Q} generated numerous Beta_{III}-Tubulin- and GABA-positive neurons; however, after 15 days the cellular architecture of the differentiated Htt^{140Q/140Q} cultures changed compared to Htt^{7Q/7Q} cultures and included a marked increase of GFAP-positive cells. Our findings suggest that NS cells expressing endogenous mutant Htt will be useful for study of mechanisms of HD and drug discovery.

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Keywords

embryonic stem cell; mutant huntingtin; neural stem; cholesterol; reactive oxygen species (ROS); reactive oxygen intermediates (ROI); motility; actin; GFAP; astrocytes

Introduction

Huntington Disease (HD) is a fatal neurodegenerative disease caused by an expansion of a normal CAG repeat in the gene encoding the protein Huntingtin (HDCRG, 1993). HD is inherited with autosomal dominance and greater than 38 CAGs is pathogenic. Increasing CAG length correlates with decreased age of onset. The CAG repeat is translated to a polyglutamine (Q) tract near the N-terminus of Huntingtin (Htt), which is ubiquitously expressed. Treatment options are limited for HD patients and do not halt disease progression. Thus, study of mechanisms of HD pathogenesis and therapy are of major importance.

Primary cells cultured directly from embryonic brain tissue are an excellent model for mechanistic studies and small-scale drug screening related to neurodegenerative diseases. Since HD is caused by a single genetic mutation, a variety of mouse lines bearing the HD gene mutation are available from which primary mouse cells can be dissected and cultured. Obtaining primary cells for high throughput studies is impractical because it involves sacrifice of large numbers of animals and prohibitive costs. Furthermore, the supply of embryonic brain from some HD lines may be limited by low fertility or small litters compared to wild-type mice. For these reasons, most cell based HD studies have used immortalized cell lines (neuronal or other etiologies), which are *de facto* abnormal. Transformed cells may have altered metabolism conferring growth and survival advantages that prevent study of selective HD phenotypes seen in primary neurons. Moreover, the subcellular localization of huntingtin differs between proliferating cells and post-mitotic primary cells (Martin-Aparicio et al., 2002, Wheeler et al., 2002, Wheeler et al., 2000). Thus, in the HD field there is a need for a renewable source of primary neurons and glia that display clear disease-relevant phenotypes.

Embryonic stem (ES) and neural stem (NS) cells offer great promise to the field owing to their intrinsic property to generate multiple cell types. ES cells are more versatile and in theory permit differentiation to all cell types, however they are labor intensive and require maintenance on feeder layers. NS cells have restricted cell fates including neurons, astrocytes and oligodendrocytes, and may even be restricted in capacity to reach a specific neuronal sub-type, but are much easier to handle. Human stem cells offer the obvious advantage of being of congruent species with the patient population. In mouse stem cells a normal or mutant gene of interest can be expressed from the endogenous alleles on an identical genetic background allowing subtle phenotypes to be detected, whereas in human iPS cells homologous recombination into endogenous alleles is difficult (Han et al., 2011). Mouse ES cells expressing endogenous mutant Htt ("Knock-in" models) have been established (Jacobsen et al., 2011, Lorincz and Zawistowski, 2009). Some phenotypes have been reported in HD ES cell models including increased neurogenesis (Lorincz and Zawistowski, 2009) and changes in ATP/ADP levels and gene expression (Jacobsen et al., 2011). Valuable information related to the normal function of Htt in mammalian cells can also be garnered from Htt "knock-out" cells (Htt^{-/-}). Studies using Htt^{-/-} ES cells suggest a role for Htt in secretion and cell adhesion (Strehlow et al., 2007), in specification of a lineage of the hematopoietic system (Metzler et al., 1999), maintenance of ATP/ADP levels (Jacobsen et al., 2011), and in regulation of the GTPase Rab11 at recycling endosomes (Li et al., 2008). NS cells derived from mouse brain expressing exogenous mutant Htt exon1 (aa

1–89) have also been described (Chu-LaGriffa et al., 2001) however no phenotypes were determined.

Ideally, NS cells for studies of HD pathogenesis should express full-length mutant Htt at endogenous levels. Here, we examined adherent EGF and FGF-2 dependent neural stem (NS) cells isolated from embryonic wild-type mice (Htt^{7Q/7Q}) and mice expressing full-length endogenous mutant Htt (Htt^{140Q/140Q}). We also differentiated NS cells from heterozygous ES cells that contain human exon1 inserted by homologous recombination into one of the mouse Htt alleles (Htt^{F7Q/7Q} and Htt^{F140Q/7Q}), and from Htt knock-out cells (Htt^{-/-}). Our findings suggest that these NS cell lines display phenotypes related to HD and may be useful for high throughput studies. Furthermore, changes in Htt null cells suggest Htt normally functions in maintenance of cholesterol levels and locomotion.

Results

Isolation of Neural Stem Cells

EGF- and FGF2-dependent NS cells were isolated from wild-type (Htt^{7Q/7Q}) mice and HD (Htt^{140Q/140Q}) knock-in mice using a modified protocol from Conti et al. ((Conti et al., 2005) and see Methods). The HD mice were created by homologous recombination of human exon1 with 140 CAG repeat into the endogenous mouse gene for Htt (*Hdh*) in ES cells and mice were bred to homozygosity (Menalled et al., 2003). Five wild-type and six HD cell lines were established each from individual embryos and at least two pregnancies. NS cells were isolated from embryonic brain tissue, expanded and propagated in EGF/FGF2 containing medium for at least 10 passages prior to characterization. One cell line from each genotype was selected for investigating HD phenotypes. By SDS-PAGE and western blot analysis, NS cells were positive for the radial glial marker Nestin as described for other NS cells (Conti et al., 2005, Lendahl et al., 1990, Okabe et al., 1996), and negative for the early neuronal marker Beta_{III}-Tubulin and the astrocytic marker Glial Fibrillary Acidic Protein (GFAP) (Figure 1a). Quantitative assessment for the radial glial cell markers RC2 and 3CB2 also showed similar levels of protein (Supplementary Figure 1). We detected wild-type Htt in the Htt^{7Q/7Q} cells and mutant Htt in the Htt^{140Q/140Q} cells migrating at the expected sizes. Immunofluorescence analysis with anti-Nestin antibody showed widespread and homogenous immunoreactivity among cells (Figure 1b). Growth curves showed an initial lag phase of growth at 1 day as expected for cells plated at low density in culture (Figure 1c). Thereafter, HD Htt^{140Q/140Q} NS cells had a much slower rate of growth compared to WT Htt^{7Q/7Q} NS cells. Increasing the initial plating density did not alter the growth of Htt^{7Q/7Q} and Htt^{140Q/140Q} cells (data not shown) suggesting this characteristic is cell intrinsic and not dependent on cell density. To distinguish between decreased rates of growth versus increased rates of cell death, we determined the proportion of cells in the total population that incorporated the thymidine analogue BrdU, which marks S-phase, for both WT Htt^{7Q/7Q} and Htt^{140Q/140Q} NS cells. Results showed significantly fewer Htt^{140Q/140Q} NS cells were in S-phase during the labeling period compared to Htt^{7Q/7Q} NS cells (40.4 ± 8.9% of total cells incorporated BrdU for Htt^{7Q/7Q} versus 26.6 ± 5.9% of total cells Htt^{140Q/140Q}; p < 0.001, unpaired t-test, n = 15 fields). Western blot analysis showed no increased activation of caspase 9 or caspase 2 (data not shown), suggesting increased cell death does not contribute to the depressed growth curve of HD Htt^{140Q/140Q} NS cells.

We also established NS cells from mouse ES cells that were engineered by homologous recombination to express no Htt (Htt^{-/-}) or Htt with normal or expanded polyglutamine repeats. In the Htt expressing ES cells, the first exon of one of the mouse *Hdh* alleles has been replaced with human exon1 bearing an N-terminal FLAG epitope tag and encoding either 7 CAGs (Htt^{F7Q/7Q}) or 140 CAGs (Htt^{F140Q/7Q}). NS cells were differentiated from the Htt^{-/-}, Htt^{F7Q/7Q}, and Htt^{F140Q/7Q} ES cells using techniques established for normal ES cells

(Conti et al., 2005). SDS-PAGE and western blot analysis showed the resulting NS cells expressed wild-type Htt in the Htt^{F7Q/7Q} cells, and both normal and mutant Htt in the Htt^{F140Q/7Q} cells as expected for heterozygous cells (Figure 1a). No Htt was detected in Htt^{-/-} cells. The NS cells were positive for Nestin, and negative for BetaIII-Tubulin and GFAP (Figure 1a). Immunofluorescence with anti-Nestin antibody showed homogeneous staining of cells in all three of the ES-derived NS cells (Figure 1d). BrdU labeling also showed reduced incorporation in Htt^{F140Q/7Q} NS cells compared to Htt^{F7Q/7Q} NS cells (36.0 ± 10.1% of total cells incorporated BrdU for Htt^{F7Q/7Q} versus 29.3 ± 5.8% of total cells Htt^{F140Q/7Q}; p<0.05, unpaired t-test, n=15 fields).

Having established NS cells from brain and from ES lines with appropriate molecular markers, we next looked for disease phenotypes in HD NS cells.

HD Htt^{140Q/140Q} and Htt^{-/-} NS cells have altered levels of cholesterol

Marked changes in the levels of cholesterol have been identified in HD patient brain and in HD cell and animal models (reviewed by (Valenza and Cattaneo, 2011)). To determine if cholesterol levels were also altered in Htt^{140Q/140Q} mice, we compared levels of total cholesterol in primary cells cultured from embryonic brains of wild-type Htt^{7Q/7Q} and HD Htt^{140Q/140Q} mice. Total cholesterol levels were significantly lower in primary neurons from HD Htt^{140Q/140Q} mice compared to WT mice: 0.758 ± 0.059 for WT and 0.528 ± 0.079 for HD neurons (mean ± SD uM cholesterol/ug protein), p=0.02, n=3, unpaired t-test and also in primary astrocytes from HD Htt^{140Q/140Q} mice compared to WT mice: 0.465 ± 0.077 for WT and 0.260 ± 0.062 for HD (mean ± SD uM cholesterol/ug protein), p=0.006, n=4, unpaired t-test). Therefore, we asked if HD Htt^{140Q/140Q} NS cells also showed changes in cholesterol levels. NS cells were examined in the undifferentiated stage with normal growth media (+EGF and +FGF2), and in an early differentiation stage when EGF is withdrawn for 3 days (D1 media). When grown in normal defined medium, wild-type Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells had similar cholesterol levels (results not shown). When grown in D1 medium, HD Htt^{140Q/140Q} cells had significantly reduced cholesterol levels compared to wild-type Htt^{7Q/7Q} (Figure 2a; p<0.01, n=3 wells, unpaired t-test). However, levels of cholesterol were not significantly different between the ES-derived Htt^{F7Q/7Q} and HD Htt^{F140Q/7Q} NS cells cultured in D1 medium (Figure 2b; 1-way ANOVA). Unexpectedly, the Htt^{-/-} cells had significantly elevated levels of cholesterol compared to Htt^{F7Q/7Q} (Figure 2b; 1-way ANOVA p=0.0115; Bonferroni's multiple comparison p<0.05, n=3 wells). These results show that changes in cholesterol levels emerge early during differentiation of NS cells that are homozygous for mutant htt or devoid of Htt compared to wild-type.

HD and Htt null NS cells have increased levels of reactive oxygen species

Evidence for oxidative damage has been identified in the HD brain and in HD cell models. (Charvin et al., 2005, Choo et al., 2005, De Luca et al., 2008, Dong et al., 2011, Li et al., 2010, Perez-Severiano et al., 2000, Valencia et al., 2012). Primary cultured cortical neurons from HD^{140Q/140Q} embryonic mouse brains have increased levels of reactive oxygen species (ROS) compared to wild-type cortical neurons (Li et al., 2010). To determine if changes in ROS exist in HD NS cells, live-cell measurements of ROS levels were determined using carboxy-DCFDA-AM, a cell permeable dye, which becomes fluorescent upon oxidation to dichlorofluorescein (DCF) diacetate. We found a significant increase in DCF fluorescence in Htt^{140Q/140Q} compared Htt^{7Q/7Q} cells (Figure 2c; p<0.0001, n=15 cells from 3 coverslips, unpaired t-test). DCF fluorescence was also significantly elevated in the Htt^{F140Q/7Q} cells compared to Htt^{F7Q/7Q} cells (Figure 2d; 1-way ANOVA p=0.0007, Bonferroni's multiple comparison p<0.001, n=5 cells). There was no difference in DCF fluorescence between the

Htt^{-/-} and the Htt^{F7Q/7Q} NS cells (Figure 2d). These results provide evidence that NS cells expressing mutant Htt have increased levels of ROS.

Motility is reduced in HD NS cells and Htt null NS cells

Studies show that mutant Htt interferes with actin dependent cellular remodeling. Mutant Htt fragments interact abnormally with actin binding proteins (Angeli et al., 2010, Kaltenbach et al., 2007). Overexpression of mutant Htt exon1 causes aggregation of the actin binding protein profilin which can be decreased by inhibitors of ROCK kinase (Angeli et al., 2010, Burnett et al., 2008, Munsie et al., 2011, Shao et al., 2008). On the other hand, knockdown of endogenous Htt in Dictyostelium resulted in stage-specific defective migration of cells (Myre et al., 2011, Wang et al., 2011), suggesting a role for normal Htt in actin-dependent processes. To study motility in NS cells, we introduced living NS cells into a microfluidic capillary chamber, recorded images of the moving cells using time-lapse microscopy, and calculated the velocity of cells (as described in Methods). The mean velocity of brain-derived Htt^{140Q/140Q} NS cells was significantly lower than that of Htt^{7Q/7Q} cells (Figure 2e; $p < 0.05$, unpaired t-test, $n = 36$ cells). For the ES-derived NS cells, the velocities of the Htt^{F140Q/7Q} and Htt^{-/-} cells were also significantly decreased compared to the Htt^{F7Q/7Q} cells (Figure 2f; $p = 0.017$, 1-way ANOVA, Bonferroni's multiple comparison, $p < 0.05$, $n = 15$ cells). The results show that mutant Htt or absence of Htt interferes with motility in mammalian cells.

Homozygous expression of mutant Htt affects neuron yield and morphology of cultures compared to WT cultures

NS cells isolated from embryonic brain (passaged 15–35 times) were differentiated using a protocol in which EGF is withdrawn and FGF2 concentrations are decreased as brain derived growth factor (BDNF) is introduced and incrementally increased (Spiliotopoulos et al., 2009). The differentiation was monitored by labeling for the neuronal marker Beta_{III}-Tubulin. The majority of Htt^{7Q/7Q} cells passaged 15 or 35 times and differentiated 5, 15 or 23 days robustly expressed Beta_{III}-Tubulin (about 75%–85% of the final culture), and developed the polarized shape and growth of processes characteristic of neurons (Figure 3a **top shows passage 35, differentiation day 15**; Figure 4c, d). Cells expressing Beta_{III}-Tubulin continued to express Nestin detected by immunofluorescence (Figure 3a). Immunoreactivity for the neurotransmitter GABA examined in separate cultures was detected to the same extent as Beta_{III}-Tubulin suggesting that the differentiation protocol produced GABAergic neurons (Figure 3b). There was no staining detected for a marker of oligodendrocytes using anti-Rip antibody or for microglia using anti-Iba1 antibody (results not shown). Htt^{140Q/140Q} NS cells passaged 15 or 35 times and differentiated 5–23 days also showed a robust labeling for Beta_{III}-Tubulin (Figure 3a, **for cells at passage 35, differentiation day 15**) and for GABA (Figure 3b) and exhibited a neuronal-like morphology with these markers. However, there was a striking difference in the cellular architecture of the Htt^{140Q/140Q} NS cultures compared to the Htt^{7Q/7Q} cultures when they were differentiated for longer than 15 days (Figure 3c). In the Htt^{7Q/7Q} cultures Beta_{III}-Tubulin positive cells grew relatively flat and had discernable bipolar or multipolar shapes. Neurites could be followed for long distances within the two-dimensional plane of the substrate. Embryoid bodies (EBs) containing Nestin-positive cells and Beta_{III}-Tubulin positive cells also accumulated in the cultures (not shown). By contrast, Beta_{III}-Tubulin positive cells in Htt^{140Q/140Q} NS cultures had broader and flatter cell bodies with less discernable bipolar and multipolar features. Neurites had a more tortuous path and extended into a larger 3 dimensional space away from the cell body within flat, multi cell layers (Figure 3c). Embryoid bodies containing Nestin and Beta_{III}-Tubulin immunoreactive cells were not detected in the differentiated Htt^{140Q/140Q} NS cultures. These growth characteristics of Htt^{7Q/7Q} and Htt^{140Q/140Q} were evident in five independent differentiation

experiments. We measured the soma area and neurite length (longest neurite per cell) of neurons (GABAergic or β -Tubulin-positive cells) in low density cultures; in HD Htt^{140Q/140Q} differentiated cultures, microscopic fields on the edge of the cellular matrix were selected so that neurites could be followed. The average area of the cell soma measured per microscopic field was significantly larger in for Htt^{140Q/140Q} NS neurons compared to Htt^{7Q/7Q} NS neurons (428 ± 34.9 sq pixels for Htt^{7Q/7Q} $n=6$ 40X fields, compared to 628.5 ± 172.3 sq pixels for Htt^{140Q/140Q} $n=4$ 40X fields; $p < 0.05$ unpaired t-test) (Figure 4a). In contrast, no significant difference in neurite length occurred between Htt^{7Q/7Q} and Htt^{140Q/140Q} neurons (Figure 4b). These results showed that Htt^{7Q/7Q} and Htt^{140Q/140Q} NS cells differentiate into neurons with neurites of normal length, but the presence of the HD mutation affects cellular architecture in the NS cultures.

Quantitative analysis of the differentiated cultures showed that Htt^{7Q/7Q} NS cells produced similar high yield of neurons (>80% of total cells) at passage 15 or passage 35 (Figure 4c; 23 days in culture). However, Htt^{140Q/140Q} NS cells of passage 35 that were differentiated for 20 days had a significantly lower percentage of β -Tubulin cells compared to Htt^{7Q/7Q} cells of the same passage and differentiation time (Figure 4c; $p < 0.05$, $n=5$ fields unpaired t-test, 20 or 23 days in culture). From day 5 to day 20 of differentiation, the mean percentage of neurons in the Htt^{7Q/7Q} and the Htt^{140Q/140Q} cultures significantly declined (Figure 4d $p < 0.05$, $n=5$ fields unpaired t-test); from day 5 to day 20, decreases in the mean percentage of neurons occurred for both the differentiated cultures (Figure 4d; $p < 0.05$, $n=5$ fields unpaired t-test, 5 and 20 days in culture). These results show that later passages of Htt^{140Q/140Q} NS cultures yielded fewer neurons than later passages of Htt^{7Q/7Q} cultures. The number of nuclei labeled with Hoechst dye and the number of β -Tubulin positive cells were also compared in the differentiated Htt^{7Q/7Q} and Htt^{140Q/140Q} cultures (Figure 4e and f). In the Htt^{7Q/7Q} cultures, the average number of cell nuclei per microscopic field and the mean number of β -Tubulin positive cells per microscopic field were not significantly changed with duration of differentiation (day 5 versus day 20) (Figure 4e and f). Compared to differentiation at 5 days, the Htt^{140Q/140Q} cultures differentiated at 20 days had a significant increase in total cell numbers per microscopic field (Figure 4e; $p < 0.05$; paired t-test, $n=5$ fields), but no significant change in the total number of β -Tubulin positive cells per field (Figure 4f). These results suggested that with an extended period of differentiation Htt^{140Q/140Q} cultures produced a cell type with a non-neuronal phenotype.

Differentiated Htt^{140Q/140Q} NS cultures have increased GFAP positive cells compared to Htt^{7Q/7Q} NS cultures

Our analysis suggested that during differentiation, a non-neuronal cell proliferated only in the Htt^{140Q/140Q} NS cultures. To determine the identity of the proliferating cells, SDS-PAGE and western blot analysis was performed with lysates of cultures that were differentiated for 23 days. Similar levels of β -Tubulin protein were detected in the lysates from the Htt^{7Q/7Q} NS cultures and the Htt^{140Q/140Q} NS cultures (Figure 5a), consistent with findings in Figure 4d. However, there was a marked accumulation of filamentous GFAP present in lysates from the HD Htt^{140Q/140Q} cultures that was not detected in the wild-type Htt^{7Q/7Q} cultures. Htt^{7Q/7Q} lysates also contained Nestin whereas the Htt^{140Q/140Q} lysates did not (Figure 5a). Immunofluorescence analysis with anti-GFAP antibody showed the presence of numerous large, flat cells immunoreactive for GFAP in the HD Htt^{140Q/140Q} cultures but not in the Htt^{7Q/7Q} cultures supporting the biochemical data (Figure 5b). Some flat cells were also present in the wild-type cultures, but were smaller in size and were not immunoreactive for GFAP (Figure 5b). Quantification of the day 20 cultures confirmed that there was a significant increase in the percentage of GFAP-positive cells in the Htt^{140Q/140Q} NS cultures compared to the Htt^{7Q/7Q} cultures (Figure 5c; $p < 0.005$,

unpaired t-test, $n=5$ fields). These results suggest that HD Htt^{140Q/140Q} cultures accumulate cells with a phenotype (large, flat and GFAP-positive) consistent with astrocytes.

Differentiation to an astrocytic phenotype is controlled in part through the Jak-STAT signaling pathway resulting in increased binding of STAT3 to the GFAP promoter (Asano et al., 2009, Cheng et al., 2011, Fan et al., 2005). We inquired whether activation of STAT3 and its association with the GFAP promoter in NS cells was responsible for the increased numbers of GFAP-positive cells observed in our differentiated HD Htt^{140Q/140Q} cultures. STAT3 association with the GFAP promoter was measured using chromatin immunoprecipitation (ChIP) from wild-type Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells with an antibody against STAT3, followed by quantitative PCR (qPCR) using primers spanning the STAT3 binding region of the GFAP promoter. In both cell types, there was more DNA immunoprecipitated with the STAT3 antibody than the mock no antibody control. A small amount of the GFAP promoter precipitated specifically with the STAT3 antibody from both the WT Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells (Figure 5d). However, there was no significant difference in the amount of DNA amplified by qPCR between the two cell lines. These data suggest that the HD Htt^{140Q/140Q} NS cells are not predisposed to differentiating to astrocytes due to increased basal levels of Jak-STAT3 signaling and binding of STAT3 to the GFAP promoter.

The addition of recombinant BDNF greatly improves survival of neurons differentiated from NS cells in culture (Spiliotopolous et al., 2009, and our observations). Disruption of BDNF transcription, trafficking and signaling has been implicated in HD (Roze et al., 2008, Zuccato and Cattaneo, 2007). Therefore, the reduced differentiation capacity of HD NS cells might be due to a lowered response to recombinant BDNF. Activation of the BDNF pathway includes ligand binding to the TrkB receptor and activation of first PI 3-kinase, then PDK1, which phosphorylates the pro-survival effector molecule AKT. To assess the responsiveness of the NS cells to BDNF stimulation, we measured levels of phospho-AKT (pAKT)/AKT by densitometry analysis of western blots (Figure 5e and f). The experiment was performed during the early differentiation phase, after withdrawal from EGF and with low FGF when BDNF is first introduced during the differentiation protocol (see methods). HD Htt^{140Q/140Q} NS cells had a significantly elevated level of pAKT compared to WT Htt^{7Q/7Q} cells in the un-stimulated condition (Figure 5e, **white bars for each cell type**). After stimulation with BDNF for 1 hour, WT and HD Htt^{140Q/140Q} NS cells had significantly elevated ratio of pAKT/AKT (Figure 5e, **grey bars for each cell type**). However, the fold increase was greater for HD Htt^{140Q/140Q} NS cells compared to WT NS cells. These results suggest that HD Htt^{140Q/140Q} NS cells have increased basal maintenance of the AKT survival pathway and are hyper-responsive to BDNF-stimulation.

Karyotype analysis suggests mutant Htt affects mouse neural stem cell stability

Karyotype analysis (normal G-band analysis) of the brain-derived NS cells was performed at early and late passages. Neither Htt^{7Q/7Q} nor Htt^{140Q/140Q} had normal karyotypes at either early or late passages. Htt^{7Q/7Q} cells at p17 were female, diploid, and consisted of three clones that had acquired 1, 2 and 3 chromosomal changes respectively (including trisomy 8, known to confer growth advantage in culture conditions, in clone 2 and 3). At p67, the WT Htt^{7Q/7Q} cells were composed largely of one stable clone derived from clone 2; this clone had now accumulated two additional changes. HD Htt^{140Q/140Q} cells at p16 were female, tetraploid and highly unstable with numerous changes in individual chromosome numbers, but with few structural aberrations. At p65, the HD Htt^{140Q/140Q} was still tetraploid with multiple non-clonal chromosomal gains and losses. We also analyzed another HD Htt^{140Q/140Q} NS cell line (HD6) at p14 that was established from an embryo from a separate litter as the original HD cell line. The karyotype analysis revealed that it, too, was female

and tetraploid; HD6 had 4 consistent chromosomal gains and 6 consistent losses (from the tetraploid state), as well as non-clonal gains and losses consistent with instability. The results suggest that Htt^{140Q/140Q} mouse NS cells tend to become tetraploid in tissue culture and exhibit changes consistent with mitotic instability.

We also had noticed that when the original Htt^{F140Q/7Q} ES cells were cultured in the absence of feeders at low density, numerous tetraploid or multinucleate cells could be seen by light phase microscopy whereas in wild-type cells only one nucleus per cell could be observed. Furthermore, when grown on feeder cells, Htt^{F140Q/7Q} ES cell clones were extremely heterogeneous in nature with multiple cell types differentiating at the edges of clones, whereas the Htt^{F7Q/7Q} and Htt^{-/-} ES cell clones grew as homogenous clumps with sharp borders and easily maintain their immature morphology. These results suggest that endogenous mutant Htt may lend instability to stem cell models in culture.

Discussion

We have isolated EGF/FGF-2-dependent NS cells from the embryonic brain of mice expressing endogenous wild-type or mutant Htt and also from ES cells expressing FLAG tagged wild-type Htt or mutant Htt, or no Htt. Compared to wild-type NS cells, HD NS cells had increased levels of ROS, impaired motility, and decreased cholesterol, although the decreased cholesterol level for heterozygous HD cells was not significant. The homozygous HD cells also had slower growth kinetics compared to wild-type cells. The wild-type NS cells were sustained through multiple passages and could be differentiated to a high proportion of cells immunoreactive for Beta_{III}-Tubulin and GABA (>80%) even at high passages (>35). However, as passage number increased the HD NS cells yielded fewer neurons. The differentiated HD cultures developed an aberrant cellular architecture, which included a proliferation of astrocytes. Our findings in wild-type cells agree with previous data indicating that EGF/FGF-2-dependent NS cells from normal mice have potential for developing into GABAergic neurons (Conti et al., 2005). Our data support the feasibility of using HD NS cells to study the effects of endogenous full-length mutant Htt in neurons and glia and for high throughput analysis.

Changes in ROS and in cholesterol that we identified in HD NS cells have been described in the HD brain or in vitro primary neuron models of HD (Charvin et al., 2005, Choo et al., 2005, De Luca et al., 2008, Diaferia et al., 2011, Li et al., 2010, Perez-Severiano et al., 2000, Valencia et al., 2012). We also measured decreased levels of cholesterol in primary neurons and astrocytes from HD^{140Q/140Q} mice, further correlating our findings with an additional HD model. Moreover we found that cholesterol and motility were also affected in NS cells devoid of Htt; these results are consistent with genetic findings in which changes in similar pathways occurred with mutant Htt and with absence of Htt in ES cells (Jacobsen et al., 2011), and indicate that Htt may function in cholesterol homeostasis and maintenance of redox levels.

HD NS cells had decreased motility compared to wild-type cells. This phenotype may be attributed in part to altered actin kinetics (Angeli et al., 2010, Burnett et al., 2008, Munsie et al., 2011, Shao et al., 2008). Several proteins known to be involved in actin dynamics co-precipitate with Htt fragments (Kaltenbach et al., 2007) and mutant Htt may alter their function. Of interest, Htt interacts with specific phosphoinositides that regulate actin dynamics and mutant Htt has increased interactions with some of these lipids (Kegel et al., 2009). Therefore, the signaling pathways that control actin kinetics may be altered. We found that the motility deficit in HD NS cells was less pronounced in homozygous brain-derived Htt^{140Q/140Q} NS cells than in the heterozygous ES-derived Htt^{F7Q/140Q} NS cells. The brain-derived Htt^{140Q/140Q} NS cells may have adapted in culture to changes induced by

mutant Htt or have benefitted from their differentiation to NS cells *in vivo* prior to their isolation in culture.

HD NS cells grew more slowly than wild-type cells. For both the brain-derived Htt^{140Q/140Q} NS cell line and the ES-derived Htt^{F7Q/140Q} NS line, fewer cells incorporated BrdU compared to their respective wild-type control cell lines, indicating fewer cells were in S-phase. These results are consistent with previous findings showing that mutant NS cells isolated from Q111/Q111 knock-in mouse brains and cultured *ex vivo* as neurospheres have a reduced proliferative capacity compared to wild-type (Molero et al., 2009). At day 5 of differentiation we saw no differences in total cell numbers between Htt^{7Q/7Q} and Htt^{140Q/140Q} cultures when plated at equal densities suggesting no change in NS self-renewal rate. In contrast, Lorincz and Zawistowski (2009) found increased production of neural progenitors using a full-length knock-in ES model of HD (Q150/Q150) (Lorincz and Zawistowski, 2009). This discrepancy may be due to the origin of the NS cells (embryonic neural tissue in our study versus postnatal neural tissue or ES cells in their study). Other factors also can affect production of neural progenitors. NS cells derived from the brains of symptomatic HD transgenic mice that over-express a small fragments of Htt (R6/2 model) exhibit more neurogenesis *in vitro* than NS cells isolated from brains of mice that are pre-symptomatic (Batista et al., 2006). However, mouse iPS cells created from R6/2 fibroblasts showed no changes in cell cycle (Castiglioni et al., 2011). Molero et al. suggest that proliferation capacity of NS cells from wild-type and HD Q111/Q111 mice reverse with embryonic age such that wild-type NS cells are increased compared to HD at embryonic day 14.5 but decreased compared to HD at embryonic day 15.5 (Molero et al., 2009). These results suggest that the source of stem cells and developmental timing of isolation might alter effects of mutant Htt on cell growth characteristics.

Our results showed that in later passages (35), Htt^{140Q/140Q} NS cells yielded fewer Beta_{III}-Tubulin positive neurons than Htt^{7Q/7Q} cultures when plated at equal densities. Lorincz and Zawistowski found increased production of Beta_{III}-Tubulin positive neurons using the full-length knock-in ES model of HD (Q150/Q150) or NS cells isolated from postnatal subventricular zone from this mouse model (Lorincz and Zawistowski, 2009). The differing results may be due to the method of analysis (direct cell counts in our study versus neurosphere number and Beta_{III}-Tubulin levels in the study by Lorincz and Zawistowski). Alternatively, the origin of NS cells might also contribute to fate commitment of a particular neuronal progenitor and neuronal specification. The differentiation protocol we used with the FGF-2/EGF dependent NS cells was tailored to produce GABAergic projection neurons (Spiliotopoulos et al., 2009) and is known to be restrictive for other neuronal cell types (Elkabetz et al., 2008). Accordingly we found that the majority of Beta_{III}-Tubulin positive cells in our differentiated cultures were GABAergic. In their study, Lorincz and Zawistowski found the level of mRNA for a marker of GABAergic medium spiny neurons, Islet 1, to be reduced in HD150Q/150Q NS cultures compared to WT cultures whereas mRNA levels for a dorsal telencephalon neuronal marker, EMX1, were increased in the HD cultures. Thus, in our HD NS cells and those studied by Lorincz and Zawistowski, the HD mutation may reduce the number of GABAergic neurons.

We found that with increased differentiation time, Htt^{140Q/140Q} NS cultures underwent marked morphological changes compared to wild-type Htt^{7Q/7Q}, and had proliferation of GFAP positive cells. Reactive astrogliosis is present in human HD striatum but it is unclear why this occurs (Vonsattel and DiFiglia, 1998). Neurons expressing mutant Htt in the NS cultures may emit a distress signal to surrounding Nestin-positive glia, inducing their differentiation to astrocytes which may be better equipped to support neurons. In support of this idea astrocytes may augment cholesterol production for HD neurons (Valenza and Cattaneo, 2011). Alternatively, mutant Htt may directly impact differentiation to astrocytes

in vitro. Differentiation to astrocytes is controlled in part by the distribution of NCoR between the nucleus and the cytoplasm with increased cytoplasmic localization of NCoR favoring astrocyte differentiation (Hermanson et al., 2002). Htt interacts with NCoR and mutant Htt interacts more tightly with NCoR causing its retention in the cytoplasm (Boutell et al., 1999). In theory activation of the Jak-STAT pathway in HD NS cells, which also regulated differentiation to astrocytes, could explain the increased numbers of astrocytes in the HD cultures. Our ChIP experiment showed no significant change in binding of STAT3 to the GFAP promoter in WT versus HD NS cells, however, diminishing support for this hypothesis.

For the differentiation of NS cells in our study, we used the protocol of Spiliotopoulos et al., which requires the incremental introduction of BDNF to the NS cultures (Spiliotopoulos et al., 2009). We found the addition of BDNF produced a higher neuron yield for both wild-type and HD NS cells compared to cultures lacking BDNF (our observations). However, the yield of neurons for HD cultures had a trend to be lower at p15 and was significantly lower at p35. We asked if the reduced neuron yield was due to a depressed response to BDNF using phosphorylation of AKT as a surrogate marker. Surprisingly, we found an increased basal level of phosphoAKT in the HD cells. Both the wild-type and HD cells responded appropriately to BDNF stimulation, although the level of activation was higher in the HD cells. This change in response to BDNF signaling may reflect an adaptive change induced by mutant Htt. BDNF levels are reduced in HD brain and loss of the neurotrophin is thought to contribute to neuronal degeneration (Gauthier et al., 2004, Zuccato et al., 2001). In studies of HD^{140Q/140Q} primary cortical neurons the addition of BDNF to the cultures increases the viability of differentiated HD neurons to the level of wild-type neurons (Valencia et al., 2012). Despite these findings, when BDNF was withdrawn from the differentiated NS cultures, we did not observe increased cell death of HD cells (unpublished studies). One potential explanation is that the accumulating glial cells in the differentiated HD NS cultures have acquired the ability to provide trophic support so that dependence on BDNF is no longer required.

Recently Biunno and colleagues reported that mouse NS cells undergo changes in karyotype with increased passage number and that genetically manipulated cell lines showed increased instability (Diaferia et al., 2011). In agreement with these findings, we found that both the wild-type and HD NS cells had abnormal karyotypes even at low passage number. Whereas the Htt^{7Q/7Q} cells were diploid, stable and had few changes, the HD cells were tetraploid and unstable with numerous non-clonal chromosomal gains and losses, consistent with mitotic instability. While numerous chromosomal abnormalities were detected in the Htt^{140Q/140Q} HD cell line even at the early passage number, the changes were random among cells and no single clone of cells predominated. Since no single clone predominated in the HD cultures, it is unlikely that the phenotypic effects we observed are due to any one chromosomal abnormality. Furthermore, the HD related differences we observed were also observed in the ES-derived NS cells. We cannot rule out that tetraploidy may affect phenotypic readouts. However, the effects of tetraploidy are often neutralized by “balancing” and can be protective when gene redundancy can shelter from the effects of certain mutations (reviewed by (Comai, 2005, Semon and Wolfe, 2007). Indeed, since an additional HD cell line was also tetraploid, the homozygous HD lines may induce tetraploidy in culture in an attempt to protect themselves from the ill effects of mutant Htt. Alternatively, mitotic instability and tetraploidy may be due to a disruption in normal function of Htt in neuroblasts to orient the mitotic spindle during mitosis (Godin et al., 2010). Thus, additional phenotypes identified in these cell lines should be rigorously validated in other HD models such as primary neurons. Despite the karyotypic changes, phenotypes consistent with those seen in primary neurons or HD brain (decreased cholesterol and increased ROS) were still measurable in the HD NS cell lines.

Few HD ES cells or induced pluripotent stem (iPS) cells have been published thus far for use as HD model systems and limited phenotypes have been reported (Niclis et al., 2009, Park et al., 2008, Zhang et al., 2010). Our findings suggest that mouse HD NS cells expressing endogenous mutant huntingtin have multiple disease relevant phenotypes and therefore may be valuable for investigating mechanisms of disease pathogenesis.

Methods & Materials

Isolation and maintenance of EGF and FGF-2 dependent NS cells

The animal protocol was reviewed and approved by the MGH Subcommittee on Research Animal Care (SRAC)-OLAW Assurance # A3596-01. The protocol was submitted and reviewed conforms to the USD Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, the “ILAR Guide for the Care and Use of Laboratory Animals” and other applicable laws and regulations. Wild-type mice were C57BL/6 strain background. HD mice were of the same strain but had human exon1 with an expanded polyQ (140Q) introduced by homologous recombination into the endogenous mouse allele (Menalled et al., 2003). Homozygous 140Q/140Q mice were used to isolate neural stem cells.

Adherent NS cells were derived from embryonic mouse brain tissue using procedure adapted from Conti et al. based on Okabe et al. for ES cells (Conti et al., 2005, Okabe et al., 1996, Ying et al., 2003). Time-pregnant mice were anesthetized with Avertin (250mg/kg) and embryos (embryonic day 16) collected in a Petri dish and placed on ice. Dissections were performed under a stereomicroscope in ice cold PBS and meninges removed. Whole brains were cut into pieces using forceps, and collected in a 15 ml Falcon tube. The tissue was homogenized by repeated pipetting with a fire-polished Pasteur pipette in dissection medium. Cells were centrifuged at 4°C for 5 minutes at 1000xg and resuspended in modified Neural Stem Cell Expansion Medium (NSEM): 500 ml of DMEM/F12 (Invitrogen, #12634), 1 bottle of N2 supplement (Invitrogen, #17502-048), 1 bottle of B27 *without retinoic acid* (Invitrogen, #12587-010), 5 ml Glutamax (Invitrogen, #35050-079), 5 ml Pen/Strep (Invitrogen, #15070-063), plus the growth factors at the final concentrations of 10 ng/ml FGF (Invitrogen, #PHG0026) and EGF 10ng/mL (Invitrogen, #PMG8045). Cells were plated on gelatin-coated plastic (Millipore, #ES-006-B). Half the medium was changed the following day to remove debris and non-adherent tissue. Cell were split depending upon the growth rates of each cell line and ranged from 1:5 to 1:12. For passaging, cells were washed once with HBSS (10mL/75cm²) and dissociated with Accutase (Sigma, #A6964) for 2 minutes or until detachment occurs. Cell suspension was centrifuged at 1,200 rpm for 3 minutes. Half of medium was changed every other day.

ES cells have been described previously (Li et al., 2008) and were “neuroconverted” as exactly as described (Conti et al., 2005) except modified NSEM medium (described above) was used as the final medium.

Neuronal Differentiation Procedure

A protocol for differentiation of neural progenitor cells into post-mitotic neurons was adapted from Spiliotopoulos et al., which slowly reduces FGF while introducing BDNF (Spiliotopoulos et al., 2009). The differences are as follows: Advanced DMEM/F12 (Invitrogen, #12634) was used in place of EUROMED-N medium and used as DMEM/F12 in differentiation mediums. See supplementary information for Medium D1, A, and B formulations. Cells were plated in uncoated flasks in D1 medium (without EGF) at 1–1.5 × 10⁵ cells/cm². On the third day in D1 medium, half of the medium was changed before replating. On the fourth day of differentiation, cells were treated with Accutase, collected by centrifugation (1,200 rpm for 3 minutes) and the pellet resuspended in prewarmed Medium

A. Cells were plated at 6×10^3 cell/cm² on coverslips or plastic that were coated overnight with laminin (3 µg/ml, Invitrogen, #23017-015). On the sixth day (the third day in Medium A), half of Medium A was replaced with fresh Medium A. On the seventh day of differentiation, half of Medium A was replaced with Medium B. The medium was changed every other day, thereafter with Medium B.

For experiments assessing AKT activation in response to BDNF, NS cells were grown for 24 hours in Medium B (which contains 5 ng/ml FGF2 and no EGF) but with no BDNF to allow for suppression of PI 3-kinase signaling pathways. After 24 hours, 1/10 volume of 1.1 µg/ml BDNF diluted in the same medium was added directly to each well for a final concentration of 100 ng/ml BDNF; cells were incubated for 1 h then harvested for western blot. For some differentiation experiments, BDNF (Invitrogen, #PHC7074) concentration reduced from the normal 30 ng/mL to test effects of BDNF withdrawal. In this case, BDNF was at the normal concentration for 10 days in Medium B prior to withdrawal. All media were made in 50 mL batches, weekly.

Immunofluorescence and confocal microscopy

Cells were plated onto poly-L-lysine coated glass coverslips in normal growth medium at low density. After 24 hours, cells were washed in 1xPBS containing Mg²⁺ and Ca²⁺ ions and fixed with 4% PFA in the same buffer for 12 minutes. Cells were washed with PBS three times then incubated in blocking buffer (4% normal goat serum in PBS) for 1 hour. Cells were incubated in primary antibodies overnight in blocking buffer at 4°C at the following concentrations: anti-Nestin (1:200; monoclonal Ab clone rat-401, Millipore); anti-Beta_{III}-Tubulin (1:500; polyclonal, Sigma), anti-GABA (1:500; Sigma); anti-GFAP (1:50; Sigma); anti-Htt 1–17 (1 µg/ml; polyclonal antibody (DiFiglia et al., 1995)); anti-MAP2 (1:500; polyclonal Ab, Millipore), anti-Rip (1:25; monoclonal, Developmental Studies Hybridoma Bank, University of Iowa), anti-IBA1 (1 µg/ml, WAKO Pure Chemical Industries, Osaka, Japan). After washing in PBS, coverslips were incubated with secondary antibodies: Bodipy-labeled anti-rabbit (1:500; Invitrogen) and rhodamine red X labeled anti-mouse (1:500; Jackson Immunolabs). Nuclei were stained with Hoechst dye (1:10,000; Molecular Probes). Confocal images were acquired using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Biorad Radiance 2100 confocal laser. Sequential images were obtained with excitation at 405 nM, 488 nM, and 568 nM and using standard fluorescent filter sets for AMCA, FITC and rhodamine. Images were merged in Adobe Photoshop. The area of neuronal somata and the length of neurites was measured using ImageJ software (NIH) from confocal images acquired using identical settings for both cell lines. For neurite length, the longest neurite per neuron was measured.

SDS-PAGE and Western Blot Analysis

Total protein lysates from cells were collected in the following buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 1% NP-40, pH 7.5) plus protease inhibitors (EDTA-free mini complete, Roche). Protein concentrations were determined by Bradford Assay (Biorad). Equal amounts of protein (10 or 20 µg per lane) were separated on precast 3–8% Tris-acetate or 4–12% Tris-glycine gels (Invitrogen) and transferred onto nitrocellulose using an iBlot system (Invitrogen). Blots were blocked in 5% non-fat milk/PBS and incubated overnight in primary antibody in blocking buffer. Nestin and RC2 antibodies were incubated in TBST instead of block overnight. Secondary antibodies were peroxidase labeled anti-rabbit or anti-mouse (Jackson Immunolabs, 1:5000). Blots were developed using ECL (Pierce). Primary antibody concentrations: anti-Htt 1–17 (Ab1, 0.5 µg/ml), anti-Nestin (1:100; Millipore), anti-GFAP (1:500; Sigma), rabbit polyclonal anti-Beta_{III}-Tubulin (1:500; Sigma), mAb anti-RC2 clone (1:200; Developmental Studies Hybridoma Bank, University of Iowa), mAb anti-3CB2 (1:200; Developmental Studies Hybridoma Bank, University of Iowa), anti-

Caspase 2 (1:500; Abcam), anti-Caspase 9 (1:500; Abcam), phospho-AKT (1:500; Cell Signaling), and AKT (1:500; Cell Signaling mAb AKT5G3).

Cholesterol Measurements

Cholesterol was measured using Amplex Red Cholesterol Assay Kit (Invitrogen/Molecular Probes). Lysates for assays were standardized by protein content measured by using Bradford assay.

ROS Detection Procedure

Carboxy-DCFDA-AM (5-(and-6)-carboxy-2',7'-dichloro fluorescein diacetate; Invitrogen) fluorescence was used as a marker of ROS and was measured in live cells with a 60X oil objective on a Nikon Eclipse TE00 inverted microscope equipped with a Biorad Radiance 2100 confocal laser. Cells were incubated in 2 μ M carboxy-DFCDA-AM at 37°C for 30 minutes, then washed with pre-warmed PBS containing 1 mM CaCl₂ and 1 mM MgCl₂. Confocal images were acquired with excitation at 488 nm using Lasersharp software from 3 coverslips and analyzed using Image J software (NIH). Cells were traced and average intensity per area for each individual cell was determined.

Karyotype Analysis

Standard G-banded karyotype analysis was performed by Cell Line Genetics.

Cell Growth Kinetics

Cells were counted using a hemocytometer and plated in plastic gelatin-coated 6-well plates at equal densities. At each time point, 3 wells per cell line were treated with Accutase to dislodge cells. Cells were pelleted and re-suspended in growth medium. Aliquots of cells were incubated with trypan blue then counted. Growth kinetics were monitored from cells plated at two different densities on day 0.

BrdU Incorporation

Cells were plated at 5×10^5 cells/well onto coverslips in 24 well plates and grown for 48 hours to allow cells to enter log phase growth. Cells were then incubated with 10 μ M BrdU for 1 hour at 37°C under normal culture conditions. Medium was removed and cells were washed with PBS containing calcium and magnesium, fixed for 15 minutes with 4% paraformaldehyde in PBS, washed in PBS and made permeable with 0.1% Triton X100 for 15 minutes. After another wash in PBS, cells were incubated in 2N HCL for 1 hour at 37°C. Acid was removed and cells washed with PBS for 15 minutes three times. Coverslips were then blocked in 4% normal goat serum and incubated overnight at 4°C with a rat monoclonal antibody to BrdU (1:200; Abcam #6323) diluted in block. Cells were washed with PBS, incubated in cy3-conjugated anti-rat antibody (1:500; Jackson Immunolabs) for 2 h at RT, then washed again and mounted using Prolong (Invitrogen). Cell counts were performed using a 40X objective. BrdU-positive cells and total cells per field (counted using phase light microscopy) were counted for 5 fields per coverslip over 3 coverslips. At least 500 cells were counted per cell line.

Cell motility experiments

Cells suspended in normal growth medium were seeded into the “cell seeding compartment” of microfluidic devices placed in 24 well-plates, at a density of approximately 2000 cells/compartment (as previously described in detail (Irimia and Toner, 2009)). The surface of the microfluidic devices was coated with laminin at 20ng/mL for 10 minutes before the migration assay. Within 24 hours after seeding, the cells entered and moved spontaneously through the arrays of 200 parallel channels (10 $\times$ 10 μ m cross section) connecting the cell-

seeding compartment and the larger well. Time-lapse images of the cells in channels were taken every 15 minutes using a Nikon TiE microscope with phase contrast illumination for 24 hours. Individual cells were tracked using Image J software (NIH) with Cell Tracking plug-in. The average velocity of each cell was calculated on the rate of movement from entry to the channel until its first stop. The average velocities of at least 15 cells per condition were compared among cell lines. Statistical analysis was performed using an unpaired t-test or one-way ANOVA.

Chromatin Immunoprecipitations (ChIPs) and quantitative PCR

A modified ChIP technique was adapted from previously published studies from our laboratory in order to analyze DNA/protein complexes in dissected brain tissue (Sadri-Vakili et al., 2007). STAT3 antibody 9D8 (Abcam #119352) was added to isolate protein-DNA complexes to look for DNA bound to STAT3 protein. GFAP promoter DNA spanning the STAT3 binding site was detected in the resulting ChIP-DNA by quantitative real-time PCR (qPCR) using specific primers based on previously published sequences (Fan et al., 2005); primers: GFAP forward 5' TAA GCT GAA GAC CTG GCA GTG 3' and GFAP reverse 5' GCT GAA TAG AGC CTT GTT CTC3'). Quantitative real time-PCR was performed using 50 PCR cycles (95°C for 30 s, 57°C for 60 s, 72°C for 90 s) in an iCycler (Bio-Rad) with the use of SYBR-green PCR Master Mix (Applied Biosystems). Threshold amplification cycle numbers (T_c) using iCycler software were used to calculate IP DNA quantities as percentage of corresponding inputs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of NS cells isolated from E16 brain tissue (Brain-derived NS) or converted from ES cells to a neural lineage (ES-derived NS)

(a) Western blot analysis with anti-Htt antisera Ab1 (top blots) confirmed the Htt genotypes of NS cells. Adult wild-type (WT) mouse brain was used to control for antibody reactivity. Mutant Htt with the expanded polyQ tract has a slower mobility on SDS-PAGE. Parallel blots analyzing the same lysates showed that cells are positive for the neural stem cell marker Nestin and negative for the neuronal marker Beta_{III}-Tubulin. Re-probing the Htt blots gave negative results for GFAP, an astrocyte marker, as expected. 20 µg loaded per lane. **(b)** Confocal immunofluorescence showed homogeneous staining for Nestin in Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells. **(c)** WT Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells have different growth kinetics in culture. Cells were plated at equal densities in 6-well plates then harvested using trypsin, suspended in equal volumes and counted using trypan-blue exclusion on days indicated. Graph shows growth curves for Htt^{7Q/7Q} and HD Htt^{140Q/140Q} NS cells. Mean ± SD, n=3 wells per time point. **(d)** Confocal immunofluorescence showed homogeneous staining for Nestin in Htt^{-/-}, Htt^{F7Q/7Q} and HD Htt^{F140Q/7Q} NS cells.

Figure 2. HD related phenotypes of NS cells

(a and b) Cholesterol levels of NS cells grown in medium lacking EGF were measured using Amplex Red Cholesterol Assay Kit (Invitrogen/Molecular Probes). For a, * Indicates $p < 0.01$, $n = 3$ wells, unpaired t-test; for b, $p = 0.0115$, $n = 3$ wells, ANOVA, * indicates $p < 0.05$ posthoc Bonferroni's multiple comparison test). (c and d) Carboxy-DCFDA-AM (5-(and-6)-carboxy-2',7'-dichloro fluorescein diacetate; Invitrogen) fluorescence was used as a marker of ROS and was measured in live cells with a 60X oil objective. For c, * Indicates $p < 0.001$, $n = 15$ fields, unpaired t-test; for d, $p = 0.0007$, $n = 5$ fields, ANOVA. * indicates $p < 0.001$, Bonferroni's multiple comparison test. (e and f) Cell motility was measured by plating cells in devices with microfluidic channels and monitoring movement with time-lapse microscopy. Cells were manually tracked using ImageJ software and the average velocity for each cell calculated from entry to the channel until the cell's first stop (see Methods). Bar graph shows mean velocity $\pm$ SD. For e, * indicated $p < 0.05$, $N = 20$ cells, unpaired t-test; for f, * indicates $p < 0.05$, $N = 15$ cells, ANOVA.

Figure 3. Immunofluorescence of differentiated Htt^{7Q/7Q} and Htt^{140Q/140Q} cells at 15 and 20 days in culture

(a) Confocal images of cultures at 15-days after initiation of differentiation showed immunoreactivity for Beta_{III}-Tubulin (Green) and Nestin (Red) in both Htt^{7Q/7Q} and Htt^{140Q/140Q} cultures in polarized cells bearing long processes. Nuclei stained with Hoechst (Blue). The NS cells were passaged 35 times prior to differentiation. **(b)** Confocal images of cultures at 15 days after initiation of differentiation showed immunoreactivity for GABA (Green). Nuclei stained with Hoechst (Blue). Control reactions omitting the primary antibody and staining with the secondary antibodies are also shown. **(c)** Confocal images of cultures at 20 days after initiation of differentiation showed immunoreactivity for Beta_{III}-Tubulin (Green) and Nestin (Red) in both Htt^{7Q/7Q} and Htt^{140Q/140Q} cultures. Nuclei stained with Hoechst (Blue). NS cells were induced to differentiate as in b and on the same day. HD Htt^{140Q/140Q} cultures showed increased numbers of Hoechst-positive nuclei compared to 15 days (compare to Hoechst staining in “a”), and cells are flatter compared to those in Htt^{7Q/7Q} cultures at 20 days. Sequential images were acquired using the same confocal exposure settings for Htt^{7Q/7Q} and Htt^{140Q/140Q} with a 60X oil objective and merged in Adobe Photoshop (Merged).

Figure 4. Quantitative analysis of neuronal attributes and neural subtypes in differentiated Htt^{140Q/140Q} and Htt^{7Q/7Q} cultures

(a) **Somal area of neurons.** Graph depicts average area of soma for GABA-positive cells with morphology characteristics of neurons (polarized bipolar or multipolar cells) from NS cells at passage 35 (p35) for WT and p31 for HD on differentiation day 5. Reported are mean area $\pm$ SD; * indicates $p < 0.05$, unpaired t-test; $n = 4$ fields; (b) **Neurite length of neurons.** Graph depicts length of longest neurite per cell measured for cells immunolabeled for GABA or for BetaIII-Tubulin from NS cells at passage 35 (p35) for WT and p31 for HD on differentiation day 5. Reported are mean length $\pm$ SD; unpaired t-test showed no significant differences; $n = 4$ fields; (c) **Percent neurons with passage number.** A significant decrease in percent neuron yield occurred in HD cultures compared to WT cultures at passage 35. Graph depicts yield of BetaIII-Tubulin positive cells as a percent of total cells counted by Hoechst-positive nuclei in final cultures on day 20 and 23 differentiated from NS cells at passage 15 (p15) or passage 35 (p35). Reported are mean $\pm$ SD; * indicates $p < 0.05$, unpaired t-test; $n = 5$ fields (> 500 cells counted); (d) **Percent neurons with length of differentiation time (age of culture).** Graph depicts yield of BetaIII-Tubulin positive cells as a percent of total cells counted by Hoechst-positive nuclei in final cultures on day 5 and 20 differentiated from NS cells at passage 35 and plated for differentiation on the same day. Reported are mean $\pm$ SD; * indicates $p < 0.05$, unpaired t-test; $n = 5$ fields (> 500 cells counted); (e) **Total cell number with differentiation time.** Graph depicts total cells per microscopic field determined by counting Hoechst-positive nuclei in final cultures on day 5 and 20. NS cells at passage 35. Reported are mean $\pm$ SD; * indicates $p < 0.05$, paired t-test; $n = 5$ fields (> 500 cells counted); (f) **Total neurons with differentiation time.** Graph depicts total neurons (BetaIII-Tubulin cells) per microscopic field in final cultures on day 5 and 20. NS cells at passage 35. Reported are mean $\pm$ SD.

Figure 5. GFAP-positive cells accumulate in Htt^{140Q/140Q} but not wild-type Htt^{7Q/7Q} cultures with time

(a) Western blot analysis of differentiated cultures (23 days). Blots were probed for Htt then reprobed with indicated antibodies. (b) *Left column*, immunofluorescence of cultures (20 days) stained for GFAP shows that flat cells in wild-type cultures are GFAP-negative and have small nuclei, whereas in HD cultures flat cells are GFAP-positive and also have morphological similarities to astrocytes including large flat nuclei. *Right*, a control reaction was performed where cells were incubated with normal goat serum in place of primary antibody, then incubated with secondary antibody. (c) Graph depicts cell counts of GFAP-positive cells as a percent of total cell number determined counting Hoechst-positive nuclei. Bars report mean percents $\pm$ SD. *indicates $p < 0.005$, $n = 5$ fields (> 500 cells counted), unpaired t-test. (d) Chromatin Immunoprecipitations show similar binding of STAT3 to the GFAP promoter in WT and HD NS cells. Graph shows crossing thresholds for quantitative PCR from $n = 4$ IPs for WT and $n = 3$ IPs for HD, standardized to input DNA. Bars are mean $\pm$ SD. Mock reactions were IPs from WT cells omitting primary antibody ($n = 2$ IPs). Unpaired t-test showed no significant difference in GFAP promoter DNA precipitated with an anti-STAT3 antibody. (e, f) Stimulation of the BDNF pathway in WT and HD NS cells. Graph shows the ratio of densitometry results from western blots for phospho-AKT to total AKT of cells $\pm$ BDNF. Bars are mean $\pm$ SD. Statistical comparisons between treated and untreated in the same cell line were paired t-tests. * $p < 0.005$. Statistical comparisons between WT and HD were unpaired t-tests. * $p < 0.005$ $N = 3$ wells for each condition.

Table 1

Phenotypes of Neural Stem Cells

Htt Genotype	Source	Cholesterol	ROS	Motility	Differentiation
7Q/7Q	E16 mouse brain	-	-	-	-
140Q/140Q	E16 mouse brain	↓	↑	↓	↑ GFAP ⁺ cells
F7Q/7Q	ES cells	-	-	-	NE
F140Q/7Q	ES cells	-	↑	↓	NE
-/-	ES cells	↑	-	↓	NE

NE, not examined