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Expanded CAG repeats in the murine Huntington's disease gene increases neuronal differentiation of embryonic and neural stem cells

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Abstract

Huntington's disease is an uncommon autosomal dominant neurodegenerative disorder caused by expanded polyglutamine repeats. Increased neurogenesis was demonstrated recently in Huntington's disease postmortem samples. In this manuscript, neuronally differentiated embryonic stem cells with expanded CAG repeats in the murine Huntington's disease homologue and neural progenitors isolated from the subventricular zone of an accurate mouse Huntington's disease were examined for increased neurogenesis. Embryonic stem cells with expanded CAG repeats in the murine Huntington's disease homologue were demonstrated to undergo facilitated differentiation first into neural progenitors, then into more mature neurons. Neural progenitor cells isolated from the subventricular zone of a Huntington's disease knock-in animal displayed increased production of neural progenitors and increased neurogenesis. These findings suggested that neuronally differentiating embryonic stem cells with expanded CAG repeats is a reasonable system in to identify factors responsible for increased neurogenesis in Huntington's disease. Expression profiling analysis comparing neuronally differentiating embryonic stem cells with expanded CAG repeats to neuronally differentiating embryonic stem cells without expanded CAG repeats identified transcripts involved in development and transcriptional regulation as factors possibly mediating increased neurogenesis in response to expanded CAG repeats.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by a mid-life onset of a choreoathetotic motor disorder, psychiatric symptoms, and cognitive decline. Pathologically, HD is characterized by preferential loss of medium spiny striatal projection neurons, but is accompanied by widespread neuronal dysfunction and degeneration (Vonsattel and DiFiglia, 1998). The clinical course of HD typically progresses over 10-20 years from a presymptomatic state to complete disability and death. There are no disease altering treatments and symptomatic therapy has limited benefit. HD is caused by an expanded polymorphic CAG repeat which is transcribed into a polyglutamine stretch at the amino terminus of the ~350 kDa huntingtin (htt) protein. Neither the normal function of htt nor the mechanism(s) by which the expanded polyglutamine domain causes HD are clearly defined.

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In the mammalian brain, adult neurogenesis was first demonstrated in 1962, and is now recognized to occur primarily in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) adjacent to the lateral ventricles (Altman, 1962; Ming and Song, 2005). Newborn neurons of the SVZ populate the rostral migratory stream, migrate to the olfactory bulb (OB), differentiate into OB granule or periglomerular neurons, and are thought to play a role in olfactory discrimination. Newborn hippocampal neurons migrate a shorter distance, become dentate gyrus (DG) granule neurons, and may play a role in learning. Although the mechanisms controlling and role of adult neurogenesis are unknown, newborn OB and hippocampal neurons functionally integrate into the adult CNS (Ming and Song, 2005). In post-mortem HD brain, Curtis et al. describe increased SVZ cellular proliferation and production of striatal neurons. The SVZ overlying the basal ganglia, the area of the brain primarily involved in HD, is 2.8 fold thicker than control (Curtis et al., 2005b). Increased SVZ thickness is attributed to an increase in the number of proliferating cells, and overall cell proliferation was shown to have a positive correlation with increasing HD pathologic grade and increasing CAG repeat size (Curtis et al., 2003; Curtis et al., 2005b). Increased cellular proliferation was primarily the result of increased neural stem cells, but a 2.6 fold increase in the number of new neurons, identified by co-expression of PCNA and β -tubulin, was also identified (Curtis et al., 2005a). These studies suggest that increased proliferation in the SVZ occurs in response to pathologic processes occurring in the HD brain. Although it has been demonstrated that newborn SVZ neurons can functionally integrate into the mature striatum in response to injury (Arvidsson et al., 2002; Parent et al., 2002b), in HD, the mechanisms underlying increased cellular proliferation, increased neural stem cells, and increased neurogenesis are unknown.

As a step toward understanding the production of new neurons in HD we developed two stem cell models of HD neurogenesis (Lorincz, 2006). In the first model, under conditions that favor neuronal differentiation, embryonic stem cells (ESC) with expanded CAG repeats in the murine HD homologue transitioned from ESC to dividing neural progenitors and then to a neuronal phenotype more rapidly and in higher number than control ESC without expanded CAG repeats. In the second model, during neuronally differentiating murine *Hdh* CAG150 SVZ neural stem cells exhibited facilitated production of dividing neural progenitors and increased neurogenesis compared to control. Comparison of expression profiles from neuronally differentiating murine ESC with and without expanded CAG repeats identified transcripts involved in development and transcriptional regulation as likely mediators of increased neurogenesis. Results obtained from ESC with expanded CAG repeats and *Hdh* CAG150 SVZ neural stem cells suggest that the proximate cause of enhanced neurogenesis is the expanded CAG repeat in the murine *Hdh* gene.

Results

Expanded CAG repeats do not alter expression of lineage markers and do not alter cell cycle parameters of undifferentiated embryonic stem cells

Semiquantitative RT-PCR was performed to determine whether expanded CAG repeats alter expression of lineage fate markers in undifferentiated ESC (Figure 1A). Following standard propagation, ESC with the normal number of CAG repeats in the murine HD homologue (*Hdh* CAG7) and ESC with 150 CAG repeats in the murine HD homologue (*Hdh* CAG150) displayed robust expression of the embryonic stem cell markers Oct-4 and Nanog (Chambers et al., 2003; Nichols et al., 1998) with little expression of the neuronal precursor marker Nestin, and no detectable expression of the neuronal marker synaptophysin. There was no differential expression of the examined markers.

In order to investigate growth kinetics of *Hdh* CAG7 and *Hdh* CAG150 ESC, cell cycle analysis was performed (Figure 1B). ESC were propagated utilizing a standardized passage protocol

prior to neuronal differentiation. Synchronized cultures of *Hdh* CAG7 and *Hdh* CAG150 ESC were analyzed for cell number and cell cycle parameters on day 6 post-thaw. There was no significant difference in the number of cells per well between *Hdh* CAG7 and *Hdh* CAG150 (mean of 5 replicate wells $\pm$ SE, $24.46 \pm 1.03 \times 10^5$ cells/well (9.6cm^2) and $26.32 \pm 0.67 \times 10^5$ cells/well respectively, $p=0.16$). The proportion of cells in the cell cycle phase G1, or S and G2/M, was not significantly different between *Hdh* CAG7 and *Hdh* CAG150 ESC (Figure 1B and Table 1).

Expanded CAG Repeat Increased Neurogenesis

In order to investigate neurogenesis in HD, we developed a system in which ESC were differentiated in monolayer under serum free conditions producing highly neuronally enriched cultures (Lorincz, 2006). Rapidly dividing *Hdh* CAG7, *Hdh* CAG77, and *Hdh* CAG150 ESC were seeded at 0.8×10^5 cells/cm² in serum free N2:B27 medium, allowed to differentiate, and were analyzed at sequential time points by immunohistochemistry (IHC) for expression of a marker of dividing neural precursors, Sox3 (Wang et al., 2006), and expression of the neuronal marker, neuron specific β -tubulin (Menezes and Luskin, 1994) (Figure 2). Following 4 days of neuronal differentiation (Figure 2A-D) the cultures were characterized by small cellular clusters with an abundance of Sox3 expressing cells. Within cellular clusters there were also cells that expressed Sox3 and neuron specific β -tubulin (Sox3+/ β -tubulin+) and few cells that expressed only neuron specific β -tubulin (Sox3-/ β -tubulin+) (Figure 2A-D). Following 4 days of neuronal differentiation, obvious distinctions in the expression of Sox3 and neuron specific β -tubulin between *Hdh* CAG7 and *Hdh* CAG150 cells were not readily apparent. Following seven days of neuronal differentiation distinctions between neuronally differentiated ESC with expanded CAG repeats and those without an expanded CAG allele became visually apparent (Figure 2E-M). By day seven, the cultures were characterized by enlarging Sox3+ cellular clusters with increasing numbers of cells stained with neuron specific β -tubulin. Cellular clusters at this stage often took on a rosette appearance (Figure 2H, 2J, and 2M) with Sox3+ or Sox3+/ β -tubulin+ cells at their center with surrounding neuron specific β -tubulin+ cells, a morphology previously described as a sign of ESC to neuronal conversion in adherent cultures (Ying et al., 2003). After seven days of neuronal differentiation, it appeared that there were more Sox3+ cells, and more neuron specific β -tubulin+ cells in *Hdh* CAG77 and *Hdh* CAG 150 cultures. By day 11 the cultures were characterized by cells expressing neuron specific β -tubulin with more elaborate processes than those observed on day 4 or 7, but continued to contain cells expressing Sox3 (Figure 2N-Q). As the cultures become more mature, the distinctions between cells with or without expanded CAG repeats persisted (Figure 2N-Q). These data suggest that ESC with expanded CAG repeats transition from ESC to dividing neural progenitors, and then to neurons, more rapidly and in greater number than ESC without expanded CAG repeats.

Fluorescence Activated Cell Sorting (FACS) Analysis of Neuronal Differentiation Supports *Hdh* CAG150 ESC increased Neurogenesis

In order to test the hypothesis that ESC with expanded CAG repeats differentiate more rapidly into neural progenitors and subsequently into neurons, a FACS method to sequentially quantitate the constituents of neuronally differentiating ESC cultures was developed. A maturation progression became apparent (Figure 3A), in which ESC first differentiated into a population of cells largely expressing Sox3 but not neuron specific β -tubulin (Sox3+/ β -tubulin-), dividing neural progenitors, these cells are represented in the left upper quadrant in the FACS scatter plot. The next differentiation stage was characterized by a population of cells that co-expressed Sox3 and neuron specific β -tubulin (Sox3+/ β -tubulin+), immature neurons, these cells are represented in the right upper quadrant of the FACS scatter plot. The final differentiation stage consisted of cells that did not express Sox 3 but expressed neuron specific β -tubulin (Sox3-/ β -tubulin +), neurons,

these cells are represented in the lower right quadrant of the FACS scatter plot. Following four, seven, and 11 days of neuronal differentiation significant differences were identified between the *Hdh* CAG7 and *Hdh* CAG150 cultures (Figure 3B-D). At all timepoints there were significantly more *Hdh* CAG150 cells than *Hdh* CAG7 cells that expressed Sox3, expressed a combination of Sox3 and neuron specific β -tubulin, or expressed neuron specific β -tubulin. That a larger number of *Hdh* CAG150 cells expressed a marker of dividing neural progenitors, Sox3, early in differentiation suggests that *Hdh* CAG150 ESC underwent a more rapid transition from an ESC to neuronal precursor. As the cultures became mature, there were more neuron specific β -tubulin+ *Hdh* CAG150 cells than neuron specific β -tubulin + *Hdh* CAG7 cells, suggesting that neural progenitors (Sox3+ cells) with expanded CAG repeats had subsequently differentiated into neurons.

***Hdh* CAG150 Lineage Marker Expression Supports Facilitated Differentiation of ESC to Neuronal Precursor and Neuronal Lineages**

RT-PCR was employed to analyze ESC, neuronal precursor, neuronal, oligodendrocyte, and astrocyte marker expression following 4, 7, and 11 days of neuronal differentiation (Figure 4). Because morphological distinctions were most prominent between *Hdh* CAG7 and *Hdh* CAG150 cells following neuronal differentiation, these lines were used for further study. Expression of ESC markers, Oct-4 and Nanog (Chambers et al., 2003; Nichols et al., 1998), was greater following 4, 7, and 11 days of neuronal differentiation in *Hdh* CAG7 cells than *Hdh* CAG150 cells. Although there is persistent expression of Oct-4 and Nanog during differentiation, their expression is markedly reduced from that observed in undifferentiated ESC (Figure 1). At day 4, expression of the neural precursor markers, Musashi, Nestin, and Pax6 (Campbell and Gotz, 2002; Lee and Cleveland, 1996; Sakakibara et al., 1996) was greater in *Hdh* CAG150 cells compared to *Hdh* CAG7. Expression of Musashi remained greater in the *Hdh* CAG150 cells on day 7 compared to *Hdh* CAG7, but by day 11 there was little difference in Musashi expression. The expression patterns of Nestin and Pax6 were similar to each other. Following 4 days of neuronal differentiation expression of Nestin and Pax6 was greater in *Hdh* CAG150 compared to *Hdh* CAG7 cells. At day 7, and more evident at day 11, expression of Nestin and Pax6 was higher in *Hdh* CAG7 compared to *Hdh* CAG150 cells. A possible interpretation of these data is that *Hdh* CAG150 cells undergo a more rapid transition from ESC to neural precursor, reflected in increased day 4 neuronal precursor marker expression. Relatively decreased expression of Nestin and Pax6 on day 7 and 11 in *Hdh* CAG150 could be consistent with *Hdh* CAG150 cells differentiating into a more mature phenotype, as was suggested by IHC (Figure 2).

Neuronal marker expression was examined to determine if the pattern supported the notion that *Hdh* CAG150 cells were transitioning to a neuronal phenotype more rapidly than *Hdh* CAG7. NeuroD1, a basic helix-loop-helix (bHLH) transcription factor expressed in neural progenitors destined to become neurons, and in mature neuronal populations (Miyata et al., 1999), displayed increased expression on day 4, 7, and 11 in *Hdh* CAG150 compared to *Hdh* CAG7. Synaptophysin, a protein important in synaptic transmission (Sudhof et al., 1987), appeared earlier and was more robust in *Hdh* CAG150 compared to *Hdh* CAG7. Emx1, a homeobox containing transcription factor expressed in both dividing and post mitotic neurons of the dorsal telencephalon (Gulisano et al., 1996; Puelles et al., 2000), had greater expression in the *Hdh* CAG150 cells on day 4 and 7. By day 11 Emx1 expression was similar in *Hdh* CAG7 and *Hdh* CAG150 cells.

Not all neuronal markers are expressed earlier or at greater levels in *Hdh* CAG150 cells compared to *Hdh* CAG7 cells. Islet1 is a LIM homeodomain protein expressed in developing striatal projection neurons in the lateral ganglionic eminence (LGE) (Stenman et al., 2003). On day 4 the expression of Islet1 was similar between *Hdh* CAG7 and *Hdh* CAG150 cells. As

differentiation proceeded, *Islet1* expression in the *Hdh* CAG150 cells decreased, whereas in *Hdh* CAG7 cells *Islet1* expression remained elevated at both day 7 and 11 compared to *Hdh* CAG150 cells. *Nkx2.1*, a marker of the ventral telencephalon (Sussel et al., 1999), displayed little expression at day 4 in either line, but greater expression in *Hdh* CAG7 cells at day 7 and 11 compared to the *Hdh* CAG150 cells. *Hoxc6*, a homeobox protein expressed in the developing spinal cord (Shimeld et al., 1993), was expressed in *Hdh* CAG7 cultures on day 7 and at higher levels on day 11, but was not detectable in *Hdh* CAG150 cells.

The differentiation protocol employed resulted in little expression of the astrocytic, glial fibrillary acidic protein (GFAP), or the oligodendrocytic markers, oligodendrocyte lineage transcription factor 2 (*Olig2*) or myelin associated glycoprotein (MAG) (Eng, 1985; Quarles and Trapp, 1984; Zhou et al., 2000). Expression of the *Hdh* gene was found to be greater in *Hdh* CAG7 compared to *Hdh* CAG150 on day 4 and to a lesser degree on day 7. Amplification across the CAG repeat was also routinely performed to verify allele size and expression of the expanded allele (data not shown).

The expression pattern of ESC and neuronal precursor markers suggests that *Hdh* CAG150 ESC differentiate more rapidly into neuronal precursors than *Hdh* CAG7 cells. Interpretation of neuronal marker expression is more complex, suggesting that generic neuronal markers, synaptophysin and *NeuroD1*, were expressed earlier and at a higher level in *Hdh* CAG150 compared to *Hdh* CAG7. The expression pattern of neuronal sub-type markers, *Emx1*, *Islet1*, *Nkx2.1*, and *Hoxc6*, suggests that the effect of expanded CAG repeats on facilitated neurogenesis may be cell type specific.

Neuronal Differentiation of Wild Type and *Hdh* CAG150 SVZ Neural Stem Cells

In order to investigate whether results obtained from ESC with expanded CAG repeats could be extended to SVZ neural stem cells with expanded CAG repeats, the neuronal differentiation potential of neural stem cells isolated from the SVZ of postnatal day 20 wild type (CAG7) and *Hdh* CAG150 knock-in mice (Heng et al., 2007; Lin et al., 2001) was examined. The goal of these studies was to examine the neuronal differentiation potential of SVZ neural progenitors. Undifferentiated SVZ neural progenitors were isolated as previously described (Rietze and Reynolds, 2006). To minimize differences between neuronal differentiation of ESC and SVZ neural progenitors, the protocol of Reynolds and Weiss was modified so that undifferentiated SVZ stem cells formed neurospheres (NS), and these were differentiated in the same medium used to neuronally differentiate ESC, N2:B27. Six days following isolation, isolated SVZ cells formed nonadherent spherical cellular clusters previously described as NS (~100-200um in diameter). Individual NS were replated onto an adhesive surface, differentiated for two or six days, and examined by IHC for expression of *Sox3*, and neuron specific β -tubulin (Figure 5A-D). The expression of *Sox3* and neuron specific β -tubulin was scored without knowledge of genotype and assigned to the indicated groups. Following two and six days of differentiation, the *Hdh* CAG150 SVZ cells produced more colonies with high or moderate *Sox3* expression than WT (day 2 $\chi^2=7.48$, $p=0.024$; day 6 $\chi^2=11.13$, $p=0.004$) (Figure 5E). Following two and six days of differentiation *Hdh* CAG150 SVZ cells produced more colonies with high or moderate neuron specific β -tubulin expression (day 2 $\chi^2=3.90$, $p=0.048$; day 6 $\chi^2=10.15$, $p=0.001$) (Figure 5F). These results suggested that *Hdh* CAG150 SVZ stem cells had increased production of neural progenitors and increased neurogenesis compared to WT, in agreement with results using ESC with expanded CAG repeats.

Cell Death and Cell Cycle Parameters of Neuronally Differentiated *Hdh* CAG7 and *Hdh* CAG150 Cells

We investigated the role of cell death during neuronal differentiation of ESC by propidium iodide (PI) incorporation (Figure 6). Following 4 and 6 days of neuronal differentiation there

was no difference in PI incorporation between neuronally differentiated *Hdh* CAG7 and *Hdh* CAG150 ESC. On days 8 and 10 of neuronal differentiation significantly more *Hdh* CAG150 cells incorporated PI compared to the control *Hdh* CAG7 cells. The cell death analysis did not support a scenario in which either increased *Hdh* CAG7 cell death, or reduced *Hdh* CAG150 cell death played a major role in the ESC neuronal differentiation results.

To determine if alterations in growth kinetics were playing a role in increased neuronal differentiation of *Hdh* CAG150 cell cycle parameters were examined (Table 2). On day 4 there was a higher proportion of *Hdh* CAG7 cells in cell cycle phase S and G2/M compared to *Hdh* CAG150. On days 4, 6, and 10 there was a significantly larger proportion of *Hdh* CAG150 cells in the G1 cell cycle phase compared to *Hdh* CAG7 cells. That there were more *Hdh* CAG150 in G1 was consistent with IHC and FACS observations of more mature (Sox3-/neuron specific β -tubulin+) *Hdh* CAG150 cells and suggest that these neurons may be post-mitotic. The larger proportion of *Hdh* CAG7 cells in cell cycle phases S and G2/M at day 4 may be secondary to a larger proportion of *Hdh* CAG7 cells maintaining an undifferentiated state, a possibility supported by observations of higher Oct4 and Nanog expression in *Hdh* CAG7 compared to *Hdh* CAG150 cells.

Expression Profiling Identifies Potential Factors Responsible for Expanded CAG Repeat Facilitated Neurogenesis

Expression profiles of CAG7 and CAG150 ESC were compared following four and six days of neuronal differentiation. Expression profiling was performed through the NIH Neuroscience Microarray Consortium at the Translational Genomics Research Institute (Phoenix, AZ) according to the Affymetrix guidelines utilizing Affymetrix Mouse Genome 430 2.0 arrays (GeneChip® Expression Analysis and Data Analysis Fundamentals). For each cell line and timepoint triplicate samples were analyzed. Replicate correlation was performed within each group of three samples and R values greater than 0.93 were obtained, indicating minimal inter-sample variability. One-way Welch t-tests with Benjamini–Hochberg false discovery rate correction for multiple testing were used to identify transcripts with a greater than 2-fold change in expression at a p-value ≤ 0.05 . The biological roles of the transcripts with significantly differing expression between *Hdh* CAG7 and *Hdh* CAG150 were evaluated by grouping transcripts into gene ontology (GO) categories (Ashburner et al., 2000). The goal of this analysis was to identify unbiased biologic pathways involved in expanded CAG repeat facilitated neurogenesis. Statistical significance was determined by comparing the number of genes observed in a given GO category compared to the expected number. Table 3 displays biological and molecular GO categories having significant gene overrepresentation comparing *Hdh* CAG7 and *Hdh* CAG150 after 4 and 6 days of neuronal differentiation. Biological processes involved with development, nervous system development, migration, and morphogenesis displayed the highest degree of significance. Molecular functions involved in transcriptional regulation, the cytoskeleton, growth factor activity, and receptor binding displayed the highest degree of significance.

To identify individual transcripts that may mediate increased *Hdh* CAG150 neurogenesis, the expression profiling data was analyzed to identify transcripts having a greater than 2-fold expression change, with a p-value ≤ 0.05 following one-way Welch t-test with Benjamini–Hochberg false discovery rate correction for multiple testing. The transcripts were further filtered to remove transcripts with expression levels in the noise range. Table 4 contains all transcripts fulfilling the criteria with increased expression in *Hdh* CAG150 relative to *Hdh* CAG7. On day 4, 21 genes were identified, of which 38% (8/21) have known roles in transcriptional regulation, 33% have unknown function, 10% (2/21) were involved in Wnt/catenin signaling, 5% (1/21) were involved in axonal growth, endocytic trafficking, amino acid transport, or as a trans-membrane receptors involved in cerebral cortex patterning. On day

6, 20 genes were identified, of which 20% (5/20) had known roles in transcriptional regulation, 35% (7/20) had an unknown or uncertain function, 15% (3/20) were cytoskeletal, 15% (3/20) were involved in signal transduction, 5% (1/20) were involved in endocytic trafficking, or were RNA binding proteins. On day 4, 38% (8/21) and on day 6, 35% (7/20) of the transcripts with significantly increased expression in CAG150 have known roles in neuronal differentiation. Of all the transcripts with increased expression, four transcripts were found to have increased expression following 4 and 6 days of neuronal differentiation. Of these four genes, 75% (3/4) were involved in transcriptional regulation, and 25% (1/4) were involved in endocytic trafficking. Three of the four genes that had differential expression on day 4 and 6 have known roles in transcriptional regulation and neuronal differentiation, Pax3, Meis1, and Otx2.

The expression pattern of a subset of genes identified to have differential expression was determined by RT-PCR analysis and found to be consistent with expression profile analysis (Figure 7). The expression patterns of Otx2 and Zic1 were similar with increased expression in *Hdh* CAG150 at day 4 more so than at day 7, and no expression differences by day 11. *Hdh* CAG150 Hes5 expression was greater than *Hdh* CAG7 at day 4, no expression differences were evident at day 7, and increased Hes5 expression was observed in *Hdh* CAG7 at day 11. The expression pattern of Otx2, Hes5, and Zic1 agree with results obtained from expression profiling and suggest that their influence may be most important early in neuronal differentiation.

The results of expression profiling demonstrated increased expression of transcripts involved in neuronal differentiation, and its transcriptional control, in neuronally differentiated *Hdh* CAG150 cells compared to *Hdh* CAG7 cells; these results are consistent with IHC, FACS, and RT-PCR results. Expression profiling has identified a relatively small group of candidates that may mediate increased differentiation from ESC to NPC and from NPC to neuron in expanded CAG repeat cells.

Discussion

In the present study, results from neuronal differentiation of two independent expanded CAG repeat ESC lines and from SVZ neuronal stem cells with 150 CAG repeats support the hypothesis that expanded CAG repeats in the murine *Hdh* gene increase neuronal differentiation. Utilizing IHC, ESC with either 77 or 150 CAG repeats appear to have increased neuronal differentiation. FACS analysis was used to confirm IHC results and demonstrated increased Sox3 and neuron specific β -tubulin expressing cells in neuronally differentiated *Hdh* CAG150 cells compared to *Hdh* CAG7 cells following 4, 7, and 11 days of neuronal differentiation. The finding of increased Sox3 expressing cells following 4 days of neuronal differentiation in *Hdh* CAG150 cells compared to *Hdh* CAG7 cells, in combination with increased expression of neuronal precursor markers suggests that ESC with expanded CAG repeats differentiate more rapidly into neuronal precursors. The finding of increased neuron specific β -tubulin expressing cells, in combination with earlier and higher expression of neuronal markers, in neuronally differentiated *Hdh* CAG150 cells, compared to *Hdh* CAG7 cells, suggests that expanded CAG repeats may increase neuronal precursor to neuron differentiation. It remains a possibility that the main effect of expanded CAG repeats is to facilitate transition from ESC or neural stem cell to neuronal precursor. Greater numbers of neuronally differentiated SVZ *Hdh* CAG150 neural stem cells expressing Sox3 or neuron specific β -tubulin, compared to WT, support findings of increased neuronal differentiation in *Hdh* CAG150 ESC. Overall, the data presented are in agreement with findings demonstrating increased neurogenesis in human HD (Curtis et al., 2003; Curtis et al., 2005a; Curtis et al., 2005b) and suggest that ESC with expanded CAG repeats may be useful to identify factors responsible for increased neurogenesis in HD.

Expression profiling was employed to identify unbiased biological pathways involved in expanded CAG repeat increased neuronal differentiation. GO analysis identified development, nervous system development, migration, transcriptional regulation, morphogenesis, the cytoskeleton, growth factor activity, and receptor binding as processes mediating increased neuronal differentiation of *Hdh* CAG150 ESCs. Subsequent analysis identified 37 transcripts with significantly increased expression in neuronally differentiated *Hdh* CAG150 cells, compared to *Hdh* CAG7. This group of 37 is highly enriched for transcripts involved in development and transcriptional regulation.

Neither the normal function of htt, nor the mechanism(s) by which the expanded polyQ domain causes HD are clearly defined. Htt may normally act as a WW domain scaffolding protein involved in intracellular signaling, and evidence suggests that htt may be involved in embryonic development, neurogenesis, apoptosis, axonal transport, cholesterol metabolism, transcription, and neurotransmitter signaling (Borrell-Pages et al., 2006; Cattaneo et al., 2005). In animal models and *in vitro*, mutant htt has been demonstrated to adversely affect a wide array of cellular processes including transcription, axonal transport, autophagy, cellular energy homeostasis, trophic support, and processing of excitatory neurotransmitter signaling. The relative order, interaction, and importance of these processes is uncertain, but a growing body of evidence supports transcriptional dysregulation as a critical early mediator of polyQ-mediated pathogenesis.

Replication of disease features by nuclear location of mutant htt (mhtt), but not aggregate formation, led to the hypothesis that mhtt may abnormally interact with transcription factors, alter transcription, and result in downstream disease features (Cha, 2000; Luthi-Carter et al., 2000; Nucifora et al., 2001; Perutz et al., 1994). The polyQ domain is a common transcription factor motif and suggested htt may be involved in regulation of transcription through protein-protein interactions (Gerber et al., 1994). Mhtt has been shown to interact with a variety of transcription factors, some in a polyQ dependant manner, including nuclear co-repressor (N-CoR), C-terminal-binding protein transcriptional co-repressor (CtBP), p53, CA150, p300, p300/CBP-associated factor (P/CAF), TAFIII130, PCG-1 α , REST/NRSF, TBP, SP-1, mSin3a, Krip-1, and CBP (Boutell et al., 1999; Cui et al., 2006; Kegel et al., 2002; Li et al., 2002; Nucifora et al., 2001; Steffan et al., 2000; Zuccato et al., 2003). The net outcome is a mixture of increased and decreased gene expression, as is exemplified in gene expression profile analysis (Hodges et al., 2006; Kuhn et al., 2007; Luthi-Carter et al., 2000). The central role of transcription factors in HD pathogenesis and CNS development (Molyneaux et al., 2007; Rallu et al., 2002), in combination with prominent differential expression of transcription factors in HD ESC suggests that transcriptional dysregulation caused by expanded CAG repeats may result in differential expression of transcription factors which then mediate increased neuronal differentiation.

Although human studies have consistently found evidence for increased proliferation of neuronal stem cells and increased neurogenesis in HD (Curtis et al., 2003; Curtis et al., 2005a; Curtis et al., 2005b), studies investigating neurogenesis in HD models have yielded inconsistent results. Neurogenesis in HD animal models has been primarily investigated in transgenic mice created by insertion of human htt exon 1, under the control of the human htt promoter, with 139-148 CAG repeats (R6/2), randomly inserted into the mouse genome. The R6/2 mouse develops an early onset neurodegenerative disorder, with motor and pathologic features 5-6 weeks and death by 16 weeks (Carter et al., 1999; Hurlbert et al., 1999; Mangiarini et al., 1996). Utilizing the R6/2 mouse, investigations *in vivo* found no difference in baseline hippocampal DG or SVZ neurogenesis (Gil et al., 2005; Phillips et al., 2005), decreased DG neurogenesis (Gil et al., 2005), or increased newborn cells and neurons in R6/2 SVZ (Jin et al., 2005). These studies investigating neurogenesis in HD highlight the possibility that some

animal models may not fully replicate this aspect of human disease, and suggests that further investigations into neurogenesis in HD are warranted.

Utilizing the neurosphere assay, R6/2 neural stem cells *in vitro* demonstrated increased self-renewal capacity compared to WT, suggesting increased potential of R6/2 neural stem cells to differentiate into neural progenitors, but no differences in their potential to differentiate in astrocytes, oligodendrocytes, or neurons (Batista et al., 2006). Our NS results utilizing *Hdh* CAG150 neural stem cells are in agreement with the results of increased neural progenitors found by Batista et al., but differ with respect to their differentiation potential. Whereas no difference in neuron production was found between R6/2 and WT by Batista et al., increased neurogenesis in *Hdh* CAG150 neural stem cells compared to WT was identified. Differences in neuronal differentiation protocols and the context of the expanded CAG repeat domain likely account for the observed differences. The low neuronal differentiation percentage described by Batista et al. suggests that their conditions were less conducive to neuronal differentiation than those employed in our studies. The aggressive R6/2 phenotype may produce an environment less conducive for production of neural stem cells capable of neuronal differentiation than the *Hdh* CAG150 mouse. In contrast to the R6/2 mouse, *Hdh* CAG150 mice, ESC, and neural stem cells were created by insertion of an expanded CAG repeat into the murine *Hdh* gene and the *Hdh* CAG150 mice display a striatal specific pathology, and develop a late onset motor phenotype by ~70 weeks (Heng et al., 2007; Lin et al., 2001).

It is likely that the inciting factors and outcomes of neurogenesis are modified through an interaction between the environment and genotype. The relative contribution of environmental and genetic factors on normal adult neurogenesis and neurogenesis occurring in response to acute and chronic injury are not known. In some cases, such as environmental enrichment, physical activity, stroke, or seizure (Arvidsson et al., 2002; Kempermann et al., 2002; Parent et al., 2002a; Parent et al., 2002b), the environment may be the primary factor driving increased neurogenesis, whereas in other situations genetic differences may be the primary mediator (Kempermann and Gage, 2002; Schauwecker, 2006). In HD where development appears unaffected, factors governing neurogenesis may be able to maintain normal homeostasis until a point is reached when increased production of neurons is favored. Our results, and the results of Batista et al. suggest that in mice a postnatal potential for increased production of neuronal precursors is present, but that potential may be held in check by normal mechanisms until a threshold is crossed resulting in increased neurogenesis (Batista et al., 2006). Although our results are most consistent with the interpretation that the expanded CAG repeat in the murine HD gene is the cause of increased neuronal differentiation, neuronally differentiating stem cells create a local environment that likely plays an important downstream role in determining their neuronal differentiation potential.

Our results support the hypothesis that ESC, in addition to having tremendous therapeutic potential, may also have an important role in modeling disease. Our results suggest that neuronally differentiated ESC may be able to model neurogenesis in HD, and be used to identify factors responsible for increased neurogenesis in HD. Previous studies indicate that newborn SVZ neurons are able to differentiate into mature neurons of the type lost in HD, make functional connections in the basal ganglion, and slow disease progression in an HD animal models (Arvidsson et al., 2002; Cho et al., 2007; Jin et al., 2005; Parent et al., 2002b). If an understanding of the factors responsible for injury related adult neurogenesis could be attained, augmentation of the process may be able to forestall disease onset or delay disease progression and could have tremendous therapeutic potential for HD and other neurodegenerative diseases.

Methods

ESC propagation and neuronal differentiation

To minimize difference in propagation characteristics between the parental ESC line, *Hdh* CAG7, and the ESC lines with expanded CAG repeats inserted into the murine Huntington's disease gene (*Hdh*), as well as to minimize differences between individual experiments, ESC propagation prior to neuronal differentiation was standardized. ESC were thawed, and maintained by routine protocol, but passage number and density was kept constant between experiments. Prior to initiation of neuronal differentiation ESC were passaged four times. The day following thawing, at 3 days, and at 5 days post-thaw ESC were passaged at a density of 1.6×10^6 cells per 25cm^2 flask. On day 7 post-thaw, the day prior to initiation of neuronal differentiation, ESC were seeded at 4×10^6 cells per 25cm^2 flask and grown overnight. The following day ESC were removed from T25 flasks with enzyme-free cell dissociation buffer (Invitrogen) plus 1mM EDTA utilizing a 30-40 minute incubation at 37°C , which produced a single cell suspension following brief trituration. Disassociation buffer was inactivated by addition of a 2X volume of N2:B27 containing 10% knock-out serum replacement (Invitrogen). For neuronal differentiation ESC were seeded at 0.8×10^4 cells/ cm^2 into 6 well tissue culture dishes in N2:B27 medium. N2:B27 medium consists of equal proportions of neurobasal medium supplemented with B27 supplement and DMEM/F12 supplemented with N2 supplement, with 33.5 μL bovine serum albumin fraction V (Invitrogen; cat. no. 15260-037) and 1mL 100 mM pyruvate per 100mL. During neuronal differentiation, medium is completely removed and replaced on day 3, then every other day thereafter.

Immunohistochemistry

ESC slated to undergo immunohistochemistry (IHC) were neuronally differentiated on gelatin treated tissue-culture treated coverslips (Nunc). At each differentiation timepoint, cells were fixed with 3.7% formaldehyde in PBS for 10 minutes at RT, then stored in PBS at 4°C until processed. For staining, cells were permeabilized with 0.2% Triton X-100 in $1 \times \text{PBS}$ for 5 min, then blocked with 10% normal goat serum, 0.05% Triton X-100, and 1% BSA in $1 \times \text{PBS}$ for 1 h. Cells were incubated overnight with primary antibodies, washed with $1 \times \text{PBS}$, then incubated with secondary antibodies for 1 h at RT. Primary antibodies used were TUJ1 (Neuronal Class III β -Tubulin, mouse (1:2000) (Covance), and Sox3 (1:5000) (rabbit, courtesy of Mike Klymkowski). Secondary antibodies used were Alexa Fluor 488-conjugated anti-mouse IgG (1:800) and Alexa Fluor 594-conjugated anti-rabbit IgG (1:800) (Invitrogen). Cells were then washed with $1 \times \text{PBS}$ and stained for 2 min with 5 $\mu\text{g}/\text{mL}$ bisBenzimide (Hoechst 33258, Sigma). Coverslips were mounted onto slides with ProLong Gold (Invitrogen), and examined with an Olympus BX-51 microscope. Images were captured with an Olympus DP70 camera and Olympus DP controller software. Image postprocessing was performed in using Adobe Photoshop CS.

FACS analysis

At each differentiation timepoint, cells were harvested using enzyme-free cell dissociation buffer (Invitrogen) plus 1mM EDTA. Cells were triturated to produce a single-cell suspension, resuspended in growth media, and an aliquot was counted with a Z1 Coulter Counter (Beckman Coulter) according to manufacturer's protocol. $0.5\text{--}2 \times 10^6$ cells from each replicate (5 replicates per experimental condition) were immunostained in suspension in 1.5 mL tubes for FACS analysis. All subsequent incubations were performed on ice, or at 4°C . Cells were spun down and resuspended in $1 \times \text{PBS}$, then fixed for 10 min with ice-cold 3.7% formaldehyde in $1 \times \text{PBS}$. Cells were pelleted ($2000 \times g$ for 5 min) and resuspended in 0.1% saponin (Sigma) in $1 \times \text{PBS}$ for 10 min. Cells were then pelleted ($2000 \times g$ for 5 min), incubated with blocking buffer (10% normal goat serum, 0.1% saponin, 1% BSA, and 0.1% sodium azide in $1 \times \text{PBS}$) for one hour, then incubated overnight with primary antibodies [mouse TUJ1(1:2000) (Covance), and

rabbit Sox3 (1:5000)]. After washing with $1 \times$ PBS or wash buffer (3% goat serum, 0.1% saponin, and 0.1% sodium azide in $1 \times$ PBS), cells were incubated with secondary antibodies (Alexa Fluor 633-conjugated anti-mouse IgG (1:1200) and Alexa Fluor 488-conjugated anti-rabbit IgG, (1:1200), Invitrogen) for 30 min. Cells were then washed twice in $1 \times$ PBS, and resuspended in 0.5 mL $1 \times$ PBS in 12×75 mm tubes for analysis with a BD Biosciences FACS DiVa cell sorter (Becton Dickinson). Post processing data analysis was performed using WEASEL (Walter and Eliza Hall Institute of Medical Research) version 2.3 software. To obtain the total number of cells expressing either Sox3 or neuron specific β -tubulin the % gated positive is multiplied by the total number of cells present in each well. Means of five independent replicates performed in parallel were compared using the independent-samples t-test (SPSS14). Because of small sample size statistical significance was confirmed by comparing the groups utilizing the Mann-Whitney U test (SPSS14).

For cell cycle analysis, $\sim 5 \times 10^5$ of the fixed and permeabilized cells from each replicate were removed and resuspended in wash buffer containing 5 μ g/mL bisBenzimide. Cells were incubated for 2 min at RT, centrifuged ($2000 \times g$ for 5 min), and resuspended in 0.4 mL $1 \times$ PBS in 12×75 mm tubes; samples were kept on ice or at 4°C until FACS analysis (within 24 hours). The proportion of cells in each phase of the cell cycle was performed on a BD Biosciences FACS DiVa cell sorter (Becton Dickinson). Post processing data analysis was performed using WEASEL version 2.3 software. Statistical analysis was performed on raw data from independent experiments in five independent samples from *Hdh* CAG7 and *Hdh* CAG150 cell lines at each time point using independent samples t-test and Mann-Whitney U test (SPSS14).

For determination of viability of harvested cells, $1\text{--}2 \times 10^5$ cells from each replicate were removed before fixation, resuspended in 0.5 mL growth media in 12×75 mm tubes, and incubated on ice. 2.5 μ L propidium iodide (250 μ g/mL, Cell Technology Inc.) was added to each sample 10–20 minutes before FACS analysis to discriminate between live and dead cells. Statistical analysis was performed on raw data in five independent samples from *Hdh* CAG7 and *Hdh* CAG150 cell lines at each time point using independent samples t-test and Mann-Whitney U test (SPSS14). To compare results between independent experiments, results were converted to a proportion by dividing the PI positive *Hdh* CAG150 cells by the PI positive *Hdh* CAG7 cells.

RNA isolation and RT-PCR

At each differentiation time point, ES cells were lysed and homogenized with TRIzol Reagent (Invitrogen), and frozen. Total RNA was isolated from samples according to manufacturer's instructions. For semiquantitative RT-PCR, 2 μ g RNA was treated with DNaseI (Sigma), and RT reactions were performed using oligo(dT)12–18 primer (Invitrogen) and Powerscript reverse transcriptase (Clontech). The expression level of the resulting cDNAs was analyzed by semiquantitative PCR, using the primers and conditions detailed in Table 5. PCR products were visualized on a 1.5–2% agarose/TBE gel, and images were captured using a ChemiDoc XRS system (BioRad).

SVZ neural stem cell isolation and neuronal differentiation

Neural stem cell isolation and neuronal differentiation was largely carried out as described previously (Rietze and Reynolds, 2006). P20 pups, previously genotyped to identify the presence or absence of an expanded CAG repeat allele, were asphyxiated with CO_2 . This protocol was approved by The University of Michigan Committee on the Use and care of animals. The brains were rapidly dissected and placed into place ice cold Opti-MEM. A sterile razor blade was used to remove the olfactory bulbs. The brain is placed to expose its ventral aspect and a second coronal cut just anterior to the optic chiasm was made to produce a slice

containing the SVZ. The coronal slice was then placed under a dissecting microscope at 25 X-magnification and forceps were used to carefully dissect bilateral SVZ. SVZ from individual *Hdh* CAG7 or *Hdh* CAG *Hdh* brains were pooled, minced for 1 minute, and the resultant slurry transferred to a 1.5mL tube. Following brief centrifugation, supernatant was removed and the tissue resuspended in 3mL tissue dissociation buffer and transferred to a 15mL tube at 37°C for 30 minutes. Dissociation buffer consisted of 1X HBSS, 0.25% trypsin, 0.01M HEPES, 0.5mM EDTA, and 0.0005% DNaseI. Following incubation, 3.5mL trypsin inhibitor was added and the sample centrifuged at $\sim 1000 \times g$ for 7 minutes. Trypsin inhibitor was composed of 14mg/100mL trypsin inhibitor, and 0.001% DNaseI in DMEM. The supernatant was then removed and SVZ tissue resuspended in 500 μ L N2:B27 with 0.0004% heparin, 20ng/mL epidermal growth factor (EGF), and 10ng/mL basic fibroblast growth factor (bFGF). The resuspended tissue was then passed through a 40 μ m filter and a Beckman Z1 Coulter counter was used to determine cell counts. SVZ cells were then resuspended at a concentration of 0.67×10^5 cells/mL N2:B27 with 0.0004% heparin, 20ng/mL EGF, and 10ng/mL bFGF and placed in 12 well tissue culture wells containing tissue-culture treated coverslips. The cultures were incubated at 37° C, 5% CO₂ in a humidified incubator. Individual cells in suspension were initially apparent in the culture. Spherical clusters enlarged, and by six days in culture many were 100-200 μ m in diameter. Individual NS were picked and re-plated onto poly-ornithine coated tissue-culture treated coverslips at a density of five-seven per coverslip. Half of the N2:B27 medium was changed every three days. NS were fixed in 3.7% formaldehyde in PBS on day 2 and 6 following plating onto poly-ornithine. Prior to IHC CS were blinded so that during microscopic analysis and scoring the genotype was not known to the examiner. The blind was not removed until scoring of the CS was complete. IHC with Sox3 and Neuronal Class III β -Tubulin was performed as detailed above. Following IHC an Olympus BX-51B microscope equipped with an Olympus DP70 camera and Olympus DP controller software were used to capture images that were post-processed using Adobe Photoshop CS. For day 2 Sox3 quantitation, a total of 37 NS were scored, 16 from *Hdh* CAG7 and 21 from *Hdh* CAG 150. For day 6 Sox3 quantitation, a total of 30 NS were scored, 14 from *Hdh* CAG7 and 16 from *Hdh* CAG 150. For day 2 neuron specific β -tubulin quantitation, a total of 37 NS were scored, 16 from *Hdh* CAG7 and 21 from *Hdh* CAG 150. For day 6 neuron specific β -tubulin quantitation, a total of 30 NS were scored, 14 from *Hdh* CAG7 and 16 from *Hdh* CAG 150.

Expression Profiling

At four and six days of neuronal differentiation ES cells were lysed and homogenized with TRIzol Reagent (Invitrogen), and frozen. Total RNA was isolated from samples according to manufacturer's instructions. Expression profiling was performed through the NIH Neuroscience Microarray Consortium at the Translational Genomics Research Institute (Phoenix, AZ) according to the Affymetrix guidelines (GeneChip® Expression Analysis and Data Analysis Fundamentals). Samples were analyzed for integrity using the Agilent Bioanalyzer 2100. One microgram of total RNA was amplified and labeled using the GeneChip HT one-cycle cDNA synthesis kit (Affymetrix). Fragmented, labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays (http://www.affymetrix.com/products/arrays/specific/mouse430_2.affx) for 16 hours at 45°C. The GeneChips® were washed and stained according to the manufacturer's recommendations (Affymetrix) using the GeneChips® Fluidics Station 450 (Affymetrix). This procedure includes washing the chips with phycoerythrin-streptavidin, signal amplification by a second staining with biotinylated anti-streptavidin and a third staining with phycoerythrin-streptavidin. Each chip was scanned using the GeneChips® Scanner 3000 (Affymetrix).

The Affymetrix GeneChip® Operating Software (GCOS) version 1.4 was used to perform global scaling by bringing the overall intensity of the arrays to a target intensity value of 150 in order to normalize the data for inter-array comparisons. The signal intensity levels and

detection calls of each gene were generated using the Statistical Expression Algorithm. GeneSpring GX (www.chem.agilent.com) was used to perform the statistical analysis of the data. Lists of statistically significant genes were generated based on the following comparisons: CAG7 day 4 v. CAG150 day 4, CAG7 day 6 v. CAG150 day 6, CAG7 day 4 v. CAG7 day 6, and CAG150 day4 v. CAG150 day6. All genes on the arrays were filtered to include those genes that were present or marginal in 3 of the 6 arrays per comparison. This filtered list was further reduced by filtering for genes with a fold change of 2 or greater in treated versus control samples. A Welch t-test was performed on the filtered list to generate a final list of those genes that were statistically significant $p < 0.05$ and had a fold change of greater than or equal to 2. Two statistical corrections for multiple testing were performed on each comparison: the Benjamini-Hochberg (False Discovery Rate < 0.05) and Bonferroni (Family wise error rate < 0.05). These gene lists were imported into the Metacore software (Genego) for functional analysis.

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Figure 1.

Expression of lineage markers, and cell cycle parameters in undifferentiated *Hdh* CAG7 and *Hdh* CAG150 ESC. (A) RNA isolation and semiquantitative RT-PCR were performed to amplify markers of undifferentiated embryonic stem cells (Oct-4 and Nanog) a neural progenitor marker (Nestin), and a neuronal marker (synaptophysin). (B) Representative histograms of FACS cell cycle analysis displaying the proportion of undifferentiated *Hdh* CAG7 and *Hdh* CAG150 cells in G₁, or S and G₂/M. Statistical analysis was performed on raw data from five independent samples from *Hdh* CAG7 or *Hdh* CAG150 cell lines using independent samples t-test and Mann-Whitney non-parametric analysis (SPSS14).

Figure 2.

Expanded CAG Repeat Facilitated Neurogenesis. Representative images of the expression of Sox3 and neuron specific β -tubulin expression following neuronal differentiation of *Hdh* CAG7, *Hdh* CAG77, and *Hdh* CAG150 ESC. *Hdh* CAG7, (A and C), and *Hdh* CAG 150, (B and D), following 4 days of neuronal differentiation. *Hdh* CAG7, (E and G), *Hdh* CAG 150, (F and H), and *Hdh* CAG77 (I and J) following 7 days of neuronal differentiation. (K-L) ~60X magnification of cellular cluster from G, J, and H respectively. (K) *Hdh* CAG7. (L) *Hdh* CAG77. (M) *Hdh* CAG 150. *Hdh* CAG7 (N and P), and *Hdh* CAG150 (O and Q) following 11 day of neuronal differentiation. 10X magnification shown in (A, B, E, F, I, N, and O). 20 $\times$ magnification shown in (C, D, G, H, J, P, and Q). Sox3 expression is nuclear and displayed in red. Neuron specific β -tubulin is cytoplasmic and is displayed in green. Hoescht (bis-benzimide) stained nuclei are displayed in blue.

Figure 3.

FACS analysis of Neuronally Differentiated *Hdh* CAG7 and *Hdh* CAG 150. (A) Representative quadrant scatter plots of neuronally differentiating ESC. At each timepoint individual graph quadrants represent distinct cellular populations. The lower left quadrant delineates Sox3⁻/neuron specific β -tubulin⁻ cells. The upper left quadrant contains Sox3⁺/neuron specific β -tubulin⁻ cells. The upper right quadrant contains Sox3⁺/neuron specific β -tubulin⁺ cells and the lower right quadrant the Sox3⁻/neuron specific β -tubulin⁺ populations. Inset into the lower two panels of (A) are pictures that display examples of each cell type. (B) FACS analysis of Sox3⁺/neuron specific β -tubulin⁻ populations. (C) FACS analysis of Sox3⁺/neuron specific β -tubulin⁺ cells (D) FACS analysis of Sox3⁻/neuron specific β -tubulin⁺ cells. Statistical analysis was performed using the means of five independent replicates neuronally differentiated in parallel using the independent-samples t-test (SPSS14). Cellular populations were determined using BD Biosciences FACS DiVa cell sorter software. Post processing data analysis was performed using WEASEL version 2.3 software. Values are displayed as cell number $\times 10^5$. Error bars represent the standard error of the mean (SEM). Tuj1= neuron specific β -tubulin⁺

Figure 4.

Lineage marker expression following neuronal differentiation of *Hdh* CAG7 and *Hdh* CAG150 cells. Semiquantitative RT-PCR was performed following 4, 7, and 11 days of neuronal differentiation. ESC markers, Oct4 and Nanog. Neuronal precursor markers, Musashi, Nestin, and Pax6. Neuronal markers, neurogenic differentiation 1 (NeuroD1), synaptophysin (Syp), homolog of *Drosophila* empty spiracles (Emx1), homeobox C6 (Hoxc6). Oligodendrocyte markers, oligodendrocyte lineage transcription factor 2 (Olig2) and myelin associated glycoprotein (MAG). Astrocyte marker, glial fibrillary acidic protein (GFAP). Huntingtin (Htt). B-actin is shown as a control. All amplification was performed with appropriate positive

and negative controls, not shown. Representative images of at least three independent experiments performed in duplicate.

Figure 5.

Neuronal differentiation of wild type and *Hdh* CAG150 SVZ neural stem cells. Following isolation from either wild type (CAG7) or *Hdh* CAG150 P20 SVZ, neurospheres were neuronally differentiated for an additional 2 (A & B) or 6 (C & D) days and then stained for Sox3 and neuron specific β -tubulin. Displayed are representative images. (A) wild type day 2 (B) *Hdh* CAG150 day 2 (C) wild type day 6 (D) *Hdh* CAG150 day 6. Colonies were smaller on day 2 they are shown at 20X magnification. Day 6 magnification is 10X. Insets display a central portion of the images at higher magnification ~60X for A & B, and 30X for C and D. Blinded assignment was undertaken to perform statistical analysis of Sox3 and neuron specific β -tubulin staining. Staining of Sox3 was divided into high, moderate, and low. Because of the relatively low number of NS with robust neuronal differentiation, the staining of neuron specific β -tubulin was grouped into either, high or moderate, and low neuronal differentiation. Homogeneity of proportions was tested using Pearson's chi-square statistic (SPSS14).

Figure 6.

Cell Death and Cell Cycle Parameters of Neuronally Differentiated *Hdh* CAG7 and *Hdh* CAG150 ESC. PI incorporation following 4, 6, 8, and 10 days of neuronal differentiation. Proportions of cells in G1 or S + G2/M were calculated using BD Biosciences FACS DiVa cell sorter software (Table 2). Post processing data analysis was performed using WEASEL version 2.3 software. For cell cycle analysis the values are the mean of 5 independent samples $\pm$ SEM. Independent samples t-test were used to determine p-values. * = P-value < 0.05.

Figure 7. Semiquantitative RT-PCR of transcripts with differential expression identified by expression profiling, following 4, 7, and 11 days of neuronal differentiation. homolog of *Drosophila* orthodenticle 1 (Otx1), homolog of *Drosophila* orthodenticle 2, (Otx2), hairy and enhancer of split 5 (Hes5), zinc finger protein of the cerebellum 1 (Zic1). B-actin is shown as a control. All amplification was performed with appropriate positive and negative controls, not shown.

Table 1Cell Cycle Analysis of Neuronally Differentiated *Hdh* CAG 7 and *Hdh* CAG 150 cells

	<i>Hdh</i> 7 CAG	<i>Hdh</i> CAG 150	p value
%G1	35.56	34.75	0.08
%S + G2/M	50.79	50.55	0.79

Table 2Cell Cycle Analysis of Neuronally Differentiated *Hdh* CAG7 and *Hdh* CAG150 cells

	<i>Hdh</i> 7 CAG	<i>Hdh</i> CAG150	P value
%G1 Day 4	43.10 ± 0.38	48.82 ± 0.29	<0.00001
%S + G2/M Day 4	42.50 ± 0.33	38.04 ± 0.64	<0.00001
%G1 Day 6	47.00 ± 0.84	56.67 ± 0.33	<0.00001
%S + G2/M Day 6	20.30 ± 0.63	20.67 ± 0.43	0.65
%G1 Day 10	46.14 ± 1.32	51.07 ± 1.25	0.018
%S + G2/M Day 10	15.48 ± 0.85	14.34 ± 0.34	0.22

Table 3

The most highly overrepresented GO categories derived from transcripts with significant differential expression between CAG7 and CAG150 following 4 and 6 days of neuronal differentiation.

Gene Ontology Biological Processes Category	p-Value day 4	p-Value day 6
development	1.16E-27	1.04E-30
organ development	3.56E-23	7.91E-21
cell migration	4.66E-14	1.30E-14
morphogenesis	5.66E-14	2.70E-16
system development	7.42E-14	1.24E-12
nervous system development	1.87E-13	4.26E-13
cell motility	3.31E-13	1.17E-12
localization of cell	3.31E-13	1.17E-12
locomotion	5.07E-13	1.89E-12
locomotory behavior	1.19E-12	3.39E-11
Gene Ontology Molecular Function Category		
transcription regulator activity	4.53E-07	1.63E-08
transcription factor activity	2.10E-06	1.95E-10
structural constituent of cytoskeleton	2.94E-06	1.56E-07
protein binding	3.29E-05	4.22E-06
heparin binding	3.64E-05	8.02E-08
RNA polymerase II transcription factor activity, enhancer binding	2.69E-04	1.39E-06
glycosaminoglycan binding	4.12E-04	3.38E-09
growth factor activity	4.16E-04	3.03E-05
polysaccharide binding	8.01E-04	1.47E-08
receptor binding	3.00E-03	1.77E-04

The top 10 categories common to day 4 and day 6 of neuronal differentiation are shown. Biological processes and molecular function GO categories were ranked by p value.

Table 4

All genes with increased expression in CAG150 compared to CAG7 following 4 or 6 days of neuronal differentiation. Genes with increased expression day 4 CAG150 vs CAG7

Transcript name (Gene Symbol)	Expression change	p-value	Potential gene function summary
adenomatous polyposis coli down-regulated 1 (Apccdd1)	3.76	0.0325	A downstream target of the WNT/beta-catenin pathway that may enhance cellular proliferation (Takahashi et al., 2002).
paired box gene 3 (Pax3)	2.95	0.0374	DNA-binding protein involved in neuronal and muscle development (Buckingham and Relaix, 2007; Nakamura et al., 2005).
naked cuticle 1 homolog (Drosophila) (Nkd1)	2.75	0.0094	A Dishevelled-binding protein that functions as a negative regulator of the Wnt -beta-catenin Tcf signaling pathway (Van Raay et al., 2007).
T-cell acute lymphocytic leukemia 2 (Tal2)	2.70	0.0201	A helix-loop-helix (HLH) protein associated with cell proliferation strongly expressed in the diencephalon and the mesencephalon, and latter in post-mitotic cells (Pinheiro et al., 2004).
dihydropyrimidinase-like 4 (Dpysl4)	2.58	0.0028	Also known as collapsin response mediator protein 3, is a brain-specific transcript that modulates growth cone collapse and axonal guidance during brain development (Jiang et al., 2007).
hairy and enhancer of split 5 (Drosophila) (Hes5)	2.58	0.0164	A basic helix-loop-helix domain containing protein, that is a down-stream Notch pathway gene critical in NSC differentiation (Basak and Taylor, 2007; Louvi and Artavanis-Tsakonas, 2006).
EH domain binding protein 1 (Ehbp1)	2.56	0.0161	Appears to function in endocytic trafficking (Naslavsky and Caplan, 2005).
zinc finger protein 503 (Zfp503)	2.44	0.0207	A zinc finger protein preferentially expressed at high levels in differentiating progenitors of striatal projection neurons (Chang et al., 2004).
RIKEN cDNA 1200015N20 gene	2.37	0.0085	Unknown
sorbin and SH3 domain containing 2 (Sorbs2)	2.29	0.0117	Unknown
RIKEN cDNA 2510009E07 gene	2.25	0.0116	Unknown
engrailed 1 (En1)	2.23	0.0239	A homeobox protein important during development in segmentation, and in differentiation of midbrain dopaminergic neurons (Simon et al., 2001).
DNA segment, Chr 1, ERATO Doi 471, expressed	2.22	0.0067	Unknown
odd Oz/ten-m homolog 4 (Drosophila) (Odz4)	2.20	0.0379	A transmembrane receptor involved in patterning the cerebral cortex (Li et al., 2006).
orthodenticle homolog 2 (Drosophila) (Otx2)	2.18	0.0088	Otx2 is expressed in the mouse epiblast by day E5.5. Its expression is subsequently localized to the anterior neuroectoderm where it is involved in specification of mid- and forebrain neurons (Acampora et al., 2005).
RIKEN cDNA 2310047C04 gene	2.14	0.0432	Unknown
zinc finger homeodomain 4 (Zfx4)	2.12	0.0054	A homeodomain and zinc finger protein involved in neuronal differentiation (Hemmi et al., 2006).
solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (Slc1a4)	2.04	0.0194	A neutral amino-acid transporter (Yamamoto et al., 2004).
RIKEN cDNA A930038C07 gene	2.03	0.0188	Unknown
myeloid ecotropic viral integration site 1 (Meis1)	2.02	0.0085	Homeobox protein important in brain development (Toresson et al., 2000).
doublesex and mab-3 related transcription factor like family A2 (Dmrta2)	2.01	0.0262	Unknown, maybe important in sex determination in fish.
Genes with increased expression day 6 CAG150 vs CAG7			
Transcript name (Gene Symbol)	Expression change	p-value	Potential gene function summary
zinc finger protein of the cerebellum 1 (Zic1)	6.18	0.0081	A zinc finger transcription factor important in posterior forebrain development (Inoue et al., 2007; Merzdorf and Sive, 2006).
orthodenticle homolog 2 (Drosophila) (Otx2)	3.79	0.0157	Expressed in the mouse epiblast by day E5.5. Its expression is subsequently localized to the anterior neuroectoderm where it is critically involved in specification of mid- and forebrain neurons (Acampora et al., 2005).
contactin associated protein-like 2 (Cnnap2)	3.06	0.0163	A member of the neuroligin superfamily, a group of transmembrane proteins that mediate cell-cell interactions in the nervous system involved in patterning corticostriatal thalamic circuit (Abrahams et al., 2007).
nucleosome assembly protein 1-like 5 (Nap1l5)	3.01	0.0374	Homologous to the yeast nucleosome assembly protein, widely expressed in the rodent brain.
paired box gene 3 (Pax3)	2.85	0.0069	DNA-binding protein involved in neuronal and muscle development (Buckingham and Relaix, 2007; Nakamura et al., 2005).
bruno-like 4, RNA binding protein (Drosophila) (Brunol4)	2.59	0.0096	A brain-specific RNA-binding protein involved in differentiation and maintenance of neuronal structures (Meins et al., 2002; Yang et al., 2007).
RIKEN cDNA C030036D22 gene	2.46	0.0144	Unknown
RIKEN cDNA C130071C03 gene	2.46	0.0281	Unknown
SPARC related modular calcium binding 1 (Smoc1)	2.41	0.0178	A calcium binding protein localized to the basement membrane expressed in the brain of uncertain function (Vannahme et al., 2002).
EH domain binding protein 1 (Ehbp1)	2.38	0.0198	Appears to function in endocytic trafficking (Naslavsky and Caplan, 2005).
achaete-scute complex homolog-like 1 (Drosophila) (Mash1)	2.31	0.0163	A basic helix-loop-helix factor expressed in the ganglionic eminences and is important in specification of neuronal precursors (Casarosa et al., 1999).
sorbin and SH3 domain containing 2 (Sorbs2)	2.25	0.0131	Unknown
microtubule-associated protein tau (Mapt)	2.17	0.0329	A cytoskeletal protein that stabilizes microtubules. Hyperphosphorylated tau tangles are a pathologic hallmark of Alzheimer's and other neurodegenerative diseases (Ballatore et al., 2007).
bone morphogenetic protein receptor, type 1B (Bmpr1b)	2.17	0.0115	Signaling through the Bmpr1b is critical in vertebrate nervous system development (Liu and Niswander, 2005).
calcium channel, voltage-dependent, gamma subunit 4 (Cacng4)	2.11	0.0340	Voltage gated calcium channel highly expressed in the developing mouse brain (Letts et al., 2005).
cDNA sequence BC030477	2.05	0.0207	Unknown
neuron specific gene family member 2 (Nsg2)	2.05	0.0157	Unknown
microtubule-associated protein 1 B (Mtap1b)	2.04	0.0332	Involved in maintaining the integrity of cytoskeletal structures and promoting neuronal stability (Ballatore et al., 2007).
microtubule-associated protein 2 (Mtap2)	2.03	0.0344	Involved in maintaining the integrity of cytoskeletal structures and promoting neuronal stability (Ballatore et al., 2007).
Myeloid ecotropic viral integration site 1 (Meis1)	2.01	0.0200	Homeobox protein important in brain development (Toresson et al., 2000).

One-way Welch t-tests with Benjamini Hochberg false discovery rate correction for multiple testing were used to identify transcripts with a greater than 2-fold change in expression at a p value less than 0.05. These transcripts were further filtered to remove genes with signal in the noise range. Genes highlighted in yellow displayed differential expression at day 4 and 6. Transcripts with differential expression on day 4 and 6 with known roles in transcriptional regulation and nervous system development are highlighted in orange. Transcripts boxed in red are involved in transcriptional regulation, those boxed in blue are cytoskeletal.

Table 5**Primer Sequences and PCR Conditions**

	Forward primers	Reverse primers	Product size (bp)	AT
β-actin	5'-AACCTAAGGCCAACCGTG-3'	5'-CAGGATTCCATACCCAAGAAGG-3'	494	57°
Musashi-1	5'-ATGGTGGAATGCAAGAAAGC-3'	5'-TAGGTGTAACCAGGGCAAG-3'	191	55°
Nanog	5'-AGGGTCTGCTACTGAGATGCTCTG-3'	5'-CAACCACTGGTTTTCTGCCACCG-3'	363	53°
Nestin	5'-GGAGTGTGCTTAGAGGTGC-3'	5'-TCCAGAAAGCCAAGAGAAGC-3'	320	55°
Oct4	5'-GATGGCATACTGTGGACCTCAG-3'	5'-GCTTCGGGCACCTCAGAAAC-3'	541	55°
Pax6	5'-AGTCACAGCGGAGTCAATCAGC-3'	5'-AGCCAGGTTGCGAAGAACTCTG-3'	425	56°
Syp	5'-GCCTGTCTCCTTGAACACGAAC-3'	5'-TACCGGAGAACAACAAAGGGC-3'	290	60°
Olig2	5'-GGCGGTGGCTTCAAGTCATC-3'	5'-TAGTTTCGCGCCAGCAGCAG-3'	260	60°
MAG	5'-GCCCGAATTGAGAAATCTCTGG-3'	5'-TTCTGCATACTCAGCCAGC-3'	239	58°
GFAP	5'-GCGCTCAATGCTGGCTTCAA-3'	5'-ACGCAGCCAGGTTGTCTCT-3'	346	58°
Htt	5'-CAAGTGGAACGGCTCTCTC-3'	5'-TGGACAGGAGAGCAAGTGTG-3'	325	55°
NeuroD1	5'-GCACATGGACCCCATGTCTT-3'	5'-CCTCTAATCGTCAAAGATGGCATT-3'	382	55°
Otx1	5'-AGCAGACACATCGAAACCTTC-3'	5'-CACTTGGGATTTTGCACCCTC-3'	300	55°
Otx2	5'-CCATGACCTATACTCAGGCTTCAGG-3'	5'-GAAGCTCCATATCCCTGGGTGGAAAG-3'	211	59°
EMX1	5'-TGAGAAGAATCACTACGTGG-3'	5'-AGGTGACATCAATGTCCTCC-3'	229	56°
Islet1	5'-GTCCGGAGAGACATGATGGT-3'	5'-TTCCCACTTCTCCAACAGG-3'	312	55°
Nkx2.1	5'-GCCCTACAGGTTCACTCCAG-3'	5'-ACTGGGACTGGGGTTCTTTT-3'	150	58°
Hoxc6	5'-ATTCGCCACAGGAGAATGTC-3'	5'-CCGAGTTAGGTAGCGGTTGA-3'	340	55°

Octamer binding transcription factor 4 (Oct4), paired box gene 6 (Pax6), synaptophysin (Syp), oligodendrocyte lineage transcription factor 2 (Olig2), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), huntingtin (Htt), neurogenic differentiation 1 (NeuroD1), homolog of Drosophila orthodenticle 1 (Otx1), homolog of Drosophila orthodenticle 2, (Otx2), homolog of Drosophila empty spiracles (Emx1), homeobox C6 (Hoxc6).
Annealing temperature (AT)