Impaired Mitochondrial Dynamics and Nrf2 Signaling Contribute to Compromised Responses to Oxidative Stress in Striatal Cells Expressing Full-Length Mutant Huntingtin

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Abstract

Huntington disease (HD) is an inherited neurodegenerative disease resulting from an abnormal expansion of polyglutamine in huntingtin (Htt). Compromised oxidative stress defense systems have emerged as a contributing factor to the pathogenesis of HD. Indeed activation of the Nrf2 pathway, which plays a prominent role in mediating antioxidant responses, has been considered as a therapeutic strategy for the treatment of HD. Given the fact that there is an interrelationship between impairments in mitochondrial dynamics and increased oxidative stress, in this present study we examined the effect of mutant Htt (mHtt) on these two parameters. STHdhQ111/Q111 cells, striatal cells expressing mHtt, display more fragmented mitochondria compared to STHdhQ7/Q7 cells, striatal cells expressing wild type Htt, concurrent with alterations in the expression levels of Drp1 and Opa1, key regulators of mitochondrial fission and fusion, respectively. Studies of mitochondrial dynamics using cell fusion and mitochondrial targeted photo-switchable Dendra revealed that mitochondrial fusion is significantly decreased in STHdhQ111/Q111 cells. Oxidative stress leads to dramatic increases in the number of STHdhQ111/Q111 cells containing swollen mitochondria, while STHdhQ7/Q7 cells just show increases in the number of fragmented mitochondria. mHtt expression results in reduced activity of Nrf2, and activation of the Nrf2 pathway by the oxidant tBHQ is significantly impaired in STHdhQ111/Q111 cells. Nrf2 expression does not differ between the two cell types, but STHdhQ111/Q111 cells show reduced expression of Keap1 and p62, key modulators of Nrf2 signaling. In addition, STHdhQ111/Q111 cells exhibit increases in autophagy, whereas the basal level of autophagy activation is low in STHdhQ7/Q7 cells. These results suggest that mHtt disrupts Nrf2 signaling which contributes to impaired mitochondrial dynamics and may enhance susceptibility to oxidative stress in STHdhQ111/Q111 cells.

Introduction

Huntington disease (HD) is a devastating inherited neurodegenerative disease caused by a CAG trinucleotide repeat expansion in exon 1 of huntingtin (Htt) gene. Although the mechanisms by which mutant huntingtin (mHtt) causes neurotoxicity have been widely studied, the pathological processes have not yet been fully elucidated. mHtt-induced impairment of the cellular responses to oxidative stress has been suggested as a crucial contributing factor in the progression of HD. Indeed, there is clear evidence of increased oxidative stress in HD. Further, defects in mitochondria, which are both a source of oxidative stress and a target, are apparent in HD and HD models [1,2].

Neurons are highly dependent on mitochondria for not only energy production but also Ca2+ buffering, and reactive oxygen species (ROS) regulation. Studies using mouse and cell models for HD have shown mitochondrial impairment and bioenergetic deficits, reminiscent of the pathological characteristics of HD [2–4]. In addition, increased oxidative stress is apparent in HD cases [5–7]. Mitochondria from STHdhQ111/Q111 cells show impaired function and significant increases in ROS production compared to STHdhQ7/Q7 cells [8–10]. Our previous studies showed that the PPARγ pathway, which regulates the expression of metabolically important genes, is severely compromised in STHdhQ111/Q111 cells which coincides with an increased sensitivity to thapsigargin induced loss of mitochondrial membrane potential (∆Ψm), and increased cell death at higher concentrations of thapsigargin. Activation of the PPARγ pathway attenuated thapsigargin-induced ∆Ψm loss and cell death in STHdhQ111/Q111 cells [8,11].

Mitochondria are dynamic organelles that are constantly undergoing fission and fusion, which is essential for normal cellular function. Imbalances between mitochondria fission and...
Impaired Mitochondrial Dynamics and Nrf2 in HD

Fusion have been shown to negatively impact the physiology and viability of neuronal cells [12,13]. Key mediators of mitochondrial fission/fusion include the GTPases Dynamin-Related protein 1 (Drp1), which is essential for fission, and Optic Atrophy Type 1 (Opa1) and the Mitofusins (Mfn1 and Mfn2) which mediate fusion. mHtt has been reported to directly bind Drp1 and increase its activity, suggesting that this may contribute to the apparent mitochondrial fragmentation and dysfunction [14]. Indeed, fragmentation of mitochondria results in increased ROS production in cell models [15,16]. Mutations of Opa1 result in autosomal dominant optic atrophy [17]. In addition, the levels of Opa1, as well as Mfn1/2 were shown to be decreased in HD cases relative to controls [18]. There is also data to indicate that alterations in mitochondrial morphology in HD enhance cellular susceptibility to apoptosis [14,19].

Nuclear factor erythroid 2-related factor 2 (Nrf2), a major transcription factor for antioxidant and cytoprotective responses, is normally sequestered in the cytosol by Kelch-like ECH-associated protein 1 (Keap1), an adaptor of a ubiquitin ligase complex, and constitutively degraded through the ubiquitin-proteasome system [20]. Upon oxidative stress, Nrf2 dissociates from Keap1, translocates into nucleus, and binds to antioxidant response elements (AREs), which in turn activates genes related to antioxidant responses such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (Nqo1) and nuclear respiratory factor-1 (Nrf1) [21–24]. Activation of Nrf2 signaling has been shown to regulate mitochondrial biogenesis in rat brain, mouse cardiomyocytes, and a sepsis mouse model [23,25,26]. Nrf2 signaling or expression is altered in various neurodegenerative diseases and animal models [27,28]. Pharmacological activation of Nrf2 has beneficial effects in an HD mouse model induced by 3-nitropropionic acid (3-NP), and transgenic mouse models including N171-82Q, R6/2, and YAC128 [29–31]. However, whether mHtt affects Nrf2 signaling is not known.

In the present study, we analyzed mitochondrial dynamics and how they are affected by oxidative stress in a striatal cell model of HD. The cells used in this study are homozygous for either the wild type or mutant Htt gene. Although HD is an autosomal dominant disease, the effects of mHtt are often studied in cell and animal models that are homozygous for mHtt to facilitate the dissection of the pathogenic mechanisms involved [1–4]. Although these knock-in models are imperfect, they are advantageous in that they express two copies of the mutant allele, rather than just one as in HD, needs to be carefully considered when evaluating the data obtained with these models.

In this study we found that STHdh Q111/Q111 cells exhibit alterations in mitochondrial dynamics compared to STHdh Q7/Q7 cells. Determination of static mitochondrial morphology along with analysis of dynamic mitochondrial fusion processes revealed that STHdh Q111/Q111 cells exhibit an increase in fragmented mitochondria concomitant with a decrease in fusion. Further STHdh Q111/Q111 cells show impairment of the mitochondrial response to oxidative stress. Given that Nrf2 is not only a key regulator for antioxidant system but also an emerging target to counter mitochondrial dysfunction in many disease models [25,26,32–34], we investigated whether Nrf2 signaling was compromised in this cell model of HD and found that the presence of mHtt results in defects in Nrf2 signaling. Significant impairment in oxidative stress-induced Nrf2 activation was observed in STHdh Q111/Q111 cells. Our study provides important insights into mHtt-induced mitochondrial impairment, with activation of Nrf2 signaling being a potential therapeutic strategy.

Results

STHdh Q111/Q111 Cells Show Altered Mitochondrial Dynamics

Mitochondrial morphology is an important component of cellular metabolism and pathogenic processes. Mitochondria are dynamic and display a range of morphologies. Maintaining the appropriate proportion of each mitochondrial morphological state is essential for appropriate functioning of the cell. To gain insight into mitochondrial dynamics in mHtt expressing cells, we transfected mitochondria-targeted GFP into STHdh Q7/Q7 and STHdh Q111/Q111 cells. Cells were divided into four groups based on the morphology of the mitochondria: tubular, fragmented, mixed, and swollen as previously described [35]. In most cells of both genotypes the mitochondria (about 60%) exhibited mixed forms with predominantly short tubular shapes. Approximately 20% of STHdh Q7/Q7 cells had predominantly tubular mitochondria while less than 10% of STHdh Q111/Q111 cells showed this mitochondrial morphology. Interestingly, more than 30% of the STHdh Q111/Q111 cells presented with fragmented mitochondria which was significantly greater than the STHdh Q7/Q7 cells. Both cell types exhibited very few cells with swollen mitochondria (Fig. 1A). Similarly, a significant increase in fragmented neuronal mitochondria in the striatum of R6/2 mice was also observed (Figure S1). Next, we examined whether there was a difference in the expression of mitochondrial fusion or fission regulators between the two cell types. Immunoblot analyses revealed that Drp1 levels were significantly lower in STHdh Q111/Q111 than in STHdh Q7/Q7 cells. In addition Drp1 phosphorylated at serine 616, which is a more active form [36,37], was also lower in STHdh Q111/Q111 cells compared to STHdh Q7/Q7 cells. Of interest, Opa1 expression was also significantly decreased in STHdh Q111/Q111 cells, while Mfn2 appears to be at a similar level between the two cell types (Fig. 1B and C). Together these results indicate that proteins involved in the dynamics of mitochondrial fission/fusion are altered in STHdh Q111/Q111 cells.

Mitochondrial Fusion is Impaired in STHdh Q111/Q111 Cells

To gain further insight into dynamic changes in mitochondrial morphology, we investigated whether mitochondrial fusion processes were altered in STHdh Q111/Q111 cells compared to STHdh Q7/Q7 cells. To this end, striatal cells were fused using PEG after transfection with either mitoCherry or mitoGFP as described in materials and methods. Cell fusion allows two individual cells to share cellular components including mitochondria. If a mitochondrion expressing mitoCherry undergoes fusion with a mitochondrion expressing mitoGFP, the resulting fused mitochondrion becomes yellow. STHdh Q111/Q111 cells exhibited significantly lower levels of fused mitochondria compared to STHdh Q7/Q7 cells (Fig. 2, A and B). Since this cell fusion assay requires the use of cycloheximide to prevent de novo protein synthesis which would generate mitochondria containing both mitoCherry and mitoGFP without the fusion process, it can be speculated that inhibition of protein synthesis might differentially affect mitochondrial fusion process in the two cell types. Therefore we used a second method to monitor mitochondrial fusion, a photoconvertible GFP (Dendra) targeted to mitochondria. Dendra, which in the unconverted state fluoresces green, was stably converted in a given area of the cell by light illumination at 400 nm and thus showed red fluorescence. When mitochondria in the cell emitting red fluorescence fuse with mitochondria emitting
green fluorescence, the fluorescence becomes yellow. Using this approach, more mitochondrial fusion events were again observed in the STHdhQ111/Q111 cells compared to STHdhQ7/Q7 cells (Fig. 2, C and D). Although neither method estimates the mitochondrial fission process, these results provide strong evidence that there is reduction in mitochondrial fusion events in STHdhQ111/Q111 cells.

Oxidative Stress Induces Differential Responses in Mitochondria Morphology

It has been shown that mitochondria dynamically change their morphologies in response to oxidative stress [38–40]. Therefore, we tested whether the two striatal cell lines showed different pattern changes in mitochondrial morphology in response to H2O2. In both cell types there were dramatic reductions in tubular and mixed forms of mitochondria in response to H2O2 in a dose dependent manner (Fig. 3). Importantly, STHdhQ111/Q111 cells showed significant increases in swollen mitochondria while the percentage of cells with fragmented mitochondria slightly decreased as the concentration of H2O2 was increased (Fig. 3). In contrast, STHdhQ7/Q7 cells showed a rapid increase in the population of fragmented mitochondria while swollen mitochondria only slightly increased with the increasing concentrations of H2O2. It is noteworthy that swollen mitochondria are shown to be functionally deficient and indicative of pathogenesis [41,42]. Therefore, this result suggests that mitochondria in STHdhQ111/Q111 cells show greater susceptibility to oxidative stress compared to STHdhQ7/Q7 cells.

Mitochondria in STHdhQ111/Q111 Cells are More Oxidized

Mitochondria play important roles in regulation of cellular redox state and defense against oxidative stress. In addition, our results show that oxidative stress dramatically affects mitochondrial morphologies. Given these data, we next determined if the two striatal cell displayed differences in in the redox states of

Figure 1. Mitochondrial dynamics is altered in STHdhQ111/Q111 cells. A, Mitochondria-targeted GFP (mitoGFP), was transfected in the striatal cells to visualize the mitochondria and cells were then assigned to one of four groups based on the morphology of their mitochondria (tubular, mixed, fragmented or swollen). STHdhQ111/Q111 cells had more fragmented mitochondria and less tubular mitochondria compared to STHdhQ7/Q7 cells, while mixed and swollen mitochondria were at similar levels in the two cell types. More than 200 cells of each cell type were assessed for each independent experiment. n = 3. B, Immunoblots revealed that STHdhQ111/Q111 cells exhibit reduced levels of Drp1, its active form which is phosphorylated at Ser616, and Opa1 compared to STHdhQ7/Q7 cells, whereas Mfn2 appears to be similar in both cell types. C, Quantitative analysis of immunoblots shows a significant reduction in Drp1, phosphorylated Drp1, and Opa1. n = 4. Data shown are mean ± SE. **P<0.01, ***P<0.001 vs. STHdhQ7/Q7. doi:10.1371/journal.pone.0057932.g001
mitochondria in the basal condition, which could ultimately contribute to different distributions in mitochondrial morphologies. To this end, mitochondrial targeted GFP redox sensors, rosGFP1 or rosGFP2 were transfected into striatal cells [43]. The fluorescence emission at 525 nm was monitored at excitation wavelengths of 470 nm and 400 nm and the ratio of emission intensities (470/400) was used to evaluate mitochondrial matrix redox state as described in methods and materials. The results from these two redox sensors indicate that mitochondria in STHdhQ111/Q111 cells are in a more oxidized state compared to STHdhQ7/Q7 cells (Fig. 4).

Figure 2. Mitochondrial fusion is significantly reduced in STHdhQ111/Q111 cells. A, Striatal cells expressing mitoCherry or mitoGFP were fused and mitochondrial fusion was analyzed as described in materials and methods. STHdhQ111/Q111 cells exhibit substantially lower levels of mitochondrial fusion compared to STHdhQ7/Q7 cells. B, Quantitative analysis reveals that STHdhQ7/Q7 cells exhibit ~60% mitochondrial fusion on average while STHdhQ111/Q111 cells display ~20% fused mitochondria. The data was acquired by analysis of more than 60 cells of each cell type from seven independent experiments. The white dashed line indicates the contact region between the two cells. C, To monitor mitochondrial fusion without cell fusion, mitoDendra was expressed in the striatal cells. The mitochondrial fusion process was observed after the photoconversion of green to red fluorescence in a small population of mitochondria as described in methods and materials. The arrow head indicates the yellowish mitochondria formed by the fusion between red and green mitochondria. D, The quantitative data shows that the mitochondrial fusion rate in STHdhQ111/Q111 cells is significantly reduced compared to STHdhQ7/Q7 cells. The data was acquired by analysis of more than 20 cells of each cell type from five independent experiments. Data shown are mean ± SE. **P<0.01, ***P<0.001 vs. STHdhQ7/Q7.

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Nrf2 Signaling Pathway is Impaired in STHdh<sup>Q111/Q111</sup> Cells

Nrf2 is a key player in redox regulation and defense mechanisms against ROS [44,45]. Since STHdh<sup>Q111/Q111</sup> cells show altered mitochondrial dynamics and increased susceptibility to oxidative stress, we investigated whether the Nrf2 signaling pathway was compromised in STHdh<sup>Q111/Q111</sup> cells. Nrf2 activity was first assessed using an ARE luciferase reporter. STHdh<sup>Q111/Q111</sup> cells exhibited significantly reduced ARE activity at the basal level compared to STHdh<sup>Q7/Q7</sup> cells (Fig. 5A). Next, we tested whether the Nrf2 signaling pathway was compromised in STHdh<sup>Q111/Q111</sup> cells. The overexpression of Nrf2 resulted in a significantly greater increase in ARE activity in STHdh<sup>Q7/Q7</sup> cells compared to STHdh<sup>Q111/Q111</sup> cells, indicating an impairment in the Nrf2 signaling pathway in STHdh<sup>Q111/Q111</sup> cells (Fig. 5B). To confirm that the impaired Nrf2 signaling is due to the presence of mHtt, we measured ARE activity in additional striatal cell lines. Two new striatal cell lines expressing mHtt (1A and 6L) exhibited a substantial reduction in ARE activity in the absence or the presence of the Nrf2 agonist sulforaphane compared to two new striatal cell lines expressing Htt (B3 and E4) (Fig. 5C). Furthermore, the ectopic expression of N-terminal mHtt led to a significant reduction in ARE activity in STHdh<sup>Q7/Q7</sup> and HEK cells (Fig. 5D). These results demonstrate that the presence of mHtt leads to the disturbance of Nrf2 signaling.

Nrf2 Activation in Response to Oxidative Stress is Impaired in STHdh<sup>Q111/Q111</sup> Cells

Nrf2 is activated by oxidative stress. Therefore, we next determined whether mHtt affects Nrf2 activity in response to oxidative stress. Striatal cells were treated with tert-butylnhydroquinone (tBHQ) and Cu<sup>2+</sup> and ARE activity was measured as described in materials and methods. STHdh<sup>Q7/Q7</sup> cells showed a dramatic increase in ARE activity in a dose dependent manner.
in response to oxidative stress, while STHdhQ111/Q111 cells showed significantly less of an increase in ARE activity at low concentrations of tBHQ and did not exhibit further increases in ARE activity when higher concentrations were used (Fig. 6). This result suggests that the disturbance of Nrf2 signaling renders STHdhQ111/Q111 cells more susceptible to oxidative stress.

Disturbed Signaling of Nrf2 in STHdhQ111/Q111 Cells is not due to its Expression Level

Although Nrf2 activity was compromised in the STHdhQ111/Q111 cells, there were no detectable differences in protein expression levels between the two cell types (Fig. 7A). Since Nrf2 activity was examined in the two additional lines for each striatal cell type, STHdhQ111/Q111 cell lines (1A and 6L) show significantly reduced ARE activity as the basal level compared to STHdhQ7/Q7 cells (B3 and E4). Treatment with sulforaphane (SFN), an agonist of Nrf2, increases ARE activity in STHdhQ7/Q7 cells to a greater extent than in STHdhQ111/Q111 cells. n = 3 D. Transient transfection of mHtt with 63 N-terminal amino acids results in a significant reduction in the ARE luciferase activity in STHdhQ111/Q111 as well as in HEK cells. n = 3. Data shown are mean ± SE. **P < 0.01, ***P < 0.001 vs. mCherry.

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STHHdhQ7/Q7 cells show a dramatic increase in ARE activity in response to 
levels of p62 were not different between the two cell types (Fig. 7) 
reduced protein levels may indicate increased signaling of 
and B)

phRL-TK were transfected into striatal cells and oxidative stress was 
shown, p62 expression results in a slight but significant increase in 
autophagy can increase Nrf2 activity [54]. Therefore, we 
signaling negatively interacts with autophagy as inhibition of 
both autophagy or proteasome [52]. p62 can also activate Nrf2 
molecule that targets polyubiquitylated proteins for degradation by 

using the striatal cell lines, showed a decrease in these genes [8,47– 
49]. This discrepancy is likely ascribable to the different forms of 
mHtt that were expressed (full-length vs highly truncated) as well 
as the different cell types that were used. Nonetheless, the findings 
in the different models all indicate that a pathological poly-
glutamine expansion results in increased oxidative stress. In addition, and unexpectedly, mRNA levels of Keap1 were 
significantly increased in STHdhQ111/Q111 cells, while mRNA 
levels of p62 were not different between the two cell types (Fig. 7D), 
despite a significant reduction in their protein expression (Fig. 7, A 
and B). Since Keap1 and p62 are degraded by autophagy, the 
reduced protein levels may indicate increased signaling of 
autophagy in STHdhQ111/Q111 cells [50,51], p62 is an adaptor 
molecule that targets polyubiquitylated proteins for degradation by 
either autophagy or proteasome [32], p62 can also activate Nrf2 by 
inhbiting the binding of Keap1 to Nrf2 [53]. In addition, Nrf2 
signaling negatively interacts with autophagy as inhibition of 
autophagy can increase Nrf2 activity [54]. Therefore, we 
examined whether the modulation of autophagy signaling can 
differentially influence Nrf2 activity in striatal cells. As previously 
shown, p62 expression results in a slight but significant increase in 
Nrf2 activity in both cell types (Fig. 7E) [53,54]. Inhibition of 
autophagy by treatment with 3-methyladenine (3-MA) or chloro-
quine (CQ) resulted in a significant increase in Nrf2 activity 
in both cell types, while the activation of autophagy by treatment 
with rapamycin (RP) or triluoroperazine (TFP) did not change Nrf2 
signaling (Fig. 7E). However, the ectopic expression of p62 or 
inhibition of autophagy failed to recover Nrf2 signaling in 
STHdhQ111/Q111 cells to the levels observed in STHdhQ2/Q2 
cells. Together, this result suggests that p62 and autophagy 
signaling are not the major contributing factors to impaired Nrf2 
signaling in STHdhQ111/Q111 cells.

Nrf2 Expression Attenuates the Fragmentation of 
Mitochondria

Given that STHdhQ111/Q111 cells display an increase in 
fragmented mitochondria and more oxidized mitochondria, we 
investigated whether Nrf2 expression modifies mitochondrial 
morphology (Fig. 8). Striatal cells were transfected with mito-
mCherry and Nrf2, Keap1, p62, or GFP as a control. Nrf2 
expression results in a significant reduction in fragmented 
mitochondria in STHdhQ111/Q111 cells and a similar trend of 
reduction in STHdhQ2/Q2 cells. Conversely, Keap1 expression 
appeared to increase the number of cells with fragmented 
mitochondria, although it was not statistically significant. The 
expression of p62 did not lead to any obvious alteration in 
mitochondrial morphology in either cell type. This result suggests 
that oxidative stress which can be in part modulated by Nrf2 signaling. 
This observation is in line with the previous study showing that 
oxidative stress caused by 3-NP increases mitochondrial fragmen-
tation, leading to neuronal cell death and that blockade of ROS 
oblishes mitochondrial fragmentation as well as cell death [55].

Autophagy Signaling is Highly Activated in STHdhQ111/ 
Q111 Cells

We next examined whether autophagy regulation is altered in 
STHdhQ111/Q111 cells compared to STHdhQ2/Q2 cells. First, we 
looked at the conversion of LC3-I into LC3-II as an indicator of 
autophagy activation. Although the total level of LC3 is 
significantly reduced in STHdhQ111/Q111 cells, the ratio of LC3-
II to LC3-I appears to be increased in STHdhQ111/Q111 cells 
compared to STHdhQ2/Q2 cells (Fig. 9, A and B). To further assess 
the autophagy activation, we transfected GFP-LC3 into striatal 
cells and monitored the cellular distribution of GFP-LC3. As 
The activation of autophagy increases, GFP-LC3 relocates from 
the cytosol into autophagosome, resulting in the punctate pattern. We 
found that STHdhQ111/Q111 cells show numerous GFP-LC3 
positive punctae, while STHdhQ2/Q2 cells showed only a few 
GFP-LC3 positive punctae (Fig. 9C). Together, these results 
indicate that STHdhQ111/Q111 cells exhibit highly activated 
autophagy signaling.

Discussion

Mitochondrial impairment is emerging as a contributing factor to 
the pathogenesis of HD. A growing number of studies using 
mouse and cell models for HD, as well as tissues from HD patients, 
have shown that mHtt causes mitochondrial functional impair-
ment such as hypersensitivity to Ca2+ induced permeability 
transition pore opening, reduced Ca2+ buffering capacity, deficits 
in the electron transport chain complexes and impaired mito-
chondrial bioenergetics [2–4]. An important factor mediating 
mitochondrial function is mitochondrial dynamics. An appropriate 
balance in mitochondrial fusion and fission is essential for cells to 
maintain metabolic states and homeostasis, and to respond to 
cellular stresses [12]. Recently, it demonstrated that the presence 
of mHtt leads to mitochondrial fragmentation in in vitro and in vivo 
HD models including HeLa cells and rat cortical neurons 
expressing mHtt, fibroblasts and lymphoblasts from HD patients, 
striatal progenitor cell lines, and YAC128 mouse [14,19,56]. 
Our finding of an increase in fragmented mitochondria with decreased 
level of tubular mitochondria in STHdhQ111/Q111 cells, as well as 
increased fragmented mitochondria in the striata of R6/2 mice, is 
in line with these previous studies. In addition, studies from two 
recent papers indicate that enhanced activation of Drp1 by mHtt 
causes mitochondrial fragmentation. Song et al. suggested that 
mHtt directly interacts with Drp1, which in turn increases its 
activity, whereas Costa et al. proposed the increased activity of 
calcineurin, a phosphatase which dephosphorylates Drp1, as 
a cause of increased Drp1 activity [14,19]. Increased steady state 
levels of mitochondrial fragmentation can be induced by increased 
fission, reduced fusion, or both. Their studies strongly support the
idea of increased fission as one possible route to mitochondrial fragmentation. However, these studies do not rule out reduced fusion as an alternative pathway that contributes to mitochondrial fragmentation. To our knowledge we provide the first evidence that the expression of mHtt in striatal precursor cells negatively impacts mitochondrial fusion. Furthermore, we found that the overall levels of Opa1 are significantly reduced in STHdh^{Q111/Q111} cells compared to STHdh^{Q7/Q7} cells, suggesting that reduced Opa1 could be causally involved in the reduced fusion process. Moreover, it has been shown that Opa1 expression attenuates the apoptosis in HD models, but Mfn1 expression did not despite correction of mitochondrial fragmentation, suggesting deficits in Opa1 may be a contributing factor to the mitochondrial fragmentation in HD [19].

It has been speculated that enhanced oxidative stress may play a significant role in HD pathogenesis [5,57]. Mitochondria play important roles in the defense against oxidative stress. A previous study showed that oxidative stress increases mitochondrial fragmentation in HeLa cells expressing mHtt [56]. In contrast, we observed a rapid increase in the population of STHdh^{Q111/Q111} cells containing swollen mitochondria rather than fragmented mitochondria in response to oxidative stress. STHdh^{Q7/Q7} cells actually display an increase in fragmented mitochondria in response to oxidative stress, followed by a slow increase in swollen mitochondria. This result implies that upon cellular stresses, mitochondrial fragmentation would be a prerequisite for mitochondrial swelling, an event that leads to the pathogenesis. Dysregulation of transcriptional processes that govern mitochondrial biogenesis and antioxidant system would result in mitochondrial impairment that would be exacerbated by cellular oxidative stress. PGC-1α is a transcriptional coactivator that plays important roles in mitochondrial biogenesis as well as defense mechanism against oxidative stress and was discovered to be repressed in models of HD [58]. In addition, we previously found that STHdh^{Q111/Q111} cells exhibit a deficit in the PPARγ pathway [8,11] that regulates the expression of genes involved in mitochondrial function, ROS regulation, and fatty acid metabolism [59,60]. Given that transcriptional dysregulation is tightly linked with mitochondrial impairment as well as defects in antioxidant response, we sought to find additional transcriptional regulator that play important roles in these pathways and may be compromised in HD. Nrf2 has been considered as a key regulator in ROS defense mechanism and mitochondrial function. We discovered that Nrf2 signaling is severely compromised by the presence of mHtt. More importantly, STHdh^{Q111/Q111} cells fail to activate Nrf2 in response to oxidative stress. This result provides...
Seem to have any effect. More than 200 cells of each cell type were mitochondria in the both cell types, while p62 expression does not a strong trend of increase in the number of cells with fragmented mitochondria in the striatal cells. In contrast, Keap1 expression results in expression of Nrf2 reduces the number of cells with fragmented morphology of mitochondria was assessed. A-B, The exogenous expression of Nrf2 reduces the number of cells with fragmented mitochondria in the striatal cells. In contrast, Keap1 expression results in a strong trend of increase in the number of cells with fragmented mitochondria in the both cell types, while p62 expression does not seem to have any effect. More than 200 cells of each cell type were assessed for each independent experiment. n = 4. Data shown are mean ± SE. *P < 0.05 vs. GFP.

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Figure 8. Nrf2 expression attenuates the fragmentation of mitochondria in striatal cells. Striatal cells were transfected with mito-mCherry as well as GFP, Nrf2, Keap1, or p62. Two days after transfection, cells were fixed with 4% paraformaldehyde and the morphology of mitochondria was assessed. A-B, The exogenous expression of Nrf2 reduces the number of cells with fragmented mitochondria in the striatal cells. In contrast, Keap1 expression results in a strong trend of increase in the number of cells with fragmented mitochondria in the both cell types, while p62 expression does not seem to have any effect. More than 200 cells of each cell type were assessed for each independent experiment. n = 4. Data shown are mean ± SE. *P < 0.05 vs. GFP.

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important insight into a potential contributing factor to the apparent impaired ability of cells to cope with oxidative stress when mHtt is present.

Two well known regulators of Nrf2 signaling are Keap1 and p62. Keap1 is an inhibitor that binds to Nrf2, leading to degradation through the ubiquitin-proteasome system. p62 is an activator that competes with Nrf2 for Keap1 binding and sequesters Keap1 into aggregates [53,54]. To gain insights into the mechanism of how Nrf2 signaling is compromised in STHdhQ111/Q111 cells and to provide insights into involvement of defects in mitochondrial dynamics in the pathogenic mechanisms of mHtt.

Materials and Methods

Ethics Statement

All animal protocols have been approved by the UCAR at the University of Rochester (UCAR# 2006-102R).
Materials and Antibodies

Sulforaphane (SFN), 3-methyladenosine (3-MA), and rapamycin (RP) were purchased from Calbiochem. tert-butylhydroquinone (tBHQ), chloroquine (CQ), and trifluoperazine (TFP) were obtained from Sigma. All other chemicals were purchased from Sigma (St. Louis, MO, USA), if not otherwise indicated. Anti-Nrf2, anti-Keap1, anti-Mfn2, anti-p62/SQSTM1, anti-GFP, and anti-actin antibodies were purchased from Abcam, Proteintech, Sigma, Biomol, Roche, and Chemicon, respectively. Antibodies for pDrp1 (S616) and LC3 were acquired from Cell Signaling Technology. Antibodies for Opa1 and Drp1 were purchased from BD Biosciences.

Plasmid Constructs

rosGFP1 and rosGFP2 were kindly provided from Dr. Roderick A. Capaldi [43]. pEGFP-mitoGFP was a kind gift from Dr. Yisang Yoon. pGL3-ARE was a kind gift from Dr. Martin Leonard [70]. pcDNA3-EGFP-C4-Nrf2 and pcDNA3-HA2-Keap1 were obtained from Addgene [71]. Myc-p62 and myc-p62 (G352A) were kindly provided by Dr. Terje Johansen [61]. Mito-mCherry and

Figure 9. STHdhQ111/Q111 cells exhibit an increase in autophagy activity. A–B. The increase of LC3-I into LC3-II is a typical indicator of the autophagy. The total amount of LC3 including type I and II is significantly higher in STHdhQ7/Q7 cells than STHdhQ111/Q111 cells. In addition, the ratio of type II to type I seems to be greater in STHdhQ111/Q111 cells than in STHdhQ7/Q7 cells. n = 4. Data shown are mean ± SE. *P<0.05 vs. STHdhQ7/Q7. C. Cells were transfected with GFP-LC3 (microtubule-associated protein 1 light chain 3) and 24 h later GFP fluorescence images were monitored. LC3-I is a cytosolic form of LC3 and LC3-II is a membrane-bound form of LC3 that is implicated in autophagy. The localization of GFP-LC3 is the diffused cytosolic pattern in most of STHdhQ7/Q7 cells, while STHdhQ111/Q111 cells exhibit punctuate pattern of GFP-LC3 that indicates the active autophagic vesicles.

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photoconvertible mitoDendra were constructed by subcloning the mCherry from pH6.II-mCherry (a kind gift of Dr. Keith Nehrke) and the Dendra from pDendra2-C (Evrogen) using PCR. The resulting PCR products were ligated into the AgeI and BsrG sites of pEGFP-mitoGFP. GFP-LEC3 was previously described [72]. Expression constructs of truncated huntingtin with N-terminal 63 amino acids, pcDNA3.1-N63-Q19 and pcDNA3.1-N63-Q99 have been previously reported [73].

Cell Culture
The immortalized homozygote striatal cell lines, STHdhQ7/Q7 (the original one, B3, E4) and STHdhQ111/Q111 (the original one, A1, A4) made from striatal primordia of E14 mouse embryos expressing Htt with 7 polyQ or mHtt with 111 polyQ were kindly provided by Dr. Marcy MacDonald [74]. Cells were cultured in DMEM containing 25 mM glucose and 4 mM glutamine (Invitrogen) supplemented with 4% fetal bovine serum (FBS, HyClone, Waltham, MA, USA) and 4% bovine growth serum (BGS, HyClone), and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and maintained at 33°C and 5% CO2.

HEK 293-TN cells were grown in DMEM supplemented with 10% FBS, and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Cell Fusion Assay
To monitor mitochondrial fusion, cell fusion was carried out as previously described with modification [7,75]. In brief, STHdhQ7/Q7 or STHdhQ111/Q111 cells were plated in two of 35 mm dish. The next day, mitoGFP or mito-mCherry was transfected in each dish using Lipofectamine 2000 following the manufacturer’s protocol. After 24 h, cells in both dishes were trypsinized, combined, and plated together onto 2–3 wells of 12 well plate containing a glass-coverslip in each well. The next day, cells were treated with 50 μg/ml of cycloheximide for 30 min prior to cell fusion to prevent protein synthesis. For fuse cells, prewarmed 50% w/v polyethylene glycol (PEG) 4000 in G-HBSS (20 Hepes, pH 7.4, 137 NaCl, 5 KCl, 0.5 KH2PO4, 0.5 Na2HPO4, 10 mM glucose) was added to cells for 90 s. The cells were washed twice, harvested in ice cold phosphate-buffered saline (PBS) and lysed with modified RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.4% SDS, 0.2% sodium deoxycholate, 5% glycerol, 1 mM EDTA, 2 mM NaF, 2 mM Na3VO4) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). The lysates were sonicated, cleared by centrifugation, and assayed to determine protein concentration using BCA assay (Pierce Bio-technology, Waltham, MA, USA). Proteins (10–100 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), and incubated with the specific antibodies in TBST containing 2% BSA or skim milk at 4°C overnight. After washing three times, HRP-conjugated secondary antibody (1:3000) in TBST containing 5% skim milk was applied and the blot was visualized by chemiluminescence. The intensity of immunoreactive bands was quantified by using Image J software.

Mitochondrial Fusion Assay with MitoDendra
Striatal cells were plated on 12 well plates containing a glass coverslip in each well. MitoDendra was transfected 1–2 d after plating. The next day, a glass coverslip was placed on the microscope (Observer D1, Zeiss) and the green/red fluorescence of mitochondria was observed with a 63× oil objective. To photoconvert a small area in mitochondria was chosen and the aperture was minimized. The green fluorescence was photoconverted into the red fluorescence by illuminating mitochondria using a 400 nm LED with 100% power controlled by Colibri system (Zeiss) for 6 min. The mitochondrial images were taken by a digital CCD camera (ORCA-ER, Hamamatsu Photonics) every 30 s for 30 min with 2 digital gain factor. The green fluorescence was obtained by using a 470 nm LED with 3–5% power for 20–30 ms exposure and a 525/20 nm emission filter. The red fluorescence was imaged with a 530 nm LED with 100% power for 10–20 ms exposure and a 630/75 emission filter. The time-lapse merged images from green and red channels were generated using imageJ. A green mitochondrion which encounters a red mitochondrion resulting in colocalization (yellow mitochondrion) was considered as a mitochondrial fusion event.

Ratiometric Measurement of Mitochondrial Matrix Redox State using Mitochondrial Targeted GFP Sensors
Cells were plated on glass coverslips and transfected with rosGFP1, or rosGFP2 [43] using Lipofectamine 2000 according to manufacturer’s instruction. Th next day, media were removed and replaced with G-HBSS for 30 min at 37°C. The fluorescence from mitochondria was aged at 63× magnification on an Observer D1 microscope (Zeiss) coupled with a digital CCD camera (ORCA-ER, Hamamatsu). The emission was detected through a 525/20 nm filter with excitation consecutively from a 470 nm LED and a 400 nm LED controlled by a Colibri system (Zeiss) with the same exposure time. Fluorescence images were background corrected, mitochondrial images were selected, and emission intensities from 470 nm and 400 nm were acquired using ImageJ software. The ratio of emission intensities (470/400) was calculated in Microsoft Excel software. To generate a standard curve for the measurement of matrix redox state, cells were treated for 30 min in G-HBSS containing 5 mM H2O2 or 10 mM DTT to obtain the maxima of oxidation and reduction, respectively. The standard curves were generated using Sigmaplot (Systat Software).

Western Blot Analysis
Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.4% SDS, 0.2% sodium deoxycholate, 5% glycerol, 1 mM EDTA, 2 mM NaF, 2 mM Na3VO4) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). The lysates were sonicated, cleared by centrifugation, and assayed to determine protein concentration using BCA assay (Pierce Bio-technology, Waltham, MA, USA). Proteins (10–100 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), and incubated with the specific antibodies in TBST containing 2% BSA or skim milk at 4°C overnight. After washing three times, HRP-conjugated secondary antibody (1:3000) in TBST containing 5% skim milk was applied and the blot was visualized by chemiluminescence. The intensity of immunoreactive bands was quantified by using Image J software.

Nuclear Fractionation
Nuclear fractionation was carried out as previously described [76] with slight modifications. Briefly, STHdhQ7/Q7 and STHdhQ111/Q111 cells were washed twice, harvested in ice cold PBS and the cell pellets were resuspended in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 0.05% Nonidet P-40, 1 mM EGTA) with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each of aprotinin, leupeptin, pepstatin A). A sample was taken and used as whole cell lysate. The cells were triturated followed by centrifugation at 380 × g for 5 min at 4°C. The supernatants were collected and used as the cytosolic fractions. The pellets were washed once in lysis buffer and twice in wash buffer (30 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl2, 1 mM EGTA, 25 mM NaCl) with protease inhibitors. The crude nuclei were overlaid on the top of 0.8 M
sucre with protease inhibitors, and spun at 1200 x g for 10 min at 4°C. The pellets were collected and resuspended in buffer B (300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 1 mM EGTA, 25 mM NaCl, 0.5% Triton X-100) with protease inhibitors and used as the nuclear fractions. 20 μg of protein from each sample was visualized by immunoblot analysis.

Dual-luciferase Reporter Assays

Cells were plated in 24 well plates. The next day, a reporter plasmid pGL3-ARE and a normalizing plasmid phRL-TK (Promega) were transfected using Lipofectamine 2000. The next day, cells were treated with vehicle control or drug. After 24 h, cells were lysed with Passive lysis buffer (Promega), and the reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The reporter activity from Firefly luciferase was normalized with the Renilla luciferase activity.

RNA Isolation, Reverse Transcription, and Real-time PCR

Cells were plated in 60 mm dish and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instruction. Extracted total RNA was treated with RNase free-DNaseI Amplification Grade (Invitrogen) to remove contaminating DNA. Two micrograms of total RNA was reverse transcribed using SuperScriptIII reverse transcriptase and random hexamers using DNA. Two micrograms of total RNA was reverse transcribed into cDNA using TRIzol (Invitrogen) according to the manufacturer’s instructions. The reaction mixture was diluted with 500 μl of DEPC-treated H₂O. The PCR reaction was prepared in triplicate containing 10 μl of diluted cDNA, 2.5 μl of 2.5 μM primer mixture (forward and reverse), and 12.5 μl of SYBR GreenER qPCR SuperMix (Invitrogen) in 96 well optical PCR reaction plate (Bio-Rad). PCR reactions were performed in Mastercycler ep real-time PCR system (BioRad). Amplification conditions consisted of an initial hot start at 95°C for 15 s, 60°C for 1 min followed by amplification of 45 cycles (95°C for 1 s, 60°C for 20 s, and 72°C for 40 s). Melting curve analysis was performed immediately after amplification. The relative amount of mRNAs was calculated by using the ΔΔCt (Ct, threshold cycle) method. The Ct value of β-actin was used for normalization. The sequences of primers are as follows. Nrf2-F: TCACACGAGATGAGCTTGGGCA, Nrf2-R: TA- CAGTTCTGGGCGGACATTTAT; Keap1-F: TTAAGGC- CATGTTCACCAACGGGC, Keap1-R: TTAAGGCGCATTT- CACCAACGGGC, p62-F: TGAAATCTGAGCATTGTCGAGC, p62-R: ACATGGG- GATCTTCTGGTGGAGCA; HO-1-F: TCCTGTCAGAT- GAACACTCTTGA, HO-1-R: TGTGTCTTCTTGTCAG- CATCACCT; Nqo1-F: CAAATCCCTGTCAGGACACT; Nqo1-R: TGTGATGTTGGGAATGGGTCAGAA, Nqo1-R: TGTGATGGCCAGATCTTCTCCATGT.

Measurement of Mitochondria in R6/2 and Wild Type Mouse Brain

Approximately 13 weeks old transgenic R6/2 mice over-expressing exon 1 of mHtt and their wild type littermates were transcardially perfused with paraformaldehyde/glutaraldehyde. Their brains were subsequently processed and sectioned at 100 μm thickness. Striatal sections were incubated with an antibody (EM48, Chemicon) recognizing huntingtin followed by a secondary antibody conjugated to 1.4 nm gold particles. Sections were silver intensified, osmicated in 1% OsO₄, dehydrated and embedded in Eponate 12. Ultrathin sections (30 nM) were cut and counterstained with 5% aqueous uranyl acetate followed by Reynolds lead citrate prior to being examined and captured by the Transmission Electron Microscope Hitachi 7650. Each mitochondrion was measured using Image-Pro. All mitochondria were grouped into different size bins (C) and or shape from 2 mice/genotype). Their brains were subsequently processed and sectioned at 100 μm thickness. Striatal sections were incubated with an antibody (EM48, Chemicon) recognizing huntingtin followed by a secondary antibody conjugated to 1.4 nm gold particles. Sections were silver intensified, osmicated in 1% OsO₄, dehydrated and embedded in Eponate 12. Ultrathin sections (30 nM) were cut and counterstained with 5% aqueous uranyl acetate followed by Reynolds lead citrate prior to being examined and captured by the Transmission Electron Microscope Hitachi 7650. Each mitochondrion was measured using Image-Pro. All mitochondria were grouped into different size bins (C) and shape from 2 mice/genotype). The Ct value of β-actin was used for normalization. The sequences of primers are as follows. Nrf2-F: TCACACGAGATGAGCTTGGGCA, Nrf2-R: TTAAGGC- CATGTTCACCAACGGGC, Keap1-F: TTAAGGCGCATTT- CACCAACGGGC, p62-F: TGAAATCTGAGCATTGTCGAGC, p62-R: ACATGGG- GATCTTCTGGTGGAGCA; HO-1-F: TCCTGTCAGAT- GAACACTCTTGA, HO-1-R: TGTGTCTTCTTGTCAG- CATCACCT; Nqo1-F: CAAATCCCTGTCAGGACACT; Nqo1-R: TGTGATGTTGGGAATGGGTCAGAA, Nqo1-R: TGTGATGGCCAGATCTTCTCCATGT.

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