HD CAG repeat implicates a dominant property of huntingtin in mitochondrial energy metabolism

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The ‘expanded’ HD CAG repeat that causes Huntington’s disease (HD) encodes a polyglutamine tract in huntingtin, which first targets the death of medium-sized spiny striatal neurons. Mitochondrial energetics, related to N-methyl-D-aspartate (NMDA) Ca2+-signaling, has long been implicated in this neuronal specificity, implying an integral role for huntingtin in mitochondrial energy metabolism. As a genetic test of this hypothesis, we have looked for a relationship between the length of the HD CAG repeat, expressed in endogenous huntingtin, and mitochondrial ATP production. In STHdhQ111 knock-in striatal cells, a juvenile onset HD CAG repeat was associated with low mitochondrial ATP and decreased mitochondrial ADP-uptake. This metabolic inhibition was associated with enhanced Ca2+-influx through NMDA receptors, which when blocked resulted in increased cellular [ATP/ADP]. We then evaluated [ATP/ADP] in 40 human lymphoblastoid cell lines, bearing non-HD CAG lengths (9–34 units) or HD-causing alleles (35–70 units). This analysis revealed an inverse association with the longer of the two allelic HD CAG repeats in both the non-HD and HD ranges. Thus, the polyglutamine tract in huntingtin appears to regulate mitochondrial ADP-phosphorylation in a Ca2+-dependent process that fulfills the genetic criteria for the HD trigger of pathogenesis, and it thereby determines a fundamental biological parameter—cellular energy status, which may contribute to the exquisite vulnerability of striatal neurons in HD. Moreover, the evidence that this polymorphism can determine energy status in the non-HD range suggests that it should be tested as a potential physiological modifier in both health and disease.

INTRODUCTION

Huntington’s disease (HD) is inherited as a autosomal dominant disorder, which features progressive writhing movements and cognitive and psychiatric decline caused by the inexorable demise of neurons in the brain, which is first manifested in the striatum (1). All HD cases possess, in at least one of their two copies of the HD gene, a version of a polymorphic CAG repeat tract in exon 1, which is ≥35 units in length (2). These ‘expanded’ CAG tracts, together with penultimate CAA and CAG codons, encode a polyglutamine segment of 37 (or more) residues in huntingtin (2), a large HEAT domain protein (3), which initiates the disease process in a dominant, polyglutamine-size dependent manner (4).

A fundamental question that promises insights into the nature of the trigger mechanism is why medium-sized spiny striatal neurons are the first to succumb to the effects of the HD CAG, even though normal and mutant huntingtins are found in most, if not all, cells (2,5). As CAG encoded ‘expanded’ polyglutamine tracts in unrelated protein contexts target different brain neurons, causing distinct clinical entities (6), we have argued that the neuronal specificity of HD must reflect a dominant huntingtin-context dependent activity of the polyglutamine tract, which in conjunction with some
special features of striatal cells conspires to kill them before other cells (4).

Medium-sized spiny striatal neurons are especially vulnerable to systemically administered mitochondrial toxins, revealing an extraordinary response of these cells to energy deficit (7,8). This may involve chronic activation of N-methyl-D-aspartate (NMDA) receptors, due to decreased voltage gating, with sustained calcium influx producing cellular ‘excitotoxicity’ (9–11). The circumstantial evidence to support this explanation is considerable. The activities of respiratory chain complexes are decreased in HD postmortem brain (12), and markers of energy metabolism are altered in HD brain, muscle (13–18) and lymphoblastoid cell lines (19). Moreover, genetic HD mouse models that express full-length mutant huntingtin, HD 4p16.3 YAC transgenic mice and lines of Hdh CAG knock-in mice carrying juvenile onset HD CAG repeats, exhibit a host of striatal specific disease phenotypes that precede overt striatal pathology (20). Among the earliest are enhanced NMDA receptor activation and aberrant mitochondrial and intracellular Ca2+ handling (21–26).

A key prediction of the energy-excitotoxicity hypothesis of HD is that the dominant HD CAG repeat mechanism that triggers disease onset will be found to influence mitochondrial ATP synthesis, with a response in striatal cells that entails enhanced NMDA receptor activation and Ca2+-influx.

To critically evaluate this hypothesis, we have performed genotype–phenotype studies in accurate genetic HD cell systems, investigating the relationship between the HD CAG repeat and mitochondrial energy metabolism. We have used immortalized STHdhQ111 striatal cells (27), from HdhQ111 knock-in mice, to examine the potential impact on NMDA receptor activation. These cells express endogenous mutant huntingtin with 111-glutamines and exhibit decreased cellular ATP and [ATP/ADP] and a marked vulnerability to mitochondrial ADP-uptake from the cytosol.

Our findings implicate huntingtin, and the dominant mechanism that triggers the disease process, in the regulation of mitochondrial energy metabolism, even in normal cells, providing essential support for the energy-excitotoxicity hypothesis of the striatal specificity of the HD mutation and suggesting that wild-type huntingtin alleles may act as modifiers of energy-dependent phenotypes in human.

RESULTS

Juvenile onset HD CAG repeat is associated with decreased mitochondrial ATP-uptake

As mentioned earlier, immortalized STHdhQ111 striatal cells, generated from HdhQ111 knock-in mice and their wild-type counterparts, STHdhQ7Q7 striatal cells provide an accurate genetic HD model system (27). Mutant STHdhQ111/Q111 striatal cells, compared with wild-type STHdhQ7Q7 striatal cells, exhibit low cellular ATP. To investigate whether this reduction might reflect decreased mitochondrial ATP production, we performed HPLC analysis of adenine nucleotides in extracts of cells incubated in media with various carbon sources. As shown in Figure 1A (left panel), the [ATP/ADP] ratio in wild-type cells cultured in glucose plus pyruvate was dramatically higher than the [ATP/ADP] ratio determined in the presence of oligomycin, an inhibitor of the mitochondrial F1F0 ATP synthase, or the [ATP/ADP] ratio determined in the presence of pyruvate as the sole carbon source. Thus, these cells generate ATP via mitochondrial respiration as well as glycolysis. In contrast, mutant cells displayed low [ATP/ADP] under all conditions, with the lowest [ATP/ADP] in pyruvate alone, demonstrating that these cells exhibit limited mitochondrial ATP synthesis.

In addition, the nucleotide data (Fig. 1A, middle and right panels) also revealed that the lower mutant cell [ATP/ADP] ratios reflected both low ATP (Fig. 1A, middle panel) and abnormally high cellular ADP, the substrate for the mitochondrial F1F0 ATP synthase (Fig. 1A, right panel). To determine the basis of this surfeit, adenine nucleotide levels were determined in purified mitochondrial preparations, which were predictably enriched in the mitochondrial marker COX4 (Fig. 1B, left panel). Despite high cellular ADP, extracts of mutant mitochondria exhibited dramatically diminished ADP and ATP (Fig. 1B, right panel), implying a block to mitochondrial ADP-uptake from the cytosol.

To explore this scenario, we assessed whether isolated mutant mitochondria could synthesize ATP if intramitochondrial ADP levels were raised. As shown in Figure 1C, exposure to very high non-physiological concentrations of exogenous ADP raised the intramitochondrial [ADP] (left panel), and supported ATP production that approached the levels detected in wild-type mitochondria (right panel), consistent with inhibition of ADP–ATP exchange at physiological cellular ADP levels. Therefore, we next examined the adenine nucleotide transporter (ANT), involved in cytosolic–mitochondrial ADP translocation, in mitochondria isolated from mutant and wild-type striatal cells. However, we did not find significant genotype-specific differences either in ANT activity or in the sensitivity to an inhibitor, atractyloside (Fig. 1D), suggesting that the block in whole mutant cells might stem from signaling pathways that regulate mitochondrial ADP–ATP exchange.

Decreased [ATP/ADP] is linked to enhanced NMDA receptor Ca2+-influx

Adenylate kinases (AKs) catalyze the fast reversible reaction [ATP] + [AMP] = 2[ADP] to facilitate ADP–ATP exchange between the cytosol and mitochondria, adjusting ATP synthesis to cellular energy demand. Consistent with high cytosolic ADP, semi-quantitative reverse transcription (RT)–PCR analysis (Fig. 2A) revealed that mutant striatal cells expressed increased levels of the brain specific cytosolic AK5 mRNA, whereas levels of the general cytosolic form (AK1) and the mitochondrial forms (AK2, AK3L1, AK3) did not differ significantly from wild-type. Moreover, we then found that parameters that regulate the membrane permeability transition pore (MPTP), such as membrane potential and Ca2+ (29), were significantly altered in mutant cells. Mitochondrial membrane potential (ΔΨm), measured by flow cytometry of
Figure 1. **STHdh^{Q111/Q111}** cells exhibit low mitochondrial ATP production because of decreased mitochondrial ADP-uptake. (A) Histograms show [ATP/ADP], [ATP] and [ADP] measured by HPLC in extracts of wild-type (open bars) and homozygous **STHdh^{Q111}** (closed bars) cells, after incubation in media with various substrates. (B) Immunoblot analysis (left panel) of α-tubulin and COX4 detected in extracts from whole cell, mitochondria and cytoplasmic fractions prepared from wild-type (ST7/7) and **STHdh^{Q111}** homozygote (ST111/111) striatal cells, showing that mitochondria were isolated without cytosolic components. Histograms (right panel) show decreased [ADP] and [ATP] in mitochondria isolated from wild-type **STHdh^{Q7/Q7}** (open bars) and **STHdh^{Q111/Q111}** (closed bars) striatal cells. (C) Histogram shows effects of external ADP treatment on ADP import (left panel) and ATP production (right panel) in mitochondria isolated from wild-type **STHdh^{Q7/Q7}** (open bars) and **STHdh^{Q111/Q111}** (closed bars) striatal cells. Addition of digitonin (Dig), to disrupt the outer mitochondrial membrane, eliminated the wild-type and mutant genotype differences [ADP] and [ATP]. (D) Mitochondria isolated from wild-type and mutant striatal cells were loaded with ^3^H-labeled ATP, washed and incubated in the presence or absence of variable doses of non-radioactive ADP, followed by the determination of ATP release into the mitochondrial suspension (left panel), with the calculated (%) ADP–ATP translocation plotted against ADP concentration for wild-type **STHdh^{Q7/Q7}** (open squares) and **STHdh^{Q111/Q111}** (closed squares). The inhibition rate of ADP import versus atractyloside concentration in isolated mitochondria is plotted (right panel), showing the relative inhibition for wild-type **STHdh^{Q7/Q7}** (open squares) and **STHdh^{Q111/Q111}** (filled squares). Results are representative of three independent determinations. Values are the mean ± SD of triplicate determinations. Statistical significance: *P < 0.05; **P < 0.005.
tetramethylrhodaminemethylester (TMRM) fluorescence, was significantly decreased (Fig. 2B), whereas intracellular Ca$^{2+}$, measured by flow cytometry of Fluo-3 dye treated cells, was significantly elevated (Fig. 2C), implicating high intracellular Ca$^{2+}$ in the block to mitochondrial ADP-uptake.

To evaluate this possibility, we assessed the response of mutant and wild-type striatal cells to NMDA. As shown in Fig. 2C, mutant cells displayed enhanced activation of NMDA receptor Ca$^{2+}$-channels, as revealed by a heightened response to NMDA, which further elevated the abnormally
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**Figure 3.** HD CAG size determines [ATP/ADP] in lymphoblastoid cells. (A) Scatter plot of [ATP/ADP] ratio detected in extracts of lymphoblastoid cells established from five normal individuals (control) and two homozygous HD individuals (HD). The control group yielded a mean [ATP/ADP] of 3.72 ± 0.60, and HD group yielded a mean [ATP/ADP] of 1.79 ± 0.22. Values are the mean ± SD of triplicate determinations. Statistical significance: *P < 0.005. (B) [ATP/ADP] values were plotted against the longer of the two CAG repeats for each of 40 control and HD heterozygote lymphoblastoid cell lines (open squares), showing the relationship between [ATP/ADP] ratio and the number of HD CAG triplets. The values obtained for the HD homozygote cell lines are plotted for the longer of the two expanded HD CAG alleles (closed squares). Error bars indicate mean ± SD for quadruplicate determinations for each sample.

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**Dominant HD CAG dependent mechanism determines energy status**

To critically assess the role of huntingtin and to test the relevance of the energy phenotype observed in the homozygous mutant striatal cells to HD, we examined the relationship of the HD CAG to [ATP/ADP] ratios measured in human lymphoblastoid cell lines. The potential effect of disease-causing alleles was assessed in three HD homozygote cell lines (23–50 CAGs) and five control cell lines (16–29 CAGs). The results, shown in Fig. 3A, disclosed a significant ~2-fold lower mean [ATP/ADP] in HD homozygote samples, confirming that two copies of the HD mutation are associated with a decreased cellular energy status.

To determine whether the underlying process might fulfill the HD genetic criteria delineated previously for the onset of clinical symptoms, [ATP/ADP] ratio was assayed in extracts of 40 HD CAG heterozygote lymphoblastoid cell lines, of which 17 had CAGs spanning only in the normal range (16–34 units) and 23 had CAGs in the disease range (35–70 units), in addition to a normal allele. The mean [ATP/ADP] for the group with disease-range CAGs was significantly lower than that for the samples with HD CAGs in the normal range (2.37 ± 0.31 and 3.34 ± 0.55, respectively), indicating that a single expanded allele was sufficient to yield low energy values. In addition, when the relationship to HD CAG repeat size was examined, as illustrated in the plot of [ATP/ADP] versus the length of the longer HD CAG allele in each sample (Fig. 3B), the energy values decreased inversely with repeat size. Moreover, the HD homozygote [ATP/ADP] ratios did not differ greatly from those measured in HD heterozygote lines, with a single similar sized CAG repeat (Fig. 3B), consistent with a dominant mechanism. Notably, [ATP/ADP] was not significantly related to other parameters, such as the size of the shorter HD CAG allele, gender or age when the cell line was established (Materials and Methods).

These observations demonstrate that the mechanism that determines cellular [ATP/ADP] fulfills the genetic HD criteria, operating in a dominant manner, which is more sensitive to CAG size than to gene dosage. These data provide strong support that reduced cellular [ATP/ADP] may be a consequence of the same property of the polyglutamine tract in huntingtin, which triggers the striatal specific HD disease process. However, in contrast to the HD CAG threshold for the onset of clinical symptoms, the apparently continuous relationship with [ATP/ADP] reveals that the dominant HD CAG-dependent mechanism may act over the entire range of allele sizes, strongly implicating wild-type huntingtin in mitochondrial energy metabolism.

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**DISCUSSION**

The selectivity of the HD CAG mutation for medium-sized spiny neurons in the striatum has long been thought to
reflect a special response of these cells to decreased mitochondrial ATP production, with elevated activation of NMDA receptors leading to chronic high intracellular Ca\(^{2+}\) levels and concomitant ‘excitotoxicity’ (11,30). This hypothesis predicts that huntingtin, because of a dominant property of the polyglutamine tract, will be found to play a role in mitochondrial energy metabolism, with consequences for NMDA receptor Ca\(^{2+}\)-influx. The purpose of this study was to provide a genetic test of these predictions by assessing mitochondrial ATP synthesis in cells expressing endogenous mutant huntingtin with polyglutamine segments associated with adult or juvenile onset HD, as well as those that do not cause overt disease symptoms.

We have discovered a relationship between the HD CAG repeat and mitochondrial ATP production, such that in the presence of the shorter allele, [ATP/ADP] worsens with added repeats, whether in the normal or the disease-causing range. Thus, the HD CAG repeat directly implicates huntingtin in the regulation of mitochondrial energy metabolism, via a property of the polyglutamine tract that conforms to the HD genetic criteria, even below the threshold for causing overt disease symptoms in a normal lifetime.

In STHdh\(^{Q111}\) striatal cells, the low [ATP/ADP] conferred by 111-glutamine huntingtin stems partly from enhanced Ca\(^{2+}\)-influx through NMDA receptors, as MK-801 blockade partially normalized mitochondrial ATP production. This supports a cycle in which the energy status, determined by polyglutamine tracts in huntingtin, may facilitate NMDA receptor Ca\(^{2+}\)-transport, further decreasing mitochondrial ATP synthesis. In vivo, the functionality of medium-sized spiny striatal neurons is determined by the feature that defines these neurons: the abundant (dynamic) dendritic spines that receive glutamatergic input from cortical and thalamic neurons. Moreover, spine energy status is a key regulator of synaptic plasticity and gene expression, forming the output of the projections to the globus pallidus and substantia nigra, with a transition to decreased membrane conductance state switching Ca\(^{2+}\)-signaling from AMPA receptors to NMDA receptors (31). Therefore, it seems reasonable to speculate that in vivo, above about 37-glutamines, the dominant huntingtin mechanism may confer a sufficiently compromised energy status, which sets the intrinsic spine membrane properties to favor Ca\(^{2+}\)-influx through NMDA receptors, further depressing mitochondrial energy production to ultimately shorten the lifespan of these cells before all other neurons.

Although other scenarios are possible, the differential vulnerabilities of neuronal cells in other brain regions and of cells in the periphery, in HD, may be determined by cell-type specific responses to the mitochondrial energy status conferred by huntingtin. For example, distinct populations of neuronal cells may exhibit different energy thresholds for membrane conductance states, influencing the degree to which Ca\(^{2+}\)-influx exacerbates mitochondrial ATP synthesis. In this scenario, the special reliance on membrane-energetics and their sensitivity to Ca\(^{2+}\)-toxicity would explain why neuronal cells succumb to the effects of the HD CAG repeat before peripheral cells, which appear to be relatively spared even at very long polyglutamine lengths.

Our studies did not reveal the process by which the polyglutamine tract in huntingtin regulates mitochondrial ATP synthesis. However, our findings in STHdh\(^{Q111}\) striatal cells point to decreased mitochondrial ADP-phosphorylation, as a consequence of a regulatory block to ADP-uptake from the cytoplasm. This mechanism is consistent with a recent report of decreased ADP-dependent mitochondrial respiration in STHdh\(^{Q111}\) cells, in the absence of deficits in isolated respiratory chain complexes (32). The block to mitochondrial ADP-uptake can be partially relieved by either MK-801 or forskolin, suggesting that Ca\(^{2+}\) from NMDA receptors may regulate mitochondrial ADP-uptake in a manner that can be modulated by CREB signaling. Although isolated mitochondria did not exhibit altered ANT activity, decreased ADP-uptake in whole striatal cells may involve in regulation of ANT activity (29), perhaps via effects on mitochondrial membrane potential and Ca\(^{2+}\)-handling characteristics (33), as suggested by observations with mitochondria isolated from HD lymphoblastoid cells (26,34). Another attractive possibility for the block to ADP-uptake is nuclear or mitochondrial Ca\(^{2+}\)-dependent calcium/CREB-signaling, which has been implicated in mitochondrial gene regulation (35,36). In any case, our findings provide the first demonstration that the consequences of the dominant huntingtin mechanism mirror the major consequence of glutamate exposure, namely, NMDA receptor mediated Ca\(^{2+}\)-dependent impairment of ADP phosphorylation, which contributes to decreased ATP-production that precedes the commitment to cell death (37).

It is worth re-iterating, given the prevailing notion that HD is initiated by aggregates of a short N-terminal expanded polyglutamine-peptide (38), that the dominant association of HD CAG repeat with mitochondrial energy status is manifested in STHdh\(^{Q111}\) striatal cells and lymphoblastoid cell lines in the absence of overt pathological markers (for example, N-terminal fragment and intranuclear inclusions) and indeed is evident even in cells bearing only alleles in the normal range. Thus, although in vivo exposure of mitochondria to polyglutamine-peptide can induce size-dependent aberrant Ca\(^{2+}\)-handling (34), our findings provide strong evidence that in vivo, the effects on mitochondrial energy metabolism are likely to result from a dominant activity of the polyglutamine tract while it is embedded in the full-length 350 kDa huntingtin protein. Possibilities include a direct interaction of huntingtin with the mitochondrion (26,39), with NMDA receptors (40) or with the endoplasmic mitochondrial Ca\(^{2+}\)-release/sensing machinery (25,41). Alternatively, mitochondrial ADP-regulation may reflect an indirect consequence of huntingtin on nuclear or mitochondrial gene transcription (42–45), autophagy (46,47) or an effect on microtubule transport (48,49), which may influence the subcellular localization of mitochondria, affecting mitochondrial biogenesis and function.

Our findings are also significant for revealing that HD can be viewed as a consequence of a fundamental shift in metabolic status. We have focused here on the impact on NMDA receptor Ca\(^{2+}\)-signaling to explain the outstanding vulnerability of medium-sized spiny striatal neurons. Even a subtle shift in metabolic status may be expected to alter many cellular processes. For example, in striatal cells and HD lymphoblastoid cells, we have found that high-energy guanidine nucleotides (GTP and GDP), which are synthesized from adenine precursors, mirror the adenine nucleotide levels...
This finding suggests that the static control of energy metabolism and stress response (52), as these two pathways have been linked to the homeostatic control of energy metabolism and lifespan in normal and disease. Evident possibilities are physiological pathways that regulate energy metabolism and the HD locus, via the polyglutamine tract in huntingtin, in the STHdhQ111 immortalized striatal neurons.

Notably, the recognition of a relationship between wild-type HD CAG alleles and cellular energy status strongly implicates the HD locus, via the polyglutamine tract in huntingtin, in the physiological pathways that regulate energy metabolism and lifespan in normal and disease. Evident possibilities are huntingtin’s proposed roles in brain derived neurotrophic factor (50) and insulin-like growth factor/Akt signaling (22,51), as these two pathways have been linked to the homeostatic control of energy metabolism and stress response (52). This finding suggests that the HD CAG repeat, which is highly polymorphic in all populations, might be an excellent candidate to test as a potential genetic modifier of phenotypes associated with ‘metabolic syndrome’ such as cardiovascular disease, obesity and type 2 diabetes and with age-related neurodegeneration such as Parkinson’s disease and Alzheimer’s disease.

In conclusion, we have showed an activity of huntingtin that conforms to the genetic HD criteria for causing clinical onset of HD in the regulation of mitochondrial energy metabolism, unequivocally supporting ‘excitotoxicity’ in the pathogenesis of HD. The nature of this property demands a conceptual change, away from the idea that the disease mechanism acts only on certain cells, which are normal until the disease process is triggered. Instead, the HD CAG repeat may act in all cells that express huntingtin, for the lifetime of the cell, to confer a CAG-size dependent energy status, but that this status results in death only for susceptible cells. The identification of the genetic and pharmacological factors that may modify the dominant effects of huntingtin on measures of cellular energy and the delineation of cell specific susceptibility factors will offer the opportunity to test this metabolic hypothesis of HD pathogenesis. Moreover, this study reveals a dominant role for huntingtin’s polyglutamine segment in normal energy homeostasis, opening interesting new possibilities for investigating huntingtin’s normal function.

MATERIALS AND METHODS

Immortalized STHdhQ111 striatal neuronal cell lines

Conditionally immortalized wild-type STHdhQ7/Q7 striatal cells and homozygous mutant STHdhQ111/Q111 striatal cells, generated from HdhQ111/Q111 and HdhQ7/Q7 littermate embryos, were described previously (27). The striatal cells of low passage number (<10 splittings) were grown at 33°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM l-glutamine and 400 µg/ml G418 (Geneticin; Invitrogen).

Nucleotide extraction and HPLC analysis

HPLC analysis for measuring nucleotides was performed on striatal cell lines and human lymphoblastoid cells. Nucleotide extraction was as previously described (22,23). To normalize the amount of nucleotide, each pellet was solubilized with 1 N NaOH, and the protein content was analyzed by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). HPLC-grade nucleotide standards (Fluka, Switzerland) were used to calibrate the signals. Internal standards were occasionally added to the samples to test recovery, which exceeded 90% for all nucleotides. All data were quantified by Breeze software (Waters, Milford, MA, USA).

Nucleotide measurements from isolated mitochondria

Mitochondria were isolated from striatal cells using a differential centrifugation method (53,54) and prepared for HPLC analysis. For incubation with added ADP or atractyloside, the mitochondrial pellets were resuspended in buffer A (200 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.6) and normalized for protein concentration, using Bio-Rad DC protein assay. Then, the mitochondria suspension was divided into 100 µl (1 mg/ml) of buffer B (200 mM sucrose, 1 mM EGTA, 20 mM HEPES, 5 mM succinate, 1.5 mM MgCl2, pH 7.6), followed by addition of ADP or atractyloside. Digitonin (Calbiochem, San Diego, CA, USA), at 0.15 mg/mg of mitochondrial proteins, was added to one of the samples. Samples were incubated on ice for 10 min with frequent shaking, and mitochondria were centrifuged at 14 000g for 10 min at 4°C. The pellets were washed twice in buffer B and also prepared for HPLC analysis.

Immunoblot analysis

The whole cell protein extracts were prepared from harvested striatal cells by lysis on ice for 30 min in a buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 0.5% Triton X-100, protease inhibitor mixture (Roche, Indianapolis, IN, USA), 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin, followed by sonication for 5 s. The total lysates were then cleared by centrifugation at 14 000g for 30 min, and the supernatants were collected. Mitochondrial and cytoplasmic fractions were prepared as described earlier and aliquoted for immunoblotting. The protein concentration was determined using Bio-Rad (detergent compatible) protein assay. Twenty-five microgram of protein extract was mixed with 4× SDS sample buffer, boiled for 2 min and subjected to 12% SDS–PAGE. After electrophoresis, the proteins were transferred to PVDF membranes (Schleicher and Schuell) and incubated for 30 min in blocking solution containing 10% non-fat powered milk in TBS-T (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 20 mM HEPES, 5 mM succinate, 1.5 mM MgCl2, pH 7.6), followed by addition of ADP or atractyloside. Digitonin (Calbiochem, San Diego, CA, USA), at 0.15 mg/mg of mitochondrial proteins, was added to one of the samples. Samples were incubated on ice for 10 min with frequent shaking, and mitochondria were centrifuged at 14 000g for 10 min at 4°C. The pellets were washed twice in buffer B and also prepared for HPLC analysis.

Semi-quantitative RT–PCR

Total RNA was isolated from striatal cells using Trizol (Invitrogen, Carlsbad, CA, USA). From total RNA, cDNA was...
ADP–ATP translocase activity in isolated mitochondria
Mitochondria were isolated according to a procedure described previously (39) and suspended in buffer A (5 mM HEPES, 3 mM MgCl2, 1 mM EGTA, 1 mM MgCl2, 3 mM K2HPO4, 20 mM HEPES, pH 7.4) and aliquoted (400 µl) of [2,8-3H]ATP in the supernatant and in the mitochondria pellet was quantitated by liquid scintillation using a Beckman LS6500 scintillation counter.

Measurements of mitochondrial membrane potential (∆Ψm) by flow cytometry
Cells were harvested by trypsinization, which was stopped by ice-cold PBS, and cell pellets (10^6 cells per each sample) were resuspended in prewarmed DMEM with 50 nM TMRM (Molecular Probes, Carlsbad, CA, USA) for mitochondrial membrane potential analysis and stained at 33°C for 15 min. Mitochondrial depolarization was achieved by incubating cells in TMRM simultaneously with 200 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma). After incubation at 33°C, cells were transferred on ice and analyzed for 30 min. Analysis was done on FACSCalibur flow cytometer (Becton Dickinson) equipped with 488 nm argon-ion laser. TMRM fluorescence was collected through a 575/30 band-pass filter. Data acquisition (10^4 cells events for each sample) and analysis were performed with the use of CellQuest software. The analysis was gated to viable cells and mean numbers were taken for analysis.

Measurements of intracellular [Ca2+] by flow cytometry
Flow cytometric analysis of intracellular [Ca2+] was performed using method of Barbiero et al. (57) with modifications. The cells (10^7 cells) were harvested and were resuspended in 10 ml of prewarmed complete medium with the addition of Fluo-3 (Molecular Probes) in cell-permeant acetoxymethyl ester form (2 µM final concentration). After incubation with Fluo-3 for 30 min in the dark at 33°C, the cells were centrifuged and the pellets were resuspended in PBS at room temperature. The cell suspension was divided into aliquots (10^6 cells), which were spun, and the cell pellets were used for Ca2+ measurement. The cells were equilibrated in 10 mM HEPES, pH 7.4 (containing 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose, 2 mM CaCl2) and analyzed on a flow cytometer using the 525/30 band-pass filter. The estimation was performed using the equation:

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\frac{[Ca^{2+}]_{\text{mut}}}{[Ca^{2+}]_{\text{wt}}} = \frac{F}{(F_{\text{max}} - F)}_{\text{mut}} \frac{F}{(F_{\text{max}} - F)}_{\text{wt}},
\]

where \(F\) represents the fluorescence of the samples and \(F_{\text{max}}\) represents the maximal fluorescence of the cells, permeabilized by Ca2+ ionophore ionomycin (2 µM final concentration).

Human lymphoblastoid cell lines
Previously genotyped lymphoblastoid cell lines (2,58) were employed in the studies in Fig. 3. Cell lines in Figure 3A were from five control individuals (HD CAG repeats: 17, 19, 18, 20, 17, 22 and 29) and two homozygous HD individuals (HD repeats: 48/41 and 50/45) with two cell lines from one of these, established 2 years apart. Cell lines in Figure 3B were from a total of 40 individuals. The HD CAG repeat size ranged from 16 to 70. Seventeen cell lines were from males and 23 were from females, ranging in age from 9 to 58 years. Twenty-three of the cell lines had an HD CAG repeat in the disease-causing range. Of these, 10 were established after a clinical diagnosis of HD and 13 were established before a clinical diagnosis was made. Seventeen of the cell lines had repeats only in the non-disease-causing range (<35 CAGs). Because [ATP/ADP] was modestly skewed (skewness = 0.75), a log-transformation was used (skewness = 0.12) for all statistical analyses. Correlations between the HD CAG repeat size and transformed [ATP/ADP] with age and gender using SAS yields non-significant Pearson coefficients of \(r = -0.04 (P = 0.67)\) and \(r = 0.07 (P = 0.41)\), respectively. A generalized estimating equation, controlling for the repeated measures (n1, n2, n3 and n4) for each sample, was used to evaluate the multivariate relationship of log-transformed [ATP/ADP] value with repeat sizes (where CAG1 represents the longer repeat for each subject) and CAG2 represents the shorter repeat for each subject), age and gender. The longer CAG repeat was a significant predictor of [ATP/ADP] \(P = 0.0001\), whereas the shorter CAG repeat \(P = 0.89\), age \(P = 0.90\) and gender \(P = 0.86\) were not. The Pearson correlation between [ATP/ADP] and the longer CAG repeat was evaluated using a bootstrap method with 1000 iterations, in which one observation was randomly selected from the quadruplicate (n1, n2, n3 and n4) values of [ATP/ADP] determined for each cell line. This method yielded a significant Pearson correlation coefficient of \(r = -0.816 (95\% \text{ CI} = -0.818 \text{ to } -0.815)\).

Statistical analysis
The relationship of [ATP/ADP] with the longer and shorter CAG repeat sizes and with age and gender were evaluated by simple Pearson correlation coefficient. A generalized estimating equation was used to control for the repeated measurements.
and to assess the relationship of [ATP/ADP] to the longer CAG repeat size, shorter CAG repeat size, affected status, age and sex. A bootstrap method with 1000 iterations was used to assess the statistical significance of the Pearson correlation of [ATP/ADP] to the longer CAG repeat size.

For other measurements (Figs 1 and 2), the statistical significance of observations from independent experiments was determined by one-way analysis of variance (ANOVA).

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