Calcium Homeostasis and Mitochondrial Dysfunction in Striatal Neurons of Huntington Disease

Received for publication, June 7, 2007, and in revised form, December 17, 2007 Published, JBC Papers in Press, December 21, 2007, DOI 10.1074/jbc.M704704200

Dmitry Lim†, Laura Fedrizzi†,#, Marzia Tartari∥, Chiara Zuccato∥, Elena Cattaneo∥, Marisa Brini§,#, and Ernesto Carafoli∥,#,‡,§

From the †Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padua, Italy, the Departments of ∥Biochemistry and §Experimental Veterinary Science, University of Padua, Viale Colombo 3, 35131 Padua, Italy, and the ‡Department of Pharmacological Sciences, University of Milan, Milan, Italy

Dysfunctions of Ca2+ homeostasis and of mitochondria have been studied in immortalized striatal cells from a commonly used Huntington disease mouse model. Transcriptional changes in the components of the phosphatidylinositol cycle and in the receptors for myo-inositol trisphosphate-linked agonists have been found in the cells and in the striatum of the parent Huntington disease mouse. The overall result of the changes is to delay myo-inositol trisphosphate production and to decrease basal Ca2+ in mutant cells. When tested directly, mitochondria in mutant cells behave nearly normally, but are unable to handle large Ca2+ loads. This appears to be due to the increased Ca2+ sensitivity of the permeability transition pore, which dissipates the membrane potential, prompting the release of accumulated Ca2+. Harmful reactive oxygen species, which are produced by defective mitochondria and may in turn stress them, increase in mutant cells, particularly if the damage to mitochondria is artificially exacerbated, for instance with complex II inhibitors. Mitochondria in mutant cells are thus peculiarly vulnerable to stresses induced by Ca2+ and reactive oxygen species. The observed decrease of cell Ca2+ could be a compensatory attempt to prevent the Ca2+ stress that would irreversibly damage mitochondria and eventually lead to cell death.

Huntington disease (HD)2 is a fatal disease characterized by chorea and psychiatric disturbance (1) caused by the expansion of CAG repeats in the first exon of the gene encoding huntingtin (Htt). In the normal gene, the repeats specify a stretch of up to 36 Gln in the N-terminal region of Htt. In the disease, the poly-Q tract is longer, and the mutant protein becomes harmful to cells. Htt is expressed ubiquitously in human tissues, but its mutation is particularly harmful to cortical and striatal medium size spiny neurons (MSNs) (2). The reasons for the specific damage to these neurons is an open problem in HD research, which reflects the incomplete knowledge on the function(s) of Htt. Htt interacts with several proteins in neurons, and plays roles in processes like axonal transport, regulation of transcription, exocytosis, calcium homeostasis, and prevention of apoptosis (3). It is easy to see that dysfunctions in any one of these processes, e.g. in Ca2+ homeostasis, could be involved in the ethiology of HD (4). As for the molecular mechanisms of the harmful effects of the mutated protein, the idea is now gaining ground that the extended poly-Q tract, cleaved off from Htt, causes the transcriptional dysfunction of genes that are essential for neuronal survival. Htt, however, could also have non-transcriptional effects.

Mitochondrial defects may have a causative role in neurodegenerative diseases, and are considered important in HD ethiology (5). They have indeed been found in the brain of HD patients (6) and in the striatal and other cells of animal HD models (7–9). The defective mitochondrial component has been suggested to be complex II of the respiratory chain. The suggestion is supported by experimental findings, for example the demonstration that the specific inhibitor 3-nitropropionic acid (3-NPA) induces a degeneration of rat striatal neurons that mimics that seen in the disease (10, 11). The alterations in Ca2+ homeostasis commonly observed in neuronal damage (12, 13) could also have a role in the HD ethiology. In neurons, the mobilization of Ca2+ from the endoplasmic reticulum (ER) through channels modulated by InsP3 produced in the phosphatidylinositol (PI) cycle is an important component of Ca2+ homeostasis. In the striatal neurons studied here the cycle is only activated by two plasma membrane agonists: ATP and Bradykinin (BK). ATP produces InsP3 through fast acting ionic (P2X) receptors, and slower acting, Gq protein-coupled P2Y receptors (14). The receptors for BK are all coupled to Gq proteins (15). The alterations of Ca2+ homeostasis, if resulting for instance in the increased release of Ca2+ from the ER, could exacerbate the mitochondrial dysfunction, as the complex II defect would limit the ability of the respiratory chain to increase activity, presumably making mitochondria peculiarly unable to retain the Ca2+ they have accumulated (16). However, the inability to retain Ca2+ could also be due to the increased pro-
penisity of the permeability transition pore to open: recent find-
ings have shown that the membrane potential (Δψm) in mito-
chondria of cells expressing mutant huntingtin is peculiarly
sensitive to Ca²⁺ (17), and that poly-Q constructs de-energize
isolated mitochondria exposed to Ca²⁺ (17).

Reactive oxygen species (ROS) also damage mitochondria,
_i.e._ they dissipate the Δψm, and are thus frequently mentioned
in the pathogenesis of neurodegenerative diseases. They are
produced by defective mitochondria, and in HD neurons they
could be generated by the dysfunction of complex II. This is
suggested by the finding that in normal neurons they increase
following the inhibition of the complex by 3-NPA (18). An
interesting development in the area of ROS has been the obser-
vation (19, 20) that the striatal neurons are particularly sensitive
to the mitochondrial damage caused by the lack of a co-activa-
tor of the transcription of the genes for ROS-scavenging
enzymes (acronym PGC-1α). PGC-1α regulates a number of
cell processes, among them the response of mitochondria to
oxidative stress. PGC-1α is down-regulated in the striatum of
HD patients (21), in striatal neurons of HD knock-in mice, and
when expressing exogenous PGC-1α in mutant neurons from HD knock-in mice were resistant to 3-NPA
administration. The concentration of ROS in the neurons increases. These
increases varied among the different mutant cells, which were examined, and found to be con-
verted off-line into [Ca²⁺] values, using a previously described computer algorithm (25).

### EXPERIMENTAL PROCEDURES

#### Cell Cultures

Clonal striatal cell lines established from E14 striatal primor-
dia of Kl-HdhQ111 and WT-HdhQ7 littermate mouse embryos
were described previously (23). The cells were grown in Dul-
becco’s modified essential medium (EuroClone, Milan, Italy)
supplemented with 10% fetal bovine serum (EuroClone), 2 mM
glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin.
They were maintained at the permissive temperature (33 °C) in
a humidified incubator with 5% CO₂.

#### Chemicals

Stock solutions in water of ATP (100 mM) and BK (1 mM)
(Sigma, St. Louis, MO) were prepared, aliquoted, and stored at
−20 °C. Submaximal final concentrations of agonists were used
in the experiments (100 μM ATP and 100 nM BK). Stock solu-
tions of FCCP (10 mM, Sigma) and cyclopasin A (CsA, 83.1
mM) were prepared in ethanol. Final concentration of ethanol
in working solutions did not exceed 0.2%.

#### Ca²⁺ Measurements

Aequorin—STHdhQ7 and STHdhQ111 cells were transfected with plasmid DNAs encoding cytosolic (cytAEQ), mitochondrial
(mtAEQ), or endoplasmic reticulum (erAEQ) aequorin
using TransFectin Lipid Reagents (Bio-Rad Laboratories, Her-
cules, CA). Ca²⁺ measurements were performed as described
elsewhere (25). All measurements were carried out at 37 °C.
The light signal was collected and stored in an IBM-compatible
computer for further analysis. The luminescence data were
converted off-line into [Ca²⁺] values, using a previously
described computer algorithm (25).

Fura-2—For Fura-2 measurements cells were plated on
24-mm coverslips in 6-well plates at a density of 400,000 cells
per well. 24-h later, cells were loaded with 5 μM Fura-2 AM
(Invitrogen, San Giuliano Milanese, Italy) in KRB (Krebs-
Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na₃PO₄,
1 mM MgSO₄, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supple-
mented with 1 mM Ca²⁺ (KRB-Ca) for 30 min at room temper-
ature. After washing with KRB-Ca cells were left for additional
30 min at room temperature for de-esterification of Fura-2. The
coverslip was then placed on the stage of a Zeiss Axiosvert 100
epifluorescence microscope, equipped with a 16-bit digital
CCD videocamera (Micromax, Princeton Instruments, Tren-
ton, NJ). Samples were alternately illuminated at 340 and 380
nm, and the emitted light (filtered with an interference filter
centered at 510 nm) was collected by the camera. Images were
acquired using the MetaFluor software (Universal Imaging,
West Chester, PA). The ratio values (1 ratio image/sec) were
calculated off-line, after background subtraction from each sin-
gle image. To quantify the differences in the peaks of the responses the data were normalized using the formula F₁(F₀/Fᵱ)
Calcium Signaling and Mutated Huntingtin

commonly used in experiments of this type. The data on basal Ca\(^{2+}\) levels are expressed as 340/380 ratio values.

**InsP\(_3\) Quantification**

For InsP\(_3\) quantification, cells were stimulated with ATP (100 \(\mu M\)) and/or BK (100 nm). The reaction was arrested at the indicated time-points with one-third volume of 1.05% PCA (Sigma). 1-h later extracts were collected and subjected to an AlphaScreen InsP\(_3\) assay according to the manufacturer’s protocol (AlphaScreen-GST and AlphaScreen InsP\(_3\) supplement, Perkin Elmer, Wellesley, MA).

**Membrane-permeable InsP\(_3\)**

A 10 mm stock solution of d-2,3-o-isopropylidene-\(\beta\)-myo-inositol 1,4,5-trisphosphate-hexakis(propionoxymethyl) ester (IInsP\(_3\)/PM, SiChem, Bremen, Germany) in Me\(_2\)SO was prepared, aliquotted, and stored at \(-20^\circ C\). ST\(\text{Hdh}^{Q7}\) and ST\(\text{Hdh}^{Q111}\) cells were loaded with Fura-2 and IInsP\(_3\)/PM was added at a final concentration of 100 \(\mu M\). Fura-2 images were collected every 3 s and the 340/380 ratio values were normalized (27).

**RNA Preparation and Reverse Transcription**

ST\(\text{Hdh}^{Q7}\) and ST\(\text{Hdh}^{Q111}\) cells were plated in 100-mm dishes and grown to 80–90% confluency. They were washed three times with cold phosphate-buffered saline and collected with 1 ml of TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer’s protocol. Striata from KI-Hdh\(^{Q111}\) and WT-Hdh\(^{Q7}\) mice were dissected, purified DNA was calculated as described (26) using hypoxanthine-55 °C for 30 s, and 72 °C for 15 s. The relative amount of amplification parameters were: 94 °C for 7 min, 45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s. The relative amount of amplified DNA was calculated as described (26) using hypoxanthine-guanine phosphoribosyltransferase mRNA as endogenous control.

**Measurement of Mitochondrial Membrane Potential (\(\Delta \psi_m\)) and of ROS**

The TMRM “diffusion” method was used, which is adequate for the comparison of the \(\Delta \psi_m\) between two populations of cells (27). The cells were loaded with 10 nm TMRM for 30 min at 37 °C in KRB containing 1 mm Ca\(^{2+}\). TMRM fluorescence was registered at 510 nm using an Olympus F-View II CCD camera mounted on an Olympus IX-81 microscope equipped with \(\times 40\) Uplan FLN objective (Olympus, Tokyo, Japan). The TMRM fluorescence intensity was analyzed off-line using MetaMorph software (Universal Imaging). In the experiments with FCCP, ROIs were positioned across the peripheral cell area, and the standard deviation of TMRM fluorescence was analyzed using MetaMorph software before and after FCCP addition. To measure ROS, ST\(\text{Hdh}^{Q7}\) and ST\(\text{Hdh}^{Q111}\) cells were treated with 3-NPA (10 mm for 40 h), and loaded with 10 \(\mu M\) 2,7’-dichlorodihydrofluorescein diacetate acetyl ester (Invitrogen) for 30 min at 37 °C in 100 \(\mu M\) KRB containing 1 mm Ca\(^{2+}\) followed by 30 min of de-esterification. Fluorescence was measured in a Fluoroskan spectrophotometer (Ascent FL, Labsystems, Thermo Electron, Waltham, MA). After background subtraction, the data were normalized to the cell number obtained by counting of nuclei stained by Hoechst 33258 (Sigma).

**Lentiviral Transduction with mitAEQ and Measurement of Mitochondrial Ca\(^{2+}\) Uptake**

To produce plasmid pLVL-mitAEQ-ires-EGFP, mitochondrial AEQ was first subcloned in a pIRES2-EGFP (Clontech, Mountain View, CA) vector, then a cassette containing mitAEQ-ires-EGFP was transferred to a pRRLsin.PPTs.hCMV.GFPpre lentiviral vector. The resulting construct was denominated as pLV-mtAEQ. Lentiviral particles were produced as described elsewhere (28). The desired lentiviral titer was obtained by infecting ST\(\text{Hdh}^{Q7}\) and ST\(\text{Hdh}^{Q111}\) cells with serial dilutions of the lentiviral stock. The minimal dilution adequate for 100% infection was used. 48–96 h after the infection AEQ was reconstituted as described in a section above. The cells were permeabilized with an intracellular buffer (100 mm KCl, 1 mm KH\(_2\)PO\(_4\), 5 mm sodium succinate, 1 mm MgCl\(_2\), 1 mm ATP, 20 mm HEPES, pH 7.0) supplemented with 10 \(\mu M\) digitonin and 50 \(\mu M\) EGTA. Cells were then perfused with Ca\(^{2+}\)-EGTA buffers containing the indicated concentrations of free Ca\(^{2+}\) (1.6–71 \(\mu M\)). FCCP (Sigma) was added in perfusion solutions at the indicated concentrations. A WebMaxLight software was used to prepare the Ca\(^{2+}\)-EGTA buffers.

**Caspase Activity Measurement**

Cells were plated in 60-mm dishes at the density of 1.2 million cells per dish. 24 h after plating, the cells were treated with 10 \(\mu M\) staurosporin for 6 h. The activity of caspase-3 was detected with the Caspase-3 Colorimetric Activity Assay kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions.

**RESULTS**

**InsP\(_3\)-linked Ca\(^{2+}\) Dynamics in Striatal Cells**—The plasma membrane of striatal cells contains P2Y receptors (29). They were challenged with ATP in a medium supplemented with 100 \(\mu M\) EGTA to exclude Ca\(^{2+}\) penetration through ionotropic P2X receptors (which are also present in the cells) and store-operated channels. ATP induced a rapid cytoplasmic Ca\(^{2+}\) increase in ST\(\text{Hdh}^{Q7}\) (control) cells peaking at 1.62 ± 0.17 \(\mu M\) (Fig. 1A), and decaying to base line in about 50 s. In ST\(\text{Hdh}^{Q111}\) (mutant) cells the peak was much lower (0.67 ± 0.15 \(\mu M\), \(p = 61e-8\), Fig. 1A). The dynamics of the mitochondrial Ca\(^{2+}\) pool was explored directly with mtAEQ: the peak of the transient triggered by ATP was much higher in ST\(\text{Hdh}^{Q7}\) than in
The simplest explanation for the differences in ATP and BK-mediated calcium signaling between control and mutant cells, as the steady-state calcium content of the ER had no role in the differences observed. The study further analyzed in the clone of Fig. 1 and in two additional clones (STHdhQ111-2 and STHdhQ111-3).

Altered Expression of Purinergic and Bradykinin Receptors—The simplest explanation for the differences in ATP and BK-mediated Ca2+ signaling would be the different amounts of receptors expressed in control and mutant cells. The differences, however, could instead have been due to the clonal nature of the cells. The expression level of the two receptors was thus analyzed in the clone of Fig. 1 and in two additional clones (STHdhQ111-2 and STHdhQ111-3).

The transcripts of the two P2Y receptor subtypes present in mouse (P2Y1 and P2Y2, Ref. 30) were indeed detected in the cells (Fig. 2, A and B). Q-RT-PCR with primers specifically designed to obtain amplicons of about 100 bp revealed a striking (about 20-fold) down-regulation of the transcription of the P2Y1 gene and a less pronounced down-regulation of the P2Y2 gene (Fig. 2, E and F) in all mutant cell lines.

The transcripts of the B1 and B2 kinin receptors were instead up-regulated (Fig. 2, C and D). In the Q-RT-PCR analysis, the B1 transcript was 3–12-fold higher in the three mutant cell lines, that of the B2 receptor 1.4–1.6-fold higher (Fig. 2, G and H).

Ca2+ Homeostasis Dysfunction in STHdhQ111 Cells—The AEQ assay shown in Fig. 1 averaged Ca2+ transients in the total population of cells expressing AEQ. The differences between control and mutant cells could thus have been due to the reduced (or enhanced) number of responding cells. Because the amount of AEQ consumed by released Ca2+ was normalized to the total amount of expressed AEQ, if the population of cells responding to stimulation had been different in control and mutant cells, a correspondingly smaller portion of AEQ would have been used for the normalization. It was thus important to control the Ca2+ changes in single cell.

The analysis of the images in single cells loaded with Fura-2 (Fig. 3) revealed that ATP induced Ca2+ transients in 88% of STHdhQ111 cells, but only in 54% of the mutant STHdhQ111 cells (Fig. 3D). The peaks of the transients in responding mutant STHdhQ111 cells were 18% lower than in control cells (0.5 ± 0.23 versus 0.61 ± 0.11 normalized Fura-2 ratios (n.r.), p =
Calcium Signaling and Mutated Huntingtin

The production of InsP₃ was delayed in ST₇. The dynamics of InsP₃ production was then explored in cells stimulated with ATP and BK for each cell type. The data are expressed as the mean for each cell type, and from four experiments for the stimulation with both ATP and BK for each cell type. The percentage of responding cells was higher in Q111 cells than in Q7 cells. When cells were stimulated with both ATP and BK the percentage of responding cells approached 100% and the height of peaks were not significantly different between Q7 and Q111 cells. The data are expressed as the mean ± S.D. Differences were significant at p < 0.05, *; p < 0.01, **; and p < 0.001, ***.

Stimulation with either ATP or BK. As shown in Fig. 4A, the production of InsP₃ was delayed in ST₇ with respect to ST₁₁₁ cells. It was lower than 30 s after stimulation with either ATP or BK, the difference only disappearing after 60 s. The difference was lower with BK. When cells were stimulated with both agonists (Fig. 4C), the increase in InsP₃ level was clearly delayed in mutant cells, reaching a maximum at 20 s in control cells, but only at 60 s in ST₁₁₁ cells.

The higher BK-induced Ca²⁺ response may seem odd, considering the decreased rate of InsP₃ production in mutant cells. Possibly, the sensitivity of the InsP₃ receptors could have been changed in the mutants. Therefore, we explored the possibility directly by treating control and mutant cells with a membrane-permeable derivative of InsP₃ (iInsP₃/PM). The experiment showed that InsP₃ receptors in ST₁₁₁ cells indeed were more sensitive than in ST₇ cells (0.64 ± 0.019 n.r., versus 0.47 ± 0.018 n.r., respectively, p < 0.001, Fig. 4D). The result nicely confirmed previous data showing increased sensitivity of the InsP₃ receptors by mutant Htt (31).

Transcriptional Regulation of InsP₃ Controlling Enzymes—The slower rate of InsP₃ production in stimulated mutant cells prompted a study of the expression of the components of the PI cycle and of the InsP₃ receptors. The striatum of the Ki-HdhQ₁₁₁ parent mouse was also studied. The transcripts of IMPA1 and inositol polyphosphate, which are rate-limiting in the cycle, were down-regulated both in ST₁₁₁ cells (by 40 and 20%, respectively, Fig. 5, A and C), and in the striatum (by 15 and 25%, respectively; Fig. 5, B and D).

The expression of the InsP₃ receptors could have been controlled by the biological significance that huntingtin has for the neuron.
Ca²⁺ Handling by Mitochondria in STHdhQ111 Cells—The driving force for mitochondrial Ca²⁺ uptake (Δψₘ), was explored with the fluorescent probe TMRM (27) in peripheral mitochondria to avoid artificial fluorescence changes due to variations in cellular thickness. Fig. 6D shows a minor, but significant (95.3% confidence, p = 0.047, unpaired 1-sided Student’s t test) fluorescence difference between resting STHdhQ7 and STHdhQ111 cells (199.7 ± 18.6 f.u. versus 192.3 ± 16.4 f.u., data collected from more than 200 cells in 15 experiments for each cell type). When STHdhQ7 cells were preincubated with the complex II inhibitor 3-NPA, the fluorescence decreased by about 10% in control cells (180.2 ± 14.3 f.u. versus 199.7 ± 18.6 f.u., p = 3e-15), and by 18% in STHdhQ111 cells (158.7 ± 9.8 f.u. versus 192.3 ± 16.4 f.u., p = 2.1e-6). Ca²⁺ uptake was monitored directly in mitochondria within control and STHdhQ111 cells, which were permeabilized with digitonin to expose them to concentrations of free Ca²⁺ precisely controlled with EGTA. To increase the efficiency of AEQ expression and thus to improve the quality of the results, in these experiments the DNA of mtAEQ was transferred into the cells using lentiviral vectors pLV-mitAEQ. As shown in Fig. 6A, perfusion with an EGTA buffer generating 1.6 μM free Ca²⁺ induced a Ca²⁺ transient which peaked at 103.6 ± 33.5 μM in STHdhQ7 cells (n = 11) and at 86.7 ± 27.7 μM in STHdhQ111 cells (n = 12). The difference was not significant (p = 0.1). Perfusion with 11 μM free Ca²⁺ evoked a significantly higher mitochondrial Ca²⁺ uptake transient in STHdhQ7 cells (300.6 ± 28.4 μM, n = 6, Fig. 6B) than in STHdhQ111 cells (172.4 ± 36.0 μM, n = 6, p = 0.00012). At these two relatively low Ca²⁺ concentrations, 3-NPA had no effect on the transients in either cell type. At 71 μM Ca²⁺, however, the peak (453.8 ± 18.6 μM, n = 6) was greatly reduced by 3-NPA (292.3 ± 53.2 μM, n = 6, p = 0.0001, Fig. 6C) in STHdhQ7 cells. In STHdhQ111 cells, the peak was much lower, (316.0 ± 25.6 μM, n = 6, p = 1e-5) and was decreased much more dramatically by 3-NPA (166.3 ± 16.8, n = 6, p = 3.1e-6). Thus, mitochondria in mutant cells were about as efficient in taking up Ca²⁺ as those in control cells when the concentration of the ion in the environment was low. As it increased, their ability to control Ca²⁺ decreased progressively in mutant cells, the difference being exacerbated if complex II was inhibited by 3-NPA.

The mitochondrial defect in HD neurons has been suggested to concern complex II. Even if no unambiguous proof of it has yet been obtained in the model cells used here, it was interesting to study whether the inhibition of the complex was specific in inducing mitochondrial damage (Δψ and Ca²⁺ uptake); i.e., whether other means of deenergizing mitochondria had the same effect as 3-NPA. STHdhQ7 and STHdhQ111 cells were thus treated with increasing concentrations of the uncoupler FCCP. As shown in Fig. 6E 0.8 μM FCCP decreased the Δψₘ by 33% in STHdhQ111 cells, but only by 6% in STHdhQ7 cells (p 2e-7, n = 6). Fig. 6F shows that the uncoupler decreased Ca²⁺ uptake more in the mitochondria of mutant cells than in those of the controls (65% versus 44%, p = 0.0049, n = 5 at 0.8 μM FCCP). Thus, mitochondria of mutant cells were more sensitive to deenergization in general, not only to that caused by inhibitors of complex II.

Mutant huntingtin, and 3-NPA, have been proposed to make the permeability transition pore (PTP) more sensitive to Ca²⁺ (16, 34–36). The preferential opening of the pore could explain the decrease of the membrane potential in the mitochondria of mutant cells challenged with high Ca²⁺ concentrations (Fig. 6, B and C). To test this possibility, the experiment of Fig. 6B was thus repeated in the presence of the PTP blocker CsA. When cells permeabilized with digitonin were perfused with a buffer containing 20 μM free Ca²⁺ the decrease of the Δψ was significantly more pronounced in mutant with respect to control cells (50%, p < 0.0001 versus 14%, p < 0.025, respectively). However, the Δψ in the mitochondria of mutant cells pretreated with CsA was not different from that of CsA-treated control cells, indicating that the greater decrease of Δψ in the mitochondria of mutant cells challenged with high Ca²⁺ concentration, was indeed due to the preferential opening of the PTP (Fig. 6G). Then experiments were performed to assess whether the preferential opening of PTP could have a role in the reduced mitochondrial Ca²⁺ uptake in STHdhQ111 (see Fig. 6B). Permeabilized cells transduced with the lentiviral vector pLV-mtAEQ were pretreated for 1 h with CsA (10 μM), and then perfused with a buffer containing 20 μM free Ca²⁺. As shown in Fig. 6H, the pretreatment completely abolished the decrease in mitochondrial Ca²⁺ uptake in mutant cells when challenged with a high Ca²⁺ concentration. Interestingly, the height of the peaks of the CsA-treated STHdhQ7 and STHdhQ111 cells (421.8 ± 118.7, p = 0.001) was decreased more in the cells pretreated with CsA (172.4 ± 36.0 μM, p = 0.00012).
Calcium Signaling and Mutated Huntingtin

63.52 μM and 407.04 ± 92.42 μM, p = 0.45), was even higher than those of the respective nontreated controls (306.13 ± 46.63 μM, p = 0.05 in STHdhQ7 cells, 228.54 ± 22.62 μM, p = 0.0052 in STHdhQ111 cells). Thus the PTP of mitochondria in mutant cells indeed was more sensitive to Ca2+. Transcriptional Regulation of ROS-scavenging Enzymes

—Huntingtin generates ROS in the striata of a HD mouse model, and 3-NPA, which generates ROS, does so in PC12 cells (37, 38). The level of ROS was thus explored in control and mutant striatal cells. Because a first set of experiments on the mutant cell clone employed employed in most of the work had failed to reveal differences in ROS level between mutant and control cells, the study was extended to two other mutant cell clones (STHdhQ111-2 and STHdhQ111-3). In all mutant clones, the concentration of ROS did not differ from the controls. However, when the cells were exposed to 3-NPA, the concentration of ROS increased as expected both in control and mutant cells. However, the increase was much more significant (even if variable) in the latter (Fig. 7A, p = 0.012, n = 4).

The transcription of the genes involved in the breakdown of ROS (Gpx1, catalase, SOD1, and SOD2) was analyzed next in the three independently generated STHdhQ111 clones (Fig. 7, B–D). The transcripts were up-regulated by 30–100% in all three mutant clones (most significantly in clone STHdhQ111-2), with the exception of SOD2, which was instead unchanged (not shown). Pilot experiments on the transcription of the ROS genes in the striata of the KI-HdhQ111 mice have so far failed to yield sufficiently reproducible results (not shown).

To measure Ca2+ cells were transduced with mitAEQ using lentiviral vectors. After AEQ reconstitution, cells were permeabilized with 10 μM digitonin (2 min) and perfused with Ca2+-EGTA buffers containing 1.6 (A), 11 (B, E, H, I, and J) or 71 (C) μM free Ca2+. D, STHdhQ7 (Q7, black bar) and STHdhQ111 (Q111, green bar) cells were loaded with TMRRM and the fluorescence of peripheric mitochondria was estimated with a CCD camera. The difference between Q7 and Q111 cells was significant at p = 0.047 (paired one-sided Student’s t test). Pretreatment with 3-NPA (20 μM, 40 h) reduced the fluorescence (10% in Q7 cells, blue bar, and 18% in Q111 cells, red bar). Data expressed as the mean ± S.D. (about 200 cells from 15 experiments for each cell type) for untreated cells and cells pretreated with 3-NPA (about 80–100 cells from six experiments for each cell type). E, cells were loaded with TMRRM and treated with the indicated concentrations of FCCP. The fluorescence was estimated with a CCD camera. The SD of fluorescence (27) was analyzed before and after FCCP addition. The data represent the mean ± S.D. of six experiments for each condition. F, cells expressing mitAEQ were permeabilized and perfused with an EGTA buffer containing 11 μM free Ca2+ and the indicated concentrations of FCCP. The data represent the mean ± S.D. of five independent experiments for each condition. G, H, and I, control (Q7) and mutant cells (Q111) were pretreated for 1 h with Ca2+ (10 μM, blue and red bars) and then perfused with buffer containing 20 μM free Ca2+. The SD of TMRRM fluorescence (27) was analyzed (G), and mitochondrial calcium (H) was measured in cells transduced with pLV-mitAEQ (see detailed description under “Experimental Procedures”). The data are presented as mean ± S.E. of 4–6 independent experiments for each condition. Differences were significant at p < 0.05; *; p < 0.01, **; and p < 0.001, ***.

3-NPA * * * 3-NPA * * * 3-NPA * * * 3-NPA * * * 3-NPA * * * 3-NPA * * * 3-NPA * * *

FIGURE 6. Mitochondrial membrane potential and Ca2+ uptake in STHdhQ7 and STHdhQ111 cells. To measure Ca2+ cells were transduced with mitAEQ using lentiviral vectors. After AEQ reconstitution, cells were permeabilized with 10 μM digitonin (2 min) and perfused with Ca2+-EGTA buffers containing 1.6 (A), 11 (B, E, H, and I, or 71 (C) μM free Ca2+. D, STHdhQ7 (Q7, black bar) and STHdhQ111 (Q111, green bar) cells were loaded with TMRRM and the fluorescence of peripheric mitochondria was estimated with a CCD camera. The difference between Q7 and Q111 cells was significant at p = 0.047 (paired one-sided Student’s t test). Pretreatment with 3-NPA (20 μM, 40 h) reduced the fluorescence (10% in Q7 cells, blue bar, and 18% in Q111 cells, red bar). Data expressed as the mean ± S.D. (about 200 cells from 15 experiments for each cell type) for untreated cells and cells pretreated with 3-NPA (about 80–100 cells from six experiments for each cell type). E, cells were loaded with TMRRM and treated with the indicated concentrations of FCCP. The fluorescence was estimated with a CCD camera. The SD of fluorescence (27) was analyzed before and after FCCP addition. The data represent the mean ± S.D. of six experiments for each cell type. F, cells expressing mitAEQ were permeabilized and perfused with an EGTA buffer containing 11 μM free Ca2+ and the indicated concentrations of FCCP. The data represent the mean ± S.D. of five independent experiments for each condition. G, H, and I, control (Q7) and mutant cells (Q111) were pretreated for 1 h with Ca2+ (10 μM, blue and red bars) and then perfused with buffer containing 20 μM free Ca2+. The SD of TMRRM fluorescence (27) was analyzed (G), and mitochondrial calcium (H) was measured in cells transduced with pLV-mitAEQ (see detailed description under “Experimental Procedures”). The data are presented as mean ± S.E. of 4–6 independent experiments for each condition. Differences were significant at p < 0.05; *; p < 0.01, **; and p < 0.001, ***.
The recent observation that the transcription of the PGC-1α gene was specifically repressed in a STHdhQ111 cell clone (21) prompted an investigation of the interplay between the transcription of the PGC-1α gene, and that of the genes of ROS-scavenging enzymes. The study was performed in the three independently generated STHdhQ111 cell clones. The results (Fig. 7E) showed great variability in the amounts of PGC-1α transcripts in the three clones. No convincing correlation was found between the levels of transcription of the PGC-1α gene and those of the 3 genes for the ROS-scavenging enzymes. In the mutant clone used in most of the experiments described here the transcript of PGC-1α increased markedly with respect to control cells (by 1.92 ± 0.27-fold, p = 0.0045), as did those of the ROS-scavenging enzymes (see Fig. 7, B–D). In another clone the amount of the PGC-1α transcript increased only marginally (by 1.23 ± 0.15-fold, p = 0.056), whereas those of the ROS-scavenging enzymes instead increased markedly. In the third clone the amount of PGC-1α transcript decreased sharply with respect to control cells (by 0.48 ± 0.083-fold, p = 0.0004), whereas those of the ROS-scavenging enzymes either increased or remained essentially unchanged. Direct measurement of ROS (Fig. 7A) showed that the increase in their concentration induced by 3-NPA did not correlate convincingly with the levels of the transcripts of the scavenging enzymes nor with that of PGC-1α. Evidently, the dynamics of ROS generation/degradation in the mutant cells was not exclusively controlled by transcriptional effects.

Susceptibility of STHdhQ111 Cells to Apoptosis—HD neurons have particular propensity to succumb by apoptosis (39, 40), and the model cell line used in this contribution has been claimed to be selectively vulnerable to treatments that cause non-apoptotic death (16). The vulnerability of three mutant cell lines to apoptotic treatments was thus investigated. Fig. 8 shows that the tendency to undergo apoptosis after treatment with the classical apoptotic agent staurosporin (10 μM for 6 h) was significantly greater in all three mutant cell lines (by 2.28 ± 0.59-, 2.84 ± 0.66-, 2.26 ± 0.37-fold in STHdhQ111.1, STHdhQ111.2, and STHdhQ111.3 cells, respectively) in respect with STHdhQ7 cells (p < 0.05 for all cases).

DISCUSSION

The accumulation of the expanded poly-Q fragments of Htt in the cytoplasm, nuclei, and axons is a hallmark of HD. As mentioned in the Introduction, the reasons for the preferential vulnerability of GABAergic MSNs to Htt, which is widely expressed in the brain and the body, is still an open problem. Mutant Htt aggregates are widely discussed with respect to their role in causing cell damage, and have even been claimed to decrease cell death risk in striatal neurons (41). Thus, they may have a number of functions, the dysregulation of the transcription of genes necessary for the function and eventual survival of striatal neurons now emerging as the most important (32, 33, 42–44). Transcription in HD neurons, however, could also be dysregulated by other agents, e.g. by ROS, the effect of the poly-Q aggregates being possibly linked to the late stages of the disease.

ROS have long been known to directly damage mitochondria: they dissipate the Δψm, impairing the production of ATP, and preferentially damage mitochondrial DNA (18, 45–47). The mitochondrial dysfunction mediated by ROS has received new impetus from recent work showing that in HD striata, and even in the model cells used in this work, a transcriptional co-activator of the genes of ROS-scavenging enzymes (PGC-1α) is transcriptionally down-regulated (21). The down-regulation has been suggested to be important in the damage to striatal neurons: however, the experiments presented here have shown that the level of PGC-1α transcript is but one of the factors active in the regulation of the level of ROS, as it does not correlate with those of the ROS-scavenging enzymes in the model cells used here.

The discovery that inhibitors of mitochondrial complex II induce cell death in striatal neurons, and produce neuronal degeneration in the striatum in vivo (11) was an important development. Complex II inhibitors, as all other mitochondrial damaging agents, also generate ROS, emphasizing their potential role in the damage to HD neurons. The finding that the complex II inhibitor 3-NPA mimics the effects of mutant Htt is an indication that somehow links the inhibition of the complex to the latter. The matter, however, still has unclear facets: recent work on cultured striatal neurons transfected with an expanded poly-Q tract have shown no down-regulation of the mRNA of two components of complex II, but have instead documented a down-regulation of their proteins (48). It thus appears probable that complex II is defective in HD neurons due to some post-transcriptional (proteolytic) effect. Whether the complex is also defective in the cell model used here is an open question. In Htt neurons the defect of complex II would not only generate ROS, but would also make mitochondria less able to respond to requests for increased activity of the respiratory chain. The defect would of course be exacerbated if the function of complex II would be further depressed by inhibitors. The canonical endogenous inhibitor of the complex is malonic acid, which has frequently been used to induce neuronal degeneration (49–51). Work now in progress in our laboratory explores the possible increase of malonic acid in Htt neurons.

The work described here has shown that the mitochondrial damage in cells expressing mutant Htt only becomes evident...
when mitochondria are exposed to stressing insults. Mitochondria in HD neurons thus appear to be in a borderline situation: this is also indirectly shown by experiments in which the per- entral administration of doses of 3-NPA that would not pro- duce striatal damage in rats, induced instead striatal neurode- generation when combined with sub-toxic doses of amphetamine (52). ROS would be likely candidates as the stressing agent(s) that would make the silent mitochondrial damage fully expressed, but Ca$^{2+}$ could be an equally plausible candidate, given its accepted role as a mediator of neuronal damage (12, 13). Ca$^{2+}$ signaling defects have indeed been a recurring theme in HD research (17, 53–55). Recent work has shown that the mitochondrial Δψ in cells expressing mutant Htt was particularly vulnerable to Ca$^{2+}$ (17, 34), and poly-Q constructs have been found to preferentially deenergize mitochondria exposed to Ca$^{2+}$ (17). However, the work presented here has shown that the damaging effect of Ca$^{2+}$ was not due to the overloadings of mitochondria with it. The damage appears instead to be linked to the continuous leakage of accumulated Ca$^{2+}$, incompletely compensated by the activity of the uptake uniporter made insufficient by the decrease of the Δψ. The Δψ of mutant mitochondria would be able to sustain the accumu- lation of a Ca$^{2+}$ pulse only lasting a short time, but not that of Ca$^{2+}$ persistently increased in their environment. As suggested by others, and directly confirmed in the present work (Fig. 6, G and H), it appears likely that the increased sensitivity of the PTP to Ca$^{2+}$ is critical to the inability of mitochondria to retain Ca$^{2+}$, thus triggering the increased Ca$^{2+}$ cycling and eventual ATP deprivation. Naturally, the drainage of ATP would have dire consequences for cell life. Peri-mitochondrial Ca$^{2+}$ could also increase by an additional mechanism because mutant Htt and the huntingtin-associated protein HAP1A form a ternary construct have been found to preferentially deenergize mito- chondria exposed to Ca$^{2+}$ (17). The resulting alterations of Peri- mitochondrial Ca$^{2+}$ transducers that control their homeostasis are important actors in their modulation in HD neurons. In the cell model described here the transcriptional alterations in the proteins that control the Ca$^{2+}$ signal are likely to be operational on a longer time scale. The resulting alterations of Ca$^{2+}$ homeostasis could reflect a compensatory mechanism developed by the cells to prevent, or at least to delay, Ca$^{2+}$ from increasing to the levels that would fatally harm their mitochondria, making their silent damage fully evident.

REFERENCES

1. The Huntington’s Disease Collaborative Research Group (1993) Cell 72, 971–983
2. Sieradzan, K. A., and Mann, D. M. (2001) Neuropathol Appl. Neurobiol. 27, 1–21
3. Cattaneo, E., Zuccato, C., and Tartari, M. (2005) Nat. Rev. Neurosci. 6, 919–930
4. Bezprozvanny, I., and Hayden, M. R. (2004) Biochem. Biophys. Res. Com- mun. 322, 1310–1317
5. Lin, M. T., and Beal, M. F. (2006) Nature 443, 787–795
6. Browne, S. E., Bowling, A. C., MacGarvey, U., Baik, M. I., Berger, S. C., Muquit, M. M., Bird, E. D., and Beal, M. F. (1997) Ann. Neurol. 41, 646–653
7. Browne, S. E., and Beal, M. F. (2004) Neurochem Res. 29, 531–546
8. Milakovic, T., and Johnson, G. V. (2005) J. Biol. Chem. 280, 30773–30782
9. Seong, I. S., Ivanova, E., Lee, J. M., Choo, Y. S., Fossale, E., Anderson, M., Gusella, J. F., Laramie, J. M., Myers, R. H., Lesort, M., and MacDonald, M. E. (2005) Hum. Mol. Genet. 14, 2871–2880
10. Brouillet, E., Jacquard, C., Bizat, N., and Blum, D. (2005) J. Neurochem. 95, 1521–1540
11. Beal, M. F., Brouillet, E., Jenkins, B. G., Ferrante, P. M., Kowall, N. W., Miller, J. M., Storey, E., Srivastava, R., Rosen, B. R., and Hyman, B. T. (1993) J. Neurosci. 13, 4181–4192
12. Schwab, B. L., Guerini, D., Didszun, C., Bano, D., Ferrando-May, E., Fava, E., Tam, J., Xu, D., Xanthoudakis, S., Nicholson, D. W., Carafoli, E., and Nicotera, P. (2002) Cell Death Differ. 9, 818–831
13. Bano, D., Young, K. W., Guerin, C. J., Lefebvre, R., Rothwell, N. J., Naldini, L., Rizzuto, R., Carafoli, E., and Nicotera, P. (2005) Cell 120, 275–285
14. Burnstock, G. (2006) Trends Pharmacol. Sci. 27, 166–176
15. Leeb-Lundberg, L. M., Marceau, F., Muller-Esterl, W., Pettibone, D. J., and Zuraw, B. L. (2005) Pharmacol. Rev. 57, 27–77
16. Ruan, Q., Lesort, M., MacDonald, M. E., and Johnson, G. V. (2004) Hum. Mol. Genet. 13, 669–681
17. Panov, A. V., Gutekunst, C. A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Stittmatter, W. J., and Greenamyre, J. T. (2002) Nat. Neurosci. 5, 731–736
18. Wang, J., Green, P. S., and Simpkins, J. W. (2001) J. Neurochem. 77, 804–811
19. Lin, J., Wu, P. H., Tarr, P. T., Lindenberg, K. S., St-Pierre, J., Zhang, C. Y., Mootha, V. K., Jager, S., Vianna, C. R., Reznicik, R. M., Cui, L., Manieri, M., Donovan, M. X., Wu, Z., Cooper, M. P., Fan, M. C., Rohas, L. M., Zavacki, A. M., Cinti, S., Shulman, G. I., Lowell, B. B., Krainc, D., and Spiegelman, B. M. (2004) Cell 119, 121–135
20. Leone, T. C., Leeman, J. H., Finck, B. N., Schaeffer, P. J., Wende, A. R., Boudina, S., Courtinot, M., Wozniak, D. F., Sambandam, N., Bernal-Mizrachi, C., Chen, Z., Holloszy, J. O., Medeiros, D. M., Schmidt, R. E., Saffitz, J. E., Abel, E. D., Semenkovich, C. F., and Kelly, D. P. (2005) PLoS Biol. 3, e101
21. Cui, L., Jeong, H., Borovecki, F., Parkhurst, C. N., Tanese, N., and Krainc, D. (2006) Cell 127, 59–69
22. Weydt, P., Pineda, V. A., Torrence, A. E., Libby, R. T., Satterfield, T. F., Lazarowski, E. R., Gilbert, M. L., Morton, G. J., Bammler, T. K., Strand, A. D., Cui, L., Beyer, R. P., Easley, C. N., Smith, A. C., Schmid, R. E., Fitchett, A. D., Cui, L., and Hayden, M. F. (2000) Cell 100, 279–289
23. Zuccato, C., and Cattaneo, E. (2007) Prog. Neurobiol. 81, 294–330
24. Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., and Rizzuto, R. (1995) J. Biol. Chem. 270, 9896–9903
25. Pfaffl, M. W. (2001) Nucleic Acids Res. 29, e45
26. Duchen, M. R., Surin, A., and Jacobson, J. (2003) Methods Enzymol. 361, 353–389
27. Poleni, A., and Naldini, L. (2002) Methods Mol. Med. 69, 259–274
28. Schemes, E., Duval, N., and Meda, P. (2003) J. Neurosci. 23, 11444–11452
29. Kugelgen, I., and Wetter, A. (2000) Naunyn Schmiedeberg's Arch Phar- macol. 362, 310–323
30. Tang, T. S., Tu, H., Chan, E. Y., Xiao, M., Wang, Z., Wellington, C. L., Hayden, M. R., and Beazovsky, I. (2003) Neurom. 39, 227–239
31. Luthi-Carter, R., Strand, A., Peters, N. L., Solano, S. M., Hollingsworth, Z. R., Menon, A. S., Frey, A. S., Spetzor, B. S., Penney, E. B., Schilling, G., Ross, C. A., Borchelt, D. R., Tapscott, S. J., Young, A. B., Cha, J. H., and Olson, J. M. (2000) Hum. Mol. Genet. 9, 1259–1271
32. Kuhn, A., Goldstein, D. R., Hodges, A., Strand, A. D., Sengstag, T., Koop- berg, C., Becanovic, K., Pouladi, M. A., Sathiasivam, K., Cha, J. H., Hannan, A. J., Hayden, M. R., Leavitt, B. R., Dunnett, S. B., Ferrante, R. J., Albin, R., Shellbourne, P., Delorenzi, M., Augood, S. J., Faull, R. L., Olson, J. M., Bates, G. P., Jones, L., and Luthi-Carter, R. (2007) Hum. Mol. Genet. 16, 1845–1861
34. Milakovic, T., Quintanilla, R. A., and Johnson, G. V. (2006) J. Biol. Chem. 281, 34785–34795
35. Choo, Y. S., Johnson, G. V., MacDonald, M., Detloff, P. J., and Lesort, M. (2004) Hum. Mol. Genet. 13, 1407–1420
36. Sawa, A., Wiegand, G. W., Cooper, J., Margolis, R. L., Sharp, A. H., Lawler, J. F., Jr., Greenamyre, J. T., Snyder, S. H., and Ross, C. A. (1999) Nat. Med. 5, 1194–1198
37. Perez-Severiano, F., Santamaria, A., Pedraza-Chaverri, J., Medina-Campos, O. N., Rios, C., and Segovia, J. (2004) Neurochem. Res. 29, 729–733
38. Mandavilli, B. S., Boldogh, I., and Van Houten, B. (2005) Brain Res. Mol. Brain Res. 133, 215–223
39. Tang, T. S., Slow, E., Lupu, V., Stavrovskaya, I. G., Sugimori, M., Llinas, R., Kristal, B. S., Hayden, M. R., and Bezprozvanny, I. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2602–2607
40. Steffan, J. S., Agrawal, N., Pallos, J., Rockabrand, E., Trotmann, L. C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y. Z., Cattaneo, E., Pandolfi, P. P., Thompson, L. M., and Marsh, J. L. (2004) Science 304, 100–104
41. Jiang, H., Nucifora, F. C., Jr., Ross, C. A., and DeFranco, D. B. (2003) Hum. Mol. Genet. 12, 1–12
42. Nucifora, F. C., Jr., Sasaki, M., Peters, M. F., Huang, H., Cooper, J. K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2001) Science 291, 2423–2428
43. Zhai, W., Jeong, H., Cui, L., Krainc, D., and Tjian, R. (2005) Cell 123, 1241–1253
44. Salazar, J. J., and Van Houten, B. (1997) Mutat. Res. 385, 139–149
45. Santos, J. H., Hanakowa, L., Chen, Y., Bortner, C., and Van Houten, B. (2003) J. Biol. Chem. 278, 1728–1734
46. Yakes, F. M., and Van Houten, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 514–519
47. Benchoua, A., Trioulier, Y., Zala, D., Gaillard, M. C., Lefort, N., Dufour, N., Saudou, F., Elalouf, J. M., Hirsch, E., Hantraye, P., Deglon, N., and Brouillet, E. (2006) Mol. Biol. Cell 17, 1652–1663
48. Greene, J. G., and Greenamyre, J. T. (1995) J. Neurochem. 64, 430–436
49. Lorenc-Koci, E., Golembiowska, K., and Wardas, J. (2005) Brain Res. 1051, 145–154
50. Maragos, W. F., Young, K. L., Altman, C. S., Pocernich, C. B., Drake, J., Butterfield, D. A., Seif, I., Holschneider, D. P., Chen, K., and Shih, J. C. (2004) Neurochem Res. 29, 741–746
51. Bowyer, J. F., Clausing, P., Schmued, L., Davies, D. L., Binienda, Z., Newport, G. D., Scallet, A. C., and Slikker, W., Jr. (1996) Brain Res. 712, 221–229
52. Golfer, D., Rigamonti, D., Tartarini, M., De Micheli, A., Verderio, C., Matteoli, M., Zuccato, C., and Cattaneo, E. (2002) J. Biol. Chem. 277, 39594–39598
53. Cepeda, C., Arriagada, C. A., Calvert, C. R., Flores-Hernandez, J., Chandler, S. H., Leavitt, B. R., Hayden, M. R., and Levine, M. S. (2001) J. Neurosci. Res. 66, 525–539
54. Zeron, M. M., Hansson, O., Chen, N., Wellington, C. L., Leavitt, B. R., Brundin, P., Hayden, M. R., and Raymond, L. A. (2002) Neuron 33, 849–860
55. Tang, T. S., Tu, H., Orban, P. C., Chan, E. Y., Hayden, M. R., and Bezprozvanny, I. (2004) Eur. J. Neurosci. 20, 1779–1787
56. Brini, M. (2003) Cell Calcium 34, 399–405