Involvement of Mitochondrial Complex II Defects in Neuronal Death Produced by N-Terminus Fragment of Mutated Huntingtin

Alexandra Benchoua,* Yaël Trioulier,* Diana Zala,[†] Marie-Claude Gaillard,[‡] Nathalie Lefort,[§] Noelle Dufour,* Frederic Saudou,^{||} Jean-Marc Elalouf,[‡] Etienne Hirsch,[¶] Philippe Hantraye,* Nicole Déglon,* and Emmanuel Brouillet*

*URA CEA-CNRS 2210, Service Hospitalier Frédéric Joliot, MIRCen Program, Département de Recherches Médicales, Direction des Sciences du Vivant, Commissariat à l'Energie Atomique (CEA), 91401 Orsay Cedex, France; [†]Swiss Federal Institute of Technology Lausanne, Institute of Neurosciences, EPFL, CH 1015 Lausanne, Switzerland; [‡]Service de Biochimie et de Génétique Moléculaire, Département de Biologie Joliot-Curie, CEA Saclay, 91191 Gif-sur-Yvette Cedex, France; [§]INSERM U421, Faculté de Médecine, Créteil 94010 Cedex, France; ^{||}UMR 146, Centre National de la Recherche Scientifique/Institut Curie, Centre Universitaire, 91405 Orsay Cedex, France; and [¶]INSERM U679 (formerly U289), Neurologie et Thérapeutique Expérimentale, Hôpital de la Salpêtrière, 75651 Paris Cedex 13, France

Submitted July 7, 2005; Accepted January 23, 2006
Monitoring Editor: Donald Newmeyer

Alterations of mitochondrial function may play a central role in neuronal death in Huntington's disease (HD). However, the molecular mechanisms underlying such functional deficits of mitochondria are not elucidated yet. We herein showed that the expression of two important constituents of mitochondrial complex II, the 30-kDa iron-sulfur (Ip) subunit and the 70-kDa FAD (Fp) subunit, was preferentially decreased in the striatum of HD patients compared with controls. We also examined several mitochondrial proteins in striatal neurons that were infected with lentiviral vectors coding for the N-terminus part of huntingtin (Htt) with either a pathological (Htt171-82Q) or physiological (Htt171-19Q) polyglutamine tract. Compared with Htt171-19Q, expression of Htt171-82Q preferentially decreased the levels of Ip and Fp subunits and affected the dehydrogenase activity of the complex. The Htt171-82Q-induced preferential loss of complex II was not associated with a decrease in mRNA levels, suggesting the involvement of a posttranscriptional mechanism. Importantly, the overexpression of either Ip or Fp subunit restored complex II levels and blocked mitochondrial dysfunction and striatal cell death induced by Htt171-82Q in striatal neurons. The present results strongly suggest that complex II defects in HD may be instrumental in striatal cell death.

INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an abnormal expansion of a CAG repeat located in exon 1 of the gene encoding for the Huntingtin protein (Htt; The Huntington's Disease Collaborative Research Group, 1993). The mutation induces at least in part a loss of function, since wild-type Htt plays an important role in cell survival (Zuccato *et al.*, 2003). In addition, the CAG repeat expansion leads to an abnormal polyglutamine (polyQ) tract that confers a new toxic function to full-length mutated Htt and/or short N-terminus fragments of the protein (Wellington *et al.*, 2002; Li and Li, 2004). However, the

mechanisms underlying the neuronal death induced by the polyQ expansion remain elusive.

One hypothesis is that mitochondrial dysfunction is involved in striatal cell death in HD. HD patients display early striatal hypometabolism (for review, Beal, 1992), increase in lactate brain concentrations (Koroshetz *et al.*, 1997; Jenkins *et al.*, 1998), and reduced production of ATP in muscles (Lodi *et al.*, 2000). Mitochondria isolated from cell expressing mutated Htt show decreased membrane potential, a defect in Ca²⁺ homeostasis, and higher susceptibility to Ca²⁺-induced permeability transition (Panov *et al.*, 2002; Choo *et al.*, 2004).

The mitochondrial hypothesis is also supported by the observation that systemic administration of the complex II inhibitor 3-nitropropionic acid (3NP) produces in rats and in nonhuman primates preferential degeneration of the striatum, abnormal movements, and frontal type cognitive deficits that are highly reminiscent of HD (for review Brouillet *et al.*, 1999). In addition, complex II activity is severely impaired in postmortem brain extracts of HD patients (Gu *et al.*, 1996; Browne *et al.*, 1997; Tabrizi *et al.*, 1999).

The main component of complex II is the enzyme succinate dehydrogenase (SDH; Ackrell, 2000). Complex II/SDH is composed of four nuclear-encoded subunits: the 70-kDa Fp subunit that catalyzes the oxidation of succinate, the

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-07-0607>) on February 1, 2006.

Address correspondence to: Emmanuel Brouillet (brouille@shfj.cea.fr).

Abbreviations used: HD, Huntington's disease; Htt, huntingtin; IRE, iron responsive element; mPTP, mitochondrial permeability transition pore; 3NP, 3-nitropropionic acid; polyQ, polyglutamine; SDH, succinate dehydrogenase; UTR, untranslated region.

30-kDa Ip subunit that transfers electrons to the ubiquinone via its iron centers and two small subunits (SDH-D and SDH-C) that anchor the complex to the internal mitochondrial membrane. Thus, complex II/SDH plays a central role in the respiratory chain, the tricarboxylic acid cycle and probably the control of free radical production (Ackrell, 2000; Rustin *et al.*, 2002).

The main objective of the present study was, therefore, to determine whether complex II defects could play a causal role in degeneration of striatal cells with HD related conditions. However, investigation of the mitochondrial function in neuronal cell models of polyQ diseases was limited by the need of large amount of material necessary to perform biochemical analysis. Models obtained by transfection in postmitotic neurons, leading to transgene expression in only 5–10% of cells, do not allow such kind of study. We therefore used a lentiviral vector expressing a mutant huntingtin protein (Htt171-82Q) to generate a chronic model of HD in rat primary striatal cultures. Our previous work showed that in this model, the majority of neurons expressed the transgene so that, apart from immunocytological studies, Western blot analysis and flow cytometry measurement could be performed to establish potential sequential links between biochemical alterations and cell death (Zala *et al.*, 2005). Striatal neurons infected with lenti-Htt171-82Q show somatodendritic aggregates and intranuclear inclusions 4 wk after infection and significant loss of neuronal markers (such as NeuN) is detected on the eighth week after infection. Cell death is not seen with vectors coding for the same protein with a polyQ in the physiological range (lenti-Htt171-19Q). The huntingtin fragment used in this study codes for the first 171 amino acids of the protein; its relevance to HD pathogenesis is supported by several *in vitro* and *in vivo* observations (de Almeida *et al.*, 2002; Zala *et al.*, 2005). In particular, expression of this construct in transgenic mice leads to motor abnormalities and HD-like neuropathological changes affecting preferentially the striatum (Schilling *et al.*, 1999). Another important aspect of this Htt fragment is that it produces cell death in lentiviral-infected primary striatal cells, whereas in cortical neurons, it is inefficient to trigger degeneration (Zala *et al.*, 2005). Thus, although this Htt fragment is relatively short, it retains cell specificity to trigger striatal cell death.

Thus, using this lentiviral vector-based model that recapitulates several cellular aspects of HD, we examined the levels of expression of the molecular constituents of complex II/SDH along with indices of mitochondrial dysfunction and neurodegeneration in cultured striatal neurons. We also studied complex II subunit expression in the striatum of HD patients. In addition, we asked whether increasing complex II expression using lentiviral vectors could protect striatal cultures against the toxicity of mutated Htt.

MATERIALS AND METHODS

Human Samples

Human brain samples were obtained from INSERM U289 brain bank at the Salpêtrière hospital (Paris) according to standard legislation. Cerebral cortex, caudate, and putamen samples were obtained from 14 controls with no history of neurological or psychiatric disorders (6 males; 8 females; mean $\pm$ SD, age: 75.0 $\pm$ 22.2 years; postmortem delay: 13.9 $\pm$ 6.4 h) and 6 symptomatic HD patients clinically and neuropathologically diagnosed and assessed as grade 1, 2, or 2/3 according to the classification of Vonsattel and colleagues (Vonsattel *et al.*, 1985; 2 males, 4 females; mean $\pm$ SD, age: 64.8 $\pm$ 8.3 years; postmortem delay: 25.7 $\pm$ 14.9 h). We could also analyze the cerebellum of 3 controls and 3 HD patients. Six of the HD patients displayed choreic movements at the end of their life. One choreic HD patient also displayed signs of

amyotrophic lateral sclerosis. Tissue samples were prepared as previously described (Gourfinkel-An *et al.*, 2002).

Primary Embryonic Striatal Neurons

Cultures were prepared as previously described (Zala *et al.*, 2005). Primary striatal neurons were obtained from embryonic day 14 (E14)–E15 embryos. Time pregnant Sprague Dawley rats (Janvier, Le Genest-St-Isle, France) were killed by injection of an overdose of pentobarbital and embryos were removed quickly and dissected on ice in Hanks' balanced sodium salts (without Ca^{2+} and Mg^{2+} , Sigma, Fallavier, France). Ganglionic eminences were isolated and incubated 15 min at 37°C in 0.3 mg/ml DNase I (Sigma). Tissues were mechanically dissociated with a fire-polished Pasteur pipette, and debris were removed after decantation of the suspension. Cells were finally concentrated by centrifugation (20°C, 5 min, 1500 $\times$ g) and resuspended in serum-free culture Neurobasal medium completed with 2% B27 supplement (Invitrogen, Carlsbad, CA), 1% antibiotic mix (Sigma), and 0.5 mM L-glutamine. Cells were plated at a density of 150,000 cells/cm² in Costar multiwell plates, coated with 50 μ g/ml poly-L-lysine (Sigma). The cultures were kept in a humid incubator (5% CO₂, 37°C), and half of the medium was changed once a week.

Lentivirus Construction, Production, and Infection

The cDNA coding for the first 171 amino acids of the human huntingtin containing 19 or 82 CAG repeats (Htt171-19Q/82Q) were cloned in the SIN-W-PGK transfer vector (de Almeida *et al.*, 2002) or in a SIN-W transfer vector containing the Tet-response element (TRE; BD Bioscience Clontech, Mountain View, CA) with seven direct repeats of the TetO operator sequence, upstream of a minimal CMV promoter (Regulier *et al.*, 2003). Lentiviral vectors coding for the tetracycline-controlled transactivator tTA1 (Regulier *et al.*, 2002; tetracycline repressor tetR fused to four copies (4F) of the minimal transcriptional activation domain of VP16 under the control of the PGK promoter; Gossen and Bujard, 1992) and a tetracycline-regulated green fluorescent protein (TRE-EGFP; BD Bioscience Clontech) were used for doxycycline-regulated expression of transgenes. DNA coding for human SDH-A and SDH-B was isolated from IMAGE clones 3051442 and 4124737, respectively (Open Biosystems, Huntsville, AL) by PCR using the following primers: SDH-A 5' CAC Cgg ATC CAC CAT gTC ggg ggT Ccg ggg CC 3' and 5' CTC gAg TCA gTA ggA gCg AAT ggC TG 3', SDH-B: 5' CAC Cgg ATC CAC CAT ggC ggC ggT ggT CgC AC 3' and 5' CTC gAg TTA AAC TgA AgC TTT CTT CTC 3'. PCR products were then cloned into the entry vector pENTR/n-TOPO (Gateway Technology Invitrogen). An LR clonase reaction was then performed with the destination vector SIN-W-PGK-CASSRFA according to the manufacturer's instruction. The SIN-W-PGK-CASSRFA was obtained by inserting a Gateway conversion cassette with attR recombination sites flanking ccdB genes and a chloramphenicol-resistance gene in the SIN-W-PGK transfer vector (Deglon *et al.*, 2000). Viral production was performed in 293T cells with a four-plasmid system. The viruses were resuspended in phosphate-buffered saline (PBS) with 1% BSA and matched for particle concentration after measuring of p24 antigen content. Twenty-four hours after plating, the cell cultures were exposed to lentiviral vectors at a concentration of 10 ng p24/10⁵ cells. For the experiments using the lenti-TRE-Htt171-19Q/82Q, cells were simultaneously coinfecting (1:1 ratio) with the lentiviral vector coding for the transactivator (tTA1) and tetracycline-regulated transgenes. At 2 day *in vitro* (DIV), half of the media was replaced with freshly prepared culture medium. Previous quantitative RT-PCR analyses at 3 wk have shown that the transcriptional levels of Htt171-82Q and Htt171-19Q are similar when lentiviral vectors are matched for p24 antigen levels (Zala *et al.*, 2005). For coinfections, Htt encoding vectors were used as described above and the second infection was performed 1 wk later with one of the SDH-encoding vectors. Analysis of the levels of SDH Ip and Fp proteins by Western blot to verify efficiency of viral vectors coding SDH subunits was performed at 2 wk postinfection in 30 DIV neuron cultures.

Flow Cytometry Analysis

For TUNEL staining, 3 $\times$ 10⁵ infected cells were trypsinized for 5 min, and a solution of 10% fetal bovine serum was added to stop the reaction. The cells were collected and washed by centrifugation at 1500 $\times$ g for 10 min. Cells were fixed in 200 μ l of freshly prepared paraformaldehyde 2% solution for 10 min on ice, washed by centrifugation, and permeabilized with a 0.1% solution of saponine. TUNEL reaction was performed using the Cell Death FITC kit (Roche Pharma, Basel, Switzerland) following the manufacturer's instruction, washed by centrifugation, and resuspended in 500 μ l of PBS for analysis. One part of the cells was processed for TUNEL reaction without transferase (negative control) or preincubated with DNase I (positive control) in order to determine the relevant gate (fluorescence intensity thresholds) for FACS analysis.

Cell counts were performed on FACScalibur analyzer on four sister cultures using CellQuest software (BD Bioscience, Franklin Lakes, NJ). The gate used to determine the number of cells considered TUNEL-positive (i.e., with high FITC fluorescence) in samples to be analyzed was set to exclude all cells of the negative control (TUNEL reaction without the transferase enzyme) showing very low (endogenous) fluorescence and include cells of the positive control

(incubated with DNase to produce DNA cleavage) showing high accumulation of FITC. For each sample (one plating well) FACS count was stopped at 10,000 cells. Four samples per condition were analyzed. The number of positive cells was expressed as percentage of total cells.

For MitoTracker red staining, cells were exposed to 200 nM Mitotracker red CMX dye (Molecular Probes, Eugene, OR) 30 min at 37°C before trypsinization. Cells were fixed in 4% paraformaldehyde in PBS for 15 min, rinsed in PBS, and analyzed. Cultures that were not incubated with the Mitotracker Red dye were used as a “negative” control to determine the FACS gating. Cells with higher levels of fluorescence were “positive.” Mean fluorescence intensity per sample was determined from the intensity of cells that were considered positive (i.e., with fluorescence intensity higher than that detected in cells not incubated with Mitotracker Red). The number of cells incubated with Mitotracker Red that were considered negative was low (<5%) in control cells at 6 wk.

Western Blot Analysis

Cultures were trypsinized and collected by centrifugation (1000 × g, 10 min). The pellets were incubated on ice in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.5% Triton, 1% NP 40, and 0.5% protease inhibitor cocktail; Sigma) for 30 min with vortexing every 10 min. Homogenates were centrifuged at 13,000 × g for 30 min at 4°C and supernatants were stored at –80°C. Protein concentrations were determined with the BCA protein Assay (Pierce, Rockford, IL). Ten micrograms of protein was resolved on a 12% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes. Membranes were incubated in 5% nonfat dry milk in tris-buffered saline containing 0.1% Tween 20 (T-tris–buffered saline) for 2 h at room temperature with the following primary mouse monoclonal antibodies (Molecular Probes): anti-70-kDa subunit of complex II, 1/3000; anti-30-kDa subunit of complex II, 1/3000; and anti-cytochrome oxidase (complex IV) subunit IV, 1/500. The membranes were also probed with monoclonal anti-calbindin D-28K (1/500, Sigma, clone CB-955), monoclonal anti-cytochrome c (1/10,000, clone 7H8-2C12, BD PharMingen, San José, CA), and polyclonal anti-BclXL (H62, 1/500, Santa Cruz Biotechnology, Santa Cruz, CA). After a 1-h incubation with an anti-mouse or anti-rabbit IgG HRP-coupled secondary antibodies, antigens were revealed using Enhanced Chemiluminescence Reaction (ECL+, Amersham Pharmacia Biotech, Les Ulis, France). Blots were routinely stripped in a denaturing solution (Tris-HCl 0.5 M, pH 6.8, SDS 10%, beta-mercaptoethanol 0.8%) and reprobed with a monoclonal anti- α -subunit of mitochondrial complex V (F1-ATPase, Molecular Probes). The preservation of α -subunit of complex V in the HD striatum and cells infected with lenti-Htt171-82Q was also found when membranes were probed directly with the antibody for α -subunit of complex V without stripping procedure. Coomassie blue was used as an absolute control for total protein loading. Quantification of signal intensity was performed using TotalLab software (Amersham) and normalized to Coomassie values (Benchoua *et al.*, 2001, 2002, 2004).

Immunostaining

Cell cultures were washed with cold PBS and fixed in 4% paraformaldehyde for 20 min at 37°C. Cultures were subsequently washed with PBS and then incubated in a blocking solution of PBS supplemented with 10% normal goat serum (NGS) and 0.03% Triton X-100 (Sigma) after permeabilization in Triton X-100 0.2% for 20 min. The cells were then incubated for 3 h at room temperature in blocking solution containing a primary antibody and then for 2 h at room temperature with secondary antibodies coupled to fluorophores (FluoProbes, Interchim, Lyon, France). The antibodies and dilutions were used: rabbit polyclonal Ubiquitin, 1/500 (Dako, Trappes, France), DARPP32, 1/5000 (Chemicon International, Temecula, CA), and the monoclonal anti-huntingtin EM48, 1/500 (MAB5374, Chemicon International). Before mounting in Mowiol (Calbiochem-Merck KGaA, Darmstadt, Germany) on microscope slides, coverslips were incubated in 2 μ g/ml Hoechst 33258 (bis-benzimide; Sigma) for labeling nuclear DNA.

Cell cultures stained with ubiquitin and DARPP32 and Hoescht were analyzed by counting the number of aggregates/inclusions and positive neurons respectively, using an Olympus microscope (Alberstland; Danemark) at a magnification of ×20. For detection and counts of neuronal nuclei, image acquisition was performed with a ×20 objective using a motorized Zeiss Axioplan2 imaging microscope (Carl Zeiss France, Vésinet, France). Image analysis using the Morphostar software (Morphostar 6.0, IMSTAR, Paris, France) consisted in thresholding the image to select all nuclei, and after filtering of the size, mean pixels intensity, shape/roundity index, of all nuclei were segmented and identified (Bizat *et al.*, 2003). Distribution analysis showed that in control cultures, nuclei presenting neuronal morphology showed cross-section areas in the 30–60- μ m² range. They represented ~40% of total cells at 4 wk in our conditions. At least five randomly chosen fields of view were counted for each of the samples (one well of 24-well plates) stained with a given antibody or Hoescht and the mean number of cells was calculated. For each experimental condition, 6–8 coverslips were analyzed. Results are expressed as mean ($\pm$ SEM) number of cells per field of view.

Effector Caspase Activity Assay

Effector caspase activity (including caspase-3 and -7 and to a lesser extent caspase-6), was measured as previously described (Benchoua *et al.*, 2002,

2004). Thirty micrograms of protein, obtained as described above, was diluted to a final volume of 90 μ l in caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM DTT). The reaction was started by the addition of 10 μ l of a 2 mM stock solution of fluoro-linked caspase substrate ac-DEVD-AFC (Calbiochem). After incubation at 37°C for 2 h, the reaction was stopped, and free AFC was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm with a Fusion fluorimeter (Perkin Elmer-Cetus, Les Ulis, France). Fluorescence units were converted into nmoles of AFC released per min and per mg of proteins using a standard curve of free AFC (Sigma) and relative activation was obtained compared with noninfected cells.

SDH Activity Assay

SDH activity was measured as described in Munujos *et al.* (1993). Cells, 1.5×10^6 , were homogenized using a glass dounce homogenizer in the following mitochondrial buffer: 20 mM Tris-HCl, pH 7.2, 250 mM saccharose, 2 mM EGTA, 40 mM KCl, 1 mg/ml BSA (Gimenez-Roqueplo *et al.*, 2001). Homogenates were centrifuged at 1500 × g, 5 min at 4°C and supernatant immediately processed for SDH activity or stored at –80°C. Twenty microliters of homogenate were diluted in 2× activity buffer (100 mM Tris-HCl, pH 8.3, 1 mM EDTA) containing 20 mM succinate. Reaction was started by adding 2 mM of iodinitrotetrazolium chloride (INT, Sigma) and incubated 90 min at 37°C. The formation of a red formazan produced by INT reduction was measured at 490 nm on a spectrometer (Bio-Rad). OD values were then normalized to the quantity of proteins.

Real-Time RT-PCR

Total RNAs were extracted from 1.5×10^6 cells using a guanidium thiocyanate/phenol method (Chomczynski and Sacchi, 1987) followed by digestion with RQ1 DNase (Promega, Charbonnière, France). RT reaction was performed on 200 ng of total RNA using Superscript II reverse transcriptase (Invitrogen) followed by treatment with RNase H. Two nanograms of random-primed cDNAs was processed for real-time quantitative PCR using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and PCR products were quantified by measuring SYBR green fluorescent dye incorporation. Value obtained for mRNA SDH were normalized to their corresponding value of the reference mRNA cyclophilin A. Primers used were designed Oligo 6.3 software.

Statistical Analysis

For studies in cell culture, each experiment was performed in quadruplicate (four sister cultures) or more and reproduced at least on two independent dissections. Two groups striatal cells were analyzed each time: neurons expressing the nonpathological N-terminus Htt fragment with 19 glutamines (Htt171-19Q) and neurons expressing the N-terminus fragment of Htt with the pathological polyQ expansion (Htt171-82Q). These two groups were also compared with wild-type, noninfected neurons (NI). Data were expressed as mean $\pm$ SEM. One-way ANOVA was performed to compare differences between groups, followed by a Fischer PLSD post hoc test. Unpaired Student's *t* test was used for comparison between HD patients and controls. A *p* value <0.05 was considered significant.

RESULTS

Reduced Expression of Complex II Subunits in the Striatum of HD Patients

Because it was reported that activity of complex II was decreased in the HD striatum, we first examined whether the levels of the main constituents of the complex were altered in the striatum of HD patients. Postmortem brain extracts from Grade 1–2/3 HD patients were assessed by Western blot analysis. An equal amount of proteins was loaded for comparison. For quantitative analysis of Western blot, the levels of expression of Ip and Fp subunits were normalized to the actual amount of protein detected in the corresponding lane. This approach avoids normalization to a single housekeeping protein with expression levels potentially affected by pathological conditions (Benchoua *et al.*, 2001, 2002). Both Ip and Fp subunit levels were significantly reduced in the striatum of all HD-affected patients we analyzed (Figure 1, A and B). Depletion was slightly more severe for the Ip subunit. Reduced levels of Fp and Ip proteins were detected in both the caudate and the putamen nuclei (Figure 1, A and B). We also observed that in the cerebellum and cerebral cortex of HD patients, SDH levels were not substantially decreased compared with those of con-

Figure 1. Preferential depletion of complex II/SDH subunits in the striatum of HD patients. Levels of Ip and Fp subunits of complex II/SDH were quantified by Western-blot in caudate (A), putamen (B), cerebral cortex (C), and cerebellum (D) of controls and symptomatic HD patients after normalization to total protein content measured in each lane. Neuropathological grade of each HD patient is indicated above the lanes. Note that only the striatum shows marked depletion of SDH constituents. Data represent mean $\pm$ SEM. * $p < 0.05$ by unpaired Student's t test.

controls (Figure 1, C and D). Thus SDH defects preferentially affect the striatum in HD patients.

To examine whether the complex II/SDH changes resulted from striatal cell degeneration, we evaluated in the putamen of HD patients and control subjects the levels of calbindin, a marker of medium-size spiny neurons. The expression of calbindin as assessed by Western blot was slightly reduced in the striatum but the apparent loss was less pronounced than that seen for SDH proteins (Figure 2). To examine whether the complex II/SDH changes resulted from a general loss of mitochondrial proteins, we reevaluated in the putamen of HD patients and control subjects the levels of SDH proteins and several mitochondrial proteins that are nuclear encoded (Figure 2). Interestingly, no significant modifications were observed for α -subunit of complex V (F1-ATPase), a macromolecular complex, which like SDH is attached to the inner mitochondrial membrane. Although variable from one control individual to the other, levels of subunit IV of cytochrome oxidase (mitochondrial complex IV), another complex of the inner membrane were slightly decreased in the striatum of grade III/II HD patients. Levels of cytochrome c, which is localized in the intermembrane space, were not changed. The levels of the outer membrane protein BclXL also remained unchanged compared with controls. These results supported the view that the loss of complex II subunits in the HD striatum did not simply result from depletion in mitochondria.

These results indicated that the reduced activity of complex II in the HD striatum that was reported by others (Gu *et al.*, 1996; Browne *et al.*, 1997; Tabrizi *et al.*, 1999) likely results from a reduced expression of complex II constituents.

Time Course of Degeneration in Striatal Cells Expressing N-Terminus Fragment of Mutated Htt Using Lentivirus-mediated Gene Transfer

Because results in HD patients could not indicate whether alterations of complex II expression were a cause or a consequence of striatal cell death, we decided to study complex II in a new model of HD in primary striatal neurons in culture (Zala *et al.*, 2005).

In the present study we further evaluated the time course of degeneration induced by lenti-Htt171-82Q. Previous work showed that a 20 and 50% reduction in the number of neurons expressing the neuronal marker NeuN occurred at 6 and 8 wk postinfection, respectively (Zala *et al.*, 2005). In line with this, immunofluorescence analyses performed in the present study at 8 wk postinfection showed that lenti-Htt171-82Q produced a significant loss (-94% , $p < 0.0001$) of neurons expressing the striatal marker DARPP32 as compared with lenti-Htt171-19Q. Because mutated Htt produced severe alteration in transcription, this loss of neuronal marker may be due to a decrease in expression of the marker and/or actual degeneration (i.e., cell death). To determine whether our model was characterized by actual cell degeneration, we therefore took advantage of the fact that $\sim 90\%$ of the neurons steadily expressed the transgene, to perform biochemical and FACS analyses. We first analyzed the potential activation of the effector caspases, the proteases known to play a role in the toxicity of short N-terminus fragments of mutated Htt in cultured neurons (Saudou *et al.*, 1998). Biochemical assay using the fluorogenic caspase substrate ac-DEVD-AFC indicated that, at 8 wk postinfection,

Figure 2. Analysis of the striatal marker calbindin and mitochondrial proteins in controls and HD patients. Levels of calbindin, BclXL, subunit IV of cytochrome oxidase (COX IV), cytochrome c and α -subunit of complex V (α -CV) were analyzed by Western blot in the putamen of control and HD patients. Top, typical Western blots; bottom, results of the image analysis of the blots shown in the top panel. Note that the proteins show no profound modification of expression, whereas SDH constituents are depleted.

the proteolytic activity resembling that of caspase-3 was significantly higher in the cytoplasmic fraction prepared from neurons expressing Htt171-82Q than with neurons expressing Htt171-19Q (Figure 3A). However, the elevation of fluorescence signal was relatively small, suggesting that only a limited proportion of cells synchronously died, consistent with the progressive aspect of degeneration in our model. Because this biochemical test based on caspase activity might not be sensitive enough to detect progressive degeneration, we next examined by FACS, in a large proportion of cultured cells ($\sim 10,000$ per culture well), nuclear DNA fragmentation, a hallmark of striatal degeneration in HD (Portera-Caillau *et al.*, 1995; Thomas *et al.*, 1995; Butterworth *et al.*, 1998). In culture at 6 and 8 wk post infection, we counted cells positive for TUNEL staining, an in situ indicator of DNA fragmentation. Results showed that in noninfected neurons and neurons infected with lenti-Htt171-19Q at 6 wk in vitro, the proportion of TUNEL-positive cells was low (4–7% of total cells; Figure 3B). In cultures infected with lentivirus coding Htt171-82Q, the number of TUNEL-positive cells significantly increased to 13% of total cells (Figure 3B). Similar FACS analysis at 8 wk in vitro showed that, in cells expressing Htt171-82Q, the number of TUNEL-positive cells further increased to 18% of total cells, showing that neurodegeneration aggravated with time in culture. Noninfected cells and cells expressing Htt171-19Q showed similar

Figure 3. Time course of degeneration in striatal cells expressing Htt171-82Q using lentivirus-mediated gene transfer. Time course of caspase-3 related proteolytic activity was measured using the fluorescent substrate DEVD-AFC. Data are mean $\pm$ SEM of two independent experiments performed on three sister cultures per group. Occurrence of actual cell death was counted by FACS after labeling of 10,000 neurons per sister cultures with TUNEL. Note that compared with noninfected cultures (NI) and cells expressing Htt171-19Q (Q19) that show low levels of degeneration, expression of Htt171-82Q produces toxicity that significantly increases from 6 to 8 wk postinfection (p.i.). Data represent mean raw counts $\pm$ SEM of two independent experiments ($n = 6$ wells per condition). * $p < 0.0001$ using ANOVA followed by post hoc PLSD Fischer test.

low levels (4–7%) of TUNEL-positive cells at 6 and 8 wk in vitro. This indicated that “aging” in vitro did not markedly increase the background rate of neurodegeneration in our model and that cell death was triggered by the presence of the expanded polyQ tract in the N-terminus part of Htt.

Complex II Alterations in Neurons Expressing Htt171-82Q Accompany Cell Death

We analyzed whether expression of Htt171-82Q could alter the expression of the catalytic subunits of complex II before the bulk of cell death at 8 wk postinfection. The time course of protein expression of Fp and Ip subunits was assessed using Western blot, as described for the analysis of HD postmortem samples. Results showed that levels of Fp and Ip subunits were reduced by expression of Htt171-82Q compared with Htt171-19Q (Figure 4, A and B). The levels of Ip subunit were first decreased by ~ 35 –40% at 5 and 6 wk postinfection. Loss of Ip aggravated thereafter, reaching $\sim 70\%$ at 8 wk. Fp depletion started at 6 wk after infection and also reached 70% at 8 wk postinfection. In contrast, the expression of α -subunit of complex V remained unchanged over time indicating that the decreased expression of both complex II/SDH subunits was not due to a global decrease of mitochondrial constituents (Figure 4C). Western blot anal-

Figure 4. Expression of Htt171-82Q expression of complex II/SDH catalytic subunits. Representative immuno-blots showing Fp, Ip, and α -subunit of complex V (alpha-CV) expression in Htt171-19Q (19Q)- and Htt171-82Q (82Q)-expressing cells after 5, 6, and 8 wk in culture. Levels of expression of Fp and Ip proteins were quantified by Western blot at different time points in control cells (gray stars), and neurons infected with either lenti-Htt171-82Q (filled triangle), or lenti-Htt19Q (filled square). Expression of alpha-CV was also quantified indicating only minor changes. Typical Western blot showing the expression of SDH subunits compared with other mitochondrial proteins at 6 wk postinfection is shown in D and corresponding quantification in E. Note that SDH Ip and Fp show decreased expression, whereas the other proteins are relatively preserved. Data represent mean $\pm$ SEM of at least two independent experiments performed on three sister cultures per group for each time point ($n = 6$ wells per condition). Loss of SDH subunits at 6 wk has been observed in more than four independent experiments. * $p < 0.05$ by ANOVA and post hoc PLSD Fischer test.

ysis of the levels of BclXL, cytochrome c, and subunit IV of cytochrome oxidase (Cox IV) also supported the preferential aspect of the loss of SDH compared with other mitochondrial proteins (Figure 4, D and E). Thus, the preferential loss of complex II/SDH subunits and actual degeneration seem to progress concurrently in cells expressing Htt171-82Q.

Complex II/SDH Alterations Are Not Associated with an Altered Transcription of SDH Genes

Abnormal transcription is an early feature of HD (Sugars and Rubinsztein, 2003). Because the four subunits of the complex II/SDH are nuclear-encoded, we reasoned that Htt171-82Q may act at the nuclear level and could repress transcription of the SDH genes. Quantification of SDH subunit mRNA content using real-time RT-PCR was performed 6 wk after infecting striatal cultures. Cultures expressing Htt171-82Q showed loss of Ip and Fp proteins at this time point. However, no profound changes in mRNAs for SDH subunits were found in cultures expressing m-Htt82Q compared with mock- and Htt171-19Q-infected cells (Table 1). This indicated that, at least at the

beginning of the process (6 wk), the marked depletion in SDH protein was not associated with proportionate alterations of SDH gene transcription.

Decreased Succinate Oxidation and Mitochondrial Membrane Potential in Neurons Expressing Htt171-82Q

We studied whether loss of complex II subunits could lead to functional change in the mitochondria. Functional analysis of complex II activity was performed at 6 wk postinfection, when both subunits were decreased in cells expressing Htt171-82Q. We tested the capability of crude mitochondrial preparations to oxidize saturating concentrations of succinate in the presence of the electron acceptor INT (iodo-nitroblue tetrazolium). Results demonstrated that SDH catalytic activity (V_{max}) was significantly reduced by 30% in cells expressing Htt171-82Q compared with Htt171-19Q (Figure 5A), consistent with the ~40% loss of Ip and Fp proteins seen by Western blot at the same time point. This confirmed that the partial depletion in complex II/SDH catalytic subunits detected by Western blot was sufficient to induce a functional impairment of complex II.

Table 1. Expression of the four mRNAs encoding SDH subunits in striatal cells expressing Htt171-19Q or Htt171-82Q

Transcript	SDH-A	SDH-B	SDH-C	SDH-D
Mock	100.03 ± 5.05	100.38 ± 5.00	100.26 ± 7.02	100.41 ± 8.16
Htt-19Q	94.61 ± 9.15	77.41 ± 5.71	93.49 ± 0.32	85.44 ± 9.29
m-Htt-82Q	103.22 ± 10.46	77.05 ± 15.39	108.66 ± 7.83	95.71 ± 4.11

Levels of SDH mRNAs were assessed using real time RT-PCR. Data are expressed as mean ± SEM of percentage of expression relative to mock-infected cells. Note that no significant changes were produced by Htt171-82Q.

We next assessed whether Htt171-82Q-induced defects in the respiratory chain could produce alterations in mitochondrial homeostasis. We hypothesized that complex II defect could modify mitochondrial membrane potential. To examine this hypothesis, we carried out FACS analysis of the fluorescence resulting from Mitotracker red (CMX-ROS) accumulation in living striatal cell cultures. We chose this dye because it can be fixed using paraformaldehyde, and for biosafety reasons we had to treat infected cells with a fixative to inactivate the viral particles potentially remaining in the cultures before FACS analysis. Chloromethyl-X-Rosamine (CMX-ROS) dye accumulation in active mitochondria was demonstrated to be dependent on mitochondrial membrane potential (Pendergrass *et al.*, 2004) and the changes of fluorescence intensity resulting from mitochondrial membrane potential variations can be measured by FACS with greater sensitivity than with spectrofluorimetric techniques (Kalbacova *et al.*, 2003). However, fixative can, in certain conditions, affect the intracellular distribution of MitoTracker red (Gilmore and Wilson, 1999). For this reason, we checked in (noninfected) cultured striatal neurons that the intensity of accumulated fluorescence as a function of incubation time was only slightly (~15%) decreased by 4% paraformaldehyde fixation (Figure 5B). Even after a 30-min incubation, cells were not saturated

and fluorescence signal increased, suggesting absence of major quenching effects. We also checked that accumulation of Mitotracker Red in striatal cells was related to mitochondrial membrane potential by showing that a 5-min preincubation with 50 μ M FCCP significantly reduced MitoTracker accumulation by 70% (Figure 5B). FACS analysis showed that at 6 wk postinfection, cells expressing either Htt171-82Q or Htt171-19Q accumulated Mitotracker red dye (Figure 5C). However, cells expressing Htt171-82Q exhibited a significant reduction of the fluorescence mean intensity related to Mitotracker incorporation when compared with mock or lenti-Htt171-19Q-infected cells (Figure 5, C and D). This indicated that expression of N-terminus fragment of mutated Htt could induce a functional alteration of mitochondrial membrane potential, possibly as a consequence of the complex II/SDH defect.

Overexpression of SDH Subunits Restores Mitochondrial Membrane Potential and Blocks Neuronal Death Induced by Htt171-82Q

We next wanted to determine whether complex II/SDH defects were solely a consequence of degeneration or could play a causal role in neurodegeneration produced by mutated N-terminal fragment of Htt. We reasoned that if

Figure 5. Expression of Htt171-82Q induces mitochondrial dysfunction. Dehydrogenase activity of complex II/SDH was measured in Htt171-19Q- and Htt171-82Q-expressing cells at 6 wk postinfection. Activity of complex II (succinate oxidation) was evaluated using the colorimetric substrate INT. Data represent mean ± SEM of two independent experiments with three sister cultures per group. (B) Fluorescence of MitoTracker red in living cells (■) or after PFA fixation of cultures (△) as a function of incubation time in presence of the dye. For these methodological controls, fluorescence accumulated in cells was determined using a plate reader. Incubation with the ionophore FCCP (50 μ M) produces marked reduction of the accumulation of MitoTracker red, demonstrating that accumulation of the dye is highly sensitive to the loss of mitochondrial membrane potential. (C) Detection of MitoTracker red levels in individual cells after PFA fixation was performed by FACS, showing the typical distribution of fluorescence levels in cells expressing Htt171-82Q (82Q) and Htt171-19Q at 6 wk postinfection. Note the shift to the left of fluorescence distribution in cells expressing Htt171-82Q, indicating reduced fluorescence

mean intensity (FMI) in the culture. (D) Histograms representing the quantification of FMI measured in two independent experiments (n = 6 wells per condition). Data are mean ± SEM. *p < 0.05 by ANOVA and post hoc PLSD Fischer test.

Figure 6. Overexpression of complex II/SDH subunits restores mitochondrial membrane potential and rescues neuronal death induced by Htt171-82Q. (A) Representative Western blots of 30 DIV neurons transduced with SDH encoding lentiviral vectors 2 wk after infection. (B) Overexpression of Fp (SDH-A virus) or Ip (SDH-B virus) suppresses mitochondrial membrane potential alteration induced by Htt171-82Q expression at 6 wk postinfection. (C) Determination of the number of TUNEL-positive cells at 8 wk in culture indicates that both infection with lentiviral vectors coding SDH subunits significantly reduces Htt171-82Q-induced cell death. Data are mean $\pm$ SEM of two independent experiments performed on three sister cultures per group ($n = 6$ wells per condition). * $p < 0.05$ by ANOVA and post hoc PLSD Fischer test.

depletion of SDH subunits was causal in HD associated neuronal death, restoring SDH levels using lentivirus-mediated gene overexpression should protect striatal neurons against Htt171-82Q toxicity. To test this hypothesis, we designed two lentiviral vectors encoding either human Fp subunit (lenti-SDH-A) or human Ip subunit (lenti-SDH-B). All potential posttranscriptional regulatory elements (UTRs) were deleted from human SDH-A and SDH-B encoding cDNA before insertion in the lentivirus transfer construct. Western blot analysis of striatal neurons showed that 2 wk after infection (Figure 6A), both viral vectors were able to transduce expression of exogenous Fp subunit (lenti-SDH-A) or Ip subunit (lenti-SDH-B). The antibodies we used to detect SDH Ip and Fp subunits similarly recognize the human and rat proteins so that we could not separately detect recombinant and endogenous SDH proteins. However, Western blot analysis showed that the overall levels of SDH subunits (endogenous plus recombinant) were increased in neurons transduced with lenti-SDH-A and lenti-SDH-B. Interestingly, lentivirus-mediated expression of only

one of the two exogenous subunits was sufficient to produce an increased expression of the other endogenous subunit of the complex II/SDH complex.

We next addressed whether restoring the levels of SDH catalytic subunits was able to preserve the mitochondrial membrane potential in neurons expressing Htt171-82Q. In these experiments, neurons were first infected with lenti-Htt171-82Q or lenti-Htt171-19Q or were mock-infected, and 1 wk later, they were mock-infected or exposed to lenti-SDH-A or lenti-SDH-B. In cells expressing Htt171-19Q, MitoTracker incorporation remained unchanged compared with mock-infected cells (Figure 6B). The double infection of neurons expressing Htt171-19Q with lenti-SDH-A or lenti-SDH-B did not modify dye incorporation either. In cells expressing Htt171-82Q, a significant reduction of MitoTracker incorporation was detected at 6 wk postinfection as seen in the experiment described above (Figure 6B). Importantly, the reduction of dye incorporation in cell expressing Htt171-82Q was totally abolished by overexpressing either Ip or Fp SDH subunits. This indicated that SDH depletion could be causal in Htt171-82Q-induced loss of mitochondrial membrane potential.

We finally asked whether this mitochondrial improvement was associated with a reduction of the toxic properties of Htt171-82Q in neurons. Consistent with experiments reported above, a significant elevation in the number of TUNEL positive cells (i.e., 3 times the levels seen in controls) was detected in neurons expressing Htt171-82Q at 8 wk postinfection (Figure 6C). In sister cultures that were also infected 1 wk later with lenti-SDH-A and lenti-SDH-B, the Htt171-82Q produced no elevation in the number of TUNEL-positive cells. Thus, overexpressing SDH subunits could block cell death induced by Htt171-82Q. This data suggests that complex II/SDH depletion plays an instrumental role in the slow toxicity of the N-terminus fragment of mutated Htt expressed under the PGK promoter.

Analysis of cell death and neuroprotection at later time points in vitro (e.g., 10 wk) could not be easily performed in our lentivirus-based model using the PGK promoter because cultures tend to become exquisitely vulnerable after 9–10 wk in vitro. Therefore, to check that Htt171-82Q could produce degeneration independently of “aging” of culture in vitro and that SDH-A and SDH-B could be neuroprotective in a more rapid model of degeneration, we used tetracycline-regulated (TRE) Htt171-19/82Q vectors (Regulier *et al.*, 2003). This system leads to higher expression levels of the transgene compared with PGK and therefore more rapid degeneration (Regulier *et al.*, 2003). We examined cultures at different time points after infection with both the lentivirus coding for the transactivator protein tTA1 and lentiviral vectors expressing TRE-Htt171-82Q, TRE-Htt171-19Q, or the green fluorescent protein (TRE-GFP). The number of EM48-positive huntingtin-containing inclusions/aggregates increased rapidly from 2 to 3 wk postinfection in cells expressing Htt171-82Q under the TRE promoter and plateaued thereafter. On the fourth week postinfection, numerous ubiquitin- and EM48-positive intranuclear inclusions were seen (Figure 7, A and B). The number of DARPP32-positive cells was decreased by more than 90% in cultures infected with lenti-TRE-Htt171-82Q compared with lenti-TRE-Htt171-19Q (Figure 7C). The number of neuronal nuclei was decreased by 35% in cultures infected with lenti-TRE-Htt171-82Q compared with lenti-TRE-Htt171-19Q and lenti-TRE-GFP (Figure 7D). Thus, expression of Htt171-82Q under the tetracycline-regulated promoter produced neurodegeneration more rapidly compared with Htt171-82Q under the PGK promoter.

Figure 7. Overexpression of complex II/SDH subunits protects striatal cell loss rapidly induced by expression of Htt171-82Q under the tetracycline-regulated promoter. The neuroprotective effect of lenti-SDH-A and lenti-SDH-B was tested in a model of degeneration induced by infection with lentiviral vectors coding for Htt-171-82Q under the tetracycline-regulated promoter (TRE). (A) Time course of accumulation of Htt-containing inclusions/agggregates detected by immunofluorescence with the EM48 antibody. Note that inclusion accumulation culminates at 3–4 wk postinfection (p.i.). (B) Inclusions/agggregates in cells expressing TRE-Htt-171-82Q at 4 wk postinfection were detected by immunofluorescence with an anti-ubiquitin antibody. (C) Loss of DARPP32-positive cells at 4 wk postinfection with lenti-TRE-Htt-171-82Q compared with lenti-TRE-Htt171-19Q. The coinfection with lenti-SDH-A and lenti-SDH-B did not markedly modify the density of inclusions and the density of DARPP32 cells. (D) Neuronal nuclei were identified and counted after DNA staining at 4 wk postinfection with lenti-TRE-Htt-171-82Q, lenti-TRE-Htt171-19Q, and lenti-TRE-GFP. When indicated, cultures were coinfecting with lenti-SDH-A or lenti-SDH-B. Note that the significant cell loss evidenced in cultures expressing Htt171-82Q is blocked by expression of SDH-A and SDH-B. * $p < 0.05$ compared with all other groups.

We next tested the effects of lenti-SDH-A and lenti-SDH-B against the neurotoxicity of lenti-TRE-Htt171-82Q. Overexpression of SDH-A nor SDH-B did modify neither the number of ubiquitin-containing inclusions (Figure 7B) nor the Htt171-82Q-induced decrease in DARPP32. However, the reduction of the number of neuronal nuclei in cultures expressing Htt171-82Q was not seen in cultures coinfecting with Htt171-82Q plus SDH-A or SDH-B.

Our results further support the view that restoring complex II/SDH levels did not modify early signs of degeneration/dysfunction (e.g., loss of DARPP32 expression, aggregates formation) but could efficiently block the execution phase of cell death induced by the N-terminus fragment of mutated Htt.

DISCUSSION

There are several hypothetical mechanisms that could underlie striatal cell death in HD. Main hypotheses include abnormal transcription (Sugars and Rubinshtein, 2003), increased transglutaminase activity (Lesort *et al.*, 2002), early axonal transport dysfunction (Li and Li, 2004), and disruption of Ca^{2+} homeostasis (Bezprozvanny and Hayden, 2004). Protein misfolding, reduction of autophagy, and proteasome dysfunction play important roles in HD (Meriin and Sherman, 2005). In addition, mitochondrial dysfunction could also contribute to neurodegeneration (Brouillet *et al.*, 2005). However, there existed no direct evidence that mitochondrial change could play a causal role in HD.

In the present study, we hypothesized that an early decrease in the levels of one or more of the four subunits constituting complex II/SDH could, at least in part, be responsible for mitochondrial dysfunction in HD. Taking advantage of a newly developed model of progressive striatal degeneration in primary cultures (Zala *et al.*, 2005), we examined this hypothesis using biochemical and cytological methods.

Our results show that expressing the N-terminus part of Htt with a pathological polyglutamine expansion in cultured striatal primary neurons reduced the levels of complex II/SDH subunits Ip and Fp and the dehydrogenase activity of the complex. We also found similar molecular defects in complex II/SDH in the HD striatum. No major changes in the expression of SDH subunits were found in the cerebral cortex and cerebellum, two regions less vulnerable to degeneration in HD patients. Our data are consistent with the reduced activity of complex II-III in HD patients reported in the literature. A decrease in succinate oxidation ranging from 39 to 59% has been observed in the caudate nucleus of HD patients (Butterworth *et al.*, 1985; Brennan *et al.*, 1985; Mann *et al.*, 1990; Gu *et al.*, 1996; Browne *et al.*, 1997; Tabrizi *et al.*, 1999). In the putamen, succinate oxidation showed an average 69% decrease (Browne *et al.*, 1997). In line with this, striatal cell lines expressing the full-length Htt with 111 polyglutamine (Knock-in 111) were found highly vulnerable to the complex II inhibitor 3NP (Ruan *et al.*, 2004). In the present study, neither striatal cells expressing Htt171-82Q nor the HD striatum showed profound alterations in α -subunit of complex V, cytochrome c, and BclXL. Levels of subunit IV of cytochrome oxidase (complex IV) and calbindin were slightly reduced in III/II HD patients, consistent with striatal degeneration. However, depletion of these proteins appeared less pronounced than that of SDH subunits. In cell expressing Htt171-82Q at 6 wk postinfection, we found no modification of subunit IV of cytochrome oxidase (complex IV). These observations support the view that HD may be preferentially associated with complex II defects. Our results obtained in cell culture also suggest that the effect of the short fragment of mutated Htt is likely not a nonspecific toxic effect but has some relevance to the human disorder. In addition, SDH/complex II alterations are probably not simply the consequence of neuronal loss because the severity of reduction of the complex is disproportionate

in comparison with the extent of cell degeneration (~20% range; see present data and Zala *et al.*, 2005).

The mechanisms that could underlie the loss of complex II/SDH in our *in vitro* model have not been elucidated. Interestingly, the present results indicate that the short N-terminus fragment of mutated Htt alone is sufficient to produce complex II/SDH defects. This is in agreement with the hypothesis that, at least in part, N-terminus fragments of mutated Htt generated from the cleavage of the full-length protein contribute to the neurotoxicity caused by the mutation (Li and Li, 2004; Wellington *et al.*, 2002). The present study shows that although protein levels and activity of complex II/SDH were markedly altered, the levels of mRNA of subunits were not decreased, suggesting the involvement of posttranscriptional regulation. Our results suggest that the loss of Ip protein precedes the loss of Fp protein. In human, mutations in any of the SDH subunits cause the complex II to fully disassemble (Rustin *et al.*, 2002). Thus, it is possible that short fragments of mutated Htt produce their effect preferentially on the Ip subunit, triggering destabilization of the entire complex, and in turn the loss of the Fp protein. Given that mutated Htt is located in the cytoplasm and can bind the outer mitochondrial membrane (Panov *et al.*, 2002; Choo *et al.*, 2004), many speculative mechanisms can be proposed to explain how mutated Htt reduces posttranscriptional Ip levels. For instance, mutated Htt could induce a decrease in the import of Ip into the mitochondria, an increase in degradation, or an abnormal assembly. One interesting mechanism may be related to p53. A role for the accumulation of p53 in mitochondrial abnormalities and degeneration in HD has been recently demonstrated (Bae *et al.*, 2005). The accumulation of p53 could possibly result from proteasome dysfunction induced by short fragments of mutated Htt (Jana *et al.*, 2001; Waelter *et al.*, 2001). Apart from its role as a transcription factor, p53 interacts in the cytosol with proteins (e.g., Bax and BclXL) involved in regulation of the mitochondrial pathway of apoptosis (Chipuk *et al.*, 2005). p53 might be even localized within the mitochondria (Heyne *et al.*, 2004). p53 is also involved in oxidative stress (Culmes and Mattson, 2005), and SDH Ip is often considered as a "sensor" of the ubiquinone pool, a major source of reactive oxygen species in the cells (Rustin *et al.*, 2002). Thus, it is possible that p53 could be involved in the reduction of complex II/SDH levels induced by mutated Htt.

Another interesting possibility is related to the Ip protein mRNA structure. Ip mRNA possesses an UTR named IRE (iron responsive element) sequence to which specific proteins (IRP-1 and IRP-2) can bind, regulating translation (Gray *et al.*, 1996). The presence of mutated Htt could interfere at this level to modify posttranscriptionally SDH expression. Supporting this hypothesis, huntingtin has been implicated in regulation of iron homeostasis (Hilditch-Maguire *et al.*, 2000). Of interest, IRP-2 levels are regulated by the ubiquitin-proteasome pathway (Guo *et al.*, 1995). Accumulation of IRP2 might reduce translation of mRNAs coding SDH Ip subunit, leading to depletion of the protein. Ongoing experiments will examine the role of IRPs.

The present results show that loss of complex II/SDH subunits and activity was accompanied by a significant decrease in mitochondrial membrane potential in striatal neurons expressing Htt171-82Q. Our observation is in agreement with the results obtained in mitochondria of HD patients and transgenic YAC72 mouse HD model that showed abnormal mitochondrial transmembrane potential (Sawa *et al.*, 1999; Panov *et al.*, 2002). Noticeably, Bae *et al.* (2005) showed reduced accumula-

tion of Mitotracker Red in striatal neuronal cells expressing mutated Htt, along with changes in JC-1 fluorescence, indicating anomalies in mitochondrial membrane potential. Reduction of SDH activity could reduce the proton gradient produced by complex III and complex IV across the inner mitochondrial membrane. Interestingly, we found that overexpressing Ip or Fp proteins using lentivirus could prevent Htt171-82Q-induced loss of mitochondrial membrane potential. This suggests that loss of mitochondrial membrane potential in neurons expressing short N-terminus fragment of mutated Htt could be, at least in part, a direct consequence of complex II/SDH defects.

We showed that correcting the molecular defect of SDH complex blocked death of striatal cells induced by expressing low levels of the N-terminus part of mutated Htt. When Htt171-82Q expression levels were increased using the tetracycline-regulated promoter, overexpression of SDH-A or SDH-B modified neither the reduction of DARPP32 expression in striatal cultures nor the levels of ubiquitin-positive inclusions. However, the rescuing effect of SDH-A and SDH-B overexpression from cell death was seen. These results indicate that loss of complex II/SDH is probably not responsible for loss of a number of striatal markers and does not play a direct effect on sequestration/detoxification of mutated Htt fragment. Importantly our results provide evidence that complex II/SDH subunits are critical for the execution of cell death induced by toxicity of N-terminus fragment of mutated Htt.

The observation that overexpression of one SDH subunit leads to an increased expression of the entire complex may sound surprising at first. It is probable that this phenomenon is due to an increased "stabilization" of the SDH complex. In humans, mutations in any of the SDH subunits cause the complex II to fully disassemble (Rustin *et al.*, 2002). One elegant study showed that, in lymphoblast cells isolated from a patient with homozygous Fp mutations leading to major loss of SDH (Bourgeron *et al.*, 1995), overexpression of a wild-type Fp protein led to a fully functional complex (Parfait *et al.*, 2000).

In conclusion, the present study shows preferential loss of complex II/SDH subunits in the HD striatum and a cellular model of the disease and provides the first proof of concept that regulating complex II/SDH expression may be of therapeutic interest to slow down striatal degeneration in HD. Animal experiments are awaited to determine whether such an experimental therapy could be effective *in vivo*.

ACKNOWLEDGMENTS

We thank Pierre Rustin and Sandrine Humbert for having shared with us their scientific expertise. We thank Carl Johnson of the Hereditary Disease Foundation for advice and encouragement. We are grateful to Marc Peschanski for his constructive comments on the manuscript. We thank Dr Jason Wray for critical reading of the manuscript and language corrections. This work has been supported by Commissariat à l'Énergie Atomique (CEA) and Centre National de la Recherche Scientifique. A.B. is a recipient of postdoctoral fellowship from CEA. Y.T. holds a postdoctoral fellowship from the Hereditary Disease Foundation.

REFERENCES

- Ackrell, B. A. (2000). Progress in understanding structure-function relationships in respiratory chain complex II. *FEBS Lett.* 466, 1-5.
- Bae, B. I. *et al.* (2005). p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. *Neuron* 47, 29-41.
- Beal, M. F. (1992). Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann. Neurol.* 31, 119-130.

- Benchoua, A., Braudeau, J., Reis, A., Couriaud, C., and Onteniente, B. (2004). Activation of proinflammatory caspases by cathepsin B in focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 24, 1272–1279.
- Benchoua, A., Couriaud, C., Guegan, C., Tartier, L., Couvert, P., Friocourt, G., Chelly, J., Menissier-de Murcia, J., and Onteniente, B. (2002). Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2. *J. Biol. Chem.* 277, 34217–34222.
- Benchoua, A., Guegan, C., Couriaud, C., Hosseini, H., Sampaio, N., Morin, D., and Onteniente, B. (2001). Specific caspase pathways are activated in the two stages of cerebral infarction. *J. Neurosci.* 21, 7127–7134.
- Bezprozvanny, I., and Hayden, M. R. (2004). Deranged neuronal calcium signaling and Huntington disease. *Biochem. Biophys. Res. Commun.* 322, 1310–1317.
- Bizat, N., Hermel, J. M., Boyer, F., Jacquard, C., Creminon, C., Ouay, S., Escartin, C., Hantraye, P., Kajewski, S., Brouillet, E. (2003). Calpain is a major cell death effector in selective striatal degeneration induced in vivo by 3-nitropropionate: implications for Huntington's disease. *J. Neurosci.* 23, 5020–5030.
- Bourgeron, T., Rustin, P., Chretien, D., Birch-Machin, M., Bourgeois, M., Viegas-Pequignot, E., Munnich, A., and Rotig, A. (1995). Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. *Nat. Genet.* 11, 144–149.
- Brennan, W. A., Jr., Bird, E. D., and Aprille, J. R. (1985). Regional mitochondrial respiratory activity in Huntington's disease brain. *J. Neurochem.* 44, 1948–1950.
- Brouillet, E., Conde, F., Beal, M. F., and Hantraye, P. (1999). Replicating Huntington's disease phenotype in experimental animals. *Prog. Neurobiol.* 59, 427–468.
- Brouillet, E., Jacquard, C., Bizat, N., and Blum, D. (2005). 3-Nitropropionic acid: a mitochondrial toxin to uncover physiopathological mechanisms underlying striatal degeneration in Huntington's disease. *J. Neurochem.* 95, 1521–1540.
- Browne, S. E., Bowling, A. C., MacGarvey, U., Baik, M. J., Berger, S. C., Muqit, M. M., Bird, E. D., and Beal, M. F. (1997). Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Ann. Neurol.* 41, 646–653.
- Butterworth, N. J., Williams, L., Bullock, J. Y., Love, D. R., Faull, R. L. M., and Dragunow, M. (1998). Trinucleotide (CAG) repeat length is positively correlated with the degree of DNA fragmentation in Huntington's disease striatum. *Neuroscience* 87, 49–53.
- Butterworth, J., Yates, C. M., and Reynolds, G. P. (1985). Distribution of phosphate-activated glutaminase, succinic dehydrogenase, pyruvate dehydrogenase and gamma-glutamyl transpeptidase in post-mortem brain from Huntington's disease and agonal cases. *J. Neurol. Sci.* 67, 161–171.
- Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D., and Green, D. R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science* 309, 1732–1735.
- Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Choo, Y. S., Johnson, G. V., MacDonald, M., Detloff, P. J., and Lesort, M. (2004). Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. *Hum. Mol. Genet.* 13, 1407–1420.
- Culmsee, C., and Mattson, M. P. (2005). p53 in neuronal apoptosis. *Biochem. Biophys. Res. Commun.* 331, 761–777.
- de Almeida, L. P., Ross, C. A., Zala, D., Aebischer, P., and Deglon, N. (2002). Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length. *J. Neurosci.* 22, 3473–3483.
- Deglon, N., Tseng, J. L., Bensadoun, J. C., Zurn, A. D., Arsenijevic, Y., de Almeida, L., Zufferey, R., Trono, D., and Aebischer, P. (2000). Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. *Hum. Gene Ther.* 11, 179–190.
- Gilmore, K., and Wilson, M. (1999). The use of chloromethyl-X-rosamine (MitoTracker red) to measure loss of mitochondrial membrane potential in apoptotic cells is incompatible with cell fixation. *Cytometry* 36, 355–358.
- Gimenez-Roqueplo, A. P., Favier, J., Rustin, P., Mourad, J. J., Plouin, P. F., Corvol, P., Rotig, A., and Jeunemaitre, X. (2001). The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. *Am. J. Hum. Genet.* 69, 1186–1197.
- Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* 89, 5547–5551.
- Gourfinkel-An, I., Vila, M., Faucheux, B., Duyckaerts, C., Viallet, F., Hauw, J. J., Brice, A., Agid, Y., and Hirsch, E. C. (2002). Metabolic changes in the basal ganglia of patients with Huntington's disease: an in situ hybridization study of cytochrome oxidase subunit I mRNA. *J. Neurochem.* 80, 466–476.
- Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996). Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proc. Natl. Acad. Sci. USA* 93, 4925–4930.
- Gu, M., Gash, M. T., Mann, V. M., Javoy-Agid, F., Cooper, J. M., and Schapira, A. H. (1996). Mitochondrial defect in Huntington's disease caudate nucleus. *Ann. Neurol.* 39, 385–389.
- Guo, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995). Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J. Biol. Chem.* 270, 21645–21651.
- Heyne, K., Mannebach, S., Wuertz, E., Knaup, K. X., Mahyar-Roemer, M., and Roemer, K. (2004). Identification of a putative p53 binding sequence within the human mitochondrial genome. *FEBS Lett.* 578, 198–202.
- Hilditch-Maguire, P., Trettel, F., Passani, L. A., Auerbach, A., Persichetti, F., and MacDonald, M. E. (2000). Huntingtin: an iron-regulated protein essential for normal nuclear and perinuclear organelles. *Hum. Mol. Genet.* 9, 2789–2797.
- Jana, N. R., Zernskov, E. A., Wang, G., and Nukina, N. (2001). Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum. Mol. Genet.* 10, 1049–1059.
- Jenkins, B. G., Rosas, H. D., Chen, Y. C., Makabe, T., Myers, R., MacDonald, M., Rosen, B. R., Beal, M. F., and Koroshetz, W. J. (1998). ¹H NMR spectroscopy studies of Huntington's disease: correlations with CAG repeat numbers. *Neurology* 50, 1357–1365.
- Kalbacova, M., Vrbacky, M., Drahota, Z., and Melkova, Z. (2003). Comparison of the effect of mitochondrial inhibitors on mitochondrial membrane potential in two different cell lines using flow cytometry and spectrofluorometry. *Cytometry A* 52, 110–116.
- Koroshetz, W. J., Jenkins, B. G., Rosen, B. R., and Beal, M. F. (1997). Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. *Ann. Neurol.* 41, 160–165.
- Lesort, M., Chun, W., Tucholski, J., and Johnson, G. V. (2002). Does tissue transglutaminase play a role in Huntington's disease? *Neurochem. Int.* 40, 37–52.
- Li, S. H., and Li, X. J. (2004). Huntingtin and its role in neuronal degeneration. *Neuroscientist* 10, 467–475.
- Lodi, R., Schapira, A. H., Manns, D., Styles, P., Wood, N. W., Taylor, D. J., and Warner, T. T. (2000). Abnormal in vivo skeletal muscle energy metabolism in Huntington's disease and dentatorubropallidolysian atrophy. *Ann. Neurol.* 48, 72–76.
- Mann, V. M., Cooper, J. M., Javoy-Agid, F., Agid, Y., Jenner, P., and Schapira, A. H. (1990). Mitochondrial function and parental sex effect in Huntington's disease. *Lancet* 336, 749.
- Meriin, A. B., and Sherman, M. Y. (2005). Role of molecular chaperones in neurodegenerative disorders. *Int. J. Hyperthermia* 21, 403–419.
- Munujos, P., Coll-Canti, J., Gonzalez-Sastre, F., and Gella, F. J. (1993). Assay of succinate dehydrogenase activity by a colorimetric-continuous method using iodinitrotetrazolium chloride as electron acceptor. *Anal. Biochem.* 212, 506–509.
- Panov, A. V., Gutekunst, C. A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Strittmatter, W. J., and Greenamyre, J. T. (2002). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat. Neurosci.* 5, 731–736.
- Parfait, B., Chretien, D., Rotig, A., Marsac, C., Munnich, A., and Rustin, P. (2000). Compound heterozygous mutations in the flavoprotein gene of the respiratory chain complex II in a patient with Leigh syndrome. *Hum. Genet.* 106, 236–243.
- Pendergrass, W., Wolf, N., and Poot, M. (2004). Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry A* 61, 162–169.
- Portera-Caillau, C., Hedreen, J. C., Price, D. L., and Koliatsos, V. E. (1995). Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *J. Neurosci.* 15, 3775–3787.
- Regulier, E., Pereira de Almeida, L., Sommer, B., Aebischer, P., and Deglon, N. (2002). Dose-dependent neuroprotective effect of ciliary neurotrophic factor

- delivered via tetracycline-regulated lentiviral vectors in the quinolinic acid rat model of Huntington's disease. *Hum. Gene Ther.* 13, 1981–1990.
- Regulier, E., Trotter, Y., Perrin, V., Aebischer, P., and Deglon, N. (2003). Early and reversible neuropathology induced by tetracycline-regulated lentiviral overexpression of mutant huntingtin in rat striatum. *Hum. Mol. Genet.* 12, 2827–2836.
- Ruan, Q., Lesort, M., MacDonald, M. E., and Johnson, G. V. (2004). Striatal cells from mutant huntingtin knock-in mice are selectively vulnerable to mitochondrial complex II inhibitor-induced cell death through a non-apoptotic pathway. *Hum. Mol. Genet.* 13, 669–681.
- Rustin, P., Munnich, A., and Rotig, A. (2002). Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. *Eur. J. Hum. Genet.* 10, 289–291.
- Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M. E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 95, 55–66.
- 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). Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nat. Med.* 5, 1194–1198.
- Schilling, G. *et al.* (1999). Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum. Mol. Genet.* 8, 397–407.
- Sugars, K. L., and Rubinsztein, D. C. (2003). Transcriptional abnormalities in Huntington disease. *Trends Genet.* 19, 233–238.
- Tabrizi, S. J., Cleeter, M. W., Xuereb, J., Taanman, J. W., Cooper, J. M., and Schapira, A. H. (1999). Biochemical abnormalities and excitotoxicity in Huntington's disease brain. *Ann. Neurol.* 45, 25–32.
- The Huntington's Disease Collaborative Research Group. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72, 971–983.
- Thomas, L. B., Gates, D. J., Richfield, E. K., O'Brien, T. F., Schweitzer, J. B., and Steindler, D. A. (1995). DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. *Exp. Neurol.* 133, 265–272.
- Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., and Richardson, E. P., Jr. (1985). Neuropathological classification of Huntington's disease. *J. Neuropathol. Exp. Neurol.* 44, 559–577.
- Waelter, S., Boeddrich, A., Lurz, R., Scherzinger, E., Lueder, G., Lehrach, H., and Wanker, E. E. (2001). Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol. Biol. Cell* 12, 1393–1407.
- Wellington, C. L. *et al.* (2002). Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J. Neurosci.* 22, 7862–7872.
- Zala, D., Benchoua, A., Brouillet, E., Zurn, A. D., Aebischer, P., and Déglon, N. (2005). Progressive and selective striatal degeneration in primary neuronal cultures using lentiviral vector coding mutant huntingtin fragment. *Neurobiol. Dis.* 20, 785–798.
- Zuccato, C. *et al.* (2003). Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat. Genet.* 35, 76–83.