Huntington disease (HD) is a neurodegenerative disease that is inherited in an autosomal dominant manner. It belongs to a family of CAG expansion diseases and is caused by the pathological elongation of the CAG repeats in one of the huntingtin protein gene (1). Symptoms and disease progression are caused by dysfunction and loss of neurons starting in the striatum (specifically medium spiny neurons), but progressing to cortex and to a lesser extent to other brain regions in the later stages of the disease (2). Disease is caused by the toxic gain of function of mutant protein but some loss of function may also contribute to the pathogenesis (for review see Ref. 3). The toxic gain of function of mutant huntingtin has not been clearly defined, but there are findings suggesting that mutant huntingtin causes transcriptional dysregulation (4), ubiquitin-proteasome system dysfunction (5), \( \text{Ca}^{2+} \) homeostasis dysfunction (6, 7), and mitochondrial dysfunction (7–10).

Mitochondrial dysfunction in HD has been suggested primarily by the studies showing impairment of mitochondrial complexes (II, III, and IV) specifically in the striatum in the late stages HD patients (8–10). Administration of the mitochondrial complex II inhibitor 3-nitropropionic (3-NP) in both rodents and nonhuman primates resulted in symptoms and neuropathology that resemble HD (11, 12). Further, studies have shown impairment of mitochondrial \( \text{Ca}^{2+} \) buffering in HD lymphoblast cell line and brain mitochondria from the full-length mutant huntingtin transgenic mice (YAC72) (7, 13).

Striatum, the primary region to get affected in HD is highly innervated by cortical glutamnergic projections (2). Previously it has been demonstrated that mitochondrial dysfunction can lead to neuronal sensitization to glutamate leading to excitotoxic cellular dysfunction and cell death (14, 15). Hence, even though HD is not a classic mitochondrial disease (16), elucidation of mitochondrial dysfunction mechanisms would likely provide important insight in HD pathogenesis.

To study the effects of mutant huntingtin on mitochondrial function, conditionally immortalized cells of striatal origin that express endogenous, comparable levels of either wild type (STHdh\(^{Q7/Q7}\)) or mutant (STHdh\(^{Q111/Q111}\)) huntingtin were used (17). These cell lines are prepared from wild type (Hdh\(^{Q7/Q7}\)) and mutant huntingtin knock-in mice (Hdh\(^{Q111/Q111}\)) (17) and therefore the STHdh\(^{Q111/Q111}\) cell line is a genetically accurate cell model of HD. In our previous study (18), we investigated the effects of mutant huntingtin on mitochondrial electron transport chain complexes using STHdh\(^{Q7/Q7}\) and STHdh\(^{Q111/Q111}\) cell lines. Given the fact that the metabolic thresholds and enzyme activities of electron transport chain complexes were not different between the two cell lines, it is likely that the mitochondrial complex deficits are a later event in the course of HD pathogenesis, indeed in low grade HD cases no deficits in the enzyme activities of electron transport chain complexes were observed (19).
In the present study, we examined the effects of Ca\(^{2+}\) on mitochondria from ST
dh\(^{Q7/Q7}\) and ST
dh\(^{Q111/Q111}\) cells. Isolated mitochondria were treated with increasing Ca\(^{2+}\) con-
centrations and mitochondrial function was assessed using dif-
ferent assays. We determined that mutant huntingtin-express-
ing cells have decreased Ca\(^{2+}\) uptake capacity, and exhibit
increased sensitivity to Ca\(^{2+}\)-induced decreases in respiration and ΔΨm. The ΔΨm defect was attenuated in the presence of
ADP and the decrease in Ca\(^{2+}\) uptake capacity was abolished in
the presence of Permeability Transition Pore (PTP) inhibitors.
This study clearly demonstrates that mitochondrial Ca\(^{2+}\) buff-
ering capacity in ST
dh\(^{Q111/Q111}\) cells is compromised, and
suggests increased sensitivity to Ca\(^{2+}\)-induced mitochondrial
permeabilization as a mechanism of mitochondrial dysfunction in
HD.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were from Sigma-Aldrich unless otherwise noted. All buffers used in experiments with crude mitochondria
preparations were prepared in water (Sigma, catalogue no. 95305) that is standar-
dized for Ca\(^{2+}\) content ([Ca\(^{2+}\)] = 0.000001%).

**Cell Culture**—In this study, conditionally immortalized striatal progenitor cell lines: ST
dh\(^{Q7/Q7}\) cell line expressing endogenous wild type huntingtin and the homozygous
mutant ST
dh\(^{Q111/Q111}\) cell line expressing comparable lev-
els of mutant huntingtin with 111 glutamines were used. Cell
lines were prepared from wild type mice and homozygous
Hdh\(^{Q111/Q111}\) knock-in mice and were described previously
(17). Culturing conditions were the same as described in our
previous study (18).

**Isolation of Mitochondria**—Cells were grown on 150-mm plates until ≈ 80–90% confluency, washed twice with cavi-
tation buffer (250 mM sucrose, 5 mM HEPES, 3 mM MgCl\(_2\), 1 mM
EGTA, pH 7.3 corrected with 5 M KOH) and scraped into cavi-
tation buffer using soft rubber scrapers. Cells were opened
using N\(_2\) cavitation for 5 min at 250 psi on ice, and samples were
additionally homogenized with 1 stroke in a glass Dounce homogenizer. Homogenates were centrifuged at 7000 X g for
10 min at 4 °C. Supernatants were aspirated, and pellets were
resuspended in cavi
tation buffer and used as crude mitochondria
preparations. Protein concentrations in crude mitochondria
preparations were determined using the bicinchoninic
acid assay (Pierce), and aliquots were then prepared that con-
tained the indicated protein content for each measurement.
Aliquots were centrifuged at 7000 X g for 10 min and kept on
ice in cavitation buffer until use in each assay.

**Measurement of Mitochondrial Respiration**—Respiration
rates were measured using an oxygraph (Hansatech Instru-
mants) as described previously (18). Crude mitochondrial prep-
arations (0.5-mg aliquots) were resuspended in respiration
buffer (130 mM KCl, 20 mM HEPES, 2 mM MgCl\(_2\), 2 mM EGTA,
2 mM potassium phosphate (KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\), 1:1.78), 1% es-
sentially fatty acids free bovine serum albumin, pH 7.2
adjusted with 5 M KOH) to a final concentration of 1 mg/ml.
The mitochondrial suspension (0.5 ml volume) was placed in
the respiratory chamber and allowed to equilibrate for 2 min.
Respiratory substrate (glutamate (10 mM) plus malate (10 mM)
or succinate (5 mM) with rotenone (10 μM)) was then added and
state 4 respiration was measured for 2 min, ADP (1.5 mM) was
then added, and state 3 respiration was measured for a further
2–4 min. Rates were normalized to citrate synthase activity in
the same samples. Citrate synthase activity was determined as
previously described (18).

**Ca\(^{2+}\) Titration Experiments**—Respiration buffers containing
specific free Ca\(^{2+}\) concentrations (Ca\(^{2+}\)-EGTA respiration
buffers) were prepared on the day of the experiment. To cal-
culate the amount of total Ca\(^{2+}\) that was needed to achieve the
appropriate free Ca\(^{2+}\) concentration in the respiration buffer
that contained 2 mM EGTA we used MaxChelator software
(20). Each Ca\(^{2+}\)-EGTA respiration buffer was prepared sep-
arately by diluting each specific 100× CaCl\(_2\) stock in the
respiration buffer and correcting its pH to 7.2 using 0.1 M
KOH in the respiration buffer. CaCl\(_2\) stocks were prepared
from CaCl\(_2\)-2H\(_2\)O (minimum 99%), that was dried overnight
and stored in a desiccation chamber until use. Crude mitochondria
preparations were dissolved in prepared Ca\(^{2+}\)-EGTA respiration
buffers, and respiration rates were measured as described above. The period between buffer addition to the
mitochondrial preparation and initiation of state 3 was ~5 min.

Free Ca\(^{2+}\) concentrations in the Ca\(^{2+}\)-EGTA buffers were
checked using a calibrated Ca\(^{2+}\) electrode on the day of the
experiment. Measured concentrations were averaged and pre-
sented on the X-axis of Ca\(^{2+}\) titration experiments graphs.
Actually concentrations were always slightly higher than those
calculated by software.

**Cytochrome c and NADH Respiration Experiments**—Respi-
ration experiments were performed as described above. State 3
respiration was measured for 2 min prior to the addition of
cytochrome c (30 μM), and respiration was monitored for an-
other 2 min. This was followed by the addition of NADH (5
mM), and respiration was monitored for an additional 2 min.

**Determination of Mitochondrial Ca\(^{2+}\) Uptake Capacity**—
Ca\(^{2+}\) uptake capacities were measured using a Ca\(^{2+}\) electrode
(World Precision Instruments). Crude mitochondrial prepara-
tion was resuspended in Ca\(^{2+}\) uptake buffer (130 mM KCl, 20
mM HEPES, 2 mM MgCl\(_2\), 2 mM potassium phosphate (KH\(_2\)PO\(_4\)/
K\(_2\)HPO\(_4\), 1:1.78), 1% bovine serum albumin, pH 7.2
adjusted with 5 M KOH) and placed in the oxygraph respiratory
chamber. The respiratory chamber was thermostatted at 37 °C,
and its contents were constantly mixed with an electromag-
netic stirrer bar. Glutamate (10 mM) and malate (10 mM) were
added as respiratory substrates. Ca\(^{2+}\) and reference electrodes
were added to the chamber from the top. Starting volume of the
reaction was 2 ml. The chamber was kept open during an exper-
iment. Ca\(^{2+}\) additions were performed using fine tubing and a
Hamilton syringe. 5, 10, and 20 mM CaCl\(_2\) stocks were used to
make 10, 20, 40, or 80 nmol of Ca\(^{2+}\) additions. The Ca\(^{2+}\) elec-
trode measures extramitochondrial Ca\(^{2+}\) and increases in the
signal present as downward deflections on the traces.

To observe the effects of PTP inhibition on Ca\(^{2+}\) uptake capacity we used cyclosporine A (1 μM) plus ADP (50 μM) plus
oligomycin (2 μg/ml). The PTP inhibitors were added to the
respiratory chamber prior to Ca\(^{2+}\) additions (21).

To calculate Ca\(^{2+}\) uptake capacity, we counted number of
Ca\(^{2+}\) additions until the addition after which no uptake was
observed (trace horizontal). The number of additions was multiplied by the nmol of Ca\(^{2+}\) per addition, and normalized to protein content.

**Mitochondrial Membrane Potential (ΔΨm) Determination in Live Cells**—Mitochondrial membrane potential was estimated using the specific mitochondrial probes: Mitotracker Red (CM-H\(_2\)TMRos) and tetrachromidine ethyl ester (TMRE) (Molecular Probes) (22–26). Cells were grown on poly-l-lysine-coated plates and cultured for 3 days. The cells were then loaded for 30 min with CM-H\(_2\)TMRos in KRH-glucose, washed, and allowed to equilibrate for 15 min. Cell plates were then mounted in a chamber on the stage of a confocal laser scanning microscope (Leica model TCS SP2). Quantitative measurements of CM-H\(_2\)TMRos fluorescence were performed by confocal microscopy (Leica model TCS SP2), using a 40× water immersion lens. CM-H\(_2\)TMRos fluorescence images were obtained by excitation at 563 nm, reflection off a dichroic mirror with a cut-off wavelength at 564 nm, and longpass emission filtering at 590 nm. For TMRE, experiments, cells were loaded with TMRE (100 nM) for 45 min in KRH-glucose, and then mounted on the stage for confocal microscopy. TMRE fluorescence was detected exciting with a 561 nm He-Ne laser line very heavily attenuated (10% laser power), and the emission was collected at >563 nm (22). Signal from control cells and cells treated with different stimuli were compared using identical settings for laser power, confocal thickness, and detector sensitivity for each dye and separate experiment (23, 25, 26). The images were analyzed with LCS Leica confocal software and recorded as the mean Mitotracker Red or TMRE fluorescence signal per live cell.

**Measurement of Mitochondrial Membrane Potential (ΔΨm) in Mitochondrial Preparations**—ΔΨm was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo cyanine iodide (JC-1; Molecular Probes) according to a published protocol with modifications (27). Modifications were made so that method could be used with isolated mitochondria. To measure ΔΨm at different Ca\(^{2+}\) concentrations, crude mitochondrial preparation was aliquoted into the wells of 96-well plate (50 μg/well). The plate was centrifuged at 3220 × g for 10 min at 4 °C, and supernatants were carefully aspirated. Ca-EGTA respiration buffers with 0, 0.4, or 0.6 μM (software calculated) free Ca\(^{2+}\) or Ca\(^{2+}\) uptake buffer with 150, 500, or 1000 μM Ca\(^{2+}\) each supplemented with glutamate (10 mM), malate (10 mM), Amplex Red (50 μM), horseradish peroxidase (0.01 units/ml or 0.1 units/ml), and with (for state 3) or without (for state 4) ADP (1.5 mM). Plate was read in the kinetic mode for 30 min at excitation/emission wavelengths 530/590 nm at 37 °C. Rates of H\(_2\)O\(_2\) production were determined using a standard curve.

**Statistical Analysis**—Results were analyzed using ANOVA, Student’s t test, or paired t test as indicated. Differences were considered significant if p ≤ 0.05.

**RESULTS**

**Effects of Ca\(^{2+}\) on Respiration in Mitochondria from STHdhQ\(^{77/Q7}\) (Wild Type) and STHdhQ\(^{111/Q111}\) (Mutant) Cells**—It has been shown previously that at the free concentrations higher than 1 μM, Ca\(^{2+}\) causes strong inhibition of oxidative phosphorylation (29). To determine the effects of Ca\(^{2+}\) on oxidative phosphorylation in mitochondria isolated from the cells expressing endogenous levels of wild type (STHdhQ\(^{77/Q7}\)) or mutant (STHdhQ\(^{111/Q111}\)) huntingtin, we measured state 4 and state 3 respiration rates in respiration buffers containing increasing free μM Ca\(^{2+}\) concentrations. In these experiments, EGTA-based respiration buffer was used for 0 μM Ca\(^{2+}\) and Ca\(^{2+}\)-EGTA respiration buffers were prepared as described under “Experimental Procedures.” At 0 μM Ca\(^{2+}\), we observed no differences in the state 4 or state 3 respiration rates between wild type and mutant cells when glutamate plus malate (complex I substrate) or succinate (complex II substrate) were used as substrates (Fig. 1A). As described earlier (29), with increasing free μM Ca\(^{2+}\) concentrations decreases in the state 3 rates were observed (Fig. 1B). However, this decrease was more pronounced in the mitochondria from the mutant huntingtin-expressing cells, reaching significance at lower Ca\(^{2+}\) concentrations than in the wild type (Fig. 1B). State 4 rates increased with increasing Ca\(^{2+}\) concentrations, reaching significance only in the mutant at the highest Ca\(^{2+}\) concentration used (Fig. 1B). To describe overall changes in the respiration rates, we calculated Respiratory Control Ratios (RCRs) at the different Ca\(^{2+}\) concentrations. RCR was calculated as the ratio between state 3 and state 4 rates. A decrease in RCR was observed with increasing Ca\(^{2+}\) concentrations and was more pronounced in mitochondria from mutant cells, reaching significance at the lower Ca\(^{2+}\) concentrations than in the wild type cells (Fig. 1C). These results indicate that mitochondria from STHdhQ\(^{111/Q111}\) (mutant) cells are more sensitive to Ca\(^{2+}\)-induced changes in oxidative phosphorylation than mitochondria from STHdhQ\(^{77/Q7}\) (wild type) cells.

**Ca\(^{2+}\) Uptake Capacity in Mitochondria from STHdhQ\(^{77/Q7}\) (Wild Type) and STHdhQ\(^{111/Q111}\) (Mutant) Cells**—Several studies have suggested that there is reduced mitochondrial Ca\(^{2+}\) buffering capacity in HD. Panov et al. (7, 13) demonstrated diminished Ca\(^{2+}\) uptake capacity in mitochondria from HD lymphoblast cell lines, and brain mitochondria from the full-length mutant huntingtin-overexpressing mice (YAC72) (7), whereas others demonstrated diminished Ca\(^{2+}\) uptake in
muscle mitochondria from R6/2 mice (30). To comprehensively describe the effects of Ca²⁺ on mitochondria in our model, we determined mitochondrial Ca²⁺ uptake capacity in STHdhQ111/Q111 (mutant) cells. For these experiments we used a Ca²⁺-sensitive electrode, as described under “Experimental Procedures.” To determine, mitochondrial Ca²⁺ uptake, isolated mitochondria (1.5 mg/2 ml) were placed in a 37 °C thermostatted chamber and challenged with 10-nmol Ca²⁺ pulses every 3 min. Representative traces are shown in Fig. 2C. Ca²⁺ uptake capacity was calculated as described under “Experimental Procedures.” We observed that mitochondria from STHdhQ111/Q111 (mutant) cells have significantly diminished Ca²⁺ uptake capacity compared with mitochondria from STHdhQ7/Q7 (wild type) cells (Fig. 2A). To determine the “initial uptake” rates we calculated the average of the rates after the second, third, and fourth additions of Ca²⁺ and determined that the “initial uptake” rates were significantly diminished in the mitochondria from the mutant cells (Fig. 2B). These results indicate that mitochondria from STHdhQ111/Q111 (mutant) cells have a Ca²⁺-buffering defect, as they can take up less Ca²⁺ than the mitochondria from wild type cells. Because ER contamination of the mitochondrial preparation was a possibility, we confirmed the mitochondrial nature of the Ca²⁺ uptake in our mitochondrial preparations, as the addition of uncoupler (FCCP) caused release of Ca²⁺, and pretreatment of the cells with thapsigargin (which blocks the Ca²⁺ uptake pump of the ER Ref. 31) did not produce any change in the Ca²⁺ uptake capacity (Fig. 2D).

**FIGURE 1. Effects of Ca²⁺ on respiration in mitochondria from STHdhQ7/Q7 (wild type) and STHdhQ111/Q111 (mutant) cells.** A, state 4 (st4) and state 3 (st3) respiration rates measured in crude mitochondrial preparations in EGTA-based respiration buffer. Crude mitochondria from wild type and mutant cells were incubated with either glutamate plus malate (glu. + mal.) or succinate with rotenone (succ. + rot.) as respiratory substrates, and respiration rates were determined as described under “Experimental Procedures.” Rates were normalized to citrate synthase activity measured in the same samples. No significant differences were observed. B, respiration rates measured in the presence of increasing free Ca²⁺ concentrations. Crude mitochondria were resuspended in Ca-EGTA buffers with the indicated free Ca²⁺ concentrations, and state 4 and state 3 rates were determined as described under “Experimental Procedures.” Glutamate plus malate was used as the respiratory substrate. Results are expressed as percentage of the state 3 rates at 0 Ca²⁺ for each of the cell lines. Significant decreases in state 3 rates at the low μM free Ca²⁺ concentrations were observed, with the decreases occurring at the lower Ca²⁺ concentrations in mitochondria from the mutant cells compared with wild type mitochondria. State 4 rates showed a trend toward increasing with increasing free Ca²⁺ concentrations, with significance being reached only at the highest Ca²⁺ concentration used in the mutant cells. C, RCRs in the presence of different free Ca²⁺ concentrations were calculated as ratios between state 3 and state 4 rates presented in B. The decrease in RCR reached significance at lower Ca²⁺ concentrations in mutant cells than in the wild type group. All data are mean ± S.E. of 3–4 independent experiments. For statistical analyses ANOVA followed by the Tukey post test (*, p < 0.05) was used.
Analysis of Mitochondrial Membrane Integrity Before and After Ca\(^{2+}\)/H\(_{11001}\) Addition in STHdh\(^{Q7/Q7}\) (Wild Type) and STHdh\(^{Q111/Q111}\) (Mutant) Cells—Ca\(^{2+}\)/H\(_{11001}\) overload of mitochondria results in increased mitochondrial membrane permeability (32). To further study the cause of differences between mitochondria from wild type and mutant cells in their sensitivity to Ca\(^{2+}\), we wanted to determine if the decrease in respiration observed in the presence of free \(\mu\)M Ca\(^{2+}\) concentrations was associated with increased permeability of the mitochondrial membrane. First, we analyzed the integrity of mitochondrial membrane in the basal conditions. NADH is the substrate for mitochondrial complex I. However, the inner mitochondrial membrane is not permeable to exogenous NADH (33). When pyruvate plus malate was added to provide reduced adenine dinucleotides (NADH, FADH\(_{2}\)) inside the mitochondria we observed significant state 3 rates upon ADP addition (Fig. 3A). However, when NADH was used as the respiratory substrate, we did not observe induction of state 3 respiration in mitochondria from any of the cell lines (Fig. 3A). Representative traces are shown (Fig. 3A). This indicates good integrity of mitochondrial inner membrane in basal conditions in mitochondria from both cell lines.

It has been suggested that damage of the outer mitochondrial membrane results in the activation of alternative respiratory pathway in the presence of exogenous NADH and cytochrome c (34–37). In this pathway, NADH is oxidized at the outer mitochondrial membrane leading to reduction of exogenous cytochrome c. As described previously, if outer membrane is being compromised, cytochrome c will translocate to the complex IV and stimulate respiration (35). To assess mitochondrial membrane integrity, we measured the effects of NADH and cytochrome c on state 3 respiration in the absence...
Ca\textsuperscript{2+} Handling Defects with Mutant Huntingtin Expression

A

|          | Wild-type: pyr+mal | Mutant: pyr+mal |
|----------|--------------------|-----------------|
| State 3  | 5.5                | 4.4             |
| State 3+cyt c | 31.5            | 29.2            |
| State 3+cyt c+NADH | 6.9            | 4.8             |

B

|          | % state 3 at 0 µM Ca\textsuperscript{2+} |
|----------|------------------------------------------|
| -Ca\textsuperscript{2+} | wild-type |
| +Ca\textsuperscript{2+} | mutant |
| -Ca\textsuperscript{2+} | -100 |
| +Ca\textsuperscript{2+} | -100 |

The state 3 respiration was decreased, cytochrome c plus NADH caused a significant increase in state 3 (Fig. 3B). Alamethicin, forms pores in the membrane, was used as a positive control for the method (not shown).\textsuperscript{3} Cytochrome c alone did not affect state 3 respiration in the presence of Ca\textsuperscript{2+} (Fig. 3B), suggesting that no substantial loss of cytochrome c is causing the decrease in state 3 rate. These results suggest that mitochondria from both wild type and mutant cells show good membrane integrity in the absence of Ca\textsuperscript{2+}. Further, the decrease in state 3 rates in the presence of µM Ca\textsuperscript{2+} concentrations is associated with the increased permeability of mitochondrial membrane but not substantial loss of cytochrome c.

Differential Effects of Ca\textsuperscript{2+} on Mitochondrial Membrane Potential (ΔΨm) in STHdhQ7/Q7 (Wild Type) and STHdhQ111/Q111 (Mutant) Cells—To determine the effects of Ca\textsuperscript{2+} deregulation on ΔΨm, the ratiometric dye JC-1 was used (38). In these experiments, isolated mitochondria were incubated with increasing Ca\textsuperscript{2+} concentrations, keeping the same [mitochondrial mass/Ca\textsuperscript{2+} buffer volume] ratio as in the respiration experiments. Ca\textsuperscript{2+} concentrations used were: 0 µM (EGTA-based respiration buffer), two low µM concentrations: 0.4 µM, 0.6 µM (software calculated) that correspond to 1.1 µM, 2.2 µM (Ca\textsuperscript{2+} electrode determined) in Fig. 1B, at which decreases in state 3 respiration was observed, and 3 high µM concentrations: 150, 500, and 1000 µM, where 150 µM corresponds approximately to the Ca\textsuperscript{2+} concentration at which we no longer observed Ca\textsuperscript{2+} uptake by the wild type mitochondria (Fig. 2). ΔΨm was also measured in Ca\textsuperscript{2+} uptake buffer (Cab), which is respiration buffer without EGTA, and contains ~10 µM Ca\textsuperscript{2+} as determined by using the Ca\textsuperscript{2+} electrode. ΔΨm was determined as described under “Experimental Procedures.” FCCP was used to induce maximal decrease of ΔΨm, as the positive control for the assay (Fig. 4). In

\textsuperscript{3} G. V. W. Johnson, unpublished observations.
Effects of Ca\(^{2+}\) on H\(_2\)O\(_2\) Production in Mitochondria from STHdh\(^{Q7/Q7}\) (Wild Type) and STHdh\(^{Q111/Q111}\) (Mutant) Cells—Increased ROS production in mitochondria is usually associated with perturbations of electron transfer in the oxidative phosphorylation process (39) and has been described in conditions of increased mitochondrial membrane permeability (40). To assess the effects of Ca\(^{2+}\) on ROS production in the mitochondria from wild type and mutant cells, a significant difference was observed when wild type and mutants were compared (Fig. 5). The presence of mitochondria from mutants in state 3 condition (ADP added), we observed increased H\(_2\)O\(_2\) production with Ca\(^{2+}\) that reached significance only in the mutant at 1000 \(\mu\)M Ca\(^{2+}\) (Fig. 5B). The trend of increased H\(_2\)O\(_2\) production with increasing Ca\(^{2+}\) concentrations was similar to what was observed for the \(\Delta\Psi\)m change. At all Ca\(^{2+}\) concentrations, mutant mitochondria displayed a greater increase in H\(_2\)O\(_2\) production than wild type (Fig. 5B). This increase was statistically significant at 0.4, 0.6, and 500 \(\mu\)M (Fig. 5B). In these experiments, rotenone-treated mitochondria were used as an assay positive control. As expected, in the wild type, rotenone treatment caused significant increase in H\(_2\)O\(_2\) production. Interestingly, mutant mitochondria generated significantly less H\(_2\)O\(_2\) upon rotenone treatment when compared with the wild type.

Effects of PTP Inhibitors on Mitochondrial Ca\(^{2+}\) Uptake Capacity in STHdh\(^{Q7/Q7}\) (Wild Type) and STHdh\(^{Q111/Q111}\) (Mutant) Cells—Diminished Ca\(^{2+}\) uptake capacity in the mutant could be explained by a lower threshold for PTP opening in the mutant cells. To test this hypothesis, Ca\(^{2+}\) uptake capacity in the presence of PTP inhibitors was measured. For these experiments, cyclosporine A plus ADP plus oligomycin was used, as this combination has been shown to be very efficient in inhibiting PTP in brain mitochondria (21). Addition of PTP inhibitors increased mitochondrial Ca\(^{2+}\) uptake capacity in both cell lines. This increase was greater and statistically significant in the mutant mitochondria (ANOVA, Tukey post test; \(p < 0.01, n = 5 \) for mutant and \(n = 8 \) for mutant plus PTP inhibitors group) (Fig. 6). In the presence of PTP inhibitors, mitochondrial Ca\(^{2+}\) uptake capacity reduction was strongly attenuated in the mutant mitochondria, as the difference between the wild type and mutant mitochondria no longer reached statistical significance (Fig. 6). These results suggest that STHdh\(^{Q111/Q111}\) (mutant) cells exhibit a lower threshold for PTP opening when compared with STHdh\(^{Q7/Q7}\) (wild type) cells.

Effects of Ca\(^{2+}\) on \(\Delta\Psi\)m in STHdh\(^{Q7/Q7}\) (Wild Type) and STHdh\(^{Q111/Q111}\) (Mutant) Cells in Situ—Prior to evaluating the effects of Ca\(^{2+}\) on \(\Delta\Psi\)m in situ with CM-H\(_2\)TMRos and TMRE,
control studies were carried out to validate the use of these fluorescence dyes. These preliminary studies demonstrated that treatment of both wild type or mutant cells with the mitochondrial uncoupler FCCP resulted in a robust decrease in CM-H2TMRos and TMRE fluorescence, indicating a loss of $\Delta \Psi m$. To determine the effects of changes in cytosolic $Ca^{2+}$ levels on $\Delta \Psi m$, cells were treated with a low concentration of the ionophore 4-BrA23187 (1 nM), which does not negatively impact cell viability (41). After treatment with the ionophore for 5 min, 4 $\mu$mol of $Ca^{2+}$ additions were made approximately every 5 min, to obtain the increasing concentrations of $Ca^{2+}$ in the medium (2, 4, 6 mM). With increasing $Ca^{2+}$ concentrations a decrease in $\Delta \Psi m$ was observed as measured with both CM-H2TMRos (Fig. 7C) and TMRE (Fig. 7D), and it was significantly more pronounced in mutant cells (Fig. 7, C and D). The difference in $\Delta \Psi m$ between wild type and mutant cells was significant starting at 2 mM $Ca^{2+}$ in the media (Fig. 7, C and D). This was in accordance with the results obtained in mitochondrial preparations as shown in Fig. 4.

**DISCUSSION**

In this study, we provide evidence for the first time that mitochondrial $Ca^{2+}$ handling defects in cells of striatal origin that express endogenous levels of mutant huntingtin result in impairment of respiration, which could contribute to neuronal dysfunction and death in HD. Treatment of isolated mitochondria from mutant cells with increasing $Ca^{2+}$ concentrations, resulted in a significant decrease in state 3 respiration at lower $Ca^{2+}$ than mitochondria from wild type cells. Further, the $Ca^{2+}$-dependent decrease of $\Delta \Psi m$ was significantly greater in the mutant cells compared with the wild type cells. However, the $\Delta \Psi m$ defect was markedly attenuated in the presence of ADP. Additionally the mitochondrial $Ca^{2+}$ uptake capacity in mutant cells was significantly lower than what was observed in mitochondria from wild type cells, which was completely abolished by the presence of PTP inhibitors. Taken together these data demonstrate that the presence of mutant huntingtin at physiologically relevant levels results in impaired $Ca^{2+}$ handling by...
mitochondria which negatively impacts their function and hence likely impairs proper neuronal function.

Excitotoxicity has been suggested as a key mechanism that is responsible for neurodegeneration in HD (42), and dysfunction at the level of the mitochondria could be a mediator of this toxicity. Mitochondrial dysfunction could result in an increase in the sensitivity of neurons to glutamate, leading to \( \text{Ca}^{2+} \)-induced cellular dysfunction and eventually cell death (the role of mitochondria in excitotoxicity is discussed in Refs. 43–45). It has been shown previously that decreases in oxidative phosphorylation and state 3 rates are early events in excitotoxicity, and occur prior to the commitment to cell death (46). Moreover, it has been shown that free \( \mu \text{M} \text{Ca}^{2+} \) concentrations cause significant decreases in oxidative phosphorylation in isolated mitochondria (29). To determine if mutant huntingtin alters mitochondrial response to \( \text{Ca}^{2+} \), we measured respiration rates in the presence of increasing \( \text{Ca}^{2+} \) concentrations in mitochondria from wild type (STHdh\text{Q7/Q7}) and mutant huntingtin (STHdh\text{Q111/Q111})-expressing cells. We observed that the decrease in the state 3 rate (ADP phosphorylation rate) occurred at significantly lower \( \text{Ca}^{2+} \) concentrations in mutant cells compared with wild type cells, suggesting an increased sensitivity to \( \text{Ca}^{2+} \).

Elevated mitochondrial \( \text{Ca}^{2+} \) levels are usually associated with the opening of PTP. However, the mechanism of the oxidative phosphorylation decrease due to elevated \( \text{Ca}^{2+} \) levels is not quite clear (discussed in Ref. 46). It has been suggested that permeabilization of the outer mitochondrial membrane leads to activation of an alternative respiratory pathway, which utilizes exogenous NADH oxidation on the outer mitochondrial membrane and translocation of subsequently reduced cytochrome c to the inner membrane where it feeds into complex IV (34, 35). In our study, addition of NADH and cytochrome c caused an increase in the state 3 rates in the presence of \( \text{Ca}^{2+} \),

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**Ca\(^{2+}\) Handling Defects with Mutant Huntingtin Expression**

**FIGURE 7. Effects of the Ca\(^{2+}\) increase in the mitochondrial potential functionality in STHdh\text{Q7/Q7} (wild type) and STHdh\text{Q111/Q111} (mutant) cells in situ.**

A, wild type and mutant cells were loaded with Mitotracker \( \text{H} \) ROS Red (200 \( \mu \text{M} \)) to measure changes in mitochondrial potential in response to 10 \( \mu \text{M} \) FCCP treatment for 30 min. FCCP induces a significant decrease in the mitochondrial potential in both cell types. Data are mean ± S.E. \( n = 3 \) separate experiments.

B, to corroborate the observations with Mitotracker \( \text{H} \) ROS Red, wild type and mutant cells were loaded with TMRE (100 \( \mu \text{M} \)) to measure changes in mitochondrial potential in response to 10 \( \mu \text{M} \) FCCP and similar results were obtained. Data are mean ± S.E. \( n = 3 \) separate experiments. C, wild type (black bars) and mutant (gray bars) cells were exposed to 1 \( \text{nM} \) 4-Bra23187 in the presence of increasing \( \text{Ca}^{2+} \) concentrations. Quantification of Mitotracker \( \text{H} \) ROS Red fluorescence, as relative units, shows significantly reduced mitochondrial potential in mutant cells pretreated with 4-Bra23187 (4-Bra) at each \( \text{Ca}^{2+} \) concentration used in comparison to wild type cells (*, \( p < 0.05; n = 3 \)). Data are mean ± S.E. \( n = 3 \) separate experiments, (*, \( p < 0.05 \) by non-paired Student’s t test).

D, wild type and mutant cells were loaded with TMRE (100 \( \mu \text{M} \)), and fluorescence changes were measured in wild type (black bars), and mutant (gray bars) cells exposed to 1 \( \text{nM} \) 4-Bra23187 in the presence of increasing \( \text{Ca}^{2+} \) concentrations. Addition of 1 \( \text{nM} \) 4-Bra23187 plus different \( \text{CaCl}_2 \) concentrations induced a significant loss of mitochondrial potential in mutant cells (*, \( p < 0.05; n = 3 \)) (gray bars) in comparison to wild type cells (black bars). Data are mean ± S.E. \( n = 3 \) independent experiments, (*, \( p < 0.05 \) by non-paired Student’s t test).
Ca\(^{2+}\) Handling Defects with Mutant Huntingtin Expression

but not in the absence, and cytochrome c alone did not increase state 3. These results indicate the Ca\(^{2+}\)-induced decrease in oxidative phosphorylation is associated with increased permeabilization of the mitochondrial membrane but not with substantial loss of cytochrome c.

In this study respiratory rates were measured in mitochondria isolated from STHdhQ111/Q111 (mutant) cells in KCl-based EGTA containing respiration buffer, and no significant differences were observed. In our previous study, respiratory rates were measured in digitonin-permeabilized cells, in a sucrose-based buffer without EGTA, and significant decreases in the state 3 rates were observed in STHdhQ111/Q111 (mutant) cells (18). In the light of the current findings, it is likely that the experimental conditions used in our previous study were permissive for the mutant huntingtin-dependent mitochondrial defects. These conditions resulted in the deficits observed in state 3 rates for the mutant cells. Indeed it is likely that the presence of free Ca\(^{2+}\) in the buffers (e.g. from the sucrose) likely resulted in an increase in mitochondrial membrane permeability to a greater extent in the mutant cells than the wild type cells, which caused the observed differences in respiration.

It has been shown that huntingtin associates with the outer mitochondrial membrane (47). We tested the integrity of outer mitochondrial membrane in basal conditions. We found that mitochondria from both cell lines have a good outer mitochondrial membrane integrity as state 3 rates did not increase in the presence of NADH and cytochrome c. The inner mitochondrial membrane was of good integrity as well, because NADH (inner mitochondrial membrane impermeable) did not work as respiratory substrate. Also, state 4 rates were comparable in the two cell lines, indicating similar levels of inner membrane proton leakage. However, it is still possible that changes in the integrity of mitochondrial membrane due to mutant huntingtin are quite subtle and could not be detected with the methods we used.

Mutant huntingtin-expressing cells (STHdhQ111/Q111) showed markedly enhanced ΔΨm reduction in response to increasing Ca\(^{2+}\) concentrations both in vitro and in situ. Mitochondrial depolarization in response to Ca\(^{2+}\) is caused by Ca\(^{2+}\) uptake itself (partial and reversible depolarization) and by opening of the PTP when Ca\(^{2+}\) uptake capacity is exceeded (complete depolarization) (38). Because in our experiments ΔΨm was measured in the population of mitochondria, determined ΔΨm values could indicate the portion of mitochondria undergoing PTP associated with complete depolarization or ΔΨm levels present in the major of mitochondria at specific Ca\(^{2+}\) concentrations. As previously described, ADP prevents PTP opening and stabilizes ΔΨm by the mechanism that includes binding and stabilization of adenine nucleotide translocator (ANT) in the conformation that prevents PTP opening (48). The differences in ΔΨm reduction between wild type and mutant cells were significantly attenuated in the presence of ADP. This suggested that differences in the threshold for PTP opening, significantly contributed to ΔΨm differences observed between wild type and mutant cells.

The reduction of ΔΨm could be observed at as low as 0.4 and 0.6 μM Ca\(^{2+}\) (concentrations at which decreases in oxidative phosphorylation were observed) in the mutant but not in the wild type cells. Because respiration was first monitored in state 4 followed by the induction of state 3, the observed differences in the state 3 rates are likely caused by the differences in the ΔΨm before the state 3 was induced. Indeed, no differences in ΔΨm were observed at 0.4 and 0.6 μM Ca\(^{2+}\) when measured in state 3 conditions.

ROS production could contribute to Ca\(^{2+}\)-induced PTP opening (discussed in Ref. 40). As described, determination of released H\(_2\)O\(_2\), is a common and the most reliable measure of mitochondrial ROS production (39). When added to isolated mitochondria, Ca\(^{2+}\) caused a dose-dependent increase in the release of H\(_2\)O\(_2\). However, no significant difference in released H\(_2\)O\(_2\) between wild type and mutant, was observed at different Ca\(^{2+}\) concentrations (state 4). Therefore, the dramatic differences in ΔΨm and Ca\(^{2+}\) uptake capacity observed between wild type and mutant are likely not caused by differences in the ROS production. However, in the presence of ADP (state 3), we observed higher levels of released H\(_2\)O\(_2\) in mutant mitochondria, which reached significance at several data points. Increased ROS in the presence of ADP would likely contribute to more pronounced decrease in state 3 rates observed in mutant mitochondria in the presence of Ca\(^{2+}\). Unexpectedly, we observed reduced H\(_2\)O\(_2\) release from mutant mitochondria upon rotenone treatment. Our results suggest that ROS production is likely not the mechanism for reduced threshold for PTP opening in the mutant cells. However, there was a trend for mitochondria from mutant cells to exhibit altered ROS homeostasis compared with wild type (modest increase in the presence of Ca\(^{2+}\) and ADP; decrease in the presence of rotenone).

In this article we observed that mitochondria from mutant cells had reduced Ca\(^{2+}\) uptake capacity compared with mitochondria from wild type cells. As suggested in multiple articles, decreases in mitochondrial Ca\(^{2+}\) uptake capacity could be caused by a decreased threshold for PTP opening (7, 49). In fact, when mitochondria from mutant cells were treated with PTP inhibitors (cyclosporine A, ADP, oligomycin), the defect in Ca\(^{2+}\) uptake was almost abolished. Decreased mitochondrial Ca\(^{2+}\) uptake has already been reported in other HD models. Panov et al. (7, 13) reported decreased Ca\(^{2+}\) uptake in mitochondria from HD lymphoblast cell line, and brain mitochondria from full-length huntingtin transgenic mice (YAC72). Recently, attenuated Ca\(^{2+}\) uptake was reported in muscle mitochondria from R6/2 mice (30). Interestingly, recombinant truncated mutant huntingtin resulted in significant mitochondrial swelling at lower Ca\(^{2+}\) loads than truncated wild type protein when added to isolated mouse liver mitochondria (47). This suggested that the mechanism of mutant huntingtin-induced mitochondrial dysfunction is possibly through its direct effects on mitochondria.

Using multiple mitochondrial functional assays, we demonstrated a mitochondrial Ca\(^{2+}\) handling defect in mutant (STHdhQ111/Q111) cells. Although Ca\(^{2+}\) overload could be the cause of the earlier PTP opening in the mutant mitochondria, our data suggest that it is the opening of the PTP at lower Ca\(^{2+}\) concentrations resulting in membrane depolarization rather than Ca\(^{2+}\) overload. ROS could contribute the PTP opening, but it did not correlate with the significant
changes in ΔΨm observed in the mutant cells, suggesting a different mechanism. Based on these and other findings, we hypothesize that the increased sensitivity of mutant mitochondria to increases in intracellular Ca\(^{2+}\) results in depolarization, and this is an upstream event in the pathological cascade followed by decreases in respiration and Ca\(^{2+}\) buffering capacity. In the light of these current findings, successful PTP inhibition would be beneficial, but, as already discussed in terms of excitotoxicity (38), limiting mitochondrial Ca\(^{2+}\) toxicity (38), limiting mitochondrial Ca\(^{2+}\)-unimporter inhibition or Na\(^{+}\)-Ca\(^{2+}\)-exchanger activation) is also likely to be beneficial in the treatment of HD.

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