Mitochondrial calcium uptake capacity as a therapeutic target in the R6/2 mouse model of Huntington’s disease

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Huntington’s disease (HD) is an incurable autosomal-dominant neurodegenerative disorder initiated by an abnormally expanded polyglutamine domain in the huntingtin protein. It is proposed that abnormal mitochondrial Ca²⁺ capacity results in an increased susceptibility to mitochondrial permeability transition (MPT) induction that may contribute significantly to HD pathogenesis. The in vivo contribution of these hypothesized defects remains to be elucidated. In this proof-of-principle study, we examined whether increasing mitochondrial Ca²⁺ capacity could ameliorate the well-characterized phenotype of the R6/2 transgenic mouse model. Mouse models lacking cyclophilin D demonstrate convincingly that cyclophilin D is an essential component and a key regulator of MPT induction. Mitochondria of cyclophilin D knockout mice are particularly resistant to Ca²⁺ overload. We generated R6/2 mice with normal, reduced or absent cyclophilin D expression and examined the effect of increasing mitochondrial Ca²⁺ capacity on the behavioral and neuropathological features of the R6/2 model. A predicted outcome of this approach was the finding that cyclophilin D deletion enhanced the R6/2 brain mitochondria Ca²⁺ capacity significantly. Increased neuronal mitochondrial Ca²⁺ capacity failed to ameliorate either the behavioral and neuropathological features of R6/2 mice. We found no alterations in body weight changes, lifespan, RotaRod performances, grip strength, overall activity and no significant effect on the neuropathological features of R6/2 mice. The results of this study demonstrate that increasing neuronal mitochondrial Ca²⁺-buffering capacity is not beneficial in the R6/2 mouse model of HD.

INTRODUCTION

Huntington’s disease (HD) is an incurable autosomal-dominant neurodegenerative disorder characterized by involuntary movements, psychiatric disorders, and dementia (1). Median age of onset occurs around 40 years of age with premature death 15–20 years after onset of symptoms with end-stage HD patients profoundly debilitated and demented. HD is caused by an unstable (2–6), expanded CAG repeat within the coding sequence of huntingtin resulting in an abnormally expanded polyglutamine (polyQ) domain in the huntingtin protein (htt) (7).

Normal huntingtin alleles contain <27 CAG repeats with a median ~18 CAG, whereas repeat numbers exceeding 39 CAG repeats result in full penetrant disease (8–10). Huntingtin is expressed ubiquitously by neurons; however, neurodegeneration exhibits a relatively selective pattern, with striatal medium spiny GABAergic projection neurons (MSNs) exhibiting early degeneration and atrophy (11). Degenerative changes in the cortex also occur in early phases of HD and careful neuropathologic examinations of advanced HD specimens reveal evidence of neurodegeneration in many brain regions (11–14).
Huntingtin is a large, phylogenetically conserved, predominantly cytoplasmic protein that may function to regulate vesicle transport (15,16). Biochemical analyses have identified huntingtin in compartments containing vesicle-associated proteins (17), in the nucleus (18,19) and associated with organelles such as mitochondria (20). The expanded polyQ domain is thought to confer a toxic gain of function on the protein (21). The detrimental effects of mutant htt are associated with several proximate pathogenic mechanisms including N-methyl-D-aspartate receptor (NMDAR)-mediated excitotoxicity, abnormal transcriptional activity (22,23), impaired axonal transport (24,25), mitochondrial dysfunction (26–28) and abnormalities of calcium homeostasis (29). In addition to its roles in ATP production and regulation of cellular metabolism, mitochondria transiently store calcium and play an essential role in cellular Ca\textsuperscript{2+} homeostasis. Pathological conditions, such as NMDAR-mediated excitotoxicity, are associated with increased cytosolic free Ca\textsuperscript{2+} and result in increased mitochondrial Ca\textsuperscript{2+} loading as a downstream mechanism for Ca\textsuperscript{2+} signaling deregulation. Studies examining isolated mitochondria provided evidence for altered mitochondrial Ca\textsuperscript{2+} buffering in HD (20,30). Mitochondria isolated from HD patient lymphoblasts depolarize in response to lower amounts of Ca\textsuperscript{2+} when compared with mitochondria of control subjects, indicating altered mitochondrial Ca\textsuperscript{2+} uptake capacity (30). Decreased Ca\textsuperscript{2+} loading capacity was also detected in brain mitochondria isolated from presymptomatic YAC72 HD-like mice, suggesting that this specific mitochondrial dysfunction is an early event in the HD pathogenic cascade (30). Ca\textsuperscript{2+}-buffering abnormalities were detected subsequently in mitochondria isolated from other genetic mouse models of HD, though these defects are not observed in all studies (31–35).

Cyclophilin D (CypD) is a nuclear-encoded peptidylprolyl isomerase residing in the mitochondrial matrix (36–39). Genetic inactivation of the Ppif gene encoding for CypD provided evidence that CypD is a key regulator of mitochondrial Ca\textsuperscript{2+}-loading capacity (36–39). Mitochondria isolated from the livers, hearts and brains of CypD knockout mice accumulate significantly higher levels of Ca\textsuperscript{2+} than mitochondria isolated from wild-type mice (37–39). These observations are consistent with a role for CypD as a key protein modulator of the Ca\textsuperscript{2+} sensitivity of mitochondria. CypD knockout mice demonstrate an important role for CypD in cell death: CypD inactivation is protective against cellular death induced by several stressors potentially relevant to HD pathogenesis such as reactive oxygen species, Ca\textsuperscript{2+} overload, and glutamate-mediated excitotoxicity (36–39). Mitochondria isolated from CypD knockouts exhibit basal and ADP-stimulated respiration rates similar to those of wild-type mitochondria, indicating that CypD ablation does not significantly impair mitochondrial bioenergetic function (39). Recent studies indicate that CypD inactivation is beneficial in Alzheimer’s disease, brain ischemia, and multiple sclerosis models. These results suggest that CypD is an attractive target for neuroprotective therapies (36,40,41). The mitochondrial Ca\textsuperscript{2+}-buffering abnormalities observed in HD and HD models and the protective effects conferred by genetic inactivation of the Ppif gene in other disease models suggest that CypD inactivation may be beneficial in HD.

In this proof-of-principle study, we examined whether an increase in mitochondrial Ca\textsuperscript{2+} capacity could ameliorate the well-characterized phenotype of the R6/2 transgenic mouse model. Because mitochondria of CypD knockout mice are particularly resistant to Ca\textsuperscript{2+} overload, we crossed R6/2 mice with CypD knockout mice and examined the effect of CypD inactivation on the mitochondrial Ca\textsuperscript{2+} capacity, behavioral phenotype, and neuropathological features of R6/2 mice. In contrast to prior in vitro work, our in vivo results indicate that increasing mitochondrial Ca\textsuperscript{2+} buffering is not beneficial in this model. Inactivation of CypD does not modify the onset and progression of the R6/2 phenotype.

RESULTS

The R6/2 transgenic model expresses truncated forms of the mutant human HD allele; the fragment expressed corresponds to exon 1 of huntingtin. Although this mouse model does not recapitulate the full spectrum of the disease, R6/2 mice recapitulate key phenotypic features of HD, including expression of cellular aggregate pathology, striatal neuron loss, weight loss, progressive motor dysfunction and gene expression abnormalities similar to HD (42–45). R6/2 mice display a relatively early-onset and robust phenotype providing clear outcome measures. R6/2 mice that are either wild type (R6/2:CypD\textsuperscript{+}+/\textsuperscript{+}), knockout (R6/2:CypD\textsuperscript{−}−/\textsuperscript{−}) and heterozygous (R6/2:CypD\textsuperscript{+}+/\textsuperscript{−}) for Ppif were generated following the breeding approach described in Materials and Methods. The CAG repeat sizes of R6/2 transgenes were 274.5 ± 6.9 for R6/2:CypD\textsuperscript{+}+/\textsuperscript{+} mice, 274.7 ± 5.2 for R6/2:CypD\textsuperscript{−}−/\textsuperscript{−} mice, and 271.9 ± 5.7 for R6/2:CypD\textsuperscript{+}+/\textsuperscript{−} mice, and were not significantly different between groups (Fig. 1A). Using quantitative real-time RT–PCR, we verified that R6/2 transgene expression levels were similar in all brain regions examined of R6/2:CypD\textsuperscript{+}+/\textsuperscript{+}, R6/2:CypD\textsuperscript{−}−/\textsuperscript{−} and R6/2:CypD\textsuperscript{+}+/\textsuperscript{−} mice (Fig. 1B).

We examined expression of CypD protein in brain of R6/2:CypD\textsuperscript{+}+/\textsuperscript{+}, R6/2:CypD\textsuperscript{−}−/\textsuperscript{−} and R6/2:CypD\textsuperscript{+}+/\textsuperscript{−} mice. Representative immunoblots of cellular lysates from striata and hippocampal formations are presented in Figure 1C. As expected, CypD immunoreactivity was not detected in the R6/2:CypD\textsuperscript{−}−/\textsuperscript{−} mouse brains and its expression level was reduced by ~50% in lysates from R6/2:CypD\textsuperscript{+}+/\textsuperscript{−} mouse brains (Fig. 1C). Immunoblot analysis for the cellular content of the mitochondrial proteins ATP synthase and porin (Fig. 1C) and subunits 30 and 70 kDa of the mitochondrial complex II (data not shown) revealed similar expression levels in R6/2:CypD\textsuperscript{+}+/\textsuperscript{+}, R6/2:CypD\textsuperscript{−}−/\textsuperscript{−} and R6/2:CypD\textsuperscript{+}+/\textsuperscript{−} mice, indicating that ablation of Ppif did not affect the mitochondrial mass of R6/2 mouse brains.

CypD inactivation increases Ca\textsuperscript{2+}-buffering capacity of brain mitochondria from R6/2 mice

Brain mitochondria isolated from mice lacking Ppif exhibit higher Ca\textsuperscript{2+}-buffering capacity than brain mitochondria from wild-type mice, requiring approximately twice the amount of Ca\textsuperscript{2+} necessary to induce MTP than wild-type mitochondria.
We determined whether an increased mitochondrial Ca\(^{2+}\)-buffering capacity occurs in brain mitochondria of R6/2:CypD\(^{-/-}\) mice. The results of a typical experiment are shown in Figure 2A. Successive additions of 10 nmol Ca\(^{2+}\) boluses demonstrated that R6/2:CypD\(^{-/-}\) mice cortical mitochondria were able to buffer substantially higher amounts of Ca\(^{2+}\) (Fig. 2A). Comparison of Ca\(^{2+}\) uptake kinetics in R6/2:CypD\(^{+/-}\) and R6/2:CypD\(^{-/-}\) brain mitochondria revealed that uptake of extramitochondrial Ca\(^{2+}\) were similar for the first two Ca\(^{2+}\) boluses (Fig. 2A and B). In R6/2:CypD\(^{+/-}\) mitochondria, however, subsequent Ca\(^{2+}\) boluses caused a rapid decrease in the rate of Ca\(^{2+}\) uptake accompanied by inability to return to baseline levels prior to addition of the next Ca\(^{2+}\) bolus. After a limited number of Ca\(^{2+}\) boluses, R6/2:CypD\(^{+/-}\) brain mitochondria were not able to sequester Ca\(^{2+}\) effectively (Fig. 2A and B). R6/2:CypD\(^{-/-}\) brain mitochondria were able to buffer significantly higher amounts of Ca\(^{2+}\) compared with R6/2:CypD\(^{+/-}\) brain mitochondria.

To confirm that similar amounts of mitochondria were present in the preparations analyzed, mitochondrial suspensions were recovered and immunoblotted for porin and ATP synthase (Fig. 2C). Porin and ATP synthase expressions were identical. As expected, CypD was not detected in mitochondrial suspensions from R6/2:CypD\(^{-/-}\) brain (Fig. 2C).

**Increased mitochondrial Ca\(^{2+}\) buffering does not modify survival of R6/2 mice**

R6/2 mice have a limited lifespan, providing a relevant outcome measure to evaluate the potential benefits of increasing mitochondrial Ca\(^{2+}\)-buffering capacity in R6/2 mice. Lifespan analysis revealed that elimination of one copy or complete ablation of Ppif did not extend survival (Fig. 3A). Average survival was not significantly different between the R6/2 groups [R6/2:CypD\(^{+/-}\) mice 170.1 ± 13.2 days, n = 17; R6/2:CypD\(^{-/-}\) mice 163.8 ± 10.51 days, n = 20; R6/2:CypD\(^{+/-}\) mice 161.3 ± 10.61, n = 21] (Fig. 3B). Gender has no significance on the average survival of R6/2 mice with different CypD genotypes (Supplementary Material, Fig. S1). Median survival was higher in R6/2:CypD\(^{+/-}\) mice (175 days) when compared with R6/2:CypD\(^{-/-}\) mice (163 days) and R6/2:CypD\(^{+/-}\) mice (157 days) (Fig. 3C). Ppif deletion did not affect survival of
Increased mitochondrial Ca\(^{2+}\) buffering does not delay the onset or ameliorate weight loss in R6/2 mice

Progressive loss of body weight is a consistent and robust feature of R6/2 mice (43). R6/2 genders lose weight at different rates. Body weight of male and female R6/2 mice within each CypD genotype group were analyzed separately (45). In both male and female R6/2 mice, there was a significant effect of age on body weight (males R6/2:CypD\(^{+/+}\) F\(_{21,181}=36.56, P<0.0001\); females R6/2:CypD\(^{+/+}\) F\(_{20,122}=14.72, P<0.0001\)). Male and female R6/2 body weights reached a maximum at \(~70\) and 100 days of age, respectively, stabilized for \(~4\) weeks, and then consistently declined until age of death (Fig. 4A and B). In both genders, we found no significant effect of CypD genotype on body weight gain or on the onset and extent of body weight loss of R6/2 mice (Fig. 4A and B). There was no significant effect of CypD inactivation on the body weight of wild-type mice (Supplementary Material, Fig. S2).

Increased mitochondrial Ca\(^{2+}\) buffering does not delay the onset of or ameliorate the behavioral phenotype of R6/2 mice

The progressive behavioral phenotype of the R6/2 model has been characterized extensively using a panel of standardized tests (45). Using these measures, we examined the consequences of increasing mitochondrial Ca\(^{2+}\)-buffering capacity on the onset and progression of the R6/2 behavioral phenotype.

Rotarod performance has been shown repeatedly to decline in R6/2 mice. Motor performances of R6/2:CypD\(^{+/+}\), R6/2:CypD\(^{+/−}\) and R6/2:CypD\(^{+/−}\) mice were analyzed using an accelerating rotarod at 60, 90 and 120 days of age. At 60 days of age, there was a significant effect of trial numbers on accelerating rotarod performance of

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Figure 2. Effects of CypD on the mitochondria Ca\(^{2+}\) uptake and release of R6/2 mouse brains. (A) Kinetics of Ca\(^{2+}\) uptake and release of mitochondria isolated from the cortex of R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{+/−}\) mice (153 days). Mitochondrial Ca\(^{2+}\)-buffering capacities were monitored in a suspension of mitochondria (0.5 mg/ml) by examining the changes in calcium green-5N fluorescence upon addition of Ca\(^{2+}\) aliquots (10 nmol/aliquots in 2 μl). Increase in fluorescence reflects increases in extra mitochondrial Ca\(^{2+}\) whereas decrease in fluorescence reflects mitochondrial Ca\(^{2+}\) uptake. In mitochondria isolated from R6/2:CypD\(^{+/+}\) mice with subsequent additions of Ca\(^{2+}\) aliquots, there was a rapid decrease in the rate of Ca\(^{2+}\) uptake that was associated with an inability to return to the baseline level prior addition of the next Ca\(^{2+}\) aliquots. After a limited number of Ca\(^{2+}\) additions, brain mitochondria from R6/2:CypD\(^{+/+}\) did not uptake Ca\(^{2+}\) anymore and released their Ca\(^{2+}\) (sustained increased in fluorescence at the end of the traces). Whereas brain mitochondria from R6/2:CypD\(^{+/−}\) mice were able to uptake Ca\(^{2+}\) more efficiently and to buffer a significantly higher amount of Ca\(^{2+}\) when compared with brain mitochondria from R6/2:CypD\(^{+/+}\). (B) Comparison of Ca\(^{2+}\) uptake and release of mitochondrial suspensions from R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{+/−}\) mouse brains [enlargement and superposition of the first nine additions of Ca\(^{2+}\) aliquots (gray area in A)]. The kinetics of Ca\(^{2+}\) uptake was slower and extra mitochondrial Ca\(^{2+}\) did not return to basal level after addition of Ca\(^{2+}\) aliquots in R6/2:CypD\(^{+/+}\) brain mitochondria when compared with R6/2:CypD\(^{+/−}\) brain mitochondria. (C) Immunoblot analysis of 10 μl of the mitochondrial suspensions with the mitochondrial proteins porin and ATP synthase revealed that a similar amount of the organelle was present in both mitochondrial preparations. As expected, CypD was not detected in the mitochondria suspension from R6/2:CypD\(^{+/−}\) mice.
R6/2:CypD+/+, R6/2:CypD−/− and R6/2:CypD+/− mice. All genotype groups were able to remain on the rod for significantly longer times with successive trials [R6/2:CypD+/+: \(F_{9,150} = 3.65, P = 0.0004\); R6/2:CypD−/−: \(F_{9,190} = 5.74, P < 0.0001\); R6/2:CypD+/−: \(F_{9,180} = 3.73, P = 0.0003\)] (Fig. 5A). At 90 and 120 days of age, however, no significant effect of trial numbers was detected as mice with different genotype groups did not improve their rotarod performances with successive trials (Fig. 5B and C). Further analysis demonstrated a significant effect of age on accelerating rotarod performances. The overall performance of R6/2:CypD+/+, R6/2:CypD−/− and R6/2:CypD+/− mice declined significantly between 60 to 120 days of age [R6/2:CypD+/+: \(F_{19,300} = 9.06, P < 0.0001\); R6/2:CypD−/−: \(F_{19,390} = 14.91, P < 0.0001\); R6/2:CypD+/−: \(F_{19,359} = 15.69, P < 0.0001\)] (Fig. 5A and C). At all time points examined, there was no significant effect of CypD genotype on declining accelerating rotarod performance of R6/2 mice (Fig. 5A and C). We further included mouse gender as a potential variable in our analysis and analyzed male and female separately. The results of this analysis are presented in Supplementary Material (Fig. S3) and revealed that, except for one trial at 120 days in the R6/2:CypD+/+ group, there was no significant effect of gender on the accelerated rotarod performance of mice of the different CypD genotypes. We also determined the potential effects of CypD inactivation on the motor performance of mice in the absence of the R6/2 transgene. At 60 days of age, there was a significant effect of the trial numbers on the accelerating rotarod performance for WT:CypD+/+ (\(F_{7,120} = 4.26, P = 0.0003\)) and WT:CypD−/− (\(F_{7,152} = 4.01, P = 0.0005\)) mice (Supplementary Material, Fig. S4A). Both groups performed similarly, with no significant difference from R6/2:CypD+/+ mice (Supplementary Material, Fig. S4A). At 90 days of age, WT:CypD−/− mice performed better than WT:CypD+/+ mice (\(F_{15,256} = 6.62, P < 0.0001\)) and R6/2:CypD+/+ mice (\(F_{15,264} = 19.71, P < 0.0001\)), with no significant difference between WT:CypD+/+ mice and R6/2:CypD+/+ mice (\(P > 0.05\)) (Supplementary Material, Fig. S4B). At 120 days of age WT:CypD−/− performed significantly better than WT:CypD+/+ (\(F_{15,248} = 3.81, P < 0.0001\)). Compared with R6/2:CypD+/+ mice, there were significant differences with both WT:CypD−/− (\(F_{15,264} = 21.13, P < 0.0001\)) and WT:CypD+/+ (\(F_{15,224} = 8.30, P < 0.0001\)) (Supplementary Material, Fig. S4C). In addition, we examined motor functions of R6/2 mice using a constant...
speed rotarod protocol (10 rpm). There was significant effect of age on fixed speed rotarod performance of R6/2:CypD\(^2/2\) and R6/2:CypD\(^+/-\) mice, but not of R6/2:CypD\(^++\) mice (\(F_{2,57} = 11.13, P < 0.0001\) and \(F_{2,54} = 2.54, P = 0.0021\)) mice, but not of R6/2:CypD\(^++\) mice (\(F_{2,45} = 1.17, P = 0.32\)). At all the time points examined, the CypD genotype has no significant effect on the fixed-speed rotarod performances of R6/2 mice (Fig. 5D). At all time point examined, gender has no significant effect on the fixed-speed rotarod performances of R6/2 mice of the different CypD genotypes (Supplementary Material, Fig. S3D). No significant difference in the fixed-speed rotarod performances of WT:CypD\(^-/-\) and WT:CypD\(^++\) mice were detected (Supplementary Material, Fig. S4D). At 60 days of age, only WT:CypD\(^-/-\) performed significantly better than R6/2:CypD\(^++\) mice (\(F_{2,49} = 3.49, P = 0.038\)), whereas both WT:CypD\(^-/-\) and WT:CypD\(^++\) mice performed significantly better than R6/2:CypD\(^++\) mice groups at 90 days of age (\(F_{2,49} = 7.76, P = 0.0012\)) and 120 days of age (\(F_{2,46} = 11.73, P < 0.0001\)) (Supplementary Material, Fig. S4D). Increasing mitochondrial Ca\(^{2+}\)-buffering capacity did not delay the onset or improve the decline in balance and motor performance of R6/2 mice, even though ablation of cyclophilin D has a tendency to ameliorate rotarod performances in the absence of the R6/2 transgene.

We examined the effect of CypD genotype on the muscular strength of R6/2 mice. Forelimb and hindlimb grip strength were assessed at 30, 60, 90 and 125 days of age (Fig. 6). Analyses showed significant effect of age on the forelimb and combined (forelimb + hindlimb) grip strength of R6/2:CypD\(^-/-\) (forelimb \(F_{3,75} = 16.68, P < 0.0001\); combined grip \(F_{3,81} = 33.88, P < 0.0001\)) and R6/2:CypD\(^++\)/+ (forelimb \(F_{3,71} = 44.07, P < 0.0001\); combined grip \(F_{3,102} = 22.49, P < 0.0001\)) and R6/2:CypD\(^++\)/+ mice (forelimb \(F_{3,55} = 10.89, P < 0.0001\); combined grip \(F_{3,87} = 48.88, P < 0.0001\)). Further analysis showed no significant effect of CypD genotype on forelimb grip strength performance of R6/2 mice at 30, 60 and 90 days of age; however, at 125 days of age, compared with R6/2:CypD\(^++\)/+ mice, pairwise comparison showed significant differences only for R6/2:CypD\(^++\)/+ mice (\(P = 0.013\)) but not for R6/2:CypD\(^-/-\) mice (\(P = 0.324\)) (Fig. 6A). At all time points examined, CypD genotype has no significant effect on the combined
grip strength performance of R6/2 mice (Fig. 6B). At all time points examined, gender also has no significant effect on forelimb and combined grip strengths of R6/2:CypD+/+ and R6/2:CypD−/− mice, whereas for R6/2:CypD+/− mice there was a significant gender effect on the 30 days of age forelimb grip strength and 60 days of age combined grip strength (Supplementary Material, Fig. S5). We further examined the effect of CypD inactivation on the grip strength in the absence of the R6/2 transgene (Supplementary Material, Fig. S6). At all time points, pairwise analyses revealed no significant difference in the combined grip strength of WT:CypD−/− and WT:CypD+/+ mice and at 125 days

Figure 5. Effects of CypD on the motor performance of R6/2 mice. (A) Times before fall from rotarod at accelerating speeds (4–40 rpm) of R6/2:CypD+/+, (n = 16), R6/2:CypD−/− (n = 22) and R6/2:CypD+/− (n = 19) mice were monitored at 60, 90 and 120 days of age. Data are presented as mean ± SEM. (B) Motor performance of R6/2:CypD+/+ (n = 16), R6/2:CypD−/− (n = 20) and R6/2:CypD+/− (n = 19) mice using a constant speed rotarod protocol (10 rpm). At different time points examined, the CypD genotype has no significant effect on both accelerated and fixed-speed rotarod performances of R6/2 mice. Data are presented as mean ± SEM.
of age both groups performed significantly better than R6/2:CypD+/+ mice (forelimb $F_{2,46} = 40.55$, $P < 0.0001$; combined grip $F_{2,46} = 81.25$, $P < 0.0001$) (Supplementary Material, Fig. S6).

R6/2 mice tend to become increasingly inactive with age. We determined whether ablation of Ppif modulates spontaneous locomotor and rhythm activities of R6/2 mice. R6/2:CypD+/+, R6/2:CypD+/− and R6/2:CypD+/− mice were acclimated for 2 days in activity monitor micro-isolator cages and beam breaks monitored continuously for three subsequent 24 h periods. Data are expressed as average of upper or bottom beams broken over a 24 h period (dark and light), or over 12 h dark and 12 h light periods. Cage activities were examined at 60 and 90 days of age. The rationale for selecting these time points was based on the outcomes of the rotarod performances.

At 60 days of age, R6/2 mice of all the different CypD genotypes performed relatively well on both accelerating rotarod (Fig. 5A) and fixed speed rotarod (Fig. 5D), but presented balance and motor coordination impairments at 90 days of age (Fig. 5B and D). We were not able to measure activity at later time points because chow mixed with water had to be placed in the bottom of cages, which was incompatible with this apparatus. At 60 days of age, there was no effect of CypD genotype on the locomotor and rhythm activities of R6/2 mice (data not shown).

At 90 days of age, a total of 7 R6/2:CypD+/+, 11 R6/2:CypD+/− and 13 R6/2:CypD+/− mice were tested. Figure 7A shows that the horizontal activities of R6/2:CypD+/+ and R6/2:CypD+/− mice were similar (pairwise comparison $P > 0.05$); however, R6/2:CypD+/− mice broke significantly fewer horizontal beams than R6/2:CypD+/+ mice ($F_{2,28} = 4.07$, $P = 0.028$ and pairwise comparison R6/2:CypD+/− versus R6/2:CypD+/+, $P < 0.05$) (Fig. 7A). The decrease in the spontaneous horizontal activity of R6/2:CypD+/− mice was due to reduced nocturnal activity when compared with R6/2:CypD+/+ mice ($F_{2,28} = 4.89$, $P = 0.015$ and pairwise comparison R6/2:CypD+/− versus R6/2:CypD+/+, $P < 0.05$), as there was no significant difference in the spontaneous diurnal activity in the different groups analyzed ($F_{2,28} = 1.82$, $P = 0.189$) (Fig. 7A). There was no significant difference in the rearing activity between the R6/2 groups ($F_{2,28} = 1.38$, $P = 0.267$) (Fig. 7B). We further analyzed the rhythm activity of R6/2:CypD+/+, R6/2:CypD+/− and R6/2:CypD−/− mice. For each groups, pairwise analysis between diurnal and nocturnal spontaneous activity revealed no significant difference in the rhythm activities for R6/2:CypD+/+ mice (pairwise comparison, horizontal activity $P = 0.436$; rearing activity $P = 0.649$); R6/2:CypD+/− mice (pairwise comparison, horizontal activity $P = 0.753$; rearing activity $P = 0.311$); and R6/2:CypD−/− mice (pairwise comparison, horizontal activity $P = 0.694$; rearing activity $P = 0.189$) (Fig. 7). Also, gender has no significant effect on the total horizontal and rearing spontaneous and rhythm activities of R6/2 mice of different CypD genotypes (Supplementary Material, Fig. S7). Next, we examined whether inactivation of CypD has any effect on the spontaneous locomotor and rhythm activities in the absence of the R6/2 transgene expression (Supplementary Material,
Horizontal activity 0.0024; rearing activity WT:CypD $\equiv$ significance difference in both the diurnal (F0.62, Pmice (Fig. S8B). In contrast to the R6/2 groups, pairwise comparison between the different groups (Supplementary Material, Fig. S8). Further, analysis revealed that the diurnal horizontal activity for WT:CypD−/− was significantly lower than both WT:CypD+/+ and R6/2:CypD+/+ mice (F2.37 = 0.99, P = 0.38; total rearing activity: F2.37 = 0.135, P = 0.87) (Supplementary data, Fig. S8A). There was no significance difference in both the diurnal (F2.37 = 2.66, P = 0.08) and noctural (F2.37 = 0.97, P = 0.39) rearing activities between the different groups (Supplementary Material, Fig. S8B). In contrast to the R6/2 groups, pairwise comparison between the diurnal and nocturnal activities revealed significant differences in the rhythm activity for both WT:CypD+/+ and WT:CypD−/− mice (WT:CypD+/+: horizontal activity P = 0.0024; rearing activity P = 0.0031) and (WT:CypD−/−: horizontal activity P < 0.0001; rearing activity P = 0.0002) mice. These results reveal a relatively early-onset abnormality in the rhythm activity of R6/2 mice characterized by the absence of difference between day and night activities. Increasing the mitochondrial Ca2+-buffering capacity did not prevent or attenuate this abnormality though inactivation of CypD tends to increase the difference between day and night activities in the absence of the R6/2 transgene.

We examined exploratory activity and anxiety-like behavior of R6/2:CypD+/+, R6/2:CypD−/− and R6/2:CypD+/+ mice using open field (Fig. 8A and C). Mouse activity in the open field test is suppressed by anxiety, and anxious animals explore the field by movement close to the borders and intermediate areas, moving only rarely into the center of the field. Open field activity of R6/2:CypD+/+, R6/2:CypD−/− and R6/2:CypD+/+ mice was recorded at 30, 60, 90 and 120 days of age. We measured the total distance walked in the arena (Fig. 8A) and distance walked in and number of entries into the central area of the open field (Fig. 8B and C). At 30 and 60 days of age, R6/2:CypD+/+, R6/2:CypD−/− and R6/2:CypD+/+ mice actively explored the borders and intermediate areas, with limited entries into the center area (Fig. 8A and C). At 90 and 120 days of age, the total distances explored by R6/2 mice of different
CypD genotypes were significantly lower than those at 30 and 60 days of age, revealing significant effects of age on the total open-field exploratory activity (R6/2:CypD+/+: $F_{3,51} = 7.58$, $P = 0.003$; R6/2:CypD−−: $F_{3,70} = 7.05$, $P = 0.0003$; R6/2:CypD+/−: $F_{3,66} = 15.95$, $P < 0.0001$) (Fig. 8A). At all time points examined, the CypD genotype has no significant effect on the total exploratory activity of R6/2 mice ($P > 0.05$) (Fig. 8A), and also gender has no significant effect on the exploratory activity of R6/2:CypD+/+, R6/2:CypD−− and R6/2:CypD+/− mice (Supplementary Material, Fig. S9). Similarly, at all time points examined, the CypD genotype of R6/2 mice has no significant effect on the exploratory activity (Fig. 8B) and number of entries (Fig. 8C) into the central area. Further, we examined the effect of CypD inactivation on the exploratory activity and anxiety-like behavior in the absence of the R6/2 transgene (Supplementary Material, Fig. S9). At 90 days of age, there were no significant differences in exploratory activity and anxiety-like behavior between WT:CypD+/+ and WT:CypD−− mice, and for all outcome measures examined both groups performed significantly better than R6/2:CypD+/+ [total exploratory activity: ($F_{2,41} = 9.59$, $P = 0.0004$); exploratory activity in

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**Figure 8.** Effects of CypD on the exploratory activity and anxiety-like phenotype of R6/2 mice. Open-field activities of R6/2:CypD+/+ ($n = 15$), R6/2:CypD−− ($n = 18$) and R6/2:CypD+/− ($n = 18$) mice. (A) Total exploratory activity of the mice in the entire arena, (B) exploratory activity and (C) number of entries of the mice into the central area of the open field. Data are presented as mean ± SEM. At all the different time points examined, there was no significant effect of the CypD genotype on the open-field activities of R6/2 mice.
Central area: \( F_{2,41} = 6.68, \ P = 0.0031 \); entries in central area: \( F_{2,41} = 8.89, \ P = 0.0006 \) (Supplementary Material, Fig. S10). At 120 days of age, however, WT:CypD\(^{2/2}\) mice exhibited significantly reduced exploratory activity when compared with WT:CypD\(^{+/+}\) mice with no significant difference with R6/2:CypD\(^{+/+}\) mice. Data are presented as mean ± SD. (B–C) Unbiased stereology analysis in the striatum of 12–13 weeks of age wild type and R6/2 mice. Histograms showing (B) the total number of striatal NeuN-positive stained cells and (C) the striatal volume of wild-type (WT/WT), R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{-/-}\) mice. Data are presented as the mean ± SEM for four animals in each genotype group. There was no significant difference in the number of NeuN-positive cells and striatal volumes between the genotype groups, and no significant effect of the CypD on any of these phenotypes in R6/2 mice (pairwise analysis R6/2:CypD\(^{+/+}\) versus R6/2:CypD\(^{2/2}\), \( P > 0.05 \)). (D) Representative photomicrographs of huntingtin protein immunostaining within the striatum of R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{-/-}\) mice. The CypD genotype did not affect the accumulation or the size of huntingtin-immunoreactive aggregates (arrows) in R6/2 mice. Scale bar equals 100 μM.

Figure 9. Effects of CypD on the neuropathological phenotype of R6/2 mice. (A) Brain weights revealed a significant gross atrophy of brains from R6/2:CypD\(^{+/+}\) (0.41 ± 0.03 g) and R6/2:CypD\(^{-/-}\) (0.39 ± 0.02 g) mice when compared with wild-type mouse brains (0.45 ± 0.03 g) \( F_{2,25} = 11.87, \ P = 0.0002 \), with no significant effect of the CypD genotype on the brain weight of R6/2 mice (pairwise analysis R6/2:CypD\(^{+/+}\) versus R6/2:CypD\(^{-/-}\), \( P = 0.1469 \)). Data are presented as mean ± SD. (B–C) Unbiased stereology analysis in the striatum of 12–13 weeks of age wild type and R6/2 mice. Histograms showing (B) the total number of striatal NeuN-positive stained cells and (C) the striatal volume of wild-type (WT/WT), R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{-/-}\) mice. Data are presented as the mean ± SEM for four animals in each genotype group. There was no significant difference in the number of NeuN-positive cells and striatal volumes between the genotype groups, and no significant effect of the CypD on any of these phenotypes in R6/2 mice (pairwise analysis R6/2:CypD\(^{+/+}\) versus R6/2:CypD\(^{2/2}\), \( P > 0.05 \)). (D) Representative photomicrographs of huntingtin protein immunostaining within the striatum of R6/2:CypD\(^{+/+}\) and R6/2:CypD\(^{-/-}\) mice. The CypD genotype did not affect the accumulation or the size of huntingtin-immunoreactive aggregates (arrows) in R6/2 mice. Scale bar equals 100 μM.

Increased mitochondrial Ca\(^{2+}\) capacity does not ameliorate the neuropathological phenotype of R6/2 mice

The R6/2 phenotype is associated with neuropathological abnormalities (43). Our secondary outcome measures examined the effects of CypD genotypes on neuropathological features of R6/2 mice. We examined whether CypD genotypes had an effect on the total brain weight (Fig. 9A). Analysis of brains from 9 R6/2:CypD\(^{+/+}\) (119.4 ± 22.9 days of age); 7 R6/2:CypD\(^{-/-}\) (106.7 ± 29.6 days of age); and 12 wild-type \( F_{2,41} = 9.59, \ P = 0.0004 \); exploratory activity in central area: \( F_{2,41} = 6.68, \ P = 0.0031 \); entries in central area: \( F_{2,41} = 8.89, \ P = 0.0006 \) (Supplementary Material, Fig. S10). These results reveal that WT:CypD\(^{-/-}\) develop an age-dependent anxiety-like phenotype that may contribute to the absence of beneficial effect of CypD inactivation in the anxiety-like phenotype developed by R6/2 mice.

Increased mitochondrial Ca\(^{2+}\) capacity does not ameliorate the neuropathological phenotype of R6/2 mice

The R6/2 phenotype is associated with neuropathological abnormalities (43). Our secondary outcome measures...
The volume of the R6/2 mouse striatum was not affected by the CypD genotype (R6/2:CypD+/+: 9.77 ± 0.64 × 10^7 μm^3, n = 4; R6/2:CypD−/−: 9.04 ± 0.55 × 10^7 μm^3, n = 4; pairwise comparison P > 0.05; Fig. 9B). The total number of neurons within the R6/2 mouse striatum was not significantly different in R6/2:CypD+/+ (11.05 ± 1.04 × 10^5, n = 4) and R6/2:CypD−/− (10.24 ± 1.87 × 10^5, n = 4) (pairwise comparison, P > 0.05). Consistent with some prior reports, striatal volume and striatal neuron number are not significantly different between R6/2 and wild-type WT:CypD−/− mice (pairwise comparison P > 0.05) (WT:WT, striatal volume: 9.91 ± 0.51 × 10^7 μm^3; striatal neurons number 10.29 ± 1.57 × 10^5, n = 4) (Fig. 9B and C). We immunostained sagittal sections for htt immunoreactivity in order to quantitatively examine the density and distribution of htt protein aggregates within the R6/2:CypD+/+ and R6/2:CypD−/− mouse striatum. Representative photomicrographs showing the striatum of R6/2:CypD+/+ and R6/2:CypD−/− are presented in Figure 9D. Mature htt immunoreactive aggregates were observed throughout the striatum of R6/2 mice and quantitative analysis revealed no significant difference in the density of NII striatal aggregates between R6/2:CypD+/+ (60 ± 12.8 NII/0.4 μm^2, n = 5) and R6/2:CypD−/− (55 ± 7.5 NII/0.4 μm^2, n = 5) and no significant difference in the striatal distribution of the NII between the two groups (data not shown).

DISCUSSION
Disruption in neuronal calcium signaling associated with defects in mitochondria calcium handling may contribute to the pathogenesis of HD. In this study, we examined the contribution of the mitochondrial calcium buffering to the pathogenesis of HD, and used a genetic approach to manipulate mitochondrial Ca^{2+}-handling capacity in a HD mouse model. We generated R6/2 mice with normal, reduced or absent CypD expression and examined the effect of increasing mitochondrial Ca^{2+} buffering on the behavioral and neuropathological features of the R6/2 model. Biochemical, functional and genetic evidence identify CypD as an important regulator of the mitochondrial Ca^{2+}-handling capacity and a key regulator of mitochondrial permeability transition (MPT) induction (36–39,46). A predicted outcome of our cross was the finding that CypD deletion significantly enhanced Ca^{2+}-buffering capacity of R6/2 brain mitochondria. Increasing mitochondrial Ca^{2+} capacity, however, failed to ameliorate either the behavioral and neuropathological features of R6/2 mice. We found no amelioration of body weight decreases, diminished lifespan, declining rotarod performances, declining grip strength, reduced overall activity and no significant effect on the neuropathological features of R6/2 mice. Several of these outcome measures exhibited trends for the R6/2:CypD−/− and R6/2:CypD+/− mice to perform worse than the R6/2:CypD+/+ mice. For assays involving an exploratory activity-like this can be explained by the moderate anxiety phenotype developed by CypD knockout mice. For other outcome measures assessed, however, the reason for these negative trends remain to be elucidated.

In association with mitochondria dysfunction, accumulating evidence supports the concept that HD is associated with an event essentially driven by the inner mitochondrial membrane. At any threshold for mitochondrial permeability transition (MPT), a significant event occurs, in which mitochondrial Ca^{2+} release occurs. The rise in cytoplasmic free Ca^{2+} above a critical level triggers Ca^{2+} release from mitochondria (30). This hypothesis is supported by morphological analyses, revealing abnormal mitochondrial ultrastructure (48) and biochemical studies demonstrating reduced activities of several mitochondrial respiratory enzymes in post-mortem HD basal ganglia (47,49). Subsequent magnetic resonance spectroscopy studies in human subjects supported this hypothesis (50). Impairments in mitochondrial ATP production are detected in HD cellular models such as lymphoblasts isolated from HD patients and striatal cell lines established from an HD knock-in mouse model (51). There are results that contradict the hypothesis of mitochondrial dysfunction in HD. Some studies suggest normal electron transport chain activity in post-mortem HD tissue (52). A recent rigorous in vivo analysis of striatal metabolism in HD subjects failed to reveal evidence of mitochondrial dysfunction (53). Mitochondria not only play an important role in ATP production but also are essential in the regulation of cellular Ca^{2+} homeostasis. When cytoplasmic free Ca^{2+} rises above a critical set point, mitochondria accumulate cytoplasmic Ca^{2+} and then slowly release Ca^{2+} when normal cytoplasmic Ca^{2+} concentrations are restored. Studies with in vitro murine and cellular models of HD suggest reduced mitochondrial Ca^{2+}-buffering capacity in HD, resulting, at least in vitro, in lower Ca^{2+} threshold for decreases in ∆Ψm and MPT induction (20,30,32–34,54). It has been proposed that the interaction of the mutant htt with mitochondria may directly affect mitochondrial Ca^{2+}-buffering properties (20,30). This hypothesis is supported by electron microscopy studies, indentifying N-terminal mutant huntingtin on neuronal mitochondrial membranes (30,55), and by rigorous sub-cellular fractionation approaches revealing that both the wild-type and mutant full-length huntingtin proteins associated with the outer mitochondrial membrane (20). Incubation of normal mitochondria with a fusion protein containing an abnormally long polyQ repeat reduced mitochondrial membrane potential and significantly decreased Ca^{2+} uptake capacity of the organelles (20,30). Mutant htt may directly compromise outer mitochondrial membrane integrity or interaction of mutant htt with the outer mitochondrial membrane may directly facilitate the Ca^{2+}-mediated MPT induction. It is uncertain, however, how interaction of mutant htt with the outer mitochondrial membrane could influence an event essentially driven by the inner mitochondrial membrane (56).
deranged Ca\(^{2+}\) signaling leading to abnormally increased cytosolic free Ca\(^{2+}\) (29). Thus, the conjunction of a reduced mitochondrial Ca\(^{2+}\) capacity with abnormal Ca\(^{2+}\) signaling may result in a deregulation of cellular Ca\(^{2+}\) homeostasis. Indeed, NMDAR-mediated excitotoxicity is a long-standing hypothesis for the pathogenesis of HD (57–59), and has been supported by numerous studies revealing the potentiating effect of the mutant htt on NMDAR-mediated calcium transients (60). When examining the contribution of the mitochondrial Ca\(^{2+}\) handling defect as a downstream mechanism for Ca\(^{2+}\) signaling deregulation, previous studies with cultured MSNs indicated that enhanced NMDAR activation produces acute signaling deregulation, previous studies with cultured MSNs (35). The cause for this shift in mitochondrial sensitivities remains to be elucidated, although it was speculated that a vulnerable mitochondrial population could be lost early during the disease progression, potentially as a consequence of an increased Ca\(^{2+}\) sensitivity of the MPT (35). Similarly, Oliveira et al. (31) did not detect a reduced Ca\(^{2+}\) capacity in forebrain mitochondria isolated from three different transgenic HD mouse models. The discrepancy between these results and prior studies indicating decreased HD mitochondrial Ca\(^{2+}\) capacity may be due to differences in the disease stages of the HD mouse model used or to variations in experimental conditions examining the mitochondrial Ca\(^{2+}\) handling (31). Further isolation of mitochondria from brain tissue inevitably results in a mixture of mitochondria from different cell types and with different functional properties, complicating the interpretation of studies using this approach (31). A rigorous test of whether an MTP takes place in intact cells, organs and living organisms remains a major challenge, and occurrence of the PTP in vivo must still be deduced by indirect means. This is due in part to the intrinsic complexity of the PTP regulation, and to the lack of the definition of the channel itself. These results point to the necessity of examining HD mitochondria in their in vivo context. To unambiguously examine the contribution of MPT induction in HD we used a genetic approach, resulting in the absent or marked reduction of CypD expression in the R6/2 HD mouse model. This approach is a powerful method for predicting the outcomes of pharmacological approaches that might target CypD and increase the mitochondrial Ca\(^{2+}\) capacity in HD. We used a well-characterized HD mouse model to examine the relevance of mitochondrial Ca\(^{2+}\) buffering to the onset and progression of HD pathology in vivo. Inactivation of CypD did not ameliorate the behavioral and neuropathological phenotypes of the R6/2 mice, suggesting that increasing the mitochondrial Ca\(^{2+}\) capacity and preventing MPT induction are not a valid therapeutic approach in this mouse model. Our observation that several outcome measures exhibited a trend for R6/2 mice with inactivation of CypD to perform worse suggests that increasing the mitochondrial Ca\(^{2+}\) capacity is detrimental in this HD mouse model. The exact mechanisms by which mitochondrial Ca\(^{2+}\) sequestration may be harmful remains to be examined and could include respiration inhibition, or increased oxidative stress compromising multiple components of cell function including Ca\(^{2+}\) extrusion (61,63,64). It is also possible that ablation of CypD inhibits a transient opening of the MPT that may serve as a mitochondrial Ca\(^{2+}\) release channel and in this way prevents detrimental effects of Ca\(^{2+}\) overload on mitochondrial functions. It is also possible that CypD may play a role as a survival signaling molecule, as CypD overexpression desensitizes cells to apoptotic stimuli (65).

A variety of HD mouse models are now available, and the absolute validity and strength of any specific model for therapeutic validation remains unknown, with each model exhibiting distinct features that confer specific advantages and disadvantages (42,66). The transgenic R6/2 mouse model is of the most widely used HD model because it displays a relatively rapid-onset and robust phenotype providing clear outcome measures. The early and strong phenotype makes the R6/2 line particularly advantageous for preclinical pharmacology and intervention experiments. In this study, we employed the R6/2B variant of the original R6/2 line; this variant has relatively long repeat numbers (~275 CAG) and is on a C57Bl/6 background. Although both R6/2 and R6/2B lines show robust and similar phenotypes, the R6/2B line exhibits a delay in the onset of behavioral deficits, has only rare early mortality due to epilepsy and delayed onset of lethality (67,68). R6/2B mice may also have some pathologic features more similar to HD (67,68). Even though the phenotype and neuropathology observed in R6/2 lines replicate several features observed in HD patients, it is possible that R6/2 mice may not model critical aspects of the pathogenic process in HD. To date, no HD model has demonstrated predictive validity, due largely to the absence of much human clinical trial data. This intrinsic limitation in the predictive validity of the R6/2 line and the fact that no HD model can be proved superior to others in all aspects (67), warrant additional evaluations of our findings in another mouse model, such as a full-length transgenic or knock-in HD model. Finally, we cannot totally exclude that the negative outcome of this study is due to adaptation of the mice to CypD ablation, thus complementary pharmacological approaches using specific MPT inhibitors are needed prior to completely ruling out therapeutic approaches that target the mitochondria Ca\(^{2+}\) in HD.
MATERIALS AND METHODS

Materials

Calcium Green-5N was obtained from Molecular Probes (Eugene, OR, USA); the bicinchoninic acid assay (BCA) and the peroxidase substrate chemiluminescence (ECL) kits were obtained from Thermo Fisher Scientific (Rockford, IL, USA); the Vectastain Elite kit was obtained from the Vector Laboratories (Vector Laboratories, Burlingame, CA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). Antibodies against the following proteins were obtained from the indicated sources: NeuN, Chemicon International (Temecula, CA, USA); α-tubulin protein, Sigma; subunits 30 and 70 kDa of the mitochondria complex II and ATP synthase from Molecular Probes (Eugene, OR, USA); CypD and porin 31HL (Ab2) were obtained from Calbiochem (San Diego, CA, USA); N-terminal of huntingtin was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA); peroxidase-conjugated goat anti-mouse IgG, and peroxidase-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Mouse maintenance and breeding

Cyclophilin D knockout in the C57Bl/6 genetic background mice were generously provided by Dr. M. Forte (Vollum Institute, Portland) and were created using homologous recombination in embryonic stem cells (39). Male chimeras were subsequently mated with black, non-agouti C57Bl/6 female mice, and F1 heterozygotes were then back-crossed into a C57Bl/6 genetic background and isogenic heterozygotes intercrossed to generate congenic homozygous CypD knockout animals (39). CypD knockout pups are born at the expected mendelian frequency, revealing that CypD is dispensable for embryonic development and viability of adult mice (39). CypD knockout mice do not show spontaneous pathology or obvious phenotype and present a normal appearing brain and cerebrovasculature (37–39). The heart, which contains ~30% mitochondria by volume, presents no signs of cardiomyopathy in the absence of CypD (38). Further, the baseline morphology and cristae organization of mitochondria isolated from CypD knockout mice is unaffected compared with wild type (38). Importantly, despite the absence of CypD, mitochondria from knockout mice displayed basal, ADP- and uncoupled stimulation rates of respiration that are indistinguishable from those of mitochondria prepared from wild-type mice (39). Analysis of CypD at the protein level revealed that its expression is reduced to the expected 50% in heterozygous CypD mice and no CypD is detected in knockout mice. Further, the levels of MPT-related proteins such as VDAC and ANT are similar in mitochondria isolated from CypD knockout and wild-type mice (38). Remarkably, mitochondria isolated from the livers, hearts, brains of CypD knockout mice are particularly resistant to Ca2+ and accumulate much higher (at least 2-fold) levels of Ca2+ prior induction of the MPT when compared with mitochondria isolated from wild-type mice (37–39). These observations are consistent with a role of CypD as a key protein modulating the Ca2+ sensitivity of mitochondria. The CypD knockout mice provide useful tools to precisely the function of the PTP regulation in both normal and pathological conditions.

The R6/2 mouse model of HD has been extensively examined and its behavioral and neuropathological phenotypes have been well characterized (43–45). R6/2 mice were initially developed in a mixed genetic background and were recently backcrossed to C57Bl/6 mice to place the R6/2 transgene on an isogenic C57Bl/6 background. Because expanded CAG repeats are inherently unstable and the CAG repeat number has a tendency to increase upon male transmission, backcrossing the R6/2 mice has resulted in an elongation of the CAG repeat size in the transgene to ~270 CAG repeats. This R6/2 variant (R6/2B) has the advantage of a reliable phenotype, lacks the epilepsy that is a common cause of early mortality in the parent R6/2 line and may potentially complicate interpretation of experiments involving survival as outcome measure. Further, the R6/2B variant exhibits pathologic features more similar to HD, and may be a more faithful model of HD than the parent R6/2 line (67,68). R6/2B mice were obtained on a C57Bl/6 genetic background from a colony maintained by the Jackson Laboratory (Bar Harbor, ME, USA) and were maintained on this background in our vivarium. To examine the effect of mutating the mitochondrial Ca2+-buffering capacity on the R6/2 phenotype, two successive matings were performed to generate R6/2 mice that were either normal (R6/2/CypD+/+), knockout (R6/2/CypD−/−) and heterozygous (R6/2/CypD+/−) for CypD. First, male R6/2 were crossed with homozygous CypD knockout females. Offspring R6/2 males from this cross (all were CypD heterozygous) were bred with heterozygous females CypD knockout. These second cross-produced littersmate that carry the R6/2 transgene at the expected mendelian ratio and were either wild type (R6/2/CypD+/+) or heterozygous (R6/2/CypD+/−) for CypD. Crossing the R6/2 line with the cyclophilin D knockout mice did not affect the original phenotype of the original R6/2. Because the objective of this study was to determine the role of the mitochondrial Ca2+-buffering in the pathogenesis of HD, data are shown only for transgenic R6/2 carriers that are CypD knockout (R6/2/CypD−/−) and heterozygous (R6/2/CypD+/−) for CypD. The results of experiments examining the effects of CypD inactivation in the absence of the R6/2 transgene are presented as Supplementary Material. Experimental mice were weaned at 3 weeks of age, separated according to sex and housed in groups of five per cage with at least one mouse of each genotype examined. Mice were housed at an ambient temperature of 23°C with a 12 h light–dark cycle; no specific environmental enrichment was added in the cages. All animals had unlimited access to water and mouse chow, and starting at 10–12 weeks of age powdered chow was mixed with water and placed in the bottom of the cages. All mice were caged for according to the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures strictly followed protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Mouse genotyping and CAG repeat size

Prior to weaning, mice were genotyped by PCR of DNA isolated from tail snips. The presence of the R6/2 transgene was
determined as described previously (44), and the genotype status of CypD was determined according to a protocol described in Basso et al. (39). Expanded CAG repeats are inherently unstable with a tendency to increase upon male transmission. To control for this potential heterogeneity, the CAG repeat size of all transgenic R6/2 animals was measured by Laragen, Inc. (Los Angeles, CA, USA). Mice with large CAG repeat size expansions or contractions were excluded from the study, and the CAG repeat size was matched between the different genetic groups analyzed.

Assessment of mitochondrial Ca2+ uptake and release

Mitochondria were isolated from mouse cerebral cortex as described previously (69). Isolated mitochondria were suspended in 5 mM HEPES (pH 7.4), 3 mM MgCl2, 1 mM EGTA, 250 mM sucrose and 0.1% fatty acid-free bovine serum albumin, briefly pelleted at 2000 g and kept on ice until measurements. The mitochondrial Ca2+-buffering capacity was monitored in a cuvette-based fluorometer using the low Ca2+ affinity Calcium Green-5N under continuous stirring. Calcium Green-5N was excited at 506 nm and emission measured at 531 nm. Mitochondria (0.5 mg/ml) were resuspended in (125 mM KCl, 1 mM MgCl2, 2 mM K2HPO4, 20 mM HEPES, pH 7.0), supplemented with glutamate (5 mM) and malate (5 mM), ADP (150 μM), oligomycin (1.2 μg/ml) and Calcium Green-5N (25 nM), and incubated for ~200 s prior to successive additions of Ca2+ aliquots (10 nmol in 2 μl) at 3 min interval.

Western blot and immunoblotting

Tissues from different brain regions were homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml concentration of each of aprotinin, leupeptin and pepstatin] with a glass/glass Dounce homogenizer, prior to being sonicated on ice and spun at 2000g for 10 min at 4°C. Protein concentration of the supernatant was determined using the BCA method, and cellular lysates were diluted to a final concentration of 1 mg/ml in 2 × reducing stop buffer [0.25 M Tris–HCl (pH 7.5), 2% SDS, 25 mM dithiothreitol, 5 mM EGTA, 5 mM EDTA, 10% glycerol and 0.01% bromophenol blue as tracking dye] and incubated in a boiling water bath for 5 min. Proteins were separated by SDS–PAGE on 12% gel then transferred to nitrocellulose. Membranes were probed with the indicated primary antibodies followed by incubation with horse-radish peroxidase-conjugated secondary antibodies according to standard protocols. Blots were developed using peroxidase substrate chemiluminescence.

Body weight analysis

Starting at 30 days of age, mice were weighed weekly. Because male and female R6/2 mice show distinct weight gain and loss rates (45), changes in the body weights of male and female mice within each genotype group were analyzed separately.

Behavioral analysis

The following set of well-established quantitative behavioral tests was used to monitor the phenotype of R6/2 mice.

Rotarod performance. Rotarod activity was assessed using an Ugo Basile 7650 accelerating Rotarod (Comerio, VA, Italy). For testing, mice were placed on the rod with an accelerating rotating speed from 4 to 40 rpm over a period of 5 min, and mice were allowed to run on the rod for a maximal period of 10 min. Rotarod tests were performed in two phases: training and testing. During the training phase (first day), mice were subjected to three trials with a period of rest of 30 min between each trial. During this training phase, mice were allowed to become accustomed to the rod. Subsequently and for the next 7 days, mice were tested with one trial per day (trials 4–10). On the last day, the rotarod performance of the mice was also assessed by placing the mice on a rod with a constant rotating speed of 10 rpm for a maximal period of 10 min.

Grip strength. Grip strength analysis was performed to assess the muscular strength or motor impersistence. The forelimbs and four limbs muscular strength of mice from the different genotype groups was analyzed using a grip strength meter as described previously (45). In brief, the maximum forces (gram) pulled by the mouse forelimbs and four limbs are recorded by the strength meter, each mouse performs three consecutive tests as low scores may be due to the mouse failing to grip the strength meter effectively; the best of the three scores was used for statistical analysis.

Cage activity. The spontaneous horizontal and rearing locomotor activities and rhythm activity of the mice was assessed by an infrared beam activity monitor. One mouse each was placed in a standard (29 cm L × 19 cm W × 13 cm H) microisolator cage with enough food and water for 1 week. A total of eight beams spanned the width of each cage. Six lower beams, evenly spaced (4.5 cm apart, 2.5 cm from cage floor), would be broken by mice normally walking the length of the cage, and two upper beams located near the cage ends (1.8 cm from each cage wall and 7.0 cm above cage floor) could only be broken by rearing or climbing upside-down on the cage rack and could not be broken by mice feeding or drinking. Each beam was checked for function before and after each trial. Mice were assessed at 60 and 90 days, the apparatus simultaneously and continuously monitored beam breaks for 20 cages (one mouse per cage) over 5 days and nights. Days and nights are defined by a periodic 12 h light and dark cycle, respectively. Data were simultaneously recorded by an IBM personal computer programmed to store data at 2 min intervals for each of the beams. Data from the first 2 days and nights were discarded as mice need to acclimate to their cage environment. Data are expressed as the average of upper or bottom beams broken over a 24 h period (spontaneous horizontal and rearing locomotor activities), or over 12 h dark and 12 h light periods (rhythm activity).

Anxiety and exploratory activity. Anxiety-like behavior was assessed by examining a task dependent on voluntary exploration in an open field. Open field consists of a square field (50 × 50 cm) limited by plexiglas walls, and a floor plexiglas. The arena is divided into three zones: center, intermediate and border. The position of the mice in the open field is...
automatically recorded by a camera for a period of 4 min and data collected using the Ethovision 3.1 software. Data are expressed as the total time spent and distance walked by the mice in each zone during the recording period.

Brain harvesting and storage
The study protocol was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Mice were deeply anesthetized and sacrificed. Brains were extracted immediately after decapitation and divided in the sagittal plane. One hemisphere was coated with embedding medium for frozen tissues (Tissue-Tek, Torrance, CA, USA), frozen in crushed dry ice and stored at −80°C. The other hemisphere was immersion fixed in 4% paraformaldehyde for 24 h, cryoprotected in 20% sucrose in 0.1 M phosphate buffer for an additional 24 h at 4°C and stored at −80°C until the time of sectioning. Fixed hemispheres were used for stereological analysis.

Immunohistochemistry
Hemispheres previously fixed in 4% paraformaldehyde as described above were serially sectioned at 40 μm sagittally throughout the entire hemisphere. Each fourth section was used for stereological analysis. Free-floating sections were then stained with a primary specific neuronal antibody, NeuN (Chemicon, Temecula, CA, USA) at a dilution of 1:1000 for 1 h. Sections were processed without primary NeuN antibody to assess background staining. No staining was visualized in these control sections. To visualize huntingtin-immunoreactive inclusions, an antibody directed against the N-terminal of huntingtin was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000. Detection of immunoreactivity was performed using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA), and diaminobenzidine substrate was used as the chromogen according to the manufacturer’s protocol. Sections were then mounted on gelatin-coated slides and air dried after dehydration with graded alcohols and xylene. Coverslips were affixed with Permount.

Stereology
Unbiased stereological counts of striatal neurons were obtained from the striatum using the StereoInvestigator software (MicroBrightField, Colchester, VT, USA). The optical fractionator method was used to generate an estimate of neuronal number with only darkly stained NeuN-immunoreactive cells counted in an unbiased selection of serial sections in a defined volume of the striatum. Striatal borders were delineated on a NeuN-stained section by reference to a mouse brain atlas (70). The striatum was defined to encompass both the dorsal and ventral striatum. Striatal volume was reconstructed by the StereoInvestigator software using the Cavalieri principle. Serially cut sagittal tissue sections (every fourth section) were analyzed for one entire hemisphere of animals in each cohort (n = 4 per group). Inclusions were counted using a ×100 objective and a grid reticle demarcating an area of 0.01 mm². For each animal, three measurements were taken per area and averaged. The inclusion density was expressed as number of inclusions/0.0004 mm² and examined in dorsolateral, dorsomedial, ventrolateral and ventromedial quadrants of the striatum. Areal measurements are an accurate index of NII density because our unbiased stereology estimates demonstrate equivalent striatal volumes among these groups.

Statistical analyses
All behavioral studies were performed blind to the CypD genotype. Comparisons between groups were performed by using repeat measures and one-way analysis of variance (ANOVA) with post-hoc comparisons made using the Tukey honestly significant difference test when P < 0.05. For pairwise comparisons Student’s t-test was performed. All immunohistochemistry and stereology studies were performed blind to treatment. Comparisons of treatment groups was performed on stereology data using a one-way ANOVA with post hoc comparisons made using the Tukey honestly significant difference test when P < 0.05. Comparison of NII striatal density between groups was performed by using a Mann–Whitney U test and further confirmed with a Student’s t-test, and the SPSS (Chicago, IL, USA) statistical software package was used.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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