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Activation of the mitochondrial permeability transition pore modulates Ca^{2+} responses to physiological stimuli in adult neurons

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Abstract

The participation of mitochondria in cellular and neuronal Ca^{2+} homeostatic networks is now well accepted. Yet, critical tests of specific mitochondrial pathways in neuronal Ca^{2+} responses have been hampered because the identity of mitochondrial proteins that must be integrated within this dynamic system remain uncertain. One putative pathway for Ca^{2+} efflux from mitochondria exists through the formation of the permeability transition pore (PTP) that is often associated with cellular and neuronal death. Here, we have evaluated neuronal Ca^{2+} dynamics and the PTP in single adult neurons in wild-type mice and those missing cyclophilin D (CyPD), a key regulator of the PTP. Using high-resolution time-lapse imaging, we demonstrate that PTP opening only follows simultaneous activation with two physiological stimuli that generate critical threshold levels of cytosolic and mitochondrial Ca^{2+} . Our results are the first to demonstrate CyPD-dependent PTP opening in normal neuronal Ca^{2+} homeostatic mechanisms not leading to activation of cell death pathways. As neurons in mice lacking CyPD are protected in a number of neurodegenerative disease models, the results suggest that improved viability of CyPD-knockout animals in these pathological states may be due to the transient, rather than persistent, activation of the PTP in mutant mitochondria, thereby shielding neurons from cytoplasmic Ca^{2+} overload.

Keywords

calcium homeostasis; cortical neurons; mitochondria; mutant mice; neurodegeneration
neuroprotection

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Introduction

It is now clear that mitochondria play a pivotal role in cellular Ca^{2+} homeostasis and thereby participate in the orchestration of a diverse range of cellular activities. Indeed, the mitochondrial proton electro-chemical gradient is used not only to synthesize ATP but also to accumulate cations such as Ca^{2+} into the mitochondrial matrix (Friel, 2000; Giacomello *et al.*, 2007; Csordas & Hajnoczky, 2009; Rizzuto *et al.*, 2009). When local cytoplasmic free Ca^{2+} levels rise, mitochondria rapidly accumulate cytoplasmic Ca^{2+} and then gradually release it as normal cytoplasmic levels are restored, amplifying and sustaining signals arising from elevation of cytoplasmic Ca^{2+} , as well as protecting cells and neurons against transient elevation in intracellular Ca^{2+} during periods of hyperactivity (Friel, 2000; Kann & Kovacs, 2007; Nicholls, 2009). As a result, the mechanisms controlling cellular and mitochondrial Ca^{2+} homeostasis, metabolism and bioenergetics must function as a tightly integrated system within the overall cellular Ca^{2+} homeostatic network (for reviews, see Bernardi *et al.*, 1999; Rizzuto & Pozzan, 2003; Giacomello *et al.*, 2007; Szabadkai & Duchen, 2008).

The Ca^{2+} uniporter mediates Ca^{2+} uptake across the inner mitochondrial membrane while exchangers (Ca^{2+} for Na^{+} and/or H^{+} Ca^{2+}) are responsible for Ca^{2+} efflux (Gunter & Gunter, 2001; Brookes *et al.*, 2004; Kirichok *et al.*, 2004; Nicholls & Chalmers, 2004; Szabadkai & Duchen, 2008; Gunter & Sheu, 2009). However, when mitochondrial Ca^{2+} loads exceed the buffering capacity of inner membrane exchangers (the mitochondrial Ca^{2+} 'set point' or threshold), an additional pathway for Ca^{2+} efflux from mitochondria may exist through opening of the permeability transition pore (PTP). The PTP is a voltage-dependent, cyclosporin A (CsA)-sensitive, high-conductance channel of the inner mitochondrial membrane (for recent reviews, see Bernardi *et al.*, 2006; Rasola & Bernardi, 2007). Indeed, the interplay between the rate of mitochondrial Ca^{2+} influx and efflux modulates mitochondrial matrix Ca^{2+} , which in turn is widely considered to be a key factor for the regulation of the PTP open–closed transitions (Bernardi, 1999). Although opening of the PTP in response to Ca^{2+} has been documented in isolated mitochondria and permeabilized cells (Bernardi *et al.*, 2006; Rasola & Bernardi, 2007), assessing opening of the PTP in intact neurons and other primary cells in response to physiological activators that dictate cytosolic Ca^{2+} has remained a major challenge. Yet, opening of the PTP is often thought to be associated with pathophysiological processes (for reviews see Rizzuto & Pozzan, 2003; Leo *et al.*, 2005; Hajnoczky *et al.*, 2006; Giorgi *et al.*, 2008). In these scenarios, activation of the PTP leads to respiratory inhibition, and thus ATP depletion, and the release of mitochondrial Ca^{2+} stores and apoptotic activators, ultimately resulting in cell death (Bernardi, 1999; Bernardi *et al.*, 2006; Rasola & Bernardi, 2007; Di Lisa & Bernardi, 2009). These have led to the idea that opening of the PTP by elevated mitochondrial Ca^{2+} is a terminal, pathologic event. Although the preponderance of research and speculation on PTP opening in neurons has been on its role in mediating neuronal death, the potential participation of the PTP in non-lethal Ca^{2+} homeostasis in cells and neurons has gone largely unexplored.

Manipulation of the PTP can be reliably achieved through the analysis of cells and mice missing cyclophilin D (CyPD), a key regulator of PTP activity and the target of CsA action (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Importantly, mice missing CyPD (CyPD-KO) are still able to form the PTP but mitochondria from CyPD-KO mice are expectedly insensitive to further desensitization by CsA (e.g. Basso *et al.*, 2005; Schinzel *et al.*, 2005). In this study, we evaluate the role of CyPD-regulated PTP in neuronal Ca^{2+} homeostasis through direct assessment of cytoplasmic and mitochondrial Ca^{2+} dynamics using high resolution imaging in individual adult cortical neurons from wild-type (WT) and CyPD-KO animals.

Materials and methods

Animals

The generation of CyPD-KO mice in which the nuclear *Ppif* gene encoding CyPD has been eliminated has been previously described (Basso *et al.*, 2005). The *Ppif*-null mouse colonies were maintained as homozygotes. All experimental procedures were conducted following NIH guidelines under an Institutional Animal Care and Use Committee-approved protocol from the Oregon Health and Sciences University.

Pericam, mitoYFP and mitoPBRf constructs

For mitochondrial Ca^{2+} imaging, cultures were transfected with mitochondrially targeted ratiometric pericam (mitoRP; 415 and 494 nm excitation, 515 nm emission), a genetically encoded, green fluorescent protein-based Ca^{2+} indicator [kindly provided by Dr Atsushi Miyawaki, Institute of Physical and Chemical Research, Japan (Nagai *et al.*, 2001)]. Mitochondrial Ca^{2+} levels were evaluated following transfection of neuronal cultures with the genetically encoded, Ca^{2+} sensor, ratiometric pericam (Nagai *et al.*, 2001). This reporter was specifically directed to mitochondria by use of a duplicated mitochondrial targeting sequence (mitoRP) (Filippin *et al.*, 2005) increasing the delivery of mitoRP constructs to the mitochondrial matrix as well as expanding the dynamic range over which mitochondrial Ca^{2+} levels can be evaluated (Filippin *et al.*, 2003). Notably, the ratiometric nature of this probe minimizes differences in the data reported by mitoRP arising from variations in the levels of expression of this genetically encoded indicator between individual neurons. To assess changes in mitochondrial matrix pH, neuronal cultures were transfected with enhanced yellow fluorescent protein (EYFP) modified as described for mitoRP (mitoYFP; 500 nm excitation, 535 nm emission). Changes in mitochondrial morphology were reported by a fluorescent probe targeted to the outer mitochondrial membrane. This reporter represents a fusion protein containing the outer membrane peripheral benzodiazepine receptor (PBR) (Papadopoulos *et al.*, 2006) coupled to a green fluorescent protein (GFP) derivative (Miesenbock *et al.*, 1998) located at the C terminus (mitoPBRf; 380 and 460 nm excitation, 515 nm emission). The C terminus of the PBR, and hence the GFP derivative, faces the cytosol (Joseph-Liauzun *et al.*, 1998).

Neuronal cell culture and transfection

For each experiment, 2- to 4-month-old, sex-matched WT and CyPD-KO mice were used. Adult neuronal cultures were prepared as outlined in Nathan *et al.* (2004). The entire cerebral cortex was dissected from the brain and placed in 2 mL B27/Hibernate A medium (B27/HA, Invitrogen, Carlsbad, CA, USA) with 0.5 mM glutamine (Sigma, St. Louis, MO, USA) at 4 °C. The cortex was sliced (0.5 mm thickness) and transferred to a 50-ml tube containing 5 mL B27/HA. After warming for 8 min at 30 °C, slices were digested with 6 mL of a 2mg/mL papain (Sigma) solution in B27/HA for 30 min at 30 °C in a gyrating water bath. The slices were transferred to 2 mL B27/HA. After 2 min at room temperature, the slices were triturated ten times with a siliconized 9-inch Pasteur pipette, and allowed to settle for 1 min. Approximately 2 mL of the supernatant was transferred to another tube, and the sediment was resuspended in 2 mL B27/HA. The above step was repeated twice, and a total of 6 mL was collected. The resultant supernatant was subjected to density gradient centrifugation at 800 g for 15 min. The density gradient was prepared in four 1-ml layers of 35, 25, 20 and 15% Optiprep (Invitrogen) in B27/HA medium (v/v). Debris above 7 mL was discarded. The rest of the fractions, excluding the bottom pellet, was collected and diluted in 5 mL of B27/HA. After centrifuging twice at 200 g for 2 min, the cell pellets were resuspended in 3 mL B27/Neurobasal A medium (Invitrogen) with 0.5 mM glutamine and 0.01 mg/mL gentamicin (Sigma). For transfection, neuronal pellets were re-suspended in 100 μL of nucleofection solution with 3 $\mu\text{g}/\text{mL}$ of each plasmid construct and electroporated

following the Amaxa electroporation system (Amaxa, Lonza, Basel, Switzerland) for neurons, modified by the use of a Ca^{2+} -free buffer immediately after the electroporation, which maintained neuronal viability. A total of 3×10^4 cells were plated in 30- μL aliquots in the center of glass cover slips (25 mm diameter) that were coated overnight with poly-D-lysine (50 mg/mL; Sigma). After 1 h incubation in a humidified incubator at 37 °C and 5% CO_2 , each cover slip was rinsed with B27/HA and transferred to a six-well plate containing B27/Neurobasal A medium. Routinely, 10% of neurons were successfully transfected and the mitoRP signal is detectable 48 h post-transfection. Within individual neurons, the mitochondrial responses were remarkably consistent (variation <5%); as a result, the responses within a neuron could be effectively averaged to generate the response for one neuron.

Use of fura-FF, TMRM and FCCP

For cytosolic Ca^{2+} measurements, cells were loaded with 5 μM fura-FF (340 and 380 nm excitation, 505 nm emission; Molecular Probes, Eugene, OR, USA) in imaging buffer (142 mM NaCl, 4 mM NaHCO_3 , 10 mM Na-HEPES, 2.5 mM KCl, 1.2 mM MgCl_2 , 2 mM CaCl_2 and 10 mM glucose, pH 7.4) containing 2% bovine serum albumin (BSA) (Sigma) for 15–20 min at 37 °C. Cells were washed with imaging buffer containing 0.25% BSA for 10 min at 37 °C before recording. For mitochondrial membrane potential measurements, cells were loaded with 10 nM tetramethyl rhodamine methyl ester (TMRM, 535 nm excitation, 575 nm emission; Sigma) and incubated in the presence of 1.6 μM cyclosporin H (Alexis Biochemicals, San Diego, CA, USA) for 30 min at 37 °C exactly as outlined in Petronilli *et al.* (2001). Neurons were perfused and imaged in ECM containing 10 nM TMRM (Sigma) and cyclosporin H. Neurons from the same culture preparation but lacking mitoRP were evaluated for cytosolic Ca^{2+} and mitochondrial membrane potential. The proximity of excitation wavelengths between fura-FF (340/380 nm) and ratiometric pericam (415/494 nm) did not allow for simultaneous imaging of both probes in the same neuron. In addition, simultaneous imaging of TMRM and mitoRP was not possible. To depolarize mitochondria, the protoionophore carbonyl cyanide *p*-trifluoromethoxy phenyl hydrazone [450 nm; FCCP (Sigma)] was added to cultures.

Fluorescence imaging

Imaging was carried out after 4–5 days in culture. Prior to stimulation, cytosolic and mitochondrial Ca^{2+} levels among neurons of either genotype were similar. In general, neurons were stimulated with adenosine triphosphate (ATP, Sigma), (S)-3,5-dihydroxyphenylglycine (DHPG, Sigma), potassium chloride (KCl, Sigma) and treated with ionomycin (Sigma) by perfusion (2 mL/min) with ECM containing each compound at 37 °C in the presence of 5% CO_2 air mixture. Imaging of mitochondrial or cytosolic Ca^{2+} , mitochondrial membrane potential, mitochondrial pH and mitochondrial morphology was carried out using an inverted microscope (Olympus IX81, 60 $\times$, UPlanSApo340, NA 1.35 oil and 150 $\times$, UPlan, NA 1.45, oil with 1.6 $\times$ magnification changer) equipped with a cooled CCD camera (Cascade II; Intelligent Imaging Innovations, Denver, CO, USA), a high-speed wavelength switcher (Lambda DG-5; Sutter Instruments, Novato, CA, USA) controlled by SlideBook software (Intelligent Imaging Innovations) and appropriate Chroma filters. For imaging, cells were transferred to ECM at 37 °C (heated stage, Warner Instruments). Exposure time was 100–200 ms and frames were taken every 0.5–2 s. After stimulation and recording, cells were not re-used. Cytosolic or mitochondrial Ca^{2+} and mitochondrial membrane potential were measured in cytosol and mitochondria, respectively, of neuronal soma only. Mitochondrial Ca^{2+} (evaluated in mitoRP-transfected neurons), mitochondrial membrane potential (evaluated by TMRM), mitochondrial pH (evaluated by mitoYFP) and mitochondrial morphology (evaluated by mitoPBRf) were all assessed in clusters of mitochondria in the regions of the soma (see Fig. 3E). Data obtained from

clusters of mitochondria were averaged to represent mitochondrial Ca^{2+} , the membrane potential or mitochondrial pH response per cell. Cytosolic Ca^{2+} was evaluated using fura-FF in the perinuclear region of a cell. In adult cortical neurons, mitoRP exhibited low photobleaching.

Image analysis

Fluorescence changes in mitochondria or cytosol were evaluated using SlideBook software version 4.2.0 (Intelligent Imaging Innovations). The background fluorescence was taken from fields not containing neurons. For imaging fluorescence changes in mitochondria, clusters of mitochondria in the soma of a neuron were chosen by using the Mask feature of this software. Movements of mitochondria were tracked throughout each recording by using the particle tracking function of Slidebook. The response from each cell was then normalized to the baseline value before stimulation using Excel software.

Immunocytochemistry and viability assays

Immunocytochemistry was carried out with antibodies for neurons (β -tubulin III, Molecular Probes), astrocytes (glial fibrillary acidic protein, Molecular Probes), oligodendrocytes (myelin basic protein, Molecular Probes) and microglia (BS lectin 1, Sigma). Cultures were routinely found to consist of 70% neurons and 30% glia. Neuronal viability was assessed by using the Biotium (Biotium, Inc., Hayward, CA, USA) CalceinAM Cell Viability assay kit.

Statistical analysis

Each experiment was replicated three times using neurons obtained from two to three different animals per genotype. There was no apparent difference in responses within a genotype from animal to animal. Data from each experiment were analysed by one-way ANOVA with SPSS statistical software (version 15.0 for Windows, IBM Corp., NY, USA), followed by Bonferroni post-hoc test when needed to analyse data between two or more groups. The data from all evaluated cells from all animals per genotype are presented as mean $\pm$ standard error of the difference (SED).

Results

Imaging cytosolic and mitochondrial Ca^{2+} in adult neurons

To assess the role of CyPD-dependent PTP activation in neuronal cytosolic and mitochondrial Ca^{2+} dynamics, cultured adult cortical neurons were prepared from isogenic WT mice and CyPD-KO (*Ppif*^{-/-}) mice (Basso *et al.*, 2005). Neuronal cultures from adults were investigated rather than the more commonly used cultures prepared from neonatal animals. Neuronal Ca^{2+} dynamics vary significantly when neurons prepared from early postnatal and adult animals are compared (e.g. Lalo & Kostyuk, 1998) and many neurodegenerative diseases target adult neurons. Indeed, following ischemic challenges, neuroprotection in CyPD-KO animals has been demonstrated to be age-dependent and is only observed in adult, not neonatal, animals (Wang *et al.*, 2009). Immunocytochemical analysis indicated that the majority of cells in these adult preparations (>70%) were neurons with characteristic morphology. To assess cytosolic Ca^{2+} levels, cells were loaded with the ratiometric indicator furaFF-AM. Mitochondrial Ca^{2+} levels were evaluated following transfection of neuronal cultures with the Ca^{2+} sensor ratiometric pericam (mitoRP) (Nagai *et al.*, 2001). Clusters containing variable numbers of mitochondria (the majority consisting of multiple mitochondria) in the soma of each transfected neuron were imaged and changes in cytosolic Ca^{2+} , as well as mitochondrial Ca^{2+} , were assessed following perfusion with various stimuli. Within individual neurons, the mitochondrial responses were remarkably

consistent (variation <5%); as a result, the responses within a neuron could be effectively averaged to generate the response for one neuron.

Changes in cytosolic and mitochondrial Ca^{2+} in response to single stimuli

Cytosolic and mitochondrial Ca^{2+} levels rise in response to the receptor-driven, inositol trisphosphate (IP₃)-mediated release of Ca^{2+} from endoplasmic reticulum (ER) stores, as has been demonstrated in many cell types (Giacomello *et al.*, 2007; Csordas & Hajnoczky, 2009; Rizzuto *et al.*, 2009). Early studies suggested that the PTP can be activated by treatment of permeabilized cultured cells directly with IP₃ (Ichas *et al.*, 1994). To directly test the role of IP₃-regulated release on PTP activation, we initially tested Ca^{2+} responses in WT and CyPD-null neurons following brief (30 s) perfusion with ATP, thereby transiently stimulating P₂Y receptors (Fig. 1A–D). No difference was observed in any aspect of cytosolic or mitochondrial Ca^{2+} dynamics following ATP stimulation of WT or CyPD-KO neurons [cytosolic Ca^{2+} as reported by fura-FF, for all the data presented the value for control (untreated) is 1, data presented as mean $\pm$ SED: WT 1.06 ± 0.02 vs. CyPD-KO 1.05 ± 0.01 , $F_{5,45} = 0.54$, $P = 0.43$, WT $n = 24$ neurons, CyPD-KO $n = 27$; mitochondrial Ca^{2+} as reported by mitoRP: WT 1.68 ± 0.23 vs. CyPD-KO 1.68 ± 0.21 , $F_{5,106} = 0.19$, $P = 0.92$, WT $n = 55$ neurons, CyPD-KO $n = 57$]. Similar stimulation with DHPG, a type I metabotropic glutamate receptor agonist coupled to the generation of IP₃, also demonstrated no differences in mitochondrial Ca^{2+} responses between WT and CyPD-KO neurons (mitochondrial Ca^{2+} as reported by mitoRP: WT 2.17 ± 0.18 vs. CyPD-KO 2.21 ± 0.15 , $F_{5,84} = 0.97$, $P = 0.24$, WT $n = 48$ neurons, CyPD-KO $n = 42$) (Supporting Information Fig. S1). Cytosolic and mitochondrial Ca^{2+} levels also rise in response to activation of plasma membrane Ca^{2+} channels following membrane depolarization (Friel, 2000). To activate voltage-dependent Ca^{2+} channels, neurons were depolarized by perfusion with KCl. Neuronal responses to various concentrations of KCl were initially tested and robust cytosolic Ca^{2+} responses were detected following brief perfusion (30 s) of adult neurons with 90 mM KCl. As expected, cytosolic and mitochondrial Ca^{2+} levels increased but, again, no significant differences were observed between WT and CyPD-KO neurons in any aspect of cytosolic or mitochondrial Ca^{2+} dynamics following transient depolarization with KCl (cytosolic Ca^{2+} as reported by fura-FF: WT 1.11 ± 0.02 vs. CyPD-KO 1.08 ± 0.03 , $F_{5,62} = 1.15$, $P = 0.12$, WT $n = 36$ neurons, CyPD-KO $n = 32$; mitochondrial Ca^{2+} as reported by mitoRP: WT 1.85 ± 0.19 vs. CyPD-KO 1.83 ± 0.20 , $F_{5,76} = 1.31$, $P = 0.09$, WT $n = 42$ neurons, CyPD-KO=40) (Fig. 1E–H). Perfusion of neurons with KCl in Ca^{2+} -free buffer eliminated cytosolic Ca^{2+} accumulation as well as mitochondrial Ca^{2+} responses (Supporting Information Fig. S2), and mitochondrial responses to receptor-generated IP₃ and depolarization were both eliminated following treatment of neurons with FCCP (Supporting Information Fig. S2). From these experiments, we conclude that the response of neurons to single stimuli does not raise cytosolic Ca^{2+} and ensuing mitochondrial Ca^{2+} levels sufficiently to open the CyPD-regulated PTP.

Mitochondrial Ca^{2+} in response to multiple stimuli

To increase cytoplasmic (and hence mitochondrial) Ca^{2+} to levels at which differences between WT and CyPD-KO mitochondria might be revealed (Rizzuto & Pozzan, 2003; Giacomello *et al.*, 2007; Szabadkai & Duchen, 2008), we next assessed neuronal responses to simultaneous release of ER Ca^{2+} and activation of plasma membrane Ca^{2+} channels. Following dual stimulation (30 s) with ATP and KCl, cytoplasmic Ca^{2+} increased in neurons of each genotype to significantly higher levels than achieved with either stimulus alone. However, in response to dual stimulation, higher levels of cytoplasmic Ca^{2+} were recorded in WT than in CyPD-KO neurons (cytoplasmic Ca^{2+} as reported by fura-FF at peak: WT 1.31 ± 0.05 vs. CyPD-KO 1.20 ± 0.07 , $F_{5,100} = 3.71$, $P = 0.006$, WT $n = 56$ neurons, CyPD-KO, $n = 50$; Fig. 2A and B). Over time, WT cytosolic Ca^{2+} levels remained at significantly

higher levels than observed in CyPD-KO neurons (cytosolic Ca^{2+} as reported by fura-FF at 1.3 min: WT 1.19 ± 0.03 vs. CyPD-KO 1.08 ± 0.05 , $F_{5,100} = 4.25$, $P = 0.003$; Fig. 2B). In contrast, mitochondrial Ca^{2+} responses to the simultaneous stimulation demonstrated that mitochondria in CyPD-KO neurons accumulated Ca^{2+} to significantly higher levels than observed in mitochondria in WT neurons (mitochondrial Ca^{2+} as reported by mitoRP at peak: CyPD-KO 3.86 ± 0.43 vs. WT 2.3 ± 0.30 , $F_{5,93} = 5.94$, $P = 0.00008$, WT $n = 51$ neurons, CyPD-KO $n = 48$; Fig. 2C and D). In addition, the return of mitochondrial Ca^{2+} to pre-stimulus levels was correspondingly slower in CyPD-KO neurons than in WT neurons (mitochondrial Ca^{2+} as reported by mitoRP at 1.3 min: CyPD-KO 3.10 ± 0.21 vs. WT 1.75 ± 0.52 , $F_{5,93} = 6.25$, $P = 0.00005$; Fig. 2D). Again, pretreatment with FCCP eliminated neuronal mitochondrial Ca^{2+} uptake in response to simultaneous activation of each pathway (Fig. 2E). Importantly, under these conditions treatment with both ATP and KCl had no effect on neuronal viability (percentage of live neurons compared with the viability of untreated cultures assessed 24 h post-stimulation; WT $98.7 \pm 0.53\%$ vs. CyPD-KO $99.6 \pm 0.85\%$, $F_{5,144} = 0.11$, $P = 0.98$, WT $n = 75$ random fields of view containing live neurons in 15 cover slips, CyPD-KO $n = 75$; Fig. 2F), nor was mitochondrial morphology noticeably altered during or immediately following simultaneous activation of each pathway (Supporting Information Fig. S3A–D). Transfection of mitochondrially targeted YFP, which exhibits changes in fluorescence in response to changes in pH (Rudolf *et al.*, 2003), demonstrated that these responses could not be due to changes in mitochondrial pH that might be reported by mitoRP (Nagai *et al.*, 2001) (mitochondrial pH value as reported by mito-YFP at 5 min: WT 0.96 ± 0.07 vs. CyPD-KO 0.98 ± 0.04 , $F_{3,24} = 2.12$, $P = 0.08$, WT $n = 13$ neurons; CyPD-null $n = 15$; Supporting Information Fig. S3E and F).

Experiments following addition of the Ca^{2+} ionophore ionomycin also support the conclusion that mitochondria in CyPD-KO neurons have a higher Ca^{2+} threshold for PTP opening. Here, in response to persistent, in contrast to transient, elevation of cytosolic Ca^{2+} , the PTP in mitochondria of WT neurons opens at lower levels of mitochondrial Ca^{2+} as documented by changes in mitochondrial Ca^{2+} and morphology when compared with mitochondria in CyPD-KO neurons. Following perfusion of neurons with ionomycin, cytosolic Ca^{2+} increased over three-fold in both genotypes (compared with only a 1.3-fold increase in response to ATP+KCl, Fig. 3A), yet was significantly higher in WT neurons than in CyPD-KO neurons (cytosolic Ca^{2+} as reported by fura-FF at peak: WT 3.62 ± 0.01 vs. CyPD-KO 2.96 ± 0.02 , $F_{5,45} = 4.82$, $P = 0.002$, WT $n = 25$ neurons; CyPD-KO $n = 26$; Fig. 3A and B). In contrast, mitochondrial Ca^{2+} reached a significantly higher level earlier in CyPD-KO neurons than in WT neurons (mitochondrial Ca^{2+} as reported by mitoRP at point 2 in Fig. 3C and D; CyPD-KO 3.60 ± 0.08 vs. WT 2.77 ± 0.12 , $F_{3,19} = 12.92$, $P = 0.0001$, WT $n = 12$ neurons, CyPD-KO $n = 11$) before they attained identical levels at saturation (e.g. point 3 in Fig. 3C and D). Differences in mitochondrial Ca^{2+} saturation were reflected in the onset of mitochondrial morphological changes associated with Ca^{2+} -driven persistent PTP activation. Although changes in mitochondrial morphology (rounding or swelling) eventually occurred in both genotypes (e.g. at point 3 in Fig. 3C), morphological changes were delayed in CyPD-KO neurons compared with WT neurons (e.g. at point 2 in Fig 3C shown in Fig. 3Ei and ii). Furthermore, these differences are associated with a significantly higher cell survival in CyPD-KO neurons than in WT neurons in response to ionomycin treatment (percentage of live neurons when compared with the viability of untreated cultures assessed 24 h post-stimulation: 1 μM ionomycin: CyPD-KO 40.0 ± 1.2 vs. WT $15.7 \pm 5.2\%$, $F_{5,74} = 7.02$, $P = 0.00001$, WT $n = 40$ random fields of view containing neurons from eight cover slips, CyPD-KO $n = 40$; 5 μM ionomycin: CyPD-KO 25.3 ± 4.1 vs. WT $7.0 \pm 1.7\%$, $F_{5,74} = 6.74$, $P = 0.00001$, WT $n = 40$, CyPD-KO $n = 40$; Fig. 3F). These results are thus consistent with the idea that opening of the PTP in mitochondria of CyPD-KO neurons requires higher levels of mitochondrial Ca^{2+} due to the desensitization of this mitochondrial

Ca²⁺ efflux pathway in mutant mitochondria and confirm the neuroprotective role of CyPD inactivation in response to elevated cytosolic and mitochondrial Ca²⁺.

Pharmacological inhibition of CyPD activity

To confirm and extend the results obtained following genetic elimination of CyPD, we tested whether similar results could be obtained following pharmacological inhibition of CyPD with CsA, a classic inhibitor of the PTP whose site of action is CyPD. Mitochondrial Ca²⁺ levels in either WT neurons or WT neurons in the presence of CsA did not differ when treated with ATP or following depolarization (i.e. KCl) alone (Supporting Information Fig. S4A and B), mimicking previous results comparing these responses in WT and CyPD-KO neurons (Figs 1 and 2). In contrast, cytosolic (Supporting Information Fig. S4C and D) and mitochondrial (Fig. 4A and C) Ca²⁺ responses following simultaneous stimulation of both pathways in WT neurons treated with CsA mimicked responses observed in CyPD-KO neurons, although somewhat attenuated compared with those observed by genetic inactivation of CyPD (cytosolic Ca²⁺ as reported by fura-FF at peak: WT 1.40 ± 0.03 vs. WT+CsA 1.30 ± 0.02 , $F_{3,21} = 4.26$, $P = 0.02$; CyPD-KO 1.22 ± 0.05 , CyPD-KO+CsA 1.19 ± 0.08 , WT $n = 13$ neurons, WT+CsA, $n = 15$, CyPD-KO $n = 12$, CyPD-KO +CsA, $n = 14$). Importantly, WT neurons treated with CsA showed significant increases in mitochondrial Ca²⁺ levels following simultaneous stimulation of both pathways (mitochondrial Ca²⁺ as reported by mitoRP at peak: WT 1.94 ± 0.13 vs. WT+CsA 2.86 ± 0.21 , $F_{5,22} = 9.47$, $P = 0.0001$, WT $n = 14$ neurons; WT+CsA $n = 14$; compare Fig. 4A and C with Fig. 2C and D). As would be expected if CyPD is the target of CsA action, no differences were observed in cellular (Supporting Information Fig. S4D and E) or mitochondrial (Fig. 4B) Ca²⁺ responses, at peak levels, or delay in return to baseline (data not shown) between CyPD-KO neurons and CyPD-KO neurons treated with CsA following simultaneous activation of both pathways (mitochondrial Ca²⁺ as reported by mitoRP at peak: CyPD-KO 4.00 ± 0.15 vs. CyPD-KO+CsA 4.08 ± 0.17 , $F_{5,22} = 9.47$, $P = 0.16$, CyPD-KO $n = 13$ neurons; CyPD-KO +CsA $n = 15$). From these studies, we conclude that pharmacological inhibition of CyPD in WT neurons is able to phenocopy the cytosolic and mitochondrial Ca²⁺ responses observed on genetic ablation of CyPD.

Assessment of $\Delta\psi$ changes

Assessment of mitochondrial Ca²⁺ levels in WT and CyPD-KO neurons supports the idea that the PTP opening in WT neurons occurs following simultaneous stimulation of two independent pathways. To assess the occurrence of PTP opening by an independent method, we measured the inner membrane potential ($\Delta\psi$) of mitochondria in WT and CyPD-KO neurons after loading neurons with the fluorescent dye TMRM and simultaneous perfusion with ATP and KCl; the inner mitochondrial membrane potential dissipates following pore opening (Nicholls & Budd, 2000). TMRM reveals changes in $\Delta\psi$ following redistribution from the cytosol to the mitochondrial matrix and consequently is a slower reporter than mitoRP (Petronilli *et al.*, 2001). Comparisons of the $\Delta\psi$ in mitochondria following stimulation of neurons with ATP or depolarization with KCl revealed no differences between WT and CyPD-KO mitochondria. However, following perfusion of neurons with ATP and KCl simultaneously, the $\Delta\psi$ of mitochondria in WT neurons decreased, as would be predicted following opening of the PTP (Fig. 5A), and returned to baseline levels on longer recording (Fig. 5B). In contrast, the $\Delta\psi$ of mitochondria in CyPD-KO neurons and in WT neurons treated with CsA was maintained in response to simultaneous activation of these pathways, reflecting the expected delay in the activation of the PTP ($\Delta\psi$ as reported by TMRM at 2 min: WT $0.65 \pm 0.05\%$ vs. CyPD-KO $0.95 \pm 0.03\%$ or WT+CsA $0.97 \pm 0.05\%$, $F_{5,86} = 6.72$, $P = 0.00001$, WT $n = 44$ neurons; WT+CsA, $n = 32$ neurons, CyPD-KO $n = 48$, CyPD-KO+CsA $n = 33$; at 5 min: WT $0.90 \pm 0.11\%$ vs. CyPD-KO $0.94 \pm 0.09\%$, $F_{5,19} = 2.11$, $P = 0.07$, WT $n = 13$ neurons; CyPD-KO $n = 12$; Fig. 5C and D). As expected, $\Delta\psi$

responses of mitochondria in CyPD-KO neurons or CyPD-KO neurons treated with CsA were identical (Fig. 5C). Furthermore, the $\Delta\psi$ of mitochondria in CyPD-KO neurons was maintained at a significantly higher level than in WT neurons in response to ionomycin, reflecting the expected delay in the activation of the PTP ($\Delta\psi$ as reported by TMRM: CyPD-KO $0.89 \pm 0.03\%$ vs. WT $0.66 \pm 0.05\%$, $F_{5,32} = 5.64$, $P = 0.007$, WT $n = 18$ neurons, CyPD-KO $n = 20$). In all cases, mitochondrial $\Delta\psi$ rapidly dissipated following addition of FCCP (Fig. 5A).

Discussion

The PTP in neuronal Ca^{2+} dynamics

Although the participation of mitochondria in cellular Ca^{2+} homeostatic networks is now well accepted, the role of the PTP in these homeostatic systems has been proposed but not directly demonstrated in the context of intact cells. Opening of the PTP in an intact cellular context has only been documented indirectly and in many cases using stimuli that disrupt mitochondrial function. Such indirect techniques include the release from mitochondria of fluorescent molecules such as calcein when cobalt is employed to quench cytosolic fluorescence (Petronilli *et al.*, 1999, 2001), by monitoring of mitochondrial NAD(P)H release following treatment with Ca^{2+} ionophores (i.e. A23187) (Dumas *et al.*, 2009) or by assessment of cytosolic Ca^{2+} levels following ER release in hepatocytes treated with CsA (Smaili *et al.*, 2001). Additional studies have monitored pore opening in response to challenges leading to cell death in neurons and cultured cells by the use of potentiometric probes such as TMRM; in this context, pore opening results in depolarization of the mitochondrial membrane potential (e.g. De Giorgi *et al.*, 2002; Abramov & Duchen, 2008; Li *et al.*, 2009).

In this report, by assessing neuronal Ca^{2+} homeostasis through direct assessment of cytoplasmic and mitochondrial Ca^{2+} dynamics in individual adult cortical neurons from WT and CyPD-KO neurons, we demonstrate several important features of the role of the PTP in normal cellular and neuronal Ca^{2+} dynamics in a whole-cell setting. First, although the role of mitochondrial Ca^{2+} accumulation following activation of IP3 receptors and plasma membrane Ca^{2+} channels is well established (Giacomello *et al.*, 2007; Csordas & Hajnoczky, 2009; Rizzuto *et al.*, 2009), we show that the response of neurons to single stimuli does not raise cytosolic Ca^{2+} and ensuing mitochondrial Ca^{2+} levels sufficiently to open the CyPD-regulated PTP. Mitochondrial Ca^{2+} only exceeds the 'set point' for PTP activation following simultaneous, transient stimulation of two pathways; activation of no single pathway is sufficient. Second, activation of the PTP following transient stimulation of two Ca^{2+} mobilizing pathways in neither CyPD-KO nor WT neurons resulted in the initiation of neuronal death pathways, as would be expected following persistent activation of the PTP, for example as demonstrated here following treatment of neurons with ionomycin. Third, activation of mitochondrial Ca^{2+} release via the PTP results in corresponding elevations in cytosolic Ca^{2+} , and both aspects of this response are reduced in CyPD-KO neurons. In contrast, mitochondria in CyPD-KO neurons can accumulate substantially higher Ca^{2+} than in WT neurons, due to the attenuation of the PTP as a Ca^{2+} efflux pathway. This conclusion is supported following persistent activation of neuronal Ca^{2+} levels after ionomycin treatment. Consequently, in a whole-cell setting, we document the higher Ca^{2+} set-point for activation of the PTP in neurons missing CyPD due to the desensitization of this mitochondrial Ca^{2+} efflux pathway in mutant mitochondria. In summary, by identifying differences in cytosolic and mitochondrial Ca^{2+} dynamics between intact WT and CyPD-KO adult neurons after transient physiological stimulation, we have been able to define for the first time a clear role for the PTP as a mitochondrial Ca^{2+} release channel in normal neuronal Ca^{2+} responses.

Mitochondria in neuronal Ca^{2+} homeostasis

It has been previously proposed that the PTP may provide a convenient fast Ca^{2+} release channel to mitochondria, which could help prevent matrix Ca^{2+} overload (Bernardi & Petronilli, 1996; Bernardi, 1999), but this hypothesis has been extremely difficult to test experimentally because of the lack of selective PTP blockers. Indeed, even the genetic ablation of CyPD does not prevent PTP opening, but rather desensitizes the pore, whose opening still occurs at higher Ca^{2+} loads (e.g. Basso *et al.*, 2005; Schinzel *et al.*, 2005). Based on these premises, we reasoned that the PTP contribution to physiological Ca^{2+} homeostasis could be tested by increasing mitochondrial Ca^{2+} loading through physiological stimuli, and comparing the response of WT and CyPD-KO mitochondria *in situ*. To date, CyPD-KO mitochondria have been demonstrated to retain higher levels of Ca^{2+} due to desensitization of PTP opening in isolated mitochondria (e.g. Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005), and after cell exposure to Ca^{2+} overload with ionophores (e.g. A23187), or to strong oxidative challenges (e.g. Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Our results are the first to document in any intact cell that the PTP is activated in normal Ca^{2+} networks rather than as a mandatory executioner in cell demise. In addition, our results demonstrate that mitochondria in intact CyPD-KO neurons have greater ability to accumulate Ca^{2+} compared with WT neurons, consistent with the neuroprotective role of CyPD inactivation in murine models that mimic human neurodegenerative conditions. Consequently, the most parsimonious interpretation of these observations is that cytosolic Ca^{2+} levels in WT and CyPD-KO neurons reflect a dynamic interplay between mitochondrial uptake and release.

Importantly, these results were not the consequence of enhanced Ca^{2+} capacity or changes in mitochondrial pH in CyPD-KO neurons, and they could be mimicked by blocking CyPD activity in WT neurons with CsA. Conversely, CsA has no effect on cytosolic or mitochondrial Ca^{2+} dynamics in CyPD-KO neurons, consistent with the prediction that CyPD is the only component of the PTP affected by CsA. As an independent measure of PTP opening, we showed that the $\Delta\psi$ of mitochondria following dual stimulation was also altered in CyPD-KO neurons compared with controls.

We also attempted to evaluate differences in cytosolic and mitochondrial Ca^{2+} dynamics between WT and CyPD-KO neurons following activation of ionotropic glutamate receptors (Kwak & Weiss, 2006). Although different glutamate concentrations were evaluated in our study of adult neurons, responses were muted or absent, probably due to our assessing adult neurons following 4–5 days in culture. Early studies of embryonic or early postnatal neurons demonstrated that effective glutamate neurotransmission and glutamate neurotoxicity is only established following several weeks in culture (Choi, 1992; King *et al.*, 2006). Moreover, in mitochondria of adult cortical neurons, mitoRP exhibits stable signals following transfection for 6 days in culture. Consequently, Ca^{2+} responses following glutamate stimulation of adult neuronal cultures might only be reliably assessed following longer times in culture to allow the formation of mature synapses.

The openings of the PTP demonstrated in our studies may represent transient openings ('flickering') or, perhaps, opening of the PTP to lower conductance states (Petronilli *et al.*, 1999, 2001). Although our results cannot distinguish between these two alternatives, they do support a role for the PTP in normal Ca^{2+} homeostasis. Importantly, we observed no differences in the viability of WT or CyPD-KO neurons following dual stimulation.

Persistent PTP activation

Consistent with the interpretation outlined above, elevated levels of cytosolic Ca^{2+} induced with the Ca^{2+} ionophore ionomycin caused more dramatic alterations in mitochondrial

morphology and PTP dynamics in WT neurons compared with CyPD-KO neurons. Morphological changes associated with mitochondrial Ca^{2+} overload and dysfunction (rounding or swelling) were delayed in CyPD-KO adult neurons. The delay in mitochondrial Ca^{2+} saturation that we observed in CyPD-KO neurons was also associated with more stable mitochondrial membrane potential and higher rates of neuronal survival. Although persistent elevation of cytosolic Ca^{2+} could still induce the death of CyPD-KO neurons, this response was significantly reduced in comparison with WT neurons. This observation is also consistent with previous studies of other cell types devoid of CyPD (e.g. Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Thus, in the context of pathological situations, PTP activation and cytosolic Ca^{2+} overload is delayed in CyPD-KO adult neurons, thereby muting the likelihood of metabolic crisis and cell death.

Our results also support the model that increases in mitochondrial Ca^{2+} may induce a spectrum of PTP responses, ranging from no activation, to transient openings that can reduce mitochondrial Ca^{2+} , to prolonged opening that triggers a cascade of events leading to cell death, as has been suggested by studies on isolated mitochondria and permeabilized cells (Bernardi *et al.*, 2006; Rasola & Bernardi, 2007). Whether PTP opening helps maintain Ca^{2+} homeostasis or promotes cell death may therefore be a function of how long the PTP remains open under different circumstances. In addition, previous studies using mitoRP (or other probes) have demonstrated that mitochondrial Ca^{2+} responses of individual mitochondria may vary, depending on their proximity to Ca^{2+} sources (e.g. ER and plasma membrane) (e.g. Csordas & Hajnoczky, 2003; Filippin *et al.*, 2003). Indeed, studies of isolated organelles have suggested that mitochondria in synaptic regions of a neuron are more susceptible to changes in cytosolic Ca^{2+} than mitochondria in other regions (Brown *et al.*, 2006; Naga *et al.*, 2007). Although we focused here on the Ca^{2+} dynamics of local groups of mitochondria within neuronal cell bodies, we anticipate that applying these methods to image Ca^{2+} responses within single mitochondria may provide a means for discriminating between mitochondrial Ca^{2+} responses within specific neuronal subdomains (Park *et al.*, 2001).

The PTP in neurodegenerative diseases

Given the ability of mitochondria to dynamically buffer cytosolic Ca^{2+} , it is easy to imagine how cells and tissues in CyPD-KO animals become resistant in many animal models of human neurodegenerative diseases in which cytoplasmic Ca^{2+} overload is envisioned as a critical event in disease progression (Mattson, 2007; Chaturvedi & Beal, 2008; Celsi *et al.*, 2009; Nicholls, 2009; Su *et al.*, 2009; Trapp & Stys, 2009). As outlined here in the context of intact neurons, and in many studies on isolated organelles, mitochondria lacking CyPD are able to accumulate higher levels of Ca^{2+} than WT mitochondria (e.g. Baines *et al.*, 2005; Basso *et al.*, 2005), possibly blunting in vivo cytosolic Ca^{2+} increases that accompany disease processes that may rely on a critical threshold of cytosolic Ca^{2+} . In turn, the pathological consequences of persistent PTP activation, leading to metabolic crisis and activation of cell death pathways, are also dampened as the Ca^{2+} set-point for PTP activation in CyPD-KO cells is increased. In support of this idea, mouse neurons lacking CyPD are resistant to the consequences of cellular and mitochondrial Ca^{2+} overload thought to play a key role in the initiation and/or propagation of the PTP activation in mouse models of ischemic brain injury (Schinzel *et al.*, 2005; Wang *et al.*, 2009), multiple sclerosis (Forte *et al.*, 2007) and Alzheimer's disease (Du *et al.*, 2008). In the course of these studies, many cell types, including neurons from CyPD-KO animals, have been demonstrated to be resistant to reactive oxygen and/or nitrogen challenges (e.g. Baines *et al.*, 2005; Schinzel *et al.*, 2005; Forte *et al.*, 2007). Moreover, neonatal neurons from CyPD-KO animals are resistant to glutamate concentrations, leading to excitotoxicity and ensuing cytosolic Ca^{2+} overload, yet in these studies mitochondrial Ca^{2+} levels were not assessed (Abramov &

Duchen, 2008; Li *et al.*, 2009). As reported here, our studies of adult neurons may more accurately reflect cellular events related to the CyPD-dependent neuroprotection tested in these adult animal models of neurodegenerative conditions. Our lack of understanding of the molecules involved in PTP formation currently prevents a rigorous testing of these hypotheses, yet our results do point to strategies inhibiting CyPD as promising neuroprotective agents.

In conclusion, we have shown that PTP opening does not necessarily induce the irreversible activation of necrotic or apoptotic cascades, nor does it automatically lead to neuronal death. In addition, by comparing both cytosolic and mitochondrial Ca^{2+} dynamics in WT versus CyPD-KO adult neurons, we show that the PTP is only activated in response to the combined action of several physiologically relevant stimuli that affect cytosolic Ca^{2+} . Our results provide the first evidence that PTP opening plays an important role in modulating cellular responses to normal cellular Ca^{2+} dynamics under physiological conditions, in addition to its involvement in the pathological events accompanying Ca^{2+} overload responses in various neurological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CsA	cyclosporin A
CyPD	cyclophilin D
CyPD-KO	cyclophilin D-knock out ($\text{Ppif}^{-/-}$)
DHPG	(S)-3,5-dihydroxyphenylglycine
ER	endoplasmic reticulum
FCCP	carbonyl cyanide p-trifluoromethoxy phenyl hydrazone
IP3	inositol trisphosphate
mitoPBRf	PBR modified with GFP derivative
mitoRP	ratiometric pericam targeted to mitochondria
mitoYFP	enhanced yellow fluorescent protein targeted to mitochondria
PBR	peripheral benzodiazepine receptor
PTP	permeability transition pore
TMRM	tetramethyl rhodamine methyl ester
WT	wild-type
$\Delta\psi$	inner membrane potential

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Fig. 1.

Cytosolic and mitochondrial Ca^{2+} responses following stimulation of adult cortical neurons prepared from WT and CyPD-KO mice with ATP and KCl alone. Neurons were perfused with 100 μM ATP or 90 mM KCl for 30 s. Cytosolic and mitochondrial Ca^{2+} levels were reported by fura-FF and mitoRP, respectively. (A) Representative traces of cytosolic Ca^{2+} responses to ATP. (B) Quantification of cytosolic Ca^{2+} levels prior to stimulation (Untreated) and at peak (ATP), $P > 0.05$. (C) Representative traces of mitochondrial Ca^{2+} responses in response to ATP. (D) Quantification of mitochondrial Ca^{2+} levels prior to stimulation (Untreated) and at peak (ATP), $P > 0.05$. (E) Representative traces of cytosolic Ca^{2+} responses to KCl. (F) Quantification of cytosolic Ca^{2+} levels prior to stimulation (Untreated) and at peak (KCl), $P > 0.05$. (G) Representative traces of mitochondrial Ca^{2+} responses to KCl. (H) Quantification of mitochondrial Ca^{2+} levels prior to stimulation (Untreated) and at peak (KCl), $P > 0.05$.

Fig. 2.

Cytosolic and mitochondrial Ca^{2+} responses following simultaneous stimulation with ATP and depolarization in adult cortical neurons prepared from WT and CyPD-KO mice. Neurons were perfused with 100 μM ATP and 90 mM KCl for 30 s. Cytosolic and mitochondrial Ca^{2+} levels were reported by fura-FF and mitoRP, respectively. (A) Representative traces of cytosolic Ca^{2+} responses. (B) Quantification of cytosolic Ca^{2+} retention following ATP and KCl stimulation. Bars represent cytosolic Ca^{2+} levels at 0.9, 1.1 and 1.3 min, at peak, $**P < 0.01$. (C) Representative traces of mitochondrial Ca^{2+} levels. (D) Quantification of mitochondrial Ca^{2+} retention following ATP and KCl stimulation. Bars represent mitochondrial Ca^{2+} levels at 0.7, 0.9, 1.1 and 1.3 min, $**P < 0.01$. (E) Pretreatment with the mitochondrial uncoupler FCCP (450 nm) for 3 min abolishes mitochondrial Ca^{2+} in neurons following perfusion with ATP and KCl. (F) Stimulation with ATP and KCl does not affect neuronal viability in adult cortical neurons prepared from WT and CyPD-KO mice. Neurons were perfused with 100 μM ATP and 90 mM KCl for 30 s and viability was assessed relative to untreated controls 24 h after stimulation. Neuronal viability was assessed as outlined in the Methods. Bars represent the total number of live neurons in five random fields of view per cover slip using a $20\times$ objective, $P > 0.05$.

Fig. 3.

Cytosolic and mitochondrial Ca^{2+} levels, mitochondrial morphology and neuronal viability of adult WT and CyPD-KO neurons following exposure to ionomycin. The responses were reported by fura-FF, mitoRP, TMRM, mitoPBRf and Calcein-AM, respectively. Neurons were perfused with 5 μM ionomycin for 30 s. (A) Representative traces of cytosolic Ca^{2+} responses. (B) Quantification of cytosolic Ca^{2+} levels prior to stimulation (Untreated) and at 0.8 min (Ionomycin), $**P < 0.01$. (C) Representative traces of mitochondrial Ca^{2+} responses. Mitochondrial Ca^{2+} saturation in WT neurons occurred earlier than in CyPD-KO neurons (compare times indicated by line 2), both followed immediately by accumulation of similar maximal levels of Ca^{2+} . (D) Quantification of mitochondrial Ca^{2+} levels prior to stimulation (Untreated) and at point 2 in C (0.8 min, Ionomycin), $**P < 0.01$. (Ei,ii) Uncropped images (magnification 240 $\times$, oil-immersion) of the neuronal soma region containing clusters of mitochondria show differences in the onset of mitochondrial swelling in WT (i) and CyPD-KO (ii) neurons, corresponding to the difference in Ca^{2+} accumulation at point 2 in C. Red outlines represent expanding (i) or normal (ii) outer border of mitochondria using deconvolution, 3D reconstruction and 3D edge detection. Arrows show single mitochondrion with swollen (i) and with normal morphology (ii) in WT and CyPD-KO neurons, respectively. Scale bar = 5 μm . (F) Neuronal viability relative to untreated controls following perfusion with 1 or 5 μM ionomycin for 30 s and viability assessed after 24 h, $**P < 0.01$. Bars represent percentage of viable neurons in random fields of view using a 20 $\times$ objective ($n = 40$ fields of view from eight cover slips per each group).

Fig. 4.

Mitochondrial Ca^{2+} responses following simultaneous stimulation with ATP and depolarization in WT and CyPD-KO adult cortical neurons treated with CsA. Neurons were pretreated with 10 μM CsA for 30 min and then perfused with 100 μM ATP and 90 mM KCl for 30 s. Mitochondrial Ca^{2+} levels were reported by mitoRP. (A) Representative traces of mitochondrial Ca^{2+} responses in WT neurons treated with CsA. (B) Representative traces of mitochondrial Ca^{2+} responses in CyPD-KO neurons treated with CsA. (C) Quantification of mitochondrial Ca^{2+} retention in WT and WT cells treated with CsA following ATP and KCl stimulation. Bars represent mitochondrial Ca^{2+} levels at 0.7, 0.9, 1.1 and 1.3 min, ** $P < 0.01$.

Fig. 5.

Mitochondrial membrane potential in response to simultaneous stimulation with ATP and depolarization, and exposure to ionomycin in adult cortical neurons prepared from adult WT and CyPD-KO mice. Neurons were perfused with 100 μ M ATP and 90 mM KCl for 30 s. Mitochondrial membrane potential was reported by TMRM. (A) Representative traces of $\Delta\psi$ following stimulation with ATP and KCl. (B) Traces demonstrating that $\Delta\psi$ in WT cells returns to baseline following transient stimulation with ATP and KCl (ATP + KCl). (C) Quantification of $\Delta\psi$ at baseline (Untreated) and at 2 min in response to ATP and KCl (ATP + KCl), ** $P < 0.01$. (D) Quantification of $\Delta\psi$ at baseline (Untreated) and at 5 min in response to ATP and KCl (ATP + KCl), $P > 0.05$.