PKA Phosphorylation of NCLX Reverses Mitochondrial Calcium Overload and Depolarization, Promoting Survival of PINK1-Deficient Dopaminergic Neurons

Graphical Abstract

Highlights
- Loss of PINK1 inhibits Ca\(^{2+}\) efflux by NCLX and triggers mitochondrial depolarization
- PKA prevents mitochondrial Ca\(^{2+}\) overload and depolarization by phosphorylating NCLX
- Phosphorylation of NCLX protects against dopaminergic neuron loss

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In Brief
Kostic et al. show that PKA activation induces phosphorylation of a mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger, NCLX. Expression of an NCLX phosphomimetic mutant (NCLX\(^{S258D}\)) prevents mitochondrial Ca\(^{2+}\) overload and depolarization in PINK1-deficient cells, suggesting a protective role of the cAMP/PKA pathway in Parkinson disease caused by loss of PINK1 via NCLX phosphorylation.

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PKA Phosphorylation of NCLX Reverses Mitochondrial Calcium Overload and Depolarization, Promoting Survival of PINK1-Deficient Dopaminergic Neurons

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SUMMARY

Mitochondrial Ca2+ overload is a critical, preceding event in neuronal damage encountered during neurodegenerative and ischemic insults. We found that loss of PTEN-induced putative kinase 1 (PINK1) function, implicated in Parkinson disease, inhibits the mitochondrial Na+/Ca2+ exchanger (NCLX), leading to impaired mitochondrial Ca2+ extrusion. NCLX activity was, however, fully rescued by activation of the protein kinase A (PKA) pathway. We further show that PKA rescues NCLX activity by phosphorylating serine 258, a putative regulatory NCLX site. Remarkably, a constitutively active phosphomimetic mutant of NCLX (NCLXS258D) prevents mitochondrial Ca2+ overload and mitochondrial depolarization in PINK1 knockout neurons, thereby enhancing neuronal survival. Our results identify an mitochondrial Ca2+ transport regulatory pathway that protects against mitochondrial Ca2+ overload. Because mitochondrial Ca2+ dyshomeostasis is a prominent feature of multiple disorders, the link between NCLX and PKA may offer a therapeutic target.

INTRODUCTION

Parkinson disease (PD) is the second most common neurodegenerative disease, characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) (Barbas, 2006; Fahn, 2003). Recent discoveries show that familial forms of PD are exposed to frequent and large Ca2+ loads, due to their autonomous pacing activity that is uniquely dependent on Ca2+ channels (Surmeier et al., 2012). The mCa2+ overload may therefore result from inability of the mCa2+ shuttling system to handle these loads (Chan et al., 2007). The mCa2+ transients in neurons are mediated by two transporters: the mitochondrial calcium uniporter (MCU), which mediates mCa2+ influx, and the mitochondrial Na+/Ca2+ exchanger (NCX), which mediates mCa2+ efflux (Baughman et al., 2011; De Stefani et al., 2011; Palty et al., 2010). We have recently identified the mitochondrial Na+/Ca2+ exchanger (NCX) family of transporters that share a common catalytic core composed of α1 and α2 repeating domains (Nicoll et al., 2013; Palty et al., 2004, 2010). However, it differs markedly in the regulatory domain region, which, in contrast to other NCX members, is much shorter and lacks allosteric Ca2+-binding domains (Cai and Lytton, 2004). The mCa2+ influx by NCX is much slower than the MCU-mediated mCa2+ influx (Drago et al., 2012). Thus, NCX is the rate-limiting system in controlling mCa2+ surges (Palty et al., 2010). The profound inhibitory effect of PINK1 deficiency on mCa2+ removal suggests that in PD the capacity of the mitochondrial exchanger to remove mCa2+ is impaired. However, it is unknown whether the effects on mCa2+ transients are mediated through direct interaction of PINK1 (PINK1) function (Gandhi et al., 2012). PINK1 is a serine/threonine kinase localized to mitochondria that exerts a neuroprotective function, and its expression has been shown to be a Ca2+-dependent process (Gómez-Sánchez et al., 2014). Loss-of-function mutations of PINK1 result in a series of mitochondrial abnormalities implicated in the etiopathology and progression of early-onset familial PD. These abnormalities include partial mitochondrial depolarization, increased oxidative stress, and mitochondrial fusion and fission defects (Valente et al., 2004; Wood-Kaczmar et al., 2008).
with NCLX or via an indirect phenomenon, such as modulation of the mCa²⁺ influx machinery. Furthermore, it is uncertain whether impaired mCa²⁺ handling and the resulting mitochondrial depolarization and neuronal death encountered with PINK1 mutations can be rescued by other signaling pathways, such as the protein kinase A (PKA) pathway, which shows diminished activity in PINK1-deficient neuronal cells (Dagda et al., 2014).

Numerous studies support a major role of the cyclic AMP (cAMP)/PKA signaling cascade in modulating mitochondrial functions such as apoptosis, mitochondrial respiration, and ATP production (Acin-Perez et al., 2009; Martin et al., 2005; Technikova-Dobrova et al., 2001). Cyclic AMP produced by plasma membrane adenyl cyclase can diffuse throughout the cell to set up localized gradients in subcellular organelles, including mitochondria (DiPilato et al., 2004). In addition, cAMP can be produced directly in the mitochondrial matrix by a soluble adenyl cyclase (Chen et al., 2000). The cAMP is postulated to activate PKA, which is detected in different mitochondrial compartments (Valsacchi et al., 2013). Interestingly, PKA exhibits a prosurvival effect in PINK1-deficient cells, which is due in part to the regulation of the mitochondrial fission protein Drp1 (Dagda et al., 2011). It is, however, unknown whether the prosurvival effects of PKA are linked to modulation of mCa²⁺ signaling. Highly coordinated interplay between cAMP and Ca²⁺ signaling, which has been demonstrated in mitochondria (Di Benedetto et al., 2013), suggests the involvement of PKA in the regulation of mCa²⁺ homeostasis.

In this study, we demonstrate that NCLX activity, impaired by PINK1 deficiency, is rescued by PKA. We show that mCa²⁺ efflux occurs via direct phosphorylation of NCLX by PKA, revealing a regulatory mode of mitochondrial Na⁺/Ca²⁺ exchange. Finally, we find that modulation of the NCLX phosphorylation site by PKA is essential and sufficient to protect PINK1-deficient neurons from mitochondrial depolarization and dopamine-induced cell death, underscoring the role of the exchanger and its regulation via PKA in the pathogenesis of PD and potentially in other neurodegenerative disorders of similar Ca²⁺-dependent pathophysiology.

**RESULTS**

**PINK1 Deficiency Causes Partial Mitochondrial Depolarization and Inhibition of mCa²⁺ Efflux**

Previous studies suggested that PINK1 mutations linked to PD lead to a partial decrease in mitochondrial membrane potential (∆Ψm) (Abramov et al., 2011; Gandhi et al., 2009). Another prominent feature of these mutations is susceptibility to mCa²⁺ overload—a hallmark event in PD as well as other neurodegenerative and ischemic diseases (Cherra et al., 2013; Surmeier and Schumacker, 2013). We hypothesized that the pathological rise in mCa²⁺ is specifically linked to impaired Ca²⁺ efflux by the mitochondrial Na⁺/Ca²⁺ exchanger (Gandhi et al., 2009), whose molecular identity was only recently revealed (Palty et al., 2010).

We first asked whether a knockdown of PINK1 in previously characterized human neuroblastoma shPINK1 SH-SY5Y cells (Dagda et al., 2009) mirrors the lowered ∆Ψm and impaired mCa²⁺ efflux, as previously reported (Abramov et al., 2011; Gandhi et al., 2009). ∆Ψm was recorded using the potentiometric fluorescent dye tetracyanoethylene methyl ester (TMRE), where a complete depolarization can be induced by addition of the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Basal ∆Ψm in shPINK1 versus control cells was determined by comparing the ∆Ψm before and after the application of FCCP. Consistent with previous findings, we recorded a significant reduction in basal ∆Ψm in stable shPINK1 SH-SY5Y cells compared to control cells expressing a nontargeting control plasmid (64% ± 11% of control) (Figures 1A and 1B).

We next compared transient mCa²⁺ responses in shPINK1 SH-SY5Y to those in control cells by using the mCa²⁺ fluorescent dye Rhod-2 AM. Application of the P2Y receptor activator, ATP, triggered cytosolic followed by mCa²⁺ transients (Palty et al., 2010) (Figure 1C). Consistent with a drop in ∆Ψm—the major driving force for mCa²⁺ uptake via MCU—the rate of mCa²⁺ influx was reduced by ~2-fold in shPINK1 cells in comparison to control cells (Figures 1C and 1D). However, the amplitude of the mCa²⁺ influx phase remained unchanged (Figures 1C and 1E), showing that, although conducted at a slightly slower rate, the capacity of mitochondria to take up Ca²⁺ remains intact. In addition, no significant difference was observed in basal mCa²⁺ levels between control and PINK1-deficient cells (Figure S1).

A much stronger effect of PINK1 knockdown was, however, observed on the mCa²⁺ efflux phase, as it triggered an ~3.5-fold decrease in mCa²⁺ efflux rate compared to control (Figures 1C and 1F). Moreover, this reduction in mCa²⁺ efflux rate was coupled to an ~4-fold reduction in the amplitude of the mCa²⁺ efflux phase (Figures 1C and 1G). Hence, whereas Ca²⁺ efflux fully recovered the mCa²⁺ to resting levels in control cells, mitochondria of PINK1-deficient cells, in contrast, failed to effectively extrude Ca²⁺ and it remained elevated above resting levels. Taken together, our results, consistent with previous studies, suggested that PINK1 deficiency reduces mCa²⁺ uptake (Gandhi et al., 2009; Heeman et al., 2011) but has a larger effect on both the rate and amplitude of the mCa²⁺ efflux phase, thereby triggering a net reduction in mCa²⁺ extrusion.

We next determined whether PINK1 knockdown caused changes in expression of NCLX in shPINK1 versus control cells. Immunoblot analysis of shPINK1 cells showed similar levels of NCLX expression but is most likely due to inhibition of NCLX in NCLX expression but is most likely due to inhibition of NCLX—binding or phosphorylation sites on NCLX. Bioinformatic analysis that we conducted using the Scan site algorithm (Obenauer et al., 2003), however, did not identify potential PINK1 binding or phosphorylation sites on NCLX. Consistent with this analysis and previous study that did not identify NCLX interaction with PINK1 (Rakovic et al., 2011), immunoprecipitation of c-myc-tagged NCLX followed by mass spectrometry (MS) analysis did not identify a pull-down of PINK1 by NCLX (Table S1), arguing against a direct interaction between these two proteins.
PKA Activation Rescues \( \Delta \Psi \) and Mitochondrial Membrane Depolarization Triggered by PINK1 Deficiency

We next sought to determine whether we could rescue \( \Delta \Psi \) and mitochondrial membrane depolarization in PINK1-deficient SH-SY5Y cells. Because the above results do not support a direct interaction of PINK1 and NCLX, we reasoned that such rescue of NCLX activity may be mediated by another signaling pathway. Among the potential signaling pathways, the PKA pathway emerged as a leading candidate able to rescue several parameters of PINK1 deficiency, most notably \( \Delta \Psi \) (Dagda et al., 2011). To determine whether PKA regulates NCLX activity, we monitored \( \Delta \Psi \) in PINK1-deficient SH-SY5Y cells versus control cells that were pre-exposed to the PKA agonist forskolin. Application of forskolin led to a full recovery of \( \Delta \Psi \) in shPINK1 cells up to the control values, but was not followed by a change in the \( \Delta \Psi \) of control cells (Figures 2A and 2B). Consistent with the role of \( \Delta \Psi \) in controlling the driving force for \( \Delta \Psi \), forskolin rescued \( \Delta \Psi \) by PKA activation was followed by recovery of \( \Delta \Psi \) rates in shPINK1 SH-SY5Y cells (Figures 2C and 2D). Importantly, activation of PKA further led to a much stronger, \( \sim 6 \)-fold increase in \( \Delta \Psi \) efflux rate in shPINK1 SH-SY5Y cells, thereby fully restoring \( \Delta \Psi \) efflux to rates monitored in control cells (Figures 2C and 2E). We also found that application of forskolin could enhance, to a lesser extent, by \( \sim 2 \)-fold, the rate of \( \Delta \Psi \) efflux but not \( \Delta \Psi \) influx of control cells, thus supporting a role of PKA in regulating NCLX rather than MCU (Figures 2D and 2E). To further ascertain that forskolin acts via PKA, cells were cotreated with forskolin and the PKA inhibitor H89. Note that their coapplication completely abolished \( \Delta \Psi \) efflux activation, both in shPINK1 and control cells (Figures 2D and 2E), lending further support that the effect of forskolin on NCLX is mediated via PKA.

Serine 258 Is the Putative PKA Phosphorylation Site of NCLX

PKA can regulate NCLX indirectly through other signaling pathways or by direct phosphorylation of the exchanger. To determine whether PKA acts directly on NCLX, we screened for putative PKA phosphorylation sites employing the Scansite 3 algorithm. A high score of probability was given to serine residue 258 (S258). The potential of S258 to undergo PKA-dependent phosphorylation was further supported by NetPhosK 1.0 (Blom et al., 2004) and pkaPS analysis (Neuberger et al., 2007), which indicated that S258 is part of a PKA signature sequence, RRXSY (where Y is a hydrophobic amino acid, leucine in this case). Figure 3A shows the predicted transmembrane topology model of NCLX depicting the putative S258 PKA phosphorylation site. This putative phosphorylation site is located within the hydrophilic loop of the exchanger, which in other NCX superfamilies
members serves as the major regulatory site responding to allo-sterically bound Ca\(^{2+}\) (the so-called CBD1 and CBD 2) (Nicoll et al., 2013). NCLX is devoid of these Ca\(^{2+}\) binding sites, but has instead the putative PKA phosphorylation site at this domain. To examine whether S258 indeed undergoes phosphorylation, and whether it is phosphorylated in a PKA-dependent manner, we employed MS analysis of c-myc-tagged NCLX treated with forskolin, with or without H89, as described in Figure 2. MS analysis identified phosphorylation of S258 in forskolin-treated but not H89-cotreated NCLX (Figure 3B). Furthermore, in vitro analysis of immunopurified NCLX revealed that S258 is phosphorylated in the presence of the PKA catalytic subunit as well, supporting direct phosphorylation of NCLX by PKA (Figure 3B).

To further determine whether phosphorylation at S258 could explain the functional effects of PKA on NCLX activity, we generated NCLXS258A and NCLXS258D mutants to mimic phosphorylation-deficient and constitutively phosphorylated residues, respectively. To ascertain that the mutation did not affect the expression or localization of NCLX, we employed immunoblot analysis of expression in total and mitochondrial cell fractions. As shown in Figure 3C, the expression and localization of the mutants were similar to that of NCLXWT.

We next monitored m\(\text{Ca}^{2+}\) efflux activity of the NCLX mutants versus NCLXWT by applying a silencing-rescue paradigm (Palty et al., 2010) of knocking down endogenous NCLX expression while expressing NCLX constructs in cells coexpressing the m\(\text{Ca}^{2+}\) sensor mito-Pericam. Consistent with our previous studies (Palty et al., 2010), silencing of endogenous NCLX expression in HEK293T cells, using small hairpin RNA (shRNA) targeted against the 3\(^{\text{UTR}}\) of NCLX, was followed by an \(\left(\text{2.5-fold}\right)\) reduction in m\(\text{Ca}^{2+}\) efflux rates compared to cells transfected with control shRNA, which was fully rescued by overexpression of NCLXWT (Figure S2A).

Similar to NCLXWT, phosphomimicking mutant NCLXS258D was able to fully rescue m\(\text{Ca}^{2+}\) efflux in shNCLX-treated cells. On the contrary, phosphodeficient mutant NCLXS258A failed to upregulate m\(\text{Ca}^{2+}\) efflux, with an \(\left(\text{3.5-fold}\right)\) decrease in activity compared to NCLXWT (Figures 3D and 3E). In addition, no difference in basal m\(\text{Ca}^{2+}\) levels was observed between the mutants and NCLXWT (Figure S2B).

The difference in activity between NCLX\(^{\text{S258A}}\) and NCLX\(^{\text{S258D}}\) mutants was further confirmed, applying the same silencing-rescue paradigm (Palty et al., 2010), in digitonin-permeabilized HEK293T cells. The mitochondria of permeabilized HEK293T cells transiently expressing mito-Pericam and shNCLX, alone or in a combination with NCLX constructs, were loaded with Ca\(^{2+}\), and Ca\(^{2+}\) efflux was initiated by addition of Na\(^{+}\), in the presence of the MCU blocker Ruthenium red, as previously
described (Palty et al., 2010). Consistent with our finding in intact cells, silencing of endogenous NCLX profoundly inhibited the Na+-dependent Ca2+ efflux, which was fully rescued by overexpression of either NCLX S258A or NCLX S258D (Figures 3F and 3G). In contrast, the NCLX S258A mutant, although demonstrating some residual activity, was inhibited by more than 2-fold compared to either NCLX S258D or NCLX WT (Figures 3F and 3G).

Taken together, these results indicate that phosphorylation at S258 mediates the effects of PKA on nCa2+ efflux.

**Figure 3. PKA Activates nCa2+ Efflux through a Phosphorylation Site on NCLX**

(A) The putative transmembrane topology model of NCLX obtained using TopPred (von Heijne, 1992). NCLX contains an N-terminal mitochondrial targeting sequence and two conserved catalytic transmembrane domains, α1 and α2. The putative PKA phosphorylation site Ser258 is located in the hydrophilic loop of the exchanger and is part of the PKA consensus sequence.

(B) Collision-induced dissociation MS/MS spectrum of the phosphorylated NCLX peptide R256RGpSLFCMPVTPSPI5DSEEDR (where p indicates phosphorylation of S258) found only in c-myc-tagged NCLX isolated from cells treated with the PKA activator forskolin (middle panel) or following direct in vitro incubation with a recombinant PKA catalytic subunit (PKA cα) (lower panel) but not in the presence of the PKA inhibitor H89 (upper panel). m/z, mass-to-charge ratio. b, y, and n denote N- and C-terminal fragments, respectively. Fragment ions that contain a mass shift as a result of phosphorylation are marked in red.

(C) Immunoblot analysis of NCLX expression in total lysate (T) and isolated mitochondria (M) in HEK293T cells transfected with either NCLX WT-, NCLX S258A-, or NCLX S258D-coding plasmids or empty vector (pcDNA3.1+). COXIV-1 was used as a mitochondrial marker. NCLX is identified as a 100-kDa dimer in the mitochondrial fraction. Similar to NCLX WT, overexpression of the constructs NCLX S258A and NCLX S258D results in mitochondrial targeted expression, and NCLX expression is not affected by the mutation.

(D) Representative fluorescence traces of nCa2+ responses upon application of ATP to HEK293T cells transiently expressing the nCa2+ sensor mito-Pericam, NCLX shRNA, and NCLX WT or mutant NCLX (NCLX S258A or NCLX S258D).

(E) Quantification of nCa2+ efflux rates of (D). NCLX WT (n = 10) and NCLX S258D (n = 10) are ~3.5- and ~3-fold faster than NCLX S258A (n = 11), respectively.

(F) Representative fluorescence traces of Na+-dependent nCa2+ efflux in digitonin-permeabilized HEK293T cells transiently expressing the nCa2+ sensor mito-Pericam, NCLX shRNA, and NCLX WT or mutant NCLX (NCLX S258A or NCLX S258D).

(G) Quantification of nCa2+ efflux rates of (F). Similar to Figure 3E, NCLX S258D (n = 3) and NCLX WT (n = 3) are more than 2-fold faster than NCLX S258A (n = 4). All bar graph data represent mean ± SEM. *p < 0.05, **p < 0.01. See also Figure S2 and Table S1.
Expression of Phosphomimetic NCLX<sup>S258D</sup>, but Not Phosphodeficient NCLX<sup>S258A</sup>, Suppresses the Inhibitory Effect of PINK1 Deficiency on mCa<sup>2+</sup> Efflux

If the S258 residue is the critical site regulated by PKA, then expressing the constitutively active NCLX<sup>S258D</sup> or the inactive NCLX<sup>S258A</sup> mutant is expected to eliminate, in both cases, the responsiveness of mitochondria that we observed for the endogenous NCLX (Figures 2C and 2E). Consistent with previous findings, we confirmed that NCLX<sup>WT</sup> is responsive to PKA activation and activates by ~2-fold when treated with forskolin (Figures 4A and 4B). On the other hand, activity of NCLX<sup>S258D</sup> in the presence of forskolin remained low and that of NCLX<sup>S258D</sup> was constitutively high, in agreement with their inactivation and activation phenotypes, respectively (Figures 4A and 4B).

If phosphorylation of NCLX at the PKA site, S258, is required for activation of mCa<sup>2+</sup> efflux, NCLX<sup>S258D</sup> should remain activated compared to NCLX<sup>S258A</sup> when expressed in shPINK1 SH-SY5Y cells. Indeed, mCa<sup>2+</sup> efflux rates of shPINK1 SH-SY5Y cells expressing phosphomimetic mutant NCLX<sup>S258D</sup> were almost 2-fold higher than the rates recorded in cells expressing phosphodeficient NCLX<sup>S258A</sup> (Figures 4C and 4D). Note that NCLX<sup>WT</sup> was also active in shPINK1 cells, indicating that the residual PKA activity observed in these cells is sufficient to activate NCLX, consistent with the critical role of intact S258 for its activation, and further showing that higher heterologous expression of NCLX<sup>WT</sup> can compensate for lesser PKA activity. Thus, modulation of the S258 site is essential and sufficient to fully recover mCa<sup>2+</sup> efflux rates from the inhibitory effect observed in PINK1-deficient SH-SY5Y cells.

NCLX Phosphomimetics Also Rescues mCa<sup>2+</sup> Overload in PINK1 Knockout Neurons

To determine the effect of the NCLX mutants in PINK1 knockout (KO) mouse neurons, intact neurons were loaded with the Ca<sup>2+</sup> indicator X-Rhod-1 and mCa<sup>2+</sup> was assessed upon provision of a physiological stimulus (Figure 5A). It should be noted that X-Rhod-1 labels mitochondrial as well as cytosolic Ca<sup>2+</sup>. However, only mitochondrial areas were selected for analysis (Figure S3). We stimulated either WT or PINK1 KO neurons with 1 μM glutamate, which caused a transient increase in cytosolic Ca<sup>2+</sup>, followed by changes in mCa<sup>2+</sup>, as mitochondria buffer this surge in cytosolic Ca<sup>2+</sup> (Figure 5B). PINK1 KO cells displayed a markedly reduced mCa<sup>2+</sup> efflux rate, by ~10-fold compared to WT neurons (Figures 5C and 5D). However, mitochondria of PINK1 KO cells overexpressing NCLX<sup>S258D</sup> and NCLX<sup>WT</sup> displayed faster Ca<sup>2+</sup> exclusion rates, similar to those observed in WT neurons (Figures 5C and 5D). In contrast, overexpression of NCLX<sup>S258A</sup> failed to rescue the aberrant mCa<sup>2+</sup> efflux in PINK1 KO neurons (Figures 5C and 5D).

To eliminate any differences in mCa<sup>2+</sup> handling due to varying cytosolic Ca<sup>2+</sup> responses, cells were permeabilized in pseudo-tracellular medium after X-Rhod-1 loading. Permeabilization of neurons allowed for precise control over Ca<sup>2+</sup> concentrations outside of the mitochondria and direct measurement of mCa<sup>2+</sup> handling (Figure 5E). Application of 5 μM Ca<sup>2+</sup> led to a transient increase and exclusion of mCa<sup>2+</sup> within 0.5–1 min in WT cells (Figures 5F and 5G). In agreement with previous data (Abramov et al., 2011; Gandhi et al., 2009), deletion of PINK1 led to altered mCa<sup>2+</sup> efflux, mirroring that observed in whole-cell experiments (Figures 5F and 5G). Similar to our results in intact PINK1 KO neurons, overexpression of NCLX<sup>S258D</sup> or NCLX<sup>WT</sup> rescued the mCa<sup>2+</sup> efflux in permeabilized neurons when compared to either PINK1 KO or PINK1 KO NCLX<sup>S258A</sup>-expressing cells (Figures 5F and 5G).

Mitochondrial Membrane Potential Is Rescued through Overexpression of NCLX<sup>S258D</sup> but Not of the NCLX<sup>S258A</sup> Mutant

Normalization of ΔΨm in PINK1 KO cells by pyruvate and succinate did not change the inhibited Na<sup>+</sup>/Ca<sup>2+</sup> exchange in these cells (Gandhi et al., 2009), suggesting that it is not the most upstream mechanism by which PINK1 deficiency affects mCa<sup>2+</sup> efflux. Because excess mitochondrial matrix Ca<sup>2+</sup> can trigger the loss of membrane potential (Vergun and Reynolds, 2005), we studied whether a recovery of mCa<sup>2+</sup> efflux via PKA-mediated activation of NCLX could lead to a rescue of ΔΨm in PINK1 KO neurons.

As expected, PINK1 KO neurons possessed significantly reduced ΔΨm (77% ± 5% of WT control) (Figure 6A). We first checked whether PKA activation by forskolin can suppress this...
reduction in ΔΨm. Similar to the effect observed in PINK1-deficient SH-SY5Y cells (Figures 2A and 2B), we observed a full rescue of ΔΨm in PINK1 KO neurons in the presence of forskolin (Figure 6A).

We then asked whether expression of phosphomimetic NCLXS258D or NCLXWT could rescue ΔΨm in PINK1 KO neurons. Notably, overexpression of NCLXS258D and NCLXWT restored ΔΨm in PINK1 KO cells back to its level in WT neurons (104% ± 8% and 110% ± 12% of WT control, respectively) (Figure 6B). In contrast, NCLXS258A expression in PINK1 KO failed to restore the ΔΨm (72% ± 5% of WT control) (Figure 6B).

**DISCUSSION**

Loss-of-function mutations of PINK1, a mitochondrially targeted serine/threonine kinase, are linked to recessively inherited PD (Gandhi et al., 2009; Valente et al., 2004). A major hallmark of PINK1 deficiency in neurons is an ΔΨm overload, which leads to neuronal cell death triggered by the opening of mPTP (Abramov et al., 2011; Akundu et al., 2011; Gandhi et al., 2009; Gautier et al., 2008). Previous findings suggested that the ΔΨm over-load related to the loss of PINK1 occurs due to specific inhibition of ΔΨm efflux from dopaminergic neurons (Gandhi et al., 2009). The prevailing route of ΔΨm efflux from neuronal mitochondria is its exchange with Na+ via the mitochondrial Na+/Ca2+ exchanger NCLX (Gunter et al., 2000). Loss of PINK1, however, strongly...
inhibits exchange activity, as neither mCa\(^{2+}\) removal nor concomitant mitochondrial Na\(^+\) influx is observed upon triggering mCa\(^{2+}\) influx in PINK1 KO neurons (Gandhi et al., 2009). Here we confirm that the mCa\(^{2+}\) efflux is significantly reduced in PINK1 KO mouse dopaminergic neurons and, furthermore, we demonstrate a similar inhibition of mCa\(^{2+}\) efflux by stably knocking down PINK1 in a human neuroblastoma cell line (SH-SYSY). The inhibition of mCa\(^{2+}\) efflux could be mediated either by a direct interaction/phosphorylation of PINK1 with NCLX or by an indirect effect of PINK1 loss that communicates with NCLX through a distinct pathway (Figure 7). The following results argue against a direct interaction of NCLX and PINK1: (1) bioinformatic analysis failed to identify any PINK1 phosphorylation site on NCLX; (2) proteomic analysis of PINK1-interacting proteins found 14 candidates, but NCLX was not among them (Rakovic et al., 2011); and (3) our MS analysis of overexpressed NCLX failed to identify interaction with PINK1 or phosphorylation of NCLX related to this kinase (Table S1).

Recent studies, however, indicate that loss of PINK1 leads to PKA inhibition (Dagda et al., 2014). We show that PKA strongly modulates mCa\(^{2+}\) efflux. This effect can be mediated indirectly or by PKA-dependent phosphorylation of NCLX. The following results support the latter mechanism. (1) The inclusion of a PKA inhibitor abolished the forskolin-dependent upregulation of NCLX activity. Our findings are consistent with early studies demonstrating a cAMP-dependent upregulation of Na\(^{+}\)-dependent mCa\(^{2+}\) efflux (Goldstone and Crompton, 1982). (2) NCLX harbors a potential PKA phosphorylation site at S258, which we now show by MS analysis is phosphorylated by activation of PKA in cells and directly by PKA in an in vitro purified NCLX preparation (Figure 3B). Importantly, when S258 is replaced by aspartate (NCLX\(^{S258D}\)), mCa\(^{2+}\) efflux by NCLX is enhanced, whereas changing it to alanine (NCLX\(^{S258A}\)) downregulates mCa\(^{2+}\) efflux. (3) The activity of NCLX\(^{S258D}\) and NCLX\(^{S258A}\) is nonresponsive to the PKA agonist, indicating that S258 is indeed the target site for PKA on NCLX (Figure 7). The S258 residue of NCLX is described here as a regulatory switch in this exchanger, and phosphorylation at this site represents a mode of regulation for mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange. Furthermore, our results indicate that mimicking phosphorylation of S258 plays a key role in preventing mCa\(^{2+}\) overload in several injury paradigms, and may therefore be an attractive therapeutic target. Notably, overexpression of NCLX\(^{WT}\) can also overcome the downregulation of NCLX activity in PINK1-deficient cells. This finding is consistent with the partial inhibition of PKA following PINK1 knockdown (Dagda et al., 2014). Thus, overexpression of NCLX\(^{WT}\) may increase the availability of the regulatory site to PKA. Indeed, overexpression of the inactive NCLX\(^{S258A}\) failed to produce a similar activation, strongly supporting the regulatory importance of this site (Figure 7).

Plasma membrane NCXs possess a regulatory mechanism involving cytosolic Ca\(^{2+}\) that is well defined. NCX has a long intracellular loop that carries two Ca\(^{2+}\)-binding domains (CBD1 and CBD2) that strongly and allosterically regulate NCX following changes in cytosolic Ca\(^{2+}\) (Nicoll et al., 2013). However, the homologous hydrophilic loop of NCLX is much shorter and lacks any obvious Ca\(^{2+}\)-binding domains. In contrast, it possesses the PKA phosphorylation site defined in the current study. Our results therefore indicate that NCLX has an entirely different mode of regulation compared to the plasma membrane NCXs—it is based on phosphorylation, not on allosteric Ca\(^{2+}\) binding.

Two distinct pools of cAMP/PKA signaling mediators are capable of reaching and regulating mitochondria. The first is associated with cytosolic cAMP that is produced by cell membrane adenyl cyclase, which can activate PKA, allowing it to translocate to the outer mitochondrial membrane and intermembrane space (Lefkimmiatis et al., 2013). The second is generated
by a soluble adenylyl cyclase that is solely contained in the mito-
genial matrix (Acin-Perez et al., 2009; Di Benedetto et al., 2013). Although the sensitivity of cytosolic PKA to forskolin sug-
gests its role in NCLX regulation, further studies are required to
determine the subcellular origin of PKA species that participate
in NCLX regulation.

Another distinctive aspect of PINK1 deficiency in neurons is a
partial mitochondrial depolarization, which significantly contrib-
utes to the increased neuronal death related to PINK1 loss (Abra-
mov et al., 2011; Dagda et al., 2011). The reduced ΔΨm in PINK1
KO neurons can be restored by provision of substrates of mito-
genrial respiratory chain complexes I and II. However, this
rescue of ΔΨm does not recover mCa2+ efflux deficits, suggest-
ing that the inhibition of the exchanger is upstream of the mito-
genrial depolarization. Here we demonstrate that mCa2+ efflux
can be fully reconstituted by expression of constitutively active
mutant NCLX S258D, which furthermore fully rescues ΔΨm in
PINK1 KO neurons. These results imply that the impairment of
mCa2+ shuffling is due to diminished NCLX activity encountered
in PINK1 deficiency, which in turn contributes, at least in part, to
the mitochondrial depolarization.

Interestingly, we previously found that PINK1-deficient cells
exhibit reduced PKA signaling (Dagda et al., 2014). Although it
possible that the two pathways operate in parallel, PINK1 and PKA exhibit features of a positive amplifying interaction.
Loss of PINK1 selectively suppresses the ability of cAMP, but
not retinoic acid, to induce neuronal differentiation (Dagda et al., 2014). This is accompanied by decreased indices of PKA
activity (CRE-luciferase and CREB phosphorylation) in PINK1
shRNA cells (Dagda et al., 2014). Conversely, PINK1 overexpres-
sion elevates CRE activity and enhances neurite outgrowth;
these effects of PINK1 are inhibited by H89 or expression of a mi-
 tochondrially targeted peptide inhibitor of PKA (Dagda et al.,
2014). These data suggest that PINK1 is necessary for full activ-
ity of PKA at the mitochondrion. Thus, the reduction in NCLX ac-
tivity observed in PINK1-deficient cells and neurons could be
due to reduced PKA activation in these cells (Figure 7). Although
it is possible that PINK1 deficiency also affects other parallel
pathways, our current results clearly indicate that PKA-mediated
upregulation of NCLX activity can break these vicious cycles in
PINK1-deficient neurons to rescue not only the mCa2+ overload
but also the ΔΨm (Figure 7).

One would expect that by rescuing both mCa2+ efflux and
ΔΨm, expression of the constitutively active exchanger
NCLX S258D would result in reduced neuronal cell death related
to PINK1 deficiency. Indeed, we recorded a significant increase
in neuronal survival rate upon expression of NCLX S258D or
NCLX WT, but not NCLX S258A, implying that modulation of the
NCLX phosphorylation site by PKA activation is essential and
sufficient to protect PINK1-deficient neurons from dopamine-
induced cell death. Taken together, our results establish NCLX
as the major player implicated in several mitochondrial defects
related to PD caused by PINK1 impairment.

Mitochondria play a major role in the regulation of cellular Ca2+
homeostasis (De Smedt et al., 2011). Impairment of mCa2+ shut-
gling results in mCa2+ overload similar to that related to PINK1
deficiency, which is implicated in a range of neurodegenerative
and ischemic diseases. This notion further highlights the impor-
tance of the exchanger and the regulatory mechanism via PKA
described in this paper. Under physiological conditions in the
steady state, NCLX and MCU Ca2+ transport must be in balance.
Under pathophysiological conditions, however, mCa2+ overload
can occur either as a result of increased mCa2+ uptake via
MCU or reduced mCa2+ removal via NCLX, as observed in
PINK1-deficient cells (Gandhi et al., 2009). In the latter case, ac-
tivation of NCLX via the PKA regulatory pathway leads to a recov-
er of impaired mCa2+ handling and neuronal rescue (Figure 7),
which opens new avenues for future targeted therapeutic strate-
gies for these disorders.

**EXPERIMENTAL PROCEDURES**

**Mice**

Wild-type and PINK1 KO mice were from breeding colonies generated by Lexicon Genetics. Animal breeding and maintenance were in accordance with the regulations described in UK Animals (Scientific Procedures) Act 1986.
Cell Culture and Transfection
HEK293T cells were as previously described (Palty et al., 2004). Stable PINK1 shRNA knockdown SH-SY5Y cell lines were generated and cultured as previously described (Dagda et al., 2009). Primary midbrain mouse neurons were isolated and cultured as previously described (Gandhi et al., 2009). Transfection of HEK293T cells was performed using the calcium phosphate precipitation protocol as previously described (Palty et al., 2004). SH-SY5Y cells and primary neuronal cells were transfected using Lipofectamine 2000 (Invitrogen) and Effectene (Qiagen), respectively, according to the manufacturers’ protocols.

Immunoblot Analysis
NCLX and PINK1 immunoblotting was performed as previously reported (Palty et al., 2010). For specific determination of NCLX expression in mitochondria, cell fractionation was performed as previously described (Bozidis et al., 2007). Immunoblot analysis was performed as described previously (Palty et al., 2010) using the following antibodies: custom-made antibody against NCLX (Palty et al., 2004; 1:1,000), rabbit polyclonal anti-PINK1 antibody (Abcam; 1:200), anti-β-actin (Sigma; 1:20,000), and anti-cytochrome c oxidase (COXIV-1) antibody (Santa Cruz Biotechnology; 1:100).

Immunoprecipitation and MS Analysis
HEK293T cells were transfected with a c-myc-tagged human NCLX-encoding plasmid. Forty-eight hours posttransfection, cells were incubated with forskolin or cotreated with H89 and forskolin and then lysed in the presence of protease inhibitors (Sigma) and phosphatase inhibitors (PhosSTOP; Roche), and the lysate was immunoprecipitated with anti-c-myc agarose beads (Pierce Antibody C-Myc agarose; Thermo Scientific) using the manufacturer’s protocol. On-bead tryptic proteolysis and MS were performed as previously described (Fukuyama et al., 2012).

Fluorescent Ca2+ and ΔΨm Imaging
In HEK293T cells, the Ca2+ levels were monitored by transiently expressed mitochondrially targeted ratiometric Pericam (mito-Pericam) as previously described (Palty et al., 2010). The Ca2+ measurement in SH-SY5Y cells was performed using Rhod-2 AM fluorescent Ca2+ dye as previously described (Jaiswal et al., 2009). Changes in inner ΔΨm were monitored using TMRM as previously described (Nita et al., 2012).

Confocal Imaging
Confocal images of X-Rhod-1-loaded neurons were obtained as previously described (Gandhi et al., 2009).

Toxicity Experiments
For toxicity assays, we loaded cells simultaneously with 20 μM propidium iodide, which is excluded from viable cells but exhibits a red fluorescence following a loss of membrane integrity, and 4.5 μM Hoechst 33342 (Molecular Probes), which gives a blue staining to chromatin, to count the total number of cells.

Statistical Analysis
All results are presented as mean ± SEM. Statistical significance was determined using t test or one-way ANOVA test followed by Tukey post hoc analysis. Values of p < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.079.

AUTHOR CONTRIBUTIONS
M.H., C.T.C., I.S., A.Y.A., H.B., M.K., and M.H.R.L. wrote the manuscript; M.K., M.H.R.L., and E.S. performed the experiments; I.S., A.Y.A., C.T.C., and M.H. provided experimental oversight; I.S., A.Y.A., C.T.C., M.K., and M.H.R.L. interpreted the data.

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