Regulation of mitochondrial calcium efflux in Parkinson's disease

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Abstract

Multiple factors are involved in the mechanism(s) of neuronal loss in neurodegenerative disorders whilst mitochondria are thought to play a central role in neurodegeneration of Parkinson's disease. Mitochondria are vital to cellular functions by supplying energy in form of ATP and affect cell physiology via calcium, ROS and signalling proteins. Changes in mitochondrial calcium homeostasis and ROS overproduction can induce cell death by triggering mitochondrial permeability transition pore opening. One of the major triggers for PTP is mitochondrial calcium overload. Mitochondrial calcium homeostasis is regulated by electrogenic calcium uptake (via calcium uniporter MCU) and efflux (in excitable cells via Na^{+}/Ca^{2+} exchanger NCLX). NCLX inhibition has been described in a familial form of Parkinson's disease where PINK-1 deficiency leads to a delayed calcium efflux and mitochondrial calcium overload in response to physiological calcium stimulation. Overexpression of NCLX in PINK-1 deficient neurons not only protects against mitochondrial calcium overload and calcium induced cell death but also restores mitochondrial bioenergetics in these neurons. Mitochondrial NCLX might therefore play an important role in the mechanism(s) of neurodegeneration in a variety of neurodegenerative disorders and activation of this exchanger may offer a novel therapeutic target.

Introduction

The most common neurodegenerative disorders – Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive and incurable diseases affecting elderly people. Considering the ageing population worldwide, this represents a serious cost to society. Many years after

these diseases were first described, much has been learnt about the pathology and pathogenesis of the disease, but a number of gaps in our understanding remain. Only by understanding the pathogenic mechanisms that underlie PD and AD, can therapeutic strategies be designed to halt or slow disease progression, rather than merely treat the symptoms. It is widely recognized that the loss of mitochondrial function is a key event leading to necrotic and apoptotic cell death under a wide range of pathological conditions (Burchell et al., 2013). Calcium signalling is fundamentally important to neuronal and glial cells and might represent either a mediator or a manifestation of pathological processes in the CNS (Abeti and Abramov, 2015; Angelova and Abramov, 2014). Calcium ions control and coordinate a diverse array of physiological functions within the cell such as muscle contraction, proliferation and neurotransmission. The importance of calcium as a secondary messenger molecule was first described by Ringer in 1883 who accidentally discovered that isolated hearts require calcium for contraction (Ringer, 1883). Subsequent studies in the 20th century underlined the importance of calcium in physiology (Carafoli, 2003). Calcium oscillations are vital for the depolarisation of neurons and synaptic transmission where basal cytosolic free calcium levels increase significantly through the influx of calcium from the extracellular space significantly after depolarisation. For neurons, it is crucial to buffer excessive calcium from the cytosol at the time of signal transmission. Calcium levels are tightly controlled by calcium-buffering proteins such as calbindin and calmodulin and intracellular stores where mitochondria are responsible for the “fine-tuning” of calcium transients (Baimbridge et al., 1992; Dupont and Combettes, 2016).

Elevated Ca^{2+} levels are then either rapidly sequestered into mitochondria and ER or extruded into the extracellular space via Na^{+}/Ca^{2+} exchanger (NCX) or Ca^{2+} ATPase are the

major calcium extrusion proteins (Lytton et al., 2002; Carafoli et al., 2001). Three plasma membrane NCX isoforms have been identified (NCX1, NCX2 and NCX3) where NCX1 is ubiquitously expressed in most tissues (elevated expression in heart and skeletal muscle) and NCX2/3 are highly expressed in brain tissue. NCX in reverse mode is thought to be neuroprotective under pathophysiological conditions such as ischemia and excitotoxicity (Jeffs et al., 2007).

Mitochondria play a vital role in a healthy calcium homeostasis whilst the calcium influx in to the mitochondria aids the bioenergetic status of the cell. They are strategically placed throughout the cell and calcium uptake stimulates dehydrogenases which in turn increase NADH and FADH levels and therefore drive ATP synthesis (Denton, 2009). Thus, a tight control of calcium transients is particularly important to high pacing cells such as cardiomyocytes and dopaminergic neurons which have a particularly high energy demand. Chronic elevated calcium levels triggered by altered calcium transient handling as described in PD may damage mitochondria, impair ATP synthesis which ultimately may lead to cell death (Gandhi et al., 2012).

The mechanism of mitochondrial calcium uptake and efflux has been extensively studied where it was shown that calcium is transported across the inner mitochondrial membrane via mitochondrial calcium uniporter (MCU). Calcium is taken up in an electrogenic manner, thus not requiring anions or cations for transport across the membrane (Gunter and Pfeiffer, 1990). Global ablation of MCU in mice is not lethal and does not result in major cardiac phenotypical suggesting a limited role in cardiac homeostasis (Pan et al., 2013; Holmstrom et al., 2015). However, a transgenic mouse model with a conditional MCU deletion in adulthood revealed that MCU is required for calcium-dependent mitochondrial metabolism

during acute stress (Kwong et al., 2015; Luongo et al., 2015). These studies suggest that mitochondrial calcium uptake under normal physiological conditions may take place via other MCU-independent calcium uptake mechanism(s).

Mitochondrial calcium is extruded in exchange with either H^+ or Na^+. It is well established that calcium exchange in excitable cells such as neurons is mediated by Na^+/Ca^{2+} exchanger (Crompton et al., 1978). Molecular identification demonstrated mitochondrial member of the Na^+/Ca^{2+} exchanger superfamily – the $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX) (Palty et al., 2004; Palty et al., 2010). Despite the discovery of the efflux mechanisms, the molecular identity remained for many years elusive. Palty et al. finally identified and characterised the mitochondrial member of the Na^+/Ca^{2+} exchanger superfamily – the $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX) (Palty et al., 2004; Palty et al., 2010). NCLX shares a common catalytic core with the NCX superfamily but its regulatory domain is shorter and lacks allosteric Ca^{2+}-binding domain. NCLX is thought to be the rate-limiting system by which it control mitochondrial Ca^{2+}fluxes since the efflux is much slower that the MCU-mediated Ca^{2+} influx (Palty et al., 2010; Drago et al., 2012). Impairment of the mitochondrial influx/efflux leads to a deregulation of mitochondrial calcium homeostasis and mitochondrial calcium overload in combination with oxidative stress are known to induce permeability transition pore opening (PTP) that is believed to be an initial trigger for apoptotic and necrotic cell death (Bernardi et al., 2015; Zhivotovsky et al., 2009). The importance of NCLX in mitochondrial calcium homeostasis and survival of excitable cells has recently been highlighted as the deletion of NCLX in the mouse adult heart leads to myocardial dysfunction and lethality within two weeks. This study provides strong evidence that mitochondrial calcium efflux (via NCLX) is indispensable for normal calcium homeostasis and cardiac function (Luongo et al., 2017).

The importance of NCLX in neuronal calcium homeostasis is yet to be fully established - this study would ideally be undertaken in a mouse model lacking neuronal NCLX.

Cellular and mitochondrial pathology in Parkinson's disease

Neurodegenerative diseases are classified as progressive degeneration and selective death of neuronal subtypes. In PD, Lewy body inclusions with a loss of dopaminergic neurons of the substantia nigra are the main histopathological hallmarks. On cellular level, oxidative stress and mitochondrial complex I deficiency have been described in many studies investigating PD pathology (Schapira et al., 1990; Dexter et al., 1989; Zhang et al., 1999; Dias et al., 2013). Neurodegenerative conditions often affect mitochondria and the bioenergetic status of the cell where mitochondria calcium dysregulation plays a key role in pathogenesis. The underlying molecular mechanism(s) are still debated whilst mitochondrial biogenetics and calcium regulation have received more attention in the recent years.

For many years, the mitochondrial complex I inhibitor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been employed to recapitulate the pathophysiology of PD in mice and assess therapeutic compounds (Meredith and Rademacher, 2011). The discovery of genes affected by mutations in familial PD has not only provided a firm link between mitochondria and PD but also improved our understanding of the underlying mechanism(s) in this debilitating disease. Among those PD-risk genes discovered by linkage analysis is α-synuclein itself, the proteins that aggregates in PD Lewy body pathology (Polymeropoulos et al., 1997; Polymeropoulos et al., 1996; Singleton et al., 2003). Alpha-synuclein has been shown to induce oxidative stress and calcium dysregulation (Deas et al., 2016; Angelova et al., 2015; Angelova et al., 2016). Further PD risk genes are protein deglycase DJ-1 (DJ-1) and

PTEN-induced putative kinase 1 (PINK1) which have both been proposed to be involved in neuronal stress-response pathways (Piccoli et al., 2008; Gandhi and Abramov, 2012). Mutations in PINK1 cause a recessive form of PD where mitochondrial phenotypes have been described by many research groups. Limited mitochondrial substrate availability and inhibition of complex I have been reported to result in impaired respiration with elevated levels of ROS (Wood-Kaczmar et al., 2008; Gautier et al., 2008; Yao et al., 2011). Furthermore, alterations in mitochondrial metabolism through inhibition of NCLX in pancreatic β-cells of PINK-1 deficient mice lead to changes in glucose sensitivity of these cells (Deas et al., 2014).

Since cytosolic calcium dysregulation has been shown to be a major pathogenic hallmark in PD and the role for mitochondria in calcium transient buffering it is not surprising that mitochondrial calcium overload was described in PINK1-deficient neurons (Schapira, 2013; Hurley et al., 2013; Surmeier, 2007; Gandhi et al., 2009). In 2009, Gandhi et al. found that PINK-1 deficient midbrain neurons are sensitive to dopamine (non-toxic to wild type neurons) and that dopamine induced mitochondrial calcium overload which in turn triggered PTP opening and cell death. The authors found that calcium extrusion in PINK-1 deficient neurons was severely inhibited leading to mitochondrial calcium overload, increase ROS production and ultimately neuronal cell death. Although functional inhibition of the mitochondrial sodium/calcium antiport was demonstrated in PINK-1 deficient neurons a direct proof of regulation or integration of NCLX and PINK1 could not be provided in this study as the molecular identity of NCLX was not established in 2009. An insight into the question to whether mitochondrial calcium or dopaminergic dysregulation are early pathogenic processes, Akundi et al. (2011) showed that increased mitochondrial calcium

sensitivity precedes dopamine dysregulations observed in a PINK1-deficient mouse model again highlighting the importance of mitochondrial calcium homeostasis in PD. Importantly, the inhibition of mitochondrial calcium efflux was also demonstrated in cells of PD patients bearing PINK-1 mutations (Abramov et al., 2011). The molecular identification of NCLX opened up avenues to study of mitochondrial calcium efflux in PD. A study in 2015, has shown that NCLX activation is able to rescue the pathogenic mitochondrial calcium efflux, MMP depolarisation and neuronal cell death in PINK-1 deficient models via a protein kinase A mediated process confirming Gandhi et al. (2009) hypothesis (Kostic et al., 2015). Furthermore, the authors provided a detailed analysis of a putative regulatory NCLX site serine 258. This study provided the first evidence that upregulation of mitochondrial calcium efflux via NCLX is able to rescue the pathogenic phenotypes observed in PD.

Whilst it is well recognised that NCLX is the main extrusion mechanism in excitable cells however, it should be noted that pharmacological inhibition (and KO) of NCLX reduces the efflux by 80% indicating the presence of other extrusion mechanism(s). NCX2 and NCX3 have been suggested to be play a role in mitochondrial calcium efflux where inhibition of NCX2/3 by siRNA or antibody-blocking led to a reduced mitochondrial calcium efflux (Wood-Kaczmar et al., 2013). This finding is supported by other studies which demonstrated a possible role for the plasmalemmal NCX in mitochondrial calcium efflux (Gobbi et al., 2007; Sisalli et al., 2014).

Considering the cytosolic calcium dysregulation observed in PD and mitochondrial calcium overload in PINK-1 deficient neurons the question remains whether mitochondrial calcium efflux is a common phenotype. Preliminary results produced by our laboratory provides further support that mitochondrial calcium homeostasis may play a central role to PD

pathology. Rat primary neuronal co-cultures overexpressing alpha-synuclein were loaded with a mitochondrial calcium dye (Rhod5N) and cells were permeabilized using pseudo-intracellular buffer containing digitonin (+5mM malate/glutamate). This approach allowed direct application of $CaCl_2$ to permeabilized neurons and recording of mitochondrial calcium handling (Fig.1A) (Abramov and Duchen, 2011). The calcium efflux in alpha-synuclein overexpressing neurons was severely impaired when compared to wild type neurons suggesting a role for NCLX in another PD model (Fig.1B). Interestingly, we have previously shown that exogenous alpha-synuclein is readily taken up by neuronal cultures and localises to the mitochondria (Ludtmann et al., 2016; Cremades et al., 2012; Angelova et al., 2016). These results warrant further investigations as to how alpha-synuclein triggers mitochondrial calcium accumulation and whether NCLX plays a role in this pathogenic process.

Mitochondrial calcium dysregulation impairs mitochondrial health and can lead to cell death. Our data on PINK-1 deficient neurons and alpha-synuclein overexpressing neurons (Kostic et al., 2015)) suggest that NCLX may play a common role in the mitochondrial pathogenesis of PD. The molecular identification of NCLX enabled studies of mitochondrial calcium homeostasis in PD models. Recent data provide evidence that the role of NCLX and mitochondrial calcium efflux in the pathogenesis of Parkinson's disease, and possibly some other neurological disorders, are underestimated.

Reference List

Abeti,R. and Abramov,A.Y. (2015). Mitochondrial Ca(2+) in neurodegenerative disorders. Pharmacol. Res. *99*, 377-381.

Abramov,A.Y. and Duchen,M.R. (2011). Measurements of threshold of mitochondrial permeability transition pore opening in intact and permeabilized cells by flash photolysis of caged calcium. Methods Mol. Biol. *793*, 299-309.

Abramov,A.Y., Gegg,M., Grunewald,A., Wood,N.W., Klein,C., and Schapira,A.H. (2011). Bioenergetic consequences of PINK1 mutations in Parkinson disease. PLoS. One. *6*, e25622.

Akundi,R.S., Huang,Z., Eason,J., Pandya,J.D., Zhi,L., Cass,W.A., Sullivan,P.G., and Bueler,H. (2011). Increased mitochondrial calcium sensitivity and abnormal expression of innate immunity genes precede dopaminergic defects in Pink1-deficient mice. PLoS. One. *6*, e16038.

Angelova,P.R. and Abramov,A.Y. (2014). Interaction of neurons and astrocytes underlies the mechanism of Abeta-induced neurotoxicity. Biochem. Soc. Trans. *42*, 1286-1290.

Angelova,P.R., Horrocks,M.H., Klenerman,D., Gandhi,S., Abramov,A.Y., and Shchepinov,M.S. (2015). Lipid peroxidation is essential for alpha-synuclein-induced cell death. J. Neurochem.

Angelova,P.R., Ludtmann,M.H., Horrocks,M.H., Negoda,A., Cremades,N., Klenerman,D., Dobson,C.M., Wood,N.W., Pavlov,E.V., Gandhi,S., and Abramov,A.Y. (2016). Ca2+ is a key factor in alpha-synuclein-induced neurotoxicity. J. Cell Sci. *129*, 1792-1801.

Baimbridge,K.G., Celio,M.R., and Rogers,J.H. (1992). Calcium-binding proteins in the nervous system. Trends Neurosci. *15*, 303-308.

Bernardi,P., Di,L.F., Fogolari,F., and Lippe,G. (2015). From ATP to PTP and Back: A Dual Function for the Mitochondrial ATP Synthase. Circ. Res. *116*, 1850-1862.

Carafoli,E. (2003). The calcium-signalling saga: tap water and protein crystals. Nat. Rev. Mol. Cell Biol. *4*, 326-332.

Carafoli,E., Santella,L., Branca,D., and Brini,M. (2001). Generation, control, and processing of cellular calcium signals. Crit Rev. Biochem. Mol. Biol. *36*, 107-260.

Cremades,N., Cohen,S.I., Deas,E., Abramov,A.Y., Chen,A.Y., Orte,A., Sandal,M., Clarke,R.W., Dunne,P., Aprile,F.A., Bertoncini,C.W., Wood,N.W., Knowles,T.P., Dobson,C.M., and Klenerman,D. (2012). Direct observation of the interconversion of normal and toxic forms of alpha-synuclein. Cell *149*, 1048-1059.

Crompton,M., Moser,R., Ludi,H., and Carafoli,E. (1978). The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. Eur. J. Biochem. *82*, 25-31.

Deas,E., Cremades,N., Angelova,P.R., Ludtmann,M.H., Yao,Z., Chen,S., Horrocks,M.H., Banushi,B., Little,D., Devine,M.J., Gissen,P., Klenerman,D., Dobson,C.M., Wood,N.W., Gandhi,S., and Abramov,A.Y. (2016). Alpha-Synuclein Oligomers Interact with Metal Ions to Induce Oxidative Stress and Neuronal Death in Parkinson's Disease. Antioxid. Redox. Signal. *24*, 376-391.

Deas,E., Piipari,K., Machhada,A., Li,A., Gutierrez-del-Arroyo,A., Withers,D.J., Wood,N.W., and Abramov,A.Y. (2014). PINK1 deficiency in beta-cells increases basal insulin secretion and improves glucose tolerance in mice. Open. Biol. *4*, 140051.

Denton,R.M. (2009). Regulation of mitochondrial dehydrogenases by calcium ions. Biochim. Biophys. Acta *1787*, 1309-1316.

Dexter,D.T., Carter,C.J., Wells,F.R., Javoy-Agid,F., Agid,Y., Lees,A., Jenner,P., and Marsden,C.D. (1989). Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J. Neurochem. *52*, 381-389.

Dias,V., Junn,E., and Mouradian,M.M. (2013). The role of oxidative stress in Parkinson's disease. J. Parkinsons. Dis. *3*, 461-491.

Drago,I., De,S.D., Rizzuto,R., and Pozzan,T. (2012). Mitochondrial Ca2+ uptake contributes to buffering cytoplasmic Ca2+ peaks in cardiomyocytes. Proc. Natl. Acad. Sci. U. S. A *109*, 12986-12991.

Dupont,G. and Combettes,L. (2016). Fine tuning of cytosolic Ca (2+) oscillations. F1000Res. *5*.

Gandhi,S. and Abramov,A.Y. (2012). Mechanism of oxidative stress in neurodegeneration. Oxid. Med. Cell Longev. *2012*, 428010.

Gandhi,S., Vaarmann,A., Yao,Z., Duchen,M.R., Wood,N.W., and Abramov,A.Y. (2012). Dopamine induced neurodegeneration in a PINK1 model of Parkinson's disease. PLoS. One. *7*, e37564.

Gandhi,S., Wood-Kaczmar,A., Yao,Z., Plun-Favreau,H., Deas,E., Klupsch,K., Downward,J., Latchman,D.S., Tabrizi,S.J., Wood,N.W., Duchen,M.R., and Abramov,A.Y. (2009). PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Mol. Cell *33*, 627-638.

Gautier,C.A., Kitada,T., and Shen,J. (2008). Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc. Natl. Acad. Sci. U. S. A *105*, 11364-11369.

Gobbi,P., Castaldo,P., Minelli,A., Salucci,S., Magi,S., Corcione,E., and Amoroso,S. (2007). Mitochondrial localization of Na+/Ca2+ exchangers NCX1-3 in neurons and astrocytes of adult rat brain in situ. Pharmacol. Res. *56*, 556-565.

Gunter,T.E. and Pfeiffer,D.R. (1990). Mechanisms by which mitochondria transport calcium. Am. J. Physiol *258*, C755-C786.

Holmstrom,K.M., Pan,X., Liu,J.C., Menazza,S., Liu,J., Nguyen,T.T., Pan,H., Parks,R.J., Anderson,S., Noguchi,A., Springer,D., Murphy,E., and Finkel,T. (2015). Assessment of cardiac function in mice lacking the mitochondrial calcium uniporter. J. Mol. Cell Cardiol. *85*, 178-182.

Hurley,M.J., Brandon,B., Gentleman,S.M., and Dexter,D.T. (2013). Parkinson's disease is associated with altered expression of CaV1 channels and calcium-binding proteins. Brain *136*, 2077-2097.

Jeffs,G.J., Meloni,B.P., Bakker,A.J., and Knuckey,N.W. (2007). The role of the Na(+)/Ca(2+) exchanger (NCX) in neurons following ischaemia. J. Clin. Neurosci. *14*, 507-514.

Kostic,M., Ludtmann,M.H., Bading,H., Hershfinkel,M., Steer,E., Chu,C.T., Abramov,A.Y., and Sekler,I. (2015). PKA Phosphorylation of NCLX Reverses Mitochondrial Calcium Overload and Depolarization, Promoting Survival of PINK1-Deficient Dopaminergic Neurons. Cell Rep.

Kwong,J.Q., Lu,X., Correll,R.N., Schwanekamp,J.A., Vagnozzi,R.J., Sargent,M.A., York,A.J., Zhang,J., Bers,D.M., and Molkentin,J.D. (2015). The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart. Cell Rep. *12*, 15-22.

Ludtmann,M.H., Angelova,P.R., Ninkina,N.N., Gandhi,S., Buchman,V.L., and Abramov,A.Y. (2016). Monomeric Alpha-Synuclein Exerts a Physiological Role on Brain ATP Synthase. J. Neurosci. *36*, 10510-10521.

Luongo,T.S., Lambert,J.P., Gross,P., Nwokedi,M., Lombardi,A.A., Shanmughapriya,S., Carpenter,A.C., Kolmetzky,D., Gao,E., van Berlo,J.H., Tsai,E.J., Molkentin,J.D., Chen,X., Madesh,M., Houser,S.R., and Elrod,J.W. (2017). The mitochondrial Na+/Ca2+ exchanger is essential for Ca2+ homeostasis and viability. Nature *545*, 93-97.

Luongo,T.S., Lambert,J.P., Yuan,A., Zhang,X., Gross,P., Song,J., Shanmughapriya,S., Gao,E., Jain,M., Houser,S.R., Koch,W.J., Cheung,J.Y., Madesh,M., and Elrod,J.W. (2015). The Mitochondrial Calcium Uniporter Matches Energetic Supply with Cardiac Workload during Stress and Modulates Permeability Transition. Cell Rep. *12*, 23-34.

Lytton,J., Li,X.F., Dong,H., and Kraev,A. (2002). K+-dependent Na+/Ca2+ exchangers in the brain. Ann. N. Y. Acad. Sci. *976*, 382-393.

Meredith,G.E. and Rademacher,D.J. (2011). MPTP mouse models of Parkinson's disease: an update. J. Parkinsons. Dis. *1*, 19-33.

Palty,R., Ohana,E., Hershfinkel,M., Volokita,M., Elgazar,V., Beharier,O., Silverman,W.F., Argaman,M., and Sekler,I. (2004). Lithium-calcium exchange is mediated by a distinct potassium-independent sodium-calcium exchanger. J. Biol. Chem. *279*, 25234-25240.

Palty,R., Silverman,W.F., Hershfinkel,M., Caporale,T., Sensi,S.L., Parnis,J., Nolte,C., Fishman,D., Shoshan-Barmatz,V., Herrmann,S., Khananshvili,D., and Sekler,I. (2010). NCLX is an essential component of mitochondrial Na+/Ca2+ exchange. Proc. Natl. Acad. Sci. U. S. A *107*, 436-441.

Pan,X., Liu,J., Nguyen,T., Liu,C., Sun,J., Teng,Y., Fergusson,M.M., Rovira,I.I., Allen,M., Springer,D.A., Aponte,A.M., Gucek,M., Balaban,R.S., Murphy,E., and Finkel,T. (2013). The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. Nat. Cell Biol. *15*, 1464-1472.

Piccoli,C., Sardanelli,A., Scrima,R., Ripoli,M., Quarato,G., D'Aprile,A., Bellomo,F., Scacco,S., De,M.G., Filla,A., Iuso,A., Boffoli,D., Capitanio,N., and Papa,S. (2008). Mitochondrial respiratory dysfunction in familiar parkinsonism associated with PINK1 mutation. Neurochem. Res. *33*, 2565-2574.

Polymeropoulos,M.H., Higgins,J.J., Golbe,L.I., Johnson,W.G., Ide,S.E., Di,I.G., Sanges,G., Stenroos,E.S., Pho,L.T., Schaffer,A.A., Lazzarini,A.M., Nussbaum,R.L., and Duvoisin,R.C. (1996). Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science *274*, 1197-1199.

Polymeropoulos,M.H., Lavedan,C., Leroy,E., Ide,S.E., Dehejia,A., Dutra,A., Pike,B., Root,H., Rubenstein,J., Boyer,R., Stenroos,E.S., Chandrasekharappa,S., Athanassiadou,A., Papapetropoulos,T., Johnson,W.G., Lazzarini,A.M., Duvoisin,R.C., Di,I.G., Golbe,L.I., and Nussbaum,R.L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045-2047.

Ringer,S. (1883). A further Contribution regarding the influence of the different Constituents of the Blood on the Contraction of the Heart. J. Physiol *4*, 29-42.

Schapira,A.H. (2013). Calcium dysregulation in Parkinson's disease. Brain *136*, 2015-2016.

Schapira,A.H., Cooper,J.M., Dexter,D., Clark,J.B., Jenner,P., and Marsden,C.D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. J. Neurochem. *54*, 823-827.

Singleton,A.B., Farrer,M., Johnson,J., Singleton,A., Hague,S., Kachergus,J., Hulihan,M., Peuralinna,T., Dutra,A., Nussbaum,R., Lincoln,S., Crawley,A., Hanson,M., Maraganore,D., Adler,C., Cookson,M.R., Muenter,M., Baptista,M., Miller,D., Blancato,J., Hardy,J., and Gwinn-Hardy,K. (2003). alpha-Synuclein locus triplication causes Parkinson's disease. Science *302*, 841.

Sisalli,M.J., Secondo,A., Esposito,A., Valsecchi,V., Savoia,C., Di Renzo,G.F., Annunziato,L., and Scorziello,A. (2014). Endoplasmic reticulum refilling and mitochondrial calcium extrusion promoted in neurons by NCX1 and NCX3 in ischemic preconditioning are determinant for neuroprotection. Cell Death. Differ. *21*, 1142-1149.

Surmeier,D.J. (2007). Calcium, ageing, and neuronal vulnerability in Parkinson's disease. Lancet Neurol. *6*, 933-938.

Wood-Kaczmar,A., Deas,E., Wood,N.W., and Abramov,A.Y. (2013). The role of the mitochondrial NCX in the mechanism of neurodegeneration in Parkinson's disease. Adv. Exp. Med. Biol. *961*, 241-249.

Wood-Kaczmar,A., Gandhi,S., Yao,Z., Abramov,A.Y., Miljan,E.A., Keen,G., Stanyer,L., Hargreaves,I., Klupsch,K., Deas,E., Downward,J., Mansfield,L., Jat,P., Taylor,J., Heales,S., Duchen,M.R., Latchman,D., Tabrizi,S.J., and Wood,N.W. (2008). PINK1 is necessary for long term survival and mitochondrial function in human dopaminergic neurons. PLoS. One. *3*, e2455.

Yao,Z., Gandhi,S., Burchell,V.S., Plun-Favreau,H., Wood,N.W., and Abramov,A.Y. (2011). Cell metabolism affects selective vulnerability in PINK1-associated Parkinson's disease. J. Cell Sci. *124*, 4194-4202.

Zhang,J., Perry,G., Smith,M.A., Robertson,D., Olson,S.J., Graham,D.G., and Montine,T.J. (1999). Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. Am. J. Pathol. *154*, 1423-1429.

Zhivotovsky,B., Galluzzi,L., Kepp,O., and Kroemer,G. (2009). Adenine nucleotide translocase: a component of the phylogenetically conserved cell death machinery. Cell Death. Differ. *16*, 1419-1425.

Figure legend

Fig.1 Impaired mitochondrial calcium efflux in neurons overexpressing alpha-synuclein. A) Representative traces of mitochondria loaded with Rhod5N exposed to $CaCl_2$ stimulus. B) The significant delay in mitochondrial calcium efflux suggests a role for NCLX in alpha-synuclein pathology. n=3 experiments; ***p<0.001 Method: Rat primary neuronal co-cultures were prepared as described in Gandhi et al. (2012) and cells were loaded with

Rhod5N before being permeabilized as described in Abramov and Duchen (2011). Confocal images were obtained using a Zeiss 710 equipped with a META detection system and a 40× oil immersion objective. Rhod-5N measurements were undertaken using the 543 nm laser line and 560 nm longpass filter. Statistical analysis and exponential curve fitting were performed using Origin 2017 software (Microcal Software Inc.). Results are expressed as means ± standard error of the mean. Student's T-tests was performed for statistical analysis.

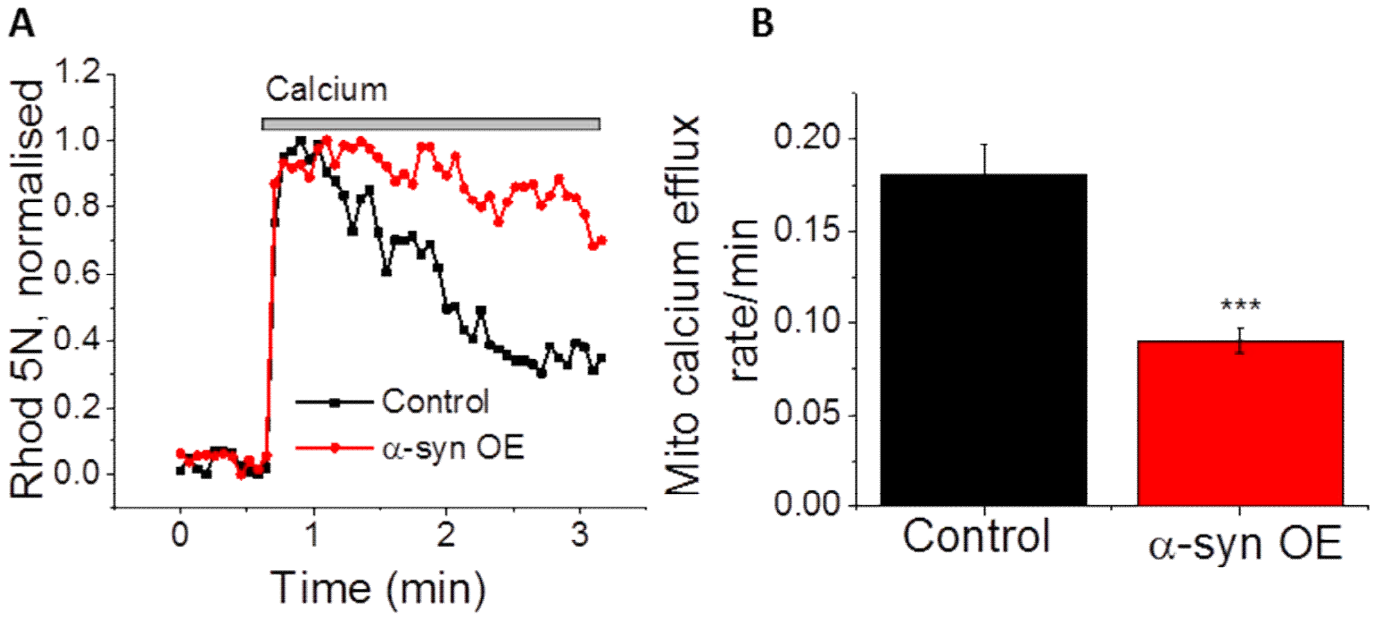
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Figure 1