Inhibition of the mitochondrial calcium uniporter rescues dopaminergic neurons in pink1−/− zebrafish

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Abstract

Mutations in PTEN-induced putative kinase 1 (PINK1) are a cause of early onset Parkinson’s disease (PD). Loss of PINK1 function causes dysregulation of mitochondrial calcium homeostasis, resulting in mitochondrial dysfunction and neuronal cell death. We report that both genetic and pharmacological inactivation of the mitochondrial calcium uniporter (MCU), located in the inner mitochondrial membrane, prevents dopaminergic neuronal cell loss in pink1−/− mutant zebrafish (Danio rerio) via rescue of mitochondrial respiratory chain function. In contrast, genetic inactivation of the voltage dependent anion channel 1 (VDAC1), located in the outer mitochondrial membrane, did not rescue dopaminergic neurons in PINK1 deficient D. rerio. Subsequent gene expression studies revealed specific upregulation of the mcu regulator micu1 in pink1−/− mutant zebrafish larvae and inactivation of micu1 also results in rescue of dopaminergic neurons. The functional consequences of PINK1 deficiency and modified MCU activity were confirmed using a dynamic in silico model of Ca2+ triggered mitochondrial activity. Our data suggest modulation of MCU-mediated mitochondrial calcium homeostasis as a possible neuroprotective strategy in PINK1 mutant PD.

Introduction

Parkinson’s disease (PD) is a common neurodegenerative disorder, resulting in both motor and non-motor symptoms (Kalia & Lang, 2015). The pathological hallmark of PD is loss of dopaminergic neurons in the substantia nigra pars compacta. Mitochondrial dysfunction was first described in post mortem tissue of patients with sporadic PD. More recently, it has been closely linked to Mendelian forms of familial PD, in particular to early onset PD due to parkin (Exner et al., 2012). Loss of PINK1 function results in mitochondrial calcium overload, thought to be due to impaired calcium efflux from the mitochondria (Gandhi et al., 2009; Marongiu et al., 2009). The reduced mitochondrial calcium capacity in PINK1 deficient neurons results in increased oxidative stress which in turn leads to reduced glucose uptake and a lowering of the threshold for the opening of the permeability transition pore (PTP). This suggests that impaired mitochondrial calcium homeostasis is at least one of the pathological mechanisms resulting from PINK1 deficiency.

Mitochondria are key modulators of intracellular calcium homeostasis. The voltage-dependent anion channel 1 (VDAC1) is located within the outer mitochondrial membrane and transports calcium into the intermembrane space (Shoshan-Barmatz & Golan, 2012). Calcium then enters the mitochondrial matrix through a dedicated channel within the inner mitochondrial membrane referred to as the mitochondrial calcium uniporter (MCU) (Baughman et al., 2011; De Stefani et al., 2011). Silencing MCU severely reduces mitochondrial calcium uptake but mitochondrial respiration and membrane potential remain fully intact. The MCU channel is part of a multisubunit modular complex that includes regulators of its activity, calcium sensitivity and expression (Murgia & Rizzuto, 2015). Additional components of this four MCU complex include mitochondrial calcium uptake 1 and 2 (MICU1, MICU2), the essential MCU regulator (EMRE) and the mitochondrial calcium uniporter regulator 1 (MCUR1) (see Murgia & Rizzuto, 2015 for review). Recently, a physical and functional interaction of MCU with VDAC1 has also been reported (Liao et al., 2015).

Zebrafish are increasingly used to study mechanisms linked to human neurodegenerative diseases. They are vertebrates and therefore more closely related to humans than Caenorhabditis elegans or Drosophila. Their genome is fully characterized and contains...
orthologues for approximately 70% of all human genes, but 82% for all human disease genes (Howe et al., 2013). We recently reported our findings in a zebrafish mutant line carrying a Stop mutation (pink1<sup>+</sup>/<sup>+</sup>, from here on referred to as pink1<sup>+/−</sup> for homozygous pink1 mutant larvae) in the kinase domain of pink1, the zebrafish orthologue of the human PD gene PINK1 (Flinn et al., 2013). pink1<sup>+/−</sup> already resulted in impaired function of the mitochondrial respiratory chain and loss of dopaminergic neurons at 3 days post fertilization (dpf). Zebrafish embryos are eminently amenable to both genetic and pharmacological manipulation.

Here, we demonstrate a rescue effect of both pharmacological and genetic MCU inhibition on the dopaminergic neurons via normalization of mitochondrial function. We further report specific transcriptional upregulation of mical in pink1<sup>+/−</sup> zebrafish larvae. Genetic inactivation of mical also results in rescue of the dopaminergic neurons. In contrast, genetic inactivation of vdac1 did not protect the dopaminergic neurons in pink1<sup>+/−</sup> zebrafish larvae.

To understand these findings on a more mechanistic level, we applied our previously developed mathematical model to investigate the role of modified Ca<sup>2+</sup> dynamics on the respiratory function (Komin et al., 2015). Incorporating the results of PINK1 on Ca<sup>2+</sup> homeostasis (Gandhi et al., 2012) into the model reveals a dramatic decrease in respiratory capacity that forms the basis of this study. Our data suggest that modulation of MICU1 and MCU may be a promising target for future neuroprotective strategies in PINK1-related PD.

Materials and methods

Animal maintenance

Adult wt (AB and TL wt lines) and pink1<sup>+/−</sup> (Flinn et al., 2013) zebrafish were maintained according to methods previously described (Matthews et al., 2002). Adult zebrafish and larvae were kept in E3 medium at 28.5 °C.

RT-PCR

RNA was isolated from a pool of 20 embryos using Tri reagent (Sigma Aldrich) and cDNA was generated using Verso cDNA RNA was isolated from a pool of 20 embryos using Tri reagent (Sigma Aldrich) and cDNA was generated using Verso cDNA synthesis kit (Thermo Scientific). RT-primers (5′-AGAGTGGCTGCAGAAAGTGTT-3′) were designed against the transcript (ENSDART00000020850) in the same samples (ΔCT = CTTarget − CTEff(αlpha)). It was further normalized with the wt control (ΔΔCT = ΔCT − CTControl). The fold change in expression was then obtained by 2<sup>−ΔΔCT</sup>. The results are representative of three biological replicates, each consisting of three technical replicates.

Chemical treatment

The pharmacological inhibitor of MCU, Ruthenium red (RR) was used to confirm the results from the functional knockdown of mcu using MO. RR (Sigma Aldrich) was dissolved in sterile water at 10 μM concentration and treated to larvae in E3 medium for 3 days (n = 10 of embryos per genotype and experiment).

Whole mount in situ hybridization

Whole mount in situ hybridization (WISH) was performed as previously described (Jowett & Lettice, 1994; Thiese & Thisse, 2008; Shimizu et al., 2015). Specific primers with artificially introduced T7 and SP6 polymerase sequence were used to perform PCR amplification of desired gene of interest. The primer sequences are as follows: th- F 5′-AATTAACCTCACTAAAGGAATGGGCGA ATTCAGACGCTCCAC-3′; R 5′-TAAATCAGCTCATATAGGGAGGAGCC-3′; 5′-TGTCCGTGTTCCACTTCTCA-3′; R 5′-GATTAGTTGCTATAGGCACCACATCCGGAAC-3′; vdac1- F 5′-TAATAGCTCAGATAGGAGACATATGAATGCAAACATGCGCC-3′; mical- F 5′-TAATACGACCTCACTATAGGGAGAGCATATGAATGCAACATGCGCC-3′; R 5′-GATTAGTTGCTATAGGCACCACATCCGGAAC-3′. The PCR

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product was used as a template for synthesis of the digoxigenin-labeled antisense RNA probe using in vitro transcription. Specifically, an RNA probe against tyrosine hydroxylase (TH) was synthesized to label dopaminergic neurons. An anti-DIG antibody coupled to alkaline phosphatase was used to detect the hybridized RNA probe. Zebrafish larvae were embedded in 3% methylcellulose and photographed. The mean number of these diencephalic dopaminergic neurons in experimental larvae were calculated over three independent experiments (n = 10 of embryos per genotype and experiment).

Mitochondrial complex I–IV assays
Assays were performed as previously described (Flinn et al., 2013). Briefly, 5 dpf zebrafish larvae were harvested into Eppendorf tubes of 20 larvae per experimental condition, and the mitochondrial fraction was isolated. Homogenates were freeze thawed three times in liquid N2 before being used for spectrophotometric assessment of respiratory chain complexes. The specific activity of complex I–IV was normalized to that of citrate synthase. Complex I activity is determined by following the oxidation of NADH to NAD+ and the simultaneous reduction of a dye which leads to increased absorbance at OD = 450 nm. All mitochondrial complex activity assays were performed in triplicate.

Data analysis
In all experiments, error bars indicate the standard error of mean (SEM, at least three independent experiments). All experiments were carried out in biological triplicate unless specifically stated otherwise. The following statistical tests were applied to determine statistical significance; Q-PCR: unpaired two-tailed, two sample T test; TH in situ hybridisation experiments (counting of dopaminergic neurons) and complex I activity assay experiments: One way ANOVA followed by Tukey’s multiple comparison test.

In silico modeling
To integrate the experimental data mechanistically, we adapted our previous developed model (Komin et al., 2015). The rate equation model takes into account the cytosolic Ca2+ dynamics that is mainly driven by Ca2+ release from the endoplasmic reticulum (ER) through inositol triphosphate receptors (IP3Rs) into the cytosol and removal of cytosolic Ca2+ back into the ER by sacro-ER Ca2+ ATPases (SERCAs) and into the extracellular space by plasma membrane Ca2+ ATPases (PMCAs) (Fig. 4A). Within the cytosol, Ca2+ is taken up by mitochondria via the MCU where it triggers SERCA and PMCA mediated fluxes (Fig. 4B). The adapted model considers the corresponding Ca2+ fluxes and the triggered mitochondrial activity is simulated by a first principle-based module (Bertram et al., 2006). The resulting cytosolic ATP concentration subsequently determines SERCA and PMCA mediated fluxes and therefore cytosolic Ca2+ dynamics. The mathematical equations were implemented in a customized Ca2+ program.

Results
MCU inactivation rescues dopaminergic neurons in PINK1 deficiency
mcu (ENSDARG00000101175), the zebrafish orthologue of human MCU (ENSG00000156026), was identified using Ensembl genome browser. mcu shares 70.7% DNA and 78.8% protein identity with human MCU. mcu was already expressed at 1 h post fertilization (hpf), expression was then constant throughout development (Fig. 1A). WISH of mcu indicated predominant expression in brain tissue (Fig. 1B). Q-PCR analysis revealed similar mcu mRNA transcript levels in pink1+/− and wild type (wt) larvae at 3 dpf (Fig. 1C). We next applied the morpholino (MO) antisense strategy to genetically inactivate mcu. Injection of a splice site MO targeted at the exon 2/intron 2 junction of mcu at single cell stage resulted in a dose-dependent skipping of exon 2 (Fig. 1D) with marked reduction in mRNA transcript levels (Fig. 1E). This MO-induced knockdown (k/d) of mcu did not alter the overt development, spontaneous motor behavior or phenotypic characteristics until 5 dpf (data not shown). As previously observed, the number of diencephalic dopaminergic neurons within the 1, 2, 4 and 5 subgroups according to the Rink-Wullimann classification were reduced in pink1+/− at 3 dpf (P = 0.0128) (Rink & Wullimann, 2001; Flinn et al., 2013). At 3 dpf, mcu k/d did not change the number of dopaminergic neurons in wt larvae, but resulted in a marked rescue of dopaminergic neurons in pink1+/− (P = 0.0085) (Fig. 2A,B). We next sought to validate the observed protective effect of genetic MCU inactivation using a different, pharmacological approach. RR has long been recognized as a pharmacological inhibitor of mitochondrial calcium influx prior to the discovery of the MCU as such (Moore, 1971). Treatment of wt larvae with RR at a concentration of 10 μM for 3 days did not have an effect on the number of diencephalic dopaminergic neurons, but – as predicted – had a marked protective effect on the seven dopaminergic neurons in pink1+/− larvae (P < 0.0001, Fig. 2C), similar to the rescue effect observed after genetic MCU inactivation. We next sought to identify the mechanism underlying the observed rescue effect of MCU inactivation in PINK1 deficiency. As mentioned earlier, we had previously observed a marked decrease in the activity of complex I in pink1+/− (Flinn et al., 2013). We hypothesized that MCU inactivation rescues dopaminergic neurons via normalization of mitochondrial respiratory chain function. As predicted, MO-mediated genetic inactivation of MCU as described above had a rescue effect on complex I activity in pink1+/− larvae at 5 dpf (P = 0.007, Fig. 2D).

The effect of VDAC1 and MCU inactivation are distinct
We next investigated whether the observed rescue effect of either genetic or pharmacological MCU inhibition on dopaminergic neurons in PINK1 deficiency is specific or could also be observed after inhibition of the outer mitochondrial membrane channel VDAC1. vdac1 (ENSDARG00000045132) the zebrafish orthologue of human VDAC1 (ENSG00000213585) shares 77.1% DNA sequence identity with VDAC1 and 85.5% protein identity. vdac1 is already expressed at 1 hpf with similar expression levels at 1, 2 and 3 dpf (Fig. 3A). Marked MO-mediated knockdown of vdac1 was achieved using a combination of two splice site MOs directed against the exon 5/intron 5 splice site and the intron 6/exon 7 (Fig. 3B). In contrast to MCU inactivation, MO-mediated k/d of vdac1 did not rescue diencephalic dopaminergic neurons in pink1+/− larvae (P > 0.99, Fig. 3C).

Altered functional regulation of MCU in PINK1 deficiency
As described above, mcu expression levels in pink1+/− larvae were similar to the expression levels observed in wt larvae but the marked rescue effect of mcu silencing suggested a possible dysregulation in MCU function. We hypothesized that this dysregulation may be due...
to altered expression levels of the MCU regulators micu1 (ENSDARG00000063358), micu2 (ENSDARG000000009939), emre (ENSDARG000000095826) or mcur (ENSDARG00000016964) in pink1<sup>+/−</sup>. We observed a 4 fold upregulation (4.01, SD ± 1.36, Fig. 3D) of micu1 (P = 0.0066) in pink1<sup>+/−</sup> compared to wt larvae at 3 dpf. mRNA expression levels of micu2, emre and mcur also varied between pink1<sup>+/−</sup> and wt larvae but the observed differences did not reach statistical significance (Fig. 3E–G). micu1 (ENSDARG00000063358) the zebrafish orthologue of human MICU1 (ENSG00000107745) shares 91.38% DNA identity and 74.3% protein identity with MICU1. We next applied the MO k/d strategy to further investigate the possible functional relevance of the observed micu1 transcriptional upregulation which suggested a possible change in the calcium trafficking properties of MCU. Splice
site MO directed against the exon 10/intron 10 boundary established k/d of *micu1* (Fig. 3H). MICU1 deficient zebrafish larvae did not develop any overt developmental, behavioural or phenotypical abnormalities (data not shown). MO-mediated knockdown of *micu1* resulted in protection of the diencephalic dopaminergic neurons similar to the effect observed after *mcu* k/d (*P* = 0.0004; Fig. 3I). Taken together, our data suggest MICU1 mediated activation of MCU in PINK1 deficiency.

**In silico modeling**

To investigate the potential role of energy metabolism as a mechanism for the observed changes in dopaminergic neuronal viability based on the selective vulnerability hypothesis (Chan et al., 2009), we simulated the different scenarios with our recently developed mechanistic modeling support of the experimental results from additional parameter scans demonstrate that the MCS1-mediated activation of *micu1* leads to a very similar picture as in the experimentally determined neuronal survival. Since neuronal survival will not depend linearly on respiratory activity, we performed comprehensive model parameter scans to test the stability of our results. Fig. 4D exhibits a parameter scan with respect to MCU activity (*P*~uni~) relative to wt conditions (*P*~uni~ = 1) and fructose-bisphosphate (FBP) that 10 depends on the glucose uptake rate. The obtained dependency of respiration on *P*~uni~ and FBP as well as similar results from additional parameter scans demonstrate that the mechanistic modeling support of the experimental findings do not depend on specific parameter combinations but are robust for a wide physiological parameter range.

**Discussion**

Calcium has repeatedly been implicated in the pathogenesis of PD. We now provide further evidence for a complex interplay between altered mitochondrial function, impaired mitochondrial calcium homeostasis and the MCU complex in PINK1 deficiency. Our observation of normal development in MCU-deficient zebrafish larvae is in keeping with the normal development of *MCU−/−* mice on the outbred, mixed CD1 genetic background (Murphy et al., 2014). Differences in the genetic background may explain the discrepancy between the lack of an effect of MCU inactivation on zebrafish development in our experiments compared to the marked effect observed by others (Prudent et al., 2013). There are ongoing concerns about potentially misleading conclusions resulting from MO-
based experiments. However, erroneous effects of MO are typically due to nonspecific toxic effects (Kok et al., 2015; Stainier et al., 2015). None of the typical morphological characteristics of these often p53-mediated, non-specific off-target effects were observed after MO-mediated k/d of mcu. Furthermore, we were able to validate our MO-based data using a completely different strategy, namely pharmacological inhibition of MCU with RR. We therefore consider it to be unlikely that the observed rescue effect of mcu k/d could be due to a non-specific off-target effect of the MOs used in our experiments. Further validation of the presented data could be undertaken in double-knockout zebrafish lines which are both pinkel and mcu mutant to further reduce the already unlikely possibility of the observed rescue effect being due to a non-specific off-target effect. MCU k/d had previously been shown to reduce excitotoxicity-induced neuronal cell death after NMDA receptor activation in vitro (Qiu et al., 2013). However, excitotoxic stress is not considered to be a key player in PD pathogenesis and modulation of excitotoxic stress is unlikely to be the underlying mechanism in our zebrafish model of PINK1 deficiency.

MICU1 is thought to have a major role in regulating MCU channel activity during high cytosolic calcium concentrations (Ahuja & Muallem, 2014). The specific increase in micul expression as well as the protective effect of micul k/d on dopaminergic neurons suggests that MICU1 activation is involved in the mechanisms leading to dopaminergic neuronal cell loss via mitochondrial calcium overload in PINK1 deficiency. Further studies in other in vitro and in vivo model systems of PINK1 deficiency as well as, ideally, assessment in PINK1 mutant patient tissue is necessary to determine whether the observed transcriptional upregulation of micul is specific for pinkel zebrafish or more generally observed in PINK1 deficiency. However, any attempts to further study the observed rescue effect of both MCU and MICU1 inactivation in higher
vertebrates will be challenging due to the absence of dopaminergic neuronal cell loss in Pink1 k/o mice (Olveras-Salva et al., 2011). Of note, lack of MCU does not protect heart cells against ischemic cell death despite an abolished effect of ischemia on the activation of the mitochondrial permeability transition pore in Muc1−/− mice (Pan et al., 2013). Further work is required to confirm that the observed beneficial effect of mcu k/d in our pink1−/− larvae is indeed due to a modulatory effect on mitochondrial calcium homeostasis with resulting normalization of mitochondrial respiratory chain function rather than exerting its effect via modulation of other mechanisms linked to the pathogenesis of PD such as an effect on mitophagy (Sousa et al., 2003; Deas et al., 2011).

All three VDAC isoforms are equivalent in allowing mitochondrial calcium loading upon agonist stimulation, but only VDAC1 silencing selectively impairs the transfer of the low-amplitude apoprtotic calcium signals (De Stefani et al., 2011). VDAC1 has been reported to physically interact with MCU (Liao et al., 2015). VDAC1 has been implicated in neuronal cell death in both Alzheimer’s disease (AD) and PD (Reddy, 2013; Alberio et al., 2014). In addition to its role in Calcium homeostasis, VDAC1 has also been implicated in PINK1/Parkin-mediated mitophagy (Geisler et al., 2010). We were therefore intrigued to observe a complete lack of a neuroprotective effect of VDAC1 inactivation in pink1−/− zebrasfish. This further suggests a specific and prominent role of MCU in PINK1 deficiency–linked neuronal cell death.

Due to the role of Ca2+ in activation of mitochondrial respiration (Chan et al., 2009), our findings point to energy metabolism as a contributing effector of neuron survival what is further supported by our mechanistic in silico studies. This perspective is in accordance with the selective vulnerability hypothesis that assumes the larger energetic load of dopaminergic neurons to be a major reason for their increased cell death rate in PD (Pissadaki & Bolam, 2013).

**Author contributions**

SS, MK MDC, HM, SS and MB undertook experiments and analysed the data; MM and AS developed the in silico model, OB and JK developed the underlying hypothesis and supervised the project. All authors contributed to the writing of the manuscript.

**Conflict of interests**

The authors declare no conflict of interest.

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**Abbreviations**

−/−, heterozygous; −/−/−, homozygous; AB, Wild type zebrafish line derived by George Streisinger by crossing Zebra fish lines A and B purchased from a pet store in Oregon.; CT, threshold cycle in qPCR experiments; DA, dopaminergic; DIO, digoxigenin, used for labelling DNA or RNA; DPF, days post fertilized; EF1ALPHA, eukaryotic translation elongation factor 1 alpha 1, like 1; EMRE, essential MCU regulator, also known as SMDC; HPF, HOURS post fertilized; MCU, mitochondrial calcium uniporter; MCU1, mitochondrial calcium uniporter regulator 1; MCU1, mitochondrial calcium uptake 1; MCU2, mitochondrial calcium uptake 2; MO, morpholino; PINK1, PTEN-induced putative kinase 1; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TH, tyrosine hydroxylase; Tl, tupfel long (wild type Zebrafish line); VDAC1, voltage dependent anion channel 1; WISH, whole mount in situ hybridisation; WT, wild type.

**References**

Ahuja, M. & Mualem, S. (2014) The gatekeepers of mitochondrial calcium influx: MCU1 and MCU2. EMBO Rep., 15, 205–206.

Alberio, T., Mannucciari, C., D’Agostino, G., Rizzuto, R. & Fasano, M. (2014) Altered dopamine homeostasis differentially affects mitochondrial voltage-dependent anion channels turnover. Biochim. Biophys. Acta, 1842, 1816–1822.

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sanacik, Y., Bao, X.R., Strittmatter, L. et al. (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature, 476, 341–345.

Bertram, R., Gram Pedersen, M., Luciani, D.S. & Sherman, A. (2006) A simplified model for mitochondrial ATP production. J. Theor. Biol., 243, 575–586.

Chan, C.S., Gertler, T.S. & Surmeier, D.J. (2009) Calcium homeostasis, selective vulnerability and Parkinson’s disease. Trends Neurosci., 32, 249–257.

De Stefani, D., Raffaello, A., Teardo, E., Szabo, I. & Rizzuto, R. (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature, 476, 336–340.

Deas, E., Wood, N.W. & Plun-Favreau, H. (2011) Mitophagy and Parkinson’s disease: the PINK1–parkin link. Biochim. Biophys. Acta, 1813, 623–633.

Exner, N., Lutz, A.K., Haass, C. & Winklhofer, K.F. (2012) Mitochondrial dysfunction in Parkinson’s disease: molecular mechanisms and pathophysiological consequences. EMBO J., 31, 3038–3062.

Flinn, L.J., Keatinge, M., Bertauf, S., Mortiboys, H., Matsuhi, S., De Felice, E., Woodroof, H.L., Brown, L. et al. (2013) TigarB causes mitochondrial dysfunction and neuronal loss in PINK1 deficiency. Ann. Neurol., 74, 837–847.

Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., Downward, J., Latchman, D.S. et al. (2009) PINK1-associated Parkinson’s disease is caused by neuronal vulnerability to calcium-induced cell death. Mol. Cell, 33, 627–638.

Gautier, C.A., Giaime, E., Caballero, E., Nunez, L., Song, Z., Chan, D., Vilaltenos, C. & Shen, J. (2012) Regulation of mitochondrial permeability transition pore by PINK1. Mol. Neurodegener., 7, 22.

Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J. & Springer, W. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol., 12, 119–131.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S. et al. (2013) The zebrafish genome sequence and its relationship to the human genome. Nature, 496, 498–503.

Jowett, T. & Lettice, L. (1994) Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. Trends Genet., 10, 73–74.

Kalia, L.V. & Lang, A.E. (2015) Parkinson’s disease. Lancet, 386, 896–912.

Kok, F.O., Shin, M., Ni, C.W., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J. et al. (2015) Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Dev. Cell, 32, 97–108.

Komin, N., Moein, M., Ellisman, M.H. & Skupin, A. (2015) Multiscale modeling indicates that temperature dependent [Ca2+]i spiking in astrocytes is quantitatively consistent with modulated SERCA activity. Neural. Plast., 2015, 683490.

Liao, Y., Hao, Y., Chen, H., He, Q., Yuan, Z. & Cheng, J. (2015) Mitochondrial calcium uniporter protein MCU is involved in oxidative stress-induced cell death. Protein Cell, 6, 434–442.

Marongiu, R., Spencer, B., Crews, L., Adame, A., Patrick, C., Trejo, M., Dallapiccola, B., Valente, E.M. et al. (2009) Mutant Pink1 induces mitochondrial dysfunction in a neuronal cell model of Parkinson’s disease by disturbing calcium flux. J. Neurochem., 108, 1561–1574.

Matthews, M., Trevorrow, B. & Matthews, J. (2002) A virtual tour of the guide for zebrafish users. Lab. Animal, 31, 34–40.

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Moore, C.L. (1971) Specific inhibition of mitochondrial Ca++ transport by ruthenium red. *Biochem. Biophys. Res. Co.*, 42, 298–305.

Murgia, M. & Rizzuto, R. (2015) Molecular diversity and pleiotropic role of the mitochondrial calcium uniporter. *Cell Calcium*, 58, 11–17.

Murphy, E., Pan, X., Nguyen, T., Liu, J., Holmstrom, K.M. & Finkel, T. (2014) Unresolved questions from the analysis of mice lacking MCU expression. *Biochem. Biophys. Res. Co.*, 449, 384–385.

Oliveras-Salva, M., Van Rompuy, A.S., Heeman, B., Van den Haute, C. & Baekelandt, V. (2011) Loss-of-function rodent models for parkin and PINK1. *J. Parkinsons Dis.*, 1, 229–251.

Pan, X., Liu, J., Nguyen, T., Liu, C., Sun, J., Teng, Y., Fergusson, M.M., Rovira, I.I. et al. (2013) The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. *Nat. Cell Biol.*, 15, 1464–1472.

Pissadaki, E.K. & Bolam, J.P. (2013) The energy cost of action potential propagation in dopamine neurons: clues to susceptibility in Parkinson’s disease. *Front. Comput. Neurosci.*, 7, 13.

Prudent, J., Popgeorgiev, N., Bonneau, B., Thibaut, J., Gadet, R., Lopez, J., Gonzalo, P., Rimokh, R. et al. (2013) Bel-wav and the mitochondrial calcium uniporter drive gastrula morphogenesis in zebrafish. *Nat. Commun.*, 4, 2330.

Qiu, J., Tan, Y.W., Hagenston, A.M., Martel, M.A., Kneisel, N., Skehel, P.A., Wyllie, D.J., Bading, H. et al. (2013) Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. *Nat. Commun.*, 4, 2034.

Reddy, P.H. (2013) Is the mitochondrial outermembrane protein VDAC1 a therapeutic target for Alzheimer’s disease? *Biochim. Biophys. Acta*, 1832, 67–75.

Rink, E. & Wullimann, M.F. (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tentorium). *Brain Res.*, 889, 316–330.

Shimizu, H., Schredelseker, J., Huang, J., Lu, K., Naghdi, S., Lu, F., Franklin, S., Fiji, H.D.G. et al. (2015) Mitochondrial Ca(2+) uptake by the voltage-dependent anion channel 2 regulates cardiac rhythmicity. *eLife*, 4, e04801.

Shoshan-Barmatz, V. & Golan, M. (2012) Mitochondrial VDAC1: function in cell life and death and a target for cancer therapy. *Curr. Med. Chem.*, 19, 714–735.

Sousa, S.C., Maciel, E.N., Vercesi, A.E. & Castilho, R.F. (2003) Ca2+-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. *FEBS Lett.*, 543, 179–183.

Stainier, D.Y., Kontarakis, Z. & Rossi, A. (2015) Making sense of anti-sense data. *Dev. Cell*, 32, 7–8.

Thisse, C. & Thisse, B. (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.*, 3, 59–69.

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