The Ca\(^2+\)/Mn\(^2+\) ion-pump PMR1 links elevation of cytosolic Ca\(^2+\) levels to \(\alpha\)-synuclein toxicity in Parkinson’s disease models

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Parkinson’s disease (PD) is characterized by the progressive loss of dopaminergic neurons, which arises from a yet elusive concurrence between genetic and environmental factors. The protein \(\alpha\)-synuclein (\(\alpha\)Syn), the principle toxic effector in PD, has been shown to interfere with neuronal Ca\(^2+\) fluxes, arguing for an involvement of deregulated Ca\(^2+\) homeostasis in this neuronal demise. Here, we identify the Golgi-resident Ca\(^2+\)/Mn\(^2+\) ATPase PMR1 (plasma membrane-related Ca\(^2+\) -ATPase 1) as a phylogenetically conserved mediator of \(\alpha\)Syn-driven changes in Ca\(^2+\) homeostasis and cytotoxicity. Expression of \(\alpha\)Syn in yeast resulted in elevated cytosolic Ca\(^2+\) levels and increased cell death, both of which could be inhibited by deletion of PMR1. Accordingly, absence of PMR1 prevented \(\alpha\)Syn-induced loss of dopaminergic neurons in nematodes and flies. In addition, \(\alpha\)Syn failed to compromise locomotion and survival of flies when PMR1 was absent. In conclusion, the \(\alpha\)Syn-driven rise of cytosolic Ca\(^2+\) levels is pivotal for its cytotoxicity and requires PMR1.

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\(\alpha\)-Synuclein (\(\alpha\)Syn) is a small, natively unfolded protein that is abundantly expressed in the central nervous system. It constitutes the major structural component of the intracellular protein inclusions termed Lewy bodies that define a pathological hallmark of Parkinson’s disease (PD).\(^1\) Mutations in or duplication and triplication of the gene coding for \(\alpha\)Syn all result in familial PD.\(^2,6\) An increasing body of evidence points towards a role for Ca\(^2+\) ions and Ca\(^2+\) -dependent processes in the pathology of PD in general\(^2,3\) and \(\alpha\)Syn-mediated neuronal death during PD in particular.\(^6,12\) The underlying mechanisms, however, remain enigmatic. A valuable tool to explore such pending questions are humanized yeast models based on heterologous expression of human \(\alpha\)Syn and pathogenic mutants, as they have not only recapitulated several features of PD but also have allowed to identify novel and evolutionary-conserved mediators and processes involved in the cytocidal consequences of \(\alpha\)Syn.\(^13–17\) As the regulation of Ca\(^2+\) homeostasis is highly conserved between yeast and mammals—with the advantage of reduced complexity and redundancy in yeast\(^18–20\) — we heterologously expressed human \(\alpha\)Syn in yeast to elucidate its effect on Ca\(^2+\) homeostasis and cell death. We could identify the Golgi-resident Ca\(^2+\)/Mn\(^2+\) ATPase PMR1 (plasma membrane-related Ca\(^2+\) -ATPase 1) as a mediator of \(\alpha\)Syn-driven changes in Ca\(^2+\) homeostasis and cytotoxicity in yeast, nematodes and flies.

Results

Heterologous expression of \(\alpha\)Syn in yeast disrupts Ca\(^2+\) homeostasis. We first quantified the basal cytosolic Ca\(^2+\) levels in yeast cells equipped with the Ca\(^2+\) -dependent reporter protein aequorin at standard external [Ca\(^{2+}\)] of \(\sim\)1 mM in the culture medium. In this setting, heterologous expression of \(\alpha\)Syn provoked an elevation of the cytosolic Ca\(^2+\) concentration, [Ca\(^{2+}\)]\(_{cyt}\), within the first 24 h of expression that subsided after 2 days of culturing (Figure 1A). Simultaneous determination of oxidative stress based on the superoxide-driven conversion of dihydroethidium to...

Abbreviations: PD, Parkinson’s disease; PMR1, plasma membrane-related Ca\(^{2+}\) -ATPase 1; GFP, green fluorescent protein; MES, 2-(N-morpholino) ethanesulphonic acid; ROS, reactive oxygen species; NAC, N-acetylcysteine; SOD, superoxide dismutase; PI, propidium iodide; RNAi, RNA interference; \(\alpha\)Syn, \(\alpha\)-synuclein; WT, wild type

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fluorescent ethidium (DHE Eth) demonstrated that the rise in \([Ca^{2+}]_{cyt}\) triggered by \(\alpha\text{Syn}\) (Figure 1a) coincided with an increase in oxidative stress. However, the \([Ca^{2+}]_{cyt}\) boost developed well before the massive production of reactive oxygen species (ROS), which started after 24 h of \(\alpha\text{Syn}\) expression (Figure 1b), and subsequent cell death (Figure 1c). This points towards a sequential course of events, where \(\alpha\text{Syn}\)-directed \([Ca^{2+}]_{cyt}\) increase occurs upstream of ROS generation and death. Immunoblot analysis revealed that \(\alpha\text{Syn}\) (driven by a galactose-promoter) was well expressed throughout the experiment, accumulating around 8 h after promoter induction (Figure 1e). The transient, \(\alpha\text{Syn}\)-induced increase in \([Ca^{2+}]_{cyt}\) was not related to a different localization of the protein itself, as – in line with
previous studies\textsuperscript{15} – green fluorescent protein (GFP)-tagged αSyn was prominently detectable at the plasma membrane within the first 2 days of expression (Figure 1f).

In addition to the disturbance in basal $[\text{Ca}^{2+}]_{\text{cyt}}$, αSyn altered the cellular response to high extracellular $[\text{Ca}^{2+}]$ pulses. Upon challenge with 150 mM $\text{Ca}^{2+}$, a rapid and transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was detectable that was largely amplified upon expression of αSyn (Figure 1d).

Addition of 2 mM ethylene glycol tetraacetic acid (a $\text{Ca}^{2+}$ chelator) to the culture medium partly inhibited the increase in basal $[\text{Ca}^{2+}]_{\text{cyt}}$ and at the same time ameliorated αSyn-induced accumulation of ROS and cell death (Figures 1g–i). Similar results were obtained using another $\text{Ca}^{2+}$ chelator, BAPTA-AM (1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester), which is cell-permeable and acts as intracellular $\text{Ca}^{2+}$ sponge (Supplementary Figures S1A and B). Thus, inhibition of the rise in basal $[\text{Ca}^{2+}]_{\text{cyt}}$ levels was largely unaffected by the addition of NAC (Figure 2d). By contrast, overexpression of cytosolic or mitochondrial superoxide dismutase (Sod1p or Sod2p, respectively) had no effect on αSyn-induced oxidative stress (Supplementary Figures S2A and B), arguing in favour of a rather broad pattern of cellular oxidative stress (that cannot by antagonized by overexpression of superoxid dismutases) as a cause of αSyn-induced cell death.

Oxidative stress and concomitant oxidation of DNA, proteins and lipids are essentially involved in neuronal cell death in various models of PD.\textsuperscript{21–23} Therefore, we tested whether the thiolic antioxidant N-acetylcysteine (NAC), a precursor of glutathione that has been shown to be protective in several PD-associated neurodegenerative scenarios,\textsuperscript{24–27} could block αSyn-induced generation of ROS and eventual cell death. Although supplementation with NAC did not prevent the first increase in ROS generation detectable within 24 h, it clearly inhibited massive ROS production and subsequent cell death occurring after 2 days of αSyn expression (Figures 2a–c). Basal $[\text{Ca}^{2+}]_{\text{cyt}}$ levels were largely unaffected by the addition of NAC (Figure 2d). By contrast, overexpression of cytosolic or mitochondrial superoxide dismutase (Sod1p or Sod2p, respectively) had no effect on αSyn-induced oxidative stress (Supplementary Figures S2A and B), arguing in favour of a rather broad pattern of cellular oxidative stress (that cannot by antagonized by overexpression of superoxid dismutases) as a cause of αSyn-induced cell death.

**Figure 2** The antioxidant NAC inhibits αSyn cytotoxicity. (a) Survival determined by clonogenicity of yeast cells expressing αSyn or harbouring the empty vector. Galactose growth medium (for promoter induction) has been supplemented or not with 20 mM or 30 mM NAC as indicated and cells were plated on YEPD agar plates at day 1 and day 2 to determine survival. Mean ± S.E.M., $n = 12–18$. Significances have been calculated for day 2, with ***$P<0.001$ and **$P<0.01$. (b) Flow cytometric quantification of oxidative stress by assessing the ROS-driven conversion of dihydroethidium to ethidium (DHE Eth) of cells described in (a). Mean ± S.E.M., $n = 8$. Significances have been calculated for day 2, with ***$P<0.001$ and *$P<0.05$. (c) Representative micrographs of dihydroethidium to ethidium (DHE Eth) staining of cells expressing αSyn or harbouring the empty vector after supplementation of galactose growth medium with 20 mM NAC for 2 days as flow cytometrically quantified in (b). (d) Determination of basal cytosolic Ca$^{2+}$ levels using aequorin-based luminescence measurement of yeast cells expressing αSyn or harbouring the empty vector after growth on galactose media supplemented or not with indicated concentrations of NAC for 20 h. Data has been normalized to equally treated vector control cells. Mean ± S.E.M., $n = 8$; **$P<0.01$ and *$P<0.05$.
PMR1 regulates α-synuclein toxicity

α-Syn cytotoxicity in yeast is facilitated by PMR1. To identify molecular determinants for α-Syn toxicity and dysregulation of Ca\(^{2+}\) homeostasis, we monitored α-Syn-induced consequences in numerous deletion mutants known to influence Ca\(^{2+}\) transport or signalling. Yeast codes for various homologues of mammalian Ca\(^{2+}\)-channels, transporters, sensors and buffers, including the plasma membrane-located voltage-dependent Ca\(^{2+}\) channel Cch1p/Mid1p, the secretory pathway and Golgi-resident Ca\(^{2+}\)-ATPase Pmr1p, the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase Cod1p, the vacuolar H\(^+\)/Ca\(^{2+}\)-exchanger Vcx1p, the plasma membrane Ca\(^{2+}\)-ATPase Pmc1p, the vacuolar cation channel Yvc1p – a putative homologue of the mammalian transient receptor potential potential canonical channels – or the general calcium sensor calmodulin as well as calciurein and calmodulin-dependent kinases.\(^{18–20}\) Using deletion mutants of these calcium-related regulators and automated quantification of ROS production, we evaluated the contribution of these proteins to α-Syn cytotoxicity. This approach identified PMR1 as a mediator of α-Syn-triggered ROS production (Figures 3a and b). In addition, the α-Syn-driven development of apoptotic and necrotic markers – assessed using AnnexinV/propidium iodide (PI) co-staining, which allows the discrimination between early apoptotic (AnnV\(^{+}\)) and late apoptotic/secondary necrotic (AnnV\(^{+}\)/PI\(^{+}\)) and necrotic (PI\(^{+}\)) cells – demonstrated that deletion of PMR1 completely inhibited α-Syn-driven increase in apoptotic and necrotic populations (Figures 3c and d). By contrast, the absence of CCH1, MID1, PMC1, VCX1, YVC1, or COD1 did neither alter α-Syn-instigated ROS-accumulation nor cell death markers as compared with wild type (WT; Figures 3a–d). Notably, deletion of these genes did not compromise α-Syn expression (Figure 3e). Determination of survival using clonogenic survival plating assays showed that the absence of Pmr1p potently inhibited α-Syn-induced cell death (Figure 3f). In addition, we generated a conditional PMR1 mutant by replacement of the promoter region of PMR1 with a tetO promoter that is active in the absence of doxycycline and prevents gene expression upon addition of 10 μg/ml of the chemical.\(^{28}\) Treatment of cells with doxycycline and thus depletion of Pmr1p largely reduced α-Syn-induced ROS production, confirming that Pmr1p is essentially involved in α-Syn cytotoxicity (Figures 3g and h).

To test whether α-Syn has any effect on the localization and/or expression levels of Pmr1p, we performed fluorescence microscopy as well as immunoblot analyses of yeast cells endogenously expressing a Pmr1p-GFP fusion protein. No obvious effect on Pmr1p protein levels or Golgi localization was apparent (Figures 4a and b). Using reverse transcription quantitative PCR, we could detect a slight upregulation of PMR1 mRNA (normalized to actin mRNA) after 14 h of α-Syn expression (Figure 4c).

We next analysed whether the rise in basal [Ca\(^{2+}\)]\(_{cyt}\) triggered by α-Syn is connected to the upregulation of a specific Ca\(^{2+}\)- influx system that has been shown to be stimulated in scenarios such as the accumulation of misfolded proteins in the endoplasmic reticulum.\(^{29}\) Different defects in vesicular trafficking,\(^{30,31}\) and depletion of Ca\(^{2+}\) from the endoplasmic reticulum, for example, upon PMR1 deficiency.\(^{32,33}\) In all of these cases, a high rate of Ca\(^{2+}\)-uptake via upregulation/activation of a high-affinity Ca\(^{2+}\)-influx system that involves Cch1p and Mid1p is triggered.\(^{34}\) Resultant elevated basal [Ca\(^{2+}\)]\(_{cyt}\) levels lead to the activation of Ca\(^{2+}\)-signalling pathways essential for initiation of compensatory mechanisms and subsequent cell survival.\(^{34}\)

Using quantitative PCR, we could indeed detect that after 14 h of expression, α-Syn significantly amplified CCH1 mRNA levels and slightly upregulated MID1 mRNA (Figure 4d). As shown for PMR1 mRNA levels (Figure 4c), this effect faded after 24 h (Figure 4d). Given these results, we tested whether proteins involved in the calmodulin/calciurein pathway and thus responsible for cellular Ca\(^{2+}\) sensing might alter α-Syn-instigated cytotoxicity. Determination of ROS production demonstrated that α-Syn toxicity was mostly unaffected by deletion of genes coding for the calciurein-responsive zinc finger transcription factor Crz1p and the calmodulin-dependent kinases Cmk1p and Cmk2p, while the absence of Cnb1p, the regulatory subunit of calciurein (and to some extent the absence of Cna2p, one of two isoforms of the catalytic subunit of calciurein), did aggravate and expedite α-Syn-induced oxidative stress (Figure 4e). Thus, an intact and functional Ca\(^{2+}\)-signalling pathway that essentially involves calciurein might contribute to a compensatory mechanism that partly counteracts and/or delays the toxic consequences of α-Syn expression.

Pmr1p mediates α-Syn-induced dysregulation of Ca\(^{2+}\) homeostasis. Next, we analysed whether the absence of proteins mediating cellular Ca\(^{2+}\)-transport modified the α-Syn-induced raise in cytosolic Ca\(^{2+}\) levels. Consistent with a link between α-Syn-mediated toxicity and dysregulation of Ca\(^{2+}\)-homeostasis, expression of α-Syn increased basal [Ca\(^{2+}\)]\(_{cyt}\) in all deletion mutants except in Δpmr1 cells (Figure 5a). In line with previous studies,\(^{35}\) deletion of PMR1 already caused increased basal [Ca\(^{2+}\)]\(_{cyt}\) as visible in the baseline recordings in Figure 5b. Furthermore, the absence of Pmr1p largely impaired α-Syn-induced amplification of rapid, transient cytosolic Ca\(^{2+}\)-peaks upon addition of 150 mM Ca\(^{2+}\) (Figure 5b). Although this remained mostly unaffected upon deletion of MID1, PMC1, VCX1 and YVC1, the absence of CCH1 or COD1 elevated the cellular response to external Ca\(^{2+}\)-pulses per se and prevented further peak amplification by α-Syn (Supplementary Figures S3A–F). Similar expression levels of aequorin were observed in all mutants (Figure 5c). Moreover, measurement of the transient elevation of [Ca\(^{2+}\)]\(_{cyt}\) following the addition of glucose to glucose-starved cells demonstrated that again α-Syn provoked an increase in [Ca\(^{2+}\)]\(_{cyt}\) peak amplitude, which was inhibited in Δpmr1 cells (Figure 5d) but present in all other deletion mutants tested (Figure 5e). Thus, Pmr1p is crucially involved in the cellular consequences following α-Syn expression, including (i) elevation of basal [Ca\(^{2+}\)]\(_{cyt}\), (ii) deregulation of the rapid cellular response to sudden external glucose or high Ca\(^{2+}\)-pulses, and (iii) accumulation of ROS and subsequent death.

Ca\(^{2+}\) rather than Mn\(^{2+}\)-transport activity of Pmr1p contributes to α-Syn toxicity. The primarily Golgi-resident pump Pmr1p not only supplies Ca\(^{2+}\) to both the Golgi complex and the endoplasmic reticulum but also constitutes
Figure 3  The Ca\(^{2+}\)/Mn\(^{2+}\) ATPase Pmr1p mediates αSyn cytotoxicity. (a) and (b) Quantification via fluorescence reader (a) and representative micrographs (b) of ROS production by assessing the ROS-driven conversion of dihydroethidium to ethidium (DHE → Eth) upon expression of αSyn for 24 h in WT yeast cells and indicated deletion mutants. Mean ± S.E.M., n = 8; ***P < 0.001 and **P < 0.01. (c and d) Flow cytometric quantification (e) and representative micrographs (d) of externalization of phosphatidylserine (AnnV\(^{+}\)) and loss of membrane integrity (PI\(^{+}\)) by Annexin V/PI co-staining of WT cells and indicated deletion mutants expressing αSyn for 48 h. Mean ± S.E.M., n = 6; ***P < 0.001. (e) Western blot analysis of αSyn expression in WT cells and indicated deletion mutants. Blots were probed with antibodies against FLAG-epitope to detect FLAG-tagged αSyn and against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. (f) Survival determined by clonogenicity of WT and Δpmr1 yeast cells expressing αSyn or harbouring the vector control after 24 h and 48 h of expression on galactose media and plating on YEPD agar plates. Mean ± S.E.M., n = 10; **P < 0.001. (g) Quantification of ROS accumulation (DHE → Eth) in yeast cells in which the promoter region of PMR1 has been replaced by a doxycycline-repressible promoter (TetO-PMR1). Doxycycline (Dox) was added in indicated concentrations and αSyn was expressed for 24 h or 48 h. Mean ± S.E.M., n = 8; ***P < 0.001. (h) Q-PCR-based quantification of PMR1 mRNA levels in yeast cells described in (g) after treatment with 10 μg/ml Doxycycline (Dox) and αSyn expression for 12 h. Data have been normalized to mRNA levels of actin. Means ± S.E.M., n = 3. Asterisks indicate significance between untreated and Doxy-treated cells, ***P < 0.001.
an important route to detoxify excess manganese, providing Mn\(^{2+}\) for protein glycosylation in the Golgi complex.\(^{36}\) Thus, deletion of PMR1 results in (i) depletion of endoplasmic reticulum Ca\(^{2+}\) stores,\(^{33}\) which in turn causes elevated [Ca\(^{2+}\)]\(\text{cyt}\) via increased Ca\(^{2+}\) uptake, as well as in (ii) hypersensitivity to manganese.\(^{36}\) Defects in cellular manganese homeostasis as well as exposure to manganese have been associated with PD and PD-like syndroms in humans\(^{37,38}\) and several model systems.\(^{39–43}\) Thus, we performed spotting assays on galactose plates containing high levels of manganese, demonstrating that exposure to manganese slightly aggravated α-Syn-instigated cytotoxicity in concentrations that did not affect isogenic control cells (2 mM and 4 mM Mn\(^{2+}\) ions, respectively; Figure 6a). Upon PMR1 deletion, Mn\(^{2+}\) was highly toxic (Figures 6a and b).

Furthermore, we generated strains that overexpress Pmr1p alone or in combination with α-Syn and subjected these strains to spotting and clonogenic survival assays on galactose plates with and without manganese. Notably, high levels of Pmr1p were per se toxic to yeast cells, and combined overexpression of Pmr1p and α-Syn killed >95% of all cells (Figures 6b and c). Complementation analyses in PMR1-deficient cells demonstrated that expression of Pmr1p could (i) restore α-Syn cytotoxicity and (ii) suppress manganese toxicity (Figures 6b and c).

To further investigate the contribution of Ca\(^{2+}\) versus Mn\(^{2+}\) transport activity of Pmr1p to the toxic consequences of α-Syn expression, we additionally transfected WT and PMR1-deficient cells with two point mutants of Pmr1p that were defective for transport of either Ca\(^{2+}\) ions (Pmr1pD53A) or Mn\(^{2+}\) ions (Pmr1pQ783A).\(^{36,44}\) Compared with native Pmr1p, the overexpression of these point mutants in the background of WT cells still was less toxic than that of native Pmr1p, and both mutated variants were slightly less effective in enforcing...
Syn cytotoxicity as determined in spotting assays and survival plating on galactose plates with and without addition of Mn$^{2+}$ (Figures 7a and b). Conducting the same experiments in PMR1-deleted cells demonstrated that native Pmr1p and Pmr1p$_{Q783A}$, which displays a selective loss of Mn$^{2+}$ transport, both efficiently reinstated aSyn toxicity, whereas the Pmr1p$_{D53A}$ variant deficient in Ca$^{2+}$ transport partly lost this ability (Figures 7a and b). Expression of the Pmr1p variants and aSyn was confirmed in all strains (Figure 7c). These data indicate that the Ca$^{2+}$ transport activity of Pmr1p – rather than that of Mn$^{2+}$ – contributes to aSyn-induced cell killing.

aSyn neurotoxicity in nematodes and flies requires PMR1. To test and challenge our finding in vivo, we analysed the effects of PMR1 depletion on aSyn neurotoxicity in the nematode Caenorhabditis elegans and in the fruit fly Drosophila melanogaster. Nematodes expressing human aSyn directed by the dopamine transporter (dat-1) promoter were examined for survival of dopaminergic neurons. In WT nematodes, expression of aSyn resulted in the death of B40% of the dopaminergic neurons, while only B20% died.

Figure 5 Pmr1p is involved in aSyn-induced dysregulation of Ca$^{2+}$ homeostasis. (a) Aequorin-luminescence-based determination of basal cytosolic Ca$^{2+}$ levels in WT cells and indicated deletion mutants expressing aSyn for 20 h. Data was normalized to corresponding isogenic vector control. Mean ± S.E.M., n = 12; ***P<0.001; NS, not significant. (b) Aequorin-equipped WT and Δpmr1 yeast cells expressing aSyn or harbouring the vector control were challenged with 150 mM CaCl$_2$ and transient [Ca$^{2+}$]$_{cyt}$ responses were observed for 50 s. Mean ± S.E.M., n = 6. (c) Western blot analysis of aequorin expression and aSyn expression in WT cells and indicated deletion mutants. Blots were probed with antibodies directed against aequorin, against FLAG-epitope to detect FLAG-tagged aSyn and against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. (d) Aequorin-equipped WT and Δpmr1 cells constitutively expressing aSyn (using the expression vector pGGE181) or harbouring the empty pGGE181 vector (Ctrl.) were starved for glucose, supplemented with low doses of Ca$^{2+}$ (10 mM) and subsequently challenged with 80 mM glucose. Transient [Ca$^{2+}$]$_{cyt}$ responses were monitored. Data represent average recordings, n ≥ 9. (e) Maximum [Ca$^{2+}$]$_{cyt}$ peak amplitude after addition of 80 mM glucose as depicted in (d) in aequorin-equipped WT cells and indicated deletion mutants upon expression of aSyn. Mean ± S.E.M., n ≥ 9; ***P<0.001; NS, not significant.
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Figure 6 Expression of Pmr1p restores αSyn cytotoxicity and suppresses manganese toxicity of PMR1-deficient cells. (a) Spotting assays of WT and Δpmr1 yeast cells expressing αSyn or harbouring the vector control. Cells were grown for 24 h in galactose media and spotted in fivefold serial dilutions onto glucose (αSyn expression repressed) and galactose (αSyn expression induced) agar plates supplemented or not with 2 mM or 4 mM Mn²⁺, respectively. (b) Spotting assays of WT and Δpmr1 yeast cells expressing either αSyn or Pmr1p alone or in combination or harbouring the corresponding vector controls. Cells were grown for 24 h in galactose media and spotted in fivefold serial dilutions onto glucose (Pmr1p and αSyn expression repressed) and galactose (Pmr1p and αSyn expression induced) agar plates supplemented or not with 4 mM Mn²⁺. (c) Quantification of clonogenic survival of cells described in (b) after plating on galactose agar plates supplemented or not with 4 mM Mn²⁺. Both Pmr1p as well as αSyn expression are driven by a galactose promoter. Mean ± S.E.M., n = 6–12; ***P < 0.001 and **P < 0.01; NS, not significant. Unless otherwise specified, asterisks indicate significances to similarly treated, isogenic control cells harbouring both empty vectors.

αSyn (Figure 8e). As αSyn is known to provoke locomotive deficits and the selective loss of tyrosine hydroxylase-positive dopaminergic neurons in Drosophila PD models, we tested for a possible involvement of SpoCk in these neurotoxic consequences. The αSyn-induced decline in negative geotaxis (which drives flies to walk upwards after being tapped to the bottom of a vial) was prevented by depletion of SpoCk (Figure 8f). Furthermore, expression of αSyn caused a significant loss of dopaminergic neurons in defined clusters of the brain, and this effect was absent when SpoCk was depleted (Figures 8g and h). Thus, the toxic consequences of αSyn expression in yeast, nematodes and flies essentially involve the Ca²⁺-ATPase PMR1.

Discussion

Diverse Ca²⁺ signals govern a myriad of vital functions, including mechanisms of fundamental neuronal biology such as synaptic transmission, plasticity, regulated neurite outgrowth and synaptogenesis as well as pivotal generic processes like proliferation, transcription, differentiation and apoptosis. In particular, the mitochondrial cell death pathway is susceptible to elevated calcium concentrations. Here, we establish that αSyn cytotoxicity is governed through sequentially occurring events, where the PMR1-dependent generation of a [Ca²⁺]cyt increase precedes a burst of oxidative radicals that ultimately triggers cell death. In fact, the cytoidal effects of αSyn are reduced by treatment of cells with Ca²⁺ chelators or by PMR1 deletion as well as via treatment with the generic antioxidant NAC. Thus, whether αSyn is able to trigger elevated basal cytosolic Ca²⁺ levels appears crucial for the subsequent cellular death. However, the transient [Ca²⁺]cyt peaks following high external Ca²⁺ or glucose pulses in αSyn-expressing cells, while remaining a good predictor of toxicity, are not fully stringent, as under specific conditions (e.g., upon deletion of COD1 or CCH1), high Ca²⁺ pulses were able to trigger massive transient [Ca²⁺]cyt peaks without αSyn expression (Figure 5 and Supplementary Figure S3). The prominent role of Ca²⁺ in αSyn-triggered cell death predicts that cellular Ca²⁺ sensing/signalling mechanisms may modulate the detrimental effects of αSyn expression. In fact, disruption of calcineurin signalling results in exacerbated toxicity, suggesting a compensatory mechanism based on the recognition of abnormal Ca²⁺ levels that partly counteracts the toxic consequences of αSyn. Cellular survival depends on tightly controlled Ca²⁺ fluxes between cellular organelles as well as across the plasma membrane. Impaired Ca²⁺ homeostasis and dysfunctional Ca²⁺ signalling are implicated in a broad variety of neurodegenerative diseases besides PD, including Alzheimer’s disease, Huntington’s disease, Glaucoma, Amyotrophic Lateral Sclerosis, Epilepsy and even the psychiatric disorder Schizophrenia. Upon Ca²⁺ overload, mitochondria readily sequester and accumulate Ca²⁺, which leads to enhanced production of ROS, and subsequently to dissipation of mitochondrial transmembrane potential, opening of the mitochondrial permeability transition pore and cellular demise. For αSyn-induced cell death in particular, where the lethal role of mitochondria has been clearly established, the molecular axis of toxicity might therefore converge in this organelle.

in PMR1-deficient (pmr-1(tm1840)) animals (Figure 8a). Quantification of cytosolic Ca²⁺ levels in αSyn-expressing dopaminergic neurons using the Ca²⁺-sensitive fluorescent reporter protein GCaMP2.0 revealed that αSyn elevated the resting [Ca²⁺]cyt in WT nematodes but not in pmr-1(tm1840) mutants (Figure 8b). In flies, the pan-neuronal elav-GAL4-driven expression of human αSyn significantly enhanced organisal death of male and female animals upon treatment with manganese. This effect was largely revised by RNA interference (RNAi)-mediated depletion of the Drosophila homologue of PMR1 (SpoCk) (Figures 8c and d). The absence of SpoCk did not affect the expression of
Our findings establish Pmr1p as a conserved mediator of aSyn cytotoxicity in yeast, nematodes and flies and indicate a toxic role for Ca\(^{2+}\) in the pathology of PD. Enhancement of cytosolic calcium levels upon aSyn expression seems crucial for subsequent toxicity, a deadly road that requires PMR1.

**Experimental Procedures**

*Saccharomyces cerevisiae* strains, plasmids and media. Experiments were carried out in BY4741 (MATa his3D1 leu2D0 met15D0 ura3D0) and corresponding null mutants Δpmr1, Δpmc1, Δcch1, Δmid1, Δcna1, Δcna2, Δcnb1, Δcnb2, Δcmk1 and Δcmk2 as well as in BY4741 harbouring endogenously GFP-tagged PMR1 (Euroscarf, Frankfurt, Germany). Strains were grown on SC medium containing 0.17% yeast nitrogen base (Difco, BD Biosciences, Schwechat, Austria), 0.5% (NH₄)₂SO₄ and 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine and 320 mg/l uracil with 2% glucose (SCD) or 2% galactose (SCG). Previously described αSyn-constructs in pESC-His (galactose promoter) or pUG23-His (methionine-repressible promoter) 14 or pGGE181 (constitutive promoter) 17 were deployed. To monitor cytosolic Ca\(^{2+}\) levels, strains were transformed with the pYX212 vector encoding cytosolic aequorin (pYX212-cytAEQ) (kind gift from E. Martegani, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy). To construct a conditional PMR1 mutant, the promoter region of PMR1 has been replaced by a tetO promoter (which prevents gene

![Figure 7](image-url)
PMR1 is critical for αSyn neurotoxicity in nematodes and flies. (a) Survival of C. elegans dopaminergic neurons in WT or PMR-1-deficient (pmr-1(tm1840)) animals expressing GFP and αSyn. Mean ± S.E.M., n > 250 individual animals; ***P < 0.001. (b) Fluorescence-based quantification of cytoplasmic Ca²⁺ levels in WT or PMR-1-deficient (pmr-1(tm1840)) nematodes expressing the Ca²⁺ indicator GCaMP2.0 and αSyn. Mean ± S.E.M., n > 150 dopaminergic neurons. ***P < 0.001. (c and d) Survival of male (c) and female (d) WT flies and of flies either expressing human αSyn or an RNAi depleting SPoCk after treatment with Mn²⁺. Means ± S.E.M., n = 12–20 with 35–40 flies per experiment; ***P < 0.001. (e) Immunoblot analysis of brain lysates obtained from flies expressing human αSyn driven by elav-GAL4 with or without co-expression of an RNAi-depleting SPoCk using antibodies directed against human αSyn or Drosophila α-tubulin as loading control. (f) Climbing activity of female flies described in (d) after 24 h of Mn²⁺ treatment. Means ± S.E.M., n = 6–10 with 8 flies per experiment; ***P < 0.001 and *P < 0.05. (g and h) Total count of tyrosine hydroxylase (TH)-immunoreactive dopaminergic neurons (TH-IR) in the DM, PM and DL1 brain clusters of female flies expressing αSyn alone or in combination with an RNAi-depleting SPoCk after treatment with Mn²⁺ for 96 h. Representative confocal microscopy images of dissected brains immunostained for TH and for Bruchpilot (BRPN1, red) to visualize brain structure are shown in (h). Neuronal counts were quantified by inspection of the individual planes of the z-stack. Means ± S.E.M., n = 5–10; **P < 0.01 and *P < 0.05.
after 2 days (YEPD plates) or 3 days (galactose plates) of growth using a Scanner
colony counter (LemnaTec, Wuerzen, Germany). To measure the level of
cellular oxidative stress, cultures were subjected to DHE staining at indicated
time points, followed by quantification using a fluorescence reader or
flow cytometry as previously described.
Externalization of phosphatidylserine and loss of membrane integrity was quantified
after 48 h of αSyn expression using Annexin V/propidium iodide co-staining as previously described.
For quantifications using flow cytometry (FACSArray, BD Biosciences, Schwechat, Austria),
30,000 cells were evaluated and analysed on BD FACSDiva software. Same
cells were visualized via epifluorescence microscopy on a Zeiss Axioskop
microscope (Zeiss, Vienna, Austria). Notably, at least four different clones were
tested after plasmid transformation to rule out clonal variations. For experiments with the Ca2+
chelators ethylene glycol tetraacetic acid and
BAPTA-AM (Sigma, Vienna, Austria) and the antioxidant NAC (Sigma), cultures
were either grown for 7 h after the shift on SCG for induction of αSyn expression
and then supplemented with 2 mM ethylene glycol tetraacetic acid or 380 μM
BAPTA-AM or treated with 20 mM and 30 mM NAC directly after the shift.
For spotting assays, cells were grown in SCG for 24 h, adjusted to 5 · 10^6 cells/ml and
spotted in fivefold serial dilutions onto glucose (expression repressed)
and galactose (expression induced) agar plates supplemented or not with indicated concentrations of Mn2+.

**S. cerevisiae cytosolic Ca2+ measurement.** [Ca2+]i was measured using yeast strains carrying the vector pYX212 encoding the bioluminescent
protein aequorin under the control of a TPI promoter. For analysis of resting, basal
[Ca2+]i and of the cellular response to high doses of external Ca2+, cells
expressing αSyn under a galactose-inducible promoter (pESC-His) and equipped
with pYX212-αCTAEQ were inoculated in SCG to OD600 0.1, grown to midlog phase
and shifted to SCG for induction of αSyn expression. At indicated time points, an
equivalent of 6 · 10^6 cells was transferred into a 96 well plate and harvested
by centrifugation. The pellets were resuspended in 200 μl SCG containing 4 μM
coelenterazine and incubated for 1 h in the dark. To remove excess
coelenterazine, the cells were washed once with fresh SCG and subsequently
incubated for further 30 min. A LUMistar Galaxy Luminometer (BMG Labotechnologies,
Offenbach, Germany) was used to measure basal [Ca2+]i as well as the response of
[Ca2+]i to external Ca2+ shocks. The basal luminescence was measured per well in 0.5-s
intervals for 25 s, whereas for kinetic luminescence measurements the signal was recorded for 70 s. In order to investigate the kinetics
of the cellular response to an external Ca2+ shock, a pump injected 40 μl of a
0.8 M CaCl2 solution into each well. During all measurements, the plate
was shaken and incubated at 28 °C. The luminescence signal was normalized to the
OD600 of each well and reported in relative luminescence units.

Reverse transcription quantitative PCR. To determine mRNA levels in yeast,
total RNA was extracted from respective strains using Qiagen RNaseasy kit
(Qiagen, Hilden, Germany) with 5 · 10^6 cells per extraction. Extracting DNA
was removed by DNase I digestion using Qiagen RNase-Free DNase Set
and RNA was cleaned up according to the Qiagen RNA cleanup and concentration
protocol. RNA concentrations were determined with a NanoDrop Spectrophotometer
(NanoDrop Technologies, Thermo Scientific, Vienna, Austria), and
100 ng were used for detection of mRNA levels of PMR1, CCH1 and MID1 and of
actin mRNA (as endogenous housekeeping gene) via reverse transcription and
quantitative PCR amplification using SensiMixTM SYBR one-Step Kit (Bioline,
Wiener Neudorf, Austria) and a Corbett Research R6000 PCR machine
(Qiagen). The following primers were used at a concentration of 300 nM: PMR1
primers 5′-TCTTATGCGGTGCTGTGCTAT-3′ (forward) and 5′-TCTTATGCGGT
GCTGTGCTAT-3′ (reverse); CCH1 primers 5′-GTACGTTAATGTTGTCGAC-3′
(forward) and 5′-GCGTCTTTCCTCTAAGTGAA-3′ (reverse); MID1 primers 5′-
GAAGGCTACCTGCAAGTCTG-3′ (forward) and 5′-GGCGAAGATGTTATCACAA-3′
(reverse), all amplifying a length between 150 and 160 bp. Cycling conditions were 10 min at 42 °C and 10 min at 95 °C,
followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C. The obtained mRNA levels were normalized to the mRNA levels of the actin
housekeeping gene within the same sample.

**Statistical analysis.** A one-way ANOVA followed by a Bonferroni post-hoc
test was used to calculate P-values. For survival of Drosophila, a two-way ANOVA
with time and strain as independent factors followed by a Bonferroni post-hoc test
was used.

**C. elegans strains and genetics.** We followed standard procedures for C.
elegans strain maintenance.36Nematode-rearing temperature was kept at 20 C,
unless noted otherwise. The following strains were used in this study: N2: WT
Bristol isolate (wt), pmr-1(tm1840), Ex[dat-GCaMP2.0], pmr-1(tm1840);Ex[dat-
GCaMP2.0], BZ555: egl-10[F3hsp-GFP], UA44: balb[F3hsp-αSyn, pGAM-GFP],
Ex[dat-GCaMP2], pmr-1(tm1840);Ex[dat-αSyn], pGAM-GFP, pGAM-GCaMP2.0]
and pmr-1(tm1840);Ex[dat-αSyn], pGAM-GFP. The BZ555 and
UA44 strains were generously provided by Guy Caldwell (Department of Biological
Sciences, The University of Alberta).

**C. elegans neurodegeneration analysis.** Seven-day-old animals were used
for αSyn-induced neurodegeneration quantification. The four CEP
dopaminergic neurons in the worm of the head were scored as described previously.23Experiments were repeated four times, and statistical analyses were
performed using the GraphPad Prism software package (GraphPad Software,
San Diego, USA). Analysis of variance (ANOVA) was used for comparisons of
multiple groups of values (in both approaches of neurodegeneration analysis),
followed by Bonferroni multiple-group comparison tests.

**C. elegans monitoring of cytosolic Ca2+ levels.** For intracellular
Ca2+ monitoring experiments, transgenic animals expressing the Ca2+ reporter
GCaMP2.0 in dopaminergic neurons were examined under a Zeiss AxioImager
22 epifluorescence microscope (Zeiss, Thessaloniki, Greece). The four CEP
dopaminergic neurons in the worm of the head were imaged. Only neurons of very
initial stages of degeneration (based on morphological features using DIC
microscopy) were used for analysis, as the expression of GCaMP2.0 ceases
during later stages of neurodegeneration. The expression intensity of GCaMP2.0
was calculated by using the ImageJ software (http://rsb.info.nih.gov/ij/).

**D. melanogaster strains, genetics and survival.** The line UAS-αSyn
was obtained from the Bloomington Stock Centre (Indiana University, USA). The
UAS-CG32451RNAi (SPock, the Drosophila homologue of PMR1) line
(transformant 110379) was obtained from the Vienna Drosophila RNAi Centre
(Vienna, Austria). Lines expressing αSyn were crossed with the RNAi
line to create the following stable stocks of flies: UAS-CG32451RNAi/ UAS-
CG32451RNAi; UAS-αSyn/UAS-αSyn. A chromosome III-linked elav-GAL4
enhancer trap line was used to drive expression. To determine survival
upon challenge with manganese, 1-3-day-old flies (both sexes, kept separately)
were incubated at 29 °C for 24 h and transferred into fresh vials with filter papers soaked
with solution containing 10% sucrose and 20 mM MnCl2. Filters were kept at
all times and numbers of dead flies were recorded at indicated time points.
experiment was performed with 35–40 flies and repeated 12–20 times (as indicated in the respective figure legend).

D. melanogaster determination of locomotive ability. To determine climbing ability upon supplementation of food with 20 mM Mn²⁺ ions for 24 h, eight female flies were placed into a vertical plastic tube with a diameter of 1.5 cm and gently tapped to the bottom. Flies reaching a specific mark (10 cm) within 5 min were considered to have reached the mark. The experiment was performed with 35–40 flies and repeated 12–20 times (as indicated in the respective figure legend).

D. melanogaster immunostaining and immunoblotting. Immunostaining was essentially performed as described before. Brains were dissected in H3L on ice, fixed in 4% PBS for 20 min and washed four times for 15 min in 0.3% PBT. After 1 h in PBT with 10% NGS at RT, brains were incubated for 2 days in PBT with 5% NGS containing primary antibodies against tyrosine hydroxylase (Millipore, Schwalbach, Germany) to detect dopaminergic neurons and against Bruchpilot (BRPNc82) to visualize brain structure and then washed in PBT four times and transferred onto slides in Vectashield (Vector laboratories, Lorrach, Germany). Image acquisition was performed with a confocal microscope (TCS SPS, Leica Microsystems, Wetzlar, Germany) using the LCS AF software (Leica Microsystems). For immunoblot analysis, 20–30 fly heads were homogenized on ice in 50 μl 2% SDS with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Equal volume of 2 × Laemmli was added, samples were incubated at 95 °C for 5 min and then kept at RT for 5 min before centrifugation for 5 min at 13000 × g and subsequent SDS-PAGE analysis. Blots were probed with primary antibodies against α-tubulin (Abcam) and α-Syn (Sigma) and respective secondary antibodies labelled with FITC or Cy3 (Invitrogen).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by the Austrian Science Fund P27490-B12 to FM and WNR, P24381-B20 to FM and TE, LIPOTOX to FM and DR, EMBO J2002: 21: 2343–2353. M. Bagh MB, Maiti AK, Jana S, Banerjee K, Roy A, Chakrabarti S. Oxidative and cytotoxic scavenging properties of N-acetylcysteine prevent dopamine mediated inhibition of Na⁺, K⁺–ATPase and mitochondrial electron transport chain activity in rat brain: implications in the neuroprotective therapy of Parkinson’s disease. Free Rad Biol Med 2008; 42: 574–581.

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