Calcineurin determines toxic versus beneficial responses to -synuclein

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Calcineurin determines toxic versus beneficial responses to α-synuclein


Calcineurin (CN) is a highly conserved Ca\(^{2+}\)-calmodulin (CaM)-dependent phosphatase that senses Ca\(^{2+}\) concentrations and transduces that information into cellular responses. Ca\(^{2+}\) homeostasis is disrupted by α-synuclein (α-syn), a small lipid binding protein whose misfolding and accumulation is a pathological hallmark of several neurodegenerative diseases. We report that α-syn, from yeast to neurons, leads to sustained highly elevated levels of cytoplasmic Ca\(^{2+}\), thereby activating a CaM-CN cascade that engages substrates that result in toxicity. Surprisingly, complete inhibition of CN also results in toxicity. Limiting the availability of CaM shifts CN’s spectrum of substrates toward protective pathways. Modulating CN or CN’s substrates with highly selective genetic and pharmacological tools (FK506) does the same. FK506 crosses the blood brain barrier, is well tolerated in humans, and is active in neurons and glia. Thus, a tunable response to CN, which has been conserved for a billion years, can be targeted to rebalance the phosphatase’s activities from toxic toward beneficial substrates. These findings have immediate therapeutic implications for synucleinopathies.

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NFAT | TORC2 | neuroinflammation | Crz1 | Slm2

Ca\(^{2+}\) homeostasis is indispensable for the well being of all living organisms. Ca\(^{2+}\) homeostasis is disrupted by α-synuclein (α-syn), whose misfolding plays a major role in neurodegenerative diseases termed synucleinopathies, such as Parkinson disease. We report that α-syn can induce sustained and highly elevated levels of cytoplasmic Ca\(^{2+}\), thereby activating a calcineurin (CN) cascade that results in toxicity. CN is a highly conserved Ca\(^{2+}\)-calmodulin (CaM)-dependent phosphatase critical for sensing Ca\(^{2+}\) concentrations and transducing that information into cellular responses. Limiting, but not eliminating, the availability of CaM, CN and/or CN substrates directly with genetic or pharmacological tools shifts the α-syn-induced CN cascade to a protective mode. This has mechanistic implications for CN’s activity and provides a therapeutic venue for the treatment of synucleinopathies.

Significance

Ca\(^{2+}\) homeostasis is indispensable for the well being of all living organisms. Ca\(^{2+}\) homeostasis is disrupted by α-synuclein (α-syn), whose misfolding plays a major role in neurodegenerative diseases termed synucleinopathies, such as Parkinson disease. We report that α-syn can induce sustained and highly elevated levels of cytoplasmic Ca\(^{2+}\), thereby activating a calcineurin (CN) cascade that results in toxicity. CN is a highly conserved Ca\(^{2+}\)-calmodulin (CaM)-dependent phosphatase critical for sensing Ca\(^{2+}\) concentrations and transducing that information into cellular responses. Limiting, but not eliminating, the availability of CaM, CN and/or CN substrates directly with genetic or pharmacological tools shifts the α-syn-induced CN cascade to a protective mode. This has mechanistic implications for CN’s activity and provides a therapeutic venue for the treatment of synucleinopathies.


The authors declare no conflict of interest.

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an ~4-fold elevation in cytosolic Ca\(^{2+}\) at 4 h and cytosolic Ca\(^{2+}\) continued to climb to an almost 65-fold increase by 8 h (Fig. 1A).

The rise in Ca\(^{2+}\) derived from intracellular stores because it was sensitive to low concentrations of the intracellular calcium chelator 1,2-Bis(2-aminoxyethoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester, but not to EGTA in the extracellular medium (Fig. S1B). Importantly, the extreme elevation in intracellular Ca\(^{2+}\) preceded the major onset of cell death in the HiTox strain (Fig. S1C). Thus, the release of Ca\(^{2+}\) from intracellular stores exhibits strong dependence on the dosage of α-syn and sustained high levels of Ca\(^{2+}\) are subsequently associated with extensive cell death.

**The Rise in Intracellular Ca\(^{2+}\) Is Accompanied by a Decrease in Free Calmodulin.** The delay between Ca\(^{2+}\) elevation and cell death in the HiTox strain suggested that high Ca\(^{2+}\) levels might initiate a cascade of events that are ultimately toxic. Calmodulin (CaM in mammalian cells and Cmd1p in yeast) is a ubiquitous node through which Ca\(^{2+}\) activates numerous downstream targets. To determine if CaM plays a direct role in α-syn toxicity, we first expressed a genetically encoded sensor for free CaM (Fig. S2A and B). This genetically encoded sensor for free CaM (BSCaM\(_{mo}\)) contains a CaM-binding site flanked by CFP and YFP (24, 25). At low Ca\(^{2+}\) levels, CaM is largely unbound and is therefore able to bind BSCaM\(_{mo}\). As cytosolic Ca\(^{2+}\) rises, CaM engages its targets and binding to BSCaM\(_{mo}\) decreases, resulting in a real-time change in fluorescence resonance energy transfer (FRET) between CFP and YFP. Given that CaM expression is not affected by α-syn in yeast (22), changes in FRET signals from BSCaM\(_{mo}\) provide a quantitative measure of changes in free CaM in our strains.
In the control strain, where cytosolic Ca\(^{2+}\) remained low (Fig. L4), free Cmdp levels remained high and constant (Fig. 1B). In the HiTox strain, which experienced a sharp rise in cytosolic Ca\(^{2+}\) (Fig. L4), free Cmdp levels plummeted (Fig. 1B). To confirm that the change in FRET with BSCaM\(_{100}\) was indeed due to a reduction in free Cmdp, we overexpressed cmd1\(_{-}\). This restored normal FRET levels in HiTox cells (Fig. 1B).

**Sequestering Free Calmodulin Ameliorates α-Syn Toxicity in Yeast.** The decrease in free CaM caused by α-syn suggests CaM is engaging downstream targets. If this contributes to cell death, the binding of CaM by BSCaM\(_{100}\) should compete with those targets and reduce toxicity. That is, BSCaM\(_{100}\) should act not only as a sensor but as a sink for CaM (25) (Fig. S2C). To test this, control and HiTox strains expressing BSCaM\(_{100}\) (or a YFP control) were assayed for growth (Fig. 1C). Indeed, expression of BSCaM\(_{100}\) conferred strong protection to the HiTox strain.

Protection by BSCaM\(_{100}\) was not due to a trivial effect on α-syn expression (Fig. S2B). Nor was it due to a general response to proteotoxic stress because cells expressing proteins associated with other neurodegenerative diseases, polyglutamine expanded huntingtin fragment (Htt) and TDP43, at similarly toxic levels were not rescued by BSCaM\(_{100}\) (Fig. S2 D and E).

Expression of BSCaM\(_{100}\) did not significantly affect Ca\(^{2+}\) levels (Fig. S2F), suggesting that its protection against cell death was primarily due to the sequestration of Ca\(^{2+}\)-CaM. Indeed, the overexpression of wild-type cmd1 restored toxicity, whereas overexpression of a cmd1 mutant with impaired Ca\(^{2+}\) binding (26) did not (Fig. 1D and Fig. S2G).

Finally, we tested a completely different CaM sink, the IQ domain from myosin cardiac light chain (CYI\(_{2}\)). This alternative CaM binder also strongly rescued cells from α-syn toxicity (Fig. S2H). These findings establish that signaling through Ca\(^{2+}\)-CaM-dependent pathways plays an important role in α-syn toxicity in yeast.

**Sequestering Free Calmodulin Ameliorates α-Syn Toxicity in Neurons.** Next, we asked if Ca\(^{2+}\)-CaM was relevant to neuronal α-syn toxicity. We used primary cells derived from rat embryonic cortical cortices as a model because they are relatively homogenous (more than 80% neurons) and more readily obtained than dopaminergic neurons. Importantly, cortical neurons are strongly affected in patients with PD (27).

Rat embryonic cortical neurons were transduced with a lentivirus that expresses α-syn A53T, a mutation causing an autosomal-dominant form of PD (28) (about 50% infected; Fig. S3A). To test the effect of α-syn on Ca\(^{2+}\) homeostasis in these neurons, we used the Ca\(^{2+}\)-sensitive dye Fluo-4. Infected neurons were visualized with either mKate (control) or an mKate-α-syn fusion regulated by the synapsin promoter. Cells expressing mKate-α-syn had increased basal Ca\(^{2+}\) and responded less strongly to KCl-induced depolarization than cells expressing mKate alone (Fig. S3 B and C). Thus, as previously shown in other primary neuronal models (2–4), overexpression of α-syn perturbs Ca\(^{2+}\) homeostasis.

To determine if CaM plays a role in the toxicity of α-syn in neurons, we coinfected A53T-expressing neurons with another lentivirus encoding BSCaM\(_{100}\) or a control protein, LacZ. Differences in viability were assessed by cellular ATP content as well as by counting the number of cells positive for microtubule-associated protein 2 (MAP2), a neuron-specific marker. BSCaM\(_{100}\) provided a highly significant, dosage-dependent rescue of α-syn toxicity (Fig. 1 E and F and Fig. S3D).

**Calcineurin Activation by Calmodulin Has both Protective and Toxic Roles.** A central player in transducing Ca\(^{2+}\)-CaM’s signals is the highly conserved phosphatase CN (14). To investigate CN’s role in α-syn toxicity, we returned to yeast. CN function can be eliminated by deleting the regulatory subunit cnb1 alone or by the combined deletion of the catalytic subunits cna1 and cna2. As expected (29), neither manipulation affected the growth of the control strain (Fig. 24 and Fig. S4A). In the HiTox cells,
deleting CN eliminated BSCaM protection (Fig. S4B). Because BSCaM acts by titrating CaM, and CaM has many targets, this result suggests that CaM’s main effects in response to α-syn toxicity are mediated through the calcineurin pathway.

Surprisingly, however, both deletion and overexpression of CN increased toxicity in the HiTox strain (Fig. 2A and B). One explanation for these apparently contradictory results is that an intermediate level of CN activation is protective against α-syn, whereas either too much or too little is detrimental.

As a genetic test of this hypothesis, we used Rcn proteins as negative regulators of CN activity. Rcn1p has a higher affinity for CN than Rcn2p (30). Whereas neither protein affected the control strain, overexpressing rcn2 (but not rcn1) rescued α-syn toxicity (Fig. 2C).

As a pharmacological approach, we used FK506. Importantly, FK506 provides a means to continuously vary CN inhibition over a very broad range. FK506 inhibits CN by precisely the same mechanism in yeast and in mammals (29, 31) although higher concentrations of this drug are required in yeast than in mammalian cells (Materials and Methods). Consistent with our hypothesis, in the HiTox strain intermediate concentrations of FK506 protected against α-syn toxicity, whereas higher concentrations eliminated this protection (Fig. 2D).

Fig. 3. Partial calcineurin activity is necessary to protect against α-syn toxicity ex vivo and in vivo. (A, Upper) MAP2 staining from representative pictures of rat primary neuronal cultures coinfected with a lentivirus carrying LacZ as control and/or α-synA53T treated with various doses of FK506 for 14 d. (Lower) Rat cortical neurons infected with α-synA53T and/or LacZ as control treated with vehicle and/or increasing concentrations of FK506 for 14 d and assayed for viability for ATP content. *P < 0.05, one-way ANOVA, Dunnett’s multiple comparison test. Each data point was normalized against control LacZ at the same FK506 dose. (B, Upper) Dopaminergic neurons from mice infected with α-synA53T and/or mKate as control treated with vehicle and/or distinct concentrations of FK506. Cell survival was measured by counting TH positive neurons after 14 d of α-synA53T infection and drug treatment. *P < 0.05, one-way ANOVA, Dunnett’s multiple comparison test. (Lower) Representative pictures of infection efficiency of mKate α-syn in TH positive neurons. (C) Representative images of C. elegans dopaminergic neurons in worms expressing GFP alone (Left, all six anterior neurons are intact) and in α-syn-expressing worms treated with RNAi control (only four anterior DA neurons remain) or with RNAi for calcineurin (CN) (Right). Arrowheads show intact dopaminergic neuron cell bodies and arrows represent areas where dopaminergic neurons are absent. (D) The population of worms with WT (wild type) numbers of neurons remaining after treatment with RNAi for calcineurin (CN) or empty vector RNAi (control) in α-syn-expressing worms. For each experiment, three independent experiments were performed, with 30 worms per trial. *P < 0.05 (Student t test). See also Fig. S5C. (E) Same assay as in D except with worms overexpressing rcn-1. Bars 1 and 2 represent independent transgenic lines. *P < 0.005, **P < 0.0005 (Student t test). See also Fig. S5B.
Importantly, rescue by intermediate concentrations of FK506 in the HiTox strain was not due to a trivial effect on α-syn expression (Fig. S4C). Furthermore, the lack of rescue at high concentrations was not due to off-target toxicity, because FK506 had no effect on control cells even at the highest concentrations used (Fig. S4D). Moreover, FK506 effects were specific to the HiTox strain where CN activity is high; the compound had no effect on the less toxic IntTox strain, which has much lower levels of cytosolic Ca\(^{2+}\) (Fig. L4 and Fig. S4E). We further confirmed FK506 effects on CN activity using BSCaM\(_{\alpha}\), which sequesters CaM and reduces CN activation. The effects of FK506 should be greatly reduced in HiTox cells carrying BSCaM\(_{\alpha}\), and indeed they were (Fig. S4F).

Thus, both genetic and pharmacologic experiments in yeast confirm that an intermediate level of CN inhibition balances the protective versus toxic effects of Ca\(^{2+}\)-CaM/CN in response to α-syn.

**Decreasing Calcineurin Activity Protects Against α-Syn Toxicity in Neuronal Models.** Next, we asked if modulating CN activities could balance toxic versus protective responses to α-syn in primary rat cortical neurons. At all concentrations, FK506 had only a minor effect on the viability of neurons expressing the control protein LacZ (Fig. 3A). Intermediate concentrations of FK506 rescued neurons from the toxic effect of α-syn expression. (Note, in keeping with the fact that neurons have many more mechanisms for buffering calcium than yeast cells do, the concentration range for rescue was much broader in neurons than in yeast, spanning a 100-fold range.) Higher concentrations of FK506 were not protective and, in fact, increased toxicity (Fig. 3A).

We next asked if FK506 was protective in dopaminergic neurons, the cell type most classically implicated in Parkinson disease. We used primary cultures containing 30–40% dopaminergic neurons assessed by tyrosine hydroxylase (TH) positivity. To allow unambiguous identification, we infected neurons with an adenovirus associated virus encoding human α-syn\(_{\Delta32}\) tagged with mKate or with mKate alone (Fig. S5A). Fourteen days after infection, ~30% of the dopaminergic neurons expressing α-syn had died. Intermediate levels of FK506 rescued cells from α-syn toxicity, whereas the protective effects of the drug disappeared at higher doses (Fig. 3B).

To begin moving into multicellular organisms, we took advantage of a previously established nematode model of α-syn toxicity (22). In these worms, GFP and α-syn are both under the transcriptional control of the dopaminergic neuron-specific dat-1 promoter. Wild-type (WT) worms invariably have six dopaminergic neurons. As previously reported (18), expression of α-syn caused an age- and dose-dependent degeneration of dopaminergic neurons (Fig. 3C). We down-regulated CN activity in this model using two methods: direct knockdown of CN with RNAi and overexpression of its negative regulator, rnr-1.

To allow RNAi targeting of CN in DA neurons, we expressed the dsRNA transporter SID-1 in these cells using the dat-1 promoter (33). Knockdown of the catalytic subunit of CN, tax-6, had no effect on the dopaminergic neurons of control worms.
However, in α-syn–expressing worms, reducing the expression of CN by knockdown protected against toxicity (Fig. 3C and D).

Nematodes have only one known negative regulator of CN, rcn-1 (34). Several independent transgenic lines were established with different levels of rcn-1 expression. In two transgenic lines, we found that moderate rcn-1 expression was nontoxic (Fig. S5B) and also protected dopaminergic neurons against α-syn toxicity (Fig. 3E). Thus, establishing a biological mechanism conserved from single cells (yeast and primary mammalian neurons) to whole worms, chemical and genetic approaches, demonstrate that moderating CN activity is protective against α-syn toxicity.

Protective and Toxic Downstream Substrates of Calcineurin. Our finding that an intermediate level of CN activity is protective against α-syn suggests that some CN downstream targets provide protection, whereas others enhance toxicity. To investigate, we returned to yeast, focusing on three of CN’s best characterized substrates: Hph1p, Slm2p, and Crz1p. Remarkably, the processes regulated by all of these proteins are disturbed by α-syn in yeast and in neurons. Hph1p facilitates the posttranslational localization of proteins, especially those involved in vacuolar ion homeostasis and vesicular trafficking (35). Slm2p acts via the target of rapamycin complex 2 (TORC2) to support cytoskeletal organization and lipid homeostasis (20, 36–38). Crz1p is a transcription factor activated by calcineurin in response to various stresses (39). Importantly, overexpression of these genes under nonstress conditions had no effect on control cells (Fig. S6A).

Overexpressing hph1 or slm2 rescued α-syn toxicity in the HiTox strain (Fig. 4A). Overexpressing crz1, however, slightly enhanced toxicity. To better characterize this enhancement of toxicity, we overexpressed crz1 in the HiTox strain in the presence of BSCaM{\textsubscript{IQ}}. Overexpression of crz1 completely abrogated the protective effects of BSCaM{\textsubscript{IQ}}, confirming the role of crz1 in enhancing α-syn toxicity and validating the effect of BSCaM{\textsubscript{IQ}} on the CN pathway (Fig. S6B). Thus, Hph1p and Slm2p drive protective pathways in response to α-syn, whereas Crz1p drives detrimental ones.

We focused further on the protective substrate Slm2p and the toxic substrate Crz1p, because Slm2p and Crz1p pathways are
conserved from yeast to humans. Because CN is a phosphatase, we began by investigating the effect of α-syn on the phosphorylation status of Slm2p and Crz1p using selected reaction monitoring (SRM)-based mass spectrometry (MS) (40). This technique avoids the use of artificial protein tags, which can interfere with protein function, and more importantly, gives information about the nature of the phosphorylation sites in a highly specific and quantitative manner.

α-syn expression increased Slm2p phosphorylation. Protective doses of FK506 further increased phosphorylation, whereas toxic doses blunted it (Fig. 4B). Although the role(s) of phosphorylation in regulating Slm2p activity is not clear, this result is consistent with an interaction between CN and Slm2p driving a protective response against α-syn toxicity. To test this, point mutations in Slm2p that selectively abrogate its interaction with CN (41) eliminated protection against α-syn toxicity, although having no effect on control strains (Fig. 4C and Fig. S6C).

Slm2p acts upon the TORC2 signaling pathway (38). To see whether this pathway is also affected by α-syn, we assayed the expression of two yeast genes that are classically up-regulated when TORC2 is inhibited (42). yhr194c and dial1 were both up-regulated in response to α-syn expression, indicating that α-syn does indeed inhibit the TORC2 pathway (Fig. 4D). As predicted from their effects on CN, overexpression of slm2 or moderate concentrations of FK506 both decreased expression of yhr194c and dial1, indicating a restoration of TORC2 function (Fig. 4D).

Activation of the transcription factor Crz1p is normally protective for yeast cells under stress (39). However, our results suggest that Crz1p drives a toxic response to α-syn. Phosphorylation of Crz1p inhibits its transcriptional activity. Using SRM to monitor Crz1p phosphorylation, we found that it was reduced in response to α-syn, whereas protective and toxic doses of FK506 increased it (Fig. 4E). A mutant of Crz1p with increased affinity for CN (43) had no effect on control cells but increased toxicity in cells expressing α-syn (Fig. 4F and Fig. S6D). This high-affinity allele of crz1 also reduced the protective effect of BSCaM10, confirming that CaM’s role in α-syn toxicity is mediated through the CN pathway (Fig. S6E). Further, deleting crz1 strongly rescued growth in HiTox cells (Fig. 4F and Fig. S6D).

If Crz1 activation is indeed driving a toxic transcriptional response to α-syn, Crz1p’s transcriptional activity should be up-regulated in the HiTox strain. Moreover, if this activity is connected to the Ca2+–CaM/CN pathway cascade, it should be down-regulated by BSCaM10, by protective concentrations of FK506 or by deletion of CN. Using a β-galactosidase reporter for Crz1p transcriptional activity (44), each of these predictions was fulfilled (Fig. 4G). Thus, α-syn activates a toxic Crz1p-regulated response to Ca2+ in yeast. Moderate inhibition of CN activity prevents activation of this toxic pathway while still allowing protective responses mediated through substrates such as Slm2p to remain intact.

Downstream Consequences of Calcineurin Activation by α-Syn in Mice. To determine whether the effects of α-syn demonstrated in yeast are conserved in the central nervous system of mammals, we turned to an α-syn transgenic mouse model where α-syn is overexpressed in various regions of the brain, including areas affected in PD such as the olfactory bulb and cortex (45, 46). As they age, these mice display phenotypes reminiscent of PD, including neuroinflammation (increased numbers of astrocytes and microglia), as well as neuronal pathology. Calcineurin is highly expressed in both glia and neurons and, hence, could potentially contribute to protective or toxic responses in both.

First, we probed the status of the TORC2 pathway (activation was protective in yeast). In the brains of 12-mo-old mice, we monitored protein kinase C alpha (PKCα) phosphorylation at serine 657, a canonical downstream substrate of TORC2. PKCα phosphorylation was reduced in brain extracts from α-syn-overexpressing mice compared with controls (Fig. 5A). Thus, the inhibition of PKCα correlates with inhibition of the TORC2 pathway in mammalian brain in response to α-syn.

Although no direct homolog of crz1 exists in mammalian cells, nuclear factor of activated T cells (NFAT) is broadly accepted as its functional analog (41). Similar to Crz1p, calcineurin dephosphorylates various isoforms of NFAT, leading to their nuclear translocation. We monitored two isoforms, NFATc4 and NFATc3, which we found to be prominent in neurons and glia, respectively. In agreement with our yeast results, the neurons from the olfactory bulbs of the α-syn transgenic mice displayed increased nuclear neuronal localization of NFATc4, consistent with its activation (Fig. 5B). NFATc3 staining intensity and nuclear localization was increased in glia (Fig. 5B), indicating that it, too, was activated. Thus, a calcineurin-mediated response to α-syn was also conserved in the mammalian brain, in both neurons and glia.

NFAT Is Activated in Human Synucleinopathies. Finally, we examined NFAT activation in fixed postmortem tissue from humans diagnosed with PD or with a more aggressive synucleinopathy, dementia with Lewy bodies (DLB). We could not detect NFATc3; however, staining of NFATc4 was highly reproducible.
and specific, as it was competed with a peptide that was used to raise the antibody (Fig. S7). Four of the five brains we analyzed from nondiseased controls exhibited cytoplasmic but no nuclear staining for NFATc4. In contrast, NFATc4 immunoreactivity was localized to nuclei in most of the eight cases of PD and DLB consistent with its activation (Fig. 6).

The nuclear staining for NFATc4 in the diseased brains was evident across several different brain regions including the substantia nigra pars compacta (SNc), hippocampus, and frontal cortex, among others. In the frontal and cingulate cortices, nuclear NFATc4 staining was most prominent in the pyramidal neurons in layers 5 and 6. In DLB, these cortical layers bear the greatest burden of α-syn pathology (27). In the hippocampus, which is also critically involved in PD pathology (47), areas CA3 and CA4 stained more strongly than CA1 or CA2 or the subiculum. These results support an association between NFATc4 nuclear localization/activation and α-syn toxicity in human disease.

Discussion
We demonstrate that increasing levels of α-syn expression proportionally increase cytosolic Ca\(^{2+}\) concentrations, paralleling the extremely strong dosage dependence of α-syn toxicity previously described in our yeast models (18, 20) and in humans (23, 48). Sustained high cytosolic Ca\(^{2+}\) levels drive a Ca\(^{2+}/\)CaM-calcineurin cascade that activates a toxic program regulated by the transcription factor Crz1p in yeast and NFAT in mammalian cells. However, calcineurin activation also triggers protective responses against α-syn involving TORC2. The balance between these responses and presumably other unidentified calcineurin substrates is critical in determining the ultimate outcome of activating the Ca\(^{2+}/\)CaM-calcineurin pathway in response to α-syn (Fig. S8). Importantly, this multifaceted calcineurin-mediated response to α-syn, although first uncovered in yeast, is conserved in nematodes as well as mammalian neurons, glia, and brains, as, indeed, are the protective effects of moderate concentrations of FK506.

How does α-syn lead to increased cytosolic Ca\(^{2+}\)? Our data clearly indicate that it is released from intracellular stores. Given the lipid-binding properties of α-syn, the protein might perturb Ca\(^{2+}\) homeostasis directly (through damage to the membrane or membrane channels) or indirectly [as a consequence of the profound block in dopaminergic neurons (ER) to Golgi trafficking]. How might increased cytosolic Ca\(^{2+}\) determine calcineurin’s switch between protective and toxic substrates in a concentration-dependent manner? Differences in the sequence and accessibility of substrate docking sites, expression levels, and the number of dephosphorylations required to alter a substrate’s activity are all likely involved. These, in turn, will interface with differences in the degree of calcineurin activation, determined by cellular levels of CaM and Ca\(^{2+}\) in different compartments within the cell. Given the complexity of this problem, many strategies, including the use of genetically tractable organisms such as yeast, will be required to achieve a full understanding.

Because inhibition of calcineurin activity is routinely exploited clinically through the use of FK506 (tacrolimus) as an immunosuppressant, we now suggest that the repurposing of FK506, a compound that readily traverses the blood brain barrier merits investigation in the management of PD. Because it persists in the central nervous system long after systemic effects have resolved (49), intermittent dosing with this already FDA-approved drug could avoid systemic immunosuppression, while still providing a readily implemented, disease-modifying treatment strategy that targets a fundamental mechanism in the pathogenesis of α-synucleinopathies.

Indeed, FK506 was previously shown to have neuroprotective properties in mammalian PD models (50–53). Because FK506 impairs calcineurin function by locking it into a complex with FKBP12, FK506’s neuroprotective effects were thought to be mediated through FKBP12. Compounds that target FKBP12, without affecting calcineurin function, prevented neurodegeneration in specific animal models (54, 55) but failed to show reversal of PD motor symptoms in humans (56). Although we have yet to address any potential contributions of FKBP12 to the protective effects of FK506 that we have seen, our findings clearly establish the importance of reducing calcineurin activity to achieve neuroprotection.

Finally, our findings help explain why in PD, DA neurons in the SNc are particularly sensitive to α-syn–related dysfunction. Adult neurons in the SNc rely on voltage-dependent Ca\(^{2+}\) channels. These channels are regulated by CaM binding (57, 58) and drive autonomous pacemaking, which leads to relatively sustained elevations in cytosolic Ca\(^{2+}\) (9, 59). Intriguingly, CaM can be cross-linked to α-syn (60, 61), suggesting that α-syn could be locally sequestering CaM to further increase cytosolic Ca\(^{2+}\) flux through these channels. Moreover, SNc neurons have higher cytosolic dopamine levels than the less susceptible ventral tegmentum DA neurons (62). This neurotransmitter renders SNc neurons more susceptible to cell death and has also been linked to increased intracellular Ca\(^{2+}\) levels (62). The enhanced sensitivity of the SNc DA neurons to Ca\(^{2+}\) stress would exacerbate the defects in vesicle trafficking (18, 63, 64), mitochondrial dysfunction (21, 22), nitrosative stress (16, 17), and metal ion homeostasis (65) caused by α-syn. The combination is poised to create the “perfect storm” that devastates the SNc of patients with PD.

Materials and Methods
Primary Cells and Strains. Yeast strains containing α-syn were generated and induced as previously described (18). Rat cortical and mouse dopaminergic neurons were infected with a lentivirus carrying α-syn as described in SI Materials and Methods. In vivo models for α-syn in Caenorhabditis elegans and mice where generated as described (32, 46). Human samples from PD and DLB patients were obtained through the Massachusetts Alzheimer Disease Research Center as described in SI Materials and Methods.

Imaging Techniques. Ca\(^{2+}\) imaging was performed using aequorin (in yeast) and Fluo-4 (in neurons). CaM measurements were performed using FRET, MAP2 and TH staining in neurons were performed by immunofluorescence. NFAT staining in mice and human samples were performed by immunohistochemistry. Detailed description of all imaging techniques can be found in SI Materials and Methods.

Viability Assays. For neurons we used ATP and MAP2 positive staining, whereas for yeast cells, spotting assays, growth curves using OD\(_{600}\), and propidium iodine incorporation by cell cytometry were performed as viability assays. Detailed description of all viability techniques can be found in SI Materials and Methods.

Biochemistry Assays. To monitor phosphorylation of substrates selected re-action monitoring assays was performed. To monitor Crz1p activity in yeast we used the Crz1-dependent reporter element-luciferase reporter (44). Detailed description of these techniques can be found in SI Materials and Methods.

Molecular Biology. RNC1 overexpression and quantitation C. elegans was performed as described (34). RNA interference (RNAi) in C. elegans was performed as described (33). CaM mutants were performed by QuikChange XL site-directed mutagenesis as described in SI Materials and Methods.

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