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Tuning beneficial calcineurin phosphatase activation to counter α-synuclein toxicity in a yeast model of Parkinson's disease

Srishti Chawla*, Mikael Molin, Thomas Nystrom

Calcineurin (CN) is a calcium- and calmodulin-dependent serine/threonine that has been studied in many model organisms including yeast, filamentous fungi, plants, and mammals. Its biological functions range from ion homeostasis and virulence in lower eukaryotes to T-cell activation in humans by human nuclear factors of activated T-cells. CN is a heterodimeric protein consisting of a catalytic subunit, calcineurin A (Cna1p), which contains an active site with a dinuclear metal center, and a regulatory Ca^{2+} binding subunit called calcineurin B (Cnb1p) required to activate Cna1p. The calcineurin B subunit has been highly conserved through evolution: For example, the mammalian calcineurin B shows 54% identity with calcineurin B from *Saccharomyces cerevisiae*. Notably, CN regulates post-translational modifications of target proteins and gene expression in parallel. The substrate selection for CN includes, but is not limited to, transcription factors, ion pumps/channels, proteins associated with mitochondria, vesicle trafficking and the polarity machinery through interactions with microtubules. The docking sites such as Short linear motifs or LxVP drive CN's dynamic interaction and selection of its targets. The CN substrate networks in yeast and animals are distinct but share a common repertoire of target kinases, such as PKA, PKC, AKT, CAMKL, and proline-directed GSK3, CDK, and MAPK kinases. Rcn1p/RCAN1p, which regulates and activates CN in a feedback loop–dependent manner during Ca^{2+} stress, is the only CN target known to be conserved in yeast and mammals (Creamer, 2020). Interestingly, altered RCAN1 expression is associated with Down's syndrome whereas its elevated expression is associated with the onset of neuronal Alzheimer's disease (AD), Huntington's disease, and Parkinson's disease (PD) pathology in human patients.

In budding yeast, CN orchestrates the responses to various environmental stresses by dephosphorylating the transcription factor Crz1p. In humans, CN acts upon the nuclear factors of activated T-cells to activate T-cells and affect N-methyl-D-aspartate and inositol-1,4,5-trisphosphate receptors to mediate Ca^{2+} release from intracellular stores. The dephosphorylation of neuronal Na^+, K^+-ATPase is also mediated through CN thereby regulating neuronal excitability, and the release of neurotransmitters and hormones. To impede the binding of CN to its targets, CN inhibitors such as cyclosporin A and FK506 are utilized clinically, mostly during organ transplantation to suppress immunological rejection and fungal virulence (Stallings et al., 2023). However, acute inhibition of CN with FK506 leads to neurotoxicity and its long-term usage could compromise kidney and heart functions.

Calcineurin in protein misfolding disorders: Apart from the functional roles of CN mentioned above, CN has been linked also to protein quality control in the endoplasmic reticulum (ER). Ca^{2+} is supplied to the ER through Ca^{2+}-dependent ATPases and Ca^{2+}, together with Mn^{2+}, is crucial for the folding, translocation, and sorting of ER proteins. ER Ca^{2+} depletion results in the activation of the unfolded protein response together with CN signaling, which is required for the long-term survival of cells undergoing ER stress. The unfolded protein response leads to attenuation of translation, induction of ER chaperones, and activation of ER-associated degradation to eliminate immature proteins. In addition, inhibitors of CN activity, such as cyclosporin A and FK506, in mice cause rapid induction of the unfolded protein response in several organs (including apoptosis in the kidney by depleting ER chaperones and inducing proapoptotic proteins) (Kitamura and Hiramatsu, 2010). It has been suggested that CN hyperactivity could cause AD pathology mechanisms, memory impairment, neuroinflammation, and neuronal death. In post-mitotic cells such as neurons, maintenance of vital proteostasis is centrally linked to CN and both inactivation and hyperactivation of CN play a central role in protein misfolding-related neurodegenerative diseases as well as non-neurological amyloid-associated disorders. Similarly, complete loss of CN leads to synaptic dysfunction, cognitive decline, and neurotoxicity (Pflugrad et al., 2020).

Mostly, CN signaling has been linked also to the pathology of AD and Huntington's disease. Importantly, the differences observed in the neurons were not caused by differences in calcineurin expression but rather, by calcineurin activity. In fact, one of the downstream effects mediated by soluble amyloid-beta (Aβ) aggregates is the hyperactivation of calcineurin characterized by hyperphosphorylated tau and neuritic plaques of Aβ. In the healthy brain, phosphorylated tau binds to the Pin1p protein, which is the only known peptidyl-prolyl isomerase known in humans. Pin1p processes amyloid precursor protein in the brain and converts the pathological trans-tau to cis-tau. Any compromise in the Pin1p activity results in the misfolding of Aβ. The rising levels of soluble Aβ lead to an aberrant rise in the cytosolic Ca^{2+} levels which results in the hyperactivation of CN responses along with several other post-translational modifications at Pin1p (Stallings et al., 2023). The cognate pairs-Akt1p kinase and CN in humans also regulate the transport of vesicles, including those containing brain-derived neurotrophic factor and amyloid precursor protein. The binding of scaffolding huntingtin to kinesin or dynein-bearing neuronal endosomes also contains the CN on the surface which navigates the delivery of those motile vesicles into anterograde or retrograde direction (Scaramuzzino et al., 2022). Therefore, dysregulation of such endosomal signaling could lead to the accumulation of Aβ resulting in a progressive intracellular boost in the CN activity impairing protein trafficking and lysosomal proteolysis.

A modest synaptic disturbance is an age-related error in human neurons. However, dendritic misfolding of α-synuclein, a protein critically linked to PD pathology, leads to its aggregation and a catastrophic progressive loss of neurons. Drastic loss of dopaminergic neurons in the substantia nigra and accumulation of intracellular inclusions known as Lewy bodies and Lewy neurites is the major outcomes in synucleinopathies and tauopathies. The pathology of PD is associated with gene duplication and gene mutations such as A30P and A53T representing familial forms of PD and each one features a distinct clinical presentation. Where A53T, unlike A30P-synuclein, has a high aggregation potential and mostly contributes to the pathogenesis of dementia in PD. As a result complex aggregated forms of α-synuclein trigger Ca^{2+} influx into neurons and glial cells, leading to pathological alterations and, ultimately, cell death (Caraveo et al., 2014; Domingues et al., 2022). However, although the role of the Ca^{2+}-CaM-Calcineurin pathway in α-synuclein -induced phenotypes has been documented, the role of CN signaling and its downstream targets is still obscure (Mackiewicz et al., 2023). As for AD, it appears that the downregulation of CN signaling could potentially be beneficial in ameliorating PD-linked pathologies.

Yeast as a model of studying protein misfolding disease mechanisms regulated by calcineurin: Various symptomatically unrelated diseases are caused by protein misfolding and to investigate their underlying molecular causes and genetic targets, studies using the yeast *Saccharomyces cerevisiae* as a cellular model have been developed (e.g., Krobitsch and Lindquist, 2000). For various protein misfolding diseases, this simple model organism has highlighted that several neurological disorders, such as AD, PD, amyotrophic lateral sclerosis, and Huntington's diseases (Caraveo et al., 2014) and type 2 diabetes (Kayatekin et al., 2018) are regulated by highly conserved molecular modulators. Thus, yeast models can be used to study various non-conserved human mutant proteins responsible for neurodegeneration, aging, and cell death and many modifiers of such maladies identified in yeast have been reliably validated in mammalian systems.

In post-mitotic cells, the maintenance of vital proteostasis is centrally linked to the presence of the Calcineurin phosphatase. It has already been established that there is a remarkable conservation of Ca^{2+}-signaling pathways from yeast to mammals where cellular homeostasis is achieved through a regulated loop of activation and de-activation of the CN phosphatase. Tacrolimus (FK506) is a Food and Drug Administration–approved drug utilized to suppress the immune system and organ rejection in saturating doses. The adverse effects of this practice include calcineurin inactivation eventually hampering its ability to activate neuroprotective responses (Zaichick and Caraveo, 2023; Pflugrad et al., 2020). Conversely, beneficial neuroprotective effects of FK506 in *in vivo* models of PD/AD were shown when administering sub-saturating doses (Caraveo et al., 2014; Stallings et al., 2023). A study by Chawla et al. (2023) approached this challenge, using yeast as a model to identify substrates and interactors of CN that could modulate α-synucleinopathy of PD. First, it was observed that depriving yeast cells of CN activity by various means, including the addition of CN inhibitors such as FK506/cyclosporin A or the removal of genes encoding Cna1p, Cna2p or the regulatory gene encoding Cnb1p, leads to an overall breakdown of Protein Quality Control and the accumulation of protein aggregates in the cytosol (Chawla et al., 2023). Second, different reporters of protein misfolding and aggregation also demonstrated that the residual level of CN signaling in non-stressed, young yeast cells is limiting for protein quality control as boosting CN signaling by either overproducing Cnb1p or deleting *CMK2*/*CMKII*, encoding a negative regulator of CN activity, reduced the basal levels of protein aggregation. The reduction in aggregate formation achieved by deleting *CMK2* required the *CRZ1* transcription factor, a nuclear factors of activated T-cells equivalent of mammals. Further, the effect of modulating CN activity on the toxicity of the PD protein α-synuclein was investigated: The expression of the wild-type form of α-synuclein and the disease-related mutant variant A53T in yeast leads to aggregation, growth arrest, and cell death in a dose-dependent manner. Interestingly, normalizing CN phosphatase activity by overproducing Cnb1p eliminated the pathological development of physiology for both

the wild type α-synuclein and the hyper-toxic allele A53T and was accompanied by normal membrane localization and a simultaneous reduced cytosolic aggregation for α-synuclein (Chawla et al., 2023; **Figure 1**). In contrast, the deletion of *CMK2*, a negative regulator of CN activity, could not replicate the phenotype due to the contrasting relative hyperactivation of CN activity observed (Chawla et al., 2023). The genetic discrepancies observed in the study underscores the molecular differences between positive and negative CN activity normalization which appeared to be critical for the yeast cells to attain equilibrium between survival and toxicity when additionally challenged by the presence of α-synuclein. The amplitude of CN stimulation in neurodegenerative diseases is poorly understood and the remarkable aggregate suppression and normal localization for α-synuclein upon moderate Cnb1p overdosing represents a unique approach for curing misfolding diseases that warrant investigation also in mammalian AD/PD models.

Final remarks: Aging is a complex process associated with the emergence of several cellular dysfunctions. One of the hallmarks of aging is the aggregation of proteins which not only seeds the misfolding of other proteins becoming the leading cause of neuropathies but at the same time molecular crowding which leads to oxidative stress. A major hallmark of aging is dysregulation of Ca^{2+} storage and flux between organelles and the cytosol leading to aberrant CN signaling. The study by Chawla et al. (2023) demonstrates that these hallmarks might be connected. Caraveo et al. (2014) highlighted that both loss and excessive CN activity is fatal for cellular survival and a recent report by Stallings et al. (2023) demonstrates that the drug FK506 has therapeutic efficacy but in a dose-dependent manner. Interestingly the study by Chawla et al. (2023) presents a selective genetic intervention where boosting Cnb1p production by exchanging its native promoter for a constitutive GPD promoter yielded a modest increment in CN activity supporting both young and old cells counteracting protein aggregation and providing resistance against the toxic effects of Parkinson's disease protein α-synuclein. However, the exact molecular mechanism by which calcium/calcineurin signaling maintains cytosolic protein homeostasis and rescues cells from synucleinopathies is unclear. The budding yeast model displaying enhanced Cnb1p production could be further utilized to delineate how dosage modulation of it kindles the protective CN phosphatase activity thereby regulating the misfolding of disease-specific proteins. Therefore, it would be intriguing to elucidate the connection between relative Calcineurin B production and CN activity and neuroprotection from protein misfolding in various neurological disorders.

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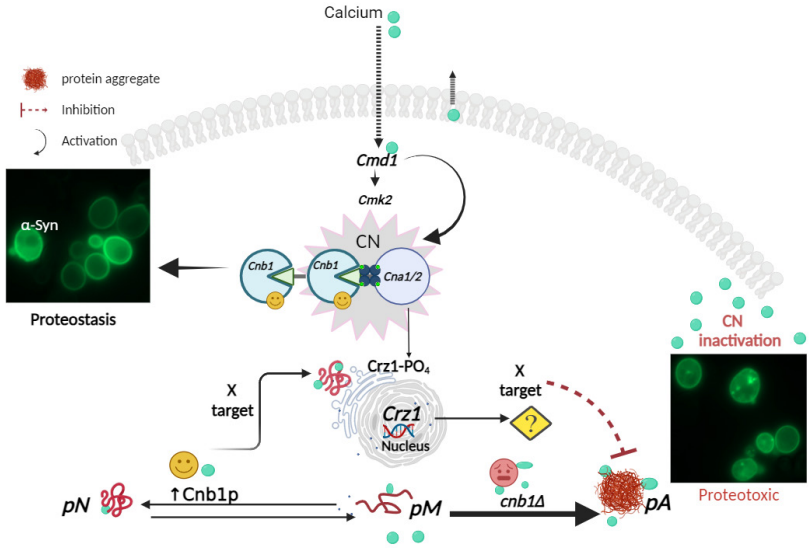

Figure 1 | Schematic representation of calcium-calcineurin signaling pathway reducing α-synuclein (α-Syn) toxicity by reducing its misfolding in *Saccharomyces cerevisiae* and enhanced membrane localization when Cnb1p overproduced.
Upon binding with calcium calmodulin-dependent kinase Cmk2p further activates calcineurin (CN) phosphatase (Cna1p/Cna2p) which dephosphorylates the downstream transcription factor Crz1p. The dephosphorylated Crz1p stimulates the expression of an array of target genes related to calcium homeostasis and also an unknown factor (?-to be identified, X). Under unstressed and calcium-replete conditions, Cnb1p leads to balanced proteostasis. Whereas, compromised Calcineurin phosphatase activity either its absence or genetic perturbation, triggers protein misfolding (pM) and shifts the flux of native protein (pN) towards proteotoxicity and protein aggregates (pA), suggesting that calcineurin activity is limiting for proteostasis under normal conditions. A flat red arrowhead represents an inhibitory effect and a pointed arrowhead (black) represents activation. CN-a Ca^{2+}/calmodulin-regulated type 2B protein phosphatase, Cna1p-Calcineurin A; one isoform (the other is Cna2p alias Cmp2p) of the catalytic subunit of calcineurin, Cnb1p-Calcineurin B; regulatory subunit of calcineurin, Cmd1p-Calmodulin; Ca^{2+} binding protein which also activates CN, Cmk2p-Calmodulin-dependent protein kinase in yeast Crz1p-Transcription factor, activates transcription of stress response genes; nuclear localization is positively regulated by calcineurin-mediated dephosphorylation and α-Syn, a Parkinson's disease associated protein. Created with BioRender.com.

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