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Loss of Functional Alpha-Synuclein: A Toxic Event in Parkinson's Disease?

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Abstract

The discovery that alpha-synuclein (α-syn) is the primary component of the neuropathological hallmarks of Parkinson's disease (PD) and the identification of α-syn mutations in numerous inherited forms of PD has positioned α-syn at the top of the list of important factors in the pathogenesis of PD. Based on the pathological accumulation of α-syn in the brains of patients, the field is currently focused on therapeutic strategies that aim to reduce or eliminate α-syn. However, recent evidence suggests α-syn is a critical protein in neuron (i.e. dopamine neurons) survival and that maintaining a certain level of biologically functional α-syn is an important consideration in targeting α-syn for therapies. Despite the widespread interest in α-syn, the normal biological functions remain elusive, but a large body of work is focused on addressing this issue. In this review, we will discuss the current evidence related to α-syn function, α-syn folding and aggregation, and α-syn's role in disease. Finally, we will propose a relatively novel hypothesis on the pathogenesis of PD that hinges upon the premises that functional α-syn is critical to cell survival and that a reduction in biologically functional α-syn, whether through aggregation or reduced expression, may lead to the neurodegeneration in PD.

Keywords

Parkinson's disease; alpha-synuclein; neurodegenerative disease; dopamine; lewy body

INTRODUCTION

Numerous neurodegenerative diseases are characterized by the selective degeneration of specific neural populations, which typically exhibit accumulations of abnormally modified, misfolded and/or aggregated forms of amyloidogenic proteins. Among these proteins is prion protein in transmissible spongiform encephalopathies (i.e. Crutzfeld-Jacob's disease), amyloid-β in Alzheimer's disease, tau protein in Alzheimer's disease and other tauopathies,

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CONFLICT OF INTEREST

The authors have none to declare.

huntingtin protein in Huntington's disease, and alpha-synuclein (α-syn) in Parkinson's disease (PD) and other synucleinopathies [1]. Typically, the presence of aggregated (i.e. fibrillar, spherical, and/or oligomeric) forms of each protein in the somata and processes (i.e. axons and dendrites) of affected neurons represent the pathological hallmarks of these diseases and often constitute the definitive post-mortem diagnosis. Debates continue as to how the disease-related changes in these proteins directly play a role in the pathogenesis of disease. Here, we will review recent findings that may shed light on how disturbances in α-syn can precipitate neuron dysfunction and death in the context of PD and other synucleinopathies.

α-Syn

The synuclein family of proteins contains three known isoforms that includes α-, β-, and γ-syn [2]. Alpha-synuclein was first cloned and isolated from the Pacific electric ray *Torpedo californica* in the pioneering work by Scheller and colleagues, who also demonstrated that α-syn is primarily expressed in neural tissue and localized to the presynaptic terminals and nuclei (i.e. the nuclear envelope) of neurons in electric rays and rats [3]. The α-syn gene (*SNCA*) is located in chromosome 4q21.3-q22 of humans [4] and encodes for a 140 amino acid protein. Although this is the primary isoform of α-syn in neurons, alternative splicing at exons 3 and 5 can generate α-syn 126 and 112, respectively [5, 6]. The primary sequence of α-syn is composed of 7 imperfect repeats of 11 residues that make up the highly basic amino-terminus (residues 1–60), a hydrophobic non-amyloid-β component (NAC; residues 61–95), and an acidic C-terminal domain (residues 96–140). The *SNCA* gene is highly conserved across species with only a few amino acids differing between the human and rodent (i.e. mouse and rat) sequence [2, 7].

Alpha-synuclein is a highly soluble intrinsically disordered protein with virtually no tertiary structure and dynamic secondary structures as a monomer [8] (Fig. 1). Fitting with its disordered nature, α-syn appears to exhibit a wide dynamic range of conformational states that varies with experimental conditions [9]. Multiple studies have confirmed the existence of long-range intramolecular interactions often involving the amino and carboxy termini [10, 11], but the precise regions of the proteins involved and whether these "global" conformations suppress [12, 13] or enhance [14, 15] aggregation is not entirely clear. Some studies suggest that the interaction between the amino and carboxy termini creates a partially folded monomer that resists aggregation by blocking the amyloidogenic portions of the protein [12, 13]. Another structural feature of α-syn is the α-helical content in the amino terminus. A large number of studies using spectroscopic techniques suggest two anti-parallel α-helices connected by a short linking segment and/or a single extended helix form in α-syn (Fig. 1B) that is significantly increased when it binds lipids [16–18]. There is little doubt that the amino terminal helices are critically important in α-syn's interaction with membranes and this interaction is influenced by the chemical composition of the membrane, ionic strength of the solutions, vesicle curvature (i.e. size), and the mass ratio of α-syn to lipids (reviewed in [19]). Thus, it seems likely that the both the intrinsic disorder of soluble α-syn monomers and the amino terminal helices are important for α-syn's biological interactions and functions [15].

Recently, the primary state of α-syn under normal physiological conditions has come under debate. A set of recent studies have isolated α-syn tetramers from human red blood cells demonstrating that α-syn may exist as a tetramer under normal physiological conditions. Interestingly, the tetramers do not readily polymerize into fibrils [20] and α-syn tetramers derived from recombinant α-syn expressed in *E. coli* do not appear to be toxic to neurons in culture [21]. Moreover, disease-related mutant forms of α-syn (i.e. A30P, A53T and E46K) appear to disrupt the formation/stability of tetramers [21]. These studies suggest that the tetramers are a "safe" form of α-syn and might represent a means to store α-syn in various subcellular compartments (i.e. the synapse or nucleus) prior to performing its biological functions.

The debate remains ongoing in light of a recent publication from a team of seven research groups that used extensive biochemical analyses of α-syn (recombinant, endogenous and exogenously expressed α-syn) derived from a long list of sources ranging from humans, rats, and mice to multiple cell types in culture [22]. These studies could not find evidence to support the existence of a tetrameric form of α-syn under both non-denaturing and denaturing conditions [22]. Perhaps, α-syn exists in a state of dynamic equilibrium between a disordered monomer and a tetrameric form, which may be facilitated by factors (still unknown) *in situ* (e.g. lipid interactions and/or chaperone interactions). While there has been substantial progress in understanding the dynamics of α-syn protein structure, many questions remain unanswered. Exactly how does structure affect α-syn function? What is the functional state of α-syn? How is α-syn structure affected in disease states? What is the structure of the toxic species of α-syn?

α-Syn expression in the CNS

During development in the CNS, the patterns of α-syn expression appear to follow a stereotypical shift in distribution from the somata to the nerve terminals as development progresses. This holds true for nearly all brain regions analyzed in humans and rats, including the cortex, hippocampus, and substantia nigra, among others [23–25]. In fact, the expression of α-syn appears at 15 weeks gestation in the cell body of human nigral neurons, which is 4 weeks after the appearance of tyrosine hydroxylase, and α-syn is localized in the processes/nerve terminals by 18 weeks of gestation [26]. In a detailed analysis of α-, β- and γ-syn protein in the adult rats, Li and co-workers demonstrated wide-spread α-syn reactivity throughout the CNS exclusively in nerve terminals/processes (no cell body reactivity was observed) [27]. Studies in mice have demonstrated that α-syn expression is detected as early as embryonic day 9.5 in areas such as the hind-brain/midbrain junction [28] and its expression rises sharply in the following days indicating that the protein is important at certain stages of neuronal differentiation and development [29]. In addition, the differential distribution of α-syn in development suggests α-syn is expressed early in development and plays a role in maintaining nerve terminals in adulthood in numerous, but select cell populations.

On the other end of the developmental spectrum, normal aged brains exhibit a redistribution of α-syn that is reminiscent of early development in the nigrostriatal system. In humans over 80 years old the level of α-syn is doubled in the cell bodies of the substantia nigra compared

to those less than 60 years old, and this effect appears relatively region-specific as no aging-related increases were seen in the frontal cortex or caudate nucleus [27]. Moreover, aging-related increases in α-syn were exaggerated in neuromelanin-containing neurons of the substantia nigra [30], which are more susceptible to degeneration in PD and non-human models of PD [31, 32]. A similar pattern of somatodendritic accumulation of α-syn was found in aged nigral DA neurons of non-human primates using immunohistochemical and densitometry measures [33]. Interestingly, transcriptional analyses of α-syn mRNA expression typically indicate an aging-related reduction in α-syn mRNA in numerous brain regions of humans, mice and rats, including the substantia nigra, striatum, cerebellum, hippocampus, and cortex [24, 34–36]. The aging-related increases in nigral neuron α-syn levels may be limited to humans and non-human primates as studies in mice report decreased levels of α-syn protein with advancing age [35].

Nonetheless, aging remains one of the strongest risk factors for developing PD [37], and thus, it is not entirely surprising that α-syn undergoes some precursory changes to those seen in PD (i.e. accumulation in the cell body). Both nitrated and phosphorylated forms of α-syn, which are prominent in PD-related α-syn pathology [38, 39], are significantly elevated in aged monkey nigral neurons [40]. In addition, the redistribution of α-syn from the terminals to the cell body may be indicative of neurons initiating maladaptive developmental plasticity-like signals [41], or age-related impairment of axonal transport of α-syn [42, 43]. Finally, the re-localization of α-syn away from the synapse removes the protein from a cellular compartment where it performs crucial neuronal functions and may represent a primary mechanism of α-syn loss-of-function in aging and disease (see below).

Role of α-Syn in disease

Mutations in *SNCA* were the first identified genetic determinant of PD, causing a small subset of familial forms of the disease [44, 45]. Moreover, genetic overexpression via gene duplication or triplication was later shown to result in a form of inherited PD [46, 47]. Importantly, higher copy-numbers of α-syn result in a more aggressive form of the disease suggesting a direct relationship between expression levels and disease severity [48]. Following the identification of disease-causing α-syn mutations, the fibrilized form of the protein was identified as the major component of Lewy bodies, the hallmark cytoplasmic inclusions in sporadic forms of the disease [49]. More recently, polymorphisms of the α-syn gene and the *SCNA* promoter have been implicated in sporadic PD [50]. Consequently, α-syn is generally thought to play an important and potentially causative role in most, if not all, forms of PD.

α-Syn aggregation

The direct mechanism(s) whereby perturbations of α-syn results in toxicity and subsequent disease are unknown. However, it is commonly proposed that the self-assembly of α-syn into soluble oligomers and/or insoluble aggregates that are directly toxic underlie its role in disease [51]. Due to the fact that the precise biological functions of α-syn are not known, the effect of “abnormal” oligomer/aggregate formation and disease-related mutations on α-syn function is difficult to determine. The ability of α-syn to undergo self-assembly into a

multitude of multimeric forms adds further complexity in understanding α-syn function and toxicity.

As discussed above, recent work suggests that the native conformation of α-syn in cells is in an aggregation-resistant helical tetramer [15, 20, 21] (Fig. 1B). When these tetramers dissociate, α-syn goes to its intrinsically disordered monomeric state where numerous dynamic ensembles likely exist [21]. Alpha-synuclein monomers exhibit a relatively high rate of intramolecular diffusion [52]; however, certain experimental conditions induce monomeric conformations (some are also enhanced by disease-related mutations [53]) with significantly slower intramolecular diffusion rates that may facilitate aggregation [52]. Indeed, partially folded α-syn is thought to be a precursor in the process of α-syn fibril formation, although once again, α-syn appears to take on a wide range of folded and/or soluble oligomeric forms depending on its environment (i.e. experimental conditions) (reviewed in [9]). It is noteworthy that monomeric α-syn more readily forms aggregates/fibrils *in vitro* when compared to its propensity to form tetramers *in vitro*, which suggests the fibrillogenic and tetrameric forms of α-syn are generated through different pathways [20, 21].

Intermediate stage prefibrillar oligomers and protofibrillar forms can induce damage in neurons both *in vitro* [54] and *in vivo* [55] presumably by forming pores in membranes [54–57]. However, intermediate oligomers are relatively unstable and the protofibrils are rapidly sequestered into the larger insoluble fibrils (i.e. Lewy bodies and Lewy neurites) [58]. Interestingly, *in vitro* studies show that the membrane permeabilizing capacity of protofibrils composed of A53T or A30P α-syn is higher than that of the wild-type α-syn protofibrils [57]. Moreover, the rate of protofibril formation is higher with these mutant forms [59, 60] and the A30P mutation stabilizes this intermediate oligomer [59], suggesting that these disease mutants indeed promote the formation of potentially toxic species of α-syn. Finally, the presence of Lewy bodies and Lewy neurites in surviving neurons in PD brains, suggests that these late-stage or mature inclusions are not inherently toxic [61, 62], and may represent a cytoprotective effort of neurons. In fact, the morphology of neurons containing LBs appears more normal compared to those neurons that do not contain these inclusions [61, 62], but it is difficult to determine how the morphological characteristics directly relate to neuron functionality in these post-mortem studies.

After certain energy barriers are overcome, the pathway of α-syn aggregation is likely a “self-propagating” event. This is supported by the polymerization kinetics observed in numerous *in vitro* studies [9, 59, 63, 64] and evidence that α-syn oligomers serve as seeds for further polymerization in a dose-dependent fashion [65–67]. This is particularly important in the context of disease pathogenesis considering recent work that suggests aggregated α-syn can spread from cell to cell and induce misfolding and oligomerization of α-syn in the recipient cell. Thus, yet-to-be defined toxic forms of α-syn may exhibit prion-like spread throughout the nervous system [68–71]. This contention supports the Braak staging hypothesis of PD, which postulates a peripheral origin of the disease that spreads to the CNS over time in a process mediated via the cell-to-cell transfer of α-syn [72]. Moreover, the demonstration that ectopically applied α-syn can cause putatively toxic changes in the endogenous protein (i.e. aggregation of α-syn in recipient cell) [65]

strengthens the hypothesis that modulation of α-syn indeed is directly causative in neuronal toxicity.

Targeting α-Syn as a therapeutic modality

Initial preclinical therapeutic approaches aimed at reducing α-syn expression either at the mRNA or protein level were based on the strong links between α-syn and PD (familial and sporadic forms), and the common conception that α-syn pathology represents a toxic gain-of-function [73]. The expression of the α-syn mRNA was successfully reduced through the use of RNAi (either as shRNA, miRNA, or direct delivery of siRNA) in rodents and primates [74–76]. However, it is noteworthy that a significant reduction of α-syn in the adult rodent can produce severe nigral neuron loss [75] (further discussed below) (Fig. 2). Alternatively, an immunization-based approach is currently being pursued as a therapeutic intervention aimed at reducing α-syn load. The α-syn vaccine (PD01A) is currently in a phase I clinical trial (June 2012) (http://www.affiris.com/html/en/presse_medien/pressemeldungen.html) in order to evaluate the safety and tolerability of its use. This method can reduce α-syn aggregation, particularly in the presynaptic boutons of immunized transgenic mice expressing human α-syn [77]. However, severe caution is recommended when pursuing strategies that might reduce and/or remove α-syn expression considering that the role of α-syn in neurons is yet to be elucidated and the protein provides a crucial function to neurons. Indeed, an improved understanding of the consequences from targeting α-syn is required and without it additional approaches should not be taken to the clinic. Perhaps a more viable approach is to breakup aggregates and/or inhibit their formation altogether (i.e. via small molecule-based approaches) [78]; however, one must be cautious not to facilitate the formation of toxic oligomeric species when taking these approaches.

Role of α-syn in disease models

Due to the prevalence of α-syn in both sporadic and heritable forms of PD, genetic manipulation of this protein in rodents and in primates has become a popular tool to model PD and other synucleinopathies. A vast array of transgenic (knock-in) rodents exist that express wildtype or disease-related mutant forms of α-syn under control of different promoters such as the TH, thy-1, or prion promoter, among others (reviewed in detail in [79, 80]). Although some of these models exhibit a distinct DAergic phenotype and some forms of α-syn aggregation, germline expression of α-syn has largely failed to recapitulate the neuronal loss observed in PD. To date, only two germline transgenic models have demonstrated nigral DAergic neurodegeneration with age. Expression of a double mutant form (A30P and A53T) from the rat TH promoter results in loss of nigral TH+ neurons starting at 2 months of age [81]. A model that produces twenty to thirty-fold overexpression (as compared to endogenous levels) of a truncated version of human α-syn (amino acids 1–130) also produces severe nigral neurodegeneration [82]. Interestingly, animals homozygous for the insertion of truncated human α-syn did not survive past the embryonic state suggesting α-syn related toxicity early in embryogenesis [82]. In contrast, conditional knock-in α-syn (A53T) mutant mice display significant SNc degeneration following induction (two-fold overexpression) in mature animals, with the accompanying motor phenotype [83]. These conditional animals exhibit a decrease in the important DAergic transcription factor Nurr1. Interestingly, Nurr1 is similarly reduced in nigral neurons

containing α-syn inclusions in human PD [84], and mutations that reduce Nurr1 expression can cause PD [85]. One Nurr1 target is the receptor tyrosine kinase RET [86], an important receptor for glial cell-line derived neurotrophic factor (GDNF)-mediated pro-survival signaling. This suggests that modulation of α-syn expression may indirectly result in the impaired trophic support of DA neurons. Similar to the conditional knock-in animals, virally-mediated overexpression of wildtype or mutant human α-syn in adult animals leads to a significant and progressive degeneration of SNc neurons both in rodents and in non-human primates. Moreover, with virally-mediated overexpression nigral loss is observed at transgene expression levels of only 2 times that of normal [87]. One interesting observation associated with virally-mediated α-syn lesion is that it is refractory to treatment with GDNF [88], a neuroprotective approach that is well established in toxin-induced models such as the 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models [89–92]. This discrepancy between treatment outcomes highlights the fundamental differences in downstream cellular consequences between different model systems. On one hand, the toxin-induced models are acute and result from the formation of toxic reactive oxygen species and the inhibition of mitochondrial respiration. On the other hand, the viral vector-mediated α-syn lesion is a chronic and progressive model that exhibits protracted cellular dysfunction, aggregate formation, and impaired axonal transport [93–95]. Thus, these models may represent distinct molecular pathologies represented in PD and potential therapeutic interventions should be tested in numerous model systems as a better predictor of treatment outcome in human disease [88].

Transgenic invertebrate α-syn models such as the fruit fly (*Drosophila melanogaster)* or the worm (*Caenorhabditiselegans*) more consistently recapitulate key aspects of PD. For example, transgenic flies exhibit a progressive loss of DA neurons with age, the formation of LB-like inclusions, and DOPA-responsive motor symptoms [96]. Similarly, overexpression in *C. elegans* is accompanied by the formation of LB-like aggregates, DAergic neurodegeneration, as well as a motor phenotype [97]. Interestingly, these invertebrates apparently lack a homologue of α-syn, and while it is tempting to suggest these data support direct α-syn toxicity the fact that a completely foreign protein is introduced into the organism makes the results of these studies difficult to interpret.

α-SYN BIOLOGICAL FUNCTIONS

Currently, the biological functions of α-syn are not known; however, there is a significant amount of ongoing work aimed at filling this gap in our knowledge. The functional repertoire of α-syn is studied largely by the way of determining the abnormalities following overexpression, loss of expression, or expression of mutant forms of α-syn. The majority of studies suggest α-syn plays a role in synaptic vesicle regulation, mitochondrial function, and/or DA handling and synthesis, but the list of other potential functions is long and continues to grow. We will highlight some of the important findings in each of these categories (Table 1).

α-syn-mediated regulation of synaptic vesicles

The initial observations that α-syn is predominately a presynaptic protein that extensively co-localized with synaptic vesicles markers hinted at an important role for α-syn in synaptic

function. In fact, α-syn is known to interact with numerous synaptic proteins including synaptophysin, synapsin I, SNAP-25, synaptobrevin 2/vesicle-associated membrane protein 2 (VAMP2) [98, 99]. However, not all studies support an interaction between α-syn and SNARE-related proteins due to a lack of co-immunoprecipitation [100], but this might be indicative of a weak and/or transient interaction.

Numerous studies have identified a link between α-syn and regulation of the synaptic vesicle reserves, vesicle trafficking, and neurotransmitter release. Some studies demonstrate that α-syn may play a role in maintaining the appropriate amount of synaptic vesicles in the reserve pool in neurons. For instance, α-syn knockout mice have less vesicles in the reserve pool [101], and hippocampal neurons exhibit signatures indicative of faster depletion of the reserve synaptic vesicle pool and slower replenishment of the pool following repetitive prolonged stimulations [101]. Overexpression of α-syn in cultured hippocampal neurons causes a reduction in vesicle mobility and the size of the reserve vesicle pool, while the opposite was found in cultured neurons derived from α-syn null mice [102]. Further supporting the role of α-syn in vesicle trafficking, studies have shown that α-syn inhibits endoplasmic reticulum-Golgi complex vesicle trafficking in yeast [103–105] and non-neuronal cell lines [106]. In PC12 cells and chromaffin cells, overexpression of wild-type and mutant α-syn causes decreased catecholamine release and an accumulation of “docked” synaptic vesicles without affecting the size of the vesicle pool [107] which is also true with virally-mediated overexpression of α-syn *in vivo* [108, 109]. Interestingly, in two α-syn null mouse lines basal and evoked DA release was enhanced [110], which is consistent with the notion that α-syn is a negative regulator of neurotransmitter release. Together, these studies suggest α-syn acts as a negative regulator of synaptic vesicle release, possibly by reducing vesicle mobility and pool size. However, some evidence suggests α-syn can enhance neurotransmitter release [111] and promote SNARE complex formation, an effect that is dependent on the C-terminal domain [99]. Moreover, SNARE complex formation is impaired when all three isoforms of α-syn are knocked out in mice [99].

Despite the somewhat discordant findings in the literature, all of these studies clearly indicate that α-syn is important in modulating synaptic function through modulating the synaptic vesicle pool, trafficking and/or vesicle exocytosis. One hypothesis that may help reconcile some of these discrepancies is that α-syn serves multiple, context-dependent functions related to synaptic vesicles. For instance, α-syn may maintain reserve pool size by modulating mobility through cytoskeletal interactions, and once a vesicle is translocated to the synaptic active zone α-syn acts in concert with other SNAP/SNARE-complex proteins to determine whether vesicles fuse and release neurotransmitters. By extension, overexpression, mutant forms, and complete loss of α-syn will likely have a dynamic detrimental effect on synaptic function and neurotransmission (an effect that will vary with experimental conditions and detection will depend upon outcome measures).

α-Syn and mitochondrial function

A compelling biological function attributed to α-syn that is coming to light is its apparent ability to influence mitochondrial function. Multiple independent reports demonstrate that α-syn localizes to mitochondria in cultured cells under various experimental conditions [11,

112–114] and in nigral DA neurons of mice [115] and humans [114]. In cultured human DAergic neurons, α-syn is imported into mitochondria in an energy and transmembrane potential-dependent fashion, localizes primarily with the inner membrane, and mitochondrial localization is dependent on a cryptic mitochondrial targeting sequence within the amino terminus (i.e. aa 1–32) of α-syn [114]. Alpha-synuclein appears to inhibit mitochondrial fusion as reducing its expression in neuronal cell lines results in increased fusion and more tubular mitochondria [116], while overexpressing α-syn in neuronal and non-neuronal cell lines induces fission leading to increased mitochondrial fragmentation [116–118]. Although investigated under conditions that produce α-syn toxicity (i.e. overexpression), the evidence that α-syn affects mitochondrial morphology and dynamics may be indicative of a normal biological role for α-syn in this capacity. These findings are interesting in light of α-syn's ability to modulate vesicle dynamics at the synapse and reduce ER-Golgi complex vesicle trafficking (as described above), which involves membrane fusion. Perhaps one of α-syn's biological functions is to regulate membrane fission and fusion events, whether it is docking of synaptic vesicles at the active zone or fission/fusion of mitochondria. Further experimentation is required to specifically address and confirm this hypothesis.

Calcium homeostasis and mitochondrial functionality have long been implicated in the pathogenic processes of PD and other neurodegenerative diseases [118, 119]. Subsequently, the findings that indicate α-syn may influence mitochondrial function through modifying calcium handling/homeostasis are particularly intriguing in the context of normal α-syn biology and disease pathogenesis. An interesting set of experiments by Cali and coworkers demonstrated that α-syn controls mitochondrial Ca^{2+} homeostasis by modulating the interaction between mitochondria and the ER [118]. In SH-SY5Y and HeLa cell lines, the expression of α-syn caused enhanced Ca^{2+} transients without affecting basal Ca^{2+} levels or mitochondrial Ca^{2+} uptake machinery, and this effect was dependent on the C-terminus of α-syn. These α-syn-mediated effects were due to an increase in ER-mitochondrial interactions, which was confirmed by knocking down α-syn expression via siRNA (i.e. caused reduced Ca^{2+} uptake). One important implication of this set of experiments is that they indicate an intricate balance of α-syn levels that is critically important to maintaining mitochondrial functionality and mitochondrial Ca^{2+} homeostasis. Alpha-synuclein also interacts with mortalin (a.k.a. Grp75) [120, 121], which is a chaperone protein involved in coupling inositol triphosphate 3 receptors to voltage-dependent anion channels on the outer mitochondrial membrane to regulate Ca^{2+} homeostasis [122]. Perhaps α-syn acts in concert with mortalin as a bridging chaperone to regulate mitochondrial Ca^{2+} levels by way of the endoplasmic reticulum.

In addition, α-syn may play a more general role in cellular Ca^{2+} homeostasis as it binds calmodulin in a Ca^{2+}-dependent fashion [123]. Moreover, long-lasting Ca^{2+} transients following both spontaneous and stimulus-induced Ca^{2+} activity are augmented in the cortex of transgenic mice expressing human α-syn [124]. These *in vivo* findings have been confirmed with *in vitro* studies using neuronal cell lines (i.e. SH-SY5Y cells), which indicate that α-syn overexpression causes an increase in stimulation-induced Ca^{2+} entry into cells [125, 126]. Again, these effects were explored in the context of being toxic to cells but they may be indicative of normal biological function of α-syn. Collectively, these studies

implicate α-syn as an important component of the mitochondrial function/Ca^{2+} homeostasis axis that is critical to neuron function and survival.

α-Syn in DA handling and biosynthesis

A substantial number of reports indicate that α-syn can regulate DA synaptic tone via modulating the dopamine transporter (DAT) and DA synthesis [127]. In fact, α-syn can regulate both the norepinephrine and serotonin transporters as well [127]. The direct interaction with DAT is mediated by the carboxy terminus of DAT [128] and the NAC region of α-syn [128, 129]. Currently, the exact nature of the relationship between α-syn and DAT function is somewhat controversial. The controversy centers around studies that indicate α-syn facilitates DAT expression and function versus those that indicate α-syn has the opposite effect. Both *in vitro* [130, 131] and *in vivo* [110] studies using loss-of α-syn models suggest that α-syn increases DAT expression and functionality. Knock down of α-syn in neuronal cell lines or knockout mice exhibit reduced DAT expression, less plasma membrane localized DAT, and impaired DA reuptake. Interestingly, eliminating or reducing α-syn increases basal and evoked levels of DA release, and increases the amount of VMAT2 on synaptic vesicles [130]. Some studies show α-syn can potentiate DA uptake via DAT modulation in neurons and non-neuronal cells [128]. The other side of the debate is supported by studies that show the overexpression of α-syn reduces DAT expression and DAT activity (i.e. DA uptake V_{max}) using a number of experimental paradigms [129, 132, 133]. Interestingly, inhibition of DAT function precedes retrograde neurodegeneration following viral-mediated delivery of α-syn to the nigrostriatal tract [109]. Alpha-synuclein likely modulates DAT functionality, at least in part, by affecting the distribution of DAT to the plasma membrane via a process that involves tethering DAT (likely on presynaptic vesicles) to microtubules [133]. At first glance, these data may seem to oppose one another; however, what if α-syn serves two functions in regulating DAT? Both sets of data could be explained if α-syn were critically important in targeting vesicles containing DAT to the appropriate subcellular localization for storage (via MT tethering) and the proper delivery of DAT to the plasma membrane to maintain membrane-associated DAT levels. Indeed, in this hypothetical paradigm both a loss of α-syn and excess α-syn would similarly lead to DAT mislocalization, reduced levels of DAT at the membrane and reduced DA reuptake.

Of note, previous studies using α-syn null mice did not observe any effects on DAT [134]. While the underlying reasons of the discrepant findings in the literature are unclear, they may be due to differences in experimental paradigms and the choice of transgenic models used. Nonetheless, it appears that α-syn can indirectly regulate DA uptake, recycling and bioavailability via modulating DAT distribution and functionality in neurons [135, 136].

Independent of DAT-related mechanisms, studies indicate that α-syn can modulate DA biosynthesis. In fact, α-syn directly interacts with TH (as indicated by co-immunoprecipitation) and acts as a negative regulator of TH activity [137]. The inhibition of TH by α-syn is mediated by a mechanism involving the dephosphorylation of TH at Ser40 by PP2A, and it appears that α-syn interacts directly with PP2A as well [138, 139]. Moreover, α-syn interacts with aromatic amino acid decarboxylase, the enzyme that converts L-dopa to DA, and causes decreased AADC activity further supporting a role of α-

syn in DA biosynthesis [140]. The role of α-syn in DA biosynthesis and DA handling may be intimately linked to disease-related malfunctions or loss-of-function in α-syn that leads to the selective dysfunction and degeneration of DA neurons in PD.

α-Syn and other potential biological functions

Certainly, the biological functions discussed above are supported by numerous studies, but there are additional potential functions of α-syn. Some insight can be gleaned from the growing list of α-syn interacting partner proteins [141, 142]. The suggestion that α-syn could act as a chaperone is based on sequence homology with 14-3-3 proteins and a direct interaction between α-syn and 14-3-3 proteins [143]. In addition to acting as a chaperone, α-syn may be regulated by chaperones. For instance, DJ-1 (a.k.a. PARK7), a gene that is mutated in autosomal recessive early-onset PD, acts as a chaperone for α-syn [144]. Indeed, DJ-1 appears to inhibit the aggregation and subsequent cell toxicity associated with overexpressing human α-syn and PD-related mutant forms of α-syn (e.g. A53T) in *in vitro* assays [145] and numerous cell-types in culture [146–148]. Interestingly, the protective chaperone activity of DJ-1 with α-syn is dependent on the redox state of DJ-1 [145, 146] and a mutant form of DJ-1 associated with inherited PD (i.e. L166P DJ-1) does not provide the protective effects of wild-type DJ-1 [146, 147]. Lastly, the protective effects of DJ-1 may involve the upregulation of Hsp70 as well [147, 148]. Whether DJ-1 is important in stabilizing non-toxic forms of α-syn under normal conditions remains unknown and future studies are required to determine whether chaperones are involved in "normal" α-syn function.

In the initial discovery of α-syn, the protein was found in the nucleus of neurons [3]. It appears that nuclear α-syn may play a role in gene expression directly through transcription factor regulation and/or indirectly through epigenetics mechanisms. Alpha-synuclein may impact transcriptional regulation via binding numerous promoters [149]. Interestingly, α-syn binds the PGC1 alpha promoter both *in vitro* and *in vivo* (including in neurons of aged and PD patients), which may have effects on mitochondrial function because PGC1 alpha is an important mitochondrial transcription activator [149]. Additionally, α-syn can bind to histones suggesting a potential functional relationship between them [150, 151], but histones can increase α-syn aggregation and the co-localization of α-syn with histones is increased in a PD model (i.e. paraquat exposure) [150]. The "normal" presence of α-syn in the nucleus suggests a biological function in this cellular compartment; however, α-syn (including mutant forms of α-syn) may mediate neurotoxicity via the nucleus by inhibiting histone acetylation [151]. Nonetheless, the precise function(s) of nuclear α-syn are still being identified, and the potential role of nuclear α-syn in normal cell function and synucleinopathies such as PD remains unresolved.

Direct interactions between α-syn and important cytoskeleton proteins, such as tubulin, tau, MAP1b, actin and Rab small GTPases, among others have been identified. It is tempting to speculate that the cytoskeletal interactions of α-syn might be intimately linked to its function in modulating synaptic vesicle distribution between the reserve pool and synaptic active zone (as discussed above), and potentially mitochondrial localization.

Alpha-syn also interacts directly with multiple kinases, such as extracellular signal-regulated kinase (ERK)1/2 and protein kinase C (PKC) [143], which are involved in a large set of biological functions. For example, PKC is linked to regulating axonal transport [152] and oxidative stress [100], among other processes, and while the exact nature of these interactions awaits further elucidation they could be indicative of an important role of α-syn in cytoskeleton functionality.

In addition to the membrane interactions outlined above, α-syn appears to interact with lipid rafts [153] and phospholipase D2 [154–156] in the plasma membrane, and thus, might play a role in regulating plasma membrane dynamics. Taken together, the majority of studies points towards α-syn as an indispensable protein likely involved in numerous cellular functions that are critical to neuron function and survival.

LOSS OF FUNCTION HYPOTHESIS

α-Syn knockout

Several germline α-syn knockout mice have been created [101, 134, 157–159], but a majority of these animals do not display neurodegeneration. However, one report evaluating the α-syn null mouse found that germline removal of the α-syn gene results in a significant but low level of neurodegeneration in the SNc early during development [158]. The observed difference in these mouse lines may be explained by the fact that phenotypes due to the targeted genetic deletions of specific genes within the DA neurons varies significantly depending on the genetic backgrounds of the mice [160]. Regardless, subtle phenotypes including impaired DA release and reduced striatal DA content have been reported in all adult and aged α-syn null mice [157, 161]. Moreover, these mice display reduced sensitivity to the neurotoxin MPTP [134, 162]. In contrast, virally-mediated delivery of shRNAs targeting α-syn in the adult rodent midbrain results in severe, dose-dependent, neurodegeneration of nigral neurons [74, 75] (Fig. 2). Further confirming the specificity of these effects, Gorbatyuk and colleagues demonstrated that shRNAs targeting different sites in the α-syn mRNA reproduced the nigral neuron degeneration [75]. Importantly, supplementation with endogenous rat α-syn rescued the neurons from degeneration, demonstrating that neuronal toxicity was explicitly due to loss of α-syn in mature neurons [75]. Similarly, neurotoxicity was seen when rat nigral neurons were simultaneously transduced with viruses to overexpress human α-syn and knockdown human α-syn mRNA (via shRNA). However, it was unclear from this study whether the shRNA had an effect on endogenous levels of rat α-syn [74]. Conversely, direct infusion of a siRNA into the non-human primate SN resulting in approximately 40% reduction of α-syn protein failed to produce neurodegeneration [76]. These findings agree with those reported by Gorbatyuk and coworkers, who demonstrated that toxicity due to loss of α-syn is dependent on the remaining levels of α-syn protein.

Unfortunately, it is difficult to determine whether germline-based approaches are appropriate for understanding sporadic, adult-onset disease-related processes. Indeed, one likely source of discrepancies between germline manipulated transgenic mice (both knock-in and knockout mice), and post-developmental α-syn manipulations (shRNA-mediated silencing, virally mediated overexpression, or conditional knock-in) is the potential

compensatory changes in other genes that occur when α-syn is manipulated in the germline. Some of these potential compensatory changes may include genes that directly compensate for crucial roles of α-syn as well as the enhancement of pro-survival pathways that prime susceptible DAergic cells providing a finite level of protection against insults such as oxidative stress [161]. Indeed, genome array comparisons between α-syn knockout and wildtype mice revealed that 369 genes were differentially expressed [163]. Among these gene changes, both ERK2 and apoptosis-related genes were affected indicating that pathways modulating neuronal survival are differentially regulated in the α-syn$^{-/-}$ mouse. Direct comparisons of genome array data from the α-syn$^{-/-}$ mouse and acute RNAi knockdown *in vitro* indicate that only 3 gene changes overlap between the two knockdown approaches when compared to control [164]. Thus, the knockout animals may have an unnaturally high tolerance for future insults, but this compensation does not appear to arise from increased expression of other synuclein family members. For example, the levels of β-syn protein in α-syn null mice are similar to that of wildtype mice [157, 159]. However, the third member of the synuclein family is upregulated in double synuclein knockout animals (α$^{-/-}$/β$^{-/-}$and α$^{-/-}$/γ$^{-/-}$) [158, 165]. Moreover, animals void of all three forms of synuclein (α, β, γ) do not display significant neuropathological changes [166, 167] supporting the compensatory effects from currently unidentified elements (e.g. pro-survival genes and/or genes that replace α-syn functions) [161]. Collectively, these findings highlight the important point that genetic manipulation in mature neurons is likely to produce a vastly different result from that of germline manipulation.

Loss-of-function hypothesis

Post-developmental loss of α-syn results in rapid neurodegeneration in the nigrostriatal system and DA depletion-related behavioral deficits clearly indicating that α-syn plays an important role in maintaining DAergic neuron function and/or survival in adulthood. Depletion in the functional pool of α-syn beyond a threshold induces neuron death [75]. Although the report by Gorbatyuk and colleagues is the only work that directly attributes neurodegeneration to loss of α-syn, work by others show that a reduction in α-syn results in the disruption of cellular processes such as mitochondrial function and autophagy [118]. This conclusion allows for an alternative explanation to the traditional toxic gain-of-function attributed to α-syn aggregation in PD. Specifically, one hypothesis of equal explanatory power posits that the incorporation of α-syn into aggregates, whether it be oligomers, fibrils, and/or mature Lewy bodies, effectively reduces the amount of functional α-syn available. Consequently, α-syn related pathology and aggregation represents a *de facto* loss-of-function mechanism of toxicity [168, 169] (Fig. 1A). This is further compounded by the fact that α-syn inclusions appear in the somata of neurons indicating that the aggregates also remove α-syn from its normal subcellular compartment (i.e. the synapse). The redistribution and accumulation of α-syn during the normal aging process [33] likely increases the probability of α-syn aggregation and is thus not met with developmental compensation. Indeed, aging, which remains the primary risk factor for developing PD [37], is characterized by reduced cellular stress defenses and compensatory capacity following injury. Moreover, the rate of aggregation may be accelerated by other factors including α-syn mutations, elevated expression, and/or post-translational modifications resulting in a more severe and rapid loss-of-function. Additionally, these other factors may directly affect

the functionality of α-syn in a manner independent of aggregation, but reducing α-syn function nonetheless. The role of aging in PD and the results from most studies can be explained through the view that a loss of α-syn function is intimately linked to neurodegeneration in PD. Finally, one can easily appreciate that a loss in functionality would lead to neuron dysfunction and death considering the list of potential biological functions attributed to α-syn, but truly confirming these hypotheses awaits a more accurate and detailed understanding of exactly what α-syn does in neurons. Importantly, the α-syn loss-of-function hypothesis does not invalidate the vast body of work that α-syn research encompasses. Rather, the majority of studies describing toxicity due to α-syn have inferred that the protein itself is directly toxic. Thus, loss-of α-syn function could serve as an alternate conclusion in most, if not all, of these instances. Although several reports directly ascribe neurotoxicity to soluble α-syn oligomers [54, 55], whether the rate of formation and sequestration of these toxic intermediate-stage protofibrils in experimental conditions truly represents what occurs in human disease remains unknown. The proposition that a mutant protein that has previously been labeled as directly toxic in neurodegenerative disease may actually confer loss of function toxicity is not unique to α-syn and its role in PD. Polyglutamine expansions in the protein huntingtin causes Huntingon's disease (HD). Initially, it was argued that huntingtin aggregates were directly toxic, but now it is clear that loss of wildtype huntingtin function is a detrimental factor to neurons (reviewed in [170]), and may be a causative pathological event in HD.

Can α-syn be a neuroprotectant?

More support for the notion that loss of α-syn function is detrimental to neurons comes from evidence that α-syn provides neuroprotection against various insults both *in vitro* [171–173] and *in vivo*. Mice deficient in synaptic cysteine-string protein α (CSPα), a synaptic protein that is important in SNARE complex assembly, exhibit neurodegeneration and the deletion produces lethality within months of life [174]. When human wildtype α-syn overexpression is superimposed on this model, neurodegeneration is significantly reduced and survival is prolonged. Conversely, when the CSPα$^{-/-}$ was crossed with a mouse void of α and β-synuclein, the pathology was exacerbated [100]. Similarly, *in vitro* studies show that subjecting primary neurons to chronic oxidative stress results in an upregulation of α-syn with the concomitant resistance to apoptosis suggesting that α-syn can protect against oxidative stress [175]. Jin and colleagues demonstrated that α-syn negatively regulates the expression of PKCδ, an important mediator of oxidative stress-mediated DAergic cell death. Moreover, the α-syn-induced down regulation of PKCδ protects against MPP+ (a DAergic neurotoxin) toxicity in N27 cells [176], and primary TH+ neurons isolated from a TH-promoter driven α-syn knock-in [177] and in α-syn overexpressing MN9D cells [173]. Interestingly, PKCδ was significantly reduced in DAergic neurons in the CSPα$^{-/-}$/α-syn$^{-/-}$/β-syn$^{-/-}$mice [100, 176]. Similarly, overexpression of α-syn may prevent cell death following oxidative stress through inhibition of c-Jun N-terminal kinase activity, an effect not seen with β-synuclein overexpression [178]. Although the direct mechanism where by α-syn potentiates neuronal survival is yet to be elucidated, a growing body of evidence demonstrates that in certain conditions α-syn serves as to promote neuronal survival.

A model for α-syn loss-of-function

A clear picture is emerging from the studies highlighted above – there is an intricate balance between too little and too much α-syn in neurons. Too little of the protein sensitizes the cell to further insults, and a dramatic loss of the protein results in rapid cell death. Conversely, both human and animal data inform us that increasing the expression, even minimally, results in a significant increase in the disease phenotype. Moreover, changing the localization of α-syn within the cell seems to be intimately tied to toxicity. Based on these premises, we propose the following model of α-syn-based neurotoxicity: In its native state, α-syn exists in a dynamic equilibrium between an intrinsically disordered monomer and a stable soluble tetramer (possibly favoring the monomeric state [22]). The tetramers may represent a state in which the cell "stores" an inert form of the protein, and the dissociation of this complex into a disordered monomer may represent its conversion to a functional state. While α-syn tetramers are fairly thermodynamically stable, they do not readily form *in vitro*, and α-syn monomers require less energy to form the soluble oligomers and intermediate protofibrils that lead to further self-assembly into insoluble fibrils. Together, these findings suggest there might be secondary factors (e.g. a chaperone) *in situ* that facilitate the formation of the stable tetramer, but currently these factors are unknown. While the mechanisms that push α-syn monomers down one path or the other (i.e. stable non-toxic tetramers versus fibrillogenic oligomers and then fibrils) are still not entirely understood, the concentration of α-syn in the neuron appears to be a clear determinant (i.e. more α-syn facilitates aggregation). With normal aging, the protein is concentrated to the cell body and when coupled with post-translational modifications (i.e. phosphorylation, nitration, truncation, among others), or enhanced expression due to genetic multiplications or promoter polymorphisms, or with mutations that increase the stability of oligomers and decrease the stability of tetramers, the likelihood of fibril formation increases. Concomitantly, once insoluble fibrils appear, the reaction will be further pushed in the favor of the production of more insoluble inclusions. Ultimately, the sequestration of α-syn into these inclusions will bring the concentration of soluble α-syn at the synapse below that which is required for proper cellular functions. Eventually, neuron dysfunction leads to neuron death and the clinical and neuropathological manifestations of PD appear.

CONCLUSION

The protein α-syn is highly conserved across species and is expressed throughout the brain starting early in CNS development. Thus, as the role of this protein in specific cellular processes is better understood it is not entirely surprising that α-syn is extremely important in normal DAergic neuron function. However using the genetic tools at hand to manipulate this protein *in vitro* and *in vivo* has in many cases yielded rather divergent results, further complicating our understanding of this protein. Although it is uncontested that α-syn plays a crucial, albeit largely unknown, part in PD pathogenesis it is likely that we will learn that α-syn intersects the molecular mechanisms underlying neurodegeneration at multiple points. The formation of toxic oligomers, the loss of functional soluble protein, and the dysregulation of crucial cellular processes may share equal burden in the formation of toxic events that either sensitize the neuron to other insults, or sets in motion a process that in itself culminates in cell death.

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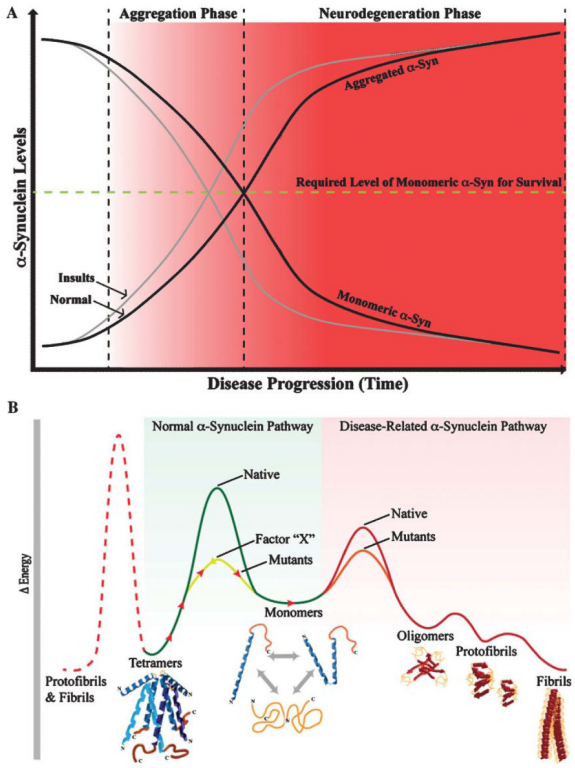

Fig. 1.

Models of the role of α-synuclein (α-syn) in Parkinson's disease (PD) and the folding/aggregation properties of α-syn. (**A**) Alpha-syn is a critical player in the pathogenesis of PD and the loss-of-function hypothesis posits that as the disease progresses the pool of functional α-syn is depleted via aggregation (in the aggregation phase). The neurons in which α-syn is being reduced succumb to death once the pool of biologically functional α-syn is depleted past a certain threshold (green dashed line). Numerous insults that are intrinsic (e.g. mutant α-syn, abnormal α-syn modifications, oxidative stress, etc.) or extrinsic (e.g. environmental toxins, neuroinflammation, etc.) to the neurons can augment the process quickening the progression of the disease (gray line). (**B**) The structure and aggregation profile of α-syn is relatively complex. Under normal conditions, α-syn exist as a disordered monomer or in a stable tetramer. Interestingly, the formation of tetramers *in vitro* is less favorable than the formation of aggregates (green line), but *in vivo* α-syn may primarily exist in the tetrameric form (yellow-green line). This suggests two possibilities. 1) The *in vitro* conditions are not representative of *in vivo* conditions, and/or 2) there are unknown factors (Factor "X") that facilitate the formation of tetramers *in vivo* (e.g. chaperones). If tetramers are dissociated they are more likely to form aggregates (dashed red arrows) than they are to reform tetramers. Disease-associated mutations of α-syn impede the formation of tetramers, and favor the formation of aggregates. The aggregation pathway is characterized by the progressive formation of soluble oligomers, protofibrils and mature fibrils (red line). Mutant forms of α-syn reduce the energy barrier for the formation of oligomers; and thus, favor the formation of fibrils (orange line). One important caveat with these models is that they tend to simplify a complicated process and make some generalizations that do not fit all of the current data. With that in mind, it is important to

view these models with the understanding that they can, and should, be modified as more data become available.

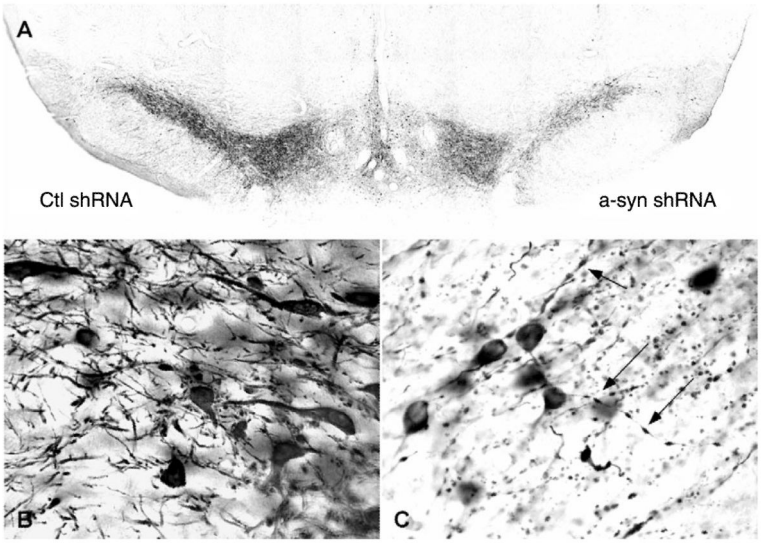

Fig. 2.

shRNA-mediated silencing in the adult rat results in loss of TH+ SN neurons. **A–C.** TH immunoreactivity. **A**. rAAV-mediated overexpression of a shRNA (2.6×10^{12} particles/ml) [75] (right hemisphere) designed against α-syn (resulting in approximately 85% loss of α-syn protein) results in severe loss of TH+ cells of the SN 28 days following viral delivery. No neuronal loss is observed in the control-shRNA injected hemisphere (left hemisphere). **B**. Neurons in control injected hemisphere appear normal; however, neurons in the α-syn shRNA treated hemisphere exhibit dystrophic and fragmented neurites (**C**), similar in morphology to dystrophic neurites observed with human α-syn overexpression in rodents [94] and non-human primates [179].

Table 1

Biological functions of α-syn protein

Proposed function	Mechanisms	Related interacting partners	References
Synaptic vesicle regulation	Maintain vesicle reserves Reduce vesicle mobility Reduce vesicle docking via SNARE complex* Reduce neurotransmitter release*	Synaptophysin, synapsin I, SNAP-25, synaptobrevin 2/VAMP2, VMAT2, CSPα	[98, 101–110]; [99, 100, 111]*
Mitochondrial function	Inhibit fusion Promote fission Mitochondrial morphology	Unknown	[11, 112–118]
Ca^{2+} Homeostasis	Mitochondrial-ER Ca trafficking Mitochondrial anion channel regulation Cytosolic Ca^{2+} regulation	Calmodulin, mortalin/Grp75	[118–126]
DA handling	Increase DA reuptake* Increase DAT function* DAT membrane localization Tethering DAT to microtubules Increase VMAT on vesicles	DAT Tubulin VMAT	[110, 128, 130, 131]; [129, 132, 133]*; [134]*
DA biosynthesis	Reduce TH activity Reduce AADC activity	TH, PP2A AADC	[137–140]
Chaperone	Unknown Prevent aggregation	14-3-3 proteins DJ-1	[143–148]
Cytoskeletal function	Unknown (Vesicle transport)	Tubulin, tau, MAP1b, actin and Rab small GTPases	[141, 142]
Protein phosphorylation	Unknown (numerous possibilities)	ERK 1/2, PKC, CK1, MARK, CaM Kinase II, PP1	[141–143]
Gene expression (Nucleus)	Histone deacetylation Transcription regulation	Histones Promoters (e.g. PGC1-alpha, & others)	[149–151]
Lipid membranes	Unknown (Lipid metabolism)	Lipid rafts, Phospholipase D2	[153–156]