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REVIEW

The contribution of alpha synuclein to neuronal survival and function – Implications for Parkinson's disease

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Abstract

The aggregation of alpha synuclein (α-syn) is a neuropathological feature that defines a spectrum of disorders collectively termed synucleinopathies, and of these, Parkinson's disease (PD) is arguably the best characterized. Aggregated α-syn is the primary component of Lewy bodies, the defining pathological feature of PD, while mutations or multiplications in the α-syn gene result in familial PD. The high correlation between α-syn burden and PD has led to the hypothesis that α-syn aggregation produces toxicity through a gain-of-function mechanism. However, α-syn has been implicated to function in a diverse range of essential cellular processes such as the regulation of neurotransmission and response to cellular stress. As such, an alternative hypothesis with equal explanatory power is that the aggregation of α-syn results in toxicity because of a toxic loss of necessary α-syn function, following sequestration of functional forms α-syn into insoluble protein aggregates. Within this review, we will provide an overview of the literature linking α-syn to PD and the knowledge gained from current α-syn-based animal models of PD. We will then interpret these data from the viewpoint of the α-syn loss-of-function hypothesis and provide a potential mechanistic model by which loss of α-syn function could result in at least some of the neurodegeneration observed in PD. By providing an alternative perspective on the etiopathogenesis of PD and synucleinopathies, this may reveal alternative avenues of research in order to identify potential novel therapeutic targets for disease modifying strategies.

Keywords: alpha-synuclein, dopamine, Lewy body, Parkinson's disease, substantia nigra, synucleinopathy.

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Research examining the role of the protein alpha synuclein (α-syn) in the etiology of Parkinson's disease (PD) began in 1997 when two seminal discoveries provided conclusive evidence that α-syn is intimately linked to PD pathogenesis. The first report described a missense mutation in the gene encoding α-syn that causes familial PD (Polymeropoulos *et al.* 1997). Shortly thereafter, aggregated α-syn was discovered as one of the primary components of Lewy bodies (Spillantini *et al.* 1997), the neuropathological hallmark of PD (Goedert *et al.* 2013). Since these definitive links between α-syn and PD were identified, research on α-syn has grown exponentially. Within this vast body of work, a preponderance of research is conducted under the prevailing hypothesis that aberrantly expressed or aggregated α-syn produces neurotoxicity through a gain-of-function mechanism. A less discussed, though equally valid hypothesis, is that the aggregation of α-syn results in neurotoxicity through a loss-of-function mechanism, following sequestration of functional α-syn protein into aggregates. Indeed, as our understanding of both the biology and pathology of

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Abbreviations used: AAV, adeno-associated virus; CMV, cytomegalovirus; DAT, dopamine transporter; MSA, multiple system atrophy; PFFs, preformed fibrils; VMAT, vesicular monoamine transporter.

α-syn expands, so too does the evidence supporting the idea that loss of α-syn function may be a critical event in PD.

Within this review, we will present a comprehensive overview of the data linking α-syn to PD, as well as the lessons learned from α-syn-based animal models of PD. Next, we will highlight how the existing data linking α-syn to PD pathology might be explained by toxicity originating from loss of necessary α-syn function, as well as providing some potential mechanisms by which loss of α-syn function could cause neurodegeneration. We will then highlight the discrepant reports in the literature that seem irreconcilable with this hypothesis. Finally, we will draw corollaries to other proteins associated with neurodegenerative disease, which were once believed to elicit toxicity directly, but are now accepted as important for neuronal survival.

α-syn: structure and aggregation

α-syn is a small protein that, along with β and γ synuclein, comprise the synuclein family of proteins (Surguchov 2008). α-syn is encoded by the SNCA gene, located on the long arm of chromosome 4 in humans (4q21.3–q22)(Chen *et al.* 1995). Full-length α-syn is a 140 amino acid protein, however, alternative splicing in exons 3 and 5 can result in 126, 112, or 98 amino acid isoforms (Uéda *et al.* 1994; Beyer *et al.* 2006). Similar to full-length α-syn, the splice variants are expressed in a region-specific manner throughout the brain, albeit at lower levels than the full-length protein (McLean *et al.* 2012). The structure of α-syn can be subdivided into a basic N-terminus (amino acids 1–60), a central ('non-amyloidogenic component') hydrophobic core (amino acids 61–95), and an acidic C-terminal tail (amino acids 96–140) (Surguchov 2008).

The N-terminus of α-syn is characterized by seven 11 amino acid repeats containing a KTKEGV consensus sequence (Jakes *et al.* 1994). This sequence is highly conserved between species and within the synuclein family itself (Surguchov 2008) and predicts an alpha helix secondary structure (Bussell and Eliezer 2003). Confirming these predictions, α-syn forms either two anti-parallel alpha helices (Chandra *et al.* 2003), or one contiguous alpha helix upon interaction with acidic lipid membranes (Davidson *et al.* 1998; Bussell and Eliezer 2003; Jao *et al.* 2004). The central portion of α-syn is highly hydrophobic and is thought to underlie the aggregate prone nature of the protein. Interestingly, the first association between α-syn and neurodegenerative disease followed the identification of a peptide fragment within plaques isolated from the brains of patients with Alzheimer's Disease (Uéda *et al.* 1993). This cleaved peptide was eventually mapped to the central hydrophobic core of α-syn, giving rise to the descriptor 'non-amyloidogenic component', or NAC region, of α-syn (Uéda *et al.* 1993). Finally, the C-terminal domain of α-syn displays the most variability within the synuclein family and between species (George 2001). The C-terminus of α-syn contains a large number of charged residues and is subject to significant post-translational modification (Hasegawa *et al.* 2002; Oueslati *et al.* 2010; Krumova *et al.* 2011), suggesting that this region may play a role in regulating α-syn function and conformation. Beyond regulation of α-syn itself, the C-terminal tail may also have a functional role in mediating interactions between α-syn and soluble NSF attachment protein receptor (SNARE) complex proteins (Burré *et al.* 2010, 2012).

α-syn is now widely considered to behave as a 'natively unfolded' protein, showing dynamic changes in conformation depending on the environment (Weinreb *et al.* 1996). Natively unfolded, or intrinsically disorder proteins, are sometimes considered a unique class of proteins in themselves, characterized by lack of a uniform tertiary structure (Uversky 2002). Recent work has suggested that natively unfolded proteins, such as α-syn, require the presence of molecular interacting partners in order take on a specific tertiary conformation (Uversky 2002; Dyson and Wright 2005). α-syn is highly soluble and intrinsically disordered under normal conditions (Weinreb *et al.* 1996), however, in the presence of acidic lipid membranes (Davidson *et al.* 1998; Bussell and Eliezer 2003; Chandra *et al.* 2003; Jao *et al.* 2004), or membranes with high curvature (Middleton and Rhoades 2010; Jensen *et al.* 2011), the N-terminus of α-syn folds into an alpha-helix that interacts with membranes. Thus, it seems as if α-syn structure may conform to its physiological structure, and thus physiological function, only in the presence of molecular interacting partners. Currently, the list of α-syn's potential interacting partners is extremely large and growing. α-syn directly interacts with lipid membranes (Davidson *et al.* 1998; Fortin *et al.* 2004), synaptic vesicles (Maroteaux *et al.* 1988), SNARE complex proteins (Chandra *et al.* 2005; Woods *et al.* 2007; Burré *et al.* 2010), proteins involved in dopamine (DA) homeostasis (Lee *et al.* 2001a; Dauer *et al.* 2002; Perez *et al.* 2002; Wersinger and Sidhu 2003a; Wersinger *et al.* 2003; Yu *et al.* 2004; Tehranian *et al.* 2006; Fountaine and Wade-Martins 2007; Fountaine *et al.* 2008), proteins involved in calcium regulation (Martinez *et al.* 2003), and the catalytic subunit of protein phosphatase 2A (PP2A) (Peng *et al.* 2005), among others too numerous to list [reviewed in (Kanaan and Manfredsson 2012)].

Beyond interacting with other proteins, α-syn can also interact with itself to form multimers, and there is evidence that physiological α-syn exists as a tetramer (Bartels *et al.* 2011; Dettmer *et al.* 2013, 2015). Moreover, under certain conditions, α-syn can self-assemble, resulting in the formation of β-pleated sheets (Serpell *et al.* 2000), followed by the formation of insoluble α-syn aggregates (Conway *et al.* 1998). Conditions that promote α-syn aggregation include genetic mutations (Conway *et al.* 1998; Narhi *et al.* 1999; Fredenburg *et al.* 2007), molecular crowding induced by high concentrations of macromolecules (Shtilerman *et al.* 2002; Uversky *et al.* 2002) or increased α-syn protein levels

(Conway *et al.* 1998; Uversky 2007), post-translational modifications [reviewed in (Uversky 2007; Stefanis 2012)], low pH (Ahmad *et al.* 2012), and oxidative conditions (Hashimoto *et al.* 1999). Under such conditions, the formation of insoluble aggregates proceeds in stereotypic manner following first-order kinetics. For example, monomeric α-syn will undertake a partial fold amenable to the formation of oligomeric species (dimers, trimers, etc.) which then progress to protofibrils, and finally to mature, insoluble fibrils (Uversky *et al.* 2001; Kanaan and Manfredsson 2012). Throughout this series of reactions, each product is more stable than the reactants, suggesting that this process is largely irreversible (Uversky and Eliezer 2009). Importantly, the progressive conversion from soluble monomeric α-syn to insoluble fibrils proceeds in a feed-forward mechanism, in which oligomers or protofibrils can act as seeds in order to accelerate the conversion of physiological α-syn into aggregates (Wood *et al.* 1999; Luk *et al.* 2009).

Although the conditions that favor α-syn aggregation have been characterized, the precise mechanism(s) that promote aggregation are unknown. It may be that simply shifting the structure of α-syn to an unfolded, or partially folded state is sufficient to result in aggregate formation (Uversky 2007). For example, manipulating the temperature or pH of the environment slows the intramolecular diffusion rate of α-syn, resulting in a partially folded conformation that increases the propensity to aggregate (Uversky *et al.* 2001; Ahmad *et al.* 2012). Mutations in the hydrophobic core of α-syn that reduce the alpha helical content also exacerbate aggregation (Burré *et al.* 2012). Further, folding induced by intramolecular interactions between the N- and C-termini are important in maintaining α-syn in a monomeric state, such that enhancing these interactions can inhibit aggregation (Bertoncini *et al.* 2005; Koo *et al.* 2008; Hong *et al.* 2011), while abolishing them by truncating the C-terminus exacerbates aggregation (Li *et al.* 2005; Burré *et al.* 2012). Thus, inhibiting the folding of α-syn, or promoting its partial folding, result in increased exposure of the amyloidogenic NAC core of α-syn, thereby providing a seed for templating and initiating the feed-forward aggregation process.

α-syn: localization and function

Consistent with its role in multiple neurodegenerative diseases, α-syn is predominantly considered a 'neuronal' protein. α-syn is expressed in high concentrations within neural tissues where it primarily localizes to the presynaptic terminal (Iwai *et al.* 1995) (Fig. 1a). Some analyses estimate that α-syn may comprise up to 1% of total cytosolic protein in the central nervous system (CNS) (Iwai *et al.* 1995). The original identification of α-syn described the presynaptic localization of the protein in the electric organ of the pacific electric ray (Torpedo californica) (Maroteaux *et al.* 1988). α-syn was cloned and identified using antisera raised against cholinergic synaptic vesicles isolated from neural tissue within the ray's electric organ. In addition to its strong presynaptic localization, Maroteaux *et al.*, also identified α-syn on the nuclear envelope, thus accounting for the moniker 'synuclein' (SYNapse + NUCLEus, synuclein) (Maroteaux *et al.* 1988). Despite some nuclear localization (Fig. 1a), α-syn is primarily a pre-synaptic protein, with relatively low levels of expression in the somatodendritic compartment or axon (Iwai *et al.* 1995). Further, although α-syn is present in some non-neural tissue, such as red blood cells (Barbour *et al.* 2008), α-syn is widely expressed throughout most neuronal tissues within both the central and peripheral nervous system, suggesting that α-syn plays a role in synaptic transmission in general.

Currently, the exact biological function of α-syn remains unclear. However, this is not to imply that there is a paucity of suggested physiological functions for α-syn. α-syn has been implicated in diverse physiological processes ranging from regulation of synaptic transmission, to calcium regulation, mitochondrial homeostasis, gene expression, protein phosphorylation, or even fatty acid binding (Sharon *et al.* 2001; Ellis *et al.* 2005). The function of α-syn has been thoroughly reviewed elsewhere [see (Kanaan and Manfredsson 2012; Stefanis 2012)] and an exhaustive description of the potential biological functions of α-syn is neither the purpose, nor within the scope of the current review. However, some understanding of the potential function(s) of α-syn is necessary in order to appreciate how a loss of α-syn function could result in neurotoxicity, and as such will be discussed briefly with a focus on neurotransmission.

The largest and most consistent body of evidence suggests that α-syn acts as a negative regulator of synaptic transmission (Fig. 2a and b). As previously mentioned, α-syn is highly enriched in presynaptic terminals throughout the CNS (Iwai *et al.* 1995). Further, α-syn interacts with synaptic vesicles and SNARE complex proteins, potentially mediating vesicular trafficking to-, and docking with, the presynaptic membrane, as well as vesicular endocytosis (Maroteaux *et al.* 1988; Burré *et al.* 2010, 2012; Vargas *et al.* 2014) (Fig. 2a and b). Ectopic expression of α-syn results in a decrease in synaptic vesicle trafficking and docking, and a corresponding decrease in neurotransmitter release (Larsen *et al.* 2006; Gaugler *et al.* 2012; Lundblad *et al.* 2012; Scott and Roy 2012). In line with these observations, α-syn null mice show decreased DA stores and increased evoked DA release (Abeliovich *et al.* 2000; Cabin *et al.* 2002), as well as decreased synaptic vesicle endocytosis (Vargas *et al.* 2014). α-syn may also have a specialized role in the dopaminergic synapse; α-syn interacts with virtually every major protein involved in DA biosynthesis and handling. α-syn interacts with and inhibits tyrosine hydroxylase (TH) (Perez *et al.* 2002; Perez and Hastings 2004; Yu *et al.* 2004) by directly decreasing TH phosphorylation, by increasing PP2A activity

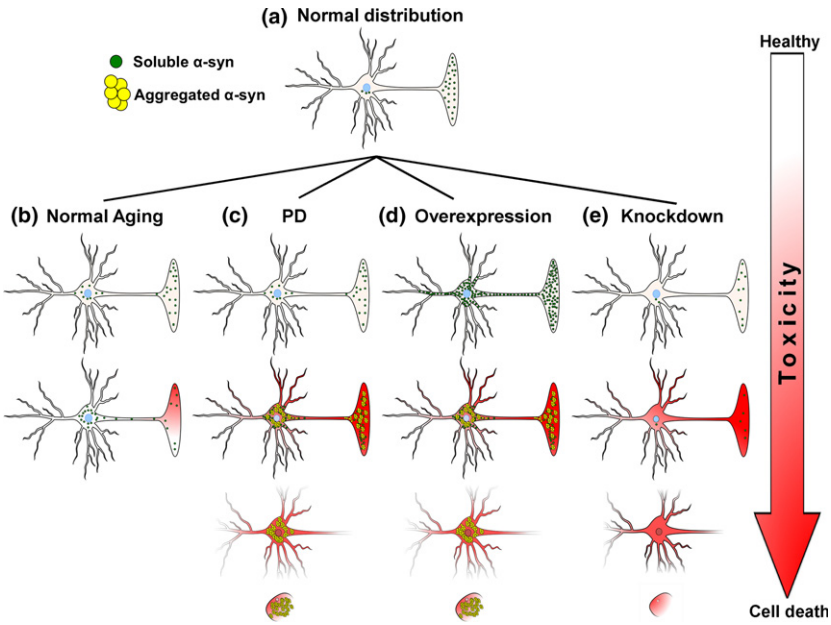
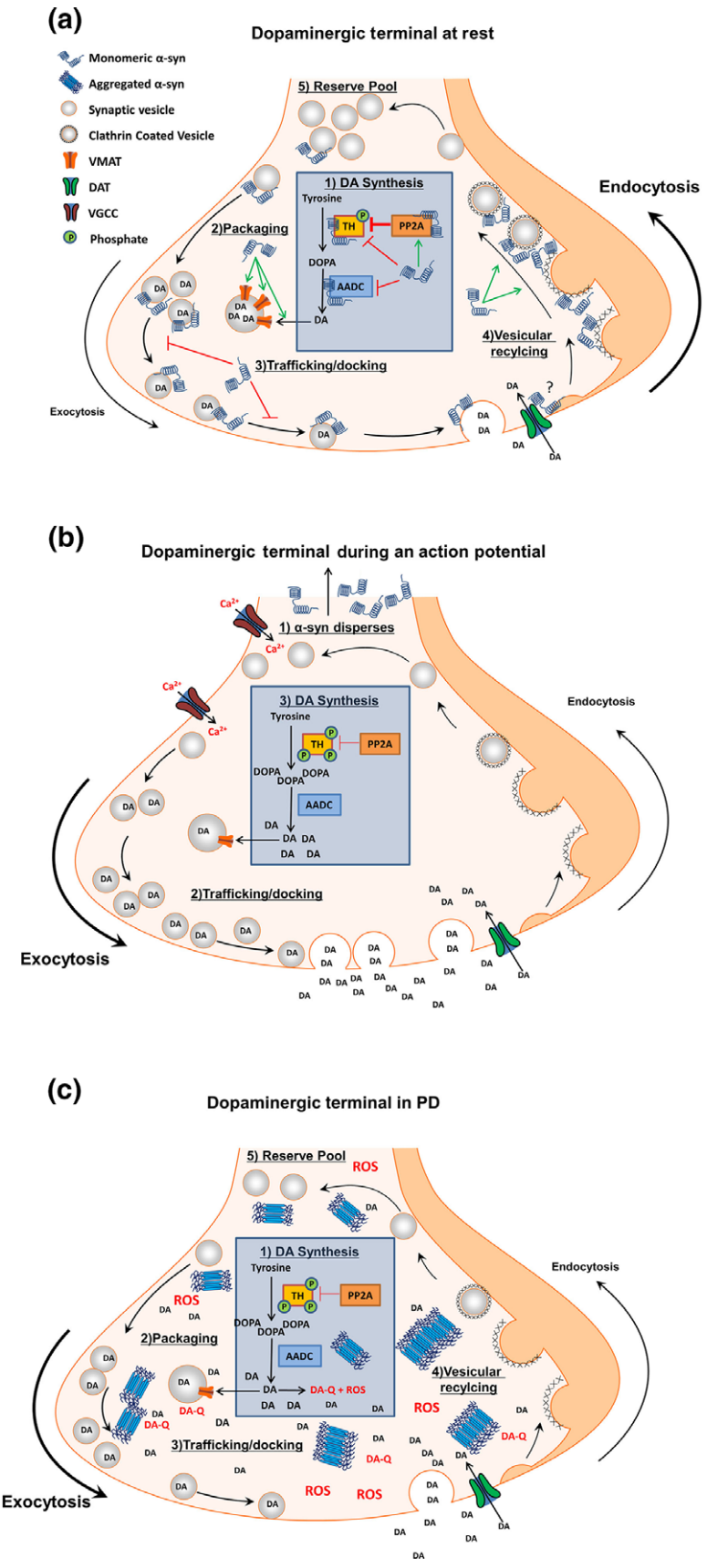


Fig. 1 Normal and pathological subcellular distribution of α-syn. (a) In healthy neurons, α-syn is highly enriched within the presynaptic terminal. (b) As humans age, there is a distribution of α-syn from the presynaptic terminal to the soma. This may predispose neurons to subsequent toxicity. (c) In PD, an initial insult (genetic mutation, oxidative stress, multiplications of SNCA gene, etc.,) induces the aggregation of α-syn, resulting in loss of α-syn function and subsequent toxicity leading to cell death. (d) Over-expression of α-syn results in increased α-syn protein throughout the entire cell. Molecular crowding induces aggregation of α-syn resulting in loss of α-syn function and subsequent toxicity leading to cell death. (e) Knockdown of α-syn decreases protein concentrations until a critical threshold is reached, below which loss of α-syn function results in toxicity and cell death.

(the major phosphatase regulating TH dephosphorylation), or by altering the binding of 14-3-3ξ protein to TH (Peng *et al.* 2005; Wang *et al.* 2009; Lou *et al.* 2010). α-syn also interacts with, and inhibits, aromatic amino acid decarboxylase (AADC) (Tehranian *et al.* 2006) (Fig. 2a and b). α-syn interacts with the dopamine transporter (DAT), increases DAT insertion into the presynaptic membrane, and modulates DAT activity (though there is a discrepancy as to whether α-syn increases or decreases DAT activity), and increases the amount of vesicular monoamine transporter

Fig. 2 Proposed model of α-syn function in the dopaminergic terminal. (a) In the absence of an action potential, α-syn concentrations are high in the presynaptic terminal where it acts as a brake on chemical neurotransmission. A1) α-syn inhibits DA synthesis by decreasing TH phosphorylation directly or by increasing PP2A activity. α-syn also interacts with, and inhibits, AADC. A2) α-syn aids in the sequestration of cytosolic DA by increasing the amount of VMAT on vesicles. A3) α-syn prevents neurotransmitter release through interactions with synaptic vesicles and SNARE complex proteins to prevent trafficking and docking of vesicles with the presynaptic membrane. (A4) α-syn facilitates the recycling of synaptic vesicles by mediating membrane bending during endocytosis, in order to (A5) maintain numbers of vesicles in the reserve (and possibly the readily releasable) vesicle pool. B1) Following neuronal stimulation and calcium influx, α-syn rapidly disperses from the presynaptic terminal (B2) providing unimpeded vesicular trafficking and exocytosis for efficient neurotransmitter release. (B3) The absence of α-syn disinhibits TH and AADC, allowing DA synthesis to replenish DA released during synaptic transmission. Upon repolarization, α-syn repopulates the terminal to perform the actions listed in panel A to terminate chemical neurotransmission. (c) The aggregation of α-syn in PD results in a loss of α-syn function and subsequent increased cytosolic DA. (C1) Loss of α-syn disinhibits TH and AADC, resulting in increased DA synthesis with a corresponding (C2) decrease in VMAT levels, and (C3) unregulated trafficking of synaptic vesicles. (C4) Loss of α-syn function impairs endocytic vesicular recycling, (C5) decreasing the size of the vesicular pool. The net result is increased cytosolic DA, with a concomitant inability to efficiently sequester DA into synaptic vesicles. Increased cytosolic DA auto-oxidizes to produce ROS and DA quinones. DA quinones react with sulfhydryl groups in proteins, forming DA-cysteinyl adducts that covalently modify proteins, impairing enzymatic function. ROS oxidize proteins and lipids. DA-cysteinyl adducts and ROS inhibit the electron transport chain, resulting in increased oxidative stress and opening of the mitochondrial permeability pore, as well as decreasing enzymatic break down of DA to DOPAC, further increasing cytosolic DA. Increased ROS and the formation of DA-α-syn adducts promote α-syn aggregation. Together, an initial loss of α-syn function can initiate a vicious cycle of toxicity ultimately resulting in cell death. Abbreviations: tyrosine hydroxylase (TH), aromatic amino acid decarboxylase (AADC), dopamine (DA), dihydroxyphenylalanine (DOPA), protein phosphatase 2A (PP2A), vesicular monoamine transporter (VMAT), dopamine transporter (DAT), voltage gated calcium channel (VGCC), reactive oxygen species (ROS), dopamine quinone (DA-Q).



(VMAT) on vesicles (Lee *et al.* 2001a; Dauer *et al.* 2002; Wersinger and Sidhu 2003b; Wersinger *et al.* 2003; Fountaine and Wade-Martins 2007; Fountaine *et al.* 2008) (Fig. 2a and b). Further, α-syn is a highly mobile protein within the presynaptic terminal, demonstrating high concentrations within the axon terminal at rest, and rapidly dispersing away from the synapse in response to neural activity (Fortin *et al.* 2005; Unni *et al.* 2010) (Fig. 2a and b).

With this scenario, α-syn may act as a brake on dopaminergic neurotransmission. For example, following neuronal stimulation, α-syn rapidly disperses from the presynaptic terminal (Fortin *et al.* 2005); allowing unimpeded vesicular trafficking and exocytosis for efficient neurotransmitter release (Fig. 2b). Upon termination of synaptic transmission, α-syn rapidly repopulates the presynaptic terminal (Fortin *et al.* 2005), presumably acting to impede the trafficking and docking of synaptic vesicles with the presynaptic membrane, thus halting chemical transmission (Fig. 2a). Further, within the dopaminergic terminal, the dispersion of α-syn from the presynaptic terminal during neuronal firing would effectively disinhibit TH and AADC, allowing *de novo* DA synthesis to replenish DA for release during synaptic transmission (Fig. 2b). Finally, as α-syn repopulates the presynaptic terminal following the termination of neuronal stimulation, α-syn could increase the rate of synaptic vesicle recycling through endocytosis and also activate DAT and increase VMAT, resulting in more efficient DA reuptake and packaging, thereby enabling a continuing level of high-fidelity neuronal signaling (Fig. 2a). With α-syn potentially performing so many critical functions within the dopaminergic synapse alone, it is easy to envision how a loss of α-syn function could result in neurotoxicity.

α-syn in PD

Although α-syn pathology is most commonly associated with PD, aggregation of α-syn is a common thread linking a spectrum of neurodegenerative disorders collectively referred to as synucleinopathies. Disorders classified as synucleinopathies include PD (Spillantini *et al.* 1997), dementia with Lewy bodies (DLB) (Kosaka 1978; Spillantini *et al.* 1998a), multiple system atrophy (MSA) (H *et al.* 1998), pure autonomic failure (Arai *et al.* 2000), Lewy body variant of Alzheimer's disease (Lippa *et al.* 1998), and neurodegeneration with brain iron accumulation (Arawaka *et al.* 1998; Wakabayashi *et al.* 1999). Synucleinopathies are clinically differentiated by the symptoms that patients manifest; however, there are overlapping symptoms that are shared by these disorders (Marti *et al.* 2003). For example, both MSA and DLB are associated with clinical Parkinsonism; yet, they differ from classical PD by the presence of additional symptoms such as autonomic failure and cerebellar ataxia in MSA, or early cognitive impairment in DLB (Marti *et al.* 2003). The differential clinical presentations of synucleinopathies are also accompanied by differences in the neuroanatomical location of pathological α-syn deposits. PD and DLB are characterized by aggregated α-syn within the soma and neurites of neurons (Spillantini *et al.* 1997, 1998a), while MSA is characterized by α-syn deposition primarily within myelinating glial cells (H *et al.* 1998). The fact that aggregation of α-syn can have deleterious effects in many distinct cell populations strengthens the argument that α-syn likely plays a crucial role in neurophysiology in general. Further, the location of α-syn deposits directly correlates with the symptomatology observed, lending support to the idea that α-syn or rather the loss of α-syn, is directly responsible for neurotoxicity.

α-syn genetics and PD

The first link between α-syn and PD was the identification of a missense mutation on chromosome 4 that segregated with an autosomal-dominant inherited form of PD (Polymeropoulos *et al.* 1997). This mutation was discovered in an Italian kindred as well as in four (ostensibly) unrelated Greek kindreds (Polymeropoulos *et al.* 1997). The actual mutation was a single base-pair change at position 209 from G to A, resulting in an alanine to threonine substitution at amino acid 53 (A53T) (Polymeropoulos *et al.* 1997). Since the seminal discovery of the A53T mutation, seven additional missense mutations in the SNCA gene have been identified to result familial PD. In the following year, the A30P mutation was described, and in 2004, the E46K mutation (Kruger *et al.* 1998; Zarranz *et al.* 2004). Most recently, the H50Q, G51D, A53E, A18T, and A29S mutations were reported (Appel-Cresswell *et al.* 2013; Hoffman-Zacharska *et al.* 2013; Lesage *et al.* 2013; Pasanen *et al.* 2014). Beyond missense mutations, genome-wide association studies have identified several single-nucleotide polymorphisms in the SNCA gene that segregate with typical PD (Satake *et al.* 2009; Simón-Sánchez *et al.* 2009; Edwards *et al.* 2010; Li *et al.* 2013). Interestingly, all currently identified mutations in the SNCA gene that result in PD occur within the N-terminus of the protein. Functional studies examining the lipid-binding characteristic or aggregation kinetics of these mutants confirm that familial mutations either disrupt membrane binding or increase the aggregation kinetics of α-syn (Conway *et al.* 1998; Fredenburg *et al.* 2007; Burré *et al.* 2012; Lesage *et al.* 2013; Fares *et al.* 2014), resulting in impaired α-syn function.

Multiplications of the SNCA gene also result in familial PD. Both duplications and triplications of the SNCA locus result in PD (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Farrer *et al.* 2004; Ibanez *et al.* 2004). Patients with a duplication of the SNCA locus present at a similar age as idiopathic PD (Chartier-Harlin *et al.* 2004; Ibanez *et al.* 2004). In contrast, triplication of the SNCA locus results in an early onset and very rapidly progressing disease phenotype (Singleton *et al.* 2003; Farrer *et al.* 2004). In line with

increased copies of the SNCA gene, triplications patients have twice the amount of protein within the blood (Miller *et al.* 2004). Interestingly, although mRNA is doubled in the brains of SNCA triplication patients, there is no increase in the amount of soluble α-syn in the brain. However, there is an increase in aggregated α-syn within the brain, suggesting that increased SNCA protein product becomes sequestered into intracellular aggregates (Fig. 1c and d) (Miller *et al.* 2004). Multiplications in the SNCA gene have been identified in several other kindreds with familial PD (Nishioka *et al.* 2006; Fuchs *et al.* 2007; Ikeuchi *et al.* 2008), and even in patients with sporadic PD (Ahn *et al.* 2008).

Mutations in the SNCA promoter are also linked to PD. Genome-wide association studies have identified several polymorphisms in the SNCA gene and promoter that are risk factors for developing PD (Farrer *et al.* 2001; Satake *et al.* 2009; Simón-Sánchez *et al.* 2009; Edwards *et al.* 2010; Li *et al.* 2013). In particular, repeats in the REP1 allele within the 5′ untranslated region of SNCA are linked to PD. REP1-SNCA is a region approximately 10kb upstream of the SNCA gene that is necessary for the proper expression of α-syn (Chiba-Falek and Nussbaum 2001). This microsatellite contains natural repeats, which are expanded in some patients with PD (Chiba-Falek and Nussbaum 2001). Longer REP1 allele lengths (263 base pairs) result in increased SNCA expression in cultured cells, transgenic animals, and in human blood and brain (Chiba-Falek and Nussbaum 2001; Fuchs *et al.* 2008; Cronin *et al.* 2009; Linnertz *et al.* 2009). In contrast, shorter REP1 allele lengths (259 base pairs) are associated with decreased SNCA expression (Maraganore *et al.* 2006). REP1 allele lengths that correlate with increased SNCA expression are risk factors for developing PD. This is in line with data from patients with multiplications in the SNCA locus, in which there seems to be a gene dosage effect, wherein increased expression of SNCA is directly correlated with disease severity.

Finally, altered α-syn expression also occurs in sporadic PD patients. There have been several studies examining the expression of the SNCA gene in the midbrain and other brain regions of patients with idiopathic PD (Neystat *et al.* 1999; Beyer *et al.* 2004; Kingsbury *et al.* 2004; Tan *et al.* 2005; Chiba-Falek *et al.* 2006; Dächsel *et al.* 2007; Gründemann *et al.* 2008). Altogether, the results from these studies are fairly inconsistent, with some results showing increased SNCA expression (Chiba-Falek and Nussbaum 2001; Gründemann *et al.* 2008), and others showing decreased expression (Kingsbury *et al.* 2004; Dächsel *et al.* 2007) or no change in SNCA expression (Tan *et al.* 2005; Quinn *et al.* 2012). These inconsistent results have been attributed to normalizing SNCA expression levels to housekeeping genes that change during disease progression. Examination of SNCA expression using more appropriate control genes provides a consensus that α-syn mRNA is decreased in the substantia nigra pars compacta (SNc) of PD patients (Dächsel *et al.* 2007). However, whether these reductions in expression represent an initial maladaptive change in SNCA expression that triggers toxicity, or merely a compensatory reduction in SNCA expression because of increased α-syn protein accumulation in the soma, is not currently clear. For example, as healthy humans age, there is an age-related redistribution of α-syn protein from the pre-synaptic terminal to the somatodendritic compartment in neuromelanin-containing neurons of SNc (Fig. 1b) (Chu and Kordower 2007; Xuan *et al.* 2011). Further, PD is characterized by perinuclear accumulation of aggregated α-syn (Fig. 1c). These increases in protein could serve as negative feedback to decrease SNCA gene expression. Alternatively, a reduction in SNCA gene expression could simply be a normal process of aging. α-syn mRNA levels are reduced in normally aged humans and rodents (Galvin *et al.* 2001; Adamczyk *et al.* 2005; Mak *et al.* 2009). Accordingly, as aging is the primary risk factor for PD (Collier *et al.* 2011), decreased SNCA expression could represent an independent comorbidity that does not actually contribute to the pathogenesis of sporadic PD. That being said, while changes in SNCA gene expression may not directly contribute to the neurotoxicity of sporadic PD, it is clear that a shift in the levels of soluble to insoluble α-syn protein is directly related to PD pathogenesis.

α-syn aggregation in PD

Along with the loss of midbrain nigrostriatal DA neurons, PD is histopathologically defined by the presence of Lewy bodies (LB) and Lewy neurites (LN) (Braak *et al.* 2003a). LB are large cytoplasmic protein inclusions that were originally identified in the brains of PD patients by Frederic Heinrich Lewy in 1912 (Goedert *et al.* 2012). For over a century, Lewy pathology has been the defining neuropathological hallmark used for postmortem confirmation of PD (Goedert *et al.* 2012). However, it was not until the advent of more sophisticated histological techniques that LB were discovered to contain aggregated α-syn (Spillantini *et al.* 1997). Shortly after the discovery that mutations in the SNCA gene result in familial PD, Spillantini *et al.*, used affinity purified anti-α-syn antiserum to label LB and LN in brain tissue from patients with PD and DLB (Spillantini *et al.* 1997). In the following years, these more sensitive histological techniques identified widespread α-syn aggregation throughout both the central and peripheral nervous systems of PD patients (Spillantini *et al.* 1998a; Galvin *et al.* 1999; Bendor *et al.* 2013). Further, extensive comparisons of normal and Parkinson diseased brains has revealed that the neural loci affected by α-syn aggregation proceeds in a relatively stereotypic manner, progressing in a caudal to rostral pattern from the brainstem to the cortex over the course of the disease (Braak *et al.* 2003b). This revelation has led to implementation of various staging schema to diagnose disease severity based on the neuroanatomical nuclei displaying α-syn aggregates (Marui *et al.* 2002; Braak

et al. 2003a). The initial sites displaying α-syn aggregates in the CNS are the dorsal motor nucleus of the vagus in the brainstem and the olfactory bulb (Braak *et al.* 2003a). As the disease progresses, aggregated α-syn next appears in the pontine tegmentum, followed by the amygdala and SNc, and eventually reaching the temporal cortex and neocortex in the terminal stages of the disease (Braak *et al.* 2003b). Although this staging schema has been useful in segregating disease progression into quantal stages, not all patients follow the staging schema, and as such the utility of this classification system has been called into question (Halliday *et al.* 2006; Jellinger 2008; Beach *et al.* 2009), with new attempts at a unified staging scheme being put forth (Beach *et al.* 2009).

In addition to synuclein pathology in the brains of PD patients, there is also widespread synuclein pathology in the peripheral nervous system (Braak *et al.* 2006; Beach *et al.* 2010). The first LBs identified outside of the brain were found in the enteric nervous system (Wakabayashi *et al.* 1988, 1990, 1993). More recently, an extensive analysis identified aggregated α-syn in the gastrointestinal system, the spinal cord, sympathetic ganglia, as well as in the sciatic and vagus nerves (Beach *et al.* 2010). The presence of aggregated α-syn in cells outside of the basal ganglia provides a potential underlying mechanism for the myriad of non-motor symptoms associated with PD (Chaudhuri *et al.* 2006). For example, GI dysfunction is one of the most common non-motor symptoms associated with PD, and GI symptoms appear years prior to the onset of motor behavior (Pfeiffer 2003). In line with this, the most consistent location of aggregated α-syn outside the brain is the enteric nervous system, with PD patients presenting α-syn aggregates throughout the entirety of the enteric nervous system, from the esophagus to the rectum (Braak *et al.* 2006; Beach *et al.* 2010). Further, aggregated α-syn appears in the enteric nervous system years prior to CNS pathology and dysfunction in PD as do many GI symptoms (Wakabayashi *et al.* 1988; Braak *et al.* 2006; Beach *et al.* 2010; Shannon *et al.* 2012a,b), suggesting that aggregated α-syn within neurons of the enteric nervous system is likely the source of this GI dysfunction. This scenario is not limited to the GI tract; other non-motor symptoms of PD include hyposmia, autonomic disturbances, impaired micturation, rapid eye movement (REM) sleep disorder, as well as dementia and depression (Chaudhuri *et al.* 2006). In line with these symptoms, aggregated α-syn is observed in the olfactory bulb, autonomic ganglia, the dorsal horn of the sacral spinal cord, glossopharyngeal nerves, other brainstem nuclei, and cortical areas, potentially providing a pathophysiological substrate for all non-motor symptoms associated with PD (Dickson *et al.* 2009; VanderHorst *et al.* 2015).

Propagation of aggregated α-syn

The fact that the appearance of α-syn pathology proceeds throughout the PD-affected brain in a spatially and temporally stereotypic pattern has led to the suggestion that misfolded α-syn may spread from cell-to-cell in a prion-like manner (Olanow and Prusiner 2009; Lee *et al.* 2014). This 'prion hypothesis' for α-syn has been supported by several observations and experimental findings. First and foremost, two independent publications report that fetal mesencephalic DA neurons engrafted into the striatum (ST) of PD patients displayed LB-like structures (Kordower *et al.* 2008; Li *et al.* 2008). These LB-like structures were composed of aggregated α-syn, suggesting that misfolded or aggregated α-syn from the patient's brain had infiltrated the grafts, potentially serving as templates to initiate aberrant folding and accumulation of normal, endogenous α-syn expressed by the engrafted neurons (Li *et al.* 2008; Kordower *et al.* 2013). This host-to-graft transmission of pathological α-syn was replicated *in vitro* and in a transgenic mouse model of PD pathology (Desplats *et al.* 2009). These observations led to significant interest in the route by which α-syn could potentially be released by 'donor' cells and subsequently taken up by 'recipient' cells. To this end, both monomeric and oligomeric α-syn are secreted from cultured neurons through unconventional exocytosis (Lee *et al.* 2005, 2014; Jang *et al.* 2010) and the released α-syn can in turn be taken up by recipient neurons and glia through endocytosis (Lee *et al.* 2008, 2014). Within cultured cells, aggregated α-syn can transmit from neuron-to-neuron via these exocytosis and endocytosis events, and can propagate aggregates by serving as a seeding template to initiate the aggregation of endogenous α-syn in recipient cells (Desplats *et al.* 2009; Luk *et al.* 2009, 2012a; Volpicelli-Daley *et al.* 2011). These results have been mirrored *in vivo*; brain homogenates from old, symptomatic transgenic (α-syn over-expressing) mice exhibiting α-syn pathology were injected into young transgenic mice, which accelerated the formation of LB-like α-syn aggregates within the young mice (Luk *et al.* 2012a). Moreover, injection of preformed α-syn fibrils into the rodent brain leads to the formation of α-syn aggregates that appear distal to the injection site months after the injection. It remains to be proven, however, whether misfolded α-syn can propagate/transmit throughout the CNS over time, akin to that seen in prion disorders, or whether these findings are the result of diffusion and transport of the ectopic synuclein seeds to these distal areas (Luk *et al.* 2009, 2012a; Paumier *et al.* 2015). Finally, the presence of aggregated α-syn within oligodendrocytes (which do not normally express α-syn) of patients with MSA has been proposed to occur through a prion-like propagation from neurons to glia (Spillantini *et al.* 1998b; Miller *et al.* 2005) Although there is considerable evidence supporting a prion-like transfer of α-syn, this area of research remains contentious and not all researchers concur with a model by which cell-to-cell transfer of α-syn causes disease.

Animal models of PD

Because of the prominent role that α-syn plays in the pathology of PD and other synucleinopathies, many animal models have been developed in an attempt to elucidate both the normal function of α-syn, and the role α-syn plays in the pathology of synucleinopathies. To date, the vast majority of work on α-syn animal models has been performed in rodents (mice and rats), however, there has been research performed in larger mammals such as non-human primates, as well as in invertebrates (Drosophila and Caenorhabditis elegans). Across species, animal models centered on α-syn can be subdivided into α-syn over-expression models, and α-syn knockdown or knockout models. The α-syn over-expression models can then be further subdivided into viral vector-mediated α-syn over-expression and α-syn transgenic over-expressing mouse lines. This section will briefly describe the pathology observed following manipulation of α-syn expression within animal models, and some insights gained therein.

Over-expression: transgenic mice

The list of transgenic human α-syn (WT or mutant) over-expressing mouse lines is currently quite large and seems to be growing (Hashimoto *et al.* 1999; Kahle *et al.* 2000; Masliah *et al.* 2000; van der Putten *et al.* 2000; Lee *et al.* 2001b, 2002; Matsuoka *et al.* 2001; Giasson *et al.* 2002; Richfield *et al.* 2002; Rockenstein *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003; Thiruchelvam *et al.* 2004; Tofaris *et al.* 2006; Wakamatsu *et al.* 2008; Lin *et al.* 2012). The majority of transgenic lines over-expressing mutant α-syn encode either the A53T or A30P familial mutations (Kahle *et al.* 2000; van der Putten *et al.* 2000; Matsuoka *et al.* 2001; Giasson *et al.* 2002; Lee *et al.* 2002; Rockenstein *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003; Lin *et al.* 2012), however, there are also double mutant transgenic lines that express both A53T and A30P mutations (Richfield *et al.* 2002; Thiruchelvam *et al.* 2004), as well as C-terminal truncated mutants (Tofaris *et al.* 2006), or even a combination of familial mutations with C- terminal truncation (Wakamatsu *et al.* 2008). One major difference between existing α-syn transgenic mice is the promoter used to control transgene expression. Existing promoters used include the Thy1 promoter (Kahle *et al.* 2000; van der Putten *et al.* 2000; Rockenstein *et al.* 2002), the human platelet-derived growth factor β promoter (Masliah *et al.* 2000; Rockenstein *et al.* 2002), the TH promoter (Richfield *et al.* 2002; Thiruchelvam *et al.* 2004; Tofaris *et al.* 2006; Wakamatsu *et al.* 2008), and the prion promoter (Giasson *et al.* 2002; Lee *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003). Additionally, a tetracycline inducible α-syn expression system was recently described (Lin *et al.* 2012). A notable exception to the use of artificial promoters to guide transgenic α-syn expression are mice lacking murine α-syn gene (mSNCA−/−) and carrying a p1 artificial chromosome expressing the entire human SNCA locus, including the endogenous SNCA promoter (Kuo *et al.* 2010).

The type of promoter used will determine the type of cells that the α-syn transgene will be expressed within. For example, many of the α-syn transgenic lines show widespread expression through the entire animal, however, some lines show more restricted expression, such as those utilizing the TH promoter (Matsuoka *et al.* 2001; Richfield *et al.* 2002; Thiruchelvam *et al.* 2004; Tofaris *et al.* 2006). Beyond determining the type of cells that the transgene will be expressed in, the choice of promoter will also determine the temporal onset of expression as well as the level of transgene expression. Currently, the range of α-syn over-expression observed in transgenic mouse lines is very large, varying from 0.3 to more than 30 fold increased expression over that of endogenous α-syn levels (Fleming *et al.* 2005). Finally, although most current transgenic mouse lines are converging toward a consensus of using the C57Bl6 strain background, not all α-syn transgenic mice are of the same strain, and this can affect the pattern and severity of pathology observed. Although this will not be discussed further, it is an important caveat to consider when interpreting data from various transgenic animals, and should thus be kept in mind.

The ectopic over-expression of α-syn in transgenic mice can result in the formation of aggregated α-syn (Fig. 1d) (Kahle *et al.* 2000; Masliah *et al.* 2000; van der Putten *et al.* 2000; Giasson *et al.* 2002; Lee *et al.* 2002; Rockenstein *et al.* 2002; Tofaris *et al.* 2006; Lin *et al.* 2012), however, this is not always the case (Matsuoka *et al.* 2001; Richfield *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003; Thiruchelvam *et al.* 2004). Differences in the aggregation profiles observed in the varying transgenic lines likely reflect the varying levels of expression achieved, as well as the type of α-syn mutation expressed. When aggregates are observed, the extent to which these aggregates recapitulate the aggregation of α-syn in PD is uncertain, however, many α-syn transgenic lines do show markers associated with LB-like pathology, such as positive staining with thioflavin or silver stain, co-labeling with ubiquitin or α-syn phosphorylated at serine 129, (Masliah *et al.* 2000; van der Putten *et al.* 2000; Giasson *et al.* 2002; Lee *et al.* 2002; Rockenstein *et al.* 2002; Tofaris *et al.* 2006). Further, in many instances in which aggregates are observed, they are not necessarily observed within the nigrostriatal system. For example, the Thy1 or prion promoter lines demonstrate extensive α-syn aggregation in the spinal cord and/or neuromuscular junction (van der Putten *et al.* 2000; Giasson *et al.* 2002; Lee *et al.* 2002; Gomez-Isla *et al.* 2003; Cabin *et al.* 2005). Other transgenic lines show extensive α-syn aggregation throughout the brain (e.g. cortex, hippocampus, cerebellum, and brainstem) with little or no aggregates found in the SNc (Masliah *et al.* 2000; Giasson *et al.* 2002; Rockenstein *et al.* 2002). Accordingly, it is likely that although many of these

mice do develop some motor impairments, those impairments are not the result of degeneration of the nigrostriatal system, but more likely represent pyramidal or motor neuron degeneration (van der Putten *et al.* 2000; Giasson *et al.* 2002; Cabin *et al.* 2005). Although the motor dysfunction in some α-syn transgenic lines may not recapitulate the motor impairment of PD with perfect fidelity, the models are still valuable for studying the effects of synucleinopathy outside the nigrostriatal system, which also occurs in PD (Beach *et al.* 2010). For example, some α-syn transgenic mice develop olfactory, GI, and autonomic dysfunction reminiscent of changes observed in PD (Fleming *et al.* 2008; Wang *et al.* 2008; Kuo *et al.* 2010; Hallett *et al.* 2012; Noorian *et al.* 2012; Farrell *et al.* 2014).

In general, a major shortcoming of current α-syn transgenic mice is the lack of degeneration of the nigrostriatal DA system (van der Putten *et al.* 2000; Giasson *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003). The loss of midbrain nigrostriatal neurons and the corresponding loss of DA innervation to the ST underlies the motor impairment which characterize parkinsonism, and as such any reliable model used to study PD pathology should recapitulate this defining feature. There are notable exceptions to this criticism. Mice expressing WT α-syn under control of the platelet-derived growth factor β promoter display decreased ST DA along with decreased TH terminal density, TH protein, and TH activity in the ST (Masliah *et al.* 2000; Rockenstein *et al.* 2002). ST pathology correlates with the presence of α-syn inclusions within neurons and glia throughout the brain, and result in motor impairment (Masliah *et al.* 2000; Rockenstein *et al.* 2002; Hashimoto *et al.* 2003). Interestingly, although there was a significant loss of TH and DA levels in the ST of these animals, the majority of α-syn inclusions were located outside the midbrain, with only occasional aggregates found in THir neurons of the SNc (Masliah *et al.* 2000). Further, there was no loss of THir neurons of the SNc, suggesting that the pathology observed reflects a loss of the TH phenotype within the nerve terminals in the absence of any nigrostriatal neuron loss (Masliah *et al.* 2000). Another commonly used α-syn transgenic mouse is the Thy1 α-syn mouse. There have been several iterations of this particular transgenic mouse with varying degrees of pathology (Kahle *et al.* 2000; van der Putten *et al.* 2000; Rockenstein *et al.* 2002). For instance, Thy-1 A53T α-syn mice display prominent α-syn inclusions that are particularly enriched in the spinal cord and neuromuscular junction, but absent in the SNc (van der Putten *et al.* 2000). These animals manifest a severe motor phenotype at an early age, which likely reflects pyramidal or motor neuron degeneration (van der Putten *et al.* 2000). In contrast, WT or A30P α-syn expressed by the Thy-1 promoter produces α-syn aggregation in the SNc and ST, as well as decreased ST DAT and VMAT, with no reported cell loss (Kahle *et al.* 2000; Rockenstein *et al.* 2002; Fleming *et al.* 2005). Expression of human α-syn under the control of the catecholaminergic specific TH promoter results in a more robust pathological phenotype within the nigrostriatal system producing a loss of TH neurons with age (Richfield *et al.* 2002; Thiruchelvam *et al.* 2004). However, loss of TH neurons was not observed in an alternative report, despite high levels of expression and the accumulation of α-syn in the SNc. (Matsuoka *et al.* 2001) Expression of a double mutant A53T+A30P α-syn under the TH promoter results in a loss of neurons in the SNc and a corresponding loss of ST DA and DA metabolites, along with reduced locomotor activity (Richfield *et al.* 2002; Thiruchelvam *et al.* 2004). Finally, TH promoter-mediated expression of C-terminally truncated human α-syn carrying the A53T mutation causes a loss of midbrain DA neurons in the SNc and a loss of DA in the ST (Wakamatsu *et al.* 2008).

In contrast to the mice described above, in which α-syn expression is initiated during development, post-developmental expression of α-syn achieved through a tet-inducible system produces a pattern of pathology that more faithfully recapitulates PD (Lin *et al.* 2012). Following the activation of transgene α-syn expression, there is a 2–4 fold increase in human α-syn expression that is largely restricted to midbrain DA neurons (Lin *et al.* 2012). Increased α-syn expression results in the formation of α-syn aggregates in the somata and terminals of TH neurons (Lin *et al.* 2012). This conditional A53T human α-syn mouse also develops a progressive loss of THir neurons in the SNc (Lin *et al.* 2012) concomitant with ST denervation and impaired DA release (Lin *et al.* 2012). Finally, this pathology results in profound motor impairment that is reversed by inactivating the transgene (Lin *et al.* 2012). The more robust and consistent pathology seen when α-syn is altered within neurons of adult animals, as opposed to germline manipulation of α-syn, highlights the confounding variable of genetic compensation observed when animals harbor a genetic aberration during development. As will be discussed in detail in later sections, with particular emphasis on α-syn null mice, the issue of genetic compensation often diminishes the conclusions that can be drawn from germline transgenic animals.

Over-expression: transgenic invertebrates

Transgenic over-expression of α-syn has also been modeled in Drosophila and C. elegans, neither of which normally express the α-syn gene. In Drosophila, transgenic flies expressing either WT, A30P, or A53T α-syn downstream of multiple binding sites for the yeast transcriptional activator protein GAL4 were crossed with flies expressing GAL4 under control of a pan neuronal promoter, or the DA neuron specific promoter DOPA-decarboxylase, resulting in an age-dependent loss of THir neurons in the dorsomedial cluster (Feany and Bender 2000). The over-expression of the α-syn transgene resulted in formation of aggregates that paralleled the temporal progression of toxicity (Feany and Bender

2000). Synucleinopathy and loss of DA neurons resulted in a motor phenotype, in the form of a reduction in negative geotactic motor behavior (Feany and Bender 2000). Interestingly, as has been observed in transgenic mice, this pathology could be completely rescued by the co-expression of the chaperone, Hsp-70 (Auluck *et al.* 2002). Co-expression of Hsp-70 did not affect the number, size, or location of the α-syn aggregates, yet prevented the neurodegeneration caused by the α-syn transgene (Auluck *et al.* 2002). Finally, either WT or A53T α-syn over-expression in the nematode, C. elegans, results in rare formation of α-syn aggregates and a loss of DA neurons (Lakso *et al.* 2003).

Over-expression: viral vectors

Though transgenic mouse models may improve our understanding of α-syn biology, to date, no mouse model accurately recapitulates the progressive neurodegenerative changes that are the hallmark of PD. In this regard, viral over-expression of α-syn may be considered a more accurate model of PD pathology. The majority of reports on virally mediated over-expression of α-syn utilize adeno-associated virus (AAV) (Kirik *et al.* 2002), however, there have also been reports of lentivirus (LV)-mediated over-expression (Bianco *et al.* 2002). As in the case of transgenic animals, the major differences between viral over-expression models is the type of promoter used to control transgene expression, as well as the use of either WT or mutant forms of α-syn. Virally mediated over-expression of α-syn is commonly driven by the highly active and ubiquitous chicken β-actin promoter (CBA; aka CAG), or the slightly less active cytomegalovirus (CMV) promoter. However, other promoters are now being utilized, such as the neuron specific synapsin promoter. Transgene expression following viral delivery is not only dependent on the promoter used, but also on the number of viral particles injected (Gombash *et al.* 2013), as well as the pseudotype of virus utilized. Accordingly, virally mediated over-expression of α-syn offers several added levels of control, which can act to spatially and temporally guide transgene expression within phenotypically distinct cells of the brain.

LV has been used to deliver both WT and mutant forms of α-syn to the SNc of mice and rats (Bianco *et al.* 2002; Lauwers *et al.* 2003). Injection of vesicular stomatitis virus glycoprotein pseudotyped LV expressing either WT, A53T, or A30P α-syn under control of the phosphoglycerate kinase (PKG) promoter into the rat SNc results in aggregation of α-syn and a corresponding degeneration of TH immunoreactive (THir) nigrostriatal soma (24–35% loss) and terminals (Bianco *et al.* 2002). The degeneration of THir neurons in the SNc is slightly progressive, increasing from 3 weeks, to peak at the 6 week time point (Bianco *et al.* 2002). In contrast, LV-mediated expression of WT or A30P α-syn in the mouse SNc produces comparably less pathology (Lauwers *et al.* 2003). Using the CMV promoter, there is a 10–25% loss of THir neurons in the SNc, with no loss of striatal DA, that requires up to 12 months of transgene expression to manifest (Lauwers *et al.* 2003). It is interesting to note that LV-mediated α-syn over-expression in TH neurons of α-syn null mice results in α-syn aggregation and loss of THir in dopaminergic neurons that are still present in CNS (Alerte *et al.* 2008). This raises the possibility that such effects may lead to erroneous interpretation of a loss of dopamine neurons if quantification of THir cells is the sole outcome measured.

AAV is the most commonly used viral vector system to over-express α-syn. The original description of the AAV-α-syn model utilized AAV serotype 2 to express either WT α-syn or the A53T mutant under control of the CBA promoter (Kirik *et al.* 2002). Surprisingly, there is no difference in the pathology observed following injection of WT versus the A53T mutant form of α-syn. In both treatments, over-expression results in the appearance of aggregated α-syn, dystrophic α-syn-immunoreactive neurites, and shrunken pyknotic soma (Kirik *et al.* 2002). Pathology is slightly progressive, beginning at approximately 3 weeks, and reaching a plateau at 6 weeks post-transduction (Kirik *et al.* 2002). Peak pathology reported was highly variable, 30–80% loss of THir neurons in the SNc, and a corresponding 50% loss of TH fibers and DA in the ST (Kirik *et al.* 2002). Further, striatal TH enzymatic activity was also reduced (Kirik *et al.* 2002). Finally, AAV2-α-syn over-expression produces a variable loss of motor behavior, likely resulting from the inconsistent amount of neurodegeneration observed in the SNc (Kirik *et al.* 2002). Interestingly, by 6 months post-surgery, striatal TH and THir neuron numbers in the SNc appear to increase, demonstrating some recovery from the neuropathology (e.g. loss of TH phenotype) induced by AAV-α-syn expression (Kirik *et al.* 2002). Other laboratories replicating this model report an increase in microglial activation, B- and T-cell infiltration (Theodore *et al.* 2008), and changes in proteins involved in axonal trafficking with a corresponding loss of proteins involved in synaptic transmission (Chung *et al.* 2009), pathological changes that are observed in PD.

A similar pattern of pathology occurs with nigral over-expression of the A30P mutant α-syn, again producing α-syn aggregates and an approximate 50% loss of SNc neurons (Klein *et al.* 2002). AAV2 expressing WT α-syn under control of the CMV promoter injected into the SNc produces a similar loss of THir nigral neurons, as well as accumulation of α-syn phosphorylated at serine 129 (Yamada *et al.* 2004). Finally, as was observed using LV, injection of the same expression cassette in mice does not elicit the same degree of pathology as compared to rats: Mice injected with AAV2 expressing WT α-syn under control of the CBA promoter do not develop obvious α-syn inclusions, with a mere 25% neurodegeneration of nigral neurons that does not appear until 24 weeks post-transduction (St Martin *et al.* 2007) [as compared with a 40–50% loss of nigral neurons by 6 weeks

in rats (Kirik *et al.* 2002)]. In this report, the proportion of THir nigral neurons transduced was also highly variable, (St Martin *et al.* 2007), and accordingly, the reduced pathology in mice may result from mistargeted injections or decreased viral expression.

Perhaps, the best-characterized and most accurate recapitulation of PD pathology reported using virally mediated over-expression is achieved using AAV2/6 expressing WT α-syn under control of the neuron-specific synapsin promoter (Decressac *et al.* 2012). This model produces robust degeneration of the nigrostriatal system that is highly specific and progressive. For example, there is a progressive loss of THir neurons of the SNc that proceeds from 50% at 3 weeks post-surgery to 80% at 8 weeks post-surgery (Decressac *et al.* 2012). The progressive loss of THir neurons of the SNc is mirrored by a progressive decline in TH fibers, TH activity, and DA concentration in the ST (Decressac *et al.* 2012). The authors also reported a progressive decline in DA release and reuptake, along with a progressive decline in motor function that could be rescued by L-DOPA administration (Decressac *et al.* 2012). The progressive nature of the SNc pathology by these investigators very nicely models human PD.

The patterns of neuropathology and behavioral deficits observed in viral rodent models have been replicated in monkeys, albeit over a much more protracted time course. Both AAV2 and AAV 2/5 expressing WT or A53T α-syn under control of the CBA promoter have been injected into the SNc of adult marmosets (Kirik *et al.* 2003; Eslamboli *et al.* 2007). AAV2 expressing α-syn produces a significant loss of neurons of the SNc and TH fibers in ST at 16 weeks post-surgery. This degeneration is accompanied by the presence of dystrophic neurites, swollen axons, pyknotic soma, and the appearance of granular α-syn aggregates (Kirik *et al.* 2003). Using AAV2/5 and a longer time course, Eslamboli *et al.* 2007 described a large amount of α-syn aggregation, loss of nigrostriatal THir neurons and fibers, as well as marked behavioral abnormalities, including impaired contralateral forelimb use and a bias in the head position test (Eslamboli *et al.* 2007).

Taken together, most pathological changes observed using either transgenic α-syn mouse lines or virally mediated over-expression of α-syn replicate changes observed in the PD brain, lending support to the construct validity of these models. However, although some aspects of PD pathology may be replicated in one particular model, the same model often lacks other pathological changes observed in PD, and currently there is no single model that replicates every aspect of PD neuropathology. For instance, virally mediated over-expression of α-syn results in a distinct pattern of neuropathology that, unlike that seen in transgenic over-expressing animals, is highly selective to the nigrostriatal system. Viewed as a whole, the use of a viral vector to express an α-syn transgene results in the aggregation of α-syn, followed by a progressive loss of THir neurons of the SNc, as well as a corresponding loss of nigrostriatal fibers and DA concentrations in the ST. However, the extent to which these models are truly progressive could be argued, as all virally mediated α-syn lesions plateau within a relatively short time (Bianco *et al.* 2002; Kirik *et al.* 2002; Klein *et al.* 2002; Yamada *et al.* 2004; Decressac *et al.* 2012), and in some instances, even seem to recover (Kirik *et al.* 2002), perhaps reflecting a transient loss of the TH phenotype induced by α-syn aggregation (Alerte *et al.* 2008).

In contrast, the neurodegeneration observed in PD is truly progressive, in that it proceeds in an inexorable sequence ultimately leading to death (Hely *et al.* 1999). Neuropathology with viral vector-mediated over-expression appears very quickly, sometimes as early as 2–4 weeks (Bianco *et al.* 2002; Kirik *et al.* 2002; Decressac *et al.* 2012), which is in stark contrast to pathology seen in transgenic animals that require months or years to achieve neurodegeneration (Masliah *et al.* 2000; Lee *et al.* 2001b; Giasson *et al.* 2002; Rockenstein *et al.* 2002). In this sense, the presentation of neuropathology in transgenic animals, in which α-syn accumulation and subsequent neurodegenerative changes occur over the lifetime of an animal, may better model the slow progression of the human disease. Because of the fact that virus is often injected directly into the SNc, the pathology observed in viral over-expression models is usually restricted to the nigrostriatal system. This localized injection can achieve very high concentrations of transgene product, somewhat exclusively within the nigrostriatal system, which presumably accounts for the much more consistent and robust nigrostriatal degeneration observed by viral over-expression (Bianco *et al.* 2002; Kirik *et al.* 2002, 2003; Klein *et al.* 2002; Yamada *et al.* 2004; Eslamboli *et al.* 2007; St Martin *et al.* 2007; Decressac *et al.* 2012), as compared to transgenic animals in which nigrostriatal degeneration is often completely absent (van der Putten *et al.* 2000; Giasson *et al.* 2002; Gispert *et al.* 2003; Gomez-Isla *et al.* 2003). Although virus is beneficial to study degeneration of the nigrostriatal system, PD is a systemic disease that affects many regions of the brain as well as the periphery (Beach *et al.* 2010), and in this sense, the α-syn expression achieved throughout the entire organism in α-syn over-expressing mice may be a more useful PD model. Finally, in many of the viral over-expression models, deficits in motor behavior are either variable or completely lacking (Bianco *et al.* 2002; Kirik *et al.* 2002; Klein *et al.* 2002; Yamada *et al.* 2004; St Martin *et al.* 2007). However, it is likely that the lack of motor deficits result from a partial lesion of the nigrostriatal system (Kirik *et al.* 2002). A critical threshold of nigrostriatal neurodegeneration is known to be necessary to induce the onset of motor deficits in humans and certain PD models (Bezard *et al.* 2001; Kirik *et al.* 2002), suggesting that most PD models fail to reach this critical threshold. This idea is further supported by a clear gene-dosage effect, whereby increased α-syn expres-

sion and accumulation cause a correspondingly increased toxicity (Masliah *et al.* 2000; Lee *et al.* 2001b; Giasson *et al.* 2002; Gispert *et al.* 2003; Decressac *et al.* 2012; Gombash *et al.* 2013).

Silencing α-syn expression: α-syn null mice

In addition to transgenic mice over-expressing human α-syn, efforts to elucidate the biological function(s) of α-syn have also focused on removing the protein from the entire animal. To this end, there have been several independent α-syn knockout (KO) mouse lines created (Abeliovich *et al.* 2000; Dauer *et al.* 2002; Schlüter *et al.* 2003; Cabin *et al.* 2005). Additionally, a spontaneous deletion of the SNCA locus was identified in a subpopulation of C57bl/6j mice from Harlan Laboratories (Specht and Schoepfer 2001; Schlüter *et al.* 2003). Overall, the α-syn null mice do not display overt neuropathological or behavioral phenotypes, however, in depth analyses reveal abnormalities that largely localize to the presynaptic terminal. α-syn null mice evaluated by Abeliovich *et al.* (2000). display increased DA release following paired electrical stimulus, a modest (18%) reduction in striatal DA content, reduced rearing in the open-field test, and an attenuation of amphetamine-induced locomotor response [the latter of these findings failed to replicate in an alternate α-syn KO model (Cabin *et al.* 2002)]. Ultrastructural analyses of the neurons of α-syn null mice generated by Cabin *et al.*, show a 50% reduction in the reserve pool of synaptic vesicles in primary hippocampal cultures and hippocampal sections from 2-month-old mice (Cabin *et al.* 2002). Some reports show that α-syn null mice have a slightly reduced number of THir neurons in SNc (Robertson *et al.* 2004), however, others failed to replicate this (Abeliovich *et al.* 2000; Cabin *et al.* 2002). Finally, still others have shown that aged (2 years) α-syn null mice develop denervated ST as reflected by decreased DA, TH, DAT, and synaptotagmin concentrations (Al-Wandi *et al.* 2010).

It was originally believed that functional redundancy between α and β synuclein could have masked some of the results in α-syn null mice. There is a high degree of homology in the primary amino acid sequences of α and β synuclein, which tend to co-localize in presynaptic terminals throughout much of the brain, suggesting that the two proteins may be functionally redundant (Surguchov 2008). In contrast, γ synuclein is highly enriched within neural tissue of the periphery, and largely absent from the brain (Surguchov 2008). Accordingly, there have also been double synuclein KO mice created, in which either α and β (Chandra *et al.* 2004) or α and γ synuclein (Robertson *et al.* 2004) were deleted, as well as triple KOs in which all three synucleins were deleted (Greten-Harrison *et al.* 2010). However, double and triple KO mice do not have a substantially different neuropathological profile from α-syn null mice. The α/β double KO mice have reduced striatal DA

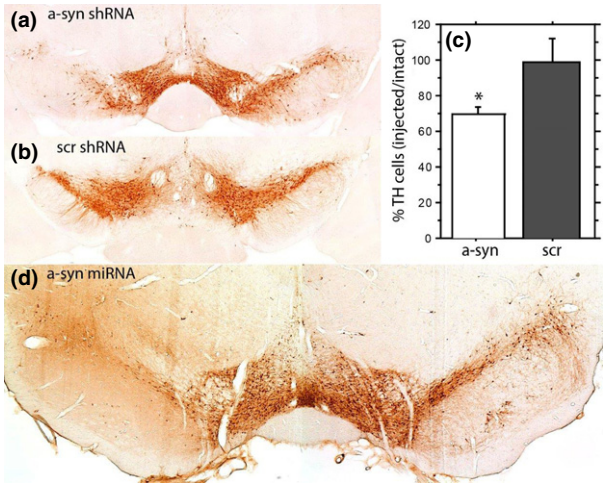


Fig. 3 Nigral neurodegeneration due to loss of α-syn translates across species and knockdown approaches. (a and b) Adult mice were injected in the left substantia nigra (SNc) with adeno-associated virus (rAAV) 2/5 expressing a shRNA targeting mouse α-syn (A) or a scrambled control shRNA(Scr) (B) (1.5 μL of 2.6×10^{12} vector genomes/mL). (c) 28-days following vector injection animals were sacrificed and numbers of tyrosine hydroxylase neurons in the SNc were quantified using unbiased stereology. rAAV-mediated expression of α-syn shRNA results in an approximate 30% loss of neurons of the SNc (expressed as percent of intact hemisphere). * Indicates significantly different than scrambled shRNA control ($p < 0.05$). (d) Several microRNA sequences targeting rat α-syn were evaluated *in vitro*. The microRNA achieving the most efficient knockdown of α-syn *in vitro* was packaged into rAAV2/5 and injected in to the left SNc of adult rats (1.5 μL of 1×10^{12} vector genomes/mL). MicroRNA-mediated knockdown of α-syn results in a robust reduction of tyrosine hydroxylase neurons within the injected SNc.

that is approximately equal to that reported in the α-syn null mouse (Chandra *et al.* 2004). Interestingly, despite a largely peripheral localization in normal animals, KO of γ synuclein alone results in a 15–20% loss of THir neurons in the SNc, and this effect is not significantly altered in the α and γ synuclein double KO mice (Robertson *et al.* 2004). The idea of functional redundancy and genetic compensation in germ line transgenic animals is further highlighted by the finding that all of the double KO synuclein animals show a compensatory increase in the remaining synuclein family member. For instance, the α/β KO mouse displays increased γ synuclein (Chandra *et al.* 2004), while the α/γ KO mice have increased β synuclein (Robertson *et al.* 2004). Some groups have reported a small, region-specific compensatory increase in β synuclein in α-syn null mice (Schlüter *et al.* 2003), however, the majority of reports saw no difference in β synuclein expression after deletion of the SNCA gene (Abeliovich *et al.* 2000; Cabin *et al.* 2002; Dauer *et al.* 2002). Finally, removal of all synucleins significantly increases the mortality of mice (Greten-Harrison *et al.* 2010). The triple α/β/γ synuclein KO mouse also develop

an age-dependent reduction in synapse size and altered synaptic transmission (Greten-Harrison *et al.* 2010). The reduction in synapse size can be rescued by crossing the triple KO with the Thy1 α-syn over-expressing mouse in order to restore α-syn function, demonstrating a specific effect of synuclein (Greten-Harrison *et al.* 2010).

In several independent reports, α-syn null mice exhibit either a complete or partial resistance to 1-methyl, 4-phenyl-1, 2, 3, 6, tetrahydropyradine (MPTP) (Dauer *et al.* 2002; Schlüter *et al.* 2003; Drolet *et al.* 2004; Robertson *et al.* 2004). MPTP is a pro-toxicant that, once metabolized to 1-meth, 4-phenylpyradinium (MPP+), is transported into dopaminergic terminals and synaptic vesicles through direct interactions with DAT and VMAT, respectively (Dauer and Przedborski 2003). Interestingly, α-syn null mice are more sensitive to the neurotoxicant rotenone (Dauer *et al.* 2002), which is lipophilic and does not require active transport via DAT or VMAT for entering dopaminergic terminals. The differential susceptibility of α-syn null mice to MPTP and rotenone suggests that the decreased MPTP sensitivity of the mice is likely mediated through changes in the dopaminergic terminal. Supporting this, α-syn null mice also display reduced MPTP-induced DA efflux (Dauer *et al.* 2002), a phenomenon that occurs as a result of reversal of DA flow through DAT, in response to large increases in cytosolic DA following DA displacement from synaptic vesicles by MPP+(Lotharius and O'Malley 2000). It is likely that changes in synaptic vesicle mobility and concentrations underlie the altered responses. For example, the reduced size of the reserve vesicular pool could potentially account for the lack of MPTP-induced DA efflux. Further, if α-syn acts as a negative regulator of vesicular trafficking and exocytosis, it is possible the MPP+ within synaptic vesicles could be purged more rapidly in α-syn null than WT mice, preventing or diminishing the amount of MPP+ available to interact with Complex I.

Silencing α-syn expression: α-syn Knock Down

In contrast to germline deletion of α-syn, knock down of endogenous α-syn in post-mitotic neurons of adult animals causes severe neurodegeneration (Fig. 1e) (Gorbatyuk *et al.* 2010; Khodr *et al.* 2011; Kanaan and Manfredsson 2012; Collier *et al.* 2016). For example, delivery of AAV expressing α-syn shRNA into the SNc of rats results in a rapid and robust loss of neurons in the SNc (Gorbatyuk *et al.* 2010). Here, there is a dose-dependent loss of nigral neurons, in which the level of knockdown of endogenous α-syn correlates with the amount of nigral neuron loss (Gorbatyuk *et al.* 2010). Importantly, the neuropathology and behavioral deficits observed are rescued by supplementation of rat α-syn (rendered insensitive to the shRNA), demonstrating that the toxicity is explicitly because of the loss of endogenous α-syn (Gorbatyuk *et al.* 2010). We have repeated this approach in mice (Fig. 3) and non-human primates (Collier *et al.* 2016), as well as by using a microRNA approach in rats (Fig. 3). In every instance, regardless of the method or species used, a removal of endogenous α-syn beyond a critical threshold produces SNc neurodegeneration. Other laboratories have also reported a pronounced loss of nigral neurons following AAV-mediated delivery of an shRNA targeting α-syn (Khodr *et al.* 2011), while an alternative report found that low level α-syn knock down results in a 20% reduction in ST DA (Zharikov *et al.* 2015). Further, knock down of endogenous α-syn in primary hippocampal neurons using antisense oligonucleotides decreases the size of the presynaptic vesicle pool (Murphy *et al.* 2000). However, not all studies show that reduction of endogenous α-syn results in neuronal loss *in vivo*. For example, no toxicity was reported after knockdown of endogenous α-syn in mouse hippocampus using naked siRNA (Lewis *et al.* 2008), however, there was no detailed analysis of cell loss or toxicity in that report. Additionally, delivery of siRNA targeting endogenous α-syn to the midbrain of squirrel monkeys does not result in appreciable nigrostriatal degeneration (McCormack *et al.* 2010). These discrepant findings likely reflect differing levels of knockdown of endogenous α-syn.

There appears to be a critical threshold of α-syn knockdown required in order to produce toxicity, and the severity of toxicity appears to be inversely proportional to the amount of endogenous α-syn remaining (Gorbatyuk *et al.* 2010; Kanaan and Manfredsson 2012). In this sense, it seems likely that alternative reports did not observe toxicity because the level of α-syn knockdown was either insufficient or not sustained for a requisite amount of time. For example, the maximum reduction in α-syn protein reported following delivery of α-syn siRNA to the squirrel monkey was 40% (McCormack *et al.* 2010). In contrast, toxicity in the rat was initially observed when α-syn protein was decreased by ~ 70% following delivery of an α-syn shRNA (Gorbatyuk *et al.* 2010). Further, although there was a 55% reduction in α-syn protein following the direct injection of siRNA into the mouse hippocampus, knockdown persisted for only 1 week, after which time endogenous α-syn rebounded to control levels (Lewis *et al.* 2008). As is the case in viral over-expression models, mice seem to require longer periods of time to develop a corresponding level of toxicity observed in a rat (Fig. 3). Finally, as is evident with the neurodegeneration observed in PD, there is a differential susceptibility of central neuronal populations to α-syn associated toxicity. It is likely that nigrostriatal neurons are particularly sensitive to loss of α-syn, possibly for reasons that will be elaborated below.

Although there are not many studies reporting neurodegeneration following knockdown of endogenous α-syn *in vivo*, there have been several descriptions of toxicity and cell loss after removal of endogenous α-syn *in vitro*. For example, knockdown of α-syn significantly decreases viability of SH-SY5Y or MN9D DA cells (Liu *et al.* 2008; Han

et al. 2011). Knockdown of endogenous α-syn in SK-MEL 28 cells increases levels of the pro-apoptotic protein Bax, and reduces levels of the anti-apoptotic proteins Bcl2 and Bcl-xl, causing significantly reduced proliferative indices and increased sensitivity to staurosporine-induced apoptosis (Choong and Say 2011). Knockdown of endogenous α-syn in HeLa cells impairs mitochondrial calcium uptake, and results in an increase in fragmented mitochondria (Cali *et al.* 2012). Within that study, an increase in α-syn expression was induced, resulting in the formation of α-syn aggregates, presumably sequestering soluble α-syn and reducing functional α-syn levels within the cell (Cali *et al.* 2012). When α-syn was sequestered in aggregates, the ability of mitochondria to buffer calcium was decreased, replicating the pathological effects observed with α-syn knockdown (Cali *et al.* 2012). Finally, other studies have demonstrated a beneficial effect of α-syn knockdown in cultured cells, which replicated studies in α-syn null mice that demonstrate a resistance to MPP+ (Fountaine and Wade-Martins 2007; Wu *et al.* 2009).

Seeding α-syn aggregation; α-syn Preformed Fibrils

In addition to genetic models of PD pathology, newer studies demonstrate that inoculating animals with preformed fibrils (PFFs) of recombinant α-syn induces a progressive pattern of pathology that strongly resembles the synucleinopathy in the PD brain. As previously mentioned regarding the prion hypothesis of α-syn, recombinant α-syn can enter cultured cells via endocytosis to induce aggregation of endogenous α-syn. Following seeding and aggregation, aberrant conformers of α-syn are then released from cells. In theory, the released, misfolded/aggregated α-syn may then initiate a new cycle of endocytosis and seeding of endogenous α-syn in recipient cells, and through this series of events, propagate misfolded/aggregated α-syn from cell to cell. α-syn aggregates induced by treatment with recombinant PFFs display many of the same phenotypic markers of LB, such as co-localization with ubiquitin, α-syn phosphorylated at serine 129, and positive staining with Thioflavin S (Desplats *et al.* 2009; Luk *et al.* 2009, 2012a,b; Volpicelli-Daley *et al.* 2011; Paumier *et al.* 2015). Additionally, similar to PD, in which the spatial and temporal pattern of toxicity is paralleled by α-syn deposition into LB, aggregation of endogenous α-syn induced by PFFs results in toxicity in experimental models. For instance, treatment of primary hippocampal neurons with PFFs results in recruitment of endogenous α-syn into aggregates, and a subsequent decrease in SNARE proteins (Volpicelli-Daley *et al.* 2011). Primary neurons exposed to PFFs also display a disruption of signaling and increased cell death (Volpicelli-Daley *et al.* 2011). Similarly, α-syn PFFs injected into either transgenic or WT mice or rats produce LB-like α-syn aggregates throughout the brain over time (Luk *et al.* 2009, 2012a,b; Paumier *et al.* 2015). Although the progressive accumulation of aggregated α-syn in different brain regions over time may reflect a prion-like spread, the proposed cell-to-cell transfer of α-syn remains contentious, an alternative hypothesis is that the injected protein travels to distant neuronal nuclei in white matter tracts, thus inducing aggregation in the distant brain regions in the absence of cell-to-cell transfer. Nevertheless, as observed in PD, the presence of aggregated α-syn throughout the brain preceeds pathology, with deposition of α-syn aggregates in nigrostriatal neurons preceeding loss of striatal DA, TH, and DAT, subsequent death of SNc neurons, and a corresponding loss in motor performance (Luk *et al.* 2012a,b; Paumier *et al.* 2015). Interestingly, the toxicity induced by α-syn PFFs, both *in vitro* and *in vivo*, is completely absent in the isolated neurons and brains of α-syn null mice, reinforcing the concept that sequestration of endogenous α-syn into aggregates appears to be a critical event in cell death associated with synucleinopathy.

Synucleinopathy in toxicant models of PD

Lastly, there have been reports of α-syn aggregation in toxicant models. Specifically, chronic administration of rotenone (Betarbet *et al.* 2000), paraquat (Manning-Bog *et al.* 2002), or MPTP (Fornai *et al.* 2005a) results in the formation of α-syn aggregates. It is likely that the dramatic increase in oxidative stress observed after toxicant exposure produces a redox environment that favors α-syn aggregation. However, results pertaining to the toxicity of these aggregates in view of the loss-of-function hypothesis are confounded by a myriad of other effects produced by these compounds. Yet, it should be noted that in some of these reports, α-syn expression was up-regulated following chronic toxicant exposure (Manning-Bog *et al.* 2002), adding to a fairly large body of literature (discussed below) that suggest a novel role for α-syn in response to cellular stress.

The α-syn loss-of-function hypothesis

Overall, the lessons learned from PD patients and animal models of synucleinopathy demonstrate discrepancies. Arguably the most consistent trend throughout all α-syn-based research is a gene-dosage effect between α-syn expression and pathology. In SNCA multiplication patients as well as in transgenic and virally mediated over-expression models, increasing the expression of α-syn increases the aggregation profile of the protein, resulting in a dose-dependent toxicity (Fig. 1d) (Masliah *et al.* 2000; Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Decressac *et al.* 2012; Gombash *et al.* 2013). In contrast, removal of endogenous α-syn produces a similar pattern of toxicity (Fig. 1e). Here, we find that there is a dose-dependent toxicity that is inversely proportional to the amount of endogenous α-syn remaining (Gorbatyuk *et al.* 2010). While these findings may at first seem discordant, it is possible that the similar pathological

profiles observed following either α-syn over-expression or knockdown reflect a single common underlying mechanism of toxicity, which is the loss of α-syn function. This mechanism is obvious in the case of the toxicity associated with removal of α-syn from neurons. However, toxicity originating from loss of necessary α-syn function may appear somewhat counterintuitive in the context of synucleinopathy or α-syn over-expression models, where α-syn seems to be abundantly present in affected cells. However, here one must distinguish between functional α-syn versus misfolded or mislocalized α-syn.

The α-syn loss-of-function hypothesis posits that as α-syn aggregation proceeds, endogenous functional α-syn becomes sequestered into inclusions. The incorporation of functional α-syn into inclusions; be they oligomers, fibrillar species, or mature LB or LN, would impede the ability of α-syn to perform normal cellular functions, producing a loss of function form of toxicity (Fig. 1) (Perez *et al.* 2002; Perez and Hastings 2004; Cookson 2006; Kanaan and Manfredsson 2012; Wu *et al.* 2012). Hypothetically, α-syn loss of function toxicity could result solely from sequestration into aggregates; however, changes in the subcellular localization of α-syn could exacerbate this process. α-syn is highly enriched in presynaptic terminals (Fig. 1a) (Iwai *et al.* 1995), while aggregated α-syn is largely present in LB located in the soma (Fig. 1c and d). As such, incorporation of α-syn into aggregates would decrease α-syn in its normal subcellular compartment, further preventing soluble α-syn from performing its normal functions. Mislocalization of α-syn from the synapse to the soma is likely exacerbated with age, as there is a redistribution of α-syn from the presynaptic terminal to soma in aging humans and monkeys (Fig. 1b) (Chu and Kordower 2007). This is in line with epidemiological studies that identify age as the primary risk factor for developing PD (Bennett *et al.* 1996; Tanner and Goldman 1996). Finally, loss of α-syn function and subsequent toxicity would be expedited by mutations or multiplications of the SNCA gene, which increase the rate of aggregation, or directly impair function, depriving the cell of normal α-syn functions (discussed more below).

Although the α-syn loss-of-function hypothesis may seem counterintuitive at first glance, there is abundant evidence from the literature to support this theory. In particular, the pattern of toxicity and changes in the localization of endogenous α-syn observed following exposure to α-syn PFFs provides incontrovertible evidence that sequestration of endogenous α-syn into aggregates is a critical event in toxicity. Indeed, as aggregation following PFF administration proceeds, a decline in cytoplasmic, soluble α-syn is observed (Volpicelli-Daley *et al.* 2011; Osterberg *et al.* 2015). An important aspect of all PFF studies reported thus far, is that the aggregates formed by treatment with PFFs, both *in vitro* and *in vivo*, are primarily composed of endogenous α-syn, with very little of the initial PFF seed comprising the resulting inclusions. When Myc-tagged α-syn PFFs are used to seed aggregation, there is a small amount myc immunoreactivity in the center of the observed aggregates, however, the majority of α-syn forming the aggregates is composed of untagged endogenous α-syn (Luk *et al.* 2009). This point is further highlighted by the fact that the formation of aggregates, the propagation of aggregates, and the toxicity produced by recombinant α-syn PFFs is completely absent in primary neurons or in the brains of α-syn null mice (that do not have endogenous α-syn to sequester) (Volpicelli-Daley *et al.* 2011; Luk *et al.* 2012a). Alternatively, hemizygous α-syn ± mice display an intermediate level of degeneration, demonstrating that the recruitment of endogenous α-syn into aggregates is an essential component of aggregate formation and toxicity (Luk *et al.* 2012a). Further highlighting the sequestration of endogenous α-syn: treatment of primary neuronal cultures with α-syn PFFs results in a relocalization of endogenous α-syn away from the synapse into perinuclear aggregates within the soma (Volpicelli-Daley *et al.* 2011). This recruitment of endogenous α-syn into aggregates results in an 80% decrease in soluble endogenous α-syn in primary neurons (Volpicelli-Daley *et al.* 2011). This result was replicated *in vivo* in an elegant study using multiphoton microscopy to image single neurons over time in living mice after they received injections of recombinant α-syn PFFs. Inclusions undergo a maturation process in which early aggregates appear alongside soluble α-syn, however, as inclusions become more compacted the normal homogenous immunoreactivity of soluble α-syn is lost (Osterberg *et al.* 2015). The loss of soluble α-syn into compact perinuclear aggregates coincides with the eventual death of the inclusion-bearing cells (Osterberg *et al.* 2015). These results demonstrate the potential toxicity that can be elicited by reducing endogenous α-syn below a critical threshold, and further supporting the α-syn loss-of-function hypothesis.

Although there is ample evidence to support the α-syn loss-of-function hypothesis, this theory is largely overlooked in favor of those ascribing α-syn toxicity to a gain-of-function mechanism. Arguments supporting a direct toxicity resulting from α-syn, and α-syn aggregates are as follow: (i) Missense mutations in the SNCA gene result in an autosomal dominant pattern of inheritance. (ii) SNCA multiplication patients show a gene-dosage effect in the severity of the PD phenotype. (iii) The pathological features of PD can be mimicked by the over-expression of WT α-syn. (iv) The brains of synucleinopathy patients show accumulation of excess α-syn. (v) Most α-syn null mice do not show overt neuropathological or behavioral phenotypes. These points provide a compelling argument for a direct mechanism of toxicity induced by α-syn and associated aggregates; however, each individual argument can also be explained by the loss-of-function hypothesis as addressed below.

(1) mutations in the SNCA gene result in an autosomal-dominant pattern of inheritance.

Mutations in the SNCA gene could result in loss of functional α-syn through two distinct means: First, mutations could impair the ability of α-syn to form alpha helices and thus promote β sheet formation, thereby promoting aggregation. For example, the A53T, E46K, and H50Q mutations show drastically increased aggregation kinetics (Conway *et al.* 1998; Burré *et al.* 2010; Ghosh *et al.* 2013). In contrast, the A30P mutation does not increase the formation of mature fibrils over WT α-syn, however, initial oligomerization of α-syn is still dramatically increased (Conway *et al.* 1998; Li *et al.* 2001). In either scenario, the increased propensity of mutant α-syn to self-assemble into either mature fibrils or oligomers, could sequester soluble α-syn into aggregates (including WT α-syn from the unaffected allele), resulting in a depletion of functional α-syn (Fig. 1c). Thus, an initial impetus promoting α-syn aggregation could act in a dominant negative fashion to decrease both mutant and WT α-syn protein levels below a critical threshold, resulting in toxicity by loss of α-syn function, which would also produce a dominant pattern of inheritance. Second, the mutation itself could directly impair the ability of mutant α-syn protein to perform its normal function. For example, the A30P, A53E, and G51D mutations impair the ability of α-syn to interact with lipid membranes or localize to presynaptic membranes, directly resulting in a loss of α-syn function (Conway *et al.* 1998; Fortin *et al.* 2004; Burré *et al.* 2010; Fares *et al.* 2014; Ghosh *et al.* 2014). Because of the myriad of vital functions of α-syn performs, as well as the severe consequences observed following partial removal of α-syn function from neurons of adult animals (Gorbatyuk *et al.* 2010), haploinsufficiency may be adequate to produce the dominant pattern of neurodegeneration observed in familial PD. This could particularly hold true for loss of α-syn function within dopaminergic neurons (discussed below).

(2) multiplication patients show a gene-dosage effect in the severity of the PD phenotype, and,

(3) pathological features of PD can be mimicked by over-expression of WT α-syn.

If PD pathology is mediated by a loss-of-function mechanism, then how can one account for the fact that increased SNCA expression causes toxicity? As mentioned above, data from patients with SNCA gene multiplications present a strong case for a gene-dosage effect in which increased SNCA expression and α-syn protein produces a more aggressive disease phenotype (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004; Ibanez *et al.* 2004; Han *et al.* 2011). Further, lessons learned from α-syn animal models show that ectopic over-expression of α-syn produces neurotoxicity in a dose-dependent fashion (Masliah *et al.* 2000; Giasson *et al.* 2002; Gomez-Isla *et al.* 2003; Fleming *et al.* 2005; Decressac *et al.* 2012; Gombash *et al.* 2013). As previously mentioned, increasing α-syn protein concentrations, as well as general increases in molecular crowding, can both increase the aggregation kinetics of α-syn (Conway *et al.* 1998; Uversky 2007). SNCA expression in multiplication patients has been confirmed to produce increased α-syn protein (Miller *et al.* 2004), while α-syn over-expression models range from approximately 0.3–30 fold increases in α-syn protein (Fleming *et al.* 2005). Accordingly, it is very likely that the increased α-syn expression in multiplication patients, or α-syn over-expression in animal models, increases the aggregation kinetics of α-syn, thus impairing the ability of α-syn to perform its physiological function after sequestration into aggregates (Fig. 1c and d). Supporting this, the brains of SNCA triplication patients do not show any increase in soluble α-syn, but exhibit a large increase in α-syn aggregates (Miller *et al.* 2004). Further, levels of soluble α-syn protein are decreased in the brains of patients with idiopathic PD (Baba *et al.* 1998; Quinn *et al.* 2012). This could also account for the much more aggressive disease phenotype seen in triplication carriers over duplication carriers, wherein increased α-syn expression would accelerate α-syn aggregation kinetics over that associated with SNCA duplication. Finally, ectopic over-expression of α-syn in animal models causes a progressive decline in soluble α-syn protein levels with a concomitant increase in insoluble α-syn, providing further support for the loss of function hypothesis (Volpicelli-Daley *et al.* 2011; Osterberg *et al.* 2015). Thus, it appears that α-syn exists in a state of tightly controlled homeostatic maintenance (Fig. 1). α-syn is one of the most abundant proteins in the brain, yet neurons are exquisitely sensitive to changes in expression levels of α-syn, with a small (fold) increase in expression able to produce widespread neurodegeneration and an aggressive disease phenotype. At the same time, small decreases in SNCA expression are also associated with disease severity. Supporting this is a recent study examining the relationship between SNCA expression and disease outcome in over 1000 well-characterized PD patients. This study demonstrates that patients with the low repeat REP1 allele (259 base pairs; resulting in decreased SNCA expression), are associated with worse motor and cognitive outcomes, while the high-repeat REP1 allele (263 base pairs; increases SNCA expression) results in better motor and cognitive outcomes (Markopoulou *et al.* 2014). Taken together, these data imply that α-syn homeostasis is constantly balanced on a knife-edge, where small perturbations that increase or decrease its expression produce neurotoxicity that is likely mediated through a common mechanism, the loss of normal α-syn function.

(4) The brains of synucleinopathy patients show accumulation of excess α-syn.

It is well documented that although the brains of synucleinopathy patients do show accumulation of α-syn, the majority of that α-syn is aberrantly folded and/or contained within aggregates (Miller *et al.* 2004; Kramer and Schulz-Schaeffer 2007; Quinn *et al.* 2012). Thus, despite the excess α-syn in the brain, there are actually reduced levels of soluble, functional α-syn available to perform its physiological functions.

A more difficult issue to resolve is whether α-syn aggregates are directly toxic. If α-syn aggregates are directly toxic, one would expect to see cell loss occurring with the initial presentation of the inclusions. If, however, aggregates produce toxicity by progressively sequestering soluble α-syn until a critical threshold is lost, toxicity would appear following a necessary lag time needed to achieve a significant depletion of the soluble α-syn pool. Indeed, there is widespread LB pathology in brain and spinal cords of presymptomatic PD patients, yet it is not until much later that symptoms and widespread cell loss are observed (Braak *et al.* 2003a; Halliday *et al.* 2006; Jellinger 2008). Next, as many as 8–12% of clinically normal individuals over the age of 60 have extensive LB pathology in the CNS, without a corresponding cell loss or PD symptoms (Forno 1969; Dickson *et al.* 2008). Further, in PD itself, there is a differential susceptibility of neuronal populations to the presence of α-syn aggregates. Although late-stage PD patients develop α-syn containing LB throughout much of the body (Braak and Braak 2000; Braak *et al.* 2003b, 2006; Beach *et al.* 2010), only a particular subpopulation of neurons with aggregated α-syn display neurodegeneration and cell death. For example, the formation of α-syn inclusion within neurons of the enteric nervous system correlates with GI dysfunction in PD, however, there has never been any documentation of neuronal cell loss in the enteric nervous system. This is also observed in animal models where over-expression of rat α-syn in rats results in α-syn aggregate formation but no cell loss (Bianco *et al.* 2002), presumably because continued expression of rat α-syn maintains synuclein function despite the presence of aggregates. Moreover, there are LB in many surviving neurons in the brains of PD patients, suggesting that mature LB may actually represent a protective effort of the cell to sequester aggregate prone α-syn away from the site (s) of action where α-syn function is needed, i.e. the presynaptic terminal. Here, it becomes important to distinguish between mature highly processed and compacted LB, and self-assembled oligomeric or fibrillar α-syn. The latter would likely perpetuate a cycle of aggregation by serving as templating seeds within the neuron. Supporting this idea is evidence demonstrating that presynaptic α-syn aggregates, not LB, cause neurodegeneration in models of synucleinopathy (Kramer and Schulz-Schaeffer 2007). Further, some studies have even reported that neurons containing LB in late-stage PD appear morphologically normal as compared to neurons without LB (Gertz *et al.* 1994; Tompkins *et al.* 1997). Finally, abundant literature describes α-syn-mediated toxicity associated with a number of key cellular processes such as mitochondrial function, calcium buffering, and protein clearance. Here, it seems possible that a loss-of-function toxicity is misinterpreted as a direct toxicity induced by α-syn. For example, as mentioned above, both aggregation and knockdown of α-syn impair the ability of mitochondria to buffer calcium, implying a common toxicity induced by loss of α-syn function (Cali *et al.* 2012). However, many of these pathological changes could actually represent downstream toxic effects caused by a loss of α-syn function. As will be discussed in the following section, loss of α-syn could also cause DA-associated toxicity, which would negatively affect virtually every organelle in a dopaminergic neuron.

(5) α-syn null mice do not show overt neuropathological or behavioral phenotypes.

Although α-syn null mice show deficits in vesicular dynamics and synaptic transmission, admittedly the pathological changes observed in α-syn null mice are modest, and do not reflect the proposed consequences resulting from loss of crucial α-syn function. However, this could result from an effect of developmental genetic compensation resulting from germline removal of the SNCA gene. This notion is supported by several studies. First, unlike germline knockout of SNCA, removal of endogenous α-syn from mature neurons in adult animals results in severe neurodegeneration (Gorbatyuk *et al.* 2010; Khodr *et al.* 2011; Zharikov *et al.* 2015; Collier *et al.* 2016) (Fig. 3). Second, genetic compensation resulting from germline removal of the endogenous SNCA gene is well documented. As compared to WT littermates, α-syn null mice have 369 transcripts differentially expressed (Kuhn *et al.* 2007). These include increases in 14-3-3 and TH expression (proteins that both interact with α-syn) (Ostrerova *et al.* 1999; Perez *et al.* 2002; Wang *et al.* 2009), decreases in the expression of apoptotic genes, altered expression of neurotrophic factors like Brain derived neurotrophic factor (BDNF) (Yuan *et al.* 2010), as well as differential expression of genes involved in vesicular function (Kuhn *et al.* 2007; Ubhi *et al.* 2010). Further, as was mentioned above, there is also a compensatory increase in other synuclein family members, which suggests functional redundancy could mask the effects of silencing α-syn expression (Schlüter *et al.* 2003; Chandra *et al.* 2004; Robertson *et al.* 2004). As a result of the plethora of differentially regulated genes in α-syn null mice, and direct evidence that manipulating α-syn expression in adult animals produces more severe consequences than those observed by germline manipulations, drawing

conclusions based solely from α-syn null animals could lead to gross underestimation of the impact of loss of α-syn function.

Potential mechanisms of α-syn Loss-of-function toxicity

Though the precise physiological function of α-syn has not been established, α-syn does play a key role in many functions involved in synaptic transmission, with a potentially specialized role in the dopaminergic presynaptic terminal. Based on a survey of the literature, α-syn plays a role in regulating the size of the vesicular pool, vesicular trafficking to- and docking with the presynaptic membrane, as well as subsequent clathrin-associated formation of synaptic vesicles. In addition, α-syn appears to play a crucial role in the regulation of DA biosynthesis and handling (see α-syn function section above). Based on our model of α-syn acting as a regulatory brake on dopaminergic synaptic transmission (Fig. 2), a loss of α-syn function would produce dysregulated DA handling and subsequent DA associated toxicity (Fig. 2c).

Loss of α-syn function has been proposed to result in increased cytosolic DA through several distinct mechanisms (Perez *et al.* 2002; Perez and Hastings 2004; Wu *et al.* 2012) (Fig. 2): First, loss of α-syn from the dopaminergic terminal can disinhibit TH and AADC, resulting in increased *de novo* DA synthesis (Perez *et al.* 2002; Perez and Hastings 2004; Yu *et al.* 2004; Tehranian *et al.* 2006; Alerte *et al.* 2008; Lou *et al.* 2010). Second, loss of α-syn decreases the size of the vesicular pool (Murphy *et al.* 2000; Cabin *et al.* 2002), as well as levels of VMAT, and causes dysregulation of synaptic vesicle trafficking and recycling (Maroteaux *et al.* 1988; Fountaine *et al.* 2008; Burré *et al.* 2010, 2012; Vargas *et al.* 2014). The net result of these effects would be increased free cytosolic DA, with a concomitant inability to sequester the excess DA in synaptic vesicles. Cytosolic DA readily auto-oxidizes to produce reactive oxygen species as well as the highly reactive DA quinone, ultimately resulting in neurotoxicity (Fig. 2c) (Graham *et al.* 1978; Hermida-Ameijeiras *et al.* 2004). Further, DA oxidation and associated changes in the cellular redox environment could inhibit the electron transport chain, resulting in further oxidative stress by opening the mitochondrial permeability pore (Berman and Hastings 1999; Khan *et al.* 2005), as well as causing a decreased ability of the mitochondrial enzymes MAO-B and aldehyde dehydrogenase to convert DA to Dihydroxyphenylacetic acid (DOPAC). Finally, increased reactive oxygen species as well as direct interactions between DA-α-syn adducts are able to promote α-syn aggregation (Hashimoto *et al.* 1999; Conway *et al.* 2001), further depleting the cell of functional α-syn. Taken together such changes initiated by a loss of α-syn function would create a feed-forward cycle of neurotoxicity, ultimately resulting in DA cell loss. Importantly, this increase in oxidative stress can produce phenomena such as mitochondrial inhibition and dysfunction of the protein clearance machinery (Höglinger *et al.* 2003; Porras and Perez 2014), many of which have been attributed to an α-syn toxic gain of function (Wang *et al.* 2009).

Our proposed mechanism of α-syn loss of function toxicity is further supported by studies demonstrating that apoptosis associated with α-syn over-expression depends on endogenous DA production, while α-syn over-expression in DA-depleted cells is not toxic, and that α-syn may actually be neuroprotective in non-dopaminergic cells (Xu *et al.* 2002). In addition, the activity of the TH-modulating protein phosphatase, PP2A, is decreased in the brains of α-syn triplication patients and patients with DLB in regions with robust α-syn aggregation (Wu *et al.* 2012), lending further support to the idea that aggregation of α-syn may cause unregulated DA production. DA associated toxicity following the loss of α-syn function could also account for the differential susceptibility of central DA neurons to synucleinopathy.

Again, aggregated α-syn is observed throughout many neuronal populations, yet only certain pools of neurons, primarily catecholaminergic nuclei, show significant cell loss. It is thus likely that the neurotransmitter used within a given neuron directly contributes to its susceptibility caused by loss of α-syn function. The associated increased cytosolic DA results in reactive oxygen species that would not occur in neuronal subpopulations that utilize other, less volatile neurotransmitters. This mechanism of toxicity is also supported by studies documenting the temporal pattern of PD pathology in which dopaminergic fiber loss precedes the actual loss of nigrostriatal neurons (Kordower *et al.* 2013). Within years of manifestating motor symptoms, there is an approximate 70–90% loss of nigrostriatal DA fibers in the ST, while at this same time, point up to 40–70% of SNc neuronal cell bodies remain intact (Kordower *et al.* 2013). This 'dying back' neurotoxicity in PD could be associated with a loss of α-syn function and subsequent DA toxicity originating in presynaptic DA terminals.

An additional mechanism whereby loss of α-syn function produces neurodegeneration is by a diminished ability to handle cellular stress. Many studies have shown that α-syn is up-regulated following cellular stress, suggesting a neuroprotective role for α-syn. α-syn is up-regulated in neuroblastoma cells in response to endoplasmic reticulum or proteasomal stress (Häbig *et al.* 2009), exposure to mitochondrial toxins *in vitro* and *in vivo* (Manning-Bog *et al.* 2002; Fountaine and Wade-Martins 2007), or drugs that specifically target catecholaminergic terminals (Fornai *et al.* 2005b). Indeed, increased α-syn expression protects against a variety of cellular stressors, including DA toxicity, oxidative stress, staurosporine-induced apoptosis, and serum deprivation (Alves da Costa *et al.* 2002; Wersinger and Sidhu

2003b; Zourlidou *et al.* 2003; Quilty *et al.* 2006). Moreover, α-syn expression drastically lowers the apoptotic response of neuronal cells by decreasing both p53 expression and p53 transcriptional activity, and this effect is lost when soluble α-syn becomes sequestered in aggregates (Alves da Costa *et al.* 2002). Taken together, loss of α-syn function likely results in an increase in DA-mediated cellular stressors, along with a concomitant decrease in the ability of neuronal cells to rebound from the same toxicity.

Discrepancies and lessons learned from other Proteins

Although the α-syn loss-of-function hypothesis can reconcile much of the conflicting published data, there are some findings that seem irreconcilable with toxicity caused by loss of α-syn function. Many of these come from data in α-syn animal models. For example, several α-syn over-expressing transgenic mouse lines develop pathology in the absence of large α-syn aggregates (Matsuoka *et al.* 2001; Richfield *et al.* 2002; Thiruchelvam *et al.* 2004). Alternatively, in one α-syn over-expressing transgenic line, large aggregates of human α-syn are detected, however, they are devoid of endogenous murine α-syn (Masliah *et al.* 2000). Aggregates containing endogenous α-syn would seem to be a prerequisite if toxicity resulted following the sequestration of functional α-syn into aggregates. However, these findings are rare and may reflect either; 1) the use of insufficiently sensitive techniques to identify species-specific α-syn or detect microaggregates or, 2) endogenous α-syn oligomers or immature aggregate species that inhibit normal α-syn function but are undetectable via histological techniques.

Another potential flaw of the loss-of-function hypothesis is studies reporting toxicity as a result of the aggregation of human α-syn in animals lacking endogenous α-syn. For example, Drosophila and C. elegans do not express endogenous α-syn, nor is there currently a known homolog of α-syn in these animals. Nonetheless, transgenic Drosophila or C. elegans expressing human α-syn develop α-syn aggregates and subsequent toxicity (Feany and Bender 2000; Lakso *et al.* 2003). Further, over-expression of mutant A53T human α-syn in α-syn null mice results in toxicity that exceeds the effects of A53T human α-syn over-expression in WT mice (Cabin *et al.* 2005). Because of the fact that these animals do not express endogenous α-syn, there is no endogenous α-syn to be lost to aggregation. Here, it is possible that an as of yet unidentified homolog of α-syn, or some other essential protein, gets sequestered into aggregates, resulting in similar pattern of loss-of-function toxicity to that proposed herein with α-syn. Supporting this possibility, a potential α-syn homolog has been identified in Drosophila (Shin *et al.* 2000). Further, co-expression of a chaperone protein such as Hsp70, completely prevents toxicity induced by human α-syn over-expression in Drosophila (Auluck *et al.* 2002). Importantly, although co-expression of Hsp70 prevents toxicity, it does not alter the size, number, or morphology of the human α-syn aggregates, suggesting that it is preventing toxicity by maintaining the proper concentrations and conformation of an endogenous Droshophila protein that, when lost, produces toxicity (Auluck *et al.* 2002).

Oligomeric or protofibrillar α-syn is thought to have the ability to puncture lipid membranes. Protofibrillar α-syn also has enhanced affinity for lipid membranes and forms pore-like structures in membranes (Volles *et al.* 2001; Caughey and Lansbury 2003). Further, mature fibrils of α-syn permeabilize membranes (Pieri *et al.* 2012), while familial mutations in α-syn enhance its tendency to form pores in membranes (Volles *et al.* 2001) Permeabilization of membranes has been hypothesized to cause membrane leakiness, allowing unregulated passage of ions and small (potentially toxic) macromolecules between the intra- and extracellular space, and/or between subcellular compartments, ultimately resulting in toxicity. Although the vast majority of work demonstrating α-syn-induced membrane leakiness has been performed *in vitro* with supraphysiological concentrations of α-syn, this could represent a potential toxic gain of function.

Finally, inhibition of the autophagy–lysosomal pathway by mutant forms of α-syn may also represent a potential gain of function toxicity. WT α-syn is selectively translocated to the lysosome for active degradation by chaperone-mediated autophagy (Cuervo *et al.* 2004). However, both the A53T and the A30P α-syn mutations appear to inhibit their own degradation, as well as the degradation of other substrates, by this pathway (Cuervo *et al.* 2004). Accordingly, the subsequent accumulation of α-syn, as well as other damaged or potentially toxic proteins, has been proposed as a possible gain of function mechanism underlying the toxicity of these α-syn mutants (Cuervo *et al.* 2004).

Taken together, these findings suggest that α-syn may have a two-pronged mechanism of toxicity developing independently and over many years during disease pathogenesis. We believe that it is likely that a significant amount of toxicity results from loss of α-syn function; however, it is also likely that a pathological cascade of protofibrillar or fibrillar α-syn exerts a degree of toxicity as well. Importantly, the idea that a single protein could cause toxicity by both loss- and gain of function is not unique to α-syn.

Other proteins associated with the pathogenies of neurological disorders have demonstrated a multifaceted mechanism of toxicity. For example, it has been hypothesized that accumulation of hyperphosphorylated tau directly results in the pathological sequellae observed in AD. Strikingly similar to the story of α-syn described above, the tau hypothesis is supported by the fact that 1) neurofibrillary tangle burden correlates with cognitive loss and disease progression (Braak and Braak 1991), 2) mutations in the gene encoding tau

result in disease-related tauopathies (Hutton *et al.* 1998), 3) tau knockout animals do not exhibit premature mortality or major neurological deficits (Morris *et al.* 2011), and 4) the induction of neurofibrillary tangles in worms, flies, and rodents mimic the neurodegeneration observed in human disease (Trojanowski and Lee 2005; Götz *et al.* 2007). However, there is now significant evidence to suggest that loss of tau function, following sequestration into Neurofibrillary Tangle (NFTs) or by inhibition following its hyperphosphorylation, contributes significantly to AD pathology (Morris *et al.* 2011; Bin Zhang *et al.* 2005; Trojanowski and Lee 2005; Santacruz *et al.* 2005). Indeed, the current debate in the tau field is centered on what function (s) of tau is lost in disease (e.g. microtubule stability or associated axonal transport) and what toxic gain of function (s) occur (e.g. impairing protein degradation machinery or signaling dysregulation) that lead to neurodegeneration. Because of these remarkable similarities between tau and α-syn, it is somewhat surprising that the contribution of decreased α-syn function to PD pathogenesis is not more widely accepted.

Conclusions

It is clear that α-syn plays an integral role in the pathology of PD; however, the mechanism(s) whereby aberrant α-syn homeostasis causes neurodegeneration remain unclear. Here, we have summarized the existing data that demonstrate how a loss of function may represent a major mechanism underlying α-syn toxicity. As science has only recently begun to understand some of the biological functions of α-syn, research examining the contribution of α-syn to PD-associated pathology has understandably been undertaken with a rather myopic focus on gain-of-function mechanisms following α-syn aggregation. However, as our understanding of the normal biology of α-syn matures, it is increasingly evident that α-syn is a multifunctional chaperone-like protein that contributes to essential cellular processes. Because of the complexity of α-syn biology and pathology, it is essential to further elucidate and validate the normal functions of α-syn in order to truly appreciate how those mechanisms could underly α-syn toxicity. In doing so, we can work to develop novel therapeutics aimed at preventing α-syn aggregation while also maintaining α-syn function (Vargas-Medrano *et al.* 2014), in order to provide the most efficacious disease modifying therapies possible.

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