frontiers in Neuroscience

REVIEW
published: 14 March 2018
doi: 10.3389/fnins.2018.00161

Can Interactions Between α-Synuclein, Dopamine and Calcium Explain Selective Neurodegeneration in Parkinson's Disease?

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Several lines of evidence place alpha-synuclein (aSyn) at the center of Parkinson's disease (PD) etiology, but it is still unclear why overexpression or mutated forms of this protein affect some neuronal populations more than others. Susceptible neuronal populations in PD, dopaminergic neurons of the substantia nigra pars compacta (SNpc) and the locus coeruleus (LC), are distinguished by relatively high cytoplasmic concentrations of dopamine and calcium ions. Here we review the evidence for the multi-hit hypothesis of neurodegeneration, including recent papers that demonstrate synergistic interactions between aSyn, calcium ions and dopamine that may lead to imbalanced protein turnover and selective susceptibility of these neurons. We conclude that decreasing the levels of any one of these toxicity mediators can be beneficial for the survival of SNpc and LC neurons, providing multiple opportunities for targeted drug interventions aimed at modifying the course of PD.

Keywords: α-Synuclein, dopamine, calcium, Parkinson's disease, substantia nigra pars compacta, locus coeruleus, multiple hits

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *28 December 2017*
Accepted: *27 February 2018*
Published: *14 March 2018*

Citation:
Post MR, Lieberman OJ and Mosharov EV (2018) Can Interactions Between α-Synuclein, Dopamine and Calcium Explain Selective Neurodegeneration in Parkinson's Disease? Front. Neurosci. 12:161. doi: 10.3389/fnins.2018.00161

DIFFERENTIAL SUSCEPTIBILITY OF CATECHOLAMINERGIC NEURONS IN PD

Parkinson's disease (PD), the second most common neurodegenerative disorder (De Lau and Breteler, 2006), is marked by slowness (bradykinesia), resting tremor, muscular rigidity, and postural instability (Lang and Lozano, 1998). Although multiple brain regions are affected in late-stage PD (Braak et al., 1995), two catecholaminergic neuronal populations degenerate early, before the onset of the motor symptoms-dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) and noradrenergic (NE) neurons of the locus coeruleus (LC). DA restoration through treatment with L-DOPA provides an effective symptomatic improvement, however, tolerance to treatment increases over time, accompanied by the development of severe side effects (L-DOPA-induced dyskinesia; Lewitt, 2015; Olanow, 2015). There is at the time no means available for delaying the progress of the disease, which is a critical goal in the field.

Rational design of disease-modifying therapies is complicated by the lack of a clear understanding of the pathophysiology of PD initiation and progression. The disease is predominantly sporadic, with an estimated 10% prevalence of familial cases (Eriksen et al., 2005). Alpha-synuclein (aSyn), encoded by the SNCA gene, plays a central role in both sporadic and

familial PD. Mutations or multiplications of the SNCA cause autosomal dominant PD (Eriksen et al., 2005). Levels of phosphorylated aSyn are increased in post-mortem brains of PD patients and in patient-derived dopaminergic neurons (Fujiwara et al., 2002; Swirski et al., 2014). Genome-wide association studies (GWASs) have reported a correlation between the SNCA locus and the risk of developing sporadic PD (Simon-Sanchez et al., 2009; Chang et al., 2017). Importantly, post-mortem PD brains show proteinaceous aSyn-positive deposits called Lewy bodies (Spillantini et al., 1997; Baba et al., 1998). Conversely, deletion of aSyn is protective in mouse and cellular models of PD (Dauer et al., 2002; Alvarez-Fischer et al., 2008). Similarly, a recent study identified β2-adrenoreceptor (β2AR) agonists as negative regulators of the aSyn gene expression, and an association was found between the use of β2AR agonist salbutamol, a brain-penetrant asthma medication, and a reduced risk of developing PD (Mittal et al., 2017). Yet, Lewy body pathology occurs throughout the nervous system in PD patients and does not correlate well with cell death (Goedert et al., 2013; Surmeier et al., 2017a), suggesting that aSyn may be necessary but not sufficient for PD neurodegeneration.

Several cellular pathways are affected in PD, resulting in endoplasmic reticulum (ER) stress and activation of the unfolded protein response, disruption of lysosomal and proteasomal protein degradation, and impaired Ca^{2+} homeostasis and mitochondrial dysfunction (Rochet et al., 2004; Stefanis, 2012; Duda et al., 2016; Michel et al., 2016). Although there does not appear to be a unifying end-point toxicity pathway, inflammatory response and both necrotic and apoptotic degeneration are often observed in PD models (Perier et al., 2012). The central question in PD neuropathology, however, is why some neurons are highly susceptible to neurodegeneration while other, even closely related populations, are much less affected. Specifically, SNpc and LC catecholaminergic neurons degenerate in PD, whereas ventral tegmental area (VTA) and tuberoinfundibular DA neurons are relatively spared in both PD patients and laboratory models of the disorder (Hirsch et al., 1988; Braak et al., 1995). Two features of SNpc and LC neurons—the presence of elevated catecholamine and Ca^{2+} concentration in the cytosol—have consistently been suggested as modulators of their sensitivity to neurodegeneration.

Due to the ability of DA to produce oxidative stress and protein damage, it has long been speculated that a dysregulation of cytosolic DA homeostasis plays a role in PD (Edwards, 1993; Gainetdinov et al., 1998; Uhl, 1998; Schmitz et al., 2001; Lotharius and Brundin, 2002; Lohr et al., 2014; Pifl et al., 2014). Spontaneous DA oxidation at neutral pH of the cytosol yields DA-o-quinone and dopaminochrome (Graham, 1978; Sulzer and Zecca, 2000), which can then react with free cysteine and exposed cysteine residues of proteins and glutathione producing 5-S-cystenyl-DA. The latter can undergo further oxidation and is toxic to cultured cells (Spencer et al., 2002) or when injected into the mouse brain (Zhang and Dryhurst, 1994). 5-S-cystenyl adducts of DA and its metabolites are used as markers of excess cytosolic DA and oxidative stress *in vivo* (Hastings and Berman, 1999; Caudle et al., 2007) and are readily detected in human SNpc and LC, consistent with DA-induced protein damage in human PD (Fornstedt et al., 1989; Montine et al., 1995; Hastings and Berman, 1999). Other mechanisms of DA-mediated neurotoxicity include reactions of DA with nitric oxide (Daveu et al., 1997), peroxynitrite (Daveu et al., 1997; Vauzour et al., 2008) and aldehydes (Collins and Bigdeli, 1975; Deitrich and Erwin, 1980; Naoi et al., 1993; Marchitti et al., 2007). Accumulation of cytosolic DA is toxic to cells *in vitro* (Mytilineou et al., 1993; Pardo et al., 1995; Sulzer et al., 2000; Xu et al., 2002; Fuentes et al., 2007; Mosharov et al., 2009) and several reports confirm that a buildup of cytosolic DA is indeed sufficient to induce progressive nigrostriatal degeneration in rodents (Caudle et al., 2007; Chen et al., 2008), although clinical studies of L-DOPA toxicity produced controversial results (Fahn et al., 2004; Olanow et al., 2004; Holford et al., 2006).

Dysregulation of Ca^{2+} homeostasis is likewise frequently observed in models of both sporadic and familial PD (Goldberg et al., 2012; Hurley and Dexter, 2012; Surmeier et al., 2017b). This includes impairment of mitochondrial Ca^{2+} maintenance (Exner et al., 2012), disrupted communication between mitochondrial and ER Ca^{2+} stores (Ottolini et al., 2013; Guardia-Laguarta et al., 2014), decreased store-operated Ca^{2+} entry (Zhou et al., 2016), and additional mechanisms that may cause toxicity due to abnormally high or low Ca^{2+} levels (Duda et al., 2016; Michel et al., 2016; Surmeier et al., 2017b). SNpc and VTA neurons express drastically different levels of calbindin-D_{28K} (Fu et al., 2012) and those expressing high levels of this Ca^{2+} buffering protein—the majority of VTA neurons and a small percentage of SNpc neurons—are spared from neurodegeneration in PD (Yamada et al., 1990; Rcom-H'cheo-Gauthier et al., 2014). Interestingly, at least some LC neurons appear to express Ca^{2+} buffering proteins calbindin-D_{28K}, calretinin and parvalbumin (Bhagwandin et al., 2013), although no comparison was made with other brain areas, such as the VTA.

SNpc neurons have long axons that extend into the striatum and arborize extensively, with many DA release sites (Matsuda et al., 2009). Physiologically, these neurons display broad action potential spikes and an autonomous tonic firing pattern governed by the activity of the L-type $Ca_v1.3$ channels (LTCCs) (Hetzenauer et al., 2006; Surmeier et al., 2010). This drives a feed-forward stimulation of mitochondrial oxidative phosphorylation that maintains ATP production during increased neuronal activity (Chan et al., 2007; Surmeier et al., 2017b). Chronically increased cytoplasmic and mitochondrial Ca^{2+} levels may however drive the production of reactive oxygen and nitrogen species (ROS and RNS), leading to mitochondrial dysfunction. While $Ca_v1.3$ channels are expressed at similar levels in SNpc and neighboring VTA dopaminergic neurons (Dragicevic et al., 2014), they do not drive pacemaking in VTA neurons (Chan et al., 2007; Duda et al., 2016) (although, this remains controversial Liu et al., 2014), suggesting post-translational regulation of their activity. Pharmacological blockade of LTCCs with dihydropyridines alleviates mitochondrial oxidative stress in SNpc neurons in *ex vivo* mouse brain slices (Chan et al., 2007), and protects them in neurotoxin-based models of PD (Chan et al., 2007). Similarly, LC neurons display broad action potential spikes and autonomous pacemaking that is dependent on $Ca_v1.2$ and $Ca_v1.3$ L-type channels (Sanchez-Padilla et al., 2014) as well as the

T-type channels (Matschke et al., 2015). Dihydropyridines also prevent mitochondrial oxidative stress in LC neurons in *ex vivo* brain slices (Sanchez-Padilla et al., 2014). Although LC neurons are selectively targeted by parkinsonian neurotoxins (Masilamoni et al., 2011), the effect of LTCC blockers on the survival of LC neurons in these models has not been studied. However, an LTCC inhibitor nimodipine was shown to protect both SNpc and LC neurons in a model of chronic neuroinflammation (Hopp et al., 2015).

Overall, SNpc and LC appear to share many of the same characteristics—a proteomic analysis identified similar changes in 61 PD-associated proteins in SNpc and LC neurons (Van Dijk et al., 2012)—and are uniquely situated with high levels of cytosolic catecholamines and Ca^{2+}, which in the presence of aSyn may underlie their higher susceptibility to neurodegeneration. Below, we focus on the interactions between these three chemicals, highlighting recent developments in their role toward cell-selective PD pathogenesis.

aSyn AND Ca^{2+}

aSyn is a protein widely expressed in the nervous system, with a subcellular localization at the presynaptic terminal. The protein is 140 amino acids in length (**Figure 1**), occurs as a helically folded tetramer under physiological conditions (Bartels et al., 2011) and is able to form oligomers, fibrils and more complex aggregates, eventually leading to Lewy bodies. The N-terminus is lysine-rich and is the site of the vesicle binding, with four lipid-binding KTK motif repeats in that region. Importantly, all known SNCA familial PD mutations to date—A30P, E46K, H50Q, G51D, A53E, and A53T—are found in this domain (Rcom-H'cheo-Gauthier et al., 2014). The central region of aSyn is known as the non-amyloid-β component (NAC) of amyloid plaques found in Alzheimer's disease patients and is responsible for aSyn aggregation and Lewy body formation (Li et al., 2002). The C-terminus is comprised of an EF-hand-like sequence that is capable of binding Ca^{2+}; however, overexpression of truncated aSyn that lacks the C-terminus is sufficient to elicit a PD-like phenotype in mice (Tofaris et al., 2006). Normally, aSyn is involved in regulation of synaptic vesicles exocytosis, although its exact function is still debated (Imaizumi et al., 2005; Larsen et al., 2006; Burre et al., 2010; Nemani et al., 2010; Bendor et al., 2013). Although gain-of-function mechanisms of aSyn toxicity due to its post-translational modifications or oligomerization have been widely reported, recent data suggest that the loss-of-function mechanisms may also play a role (Collier et al., 2016).

Effects of aSyn on Ca^{2+}

Intracellular Ca^{2+} is a potent second-messenger that triggers many cellular events, and its concentration is tightly regulated by the activities of transporters and channels of the plasma, ER, and mitochondrial membranes, as well as calcium-binding proteins such as calbindin, parvalbumin, and calretinin (Zaichick et al., 2017). Various mechanisms by which aSyn is able to interfere with Ca^{2+} homeostasis in different cellular compartments have been reviewed in more detail elsewhere (Duda et al., 2016; Michel et al., 2016; Ottolini et al., 2017; Surmeier et al., 2017b), but are described here briefly.

First, aSyn localizes at the mitochondria-associated membranes of the ER (MAMs) where it can regulate IP_3 receptor-mediated transfer of calcium (Cali et al., 2012; Guardia-Laguarta et al., 2014). Pathogenic PD mutations in aSyn result in reduced association with MAM accompanied by increased mitochondrial fragmentation and augmented autophagy (Guardia-Laguarta et al., 2014). Additionally, post-translationally modified aSyn interacts with TOM20, a translocase of the outer mitochondrial membrane, and impairs mitochondrial import of proteins required for oxidative phosphorylation both *in vitro* and in postmortem brain tissue from PD patients (Di Maio et al., 2016). Second, aSyn overexpression induces lysosomal permeability, allowing lysosomal calcium and protons to leak to the cytosol and induce cell death (Bourdenx et al., 2014). Third, aSyn can increase ion permeability of the plasma membrane or interfere with the activity of its channels resulting in dysregulated neuronal firing and Ca^{2+} dynamics patterns that precede neurodegeneration (Subramaniam et al., 2014; Angelova et al., 2016). Consistently, aSyn is required for cytosolic Ca^{2+} influx through the plasma membrane following exposure to the parkinsonian neurotoxin MPP^+ via a putative interaction with LTCCs (Lieberman et al., 2017), although the exact mechanism of this interaction needs further investigation. Fourth, a recent study showed that extracellularly added aSyn increased the activity of the $Ca_v2.2$ channel, thus increasing cytoplasmic Ca^{2+} sufficiently to induce exocytotic DA release (Ronzitti et al., 2014). Interestingly, aSyn did not increase $Ca_v2.2$ expression, but rather caused a relocation of $Ca_v2.2$ from lipid rafts to cholesterol-poor domains, providing a novel mechanism by which aSyn may change the activity of Ca^{2+} channels via the reorganization of membrane microdomains indicating an indirect interaction between aSyn and Ca^{2+}-channels. Finally, a study of the proximal aSyn intracellular partners using APEX2-based labeling found that aSyn might interact with calcineurin, a calmodulin dependent serine/threonine protein phosphatase that has ubiquitous intracellular substrates (Chung et al., 2017). This finding buttresses previous reports from the same group that demonstrated a functional relationship between aSyn levels and calcineurin activity (Caraveo et al., 2014).

Effects of Ca^{2+} on aSyn

Ca^{2+} binding seems to promote aSyn annular oligomer formation. These ring-like oligomers have been shown to insert in the membrane forming a pore, perhaps allowing more Ca^{2+} to enter the cell (Mironov, 2015). This oligomer formation is dependent on the C-terminus and is eliminated in truncated forms of aSyn (Lowe et al., 2004). Increasing internal Ca^{2+} concentration via thapsigargin or Ca^{2+} ionophore treatment causes an increase in aggregate formation, while Ca^{2+}-chelators or Ca^{2+} channel inhibitors have the opposite effect (Danzer et al., 2007; Nath et al., 2011; Follett et al., 2013). The effect of Ca^{2+} on aSyn aggregation is mediated by the Ca^{2+}-activated protease, calpain, which cleaves the C-terminus of aSyn (Dufty et al., 2007; Nath et al., 2011). This has been confirmed *in vivo* by the overexpression of the calpain inhibitor, calpastatin,

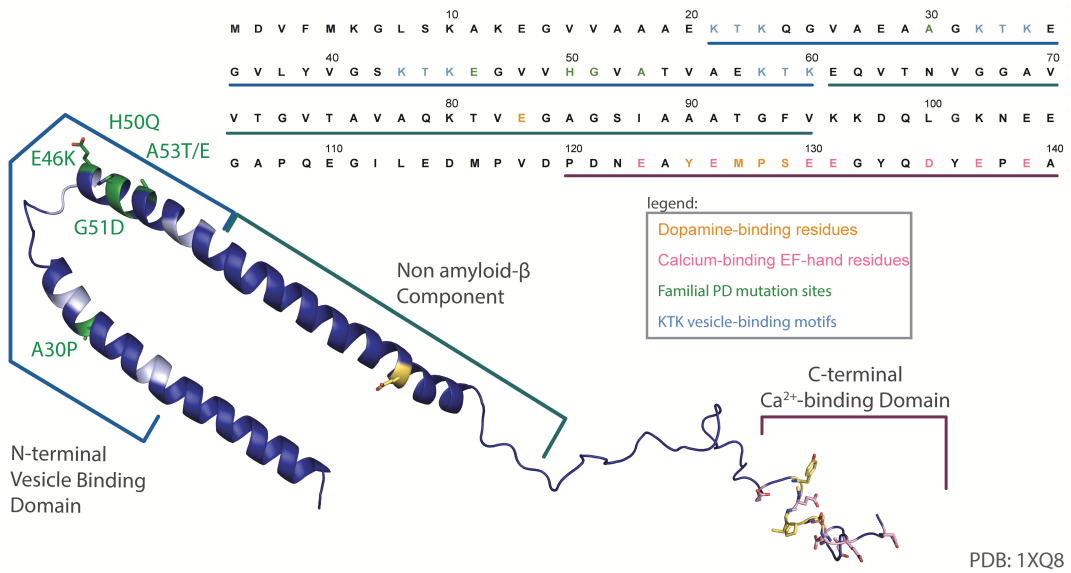

FIGURE 1 | aSyn sequence map. An annotated structure of human micelle-bound aSyn solved by solution NMR (PDB 1XQ8) (Ulmer et al., 2005) and corresponding sequence have been color coded to highlight the lipid binding N-terminal domain and associated KTK motifs (cyan), common familial PD mutations (green), acidic residues in the C-terminal Ca^{2+}-binding EF-hand-like motif (pink), and the dopamine-binding residues (yellow).

which reduces PD-like symptoms and pathology in a mouse model of A30P aSyn overexpression (Diepenbroek et al., 2014). Additionally, an indirect effect of Ca^{2+} on aSyn aggregation can be mediated by disruptions in autophagy. As elevated Ca^{2+} leads to increased mitochondrial stress, it has been suggested that this adds demand to proteostasis systems by necessitating increased mitochondrial turnover. This process then reduces cell's capacity to degrade aSyn, leading to aSyn aggregation (Surmeier and Schumacker, 2013).

aSyn AND DA

Due to the toxic potential of DA, it is not surprising that multiple cellular mechanisms exist to regulate its cytosolic concentration. It has been suggested that neuromelanin biosynthesis acts as one of the mechanisms for regulating toxic DA by-products by sequestering them into autophagic vacuoles (Sulzer and Zecca, 2000). Similarly, oxidized derivatives of both DA and NE are found in LC-derived neuromelanin (Wakamatsu et al., 2015). Other mechanisms include feedback inhibition of DA synthesis, catabolic DA cleavage and synaptic vesicle sequestration.

Catecholamines are synthesized from the non-essential amino acid tyrosine by a series of enzymatic reactions. In the first, rate-limiting step, tyrosine hydroxylase (TH) attaches a hydroxyl group to the aromatic ring of tyrosine, forming L-DOPA. TH activity is regulated on transcriptional, translational and post-translational levels (Goldstein and Lieberman, 1992; Kumer and Vrana, 1996; Fitzpatrick, 2000; Daubner et al., 2011), including phosphorylation-dependent activation of TH by various kinases and its inhibition by DA, which limits DA production when its cytosolic concentration increases. The second enzyme in DA biosynthesis, aromatic L-amino acid decarboxylaze (AADC), converts L-DOPA to DA. AADC activity can also be regulated by second messenger systems to decrease DA production when its extracellular concentration increases (Hadjiconstantinou and Neff, 2008). aSyn has been shown to co-immunoprecipitate with both TH (Perez et al., 2002) and AADC (Tehranian et al., 2006), and this interaction leads to decreased phosphorylation and activity of both enzymes. Decreased TH activity in the presence of aSyn overexpression depended on aSyn phosphorylation at Ser129 residue (Lou et al., 2010), which was modulated by the activity of protein phosphatase 2A (Peng et al., 2005). These data suggest that a loss of soluble aSyn due to reduced expression or aggregation may increase catecholamine synthesis.

Intracellular catecholamine catabolism starts with the cleavage by monoamine oxidase (MAO), which is localized at the outer mitochondrial membrane (Schnaitman et al., 1967), and produces two highly reactive compounds, hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL) (or 3,4-dihydroxyphenylglycolaldehyde for NE) (Richter, 1937). This is followed by the oxidation by aldehyde dehydrogenase (ALDH) to 3,4-dihydroxyphenylacetate (DOPAC) and 3,4-dihydroxyphenylglycol (DHPG), correspondingly. Although ALDH activity—there are both cytosolic and mitochondrial isoforms of this enzyme (Marchitti et al., 2007; Chen et al., 2014; Doorn et al., 2014) - is very high, possible neurotoxicity of the aldehyde metabolites of amines was predicted 60 years ago due to their extremely reactive nature (Blaschko, 1952). Indeed, the presence of DOPAL and its metabolites has been demonstrated both *in vitro* and *in vivo* (Burke et al., 2004; Goldstein et al., 2014). Additionally, a line of mice that are deficient for ALDH1a1 and ALDH2, the cytosolic and the mitochondrial isoforms expressed in SNpc DA neurons (Mccaffery and Drager, 1994; Galter et al., 2003), showed age-dependent, L-DOPA-responsive deficits in

motor performance, significant increases in biogenic aldehydes and a loss of SNpc DA neurons (Wey et al., 2012), confirming that impaired detoxification of biogenic aldehydes may cause PD-like degeneration.

Finally, sequestration and compartmentalization of DA inside secretory vesicles is achieved via the activity of vesicular monoamine transporters (VMAT). The enzyme responsible for the conversion of DA to NE in noradrenergic neurons, dopamine beta-hydroxylase, is located in the lumen and the membrane of synaptic vesicles. Moreover, the acidic pH of vesicles prevents auto-oxidation of DA and NE, allowing high vesicular neurotransmitter concentrations without the formation of reactive species. Importantly, synaptic vesicle membrane is "leaky" and *in vitro* and *in vivo* studies have demonstrated that leakage of catecholamines from storage vesicles is the primary source of their catabolism in the cytosol (Goldstein et al., 1988; Halbrugge et al., 1989; Tyce et al., 1995).

High cytosolic DA levels following L-DOPA treatment have been shown to induce selective SNpc neuron degeneration and the formation of neuromelanin (Sulzer et al., 2000), whereas increased loading of DA from cytosol to vesicles following overexpression of vesicular monoamine transporter 2 (VMAT2) provides neuroprotection from L-DOPA (Mosharov et al., 2009). Consistent with this, higher levels of neuromelanin are found in SNpc neurons that degenerate in PD (Zucca et al., 2014). Striatal DA synaptic vesicles from PD patients were also found to have lower levels of VMAT2 (Pifl et al., 2014), although as these patients were almost certainly treated with L-DOPA, a decrease in VMAT expression could be a compensatory response rather than a cause of PD.

Oxidized DA and other catecholamines are able to interact with aSyn, producing DA-modified aSyn, which is less likely to fibrilize and instead forms soluble oligomers (Conway et al., 2001; Rochet et al., 2004). This interaction is non-covalent, reversible and occurs at the $Y_{125}EMPS_{129}$ pentapeptide in the C-terminal region of α-Syn with an additional long-range electrostatic interaction with E83 in the nAC region (**Figure 1**, in yellow) (Mazzulli et al., 2007; Herrera et al., 2008). Using fluorescence-lifetime imaging microscopy to monitor the relative position of the N- and C- terminals of aSyn, it was shown that DA induces a conformation where the termini are closer together, which may inhibit fibril formation (Outeiro et al., 2009). Additionally, DOPAL may cross-link aSyn lysine residues, also facilitating its aggregation (Werner-Allen et al., 2016). Intracellular aSyn oligomeric species can be cytotoxic by a variety of mechanisms, including permeabilization of vesicular and plasma membranes by pore-forming fibrils (Ding et al., 2002; Gosavi et al., 2002; Lashuel et al., 2002; Mosharov et al., 2006), disruption of proteasomal protein clearance, chronic ER stress, mitochondrial dysfunction and inhibition of SNARE complex formation and neurotransmitter release (Rochet et al., 2004; Ebrahimi-Fakhari et al., 2011; Choi et al., 2013; Kalia et al., 2013; Zaltieri et al., 2015).

Monomeric DA-aSyn, however, may also be toxic by interfering with protein degradation via a lysosomal pathway called chaperone-mediated autophagy (CMA) (Cuervo et al., 2004, 2010). CMA cytosolic substrates contain a KFERQ-like motif that can be recognized by the chaperone protein cyt-Hsc70 that delivers them to a lysosomal associated membrane protein (LAMP2A). LAMP2A forms a translocation complex once bound to a substrate and the unfolded protein crosses into the lysosomal lumen where it can be degraded. While aSyn, oxidized aSyn, and a phosphomimetic S129E aSyn mutant show similar LAMP2A binding levels, lysosomal uptake of the latter is significantly diminished. DA-aSyn demonstrates a similar CMA profile when compared to phosphorylated aSyn in that it binds to the lysosome without evidence of translocation. Furthermore, unlike phosphorylated aSyn, DA-aSyn blocks both the binding and uptake of a CMA substrate GAPDH, suggesting stronger binding to LAMP2A. A mutation in the DA-interacting region of aSyn ($Y_{125}EMPS_{129}$ to $F_{125}AAFA_{129}$) nullifies the effect, further demonstrating that the interaction of DA and oxidized forms of DA with aSyn leads to this change in CMA. In primary neuronal cultures, the same CMA blockade was demonstrated after exposure to a high dose of L-DOPA, but not in neurons derived from aSyn null animals (Martinez-Vicente et al., 2008).

A hypothesis that decreased uptake of DA into synaptic vesicles should lead to PD-like nigrostriatal neurodegeneration due to increased cytosolic transmitter levels was examined in mice that displayed a 95% reduction of VMAT2 expression due to a hypomorphic allele (Caudle et al., 2007). Surprisingly, the first generation of these mice (VMAT2-deficient KA1 line Mooslehner et al., 2001) did not show any PD phenotype, despite an ∼85% reduction in brain levels of DA, NE and serotonin and their increased turnover. It was subsequently discovered, however, that this mouse line had a spontaneous deletion of the SNCA gene (Specht and Schoepfer, 2001; Colebrooke et al., 2006). After further breeding to reintroduce the wild-type aSyn gene, the resulting VMAT2-LO mice showed signs of PD-like progressive neurodegeneration, including L-DOPA-responsive motor deficits, oxidative stress and protein damage, decreased DA, DAT, and TH levels in the striatum, and pathological accumulations of aSyn and a reduced number of DA neurons in the SNpc (Caudle et al., 2007; Taylor et al., 2011). Overall, the VMAT2-LO mouse model not only demonstrated that a reduced capacity of cells to sequester cytosolic DA is sufficient to cause PD-like degeneration of neurons and their axonal projections, but also that this effect requires the presence of aSyn.

Another recent study investigated the toxic interaction between aSyn and DA *in vivo* by combining a common familial PD aSyn mutation with elevated cytosolic DA (Mor et al., 2017). Mice that overexpress PD mutant A53T aSyn were injected with a lentivirus containing TH with an $R_{37}R_{38}$ to $E_{37}E_{38}$ mutation. This mutation leads to a loss of feedback inhibition of TH by DA, resulting in increased neurotransmitter production in the cytosol (Nakashima et al., 2002). Elevation of cellular DA levels induced progressive motor impairment accompanied by nigrostriatal degeneration and increased formation of aSyn oligomers in A53T aSyn overexpressing mice but not in WT. Furthermore, in *Caenorhabditis elegans* overexpressing A53T aSyn, DA toxicity was prevented if DA-interacting residues of aSyn were mutated (Mor et al., 2017). Overall, both *in vitro* and *in vivo* data suggest that DA and aSyn have a synergetic effect on

toxicity and that decreasing the levels of either of the compounds is neuroprotective.

aSyn, DA AND Ca^{2+}

Ca^{2+} levels positively regulate the activity of both TH and AADC, providing a direct connection between synaptic activity and DA synthesis. However, because of Ca^{2+}-driven pacemaking in SNpc and LC neurons, elevated levels of Ca^{2+} also lead to chronically increased cytosolic catecholamine levels. In agreement with this, L-DOPA treatment produces higher concentration of cytosolic catecholamines in cultured SNpc (Mosharov et al., 2009) and LC (unpublished data) compared to VTA neurons, which translated into higher susceptibility of these neurons to L-DOPA-induced degeneration. The difference between these cell types was normalized by pharmacological or genetic blockade of the LTCCs, confirming their role in selective PD-like neurodegeneration. Importantly, deletion of aSyn also protected SNpc neurons from L-DOPA-induced toxicity without changing cytosolic DA concentration, demonstrated that the levels of Ca^{2+}, DA and aSyn are equally important for toxicity.

Using the same model system, we recently investigated metabolic changes in neurons exposed to the parkinsonian neurotoxin MPP^+ (Lieberman et al., 2017). Similar to the difference observed *in vivo* described above, a significantly higher level of toxicity was observed in cultured SNpc than VTA neurons. In MPP^+-treated SNpc, but not VTA neurons, neurotoxicity was caused by a transient increase in cytosolic Ca^{2+} that required the activity of LTCCs and ryanodine receptors. Combined with MPP^+-mediated inhibition of DA cleavage by MAO (Choi et al., 2015), this caused upregulation of cytosolic DA and nitric oxide levels, mitochondria oxidation, and ER stress. As with L-DOPA toxicity, SNpc neurons from aSyn deficient mice were significantly more resistant to MPP^+. Thus, in two different toxicity models we found that selective death of SNpc neurons results from a combination of "multiple hits," including the activity of the LTCCs that create high basal cytoplasmic Ca^{2+} levels, an upregulation of DA synthesis and the presence of aSyn. Similar upregulation of Ca^{2+}/NO with concomitant mitochondria oxidative stress was demonstrated in LC neurons (Sanchez-Padilla et al., 2014) and SN neurons exposed to preformed aSyn fibrils (Dryanovski et al., 2013), indicating that this pathway may be commonly activated under stress conditions.

A recent study of DA- and aSyn-mediated toxicity in human idiopathic and familial iPSC-derived DA neurons from patients with a DJ-1 mutation (PARK7) provided more evidence for the involvement of multiple factors in mediating PD-like neurotoxicity (Burbulla et al., 2017). The authors identified a DA- and Ca^{2+}-dependent toxic cascade that started with mitochondrial oxidative stress leading to lysosomal dysfunction and aSyn accumulation. Interestingly, this toxicity pathway was not present in DJ-1 deficient mice or mouse iPSC-derived

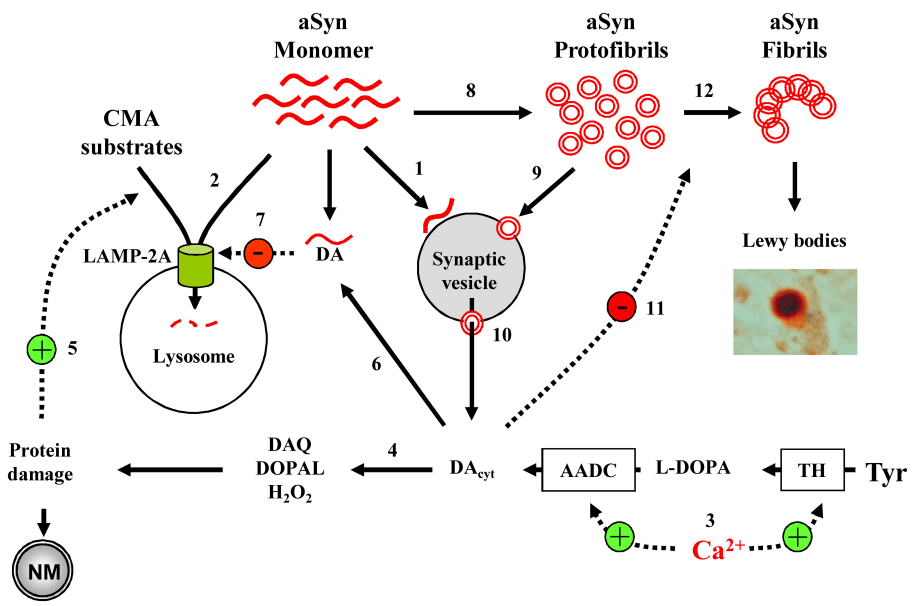

FIGURE 2 | Model of cell-selective PD pathogenesis. **(1)** A physiological function of aSyn may be to bind synaptic vesicles in a reversible manner to inhibit exocytosis. **(2)** aSyn is degraded by CMA following LAMP-2A-mediated transport into the lysosomes. **(3)** In SN and LC neurons, high Ca^{2+} levels upregulate TH and AADC, leading to increased DA concentrations and **(4)** associated oxyradical stress, which induces various cell defense mechanisms, including **(5)** CMA. **(6)** DA-modified aSyn **(7)** blocks LAMP-2A-mediated uptake of CMA substrates, including aSyn itself. **(8)** aSyn may oligomerize to toxic protofibrils, which can **(9)** bind to and **(10)** permeabilize synaptic vesicles, leading to further increase in cytosolic DA. **(11)** DA stabilizes aSyn protofibrils, inhibiting the formation of larger polymers of aSyn **(12)**. Overall, the presence of such interactions where DA and aSyn act as independent stressors that converge to produce neurotoxicity may explain why catecholaminergic neurons with Ca^{+2} channel- mediated pacemaking are more prone to produce neuromelanin and Lewy bodies, and are particularly vulnerable in PD and parkinsonian animal models. Only a few aSyn-DA-Ca^{2+} interactions discussed in the text are shown.

DA neurons generated from DJ-1 KO fibroblasts unless either DA production or aSyn expression was increased. Underlying species-specific differences may therefore explain the difficulties of creating an appropriate mouse model of PD.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

At the center of PD pathology is aSyn, which tends to form soluble oligomers and insoluble fibrils. Oligomerization is increased with increased Ca^{2+} or DA levels, while aSyn oligomers are able to increase internal Ca^{2+} and DA concentrations, forming a potential positive feedback cycle. Furthermore, DA-modified aSyn blocks CMA-mediated protein degradation, potentially causing a buildup of monomeric aSyn that then aggregates into more oligomers (**Figure 2**). These interactions demonstrate the precarious nature of SNpc and LC neuron health as, if one aspect of the homeostatic processes goes awry, the feedback loops activate and neurotoxicity ensues. Importantly, in this model it is possible to initiate the pathological sequence of events that lead to neurodegeneration by diverse insults, including elevation of Ca^{2+} levels, increased cytosolic DA unrelated to Ca^{2+}-dependent regulation, mutation or overexpression of α-Syn, inhibition of CMA activity due to aging (Schneider et al., 2014, 2015), the presence of other parkinsonian mutations or other possible mechanisms.

Therapeutically, this hypothesis provides several avenues to pursue the disease-modifying opportunities as decreasing the levels of any one of these key toxicity mediators should be beneficial for the survival of SNpc and LC neurons. Previous work has demonstrated the utility of immunotherapy to reduce aSyn levels in the CNS (Masliah et al., 2005) and prevent possible trans-synaptic spread of toxic aSyn species (Bae et al., 2012). A retrospective analysis demonstrated that the use of dihydropyridines correlates with decreased probability of developing PD (Pasternak et al., 2012), and an LTCC antagonist isradipine is currently in phase III clinical trials as a disease-modifying therapy for PD (Swart and Hurley, 2016). Combining these approaches with drugs that reduce toxic DA species might provide additional benefits. One important future focus will be the development of diagnostic tools to enable earlier disease-modifying treatments and stratification of patient populations to enhance beneficial outcomes. The level of aSyn peripherally and in the CNS (Malek et al., 2014) as well as the status of DA homeostasis (Niethammer et al., 2012) are currently the focus of studies aimed at developing bioassays and imaging approaches to identify pre-symptomatic PD cases with defined patho-physiologies to give "personalized" treatments.

AUTHOR CONTRIBUTIONS

MP, OL, and EM gave their substantial contribution to conception and design of the manuscript, its drafting and revising it critically. All authors have approved the manuscript in its present form for publication and agree to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

Supported by the National Institute of Neurological Disorders and Stroke (Grants NS075222 to EM) and the National Institute of Mental Health (F30MH114390 to OL and T32MH20004 to MP). We thank David Sulzer and Maha Subramaniam for their help during the preparation of the manuscript and Ellen Kanter for invaluable assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.