THE PROTEIN ALPHA-SYNUCLEIN: ITS NORMAL ROLE (IN NEURONS) AND ITS ROLE IN DISEASE

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THE PROTEIN ALPHA-SYNUCLEIN: ITS NORMAL ROLE (IN NEURONS) AND ITS ROLE IN DISEASE

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EDITORIAL
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Editorial: The Protein Alpha-Synuclein: Its Normal Role (in Neurons) and Its Role in Disease

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Editorial on the Research Topic

The Protein Alpha-Synuclein: Its Normal Role (in Neurons) and Its Role in Disease

The protein alpha-Synuclein (aSyn) is highly-studied due to its role in Parkinson's disease (PD) and its accrual as the major protein component of Lewy bodies/Lewy neurites (LB/LN) (Anderson et al., 2006). Though the precise role of aSyn in disease pathogenesis is not fully elucidated, aSyn toxicity is widely thought to be associated with its aggregation in LB/LN. This has led some to consider modulating aSyn expression as a means to counteract PD pathology. Yet, a multitude of studies show that altering aSyn expression or removing it from neurons has profound effects on many intracellular processes and in some cases induces neurodegeneration. This leads some to propose that normal aSyn function is crucial for particular neuronal populations and that PD results from a toxic loss of aSyn function (Perez and Hastings, 2004; Benskey et al., 2016). Indeed, aSyn is one of the most abundant proteins of the nervous system. Its role in neurotransmission at the synapse is well-established, and research also confirms roles for aSyn in neurotransmitter synthesis, calcium homeostasis, mitochondrial function, and gene regulation. Thus, it is essential to thoroughly define normal aSyn function in neurons before pursuing aSyn reducing therapies. In this Research Topic the contributions of many aSyn experts describe original research, timely reviews, or perspectives regarding the role of aSyn to wellness and disease.

Surguchev and Surguchov review evidence that a normal function of all three synuclein homologs is a regulatory role in gene expression that occurs by their interactions with nucleic acids, transcription factors, and translation factors. Their main focus is on aSyn, as it has been more extensively studied. Emerging roles have been identified for aSyn in epigenetics by its binding interactions that affect DNA methylation, RNA-associated-gene-silencing, and histone acetylation. Further, genes involved in DNA repair are also modulated by aSyn, suggesting how the loss of soluble aSyn could impair cellular function. More evidence for important normal aSyn function is seen in well-controlled research from Benskey et al. who demonstrate a role for aSyn in maintaining nigrostriatal dopaminergic neuron viability. Using adult wild type rats in which they downregulate endogenous aSyn in substantia nigra with adeno-associated-virus short hairpin RNA, neuronal dysfunction is induced followed by neuronally-mediated inflammation and then nigral dopaminergic neuron loss. Control experiments in glutamatergic neurons of the rats have no loss of viability, confirming the key importance of aSyn in dopaminergic neurons. Added support comes from Vidal-Martinez et al. who review findings confirming that aSyn inhibits dopamine synthesis in neuronal cells (Perez et al., 2002), and inhibits insulin secretion from pancreatic ß-cells by aSyn binding the Kir6.2 subunit of K-ATP channels on insulin granules (Geng et al., 2011). Moreover, Kir6.2 has been shown to inhibit brain dopamine secretion

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(Avshalumov and Rice, 2003), suggesting that therapies that sustain aSyn:Kir6.2 interactions and enhance brain derived neurotrophic factor (BDNF) expression while blocking inflammation (Vargas-Medrano et al., 2014), may protect against diabetes and PD.

Mitochondrial impairment, which is common in PD, influences energy metabolism, homeostasis, the stress response, and apoptosis (Winklhofer and Haass, 2010). Vicario et al. review data showing that aSyn directly influences mitochondria by modulating their membrane potential, calcium homeostasis, cytochrome c release, ATP production, and fusion/fission. aSyn localization on mitochondria was first demonstrated in dopaminergic cells (Perez et al., 2002), and more recently using aSyn overexpressing dopaminergic cells others show that aSyn inhibits fusion and stimulates fission of mitochondria (Kamp et al., 2010). In that same study increasing wild type PINK1 or Parkin expression in dopaminergic cells reversed their mitochondrial deficits. Research from Creed and Goldberg in PINK1 knockout (−/−) rats demonstrates age-onset aSyn accumulation in synaptic vesicle pools, as well as spontaneous accumulation of insoluble aSyn in cortex, thalamus, striatum, and ventral midbrain. Curiously, aSyn pathology arises in the rats even though aSyn is not overexpressed. The authors propose that PARK1 −/− rats nicely model sporadic PD making them useful for assessing treatments aimed at slowing PD progression. Research by Cuvelier et al. reveal metabolic changes such as reduced body mass and less adiposity in aging male Thy-1 transgenic mice that express wild type human aSyn (Thy1-aSYN mice) on a C57BL/6-DBA/2 background (Rockenstein et al., 2002; Fleming et al., 2004). Thy1-aSYN males also exhibit increased spontaneous activity, lower food intake, and reduced energy expenditure than control mice. As metabolism and mitochondria are strongly linked, it could be instructive to assess the activity of mitochondria in adipose tissue from male Thy1-aSYN mice, especially in light of data in humans with persistent low body weights having higher mitochondrial activity in white adipose tissue (Ling et al., 2019).

Further regarding aSyn in neurons, Post et al. review interactions between aSyn, dopamine and calcium that underlie the loss of substantia nigra and locus coeruleus (LC) neurons in PD. In their multi-hit model they describe synergistic interactions between aSyn, calcium ions and dopamine that cause abnormal protein turnover that leads to nigral and LC vulnerability. The review by Betzer and Jensen reconsiders the calcium hypothesis which states that elevated intracellular neuronal calcium causes their demise. They present more recent findings showing that neurons undergoing a gradual build-up of aSyn, cytosolic calcium is actually reduced as aggregated aSyn binds to the sarcoplasmic-endoplasmic reticulum (ER) calcium ATPase (SERCA). The aggregated aSyn binding activates SERCA which reduces cytosolic calcium and overloads the ER with calcium (Betzer et al., 2018). They suggest that targeting abnormally activated SERCA or preventing the aSyn:SERCA interaction could yield therapies to slow PD. Butkovich et al. describe research on how a loss of LC norepinephrine (NE) and subsequent NE-effects reduce neurotrophic factor signaling, worsen central and peripheral inflammation, and alter innate and adaptive immune responses to accelerate PD progression. They note that in NE-producing cells and related animal models that overexpress aSyn, aSyn translocates to the nucleus where it interferes with transcription of dopamine ß-hydroxylase (DBH), the final enzyme in NE biosynthesis to reduce NE production (Kim et al., 2014). Loss of NE in noradrenergic neurons could be exacerbated by the upstream inhibition of tyrosine hydroxylase (Perez et al., 2002) and amino acid decarboxylase (Tehranian et al., 2006) by aSyn, producing lower levels of dopamine to convert to NE by DBH.

A final series of articles describes tools for elucidating aSyn structure, *in vivo* modeling of sporadic PD, and aSyn interactions with vesicle proteins. Dettmer reviews the use of rationally-designed aSyn variants to define properties of aSyn that are relevant to health and disease. Describing biochemical and cell biological aSyn data from various labs, he emphasizes how intact-cell approaches show how small changes in aSyn structure can contribute to PD and other synucleinopathies. In a perspectives article, Duffy et al. support the use of preformed fibrils of aSyn to model idiopathic PD to allow exploring relationships between aSyn aggregation and cellular toxicity. Preformed aSyn fibrils can be injected intrastriatally at endogenous levels after which over time they induce LB-like inclusions, neuroinflammation, and progressive nigrostriatal degeneration. Almandoz-Gil et al. show co-localization of aSyn with SNARE proteins in primary cortical neurons by *in situ* proximity ligation assays (PLA). PLA was previously used to corroborate aSyn interactions with other proteins or identify aSyn oligomers in PD brain. In beautiful images they show aSyn proximity with VAMP-2, SNAP-25, and syntaxin-1 in cell bodies and neurites, remarkably no differences were seen in neuronal aSyn:SNARE interactions from A30P aSyn transgenic and non-transgenic mice.

In conclusion, the papers presented in this Research Topic emphasize the importance of aSyn function that when exaggerated or disrupted may impair function and viability of neurons, pancreatic ß-cells, and other cells that utilize normal aSyn function. The data further suggest that therapies that help sustain normal levels of soluble aSyn could be highly protective.

AUTHOR CONTRIBUTIONS

RP authored the editorial by making direct intellectual contributions regarding the Research Topic.

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REVIEW
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Synucleins and Gene Expression: Ramblers in a Crowd or Cops Regulating Traffic?

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Synuclein family consists of three members, α, β, and γ-synuclein. Due to their involvement in human diseases, they have been thoroughly investigated for the last 30 years. Since the first synuclein identification and description, members of this family are found in all vertebrates. Sequencing of their genes indicates high evolutionary conservation suggesting important function(s) of these proteins. They are small naturally unfolded proteins prone to aggregate, easily change their conformation, and bind to the membranes. The genes for α, β, and γ-synuclein have different chromosomal localization and a well preserved general organization composed of five coding exons of similar size. Three genes encoding synucleins are present in the majority of vertebrates, however, a variable number of synuclein genes are described in fishes of different species. An important question concerns their normal function in cells and tissues. α-Synuclein is implicated in the regulation of synaptic activity through regulation of synaptic vesicle release, while the physiological functions of two other members of the family is understood less clearly. Here we discuss recent results describing their role in the regulation of gene expression.

Keywords: gene expression, synucleins, epigenetic regulation, transcription factors, translation factors, DNA methylation, histone modifications, neurodegeneration

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INTRODUCTION

Since the discovery of the first synuclein by Maroteaux et al. (1988), the members of the synuclein family attract growing attention primarily as proteins implicated in neurodegenerative (α-synuclein) and neoplastic (γ-synuclein) diseases. The majority from almost 9,000 publications describe how these naturally unfolded proteins aggregate and propagate between neurons (α-synuclein), or cause misregulation of intracellular signaling pathways in oncological transformation (γ-synuclein). β-Synuclein, the third member of the family, received much less attention. It is less prone to form insoluble aggregates and presumably plays a protective role against α-synucleinopathies (Hashimoto et al., 2001). Since the discovery of synucleins, researchers investigate mostly α-synuclein for its association with synucleinopathies, i.e., PD, PD dementia, DLB and multiple system atrophy.

During the late 1990s the popular theory to explain mechanisms causing α-synuclein aggregation in cellular milieu was a concept of macromolecular crowding (Minton, 1993, 1998; Uversky et al., 2001). According to this concept, total cytoplasmic concentration of proteins and other macromolecules can either reach the high level of 400 g/l causing macromolecular

Abbreviations: Aβ, amyloid beta; BDNF, brain-derived neurotrophic factor; DBH, dopamine-β-hydroxylase; DLB, dementia with Lewy Bodies; DNMT, DNA methyltransferase; eEF2, translation elongation factor 2; eIF3, translation initiation factor 3; HAT, histone acetyltransferase; MMP, matrix metalloproteinase; PD, Parkinson's disease; PKCδ, protein kinase Cδ.

crowding (Uversky et al., 2001, 2002; Shtilerman et al., 2002) or be subjected to excluded volume effect (Ellis, 2001) in the neuronal cytoplasm. This induces α-synuclein and other naturally unfolded protein aggregation, which may lead to the formation of toxic aggregates, fibrils, and protofibrils.

While synuclein's role in pathology is thoroughly and systematically investigated, their normal physiological functions still remain debated despite almost 30 years of research. Some progress in unveiling α-synuclein physiological role is achieved due to the discovery of its role in synapsis. Several research teams have demonstrated that α-synuclein is implicated in the modulation of synaptic activity through modulation of vesicle release (Chandra et al., 2005; Burré et al., 2010; Bendor et al., 2013; Zaltieri et al., 2015). Numerous studies demonstrate α-synuclein's participation in vesicle trafficking, chaperoning of SNARE complex assembly (Bonini and Giasson, 2005; Chandra et al., 2005; Burré et al., 2010; Diao et al., 2013), regulation of synaptic vesicles endocytosis, presynaptic terminal topography (Vargas et al., 2014, 2017), and modulation of dopamine release (Abeliovich et al., 2000). However, the progress in understanding the physiology of β and γ-synucleins is much more limited. β-Synuclein inhibits α-synuclein aggregation caused by several types of lipids as well as α-synuclein nucleation (Brown et al., 2016). γ-Synuclein possesses chaperon activity, is involved in the regulation of monoamine homeostasis and cytoskeleton structure rearrangement. Moreover, it may have similar functions as α–synuclein in the process of synaptic vesicle turnover, neurotransmitter release (Ninkina et al., 2012; Kokhan et al., 2013), and regulation of signaling pathways (Surguchov et al., 1999, 2001, 2017; Surgucheva et al., 2006, 2008; Surguchov, 2008, 2015).

Despite the information presented above, many reviews consider the normal functions of α-synuclein to be unclear and vague, creating an impression that it does not have its own function in living cells, and settles for a supporting role in several pathways. In addition to synaptic functions, α-synuclein participation in several cellular processes has been described, e.g., suppression of apoptosis, regulation of glucose level, modulation of calmodulin level, maintenance of PUFA level, antioxidation, neuronal differentiation, and regulation of dopamine biosynthesis (Emamzadeh, 2016). However, it appears that in all these processes, synucleins are not the major players, but just "ramblers in a crowd" with limited authority and jurisdictions. Still the emerging evidence suggests, that synucleins may have a very important non-canonical function unrelated to their role in synapsis. The nuclear localization of α-synuclein and several new findings point to its essential role in the nucleus related to the regulation of gene expression.

SYNUCLEINS AS MODULATORS OF SPECIFIC GENE EXPRESSION

Gradually new results accumulate, and point out that our knowledge about synuclein's functions is incomplete, their "social network" is larger than it is currently assumed, and they play a more essential dynamic role in the regulation of intracellular processes. The current understanding of their role in cellular processes is slowly shifting from "ramblers in the crowd" to "cops regulating traffic." This regulatory role is performed through their interactions with key players governing gene expression, i.e., nucleic acids, transcription and translation factors, and histones. Moreover, synucleins may play a role in epigenetic regulation of cellular processes. Epigenetics applies to external modifications to DNA that alter expression of specific genes without changes in the DNA sequence. It can involve several mechanisms, e.g., DNA methylation, histone modifications, and RNA-associated silencing.

While the researchers still keep working out the details of synuclein's functions in synapsis and a role in vesicles formation, another page of their story emerges, attracting increasing attention. Several new methods demonstrate that synucleins are "more social" that it has been assumed earlier, and find novel partners in synuclein's "social network" not only among proteins, but also nucleic acids. Below we present several examples of synuclein's regulatory role in gene expression.

The first hint suggesting that synucleins regulate gene expression came from the analysis of the effect of their upregulation in cell cultures (Seo et al., 2002; Baptista et al., 2003). Although the effect of elevated synucleins level on expression of specific genes was significant, the exact molecular mechanisms was often unknown. In several studies a direct effect of synucleins on transcription in the nucleus is demonstrated, other publications suggest hypothetic mechanisms explaining this effect. For example, α-synuclein downregulates c-Jun N-terminal kinase protecting cells against oxidative stress, upregulates caveolin-1 expression, and downregulates ERK, affecting the pathogenesis of PD (Hashimoto et al., 2003). In another publication α-synuclein's effect in regulating apoptosis is reported. It reduced Bcl-xL expression and increased BAX expression (Seo et al., 2002).

In several studies a substitution of one amino acid in α-synuclein dramatically changed its regulatory properties suggesting that this effect was mediated by conformational alterations. For example, α-synuclein altered expression of several families of genes including genes responsible for apoptosis, stress response, transcription regulation, and membrane proteins (Baptista et al., 2003). Significant changes in expression levels are also found for genes responsible for the regulation of dopamine synthesis. Reduced expression of the orphan nuclear receptor Nurr1 suggests that the synergetic regulation of dopamine synthesis occurs through this transcription factor. These alterations in mRNAs levels identified by microarray experiments occurred on transcriptional level and were validated by quantitative RT-PCR. Interestingly, the expression levels of four genes were regulated in opposite directions in cells overexpressing wild-type protein or α-synuclein with A53T substitution (Baptista et al., 2003).

Overexpression of wild-type or mutant α-synuclein interferes with DBH transcription regulation by CRE element in catecholaminergic neurons (Kim et al., 2014). In the nucleus α-synuclein interacts with the DBH promoter including the CRE element, which interferes with forskolin-induced transcription factor CREB binding to the CRE region. Thus, α-synuclein

attenuates CRE-mediated transcription of DBH. This interaction is physiologically important, because CREB plays an essential role in survival of neurons by controlling the transcription of several genes implicated in cell protection, such as BDNF (Chalovich et al., 2006). Remarkably, mutant α-synuclein demonstrates higher tendency to nuclear translocation and interaction with the DBH promoter than the wild type.

Another example confirming that the effect of α-synuclein on transcription of specific gene may differ as a result of a single amino acid substitution is recently described (Segura-Ulate et al., 2017). Oligodendroglial cells OLN-93 stably expressing either a human wild type or a mutant A53E α-synuclein (multiple system atrophy associated mutant) reduced BDNF mRNA to practically unmeasurable levels. At the same time, another MSA-associated α-synuclein mutant, caused only a small reduction in BDNF mRNA. Therefore, point mutations in α-synuclein may change not only its physico-chemical properties and propensity to aggregation, but also alter its regulatory function (Segura-Ulate et al., 2017). Moreover, in dopaminergic neurons α-synuclein also reduces the expression of PKCδ to inhibit apoptosis, by decreasing enzymatic activity of p300 HAT.

This effect results in neuroprotection in a α-synuclein expressing dopaminergic cell model as a result of exposure to the Parkinsonian neurotoxicant MPP+. This mechanism involves modulation of both NFκB and p300 signaling pathways in transgenic mice and in neuronal culture (Jin et al., 2011). The effect of α-synuclein on histone acetylation is further described in Chapter 6.

α-Synuclein is colocalized with transcription factor Elk-1 and interacts with MAP kinase pathway (Iwata et al., 2001a,b). These findings point to the role of α-synuclein in the modulation of transcription and signaling pathways.

Another member of the synuclein family, γ-synuclein also interacts with Elk-1 (Surguchov et al., 1999). Furthermore, it plays a role as a modulator of matrix metalloproteinases (MMP-9 and MMP-2) expression upregulating both MMP-9 protein level and activity (3.2 to 7.1-fold, respectively). This upregulation takes place on transcriptional level via the activation of the AP-1 *cis*-elements in MMP-9 promoter (Surgucheva et al., 2003). Further studies demonstrated direct binding of γ-synuclein to transcription factors JunB, MECP2, CREB1, PPAR-gamma, TCEA1, and ATF3 (Surgucheva and Surguchov, 2008). Recent study using yeast two-hybrid screening, identified another transcriptional regulator – polyC binding protein 1 (PCBP1) as a γ-synuclein interacting protein (Hunkele et al., 2016).

SYNUCLEIN – DNA INTERACTIONS

α-Synuclein Binding to DNA

The existence of α-synuclein in the nucleus first found by Maroteaux et al. (1988) and later confirmed by many other studies, encouraged researchers to examine synuclein–DNA interaction and to investigate its possible functions. Several research groups demonstrated that α-synuclein could bind directly to a single copy DNA; this binding is especially efficient if DNA is active in the process of transcription and is not bound to histones (Hegde and Jagannatha Rao, 2003; Hegde et al., 2010; Vasudevaraju et al., 2012). This binding occurs preferentially to GC-box-like sequences (Vasudevaraju et al., 2012; Ma et al., 2014) and may alter properties of both protein and DNA. In particular, α-synuclein induces DNA damage by changing its stability, conformation, and by causing DNA nicking (Padmaraju et al., 2011). In turn, DNA can itself modulate α-synuclein folding (Hegde and Rao, 2007). After binding with double- or single-stranded DNA α-synuclein acquires a highly structured conformation. Circular dichroism studies show that the α-helical content of α-synuclein increases from 5 to 64% upon binding to DNA, whereas the random coil decreases from 95 to 33% (Hegde and Rao, 2007).

Interaction of α-synuclein with linear or supercoiled double-stranded DNA (dsDNA) protects DNA from digestion by restriction endonucleases. Complexes between α-synuclein and DNA as well as assembly of wild-type α-synuclein into fibrils in the presence of linear DNA are revealed by electron microscopy (Cherny et al., 2004).

The presence of synucleins in the nucleus and their binding to DNA provides a possibility that such interaction affects transcription regulation and may change neuronal function (Ma et al., 2014; Surguchov, 2014). In several studies such functional consequences of synuclein – DNA interaction have been demonstrated. For example, in neuroglioma, α-synuclein binds to DNA, and regulates the transcription of genes controlling ubiquitination and other biochemical processes linked to PD (Martins et al., 2011).

DNA Binding Is a Common Feature of Several Amyloidogenic Proteins

α-Synuclein is not a unique amyloidogenic protein possessing DNA-binding ability. Amyloid-beta (Aβ) peptides and prion proteins also have high DNA binding capacity, suggesting that DNA binding may be a common property of amyloidogenic proteins (Hegde et al., 2010). Binding of α-synuclein and other amyloidogenic proteins to DNA may affect normal DNA functions and cause genetic stress altering the normal pattern of gene expression (Jiménez, 2010). Results supporting this unifying hypothesis were obtained from the investigation which identified DNA aptamers that specifically bind to α-synuclein (Tsukakoshi et al., 2010, 2012). In this study eight aptamers specific for α-synuclein monomers, oligomers, and fibrils were characterized by a competitive screening method. Their nucleotide sequences are not conserved, but all possess guanine-rich sequences which form scaffolds-like G-quadruplex structures. Importantly, some of the aptamers recognizing α-synuclein, are also specific for Aβ oligomers. Dissociation constant for such aptamers binding is in nano- to picomolar range. The structure of these aptamers may give a clue for the search of new medications and biomarkers specific for neurodegenerative diseases. Notably, the aptamer could recognize not only the primary structure of α-synuclein, but also its conformation (Tsukakoshi et al., 2012).

Several studies demonstrate that α-synuclein is able to bind directly to promoter region of specific genes and affect their transcription. For example, α-synuclein binding to a promoter

of the mitochondrial transcriptional co-activator PGC-1α, which reduces its expression in response to oxidative stress is described (Siddiqui et al., 2012).

Another member of the synuclein family, γ-synuclein also modulates genes expression by binding to the promoter region of specific genes. For instance, upregulation of matrix metalloproteinases-9 (MMP-9) expression and activity is mediated by γ-synuclein binding to AP-1 sites at the promoter region of the MMP-9 gene (Surgucheva et al., 2003). Under stress conditions a translocation of γ-synuclein to the nucleus decreases outgrowth of neurites more efficiently than α-synuclein overexpression. Thus, γ-synuclein may alter its intracellular localization in response to stress and make appropriate alterations in the gene expression pattern (Surgucheva et al., 2003, 2006).

BINDING OF SYNUCLEIN TO RNA

α-Synuclein binds its own mRNA and prevents initiation of translation (Zanzoni et al., 2013). Interaction of proteins with cognate transcript is a known regulatory mechanism modulating gene expression at the translational level. Usually such mRNA contains a riboswitch – a regulatory segment that interacts with a small molecule, affecting the translation of the proteins encoded by the mRNA (Tucker and Breaker, 2005). However, no riboswitches have been described for synucleins yet. Such autoregulatory control is important for assurance of optimal protein expression levels, and abolition of this normal feedback may lead to various negative consequences (Tai et al., 2004; Hasnat et al., 2007). Autogenous interactions alter gene expression at the translational level and when protein production is elevated, binding to mRNA has a significant inhibitory effect on translation efficiency.

To calculate the propensity of proteins to bind RNA, catRAPID approach was put forward which allows to predict the incidence of autogenous associations in the human proteome (Zanzoni et al., 2013). This method demonstrates that α-synuclein easily binds with cognate mRNA, inhibiting its translation, preventing overexpression and thus supporting the optimal level of protein expression. Since aggregation is intrinsically concentration dependent, it is likely that autogenous interactions play a crucial role in controlling protein homeostasis.

Several recently developed methods are useful to investigate the role of RNA-protein interactions in the pathogenesis of human diseases. CatRAPID is a theoretical framework, which predicts the binding ability of protein and RNA molecules based on physico-chemical properties of nucleotide and amino acid chains. This includes hydrogen bonding, secondary structure, and van der Waals' forces to predict protein–RNA associations with a high confidence (Bellucci et al., 2011; Cirillo et al., 2013). This approach presents a novel tool in the development of RNA aptamers, a useful therapeutic instrument for the diagnostics and management of neurodegenerative diseases (Lee et al., 2006).

Garcia-Esparcia et al. (2015) described significant alterations in the machinery of protein synthesis at the specific regions of the brains of PD patients which are region- and stage-dependent. These alterations include 18S and 28S rRNA, expression of several mRNAs encoding ribosomal proteins, and altered level of translation factors eIF3 and eEF2. These alterations occur in substantia nigra and in the cerebral cortex and may be linked to a significant elevation of α-synuclein oligomers. However, there is no direct evidence to date proving this association.

Thus, there is growing understanding that interaction of α-synuclein with RNA and other protein-RNA interactions are involved in PD and other neurodegenerative diseases (Anthony and Gallo, 2010; Cirillo et al., 2013).

DATA FROM YEAST HELP TO EXPAND α-SYNUCLEIN'S HUMAN INTERACTOME DEMONSTRATING ITS CRITICAL ROLE IN THE REGULATION OF TRANSLATION

New methodologies are required to reveal how synucleins orchestrate the expression of other genes, to identify new binding partners, and to examine mechanisms of synuclein's toxicity. Classical methods, such as knockdown of a single member or all three members of the synuclein family, give only limited information about synuclein's function, confirming their role in synapse structure and physiology. Synuclein's deficiency causes age-dependent neuronal dysfunction, impaired survival and extensive alterations in synaptic dopamine neurotransmission in the nigrostriatal system (Greten-Harrison et al., 2010; Anwar et al., 2011). The results of these experiments also confirmed the existence of overlapping functions in synuclein family members (Anwar et al., 2011). These findings were important for understanding of synuclein pathology in PD and aging, but synuclein biology and their normal physiological functions remained poorly understood.

An important clue for better understanding of a protein's physiological function is the elucidation of its physical interactions. To recognize the normal biological functions and role of α-synuclein in neuropathology, several transgenic and viral overexpression models were developed in various organisms, including roundworms (nematode), fruit flies, rodents, and non-human primates. Amazingly, significant progress in the identification of new synuclein binding partners was made in budding yeast *Saccharomyces cerevisiae*, the organism without CNS or brain. Moreover, it does not even have α-synuclein ortholog. Yeast *Saccharomyces cerevisiae* have repeatedly been shown to be suitable for studies of high eukaryotic cell biology, for example, programmed cell death, mitochondria biology (Ter-Avanesyan et al., 1982; Grandin and Charbonneau, 1999; Knott et al., 2008), vesicular trafficking (Littleton and Bellen, 1995), secretory pathway (Malhotra and Emr, 2002), as a model to study neuroprotection at the cellular level (Scheper and Hoozemans, 2009). The method used in the laboratory of Susan Lindquist (Khurana and Lindquist, 2010) and other labs (Braun et al., 2010; Tenreiro et al., 2017) also helped to get better understanding of the mechanisms of α-synuclein toxicity. The 'humanized' yeast models for synucleinopathies recapitulate the fundamental properties of the pathology on

molecular and cellular level, identified in human diseases. This provides the rationale for engaging the powerful analytical instrument for various high throughput screening approaches in yeast. In yeast, genome-wide analyses can be performed, which enables the un-biased identification of genes and pathways critically modulating (either executing or preventing) the toxicity of α-synuclein (Lan et al., 2011; Tardiff et al., 2014; Khurana et al., 2017). In addition, comprehensive transcriptomic data may be collected from yeast cells expressing various levels of α-synuclein. Usually genetic screens identify response regulators, whereas transcriptomic profiling assays reveal components of metabolic processes. The ResponseNet computational method combines data from the genetic screens and gene expression analyses with the comprehensive knowledge on protein (and gene) interaction information in public yeast data bases (Yeger-Lotem et al., 2009). This generated a novel level of information on the functional α-synuclein gene/protein interaction network. The ResponseNet method allowed to reveal cellular pathways that responded to the toxicity of α-synuclein mapping both previously identified, and unrecognized pathways responding to α-synuclein toxicity. In the response to α-synuclein toxicity four *de novo* predictions identified by ResponseNet were validated, i.e., the nitrosative stress, the TOR pathway, the disruption of the sterol biosynthesis pathway and the mode-of-action of the genetic suppressor Gip2 (Yeger-Lotem et al., 2009).

Recently a new important method, TransposeNet, was developed allowing to combine genome-wide genetic screens in yeast with the comprehensive protein and gene interaction information available in yeast databases, using an improved computational method (Khurana et al., 2017). The method allows identification of protein–protein and protein–DNA interactions using a yeast interactome data. With this approach a functional protein/gene interaction network for α-synuclein has been described. An important step in this method is a transposition of α-synuclein interaction network into a human α-synuclein interaction network. This transposition is based on a significantly improved identification of human homologs of yeast genes, and on the information of the protein and gene interaction networks known from both the human system and from yeast. A very important result brought by the TransposeNet is that the mRNA translation subnetwork is relevant for α-synuclein toxicity in patient-derived neurons.

The pivotal role of mRNA translation for α-synuclein toxicity was further substantiated by a biochemical method enriching our knowledge about α-synuclein interactome (Chung et al., 2017). Here, the authors used APEX (from ascorbate peroxidase), a method based on the extremely short-lived radicals which covalently react and label amino acids in their immediate proximity (Martell et al., 2012). This method identifies even transient protein–protein interactions in living neurons by mass spectrometry. The method relies on fusing α-synuclein with APEX, which oxidizes phenol derivatives to phenol radicals (**Figure 1**).

Rat primary cortical neurons were transduced with α-synuclein fused to APEX2, a catalytically superior version of APEX. As a result, α-synuclein interacting proteins were identified by mass spectrometry. In initial screening the authors detected 225 proteins in living neurons which interacted with α-synuclein. Many of these interacting proteins were present in complexes with α-synuclein. This study in addition to anticipated interacting proteins involved in synaptic transmission and endocytosis, identifies several proteins implicated in mRNA metabolism (RNA binding, processing and translation factors). Among them are ten proteins with various functions in translation: EIF3C, EIF3D, CARS, EEF1B2, DARS, EIF3L, EEF2, RPS10, EEF1D and MARS (**Figure 2**).

Polyadenylate-binding protein 1 (PABPC1) – an mRNA binding protein which facilitates mRNA transport out of the nucleus, degradation, translation, and stability is also identified as α-synuclein interacting protein. The authors assume that α-synuclein physically associates with translation factors and sequesters them. This could occur in the synapse, where α-synuclein is localized, and the local mRNA translation plays a key role in the synaptic plasticity. This approach links many Parkinsonism and neurodegenerative disease risk factors to α-synuclein toxicity through specific molecular pathways. The most important of them are mRNA metabolism, translation, and vesicle trafficking. In this network, α-synuclein is associated with genetic modifiers related to mRNA translation, including initiation factors. In particular, it binds to translation initiation factor 4 gamma 1 (EIF4G1) and the poly(A)-binding protein (PAB or PABPC1). The latter one binds to the 3′ poly(A) tail of mRNA and is involved in poly(A) shortening and translation initiation. In this network α-synuclein is also associated with several ribosomal components. Overexpression of these genetic modifiers in the mRNA translation and mRNA processing pathways suppressed α-synuclein toxicity in bioscreen, while genetic experiments in various disease models revealed that their effects were specific. As a result, α-synuclein screens and network analysis identified a robust effect of α-synuclein toxicity on bulk mRNA translation in cellular models of synucleinopathy, which was not attributable to an ER stress response (Khurana et al., 2017). The link of α-synuclein with EIF4G1 presumably is involved in pathological alterations in PD and other neurodegenerative disease, since this translation initiation factor is linked to both PD (Chartier-Harlin et al., 2011) and Lewy body dementia (Fujioka et al., 2013). EIF4G1 functions as a scaffold in the eIF4F initiation complex, recruiting other components of translation machinery, i.e., ribosomes and tRNAs to the 5′ cap of mRNA (Sonenberg and Dever, 2003).

α-SYNUCLEIN REGULATES CELLULAR PROCESSES BY EPIGENETIC MECHANISMS

Interaction of α-Synuclein with Histones

The first evidence of α-synuclein interaction with histones was published almost 15 years ago (Goers et al., 2003). The initial data describing this interaction were obtained from the studies of neurotoxicity. After injections of the herbicide paraquat to mice α-synuclein was colocalized with histones in the nuclei of nigral

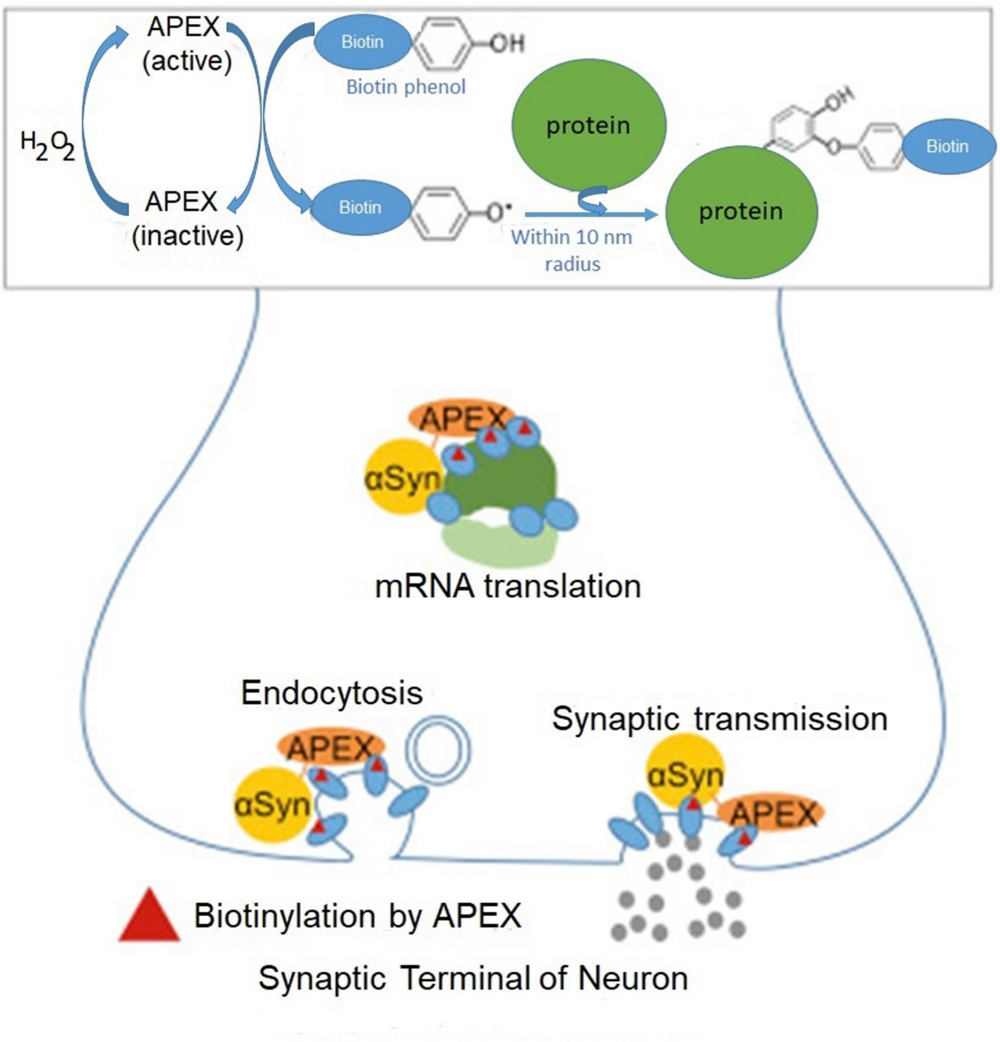

FIGURE 1 | Identification of α-synuclein-interacting proteins by peroxidase labeling technique coupled with mass spectrometry. A genetically engineered peroxidase APEX tagged to the C-terminal of wild-type α-synuclein defines the protein network in the vicinity (10 nm) of α-synuclein in living neurons. In addition to anticipated α-synuclein-interacting proteins involved in synaptic transmission and endocytosis, this method reveals several mRNA binding proteins implicated in mRNA translation. Adapted from Chung et al. (2017) with permission from the copyright holder.

neurons (Goers et al., 2003; Cherny et al., 2004). α-Synuclein formed a tight complex with histones with a molecular mass of 48,700 Da, with a stoichiometry of 2:1 (α-synuclein/histone), and a dissociation constant of about 1 μM. Hypothetically, histones enriched with arginine and lysine residues act as scaffolds bringing together molecules of α-synuclein with high content of acidic amino acids at C-terminal domain. The authors assume that translocation into the nucleus and binding of histones is one of the mechanisms underlying α-synuclein toxicity (Goers et al., 2003).

Physiological Consequences of α-Synuclein-Histone Interaction

Further studies demonstrate that interaction of α-synuclein with histones has an important physiological function. Binding of α-synuclein to histones decreases the histone H3 acetylation and reduces acetylation in HAT assays (Kontopoulos et al., 2006). Two α-synuclein mutations, A30P and A53T, that cause familial PD, display increased probability of localization in the nucleus. These data point to an importance of further research on histone deacetylase inhibitors as a potential target for the treatment of this disease.

Several lines of evidence show that interaction of α-synuclein with histones may also alter transcription of specific genes, representing epigenetic mechanism of gene expression regulation. α-Synuclein overexpression enhances mono- and dimethylation of histone H3K9, resulting in an increase in methylated form of this histone at the SNAP25 promoter, presumably disturbing SNARE complex assembly and fusion of synaptic vesicles (Sugeno et al., 2016). Histone modification

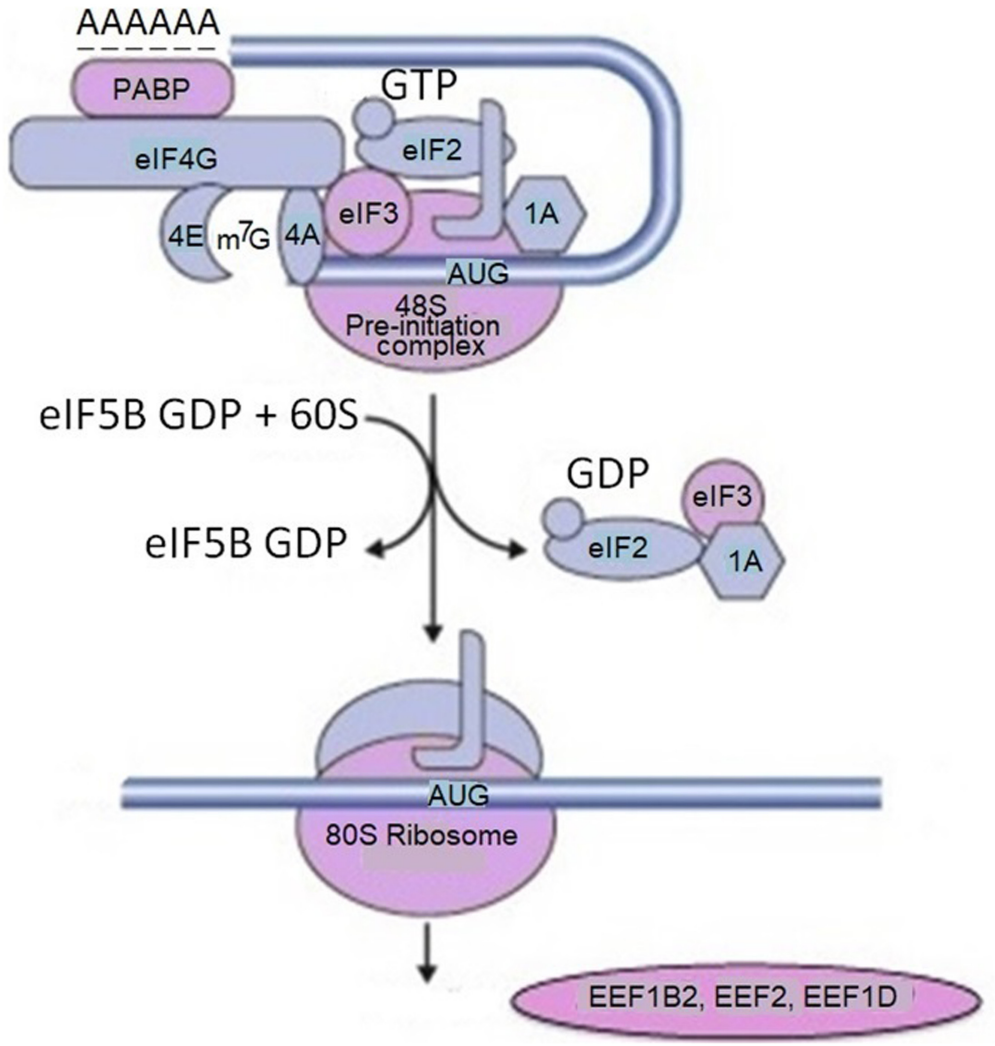

FIGURE 2 | Proteins involved in mRNA translation labeled by α-synuclein-APEX. mRNA translation-related proteins are tested for physical interaction with α-synuclein using the membrane-2-hybrid (MYTH) technique. For example, multiple subunits of translation initiation factor EIF3 are labeled with α-synuclein-APEX and stain positive for MYTH. From the list of mRNA translation hits in the APEX neuron study, five were available for testing by MYTH. Four of those, namely EIF3C, EIF3L, RPS12, and PABPC1 were confirmed. These data imply that mRNA translation machinery elements and binding proteins are in close proximal interactions with α-synuclein. Adapted from Chung et al. (2017) with permission from the copyright holder.

is an epigenetic mechanism which has a unique role in the cell, modulating the transactivation or repression of particular genes. Such mechanism may contribute to synaptic dysfunction occurring in PD (Sugeno et al., 2016).

Another group examined the role of histones in α-synuclein aggregation in cells experiencing apoptosis and in neuronal cells with nuclear membrane defects causing leakiness. The authors found that histones H1 and H3 released into the cytoplasm during apoptosis interact with α-synuclein to form cytoplasmic aggregates and play a role as a proaggregant factor. Importantly, their results demonstrate that histone-induced α-synuclein aggregates are transmissible to neurons both *in vitro* and *in vivo*. Histone-induced α-synuclein aggregates could spread to neurons and seed α-synuclein aggregation. Histone H1 less tightly associated with DNA is more important in the formation of pathological forms of α-synuclein. Histone-induced aggregates contain α-synuclein oligomers of variable size, including protofibrils and mature fibrils. The authors hypothesize that endogenous histones might facilitate internalization of α-synuclein aggregates and favor cell-to-cell propagation similar to the widely used transfection reagent lipofectamine (Jiang et al., 2017).

Importantly, binding of α-synuclein to histones not only affects histone modifications (**Figure 3**), but also it accelerates α-synuclein fibrillation.

Synuclein binding with histone H1 or the other core histones, causes the enhancement of α-synuclein fibrillation. α-Synuclein is able to reduce H3 acetylation, affecting the expression of several genes responsible for cell survival (Pavlou et al., 2017).

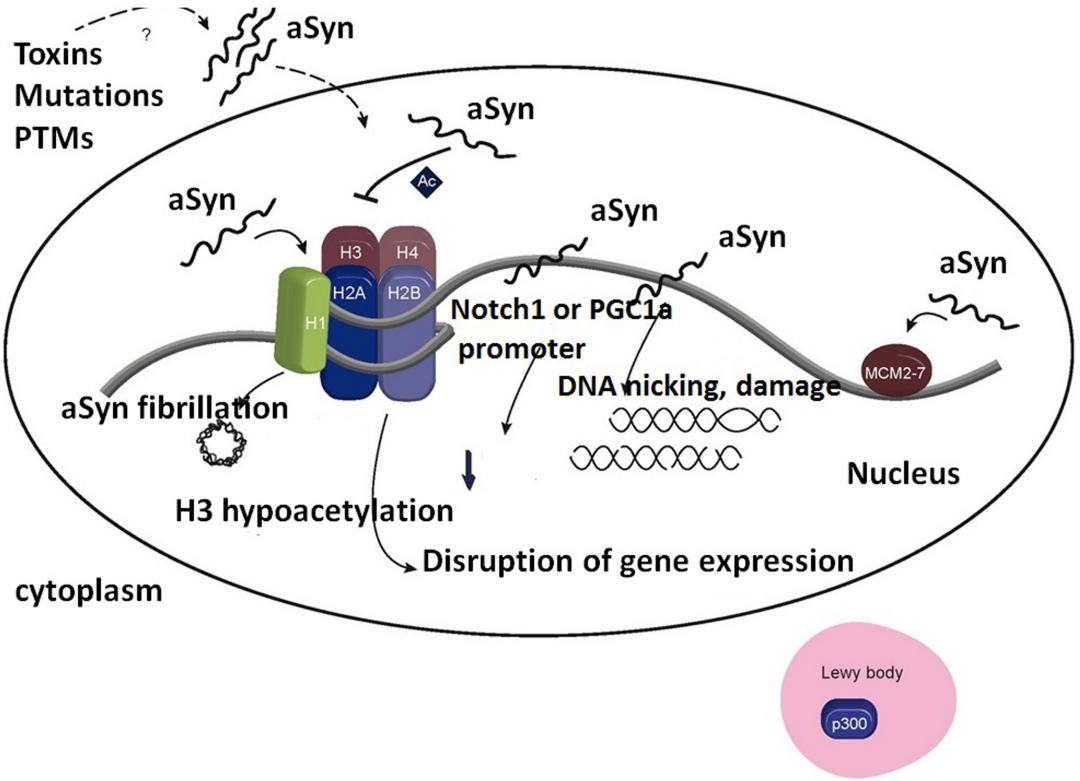

FIGURE 3 | Association of α-synuclein with histones in the cell nucleus induces formation of α-synuclein fibrils. Importantly, inhibition of H3 histone acetylation by α-synuclein affects the expression level of genes responsible for cell survival. Furthermore, association of α-synuclein with p300 decreases HAT activity. Binding of α-synuclein to DNA affects the expression of several genes, e.g., NOTCH1 and PGC-1A, and may lead to DNA damage. It allows the preservation of associated MCM2-7 complexes on the chromatin. Ac, acetylation; PTMs, post-translational modifications. Adapted from Pavlou et al. (2017) with permission from the copyright holder.

Effect of α-Synuclein on DNA Methylation

Another mechanism of α-synuclein participation in epigenetic regulation is realized via DNA methylation. α-Synuclein associates with Dnmt1 causing mislocalization and retention of Dnmt1 in the cytoplasm of neuronal cells (Desplats et al., 2011). As a result methylation in the regulatory regions of specific genes in PD and DLB brains is significantly reduced. The effect is observed both in post-mortem human brain samples and in brains of animal models of PD/DLB. Importantly, overexpression of another member of the synuclein family, β-synuclein does not cause cytoplasmic retention and Dnmt1 is not immunoprecipitated by anti-β-synuclein antibody, confirming the specificity of association between α-synuclein with Dnmt1. However, this effect could be reversed by lentivirus-mediated overexpression of Dnmt1. The finding of the inverse relation between α-synuclein oligomerization and Dnmt1 content in the nucleus suggests that the sequestration of Dnmt1 is increased by α-synuclein aggregation (Desplats et al., 2011). The loss of Dnmt1 protein from the cell nuclei is described in brains of patients with PD, DLB, and brains from transgenic mice that overexpress α-synuclein. In turn, these changes lead to the alterations in methylation pattern at the promoters of α-synuclein and several other genes, associated with neurodegenerative diseases (Desplats et al., 2011). A reduction in Dnmt1 causing mislocalization has been described in PD patient's brains. Another team (Jowaed et al., 2010) found that methylation in intron 1 decreases α-synuclein expression, whereas inhibition of DNA methylation upregulates expression. Furthermore, methylation of intron 1 in α-synuclein gene is reduced in DNA from sporadic PD patients' brain, including substantia nigra, putamen, and cortex. These results confirm epigenetic regulation of α-synuclein expression in PD (Jowaed et al., 2010). This data suggest another possible mechanism of α-synuclein interference with pathogenic processes of PD.

Epigenetic Mechanisms Regulating α-Synuclein Expression May Affect Its Regulatory Functions

As discussed in chapter 2, α-synuclein role in gene expression regulation depends on its intracellular concentration which in turn is regulated by epigenetic mechanisms controlling its levels. These mechanisms include methylation of α-synuclein promoter, post-translational modifications of histones, and epigenetic mechanisms based on non-coding RNAs (Pavlou et al., 2017).

Epigenetic mechanisms that control α-synuclein expression level may affect both its aggregation state and regulatory properties. Importantly, methylation level of CpG sites in α-synuclein gene in leukocytes correlates with the level in brain cells and therefore this analysis may be used as informative biomarker for prognosis of neurodegenerative diseases (Tan et al., 2014; Schmitt et al., 2015).

α-Synuclein and Chromatin Remodeling

The molecular interactions described above in Chapter 6, including binding of α-synuclein with histones, effect on DNA methylation, and epigenetic mechanisms could all induce chromatin remodeling.

Association of α-synuclein with histones may disturb nucleosome structure by reducing the availability of free histones for binding with DNA, or by affecting histone PTMs and leading to alterations in the pattern of gene transcription. In post-mortem PD brains the association of α-synuclein and chromatin is higher than in control samples. Furthermore, α-synuclein binds to the promoter of the master mitochondrial transcription activator, PGC1 alpha (PGC-1α), which is downregulated in PD brains (Siddiqui et al., 2012; Pavlou et al., 2017). The role of α-synuclein in chromatin remodeling is discussed in a recent review (Pavlou et al., 2017).

CONCLUSION

Here we discuss recent findings showing that in addition to their role in synapses, synucleins have non-canonical functions, and are involved in the regulation of essential cellular processes. In particular, the results presented here are consistent with a significant, although not completely understood role of synucleins in the regulation of gene expression. These mechanisms include both direct interaction of synucleins with DNA, transcription and translation factors, and less direct intervention, for example, via their effect on histone acetylation. Thus, synucleins may play a passive role of "ramblers in the crowd," but in response to stress or changing environmental conditions they become involved in the modulation of specific protein expression via mechanisms described in Chapters 2–4 and 6. Further research is required to unveil the details of these intimate mechanisms.

AUTHOR CONTRIBUTIONS

AAS wrote, and edited the manuscript; made substantial contributions to the conception of the work; made a final approval; and agreed to be accountable for all aspects of the work. AS wrote a part of the manuscript; made analysis and interpretation of data; made a final approval of the version to be published; and agreed to be accountable for all aspects of the work.

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frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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Silencing Alpha Synuclein in Mature Nigral Neurons Results in Rapid Neuroinflammation and Subsequent Toxicity

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Human studies and preclinical models of Parkinson's disease implicate the involvement of both the innate and adaptive immune systems in disease progression. Further, pro-inflammatory markers are highly enriched near neurons containing pathological forms of alpha synuclein (α-syn), and α-syn overexpression recapitulates neuroinflammatory changes in models of Parkinson's disease. These data suggest that α-syn may initiate a pathological inflammatory response, however the mechanism by which α-syn initiates neuroinflammation is poorly understood. Silencing endogenous α-syn results in a similar pattern of nigral degeneration observed following α-syn overexpression. Here we aimed to test the hypothesis that loss of α-syn function within nigrostriatal neurons results in neuronal dysfunction, which subsequently stimulates neuroinflammation. Adeno-associated virus (AAV) expressing an short hairpin RNA (shRNA) targeting endogenous α-syn was unilaterally injected into the substantia nigra pars compacta (SNc) of adult rats, after which nigrostriatal pathology and indices of neuroinflammation were examined at 7, 10, 14 and 21 days post-surgery. Removing endogenous α-syn from nigrostriatal neurons resulted in a rapid up-regulation of the major histocompatibility complex class 1 (MHC-1) within transduced nigral neurons. Nigral MHC-1 expression occurred prior to any overt cell death and coincided with the recruitment of reactive microglia and T-cells to affected neurons. Following the induction of neuroinflammation, α-syn knockdown resulted in a 50% loss of nigrostriatal neurons in the SNc and a corresponding loss of nigrostriatal terminals and dopamine (DA) concentrations within the striatum. Expression of a control shRNA did not elicit any pathological changes. Silencing α-syn within glutamatergic neurons of the cerebellum did not elicit inflammation or cell death, suggesting that toxicity initiated by α-syn silencing is specific to DA neurons. These data provide evidence that loss of α-syn function within nigrostriatal neurons initiates a neuronal-mediated neuroinflammatory cascade, involving both the innate and adaptive immune systems, which ultimately results in the death of affected neurons.

Keywords: alpha-synuclein, knockdown, major histocompatibility complex class 1 (MHC-1), microglia, neuroinflammation

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INTRODUCTION

A growing body of research suggests that inflammation, mediated by both the innate and adaptive immune systems, is a crucial event in the pathogenesis of Parkinson's disease (McGeer et al., 1988b; Croisier et al., 2005; Orr et al., 2005; Ouchi et al., 2005; Gerhard et al., 2006; Cebrián et al., 2014b). For example, increased numbers of microglia are present in virtually all brain regions affected by Parkinson's disease (Ouchi et al., 2005; Gerhard et al., 2006). Microglia within the Parkinson's disease brain express the major histocompatibility complex (MHC) class II and CD68, indicating that they are activated and phagocytic (McGeer et al., 1988b; Croisier et al., 2005). Parkinson's disease patients exhibit increased pro-inflammatory cytokines in the brain and periphery (Mogi et al., 1994a,b, 1996), and infiltration of peripheral leukocytes, such as T-cells, into the brain (McGeer et al., 1988a). Within the Parkinson's disease brain catecholamine neurons express major histocompatibility complex class 1 (MHC-1; McGeer et al., 1988b; Croisier et al., 2005; Orr et al., 2005; Ouchi et al., 2005; Gerhard et al., 2006; Cebrián et al., 2014b), and Lewy bodies and dopamine (DA) neurons label with immunoglobulin G (Orr et al., 2005; Ouchi et al., 2005; Gerhard et al., 2006). Finally, serum or immunoglobulin G isolated from Parkinson's disease patients can mediate selective destruction of DA neurons in models of Parkinson's disease (McGeer et al., 1988b; Defazio et al., 1994; Chen et al., 1998; Croisier et al., 2005).

Whether neuroinflammation is an active participant in neurodegeneration or merely a reactionary response to cell death remains contentious. Microglia are increased within disease-affected brain regions of early stage Parkinson's disease patients (Mogi et al., 1994a,b, 1996; Gerhard et al., 2006), and increased microglia remain constant over the disease duration (McGeer et al., 1988a; Croisier et al., 2005; Gerhard et al., 2006). These data suggest that an initial immunogenic signal triggers microglial activation and recruitment to disease affected brain regions early in Parkinson's disease, prior to cell death. Aberrations in the expression or function of the protein alpha synuclein (α-syn) may be one such immunogenic signal, capable of directly initiating an inflammatory cascade.

α-syn is a small protein that is expressed throughout many tissues in the body, but is highly enriched within neural tissue, where it primarily localizes to the presynaptic terminal (Maroteaux et al., 1988; Iwai et al., 1995). Missense mutations or multiplications of the α-syn gene cause familial Parkinson's disease (Polymeropoulos et al., 1997; Singleton et al., 2003), and aggregated α-syn protein is a primary component of Lewy bodies and Lewy neurites (Spillantini et al., 1997), the defining histopathological features of idiopathic Parkinson's disease. Further, perturbations in α-syn homeostasis can initiate an inflammatory response. α-syn depositions correlate, both spatially and temporally, with the presence of activated microglia (Croisier et al., 2005), and antibodies against α-syn have been isolated from the serum and cerebrospinal fluid of Parkinson's disease patients (Papachroni et al., 2007). Activated microglia are also found surrounding Lewy bodies in other synucleinopathies, suggesting that neuroinflammation is a common response to α-syn dysfunction (Streit and Xue, 2016). Supporting this idea, neuroinflammation is a common feature reported in virtually all α-syn-based animals models (reviewed in Magen and Chesselet, 2010; Sanchez-Guajardo et al., 2013). Within rodents, transgenic or virally mediated overexpression of α-syn, or injection of recombinant α-syn protein, cause microgliosis, pro-inflammatory cytokine production, and infiltration of peripheral T-cells within the brain (Theodore et al., 2008; Wilms et al., 2009; Sanchez-Guajardo et al., 2010, 2013; Barkholt et al., 2012; Watson et al., 2012; Fischer et al., 2016). Mimicking the human disease, microgliosis reported in α-syn based animal models occurs during neuronal dysfunction, but prior to cell death (Theodore et al., 2008; Sanchez-Guajardo et al., 2010), suggesting that the inflammatory response actively contributes to neurodegeneration.

An important question that remains unanswered is how perturbations in α-syn homeostasis initiate neuroinflammation. Currently, the prevailing hypothesis posits that α-syn is released from neurons (either actively or following cell death) after which it directly stimulates glia, thereby initiating the inflammatory process. Supporting this theory, recombinant α-syn or conditioned media from α-syn expressing cells stimulates a pro-inflammatory response in cultured microglia (Su et al., 2008; Lee et al., 2010; Kim et al., 2016). However, it appears that loss of normal α-syn function is also capable of eliciting a pro-inflammatory response without a direct interaction between α-syn and glia. For example, microglia isolated from α-syn germline knockout (KO) mice display increased reactivity, both basally and following stimulus, as well as increased cytokine secretion (Austin et al., 2006, 2011). Bidirectional crosstalk between neurons and glia is an integral component to both the normal homeostatic maintenance of healthy neurons and the phagocytic removal of damaged neurons (Sheridan and Murphy, 2013). Thus, we hypothesized that loss of *normal* α-syn function, resulting from aggregation or genetic silencing (Benskey et al., 2016a), leads to neuronal dysfunction and subsequent stimulation of microgliosis and inflammation. We aimed to test this hypothesis without the confounding variable of increased extracellular α-syn, which is able to directly stimulate microglia. Endogenous or overexpressed α-syn is secreted from neurons (Lee et al., 2005; Jang et al., 2010), while injection of recombinant α-syn protein directly exposes microglia to α-syn (Yu et al., 2010). Thus, to induce intra-neuronal α-syn dysfunction without directly increasing microglial exposure to α-syn, we chose to silence endogenous α-syn expression within mature nigrostriatal neurons, using adeno-associated virus (AAV) expressing a short hairpin RNA (shRNA) targeting endogenous rat α-syn.

Removal of α-syn from mature nigrostriatal neurons of adult rodents and non-human primates results in neurodegeneration that recapitulates many neuropathological aspects of Parkinson's disease (Gorbatyuk et al., 2010; Khodr et al., 2011, 2014; Benskey et al., 2016a; Collier et al., 2016). However, the time course of neuronal dysfunction and degeneration following α-syn

knockdown has never been thoroughly characterized. Thus, the purpose of this study was to: (1) establish a time line of nigrostriatal neuronal dysfunction and degeneration following removal of endogenous α-syn; and (2) examine the effects of intra-neuronal α-syn dysfunction on neuroinflammation.

MATERIALS AND METHODS

cDNA Clones and Virus Production

α-syn (GAAGGACCAGATGGGCAAG), myocardin (TGCA ACTGCAGAAGCAGAA), or scrambled (CACAAGATGAAGA GCACCA) siRNAs were designed using standard algorithms (Toro Cabrera and Mueller, 2016), and sequences were confirmed against available genomic data in order to ensure α-syn specificity. shRNAs were expressed by the H1 promoter (Benskey et al., 2014). All vectors also contained a green fluorescent protein (GFP) reporter gene under control of the hybrid chicken β-actin/cytomegalovirus promoter.

AAV was packaged through the co-transfection of human embryonic kidney 293T (HEK293T) cells with the AAV genome and an AAV5 helper plasmid. AAV was isolated from cellular lysates using an iodixanol step gradient and purified via column chromatography using a Q sepharose column (Amersham Biosciences). Virus titers were determined by dot blot and expressed in viral genomes/milliliter (vg/ml; Benskey et al., 2016b). Throughout the course of this study, several independent lots of the respective AAV vectors were created and used as needed. Results remained consistent between vector lots.

Lentivirus (LV) was packaged through the co-transfection of HEK293T cells with the respective LV genome, a LV packaging vector and a vesicular stomatitis virus glycoprotein psuedotyping plasmid. LV was concentrated from cellular media via ultracentrifugation, and resuspended in Dulbeccos modified eagles medium (Benskey and Manfredsson, 2016b). Viral titers were determined using the Lenti-X-qRT PCR kit (Clonetech).

Animals and Surgery

Experiments involving animals were conducted in accordance with the Michigan State University Institutional Animal Care and Use Committee (AUF 10/15-156-00). The protocol was approved by the Michigan State University Institutional Animal Care and Use Committee. Rat experiments were conducted using young adult (220 g) male Sprague-Dawley rats. Mouse experiments were conducted using young adult (30 g) male α-syn knockout (B6:129X1-SNCA^{tmRosl}/J; Abeliovich et al., 2000) or wild type C57Bl6/j mice. Rats were housed two per cage while mice were housed four per cage. All animals were maintained in a light-controlled (12 h light/dark cycle; lights on at 06:00 h) room, and provided food and water *ad libitum*.

Surgery was performed under 2% isoflurane anesthesia as previously described (Benskey and Manfredsson, 2016a). AAV expressing the α-syn or myocardin shRNAs were injected into the rat substantia nigra pars compacta (SNc) or cerebellum at a titer of 2.6×10^{12} vg/ml (Full) or 1.3×10^{12} vg/ml (Half) in the same volume. AAV expressing the α-syn or scrambled shRNAs were injected into the mouse SNc at a titer of 3×10^{13}vg/ml. All stereotaxic coordinates were calculated relative to Bregma. Unilateral 1.5 μl injections to the rat SNc: −5.4 mm anterior/posterior; +2.0 mm medial/lateral; −7.2 mm dorsal ventral (relative to dura). Unilateral 1.5 μl injections to the rat cerebellum: −11.3 mm anterior/posterior; +2.5 mm medial/lateral; −6.2 mm dorsal/ventral (relative to dura). Unilateral 1 μl injections to the mouse SNc: −3 mm anterior/posterior; +1.5 mm medial/lateral; −4.6 mm dorsal ventral (relative to dura). Injections were performed using an automated micropump (World Precision Instruments).

Cell Culture

Undifferentiated PC12 cells were maintained in RPMI-1640 medium supplemented with 10% horse serum, 2.5% fetal bovine serum and 1% penicillin/streptomycin. All cells were grown in a sterile incubator with 5% CO_2 at 37°C and subcultured every 2–3 days.

Tissue Collection and Processing

α-syn shRNA toxicity in the rat nigrostriatal system was analyzed at 7, 10, 14, and 21-days post-surgery. α-syn shRNA toxicity in the rat cerebellum or mouse SNc, was analyzed 1-month post-surgery. Animals were sacrificed by a lethal dose of pentobarbital and transcardially perfused with 0.9% saline. The striatum was dissected and immediately frozen in liquid nitrogen for protein and neurochemical analysis. For histological analysis, brains were post fixed in 4% paraformaldehyde, and coronal sections (40 μm) were cut through the midbrain or cerebellum using a microtome. To control for variations in inter-sample processing, the contralateral (uninjected) hemisphere is used as an internal control, and data is presented as percent of the contralateral hemisphere.

Histology

Free floating immunohistochemistry or immunofluorescence was performed by washing tissue in TBS containing 0.25% Triton-X 100 (TBS-Tx), blocking in 10% normal goat serum, and incubating in primary antibody over night at room temperature. Primary antibodies used: mouse anti-tyrosine hydroxylase (TH; Millipore MAB318), rabbit anti-TH (Millipore AB152), mouse anti-alpha-synuclein (BD Transduction Laboratories 610787) rabbit anti-calbindin (AbCam ab11426), mouse anti-HUc/d (Invitrogen A-21271), mouse anti-neuronal nuclear protein (NeuN; Millipore MAB377), rabbit anti-ionized calcium-biding adapter molecule 1 (IBA1; Wako 019-19741), rabbit anti-CD68 (Abcam ab31630), mouse anti-rat MHC-1 (Bio Rad MCA51GA), mouse anti-rat CD3 (Bio Rad MCA772), rabbit anti-cleaved caspase-3 (cell signaling 9661), and rabbit anti-vesicular monoamine transporter (VMAT; Abcam ab81855).

For immunohistochemical detection, tissue was washed in TBS-Tx and incubated in a biotin-conjugated goat anti-rabbit (Abcam ab6720), or a goat anti-mouse (Millipore AP124B) secondary antibody for 2 h at room temperature. Bound peroxidase was visualized with 0.05% 3-3′-diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide using an ABC Elite kit (Vector Laboratories). For immunfluorescent detection, tissue was washed in TBS-Tx and incubated with

alexafluor 488 (Invitrogen), alexafluor 594 (Invitrogen) or LiCor 680 (LiCor-Biosciences) secondary antibodies targeting the respective primary antibody host species.

Densitometry

Quantification of TH and VMAT protein levels in the striatum was made indirectly using an Odyssey near infrared scanner (Manfredsson et al., 2009; Benskey et al., 2016b). Briefly, striatal tissue was processed for immunodetection of TH and VMAT as described above, after which tissue was incubated in goat anti-mouse LiCor 680LT (925-68020) or goat anti-rabbit 800CW (925-32211) secondary antibodies. Tissue was scanned using the Odyssey infrared scanner using standardized scanning intensities optimized to avoid saturation. Image Studio software 5.2. (Li-COR Biosciences) was used to quantify signal intensity within the ipsilateral and contralateral striata. Signal was normalized to background (cortex), quantified as total intensity per unit area and expressed as the average signal from the ipsilateral hemisphere as a percent of the average signal from contralateral hemisphere.

Western Blot Analysis

As previously described (Benskey et al., 2013), PC12 cells were collected and homogenized in ice-cold lysis buffer (TBS containing 1% SDS, 1 mM DTT with Complete Mini Protease Inhibitor Cocktail Tablet (Roche)). Supernatants containing total cytosolic protein were assayed for protein content using the BCA assay. Protein (30 μg) was run on a 4%–20% polyacrylamide gel and transferred to a 0.2 μm nitrocellulose membrane. For detection of endogenous α-syn, membranes were then incubated in 0.4% paraformaldehyde for 30 min at room temperature. Membranes were blocked with 5% non-fat dry milk and incubated with mouse anti-alpha synuclein (BD Transduction Laboratories 610787), rabbit anti-beta synuclein (AbCam ab76111) and mouse anti-GAPDH (Cell Signaling D16H11) primary antibodies overnight at 4°C. Membranes were then incubated with goat anti-mouse LiCor 680LT (925-68020) or goat-anti mouse LiCor 800CW (925-32210). Membranes were visualized with the Odyssey infrared imager (Li-Cor Bioscience) and protein band density was quantified using Image Studio software 5.2.

Neurochemistry

Striatal tissue was homogenized in antioxidant solution and a portion of the homogenate was used for protein determination using the BCA assay (Manfredsson et al., 2007). The remaining homogenate was centrifuged at 14,000× *g* for 20 min and supernatants were used for high performance liquid chromatography to detect DA and 3,4-dihydroxyphenylacetic acid (DOPAC) as previously described (Koprich et al., 2003). Calculated concentrations of catecholamines were normalized to protein content to account for variations in sample size.

Unbiased Stereology and Manual Cell Counting

Unbiased stereological estimates were performed as previously described (Benskey et al., 2016b). Using Stereo-Investigator software (Version 4.03; Microbrightfield, Inc., Williston, VT, USA, 2000), sections were viewed on a screen at low magnification (4×) and the region of interest was delineated through the rostrocaudal extent of the respective nuclei. Every sixth section was sampled using the optical fractionator method. Counting of cells was performed using a 60× oil objective on an Olympus BX53 microscope equipped with a motorized stage. The coefficient of error for each estimate was calculated and was less than 0.1 (Gundersen, $m = 1$). The number of purkinje cells in lobule 3 of the cerebellar vermis (3Cb) was quantified by manually counting nissl+ cells located within the neuroanatomical limits of the purkinje cell layer (PCL), with a round morphology and somal diameter of least 16 μm. The large size of purkinje soma excludes other local cell types from the analysis. 16 μm was chosen based on an analysis of 50 calbindin+ purkinje cells. From these 50 cells, the average, minimum and maximum cross sectional somal diameter was 19.24 μm, 15.82 μm and 24.67 μm, respectively. Purkinje cells in the 3Cb were quantified within three adjacent sections at the level of the injection site (−11.3 mm anterior/posterior), and the mean number of purkinje cells was averaged over all sections within a single animal.

To quantify the number of MHC-1 positive neurons in the ventral midbrain, midbrain tissue was processed for immunofluorescent detection (as described above) of rat MHC-1. High magnification images of the SNc were obtained from four sections spanning the rostral-caudal axis of the ventral midbrain. The ImageJ cell counter plugin was used to manually count the number of MHC-1 expressing neurons from photomicrographs. To ensure quantification of MHC-1+ neurons, and exclude MHC-1+ microglia from the analysis, a priori criteria were set based on measurements obtained from 50 MHC-1+ cells that co-express the pan neuronal marker HUc/d (see **Figures 4Y–CC**). From these 50 cells, the average, minimum and maximum cross sectional somal diameter was 20.69 μm, 11.17 μm and 33.15 μm, respectively. Thus, inclusionary criteria stated that cells must: be MHC-1+, have a distinctive cell boundary, have a clearly discernable nucleus, have a somal diameter of at least 11 μm at its widest cross-sectional distance (this somal diameter excludes microglia which had an average cross-sectional somal diameter of 7.8 μm), and have no more than two processes to be included in the analysis. The mean number of MHC-1+ neurons was averaged over all sections within a single animal and presented as MHC-1+ neurons per ipsilateral SNc section.

Fluorescence Intensity Analysis

Tissue was processed for immunofluorescent detection (as described above) of TH and α-syn. Images were acquired using a Nikon A1 laser scanning confocal system with standardized laser and detection settings that were optimized to avoid saturation. Regions of interest were drawn around approximately 100–150 individual TH+ neurons, per hemisphere, per animal. a priori criteria were set such that all cells must be TH+, have a distinctive cell boundary, and a clearly discernable nucleus to be included in the analysis. To detect sub-regional differences in cellular TH, midbrain DA neurons were subdivided into the

medial ventral tegmental area, the lateral VTA, dorsal tier SNc, ventral tier SNc, and lateral SNc (see Supplementary Figure 5B). Outlined cells were treated as replicates that were averaged within a single animal (or within the respective midbrain subregion), and the mean fluorescence intensity was calculated for each hemisphere and expressed as the percent of the mean fluorescent intensity of the contralateral hemisphere (or the respective sub-region of the contralateral hemisphere).

To quantify the fluorescence intensity of MHC-1, tissue was processed for immunofluorescent detection (as described above) of rat MHC-1. High magnification images of the ventral midbrain were obtained from four sections spanning the rostral-caudal axis of the SNc, within the ipsilateral and contralateral hemispheres. Confocal images were acquired as described above. Nikon NIS elements software was used to perform automated-unbiased pixel fluorescence intensity analysis. The mean background fluorescence intensity in the 594 channel was measured in a subset of animals representing each experimental group to be analyzed. This mean background fluorescence intensity value was set as the lower threshold for automated fluorescence intensity analysis and was applied to all samples analyzed. The automated measurement tool in NIS elements was used to quantify the mean fluorescence intensity for all pixels above the preset, lower threshold. The mean MHC-1 fluorescence intensity was averaged over all sections within animal, and expressed as a percent of the mean fluorescence intensity of the contralateral hemisphere.

RNAscope *in Situ* Hybridization

RNAscope *in situ* hybridization for the promoter within the AAV genome was combined with immunohistochemical detection of IBA1+ microglia. Forty micrometer thick tissue was processed for RNAscope detection of the promoter within the viral genome using custom VS probes according to the manufacturer's instructions (Advanced Cell Diagnostics, Hayward, CA, USA) and previously published methods (Polinski et al., 2016). RNAscope was developed with 3-3′-diaminobenzidine tetrahydrochloride, after which tissue was counterstained for IBA1 using the immunohistochemical procedures detailed above, with the exception that the Vector SG reagent (Vector Laboratories) was used as the chromagen (Polinski et al., 2016).

Statistical Analysis

Power analyses were conducted to determine optimal sample size required to detect a statistical difference at $p < 0.05$ with a power of 0.8. *A priori* exclusion criteria stated that animals displaying a complete absence of transduction (no GFP reporter gene expression) were excluded from analysis. The experimenter was blind to all experimental conditions during data collection and analysis. One-way analysis of variance (ANOVA) tests were used to detect statistical significance between two or more groups containing a single independent variable. Repeated measure two-way ANOVAs were used to detect statistical significance between two or more groups when there were two independent variables in the experiment. A repeated measures ANOVA was used to detect subregional differences within subjects. A p value of less than or equal to 0.05 was considered statistically significant. If the ANOVA revealed an interaction of statistical significance Tukey's test was used for multiple comparisons among groups.

Data Availability

The raw data supporting the author's conclusions will be made available by the authors, without undue reservation, to any qualified researcher.

RESULTS

Specificity of α-syn shRNA-Induced Nigrostriatal Toxicity

We first sought to unequivocally confirm that nigrostriatal cell loss observed following α-syn knockdown was not due to non-specific, or off-target, RNA interference (RNAi) toxicity. The synuclein family consists of α, β and γ synuclein, three proteins which share sequence homology in the amino terminus. γ-synuclein is expressed in the brain and periphery but is the least conserved of the synucleins (George, 2001); however, α- and β-synuclein (β-syn) are largely co-expressed in the central nervous system and they appear to be functionally redundant (Chandra et al., 2004; Greten-Harrison et al., 2010). Although our α-syn shRNA specifically targets a position of the α-syn transcript that does not share sequence homology with either β- or γ-synuclein (Supplementary Figure 1A), we aimed to ensure that the α-syn shRNA did not affect expression of β-syn, as this could mask the true effects of α-syn silencing (as has been observed with several α-syn germline knockout mice (Abeliovich et al., 2000; Chandra et al., 2004; Greten-Harrison et al., 2010)). PC12 cells were transduced with LV expressing either the α-syn shRNA or a scrambled control shRNA, and α- and β-syn protein levels were quantified 5 days post-transduction. The α-syn shRNA significantly reduced α-syn protein (t-test; $t_{(10)} = 2.37$, $p = 0.039$), however there was no change in β-syn protein (Supplementary Figures 1B,C).

Next we examined the consequences of α-syn shRNA administration in α-syn germline KO and WT mice. α-syn KO or WT mice received unilateral SNc injections of AAV expressing the α-syn shRNA or a scrambled control shRNA and TH positive (TH+) nigral neuron numbers were quantified 1-month later. Expression of the α-syn shRNA significantly decreased TH+ nigral neurons in WT mice (∼45% reduction; Supplementary Figures 1D,E; ANOVA: $F_{(3,24)} = 4.465$, $p < 0.05$), but had no effect on TH+ neurons in α-syn KO mice (Supplementary Figures 1D,F). These data provide further evidence that loss of endogenous α-syn is toxic to nigrostriatal neurons, and combined with previously published controls (Gorbatyuk et al., 2010), exclude the possibility that nigrostriatal cell loss following α-syn shRNA administration is due to non-specific RNAi-induced toxicity or off-target activity.

Time Course of Nigrostriatal Pathology Following α-syn Knockdown

Two separate titers of AAV 2/5 expressing an shRNA targeting endogenous rat α-syn were unilaterally injected into the SNc

of adult rats, and nigrostriatal pathology was examined at 7, 10, 14 and 21 days post-surgery. We observed successful transduction of TH+ cells in the SNc at all time points examined (indicated by the GFP transduction marker), however, GFP transgene expression increased over the time course (Supplementary Figure 2 shows GFP in myocardin shRNA treated animals). In animals receiving the α-syn shRNA, expression of the GFP reporter gene seemed to decrease over time (data not shown), likely the result of ongoing pathology within α-syn shRNA expressing neurons.

The level of α-syn knockdown was analyzed using confocal microscopy to quantify the intensity of α-syn fluorescence within TH+ nigrostriatal soma (Supplementary Figure 3). Prior to this analysis, WT and α-syn KO brain were stained using the α-syn antibody (clone 42/α-synuclein) to confirm the specificity of the antibody. No signal was observed in α-syn KO brains (Supplementary Figures 3A,B). Further, immunoblotting of recombinant monomeric α-syn protein or striatal lysates from WT mice, α-syn KO mice or rat brain yielded a single band resolving at correct molecular weight (Supplementary Figure 3C).

There was no change in the fluorescence intensity of α-syn at any time point following injections of either the FULL or HALF titer AAV-myocardin-shRNA, thus solely 21-days post myocardin shRNA is shown (Supplementary Figures 3D–G,X–AA,RR). The α-syn shRNA (FULL) significantly reduced α-syn immunoreactivity by 44%, 48%, 50% and 57% as compared to the contralateral hemisphere at 7, 10, 14 and 21 days post-injection, respectively (Supplementary Figure 3RR; ANOVA: $F_{(7,18)} = 16.865$, $p < 0.0001$). The α-syn shRNA (HALF), significantly reduced α-syn immunoreactivity by 24%, 25%, 40% and 47% as compared to the contralateral hemisphere at 7, 10, 14, and 21 days-injection, respectively (Supplementary Figure 3RR; ANOVA: $F_{(7,20)} = 8.092$, $p < 0.0001$).

Next the time course of TH+ cell loss in the SNc following α-syn knockdown was examined (**Figures 1A–Q**). There was no change in TH+ neuron numbers at any time following expression the myocardin shRNAs. The α-syn shRNA significantly decreased TH+ cell numbers within the injected SNc (ANOVA: main effect of treatment $F_{(3,82)} = 12.36$, $p < 0.0001$), and this cell loss progressed over time (ANOVA: main effect of time $F_{(3,82)} = 4.253$, $p < 0.007$). The α-syn shRNA (Half) significantly decreased TH+ neurons in the injected SNc at 21 days post-injection (∼25% loss; **Figures 1A,Q**). The α-syn shRNA (Full) produced a progressive loss of TH+ cells in the SNc beginning at 14 days (∼30% loss; **Figures 1A,K**) and continuing to 21 days post-injection (∼50% loss; **Figures 1A,O**). Further, administration of α-syn shRNA (Full) resulted in a more pronounced loss of TH+ nigrostriatal neurons as compared to the α-syn shRNA (Half) at the 21-day time point,

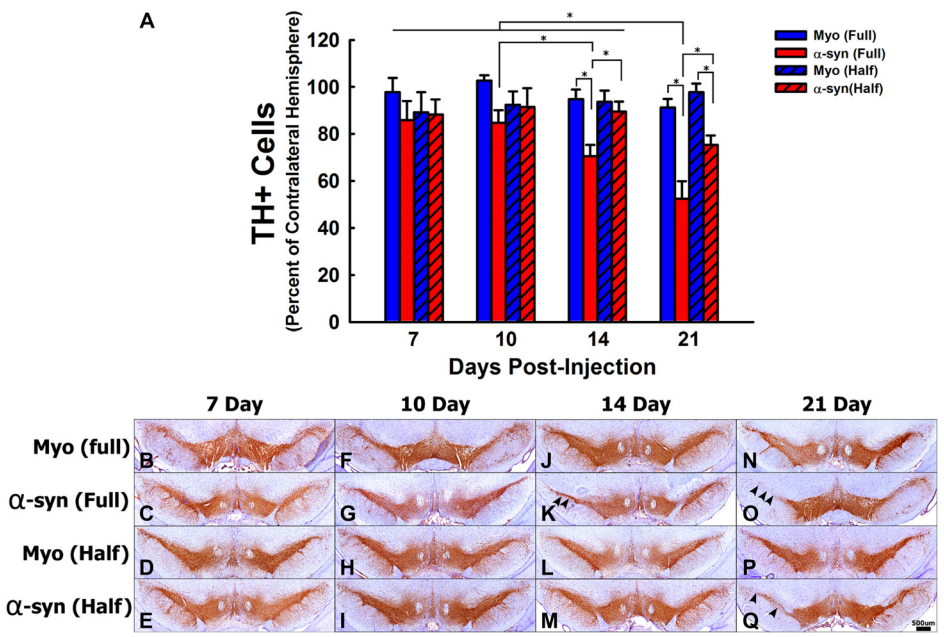

FIGURE 1 | The time course and concentration response of tyrosine hydroxylase positive (TH+) cell loss following α-syn knockdown within nigrostriatal neurons. Rats received unilateral substantia nigra pars compacta (SNc) injections of AAV2/5 expressing an α-syn short hairpin RNA (shRNA) or a myocardin (Myo) control shRNA. AAV2/5 expressing the respective shRNAs was injected at a titer of 2.6×10^{12} vg/ml (Full) or 1.3×10^{12} vg/ml (Half). Rats were then sacrificed at 7, 10, 14, and 21 days post-surgery, and the number of TH+ neurons in the SNc was estimated using unbiased stereology. Columns in panel **(A)** represent mean number of TH+ cells in the injected SNc, expressed as the percent of TH+ cells in the contralateral SNc, +1 SEM (n = 6–7/group), in animals receiving Myo Full shRNA (solid blue columns), α-syn Full shRNA (solid red columns), Myo Half shRNA (hatched blue columns), or α-syn Half shRNA (hatched red columns). *Significantly different (ANOVA: $p < 0.05$). Representative images of TH positive neurons of the SNc for all shRNA concentrations and time points are shown panels **(B–Q)**. Arrowheads indicate areas in which TH+ cell loss was observed. Scale bar in **(Q)** represents 500 μm and applies to all other panels.

demonstrating a time and concentration dependent loss of TH+ neurons following knockdown of endogenous α-syn. To determine if TH+ cell loss reflects actual nigral cell loss or merely a phenotypic loss of TH expression, we quantified total neurons within the SNc using the pan-neuronal marker HUc (Supplementary Figure 4). The total number of HUc+ neurons within the ipsilateral SNc was significantly reduced by ∼31% and ∼36% 21-days following delivery of α-syn shRNA (Half) and α-syn shRNA (Full), respectively (ANOVA: $F_{(4,24)} = 3.61$, $p < 0.01$). There was also a non-significant reduction in HUc+ neurons 14 days following delivery of the α-syn shRNA (Full; ∼27%).

Manipulation of α-syn alters TH expression (Baptista et al., 2003; Yu et al., 2004; Alerte et al., 2008), thus, we also analyzed TH fluorescence intensity within individual nigral neurons to probe for changes in TH expression (Supplementary Figures 5A–C). In an effort to detect any sub-regional differences in cellular TH intensity, we subdivided midbrain DA neurons into the medial ventral tegmental area (VTA-Medial), the lateral VTA (VTA-Lateral), the dorsal tier SNc (SNc-Dorsal), the ventral tier SNc (SNc-Ventral), and the lateral SNc (SNc-Lateral; Supplementary Figures 5A,B). There was no change in TH fluorescence intensity among the myocardin shRNA treated animals (data not shown), thus, data from these animals is presented as a single time point and treated as time zero after α-syn shRNA administration (Supplementary Figure 5C). There was no change in TH fluorescence intensity within VTA neurons in any groups analyzed. However, α-syn shRNA expression significantly decreased TH fluorescence intensity within nigral neurons over time (ANOVA: $F_{(4,16)} = 14.675$, $p < 0.0001$). Further, there was a significant interaction between time after α-syn shRNA treatment and the midbrain dopaminergic sub-region, in which TH fluorescence intensity in the ventral tier of the SNc was significantly lower than the VTA as well as the other sub-regions of the injected SNc at the 21-day time point (Supplementary Figure 5C; ANOVA: $F_{(16,40)} = 2.675$, $p < 0.01$).

We next analyzed nigrostriatal axon terminal integrity by quantifying levels of striatal TH (**Figures 2A–Q**) and VMAT (**Figures 2R–HH**) protein, and striatal concentrations of DA and DOPAC (**Figures 2II–KK**). The α-syn shRNA (Full) and α-syn shRNA (Half) significantly decreased TH+ fibers within the striatum 21-days post-injection (**Figures 2A,I,Q**; ANOVA: main of effect of time $F_{(3,82)} = 15.01$, $p < 0.0001$; interaction between shRNA treatment and time $F_{(9,82)} = 3.467$, $p < 0.001$). In contrast, only the α-syn shRNA (Full) decreased VMAT+ nigrostriatal fibers 21-day post-injection (**Figures 2R,Z**; ANOVA main effect of α-syn shRNA treatment $F_{(3,82)} = 3.214$, $p < 0.02$; main effect of time $F_{(3,83)} = 3.695$, $p < 0.01$).

Striatal DA concentrations were significantly reduced 21-days following injection the α-syn shRNA (Full) and α-syn shRNA (Half; **Figure 2II**; ANOVA: main effect of treatment $F_{(3,94)} = 2.716$, $p < 0.04$; main effect of time $F_{(3,94)} = 4.957$, $p < 0.003$; interaction effect between treatment and time $F_{(9,94)} = 2.814$, $p < 0.005$). There was no change in striatal DOPAC concentrations regardless of treatment or time point (**Figure 2JJ**). However, there was a significant increase in the DOPAC to DA ratio at 21 days following injection of the α-syn shRNA (Full) and α-syn shRNA (Half; **Figure 2KK**; ANOVA main effect of treatment $F_{(3,94)} = 3.091$, $p < 0.03$; main effect of time $F_{(3,94)} = 3.463$, $p < 0.01$).

Neuroinflammatory Response Initiated by α-syn Knockdown in Nigrostriatal Neurons

After characterizing the time line of pathology, we sought to determine if α-syn knockdown within nigral neurons elicits an inflammatory response. Here we focused on the α-syn shRNA (FULL) as this titer resulted in demonstrable neuronal dysfunction throughout the time course. In all rats we observed a moderate, and roughly equal, amount of gliosis along the needle track and the injection site at the 7-day time point (**Figures 3C,G**). This gliosis is an artifact of surgery, thus the 7-day time point was not included in additional analyses. The α-syn shRNA (Full) significantly increased the number of IBA1+ microglia within the injected ventral midbrain, beginning at 10 and 14 days post-injection, and increasing further at 21 days post-injection (ANOVA: main effect of treatment $F_{(5,30)} = 9.098$, $p < 0.0001$; **Figures 3A,D–F**). In addition, by 21 days post-injection the number of IBA1+ microglia was significantly increased in the substantia nigra pars reticulata (SNr; **Figures 3B,F**; ANOVA: main effect of treatment $F_{(5,31)} = 2.608$, $p < 0.0442$). Beyond microglia lining the needle track and injection site at early time points, microglial numbers did not change following injection of the myocardin shRNA (**Figures 3A,B,G–J**). Interestingly, at several time points post-α-syn shRNA administration the abundance of IBA1+ microglia appeared, from a purely qualitative point of view, to be increased far beyond the increase in the number of IBA+ microglia that were objectively quantified (e.g., **Figure 3B** vs. **Figures 3F,J**). This appears to result from an increase in the expression of IBA1 per cell, which exceeds the increase in actual IBA1+ cell numbers. Beginning at the 10-day time point, microglia in the α-syn shRNA injected SNc were reactive and phagocytic as indicated by increased CD68 immunoreactivity (**Figure 3K**). CD68+ microglia were in close apposition to GFP+ transduced neurons, and appeared to be extending processes to surround and engulf transduced neurons, indicative of neuronophagia of living neurons (arrowheads in **Figures 3L–N**). Further at 21 days post-α-syn-shRNA injection, we observed numerous ameboid CD68+ cells throughout the ventral midbrain (**Figures 3S–U**). The ameboid cells were in close apposition to IBA1+ microglia but displayed little to no IBA1 expression themselves (**Figures 3V,W**), indicating that they may be either ameboid microglia or infiltrating mononuclear cells. By comparison the microglia within the brains of myocardin shRNA treated animals displayed very low CD68 immunoreactivity (**Figures 3O–R**).

Mesencephalic DA neurons increase MHC-1 expression in the parkinsonian brain, and MHC-1 expression in cultured neurons results in the recruitment of immune cells and subsequent death of the affected neuron (Cebrián et al., 2014b). Thus, we next sought to determine if α-syn knockdown induces MHC-1 expression in nigral neurons. We observed either very

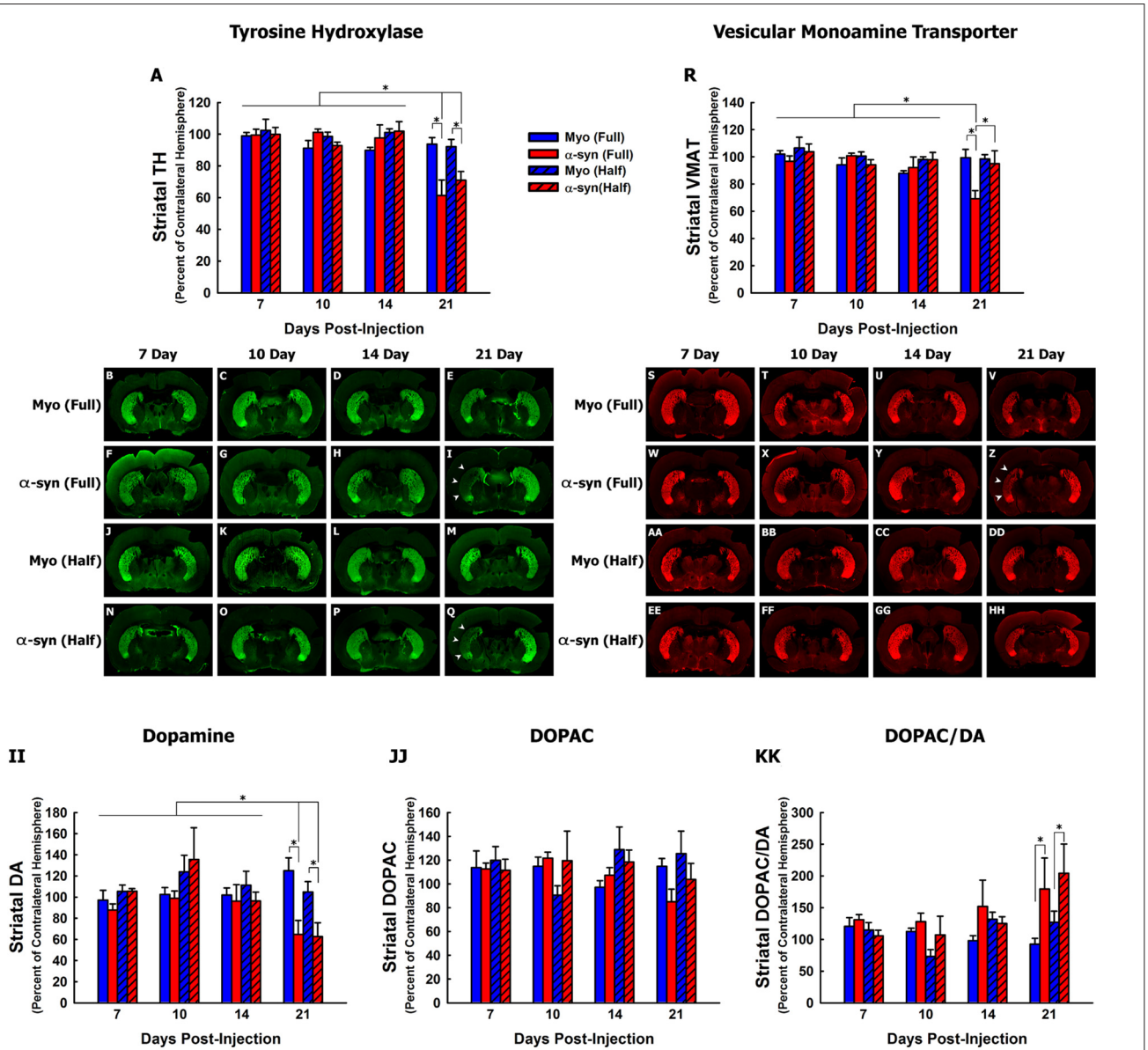

FIGURE 2 | Time course and concentration response of nigrostriatal axon terminal degeneration following α-syn knockdown. Rats received unilateral injections of AAV2/5 expressing the α-syn shRNA or a myocardin control shRNA. AAV2/5 expressing the respective shRNAs was injected at a titer of 2.6×10^{12} vg/ml (Full) or 1.3×10^{12} vg/ml (Half). Rats were then sacrificed at 7, 10, 14 and 21 days post-surgery, and striatal TH, VMAT, dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were quantified. Columns represent the mean total signal intensity of striatal TH **(A)** or VMAT **(R)**, or the mean concentration of striatal DA **(II)**, DOPC **(JJ)** or the DOPAC to DA ratio **(KK)**, expressed as the percent of the contralateral striatum, +1 SEM (n = 5–8/group), in animals receiving Myo Full shRNA (solid blue columns), α-syn Full shRNA (solid red columns), Myo Half shRNA (hatched blue columns), or α-syn Half shRNA (hatched red columns). *Significantly different (ANOVA, $p < 0.05$). Representative images of TH+ **(B–Q)** and VMAT+ **(S–HH)** striata are shown. Arrowheads in panels **(I,Q,Z)** indicate areas in which nigrostriatal terminal loss was observed.

weak, or a complete absence of MHC-1 immunoreactivity in the SNc of animals injected with the myocardin shRNA (**Figures 4A–D,I–L,Q–T**). In contrast, knockdown of α-syn increased MHC-1 expression within the SNc. Most of the cells expressing MHC-1 between 10 days (**Figures 4E–H**) and 14 days (**Figures 4M–P**) post-α-syn shRNA injection did not express TH, but resembled nigral neurons in terms of their anatomical location, size, and morphology. At the 10 and 14-day time points, all MHC-1+ neurons were almost exclusively found within the ventral tier of the SNc (**Figures 4E,M**), which is the location where we observed the most pronounced cell loss at the 21 day time point. At the 21-day time point MHC-1 immunoreactivity was no longer confined to the SNc but was observed throughout the ventral midbrain of the injected hemisphere. Further, at 21 days post-α-syn shRNA, MHC-1+ cells no longer presented a neuronal morphology,

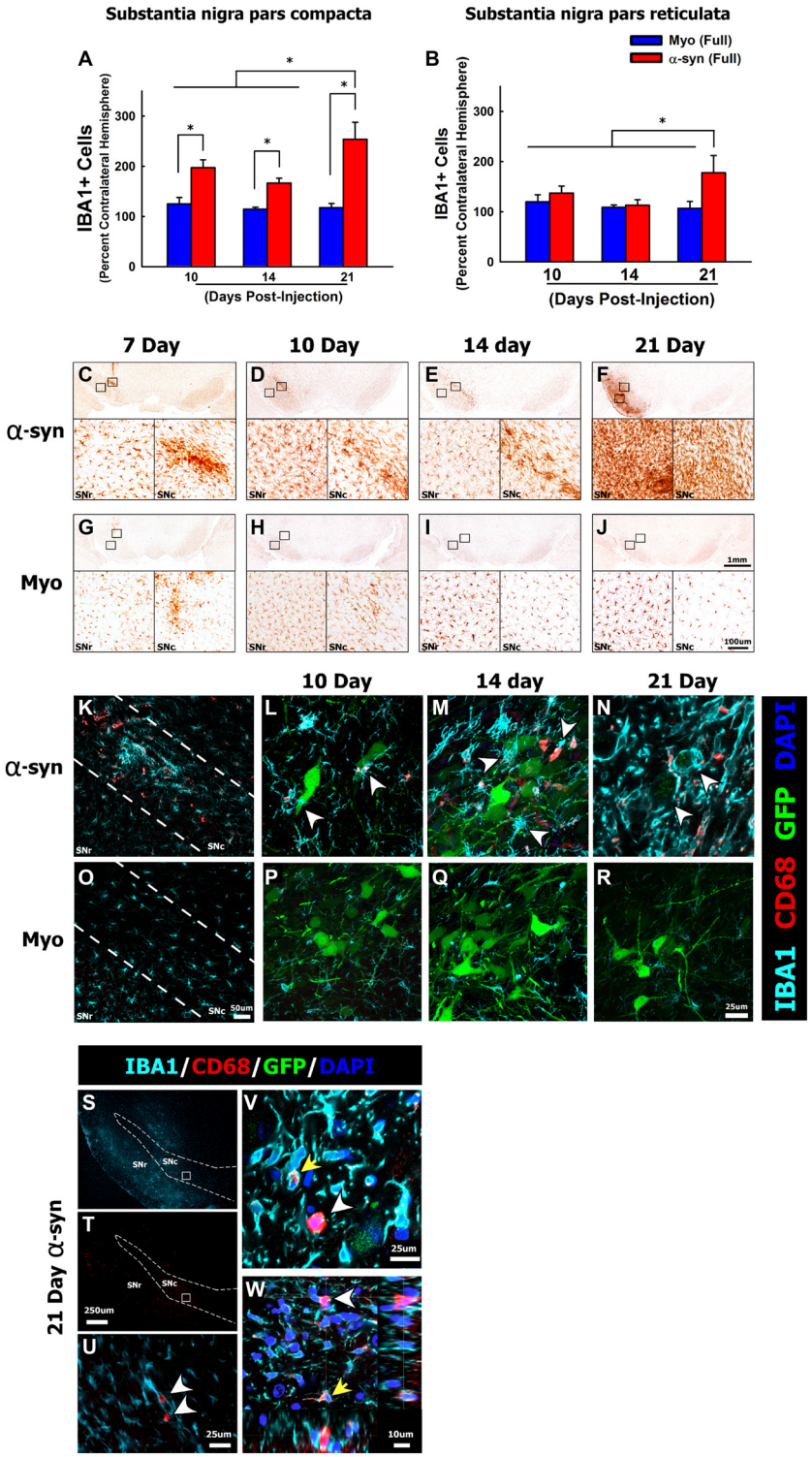

FIGURE 3 | α-syn knockdown increases reactive microglia in the SNc. Rats received a single injection of AAV2/5 (2.6×10^{12} vg/ml; Full) expressing a target α-syn shRNA or a myocardin control shRNA. Rats were then sacrificed at 7, 10, 14 and 21 days post-surgery, the number of IBA1+ microglia were quantified using unbiased stereology. Columns in panel **(A,B)** represent mean number of IBA1+ microglia in the ipsilateral SNc and substantia nigra pars reticulata (SNr), respectively, expressed as the percent of IBA1+ microglia in the contralateral SNc and SNr, +1SEM (n = 5–8/group), in animals receiving Myo Full shRNA (solid blue columns), α-syn Full shRNA (solid red columns). Representative images show low and high magnification images of IBA1+ microglia within the SNc and SNr in animals treated with the α-syn shRNA **(C–F)** or myocardin shRNA **(G–J)**. Panel **(K)** depicts CD68+ (red), IBA1+ (teal) microglia within the anatomical boundaries of the SNc 10-days

(*Continued*)

FIGURE 3 | Continued
following injection of the α-syn shRNA. Arrowheads in panels **(L–N)** depict CD68+ (red), IBA1+ (teal) reactive microglia surrounding α-syn shRNA transduced neurons (indicated by green fluorescent protein (GFP) reporter; green) at 10- **(L)**, 14- **(M)**, and 21-days **(N)** post-injection. Panels **(O–R)** depicts IBA1+ microglia that are largely devoid of CD68 immunoreactivity within the anatomical boundaries of the SNc at 10 **(O,P)**, 14 **(Q)** and 21 days **(R)** following injection of α-myocardin shRNA. Numerous round CD68+ cells, which expressed little to no IBA1, were found throughout the entire ventral midbrain 21-days post-α-syn-shRNA expression **(S,T)**. Panel **(U)** corresponds to area within the box in panels **(S,T)**. The yellow arrow in panels **(V,W)** depicts the normal punctate CD68 immunoreactivity that was observed within IBA1+ microglia. The white arrowheads in panels **(V,W)** depict ameboid cells expressing high levels of CD68 with little to no colocalization with IBA1. *Significantly different (ANOVA: $P < 0.05$). The scale bars in panel **(J)** and the inset in panel **(J)** represents 1 mm and 100 μm respectively, and apply to the corresponding panels and insets in **(C–I)**. The scale bar in panel **(O)** represents 50 μm and applies to panel **(K)**. The scale bar in panel **(R)** represents 25 μm and apply to panels **(L,M,P,Q)**. Scale bar in panel **(T)** represents 250 μm and applies to panel **(S)**. Scale bar in panels **(U,V)** represent 25 μm. Scale bar in Panel **(W)** represents 10 μm.

instead MHC-1 staining appeared diffuse or found on cells morphologically resembling microglia throughout the ventral midbrain (**Figures 4U–X**).

To confirm that MHC-1 expression was occurring on nigral neurons in the SNc, we analyzed colocalization of MHC-1, TH, and the pan-neuronal marker HUc (**Figures 4Y–JJ**). At the 10-day time point MHC-1 was expressed on HUc+ neurons that were TH- (**Figures 4Y–CC**). At the 14-day time point MHC-1 was expressed on HUc+ neurons (data not shown), in addition to neurons with limited TH expression (arrowheads in **Figures 4DD–FF**). Finally, at the 21-day time point, many of the remaining TH+ neurons in the injected SNc demonstrated cell surface MHC-1 expression (**Figures 4GG–JJ**).

Microglia also express MHC-1, and many MHC-1+ cells with distinct microglial morphology were in the α-syn shRNA injected SNc 21 days post-injection (yellow arrows **Figures 4GG–II**). As such, we wanted to confirm that MHC-1 expression was not solely an artifact of microglia surrounding transduced neurons. Ten days post α-syn shRNA injection, GFP+ transduced neurons displayed robust cell surface MHC-1 expression (**Figures 4KK–OO**). This MHC-1 expression distinctly surrounded the soma of GFP+ neurons, and although there were also IBA1+ microglia surrounding the same neurons, the IBA1 and MHC-1 signals rarely co-localized (**Figure 4OO**). At 14 days post α-syn shRNA injection, neuronal MHC-1 expression was more robust, filling the soma in addition to cell surface expression (**Figure 4PP**). Further, at the 14-day time point MHC-1+ neurons were often completely surrounded by IBA1+ microglia (**Figures 4PP–TT**), again indicative of neuronophagia.

We next quantified the number of MHC-1 expressing neurons within the ventral midbrain between 10 days and 21 days post-injection. MHC-1+ neurons were extremely rare or not present in the contralateral hemisphere, thus the contralateral hemisphere could not be used as an internal control in the analysis. Within the ipsilateral hemisphere, there was a significant increase in MHC-1+ neurons at 10, 14 and 21 days following injection of the α-syn shRNA (**Figure 4UU**; ANOVA: $F_{(5,18)} = 11.754$, $p < 0.0001$).

We also quantified MHC-1 fluorescence intensity within the ventral midbrain to detect changes in MHC-1 expression in all cell types, including microglia (**Figure 4VV**). The α-syn shRNA progressively increased MHC-1 fluorescence intensity from 10-days post-injection to peak at 21-days post-injection (**Figure 4VV**; ANOVA: $F_{(5,18)} = 48.536$, $P < 0.0001$). These collective data show that α-syn knockdown in nigral neurons induces MHC-1 expression in transduced neurons, prior to any neuronal loss, which coincides with the recruitment of reactive microglial to MHC-1 expressing neurons.

Neuronal MHC-1 expression is associated with the recruitment of T-cells in the parkinsonian brain (Cebrián et al., 2014b), thus we next investigated whether α-syn knockdown caused infiltration of T-cells to the brain. We observed a number of CD3 + T-cells within the injected SNc, solely at 21-days post-α-syn shRNA administration, many of which were in close apposition to TH+ neurons (**Figures 5A–D**). In contrast we could not detect any CD3+ T-cells in the brains of animals receiving the myocardin shRNA (**Figures 5E–H**), or at other time points following α-syn knockdown (data not shown).

Neuronophagia activates programmed cell death within affected cells. Thus we next analyzed the SNc for levels of cleaved caspase-3, a marker of apoptosis (Supplementary Figure 6). Within the myocardin shRNA treated animals there was little to no cleaved caspase-3 immunoreactivity (Supplementary Figures 6A–C,G–I). There was sparse cleaved caspase-3 immunoreactivity within a limited number of cells 14-days following α-syn shRNA expression (Supplementary Figures 6D–F), however, by the 21-day time point, punctate cleaved caspase-3 immunoreactivity within the cytosol and nucleus of cells was prevalent. Most cells displaying strong cleaved caspase-3 immunoreactivity morphologically resembled neurons, and were TH-, though there was some sparse cleaved caspase-3 immunoreactivity in TH+ neurons as well (Supplementary Figures 6J–L).

Microglial from α-syn KO mice display a reactive phenotype (Austin et al., 2006, 2011). Thus, it is possible that loss of endogenous α-syn within microglia of the SNc (following transduction with AAV expressing the α-syn shRNA) results in increased cytokine secretion, which induces nigral neurons to express MHC-1 (Cebrián et al., 2014b). To investigate this possibility, we performed a combination of RNAscope *in situ* hybridization for the promoter of the AAV genome, and IBA1 immunohistochemistry to visualize microglia, to determine if any viral genomes were located within microglia. Although there was abundant punctate signal from the AAV genome within the transduced SNc, we were unable to detect any AAV genomes within IBA1+ microglia (Supplementary Figures 7A–C), supporting previous reports that AAV5 does not transduce microglia (Rosario et al., 2016). These data support the conclusion that loss of endogenous α-syn within nigral neurons is able to stimulate a neuron-initiated inflammatory cascade without directly transducing- or manipulating levels of α-syn within microglia.

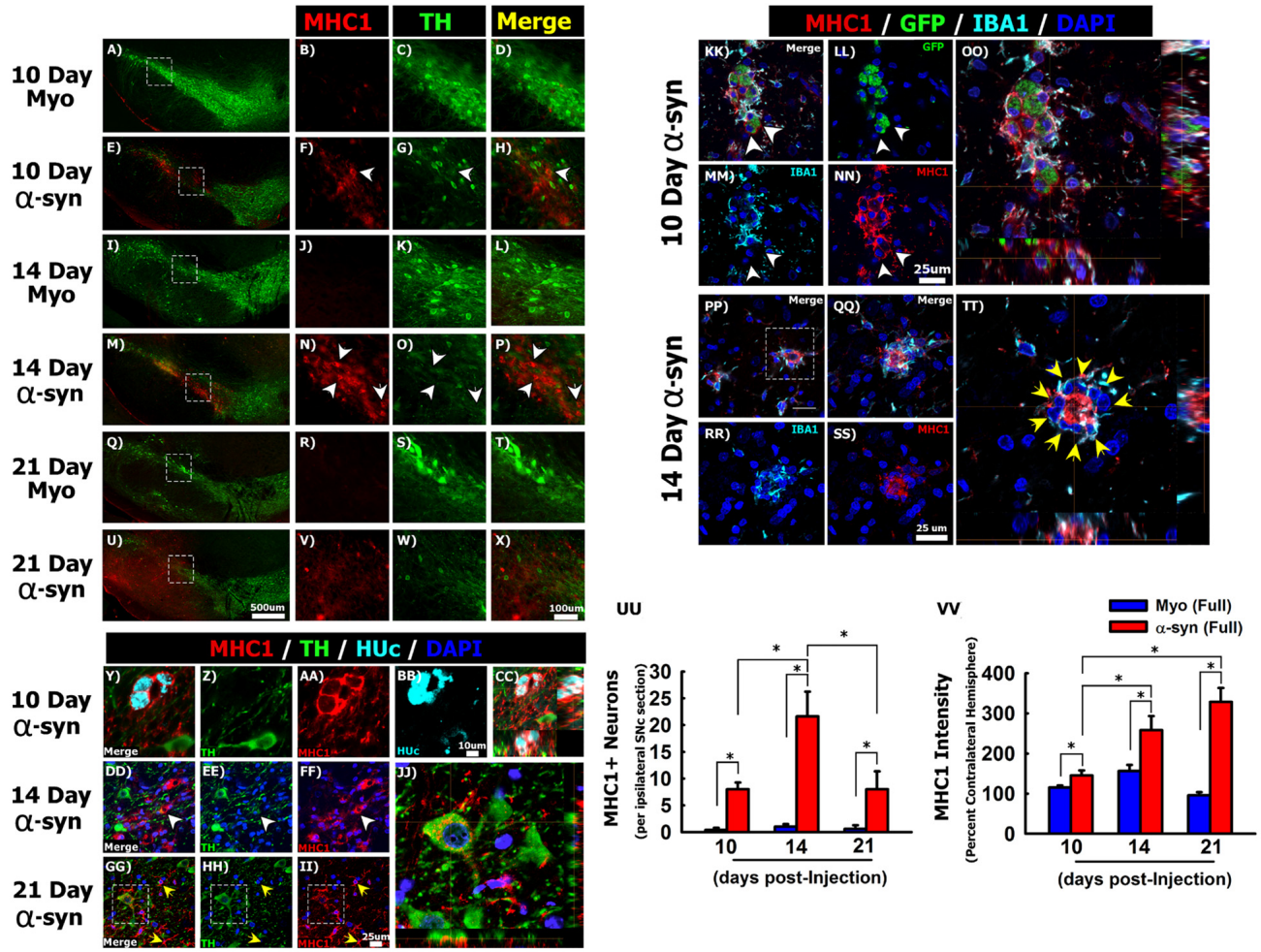

FIGURE 4 | Silencing α-syn in nigral neurons induces major histocompatibility complex class 1 (MHC-1) expression. Rats received a single injection of AAV2/5 (2.6×10^{12} vg/ml; Full) expressing a target α-syn shRNA or a myocardin control shRNA. Rats were then sacrificed at 10, 14, and 21 days post-surgery. Panels **(A–X)** show MHC-1 (red) immunoreactivity near TH (green) neurons in myocardin shRNA treated animals at 10 days **(A–D)**, 14 days **(I–L)** and 21 days **(Q–T)** post injections. MHC-1 expression in α-syn shRNA treated animals at 10 days **(E–H)**, 14 days **(M–P)** and 21 days **(U–X)** post injection. White arrowheads in **(F–H)** and **(N–P)** indicate neuronal MHC-1 expression. Panels **(Y–CC)** show co-localization of MHC-1 (red) with the neuronal marker HUc (teal) within the SNc 10 days following α-syn shRNA injection. Panels **(DD–JJ)** show co-localization of MHC-1 expression in TH+ neurons of the α-syn shRNA injected SNc. White arrowheads in **(DD–FF)** indicate MHC-1 expression in a neuron that also expresses low levels of TH. Yellow arrows in **(GG–II)** indicate MHC-1 expressing microglia. Panel **(JJ)** shows a composite z-stack image acquired using confocal microscopy, corresponding to the area within the box in panels **(GG–II)** showing MHC-1 expression on the surface of a TH+ neuron. Panels **(KK–TT)** show representative micrographs of transduced neurons (GFP+; green) within the α-syn shRNA injected SNc stained for IBA1 (teal) and MHC-1 (red). White arrowheads in **(KK–NN)** indicate cell surface expression of MHC-1 on GFP+ transduced neurons, which does not co-localize with the microglial marker IBA1 **(OO)**. Yellow arrows in **(TT)** indicate IBA1+ microglia surrounding a neuron with high MHC-1 expression. Panels **(QQ–TT)** correspond to the area within the box in **(PP)**. Panels **(OO)** and **(TT)** are composite z-stack images acquired using confocal microscopy. Columns in **(UU)** represent the mean number of MHC-1+ neurons, + 1 SEM (n = 4–5/group), counted from four sections of the ipsilateral ventral midbrain, spanning the rostral-caudal axis of the SNc. Columns in **(VV)** represent the mean MHC-1 fluorescent intensity within ventral midbrain of the ipsilateral hemisphere, expressed as a percent of the mean MHC-1 fluorescent intensity within ventral midbrain of contralateral hemisphere, +1 SEM (n = 4–5/group). *Significantly different (ANOVA: $P < 0.05$). Scale bar in **(U)** represents 500 μm and applies to panels **(A,E,I,M,Q)**. Scale bar in **(X)** represents 100 μm and applies to **(B–D,F–H,J–L,N–P,R–T,V,W)**. Scale bar in panel **(BB)** represents 10 μm and applies to panels **(Y–AA)**. Scale bar in **(II)** represents 25 μm and applies to panels **(DD–HH)**. Scale bars in **(NN)** and **(SS)** represent 25 μm and apply to panels **(KK–MM,PP–RR)**.

The Effects of α-syn Knockdown in Non-dopaminergic Neurons

To determine if α-syn knockdown toxicity and the corresponding neuroinflammatory sequela are specific to DA neurons, we analyzed the consequences of α-syn knockdown in a non-dopaminergic neuronal subpopulation. We choose to investigate the effects of α-syn knockdown within the granule cell layer (GCL) of the cerebellum, as neurons in the rat cerebellum expresses high levels of α-syn, and granule cells predominantly utilize glutamate as a neurotransmitter (Wersinger et al., 2004).

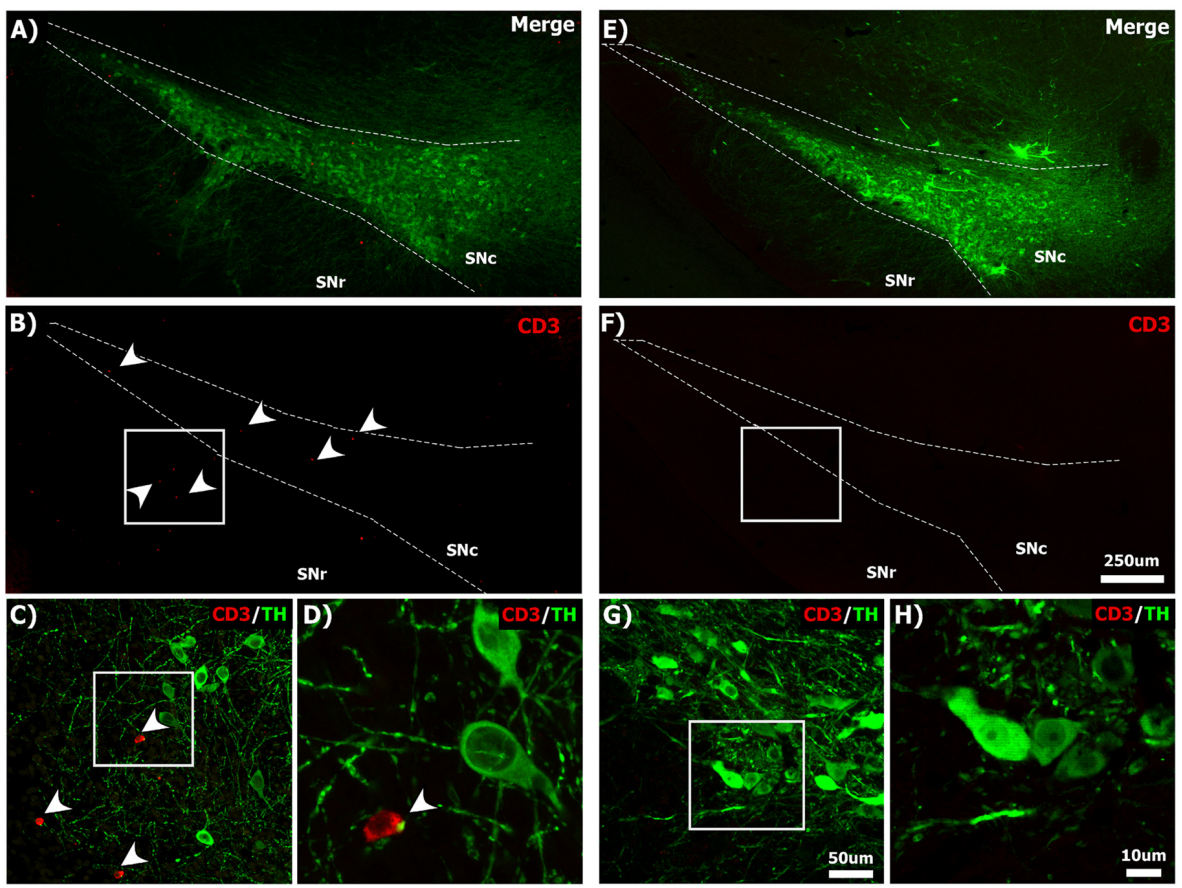

FIGURE 5 | Silencing α-syn in nigral neurons results in the infiltration of CD3+ T-cells to the SNc. Rats received a single injection of AAV2/5 (2.6×10^{12} vg/ml; Full) expressing the α-syn or myocardin shRNA and were sacrificed 21 days post-surgery. Panels show CD3+ (red) T-cells near TH+ (green) nigral neurons in the SNc of α-syn shRNA **(A–D)** and myocardin shRNA **(E–H)** treated animals. White arrowheads in panels **(B–D)** indicate CD3+ T-cells. Scale bar in panel **(F)** represents 250 μm and applies to panel **(A,B,E)**. Scale bar in panel **(G)** represents 50 μm and applies to panel **(C)**. Scale bars in panels **(H)** represents 10 μm and applies to panel **(D)**.

One-month post-surgery there was abundant transduction that was largely confined to the GCL (**Figures 6A,B**). There was no change in IBA1+ microglia within the cerebellum following α-syn knockdown (**Figures 6C–H**). There was no change in the total number of NeuN+ neurons within the cerebellum following α-syn knockdown (**Figures 6I–M**). Purkinje cells to do not express NeuN (Mullen et al., 1992), thus, we also processed cerebellar tissue for immunohistochemical detection of the common purkinje marker, calbindin D-28K (calbindin), to probe the integrity of purkinje neurons (**Figures 6N–R**). α-syn knockdown resulted in a striking loss of calbindin+ purkinje cells (**Figure 6R**; ∼50% loss; *t*-test: $t_{(13)} = -2.738$, $p = 0.016$). However, while performing calbindin+ cell counts, there were many remaining purkinje cells, which had very weak calbindin-immunoreactivity (arrows in the inset in **Figure 6Q**). This suggested that α-syn knockdown was not necessarily resulting in death of purkinje cells, but merely a phenotypic loss of calbindin expression.

To determine if calbindin negative purkinje cells were still present, we analyzed the PCL for expression of neuronal marker, HUc. **Figures 6S–V** shows colocalization of HUc and calbindin within purkinje cells of myocardin shRNA treated animals (arrows in **Figures 6T–V**). Within the α-syn shRNA injected cerebellum (**Figures 6W–Z**), calbindin immunoreactivity was almost completely absent, however, there remained strong HUc expression within cells of the purkinje layer (arrows in **Figures 6X–Z**). Indeed there was no change in the number of purkinje cells in the lobule 3 of the cerebellar vermis (an area immediately adjacent to the injection site) of α-syn shRNA treated animals (**Figure 6AA**). These data demonstrate that α-syn knockdown in cerebellar granule cells causes a phenotypic loss of calbindin expression within neighboring, non-transduced purkinje cells, suggestive of a functional deficit in the former. Importantly, our data show that α-syn knockdown does not cause neurodegeneration of the non-dopaminergic neurons of the cerebellum.

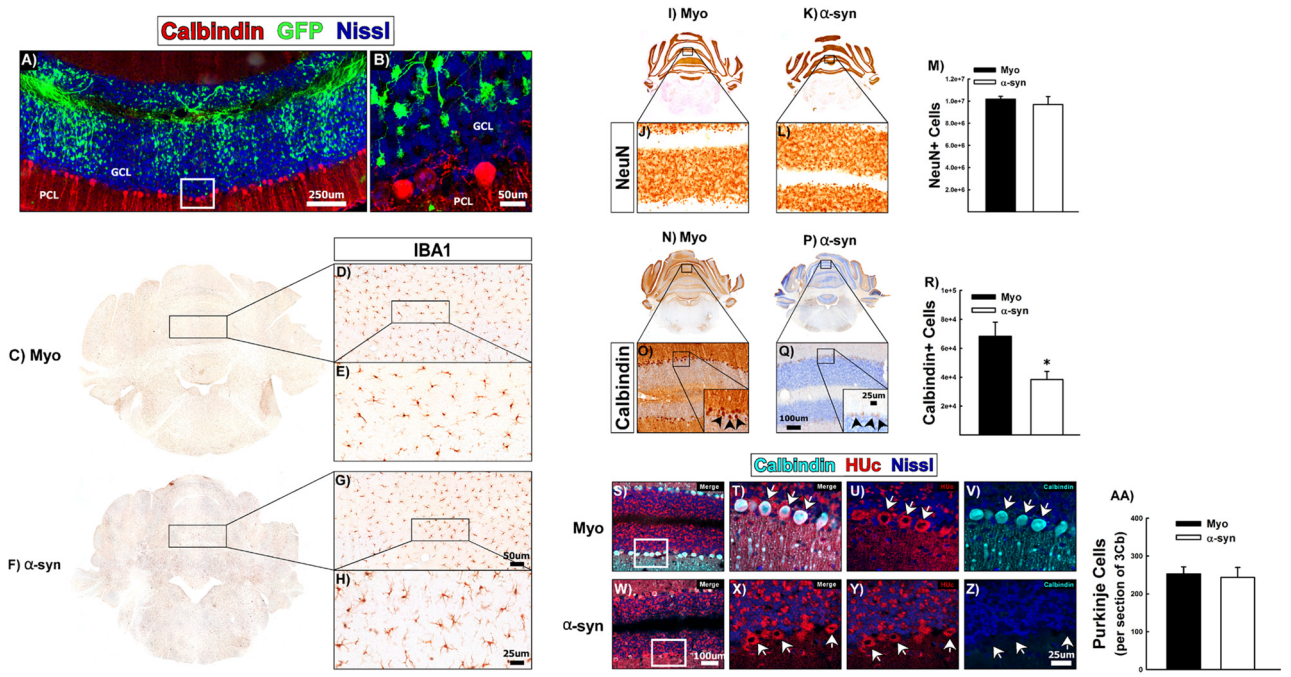

FIGURE 6 | The effects of α-syn knockdown within purkinje cells of the cerebellum. Rats received a single injection of AAV2/5 (2.6×10^{12} vg/ml; Full) expressing an α-syn shRNA or a myocardin control shRNA. One-month post-surgery rats were sacrificed. Panels **(A,B)** show transduction within the granule cell layer (GCL) of the cerebellum, directly adjacent to the purkinje cell layer (PCL). High magnification of the area within the box in panel **(A)** is shown in panel **(B)**. Panels **(C–H)** shows IBA1+ microglia in Myo **(C–E)** and α-syn shRNA **(F–H)** treated animals. Unbiased stereology was used to quantify NeuN+ and calbindin+ neurons in the cerebellum. Columns in Panel **(M)** represent the mean number of NeuN+ cells from animals treated with the myocardin shRNA (black bars) or the α-syn shRNA (white bars). Representative images of NeuN+ cells of the cerebellum are shown for myocardin shRNA **(I,J)** and α-syn shRNA **(K,L)** treated animals. Columns in **(R)** represent mean number of calbindin+ cells within the cerebellum from animals injected with the myocardin shRNA (black bars) or the α-syn shRNA (white bars). Representative images of calbindin immunoreactivity within purkinje cells of the cerebellum from animals injected with the myocardin shRNA **(N,O)** and α-syn shRNA **(P,Q)** are shown. Error bars in **(M,R)** are + 1 SEM (n = 7–8/group). *Significantly different than myocardin treated group (t-test: $P < 0.05$). Panels **(S–Z)** show co-localization of calbindin (teal) with the pan-neuronal marker HUc (red) in the PCL of animals treated with the myocardin shRNA **(S–V)** or the α-syn shRNA **(W–Z)**. High magnification images of the boxes in panels **(S,W)** are shown in panels **(T–V)** and **(X–Z)**, respectively. Arrows indicate purkinje cells that are calbindin positive **(T–V)** or calbindin negative **(X–Z)**. Columns in panel **(AA)** represent the mean number of purkinje cells in the third lobule of the cerebellar vermis (3Cb) from animals treated with the myocardin shRNA (black bars) or the α-syn shRNA (white bars). Scale bars in panels **(A,B)** represent 250 μm and 50 μm respectively. Scale bar in panels **(G)** represents 50 μm and applies to panel **(D)**. Scale bar in panel **(H)** represents 25 μm and applies to panel **(E)**. Scale bar in panel **(Q)** represents 100 μm and applies to panels **(J,L,O)**. Scale bar in the inset in panel **(Q)** represents 25 μm and applies to the inset in panel **(O)**. Scale bar in panel **(W)** represents 100 μm and applies to panel **(S)**. Scale bar in panel **(Z)** represents 25 μm and applies to panels **(T–V)** and **(X,Y)**.

DISCUSSION

Mechanistic Model of α-syn shRNA-Induced Toxicity

Here we have characterized the early pathological events that occur following α-syn knockdown in mature nigral neurons. Based on the data provided we propose a mechanistic model of toxicity elicited by α-syn silencing (**Figure 7**). Expression of the α-syn shRNA reduces α-syn protein as early as 7 days (**Figure 7B**), resulting in a progressive decrease in TH expression within nigral neurons, and an overall loss of nigral neurons over the 21-day time course. Nigral neurons up-regulate MHC-1 expression as early as 10-days following AAV-α-syn-shRNA injection, a time point prior to neurodegeneration (**Figure 7C**). MHC-1 up-regulation coincides with the recruitment of reactive microglia to affected neurons, followed by the infiltration of CD3+ T-cells to the injected midbrain. Recruited immune cells surround and engulf MHC-1+ neurons, culminating in the death of nigral neurons, and a corresponding loss of nigrostriatal terminals (**Figures 7D,E**). Cell death elicited by α-syn knockdown appears to be apoptotic and specific to dopaminergic neurons of the SNc, as neurodegeneration was not observed in the non-dopaminergic neurons of the cerebellum or in dopaminergic neurons of the VTA (Gorbatyuk et al., 2010). Finally, DA neurons in the ventral tier of the SNc were more sensitive to α-syn knockdown, as many of these metrics were exacerbated or observed solely in this population.

The Critical Threshold of α-syn Knockdown

To date, the majority of knowledge on the biological function of α-syn, and more notably the pathological role of α-syn in Parkinson's disease, has been gained by examining the

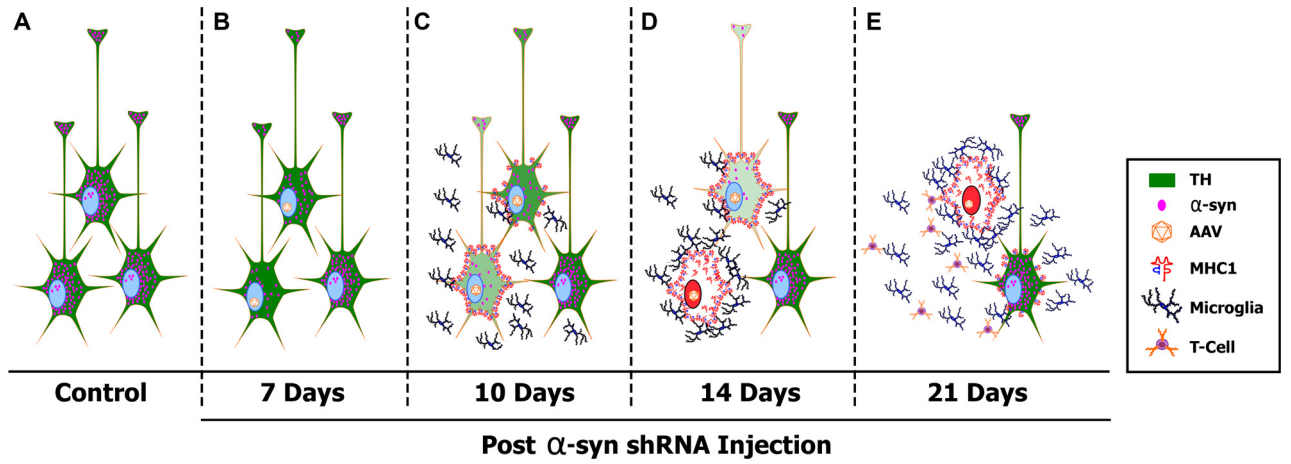

FIGURE 7 | A proposed mechanism of α-syn shRNA induced toxicity. Healthy, TH+ (green) nigral neurons express high levels of α-syn (pink dots) **(A)**. Delivery of adeno-associated virus (AAV) expressing an shRNA targeting endogenous α-syn results in a rapid knockdown of α-syn expression that was detected as early as 7 days post-delivery **(B)**. Silencing endogenous α-syn results in a progressive decrease in TH expression within individual nigral neurons, as well as an overall loss of TH+ neurons within the SNc. Coinciding with loss of TH expression, nigral neurons up-regulate cell surface expression of MHC-1 as early as 10 days post-injection **(C)**. MHC-1 up-regulation results in recruitment of reactive microglia and the infiltration of CD3+ T-cells to the injected SNc. Recruited immune cell then surround and engulf MHC-1+ neurons, culminating in the death of nigral neurons *via* the induction of apoptotic cell death (red nucleus), and a corresponding loss of nigrostriatal terminals **(D,E)**. Cytokines released from reactive microglia in the affected SNc cause neighboring, non-transduced nigral neurons to increase surface expression of MHC-1, creating a cycle of toxicity **(E)**.

consequences of increasing α-syn concentrations within a cellular environment (Chesselet, 2008). Although this approach has yielded a significant amount of information, an alternative though equally productive approach, is to examine the consequences of removing endogenous α-syn from the cell. This was initially attempted through the generation of several different lines of α-syn KO mice, however, despite modest changes in neurotransmission, germline removal of α-syn does not result in any major biological change or recapitulate Parkinson's disease pathology (Abeliovich et al., 2000; Cabin et al., 2002, 2005; Dauer et al., 2002; Schlüter et al., 2003). This lack of an effect is likely the result of developmental genetic compensation (Perez et al., 2002; Schlüter et al., 2003; Chandra et al., 2004; Robertson et al., 2004; Kuhn et al., 2007; Wang et al., 2009; Ubhi et al., 2010; Cali et al., 2012; Benskey et al., 2016a; Ludtmann et al., 2016). For example, compared to WT mice, α-syn KO mice have 369 differentially expressed genes, including genes involved in apoptosis, neurotrophic signaling, and other, functionally redundant members of the synuclein family (Schlüter et al., 2003; Chandra et al., 2004; Robertson et al., 2004; Kuhn et al., 2007). As such, genetic compensation may mask the true consequences of loss of α-syn function, including the intra-neuronal sequela ultimately leading to an induction of a severe inflammatory response. Thus, many laboratories have begun examining the consequences of removing α-syn from mature neurons (Gorbatyuk et al., 2010; Khodr et al., 2011, 2014; Zharikov et al., 2015; Benskey et al., 2016a; Collier et al., 2016).

Here we have successfully utilized this approach to provide novel insights into α-syn function and toxicity, however; previously published research has yielded discrepant findings. For example, no toxicity or inflammation was reported in two independent studies in which endogenous α-syn was silenced in the mouse hippocampus or the squirrel monkey SNc (Lewis et al., 2008; McCormack et al., 2010). Other laboratories have reported an intermediate degree of toxicity (e.g., loss of striatal DA; Zharikov et al., 2015), likely reflecting functional deficiencies (Cali et al., 2012), following acute α-syn silencing. In contrast, we and others report robust nigral cell death following α-syn knockdown in the rat, mouse, and non-human primate using both shRNA and microRNA technology (Gorbatyuk et al., 2010; Khodr et al., 2011, 2014; Benskey et al., 2016a; Collier et al., 2016). Finally, the absence of endogenous α-syn during development leads to decreased numbers of DA neurons in the mouse SNc (Garcia-Reitboeck et al., 2013). We believe that the source of discrepancy between these findings is a differing degree of α-syn knockdown, the kinetics of α-syn knockdown, and the type of cell being targeted. Here we show a dose dependent effect of α-syn knockdown, where the degree of nigrostriatal toxicity is inversely proportional to the amount of endogenous α-syn remaining within the affected neurons (Gorbatyuk et al., 2010). Thus, it is likely that alternative reports, which did not report toxicity, did not reach the critical threshold of α-syn knockdown. For example, the degree of α-syn knockdown reported in studies that did not observe toxicity ranges between 35%–55% (Lewis et al., 2008; McCormack et al., 2010; Zharikov et al., 2015). In contrast, knockdown of α-syn by 70%–90% causes severe nigral cell loss (Gorbatyuk et al., 2010). These findings are in line with the level of knockdown and toxicity observed here. A ∼50% reduction of α-syn was achieved following expression of the α-syn shRNA (FULL), whereas the α-syn shRNA (HALF) caused an initial ∼25% reduction in α-syn, that eventually progressed to

a ∼50% reduction over the course of the study. Corresponding to the differing degrees of α-syn knockdown between the two titers used, the α-syn shRNA (FULL) caused a progressive loss of TH+ neurons beginning at 14 days post-injection, while expression of the α-syn shRNA (HALF) did not produce any detectable TH+ cell loss until 21 days post-injection. An important caveat to these observations is that these are the levels of knockdown observed within nigral neurons that still retain their TH phenotype. Due to technical reason (loss of GFP reporter expression at later time points) we were unable to quantify α-syn knockdown after loss of TH expression. As loss of TH expression is one of the first pathological changes we observed following α-syn knockdown, it is likely that 50% depletion is the critical threshold of α-syn knockdown, below which a pathological cascade is initiated. Taken together, it seems that in order for nigral pathology to occur, levels of endogenous α-syn must be decreased below a certain level (likely below 50%) and this depletion must be maintained for a certain period of time (likely at least 7 days). Finally, an important limitation to our study, and others, is the fact that we are using α-syn knockdown at the level of the soma as a surrogate for estimating α-syn levels at the synapse, the presumptive functional location of α-syn. However, it is extremely difficult to accurately measure nigral terminal α-syn in the striatum given the large number of striatal terminals originating from non-nigral areas.

The Specificity of α-syn shRNA Mediated Toxicity

Nigrostriatal toxicity induced by shRNA-mediated α-syn knockdown is partially rescued by co-expression of a rat α-syn transgene rendered insensitive to the shRNA (Gorbatyuk et al., 2010). Although these data demonstrate that nigrostriatal cell loss is, at least in part, due to loss of endogenous α-syn, there is always a concern of non-specific toxicity when using RNAi. One goal of this work was to ensure that α-syn shRNA toxicity is indeed due to loss of α-syn function, and not an artifact from RNAi. To this end we examined the effects of α-syn silencing within WT and α-syn KO mice. If the toxicity we observe following expression of the α-syn shRNA is due to non-specific RNAi toxicity, than one would predict comparable toxicity within both WT and α-syn KO mice. If however, α-syn shRNA expression produces neurodegeneration by specifically decreasing endogenous α-syn, then α-syn KO mice should be immune to α-syn shRNA toxicity, as they do not express α-syn. α-syn KO mice were immune to the effects of α-syn shRNA administration, thus in conjunction with previously published controls (Gorbatyuk et al., 2010), we confidently conclude that α-syn knockdown-mediated nigral toxicity is solely mediated by loss of endogenous α-syn.

Neuroinflammation Induced by α-syn Knockdown

In the work of Gorbatyuk et al. (2010), the integrity of the nigrostriatal system was first examined at 4-weeks post-shRNA administration, at which point there was an approximate 90% reduction of endogenous α-syn and an 80% loss of TH+ nigral neurons. Here we sought to determine if intra-neuronal α-syn dysfunction, in the absence of overt cell death, could elicit a neuroinflammatory response, thus we extensively characterized the early events in the pathological process that occur following loss of α-syn from nigrostriatal neurons, but prior to neurodegeneration.

One of the most remarkable changes we observed following α-syn knockdown was an up-regulation of neuronal MHC-1 expression, coinciding with the recruitment of immune cells. Members of the MHC-1 family are expressed on virtually all nucleated cells. However, under normal conditions, neuronal expression of MHC-1 is relatively low, especially in the adult brain (Boulanger and Shatz, 2004). Neuronal MHC-1 expression is suggested to play a role in synaptic plasticity (Corriveau et al., 1998), learning and memory (Nelson et al., 2013), and following injury (Boulanger and Shatz, 2004; Oliveira et al., 2004; Cebrián et al., 2014a). Additionally, neuronal MHC-1 expression is associated with neurodegenerative disorders such as Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis and Parkinson's disease (Boulanger and Shatz, 2004; Cebrián et al., 2014a,b). Within Parkinson's disease, midbrain catecholaminergic neurons express MHC-1, while cultured midbrain DA neurons induce MHC-1 expression in response to increased cytosolic DA, oxidative stress, α-syn and cytokines released from glia. Finally, increased MHC-1 expression on cultured DA neurons triggers an antigenic response, leading to a cytotoxic T-cell-mediated death of DA neurons (Cebrián et al., 2014b).

Here we observed a near identical sequence of events following α-syn knockdown within DA neurons of the rat SNc, including neuronal MHC-1 expression, glial cell recruitment, and infiltration of T-cells. Although we observed an infiltration of T-cells into the brain of animals injected with the α-syn shRNA, it is currently unclear whether the T-cells were CD8+ cytotoxic T-cells or CD4+ helper T-cells. Previous work indicates that MHC-1 expression on catecholaminergic neurons results in the recruitment of CD8+ cytotoxic T-cells, which subsequently mediate the selective degeneration of the MHC-1+ neurons (Cebrián et al., 2014b). Although, this type of CD8+ T-cell response is in line with what we observe here, the identity of the infiltrating T-cells remains unknown. Nonetheless, the nature of the T-cell response elicited by α-syn knockdown in nigral neurons is very important, as is could provide a mechanism of cell death, and as such will be the topic of future investigation.

Examining the temporal sequence of increased neuronal MHC-1+ numbers vs. total MHC-1 expression suggests that there is a biphasic induction of MHC-1 expression within more than one cell type, specifically neurons and microglia. Neuronal MHC-1 expression increased at 10-days, peaked at 14 days, and decreased at 21-days post injection. The decrease in MHC-1+ neurons at the 21-day time point is likely due to the death of MHC-1 expressing nigral neurons. In contrast, total MHC-1 expression within the injected SNc progressively increased from 10 days to 21 days post-injection. This progressive increase in total MHC-1 expression, in the face of decreasing numbers of MHC-1+ neurons, is the result of a dramatic increase in the

number of reactive microglia expressing MHC-1 within the injected midbrain.

From this sequence of events we believe that neuronal MHC-1 expression, and the recruitment of immune cells, is not merely a generalized inflammatory reaction to cell death, but rather a cell-autonomous response to loss of α-syn function, which actively contributes to neurodegeneration. For instance, MHC-1 was up-regulated within transduced neurons 10 days post-injection, a time point that precedes any detectable amount of cell death by almost 2 weeks. At these early time points, the majority of MHC-1 expressing neurons were located in the ventral tier of the injected SNc, the anatomical location exhibiting the greatest cell loss at 21 days post-injection, and the anatomical location that where the earliest signs of pathology are seen in Parkinson's disease (Gibb and Lees, 1991). Finally, due to the fact that we silenced endogenous α-syn expression, and the inflammatory response occurred prior to cell loss, it is highly unlikely that the inflammation was initiated by a direct exposure of microglia to extracellular α-syn. We believe that these observations are consistent with microglial-mediated phagocytosis of stressed, yet still living, transduced neurons. Taken together, these data support an immune cell mediated process of neurodegeneration that is initiated by neurons, following intra-neuronal α-syn dysfunction.

Nonetheless, the initial impetus leading to MHC-1 up-regulation remains unclear. Neuronal expression of MHC-1 can be induced by viral infection (Redwine et al., 2001), however there was no increase in MHC-1 expression within AAV-myocardin shRNA transduced neurons, excluding this possibility. Further, previous work has demonstrated that a single administration of AAV is not sufficient to cause an inflammatory response (Peden et al., 2004). As mentioned, increased cytosolic DA (presumably leading to increased oxidative stress) can initiate neuronal MHC-1 up-regulation (Teoh and Davies, 2004; Cebrián et al., 2014b). α-syn regulates many aspects of chemical neurotransmission and DA biosynthesis (reviewed in Benskey et al., 2016a), thus, we predict that loss of normal α-syn function within nigral neurons results in a dysregulation of DA synthesis and handling, thereby increasing oxidative stress and MHC-1 expression (Teoh and Davies, 2004; Caudle et al., 2007; Cebrián et al., 2014b).

However, there was also a second initiation of MHC-1 expression at the 21-day time point, where non-transduced, TH+ neurons expressed MHC-1. This is likely caused by increased pro-inflammatory cytokine production within the injected SNc. Microglial-released pro-inflammatory cytokines, such as interferon gamma, are sufficient to induce MHC-1 expression in DA neurons (Cebrián et al., 2014b). Thus, it is likely that the large increase in reactive microglia observed at the 21 day time point, resulted in a corresponding increase in inflammatory cytokine production, thereby inducing MHC-1 expression within the remaining, un-transduced nigrostriatal neurons. Future research is needed to confirm this hypothesis, however, this mechanism could account for the continued nigral degeneration (80% loss of TH+ neurons) observed at the 1-month time point by Gorbatyuk et al. (2010).

Nigrostriatal Pathology Induced by α-syn Knockdown

In parallel with a major immune response, α-syn knockdown also caused nigral neuron dysfunction, in the form of a progressive loss of TH expression. The loss of TH+ neurons was dose-dependent, indicating that the degree of TH neuron loss is proportional to the degree of α-syn knockdown. Remarkably, despite early losses in TH immunoreactivity, striatal DA concentrations were unaffected until the 21-day time point. This may be the result of disinhibition of TH following α-syn knockdown. α-syn acts as a negative regulator of both TH and aromatic amino acid decarboxylase (AADC; Perez et al., 2002; Perez and Hastings, 2004; Yu et al., 2004; Peng et al., 2005; Tehranian et al., 2006; Wang et al., 2009; Benskey et al., 2016a). Thus, α-syn knockdown likely disinhibits TH and AADC, resulting in increased *de novo* DA synthesis, maintaining normal DA levels in the face of decreased TH expression. Finally, 21-days following expression of the α-syn shRNA (Half), there was no change in the levels of striatal VMAT, despite an overall reduction in the number of TH+/HUc+ neurons in the ipsilateral SNc. This is likely the effect of collateral axonal sprouting of the nigrostriatal terminals remaining in the striatum, a phenomenon that occurs following striatal denervation which has been well documented in rodent models of Parkinson's disease (Arkadir et al., 2014).

Interestingly, α-syn overexpression results in a near identical decrease in both TH expression (Baptista et al., 2003; Yu et al., 2004; Alerte et al., 2008; Li et al., 2011). Increased oxidative stress can cause the addition of carbonyl adducts to the TH protein, resulting in a loss of immunoreactivity (Dela Cruz et al., 1996). As such, the proposed (unregulated) increase in DA synthesis following α-syn manipulation, may result in DA auto-oxidation and a corresponding loss of TH protein following the addition of oxidative adducts. It is possible that both α-syn overexpression and silencing induce toxicity through a common mechanism, which is the loss of α-syn function. For example, α-syn overexpression, and subsequent aggregation, may produce toxicity by sequestering functional forms of endogenous α-syn into aggregates, causing a *de facto* loss of function (Perez and Hastings, 2004; Benskey et al., 2016a). From this viewpoint it is not surprising that both α-syn overexpression and knockdown result similar decreases in TH expression, albeit over a different time course. This theory is supported by α-syn based animal models where overexpression of α-syn, or injection of α-syn recombinant protein (preformed fibrils) increase α-syn aggregation, with a corresponding depletion of soluble α-syn (Perez et al., 2002; Perez and Hastings, 2004; Unni et al., 2010; Volpicelli-Daley et al., 2011; Cali et al., 2012; Osterberg et al., 2015). Interestingly, administration of α-syn PFFs results in an inflammatory response, similar to that observed here and in the PD brain, potentially suggesting that loss of normal α-syn function following PFF-induced aggregation can elicit an inflammatory response (Harms et al., 2017).

Is Toxicity Induced by α-syn Dysfunction Exclusive to Dopaminergic Neurons?

One important question of the current study was whether intra-neuronal α-syn dysfunction within non-dopaminergic neurons could elicit neuroinflammation and toxicity. As mentioned above we propose that loss of α-syn function may mediate toxicity via impaired DA metabolism and handling. Indeed several reports indicate that toxicity initiated through manipulation of α-syn is dependent upon the presence of intracellular DA (Xu et al., 2002). Further, antibodies isolated from the sera of Parkinson's disease patients react with proteins modified by DA oxidation. In line with these findings, no inflammation or neurodegeneration was detectable following α-syn knockdown within the cerebellum. Although this is by no means an exhaustive analysis of the effects of α-syn silencing in the myriad of non-dopaminergic neurons present in the brain, this result does support the notion that the inflammation and toxicity observed following α-syn dysfunction is specific to catecholaminergic neurons.

Interestingly, there was a phenotypic loss of calbindin expression within purkinje cells. This result was unanticipated due to the fact that there was no transduction of the PCL, and the fact that purkinje cells do not express appreciable amounts of α-syn (Lee et al., 2015). We believe that this loss of calbindin expression is the result of altered synaptic input from α-syn shRNA transduced granule cells onto purkinje cells. α-syn has been proposed to act as a negative regulator of synaptic transmission, and increasing synaptic input onto purkinje cells results in a decrease in calbindin mRNA and protein (Barmack and Qian, 2002). Thus, it is likely that removing α-syn from presynaptic granule cells increases neurotransmission at the granule-to-purkinje cell synapse, resulting in an activity dependent decrease in calbindin expression.

Is Loss of α-syn Function an Early Event in Parkinson's Disease Pathogenesis?

Based on the correlations between the data presented here and the sequence of pathological events observed in Parkinson's disease, we believe that α-syn loss-of-function toxicity may, at least in part, contribute to neurodegeneration in Parkinson's disease. Parkinson's disease pathology is characterized by a phenotypic loss of TH preceding the death of nigral neurons (Kastner et al., 1993, 1994; Kordower et al., 2013), a significant inflammatory response involving microgliosis, neuronal MHC-1 expression, and infiltration of peripheral leukocytes to the brain (McGeer et al., 1988a,b; Croisier et al., 2005; Orr et al., 2005; Ouchi et al., 2005; Cebrián et al., 2014b). Within the above experiments we describe a pathological cascade that entails all of these pathological features following acute α-syn knockdown within nigral neurons. Further, there is ample evidence to suggest that loss of normal α-syn function contributes to Parkinson's disease pathogenesis. For example, most familial SNCA mutations increase the aggregation kinetics of α-syn (Conway et al., 1998; Burré et al., 2010; Ghosh et al., 2014), while increasing cellular concentrations of α-syn (as is achieved with gene duplication) also increases the propensity of α-syn to aggregate (Conway et al., 1998; Uversky, 2007). As described above, the aggregation of α-syn could act to sequester functional forms of the protein, impairing normal function. This idea is supported by the fact that, although the brains of synucleinopathy patients do show accumulation of excess α-syn, the majority of α-syn is abberantly folded and/or contained within aggregates (Miller et al., 2004; Kramer and Schulz-Schaeffer, 2007; Quinn et al., 2012). Further, the brains of idiopathic Parkinson's disease patients have decreased levels of soluble (presumably functional) α-syn (Baba et al., 1998; Quinn et al., 2012). In addition, most mutations in the SNCA gene directly impair the ability of the mutant protein to perform its normal function (Fortin et al., 2004; Burré et al., 2010; Fares et al., 2014; Logan et al., 2017; Pozo Devoto et al., 2017), again linking loss of α-syn function to neurotoxicity. Finally, Parkinson's disease patients carrying the low repeat REP1 allele, which results in decreased SNCA expression (Chiba-Falek and Nussbaum, 2001), have worse motor and cognitive disease outcomes (Markopoulou et al., 2014). Viewed as a whole, we believe it is possible that an initial loss of function following α-syn aggregation in the Parkinsonian brain could elicit an early inflammatory response that contributes to neurodegeneration. Thus, examining the early pathological events following loss of α-syn function could provide insights into Parkinson's disease pathogenesis as well as the identification of novel therapeutic targets. Further, these data provide further support for examining early inflammatory markers as potential biomarkers for PD. Indeed, a recent study was able to identify a panel of inflammatory markers isolated from human serum or cerebrospinal fluid that could reliably distinguish between healthy controls and PD patients (Eidson et al., 2017).

Finally, these data suggest that therapeutics aimed at decreasing or ablating α-syn within Parkinson's disease-affected neurons should be undertaken with caution. From the current work it is clear that lowering α-syn levels beyond a critical threshold within nigral neurons can have deleterious consequences. Although it is theoretically possible to titer the degree of α-syn knockdown to a level that would be therapeutic in the context of PD, from a technical standpoint this could be extremely challenging if not impossible. We propose that a better therapeutic approach would be drugs or small molecules that prevent α-syn aggregation, or conversely, disassemble fibrilized α-syn to soluble, functional α-syn.

AUTHOR CONTRIBUTIONS

FPM conceived the experiments and performed surgery. MJB performed surgery, the experimentation, the data analysis and interpretation and wrote the manuscript. RCS performed stereological cell counting and surgery. IMS performed surgery. CES performed surgery and provided critical feedback on the manuscript. JWL performed HPLC analysis of neurochemicals.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00036/full#supplementary-material

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REVIEW
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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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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,

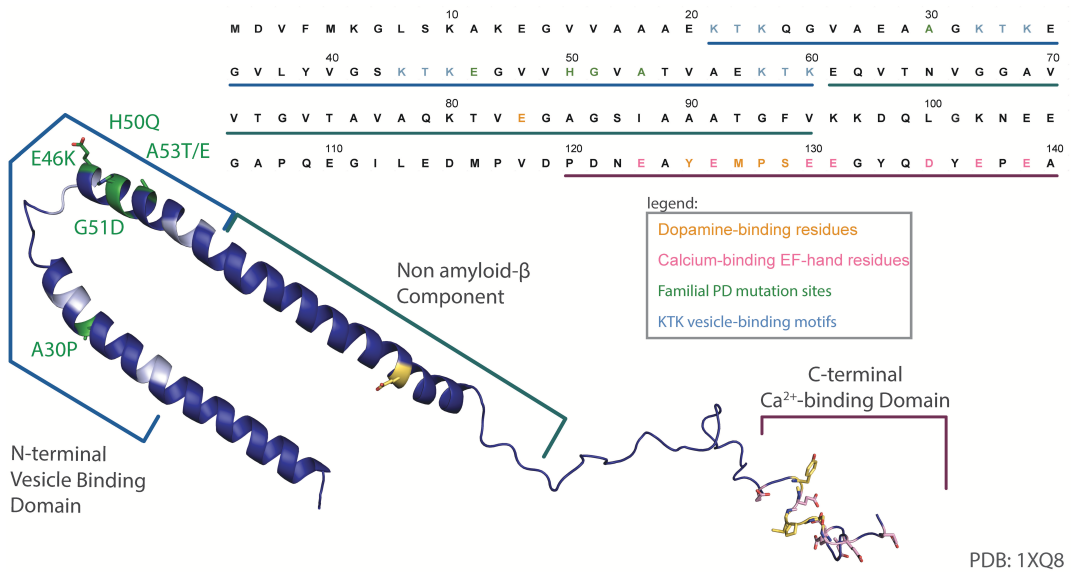

FIGURE 1 | aSyn sequence map. An annotated structure of human micelle-bound aSyn solved by solution NMR (PDB 1XQ8) (Ulmer et al., 2005) and corresponding sequence have been color coded to highlight the lipid binding N-terminal domain and associated KTK motifs (cyan), common familial PD mutations (green), 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

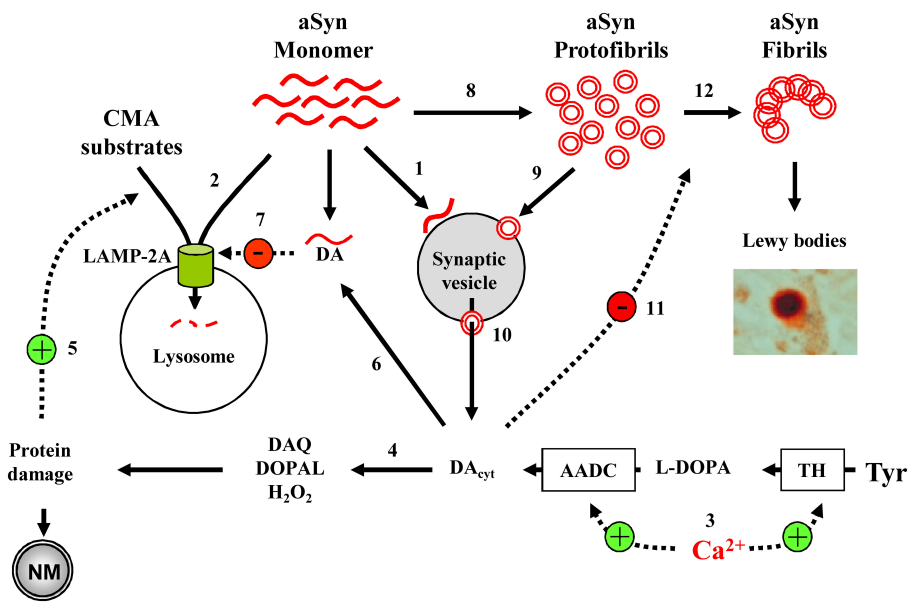

FIGURE 2 | Model of cell-selective PD pathogenesis. **(1)** A physiological function of aSyn may be to bind synaptic vesicles in a reversible manner to inhibit exocytosis. **(2)** aSyn is degraded by CMA following LAMP-2A-mediated transport into the lysosomes. **(3)** In SN and LC neurons, high Ca^{2+} 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.

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In Situ Proximity Ligation Assay Reveals Co-Localization of Alpha-Synuclein and SNARE Proteins in Murine Primary Neurons

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The aggregation of alpha-synuclein (αSyn) is the pathological hallmark of Parkinson's disease, dementia with Lewy bodies and related neurological disorders. However, the physiological function of the protein and how this function relates to its pathological effects remain poorly understood. One of the proposed roles of αSyn is to promote the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly by binding to VAMP-2. The objective of this study was to visualize the co-localization between αSyn and the SNARE proteins (VAMP-2, SNAP-25, and syntaxin-1) for the first time using *in situ* proximity ligation assay (PLA). Cortical primary neurons were cultured from either non-transgenic or transgenic mice expressing human αSyn with the A30P mutation under the Thy-1 promoter. With an antibody recognizing both mouse and human αSyn, a PLA signal indicating close proximity between αSyn and the three SNARE proteins was observed both in the soma and throughout the processes. No differences in the extent of PLA signals were seen between non-transgenic and transgenic neurons. With an antibody specific against human αSyn, the PLA signal was mostly located to the soma and was only present in a few cells. Taken together, *in situ* PLA is a method that can be used to investigate the co-localization of αSyn and the SNARE proteins in primary neuronal cultures.

Keywords: alpha-synuclein, SNARE, VAMP-2, SNAP-25, syntaxin-1, proximity ligation assay, primary neurons

INTRODUCTION

Alpha-synuclein (αSyn) is a presynaptic protein implicated in the pathology of Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy. In its native state αSyn is an unfolded monomer, but under certain circumstances it can adopt a partly folded conformation and start an aggregation cascade, resulting in oligomers of increasing sizes and finally insoluble fibrils (1). The resulting inclusions, known as Lewy bodies and Lewy neurites, are the pathological hallmark of PD and related diseases (2). The presence of these inclusions is not enough to explain the neurodegeneration in the actual disorders, as the amount of Lewy bodies does not correlate with disease severity (3, 4). It has been suggested instead that smaller αSyn aggregates can cause synaptic dysfunction and loss of synapses. For example, up to 90% of all αSyn aggregates have been described to be located at the synapses in DLB brains with an associated loss of presynaptic proteins (5).

The physiological function of αSyn has remained unclear, but due to its presynaptic localization and interactions with membrane lipids (6) it has been hypothesized that it might play a role in neurotransmitter release. Recently, αSyn has been reported to chaperone the assembly of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex by interacting with VAMP-2 (7). The SNARE proteins are critical for neurotransmitter release, as their assembly into a complex promotes the fusion of synaptic vesicles to the presynaptic membrane. They are classified in two groups: vesicle-SNAREs (v-SNAREs), which are bound to synaptic vesicles, i.e., VAMP-2, and the target-SNAREs (t-SNAREs), which are bound to the presynaptic membrane, i.e., SNAP-25 and syntaxin-1. When v-SNAREs and t-SNAREs assemble into a complex, the membranes fuse (8). Experimental evidence suggests that alpha-synuclein directly binds the N-terminus of VAMP-2 (1–28) with its C-terminus (96–140) (9).

It is unknown if the interaction between αSyn and the SNARE proteins is affected in PD and other α-synucleinopathies. Previous studies on mouse models for such disorders have shown that a 1–120 truncated αSyn can cause the redistribution of VAMP-2, SNAP-25, and syntaxin-1 in the synapse (10). One of the most widely studied αSyn transgenic (tg) models expresses human αSyn (h-αSyn) containing the point mutation A30P, which has been found to cause PD in humans (11). The tg A30P αSyn mice present PD-like motor symptoms at around 8 months of age and there are abundant αSyn aggregates found throughout the brainstem and midbrain (12–15). The motor symptoms have been associated with an increase in soluble αSyn protofibrils in the spinal cord (16). However, it is unknown whether the interaction between αSyn and the SNARE proteins is affected in these mice.

Interaction between two proteins in either cells or tissues can be visualized with the proximity ligation assay (PLA) (17). With this technique, the protein partners are targeted with two primary antibodies raised in different species and a pair of oligonucleotide labeled secondary antibodies (PLA probes). If the proteins of interest are located in close proximity to each other, two circularization oligonucleotides will be hybridized to the PLA probes and then ligated together, forming a circular DNA strand which will be the template for a rolling circle amplification step. Fluorescently labeled oligonucleotides will then be added and hybridized with the concatemeric construct.

In this study, we used PLA to investigate the molecular interaction between αSyn and the SNARE proteins in cortical primary neurons from non-tg and tg (Thy-1)-h[A30P] αSyn mice.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the animal ethics committee of Uppsala, Sweden (C75/13, C92/14). The use and care of the animals were conducted in accordance with the EU Directive 2010/63/EU for animal experiments. C57BL/6 and Tg (Thy-1)-h[A30P] αSyn mice pregnant females were used for the extraction of E14 embryos. Non-transgenic (C57BL/6) mice were obtained from Jackson laboratory (Bar Harbor, ME, USA).

Cortical Primary Neuron Cultures

Cortices from E14 embryos were dissected in Hank's buffered salt solution supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 8 mM HEPES buffer (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 90,000 cells/ml were plated on poly-L-ornithine (Sigma-Aldrich, St. Louis, MO, USA) and laminin coated (Thermo Fisher Scientific) cover slips. Cells were grown in neurobasal medium supplemented with B27, 100 U/ml penicillin, 100 µg/ml streptomycin, and L-glutamine 2 mM (Thermo Fisher Scientific). The cells were maintained at 37°C, 5% CO_2, until they were fixated with paraformaldehyde 4% 12 days later.

Cell Lysis

The cells were washed with phosphate-buffered saline (PBS) containing a protease inhibitor cocktail tablet (PIC, cOmplete, EDTA-free, Roche, Basel, Switzerland) and scraped. After centrifugation at 2,000 × *g* for 5 min, the lysis was performed incubating the cells in PBS with PIC and 1% Triton X-100 (Sigma-Aldrich) for 5 min. After centrifuging at 16,000 × *g* for 5 min, the supernatant was saved.

Sandwich Enzyme-Linked Immunosorbent Assay

A 96-well high-binding polystyrene plate (Corning Inc., Corning, NY, USA) was coated with 50 ng/well of either mouse monoclonal clone 42/alpha-synuclein antibody (BD Biosciences, San Jose, CA, USA) for total (i.e., m-αSyn and h-αSyn) αSyn detection or mouse monoclonal 4B12 anti-αSyn antibody (Eurogentec, Osaka, Japan) for detection of h-αSyn and was incubated at 4°C overnight. After blocking the plate for 2 h with PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich), the primary neuron lysates were incubated for 2 h at room temperature, together with serial dilutions of recombinant monomeric αSyn as a standard. Next the detection antibody FL-140 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was incubated for 1 h at room temperature at 1 µg/ml, followed by 1 h incubation of goat-anti-rabbit-HRP secondary antibody at 1:10,000 at room temperature. The reaction was developed with K-Blue Aqueous TMB substrate (Neogen Corporation, Lexington, KY, USA) and 1 M sulfuric acid (Sigma-Aldrich). Between every step 5× washes were performed with washing buffer (6.5 mM sodium dihydrogen phosphate monohydrate, 43.5 mM di-sodium hydrogen phosphate dihydrate, 0.3 M sodium chloride, and 0.1% Tween-20) in a HydroSpeed microplate washer (Tecan, Männedorf, Switzerland). The absorbance was measured at 450 nm using an Infinite M200 Pro microplate reader (Tecan). The reactions were performed in duplicates and their signal was averaged. The blank signal was deducted from the sample signal.

Antibodies

The following antibodies were used for the immunofluorescence and PLA experiments, with the same concentration for both techniques: mouse monoclonal mAb1338 recognizing both endogenous mouse αSyn (m-αSyn) and human αSyn (h-αSyn)

at 4 µg/ml (R&D Systems, Minneapolis, MN, USA), mouse monoclonal Syn 204 against h-αSyn at 4 µg/ml (Santa Cruz Biotechnology), rabbit monoclonal EPR12790 against VAMP-2 at 2 µg/ml (Abcam, Cambridge, UK), rabbit monoclonal EP3274 against SNAP-25 at 4 µg/ml (Abcam), rabbit polyclonal against syntaxin-1 at 1:1,000 (ABR-Affinity Bioreagents, Golden, CO, USA), rabbit polyclonal AB5622 against microtubule associate protein MAP2 at 1:200 (Merck Millipore, Burlington, MA, USA), and rabbit monoclonal C39A9 against nucleoporin NUP98 at 1:50 (Cell Signaling Technologies, Danvers, MA, USA).

Immunofluorescence

Fixed cells were permeabilized and blocked with PBS containing 0.1% Triton X-100 and 5% normal goat serum for 30 min at room temperature. The cells were incubated with primary antibodies for 1 h at room temperature in PBS with normal goat serum 0.5%. After three PBS washes, the cells were incubated with the secondary antibodies (goat anti-rabbit Alexa 488 or goat anti-mouse Alexa 594, Thermo Fisher Scientific) at 2 µg/ml in PBS with normal goat serum 0.5% for 1 h at room temperature. After three PBS washes, the cover slips were mounted with Vectashield hard set mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

"*In situ*" PLA

The *in situ* PLA was performed on fixed primary neurons with DuoLink PLA technology probes and reagents (Sigma-Aldrich), and following the manufacturers protocol. First the neurons were permeabilized with PBS + Triton X-100 0.4% for 10 min. After two PBS washes, the cells were incubated with blocking solution for 30 min at 37°C and then with the primary antibodies for 1 h at room temperature. Every experiment was performed with a pair of antibodies of different species. The cover slips were washed twice for 5 min with buffer A, followed by incubation with the PLA probes (secondary antibodies against two different species bound to two oligonucleotides: anti-mouse MINUS and anti-rabbit PLUS) in antibody diluent for 60 min at 37°C. After two washes of 5 min with buffer A, the ligation step was performed with ligase diluted in ligation stock for 30 min at 37°C. In the ligation step, the two oligonucleotides in the PLA probes are hybridized to the circularization oligonucleotides. The cells were washed with buffer A twice for 2 min before incubation for 100 min with amplification stock solution at 37°C. The amplification stock solution contains polymerase for the rolling circle amplification step and oligonucleotides labeled with fluorophores, which will bind to the product of the rolling circle amplification and thus allow detection. After two washes of 10 min with buffer B, the

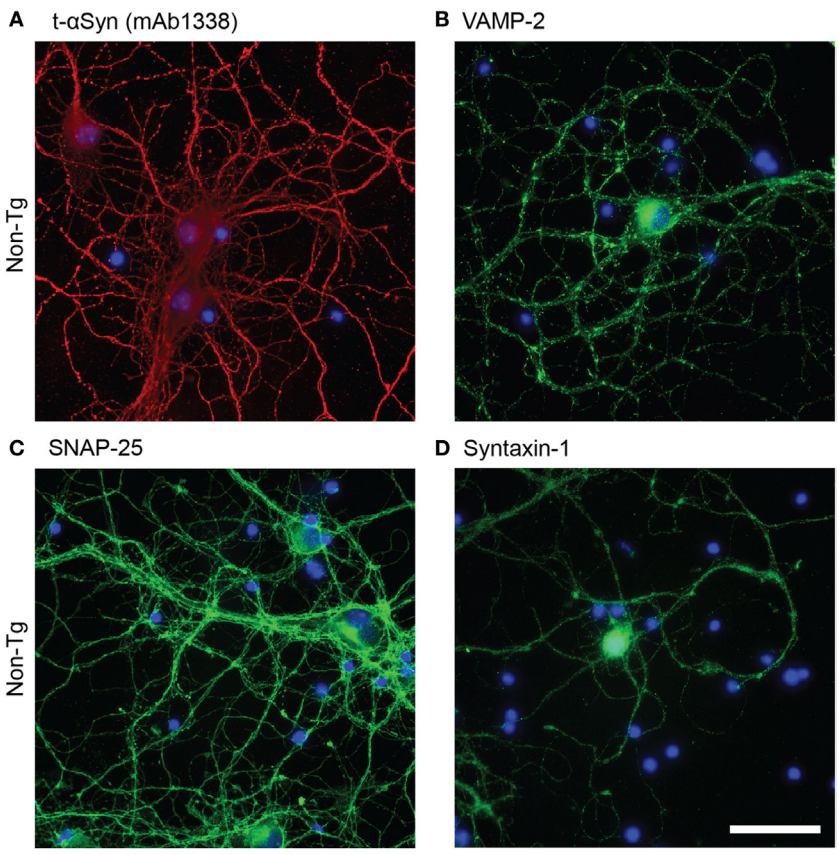

FIGURE 1 | Representative images of immunofluorescence staining of non-tg cortical primary neurons (12 DIV) using antibodies against alpha-synuclein (αSyn) [mAb1338, red, **(A)**], VAMP-2 [(green), **(B)**], SNAP-25 [(green), **(C)**], and syntaxin-1 [(green), **(D)**]. DAPI in blue. Scale bar 50 µm.

cells were incubated with FITC-conjugated phalloidin (Sigma-Aldrich) at 1.25 µg/ml. Finally, the cover slips were washed with PBS and mounted with Duolink *in situ* mounting medium containing DAPI. For every antibody, a negative control experiment was performed where only one antibody was incubated with the PLA probes. The experiments were performed three times on non-tg neurons and twice on tg neurons. Each experiment was performed with neurons originated from embryos of different mothers.

Microscopy and Image Analysis

Immunofluorescence staining was imaged with a Zeiss Axio Observer Z1 (Zeiss, Oberkochen, Germany). Quantification of h-αSyn positive cells was performed in 10 images of non-tg cells and 20 images of tg cells.

Proximity ligation assay imaging was performed with a confocal laser scanning microscope (LSM700, Zeiss). z-Stacks were captured with sections spanning entire cells. Zeiss software Zen 2.3 Blue edition was used to obtain maximum intensity projections and cross-sections of the confocal images. Quantification of PLA signal was performed on z-stack images taken systematically with a Zeiss Axio Observer Z1 microscope. Background signal was reduced by deconvolution using the Huygen software. Live cells with non-condensed nuclei were manually counted as number of neurons per image. The soma was manually outlined for each cell and number of puncta per soma was counted with ImageJ 3D Objects Counter. The number of puncta in the processes was calculated by counting total number of puncta per image and subtracting number of puncta in the soma. The number of puncta was normalized by number of cells to obtain mean PLA puncta/cell. The threshold was set automatically using ImageJ 3D Objects Counter for each image and kept constant as the puncta in the soma were measured separately. Ten z-stack images were quantified per staining. The microscope settings were kept constant for all images to enable direct comparison. The quantification was performed on one set of experiments, when all stainings were performed at the same time. A sampling of the images is included as Figure S1 in Supplementary Material, with examples of the final thresholded images that were quantified. Quantification of cells with positive PLA signal between h-αSyn and SNARE proteins was performed manually in 10 images of tg cells.

Statistical Analysis

The data were analyzed using GraphPad Prism. Two-tailed Student's *t*-test was used to compare the amount of alpha-synuclein in cell lysates. One-way ANOVA followed by Bonferroni *post hoc* test was performed to compare the PLA puncta in soma/processes of non-tg and tg (Thy-1)-h[A30P] primary neurons.

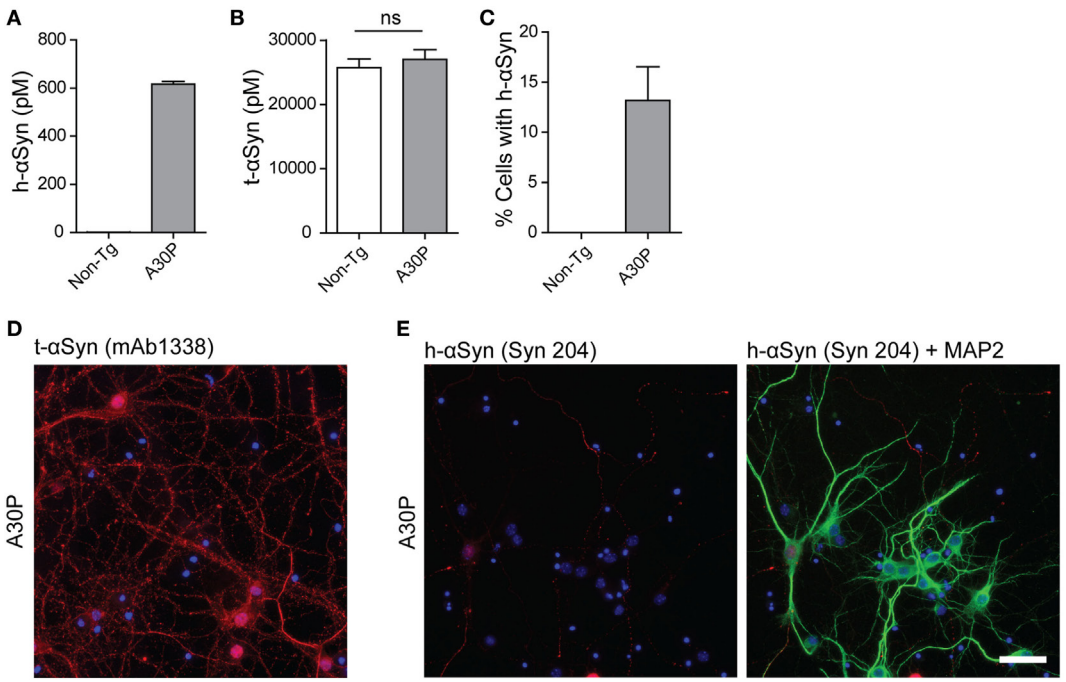

FIGURE 2 | Characterization of primary neurons from tg A30P mice. Levels of h-alpha-synuclein (αSyn) in lysates of non-tg and A30P neuronal cultures were measured by ELISA, using the h-αSyn specific antibody 4B12 as a capture antibody **(A)**. Levels of total (t)-αSyn (m-αSyn + h-αSyn) in lysates of non-tg and A30P neuronal cultures were measured by ELISA, using clone 42 as a capture antibody. Two-tailed Student's *t*-test, non-significant (ns), error bars represent the SEM **(B)**. Quantification of h-αSyn positive non-tg and A30P neurons, observed by immunofluorescence with h-αSyn specific antibody Syn 204 **(C)**. Representative images of immunofluorescence of tg A30P neurons with t-αSyn mAb1338 **(D)**. Representative images of immunofluorescence with h-αSyn specific antibody Syn 204 of A30P cortical neurons (red). MAP2 neuronal marker (green) and DAPI (blue) **(E)**. Scale bar 50 µm.

RESULTS

The aim of this study was to visualize the co-localization between αSyn and SNARE proteins in non-transgenic primary neurons and primary neurons overexpressing A30P h-αSyn using PLA. First, we cultured cortical primary neurons of non-tg mouse embryos to observe the localization of mouse αSyn (m-αSyn) and the SNARE proteins, using conventional immunofluorescence. In addition to characterizing the expression pattern of the proteins of interest, it ensured that the antibodies were suitable candidates for PLA.

Antibody mAb1338 recognizes both m-αSyn and h-αSyn and can thus be used to detect total alpha-synuclein (t-αSyn). The αSyn staining was observed throughout the cell body and processes (**Figure 1A**). Similarly, the SNARE proteins (VAMP-2, SNAP-25, and syntaxin-1) were abundantly expressed (**Figure 1B-D**), particularly in cell processes, where a more dotted pattern could be observed; presumably due to the presence in the synaptic boutons.

We next cultured cortical primary neurons from tg (Thy-1)-h[A30P] αSyn mouse embryos and analyzed the expression and localization of m-αSyn and h-αSyn by ELISA on cell lysates and immunofluorescence (**Figure 2**). Human-αSyn was detected in lysates from A30P primary neurons, at a concentration of 600 pM and, as expected, not at all in non-tg primary neurons (**Figure 2A**). The levels of t-αSyn (m-αSyn in the case of non-tg neurons and both m-αSyn and h-αSyn in tg neurons) were found to be similar in both types of primary neurons, with h-αSyn constituting approximately 2% of all αSyn

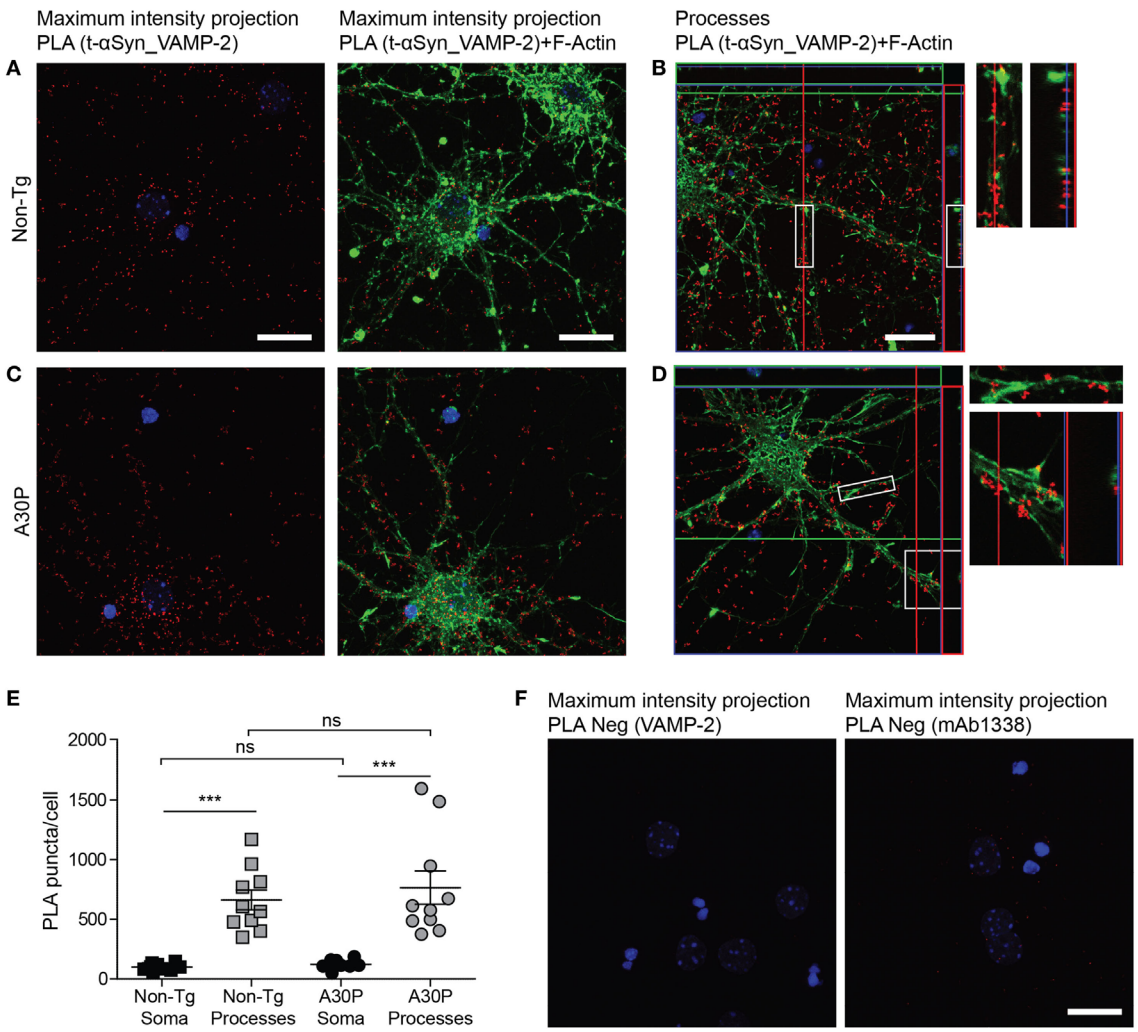

FIGURE 3 | Representative confocal images of *in situ* proximity ligation assay (PLA) between alpha-synuclein (αSyn) (mAb1338) and VAMP-2 (red) in non-tg **(A,B)** and A30P **(C,D)** cortical primary neurons. Maximum intensity projections of a confocal *z*-stack including a whole cell were performed to observe the maximum amount of PLA puncta **(A,C)**. Cross section of the processes allows visualization of the PLA puncta in an orthogonal view [**(B,D)**, inlet], scale bar 20 μm. F-actin stained by phalloidin-FITC (green) and DAPI (blue). Quantification of amount of PLA puncta per cell in the soma and processes. One-way ANOVA followed by Bonferroni *post hoc* test, non-significant (ns), ***$P < 0.001$. Error bars represent the SEM **(E)**. Negative control PLA with VAMP-2 antibody only and αSyn antibody (mAb1338) only **(F)**. Scale bar 20 μm.

detected in A30P primary neurons (**Figure 2B**). We performed immunofluorescence of the cultured A30P neurons with the h-αSyn-specific antibody Syn 204 and observed that 13% of the neurons expressed detectable levels of h-αSyn (**Figure 2C**). On the other hand, antibody mAb1338 against t-αSyn stained all A30P neurons, similarly to non-tg neurons, indicating that the distribution of m-αSyn was similar in both non-tg and tg neurons (**Figure 2D**). The h-αSyn was expressed mostly in the cell body and around the nucleus of the neurons, but rarely in processes (**Figure 2E**).

Next, we performed *in situ* PLA between αSyn and the three SNAREs in non-tg and A30P primary neurons. For these analyses, we used mAb1338 antibody, which recognized both m-αSyn and h-αSyn. Theoretically, each PLA dot is the result of the close proximity of one molecule of αSyn and one molecule of one of the SNARE proteins. As a negative control to detect potential unspecific signal, we performed the assay removing one of the primary antibodies, thus one of the secondary antibodies will have no primary antibody to bind and subsequently no PLA signal will be generated.

Abundant PLA signal (red dots) was observed in both sets of primary neurons, indicating a close proximity between αSyn and VAMP-2 (**Figures 3A–D**). F-actin staining with phalloidin-FITC (green) was performed to observe the cytoskeleton of the neurons and locate the PLA puncta within the cell. Confocal images showed abundant PLA puncta in the processes (**Figures 3B,D**), a significantly higher number than those found in the soma (**Figure 3E**). No significant differences were found in the amount

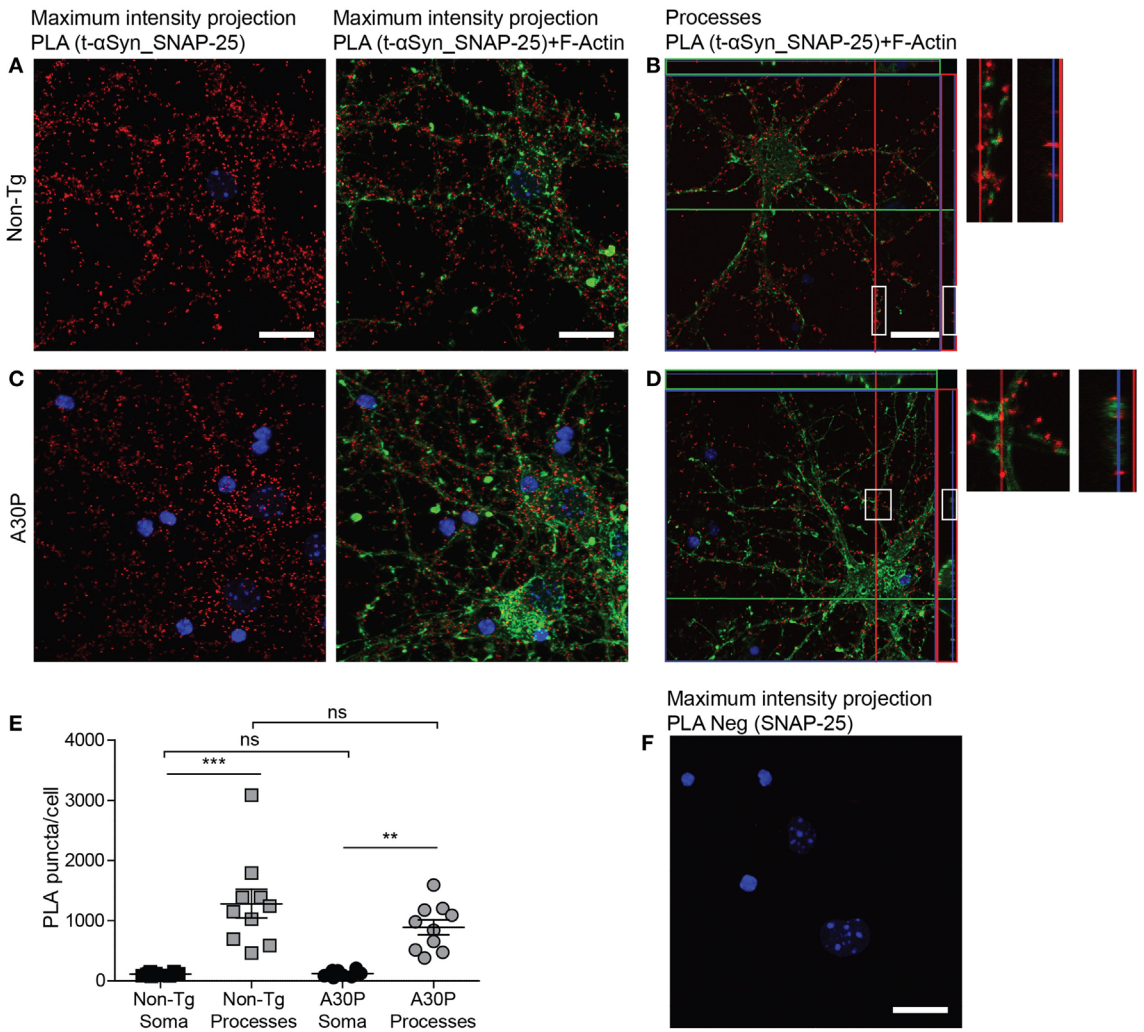

FIGURE 4 | Representative confocal images of *in situ* proximity ligation assay (PLA) between alpha-synuclein (αSyn) (mAb1338) and SNAP-25 (red) in non-tg **(A,B)** and A30P **(C,D)** cortical primary neurons. Maximum intensity projections of a confocal z-stack including a whole cell were performed to observe the maximum amount of PLA puncta **(A,C)**. Cross section of the processes allows visualization of the PLA puncta in an orthogonal view [**(B,D)**, inlet], scale bar 20 μm. F-actin stained by phalloidin-FITC (green) and DAPI (blue). Quantification of amount of PLA puncta per cell in the soma and processes. One-way ANOVA followed by Bonferroni *post hoc* test, non-significant (ns), ***$P < 0.001$. Error bars represent the SEM **(E)**. Negative control PLA with SNAP-25 antibody only **(F)**. Scale bar 20 μm.

of PLA puncta between non-tg and tg A30P αSyn neurons in either processes or the soma (**Figure 3E**). Very few PLA puncta were observed when the VAMP-2 and αSyn antibodies were used on their own, as negative controls (**Figure 3F**).

A PLA signal was also observed for αSyn and SNAP-25 (**Figures 4A–D**), and the distribution of the PLA puncta was similar to the αSyn and VAMP-2 PLA, where the PLA puncta were significantly more abundant in the processes than in the soma (**Figure 4E**). The amount of PLA puncta per cell was not significantly different between non-tg and tg cells (**Figure 4E**). Very low signals were observed in control experiments when SNAP-25 antibody was used alone in PLA (**Figure 4F**).

Proximity ligation assay against syntaxin-1 and αSyn produced a positive signal in both non-tg and tg neurons (**Figures 5A–D**), but no differences were observed in the amount of PLA puncta between the two types of cells (**Figure 5E**). As expected due to the subcellular location of syntaxin-1 (**Figure 1D**), the puncta were significantly more abundant in the processes, compared with the soma (**Figure 5E**). Very few puncta were observed when the syntaxin-1 antibody was used alone in control experiments (**Figure 5F**).

To study the difference in distribution of the interaction between the SNAREs and m-αSyn or h-αSyn, respectively, we performed PLA with the h-αSyn specific antibody Syn 204 and VAMP-2, SNAP-25 and syntaxin-1 (**Figures 6A–F**). All three antibody combinations showed similar results: only a fraction of the cells were PLA positive, in agreement with what we observed with conventional immunofluorescence with Syn 204,

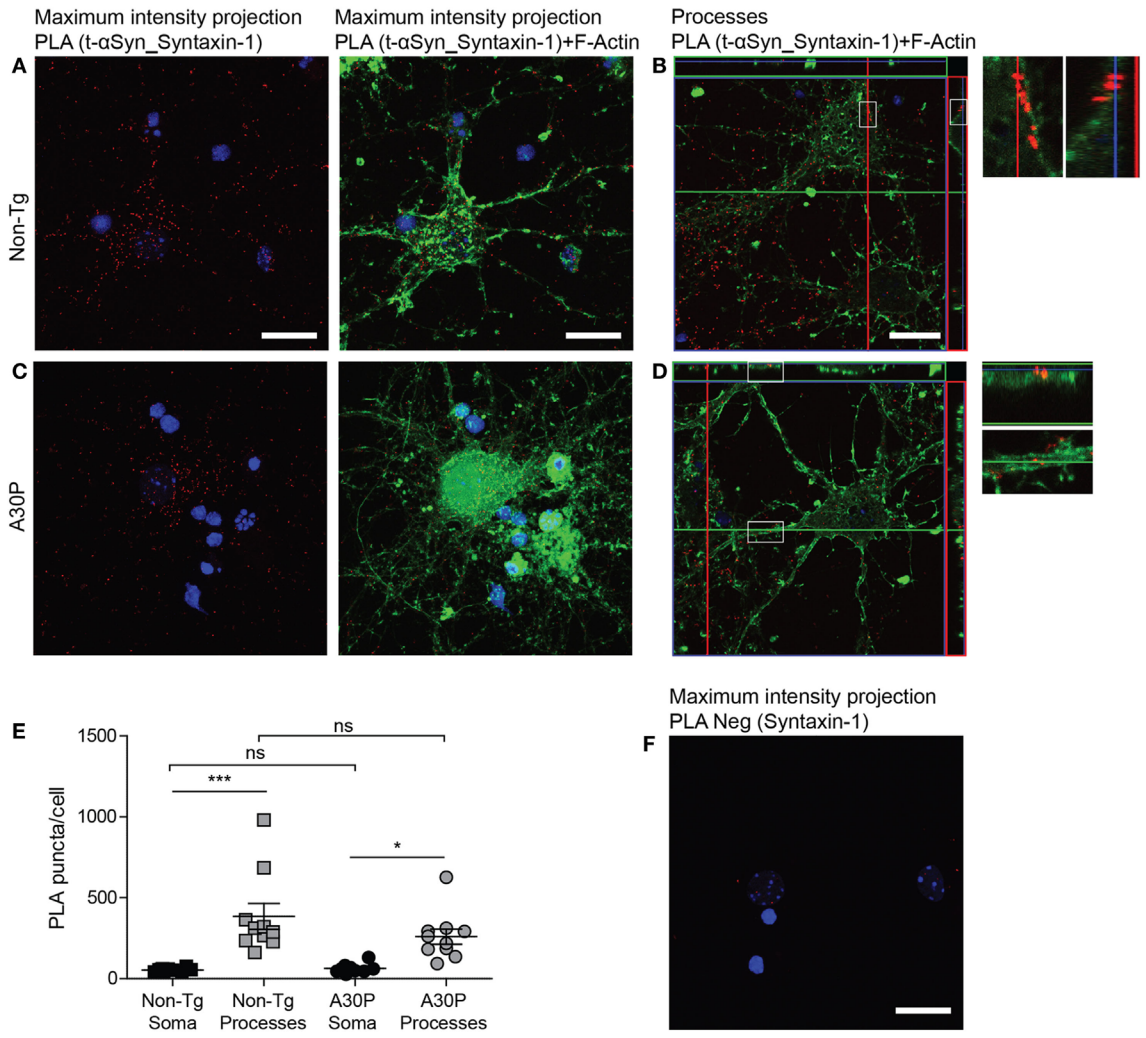

FIGURE 5 | Representative confocal images of *in situ* proximity ligation assay (PLA) between alpha-synuclein (αSyn) (mAb1338) and syntaxin-1 (red) in non-tg **(A,B)** and A30P **(C,D)** cortical primary neurons. Maximum intensity projections of a confocal z-stack including a whole cell were performed to observe the maximum amount of PLA puncta **(A,C)**. Cross section of the processes allows visualization of the PLA puncta in an orthogonal view (**B,D**, inlet), scale bar 20 μm. F-actin stained by phalloidin-FITC (green) and DAPI (blue). Quantification of amount of PLA puncta per cell in the soma and processes. One-way ANOVA followed by Bonferroni *post hoc* test, non-significant (ns), ***$P < 0.001$. Error bars represent the SEM **(E)** Negative control PLA with syntaxin-1 antibody only **(F)**. Scale bar 50 μm.

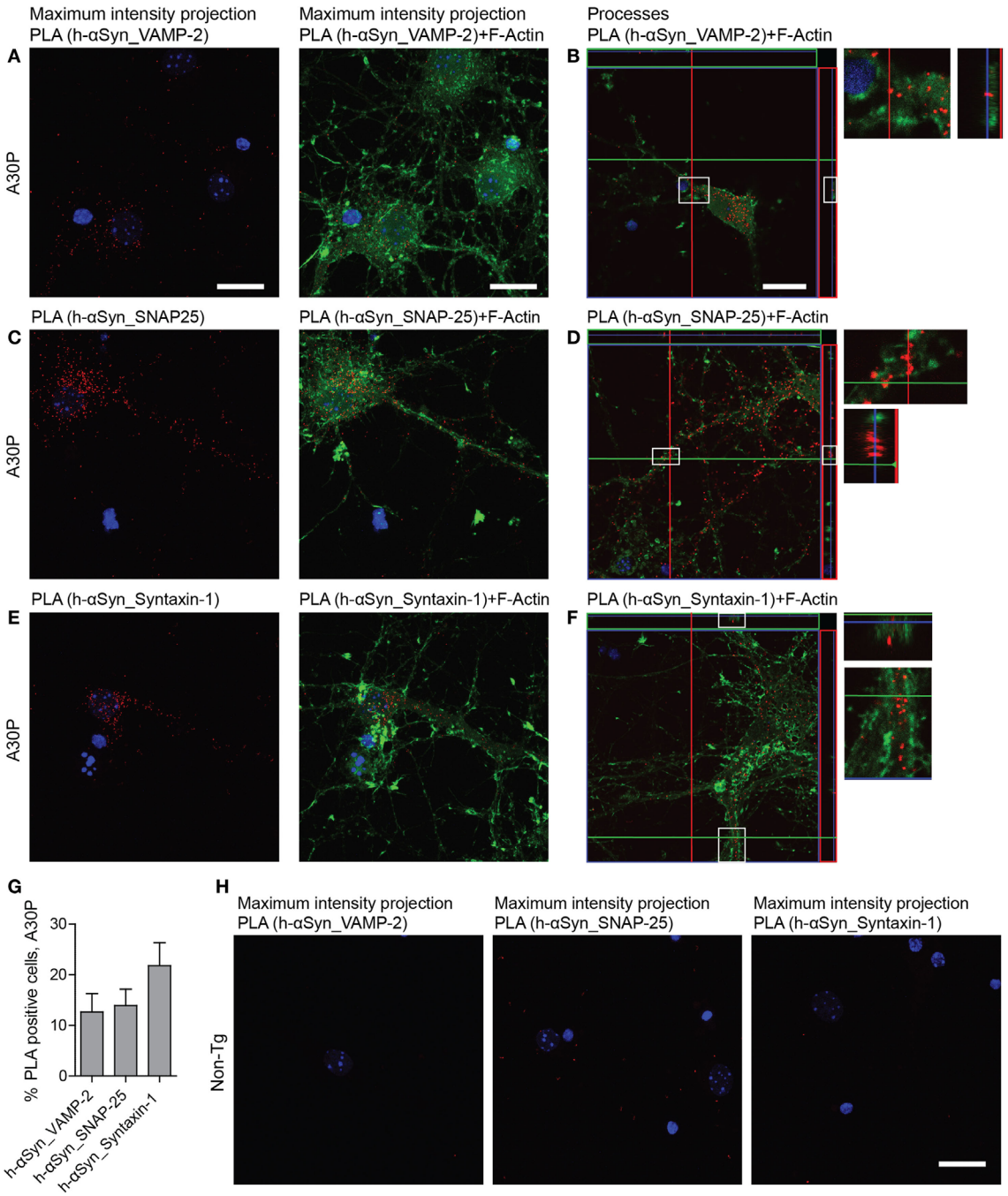

FIGURE 6 | Representative confocal images of *in situ* proximity ligation assay (PLA) (red) between h-alpha-synuclein (αSyn) (Syn 204) and VAMP-2 **(A,B)**, h-αSyn (Syn 204) and SNAP-25 **(C,D)**, and h-αSyn (Syn 204) and syntaxin-1 **(E,F)** in tg A30P neurons and in non-tg neurons **(H)**. Maximum intensity projections of a confocal z-stack including a whole cell were performed to observe the maximum amount of PLA puncta **(A,C,E)**. Cross section of the processes allows visualization of the PLA puncta in an orthogonal view [**(B,D,F)**, inlet], scale bar 20 μm. F-actin stained by phalloidin-FITC (green) and DAPI (blue). Quantification of PLA between h-αSyn and VAMP-2, h-αSyn and SNAP-25, and h-αSyn and syntaxin-1 in A30P neurons **(G)**.

where 13% of all cells expressed h-αSyn (**Figure 6G**). Most of the h-αSyn-SNARE PLA signal was observed in the neuronal body, with some rare instances where the signal could also be seen in processes. As expected, very few PLA puncta were observed in non-tg primary neurons with h-αSyn specific antibody Syn 204 (**Figure 6H**).

The nuclear pore complex protein NUP98 was used in PLA to further confirm that PLA signal is indicative of proximity between two proteins, as αSyn can be found in the nucleus (18, 19). First we performed immunofluorescence staining of the nuclear pore complex protein NUP98, which gave a perinuclear staining (**Figure 7A**). PLA with NUP98 and αSyn antibody mAb1338

only produced a few dots, indicating that those two proteins were not in close proximity to each other (**Figure 7B**). The results of the immunofluorescence staining and PLA were similar in non-tg and tg neurons.

DISCUSSION

In this study, we can for the first time visualize the co-localization between αSyn and the SNARE proteins in cultured primary neurons by using *in situ* PLA. Previous studies have demonstrated the use of PLA to study αSyn interacting with other proteins, such as the dopamine transporter and αSyn complexes in a tg mouse overexpressing αSyn 1–120 (20). Interactions with TOM20 (21) and the synaptic protein synapsin III (22, 23) have also been observed with PLA. In addition, PLA has been used to specifically detect oligomeric αSyn in brain tissue from PD patients (24).

Direct binding between proteins cannot be proven through *in situ* PLA, as the length of the probes and antibodies theoretically allows proteins with a proximity of up to 40 nm to give a positive PLA signal (25). However, Burré et al. showed that m-αSyn co-immunoprecipitates with VAMP-2, SNAP-25 and syntaxin-1 in brain lysates from non-transgenic mice (7). This observation is indeed in agreement with our PLA results, in which we can see that αSyn is in close proximity to all three SNAREs in non-tg primary neurons, presumably due to its presynaptic localization and membrane binding properties. The PLA puncta were observed to a significantly higher degree in the neuronal processes, presumably indicating the location of synaptic boutons.

We did not observe any significant differences in PLA signal between αSyn and any of the SNARE proteins when comparing primary neurons from non-tg and A30P tg mice. This can be explained by the low amount of h-αSyn present in the tg primary neurons, which according to our ELISA analysis constituted about 2% of the total αSyn detected. This is in contrast to the twofold increase of h-αSyn relative m-αSyn levels observed in adult A30P tg mice (14). The reason for the discrepancy in expressed protein *in vivo* and *in vitro*, as well as for the predominant localization of h-αSyn to the soma, is probably due to the late expression of Thy-1 promoter in the primary neurons (26).

The A30P αSyn mutation exhibits a lower binding affinity for lipid membranes and has a reduced ability to promote SNARE complex formation, compared with wt αSyn and other disease-causing mutations (27, 28). Nevertheless, in the present study we could show that although the amount of h-αSyn positive cells was low (about 13%), positive PLA signals were observed between

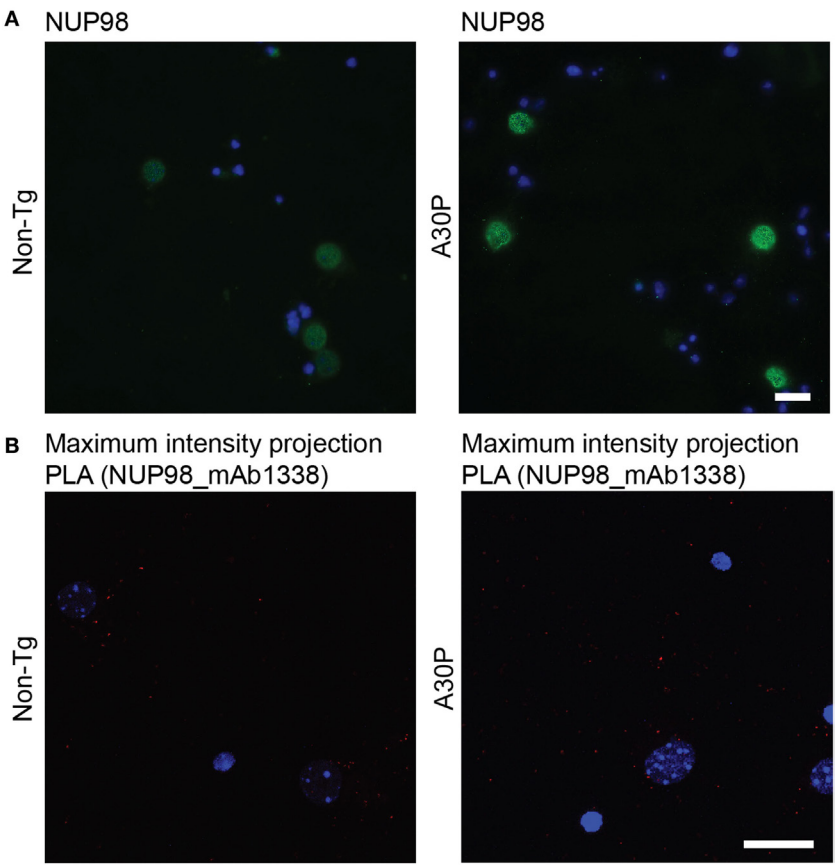

FIGURE 7 | Representative images of NUP98 immunofluorescence staining (green) in non-tg and A30P alpha-synuclein (αSyn) tg primary neurons, DAPI (blue) **(A)**. Representative confocal images (maximum intensity projection) of *in situ* proximity ligation assay (PLA) in non-tg and A30P cortical primary neurons between αSyn (mAb1338) and the NUP98 (red), DAPI (blue) **(B)**. Scale bar 20 μm.

h-αSyn and all three SNARE proteins. This could perhaps be explained by the finding that there is much smaller difference in relative binding affinity between A30P and wt h-αSyn for highly curved membranes such as synaptic vesicles (6).

Taken together, we can demonstrate that *in situ* PLA is a suitable technique for the study of αSyn co-localization with SNARE proteins in primary neurons. The finding of robust PLA signals between m-αSyn and all three SNARE proteins in the processes, suggests that non-tg primary neurons could be a useful model to study the physiological interaction between αSyn and presynaptic proteins.

ETHICS STATEMENT

This study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments. The protocol was approved by the animal ethics committee of Uppsala, Sweden (C75/13, C92/14).

AUTHOR CONTRIBUTIONS

LA-G, EP, AE, and JB designed the experiments. LA-G, EP, and VL performed experiments. LA-G, EP, VL, MI, AE, and JB analyzed data. LA-G, EP, MI, and JB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00180/full#supplementary-material.

FIGURE S1 | *In situ* PLA (red) between alpha-synuclein (αSyn) and VAMP-2 **(A)**, αSyn and SNAP-25 **(B)**, and αSyn and syntaxin-1 **(C)**. Sample maximum intensity projections of unprocessed images, which were used for quantification of *in situ* proximity ligation assay (PLA) between αSyn and SNAREs in non-tg and A30P cortical primary neurons [**(A–C)**, upper panel]. The lower panels **(A–C)** display the red channel of the same images after deconvolution, and set to the same threshold as was used for the quantification. All PLA puncta from each plane were quantified with ImageJ 3D Objects Counter on deconvolved and thresholded z-stacks. DAPI in blue. Scale bar 20 μm.

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REVIEW
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The Close Encounter Between Alpha-Synuclein and Mitochondria

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The presynaptic protein alpha-synuclein (α-syn) is unequivocally linked to the development of Parkinson's disease (PD). Not only it is the major component of amyloid fibrils found in Lewy bodies but mutations and duplication/triplication in its gene are responsible for the onset of familial autosomal dominant forms of PD. Nevertheless, the precise mechanisms leading to neuronal degeneration are not fully understood. Several lines of evidence suggest that impaired autophagy clearance and mitochondrial dysfunctions such as bioenergetics and calcium handling defects and alteration in mitochondrial morphology might play a pivotal role in the etiology and progression of PD, and indicate the intriguing possibility that α-syn could be involved in the control of mitochondrial function both in physiological and pathological conditions. In favor of this, it has been shown that a fraction of cellular α-syn can selectively localize to mitochondrial sub-compartments upon specific stimuli, highlighting possible novel routes for α-syn action. A plethora of mitochondrial processes, including cytochrome c release, calcium homeostasis, control of mitochondrial membrane potential and ATP production, is directly influenced by α-syn. Eventually, α-syn localization within mitochondria may also account for its aggregation state, making the α-syn/mitochondria intimate relationship a potential key for the understanding of PD pathogenesis. Here, we will deeply survey the recent literature in the field by focusing our attention on the processes directly controlled by α-syn within mitochondrial sub-compartments and its potential partners providing possible hints for future therapeutic targets.

Keywords: alpha-synuclein, mitochondria, Parkinson disease, neurodegeneration, bioenergetics

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting more than 50000 people each year only in the US[1,2]. Worldwide, more than 10 million people are estimated to suffer from PD. Clinically, the selective loss of dopaminergic neurons in the substantia nigra pars compacta leads to motor dysfunction and the appearance of bradykinesia, resting tremor, rigidity and postural instability. Although some approved drugs alleviate the symptoms of PD, the disease is still incurable.

More than 90% of PD cases are sporadic. A small percentage, however, is due to mutations in specific genes. *SNCA* was the first gene to be associated to familial cases of PD

[1]http://parkinson.org/Understanding-Parkinsons/Causes-and-Statistics/Statistics
[2]https://report.nih.gov/NIHfactsheets/ViewFactSheet.aspx?csid=109

(Polymeropoulos et al., 1997) and it encodes alpha-synuclein (α-syn), a small 14 kDa protein that was also found to accumulate in the Lewy bodies, the proteinaceous structures that mark the histopathology of PD (Spillantini et al., 1998). The 140 amino acids sequence of α-syn can be divided in three major regions (**Figure 1**): the amphipathic N-terminus, the hydrophobic central non-amyloid-beta component (NAC) and the acidic C-terminal domain.

The N-terminal domain is enriched in lysines and is characterized by the presence of 7 repeats of 11 residues each among which the KTKEGV motif is the core consensus sequence. The pathological mutations (A30P, A53E/T, E46K, G51D and H50Q) cluster in this domain, which is likely involved in the interaction with membranes. Indeed, the A30P mutation disrupts the interaction of α-syn with lipid rafts and causes the redistribution of α-syn in the axon (Jensen et al., 1998; Fortin et al., 2004). The NAC region was originally discovered in amyloid from the cortex of Alzheimer's disease patients (Ueda et al., 1993) and is essential for the aggregation of α-syn molecules, since deletions within this region abrogate the assembly of α-syn into filaments both *in vitro* and in intact cells (Giasson et al., 2001; Luk et al., 2009). The C-terminal tail displays many charged residues, lacks secondary structure bias and contains the pathologically relevant phosphorylation sites cluster.

The expression of α-syn is limited to the subphylum of vertebrates. It is highly expressed in both the central and peripheral nervous system and it is particularly enriched in the nerve terminal (Maroteaux et al., 1988; Yang et al., 2010; Vivacqua et al., 2011); at lower levels, it is also present in other tissues like hearth and muscles[3].

The function of α-syn within the cell is still not completely clear, but its localization at presynaptic terminal reflects a role in synaptic transmission. α-syn co-localizes with synaptic vesicles and plays a role in the fast kinetics of synaptic vesicle endocytosis, indeed, this process is impaired in αβγ-syn$^{-/-}$ neurons and rescued by α-syn reintroduction (Lee et al., 2008; Zhang et al., 2008; Vargas et al., 2014). The number of presynaptic vesicles was reduced in cultured neurons from α-syn knockout mice (KO) as well as the size of synaptic puncta in αβγ-synuclein triple KO, (Cabin et al., 2002; Greten-Harrison et al., 2010). At the synapses, α-syn promoted the assembly of the SNARE complex by interacting with synaptobrevin 2 (VAMP2) and phospholipids through its C- and N- terminus, respectively (Burre et al., 2010).

In addition to its role at synapses, α-syn plays a role in the nucleus. Its name, indeed, reflects a nuclear localization (Maroteaux et al., 1988; Mori et al., 2002; Goncalves and Outeiro, 2013). Within the nucleus, α-syn affects the expression of several genes, including downregulation of major genes involved in DNA repair leading to increased levels of phosphorylated p53 and reduced levels of acetylated histone 3 (Paiva et al., 2017) and impaired neurogenesis by modulating Notch1 expression (Desplats et al., 2012). Interestingly, upon oxidative stress induction, α-syn nuclear localization increases and the protein binds to the promoter of several genes including PGC-1alpha, a transcription co-activator involved in metabolism and mitochondrial biogenesis (Siddiqui et al., 2012).

Either gene multiplications or point mutations in the *SNCA* gene are at the basis of familial dominantly inherited PD and result in the accumulation and aggregation of the protein in Lewy bodies within the brain (Conway et al., 1998; Pandey et al., 2006; Ghosh et al., 2013; Kara et al., 2014). The finding that gene multiplication is linked to PD suggests that α-syn expression level must be kept under tight control: when it reaches a specific threshold, wild type (wt) α-syn is sufficient *per se* to trigger PD, possibly as a consequence of augmented aggregation propensity. The issue of α-syn aggregation and conformational structure is deeply investigated, but controversial reports have appeared so far. The soluble form is a natively unfolded monomer with no defined secondary structure (Weinreb et al., 1996), but the first 100 residues are predicted to be structured in an α-helical conformation (George et al., 1995; Wang et al., 2011) that can be induced upon binding to lipids (Davidson et al., 1998). The NAC domain is responsible for α-syn aggregation: long-range hydrophobic interactions between the C-terminus and this region, as well as electrostatic interactions between the C- and the N-termini, prevent α-syn aggregation. Increases in temperature or the binding of polyamine have been shown to perturb these interactions favoring aggregation (Bertoncini et al., 2005). Recently, an aggregation resistant tetrameric structure of α-syn was described (Bartels et al., 2011; Wang et al., 2011;Dettmer et al., 2013): evidence obtained in non-neuronal and neuronal cell cultures, brain tissue, living human cells and purified protein suggested that under non-denaturing conditions α-syn is a 58 kDa tetramer, whose formation is disturbed by the introduction of A30P, A53T, E46K, H50Q, and G51D PD-related mutations (Dettmer et al., 2015). However, a different study performed on human, rat and mouse brains as well as on cell lines and *Escherichia coli* demonstrated that α-syn exists rather as a disordered monomer (Fauvet et al., 2012). All together, these findings indicate that multimeric α-syn can co-exists together with the monomeric protein in a dynamic equilibrium within the cell and, when the fraction of the unfolded monomer increases, the α-syn-mediated pathology is triggered (Dettmer et al., 2015).

Regardless of the monomeric or oligomeric nature of α-syn, its aggregation represents a key pathological feature of PD. Both oligomers and fibrils have been reported to have toxic effects on the functionality and the survival of neuronal cells. A better understanding of the physiological function of α-syn within the cell as well as of the mechanisms involved in its aggregation and propagation in the brain is essential to find new therapeutic approaches for PD. A lot of effort has been put in the last 5 years in the understanding of both the process of α-syn aggregation and the pathways responsible for aggregates elimination. Recently, a new mitochondria-mediated pathway that degrades cytosolic proteins prone to aggregation has been described in yeast and human cells. It has been named MAGIC (mitochondria as guardian in cytosol) and consists in the import of aggregation-prone proteins within mitochondria, where they will be degraded by mitochondrial

[3]https://www.proteinatlas.org/ENSG00000186868-MAPT/tissue

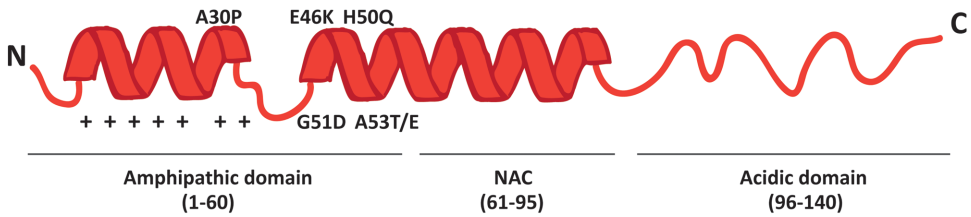

FIGURE 1 | Schematic representation of α-syn structure. The protein forms two alpha-helices when interacting with lipids and is composed of three distinct domains: the N-terminal amphipathic region, the non-amyloidogenic component (NAC) and the C-terminal acidic domain. The N-terminal domain drives α-syn to mitochondria thanks to the presence of seven positively charged lysine residues (indicated as +) and contains all the PD-related mutations.

proteases (Ruan et al., 2017). This process highlights the role of mitochondria as guardian of cell integrity and points on the necessity to deeply understand α-syn action and distribution at mitochondrial level.

ALPHA-SYNUCLEIN EFFECTS ON MITOCHONDRIA

Mitochondria are crucial players in the pathogenesis of PD. It is surprising that many (if not all) of the genes responsible for the onset of familial forms of PD, indeed, converge on mitochondria (Cieri et al., 2017) and besides the clear role played by PINK1 and Parkin as key regulators of mitochondrial integrity (Narendra et al., 2008), the list of PD-related genes linked to mitochondria is longer and α-syn is not an exception.

In this paragraph, we will briefly summarize the effects induced by α-syn on mitochondria. Several reports show contrasting results on this topic. It must be stressed, however, that differences in α-syn-induced effects may be explained by the cell type used in the experiments as well as the transfection method used, that may strongly affect protein expression levels.

α–syn itself can affect the Ca^{2+} signaling within the mitochondria as it has been repeatedly reported to influence the Ca^{2+} exchange and the physical interaction between the ER and mitochondria, despite different groups reached different conclusions (Calì et al., 2012; Guardia-Laguarta et al., 2014; Paillusson et al., 2017). Interestingly, we have also shown that addition of exogenous recombinant α-syn to cell cultures leads to a dose dependent impairment of Ca^{2+} handling, with different doses showing different effects: increased mitochondrial Ca^{2+} transients were observed upon incubation with the 4 μM exogenous α-syn, whereas a reduction was measured upon treatment with 8 μM (Calì et al., 2012). Similarly, another group reported that α-syn-induced mitochondrial fragmentation is dependent on its expression levels: whereas the expression of low levels of wt, A53T or A30P α-syn induced mitochondrial fragmentation only in the case of A53T, higher expression of the protein resulted in mitochondrial fragmentation also in the case of wt α-syn (Pozo Devoto et al., 2017).

Dynamic processes such as mitochondrial fusion/fission and axonal transport are also influenced by α-syn. Mitochondrial fragmentation induced by overexpression of mutant (A53T, A30P, E46K) α-syn has been observed, although the effect of the wt was not consistently reported (Kamp et al., 2010; Nakamura et al., 2011; Gui et al., 2012; Guardia-Laguarta et al., 2014). The expression of α-syn in sensory neurons of living zebrafish embryos resulted in the fragmentation of mitochondria, occasionally leading to their swelling within the axon (O'Donnell et al., 2014). The mitochondrial pathology is also extended to their axonal transport and to the mtDNA: mitochondrial motility was indeed reduced by α-syn expression in SH-SY5Y cells and cultured neurons derived from human embryonic stem cells (Xie and Chung, 2012; Melo et al., 2017; Pozo Devoto et al., 2017) and α-syn transgenic mice display increased mitochondrial oxidative stress and DNA lesions (Bender et al., 2013).

How α-syn induces changes in mitochondrial morphology is still unclear. Some groups have shown a direct effect on the expression of mitochondria-shaping proteins (Gui et al., 2012; Xie and Chung, 2012; Menges et al., 2017), despite a general consensus has not been reached (Kamp et al., 2010; Nakamura et al., 2011; Guardia-Laguarta et al., 2014; Pozo Devoto et al., 2017). Alternatively, a direct effect induced by the binding of α-syn to the mitochondrial membrane has been proposed. Accordingly, *in vitro* studies have shown inhibition of membrane fusion by α-syn (Kamp et al., 2010), prompting to speculate that the binding of α-syn to the mitochondrial membrane may change the curvature of the outer mitochondrial membrane (OMM) and reduce its fusion with surrounding mitochondria. This hypothesis is supported by experiments showing that the selective targeting of wt and A53T α-syn, but not A30P, to the OMM induced mitochondrial fragmentation (Pozo Devoto et al., 2017).

Endogenous α-syn has also been shown to be required for the normal activity of the respiratory chain complexes (Ellis et al., 2005; Devi et al., 2008), we thus may hypothesize that its levels must be tightly regulated and kept under control. Deviations from the threshold levels inevitably affect cellular and mitochondrial functions and lead to the alterations hereby described. Accordingly, mutations may exert their pathogenicity because, by affecting aggregation propensity, they could contribute to compromise the availability of α-syn by sequestrating it in aggregates both within the cell and in specific cellular compartments or because mutated α-syn may quickly reach the dose that is required to induce mitochondrial and cellular dysfunction.

Indeed, impairment of the complex I function and increased production of reactive oxygen species (ROS) have been consistently observed both in the absence and in the presence of overexpressed α-syn and the expression of the A53T mutation exacerbated the defects (Devi et al., 2008; Loeb et al., 2010; Reeve et al., 2015). Mitochondrial membrane potential and ATP production were also affected upon exogenous administration of the recombinant wild-type and mutant α-syn (Banerjee et al., 2010).

ALPHA-SYNUCLEIN AT THE MITOCHONDRIA-ASSOCIATED MEMBRANES (MAMs)

Mitochondria-associated membranes (MAMs) are functionally specialized regions where the endoplasmic reticulum (ER) and mitochondria come in close proximity (Hayashi et al., 2009) and are in place to regulate several cellular processes including ER stress, unfolded protein response, cholesterol and phospholipid metabolism, mitochondrial division and dynamics, Ca^{2+} signaling and apoptosis (Rizzuto and Pozzan, 2006; Wozniak et al., 2006; Csordas and Hajnoczky, 2009; Simmen et al., 2010; Friedman et al., 2011).

Impaired functionality of the MAMs as well as changes in the number of contacts between ER and mitochondria have been recently associated to neurodegenerative diseases, including PD (Calì et al., 2012, 2013; Ottolini et al., 2013; Guardia-Laguarta et al., 2014; Rodriguez-Arribas et al., 2016), amyotrophic lateral sclerosis (ALS) (Stoica et al., 2014, 2016) and Alzheimer's disease (Zampese et al., 2011; Area-Gomez et al., 2012; Hedskog et al., 2013; Schon and Area-Gomez, 2013; Area-Gomez and Schon, 2016, 2017). The presence of lipid raft-like domains at the MAMs and the intrinsic propensity of α-syn to interact with acidic phospholipids (Fortin et al., 2004), immediately suggested the possibility of a potential specific targeting and action of α-syn at these sites (**Table 1**).

One of the first evidence suggesting a potential role played by α-syn at the ER-mitochondria interface comes from the demonstration that overexpression of wt α-syn was able to sustain mitochondrial Ca^{2+} uptake by increasing the number of ER-mitochondria juxtapositions in SH-SY5Y and HeLa cells, while its down regulation impaired mitochondrial Ca^{2+} transfer and morphology (Calì et al., 2012). Of notice, in Hela cells the increase of α-syn levels over a certain threshold both by treatment with valproic acid (which acts on endogenous protein) and by incubation with exogenous recombinant TAT-α-syn leads to a reduction in mitochondrial Ca^{2+} uptake (Calì et al., 2012). This suggests the intriguing possibility that α-syn behavior at MAMs is dependent on its expression level, which is also known to affect its mitochondrial localization (see below) (Shavali et al., 2008; Liu et al., 2009).

Soon after, α-syn was found at the MAMs (Guardia-Laguarta et al., 2014; Paillusson et al., 2017) and shown to interact with the ER vesicle-associated membrane protein-associated protein B (VAPB), thus perturbing its association with the protein tyrosine phosphatase–interacting protein 51 (PTPIP51) (**Figure 2**), and consequently their ER-mitochondria tethering function (De Vos et al., 2012). Thus, increased α-syn levels induced a decrease in the number of ER-mitochondria interactions in SH-SY5Y and iPS cell-derived dopaminergic neurons from a patient harboring *SNCA* triplication, followed by impaired inositol 1,4,5 triphosphate (IP_3) receptor-mediated Ca^{2+} transfer to mitochondria and mitochondrial ATP production (Paillusson et al., 2017).

Additional evidence confirmed the pivotal role of α-syn at the MAMs, although different effects on the ER-mitochondria interface were reported for the wt and the mutant forms in different cell lines (Guardia-Laguarta et al., 2014). Whether this reflects cell line-specific features or is influenced by the different distribution within the cells of the wt and mutant proteins, remains to be elucidated.

Altogether, these data suggest that a portion of α-syn resides at the MAMs and influences some of their basic cellular activities. Altered α-syn expression, unbalanced equilibrium between the α-syn located in the MAMs, in the cytosol or mitochondria and the presence of PD-related mutations could contribute to the onset and the development of PD pathology by differentially interfering with MAMs functions (Guardia-Laguarta et al., 2015a,b).

TABLE 1 | Effects of the different sub-mitochondrial α-syn localizations on mitochondrial dynamics.

α-syn localization	α-syn effects	
	High expression	**Low expression**
Mitochondria-associated membranes (MAMs)	↓ ER-mito contacts ↓ Mitochondrial Ca^{2+} uptake ↓ ATP synthesis ↑ Mitochondrial fragmentation	↑ ER-mito contacts ↑ Mitochondrial Ca^{2+} uptake ↓ ATP synthesis
(OMM)	↑ Mitochondrial fragmentation ↓ Mitochondrial size ↑ Mitochondrial nuclear clustering ↓ Mitochondrial respiration ↓ Mitochondrial proteins import ↑ ROS production ↑ mtDNA damage ↓ Mitochondrial membrane potential ↓ ATP synthesis	
Inter-membrane space (IMS)	↑ mPTP opening ↓ Mitochondrial membrane potential ↑ ROS production ↑ Mitochondrial swelling ↑ Mitochondrial vacuolation ↓ Cristae number	
Inner mitochondrial membrane (IMM)	↑ ROS production ↓ Complex I activity	
Mitochondrial matrix	↑ ATP synthesis	

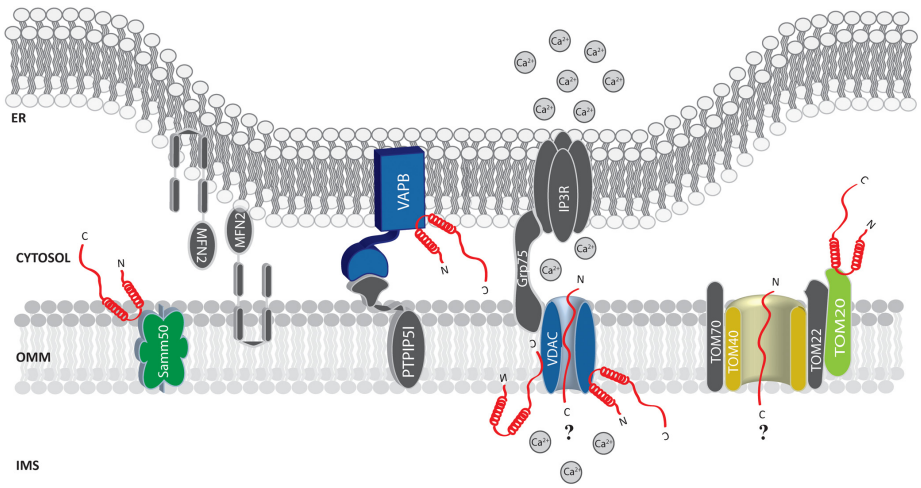

FIGURE 2 | Alpha-syn at the mitochondria-associated membranes (MAMs) and the OMM. Picture shows α-syn binding partners at the MAMs and OMM. The protein's interactors are highlighted in colors. The possible (?) mechanism/s of α-syn translocation inside mitochondria are also reported. A-syn is shown in red.

MITOCHONDRIAL ROUTES FOR ALPHA-SYNUCLEIN

Mitochondrial Localization of Alpha-Synuclein: General Evidence and Targeting Signals

The aforementioned data strongly imply that α-syn governs a plethora of mitochondrial processes. Whether these effects are regulated by α-syn directly or represent the culminating events of a signaling network arising from impairments in compartments other that mitochondria is still matter of investigation. Direct association of α-syn with mitochondria has been repeatedly and constantly observed in model cells and in different regions of the mouse brain (ventral midbrain, striatum and cortex) from α-syn transgenic mice (Subramaniam et al., 2014). Immunocytochemistry analysis (Parihar et al., 2008), immuno-gold electron microscopy (Parihar et al., 2008) and subcellular fractionation studies followed by western blot analysis (Subramaniam et al., 2014) revealed the presence of wt as well as PD-related A30P and A53T mutant α-syn in mitochondria. Interestingly, the presence of a fraction of α-syn residing in mitochondria was also found in dopaminergic neurons of substantia nigra from non-PD and PD subjects, but its accumulation was found only in mitochondria of PD patients (Devi and Anandatheerthavarada, 2010).

Albeit α-syn lacks a canonical mitochondrial targeting sequence, NMR studies and sequence alignments with cleavable N-terminal mitochondrial targeting sequences of cytochrome P450Scc and cytochrome P450 sterol 27-hydroxylase revealed that N-terminus domain of α-syn, which is rich in positively charged residues, mirrors the physico-chemical properties of mitochondrial targeting sequences and can adopt an α-helical conformation that can drive the anchoring of the protein to mitochondrial membranes (Ulmer et al., 2005; Devi and Anandatheerthavarada, 2010). The first N-terminal 32 amino acids have been shown to be fundamental for mitochondrial localization of the protein, (Devi et al., 2008) and, in particular, the lack of the first 11 N-terminal amino acids almost completely suppressed the *in vitro* binding of exogenous α-syn to isolated mitochondria from human HEK293 cells (Robotta et al., 2014).

As a matter of fact, all the PD-related mutations occur within the α-syn N-terminal domain, raising the interesting possibility that, beside their effect on protein aggregation (Conway et al., 1998; Li et al., 2001; Choi et al., 2004; Greenbaum et al., 2005; Sahay et al., 2017), they could impact on α-syn association to intracellular membranes and thus on its subcellular localization.

Interestingly, some mutants showed a greater tendency to be imported into mitochondria as compared with their wt counterpart (Cole et al., 2008; Devi et al., 2008; Guardia-Laguarta et al., 2014; Pozo Devoto et al., 2017), highlighting a potential pathogenic mechanism of action.

At the functional level their mitochondrial import was associated with broad mitochondrial defects such as increased mitochondrial Ca^{2+} levels, nitric oxide and ROS formation, cytochrome c release and apoptosis (Parihar et al., 2008, 2009), impairments of selected mitochondrial respiratory chain complexes (Subramaniam et al., 2014) and increased mitochondrial clearance (Chinta et al., 2010).

In the next sections, we will discuss the molecular basis for this intimate and functionally relevant relationship of α-syn with mitochondria, α-syn peculiar sub-organelle localization as well as the specific partners and processes that it governs at submitochondrial level (**Table 1**).

Alpha-Synuclein at the Outer Mitochondrial Membrane

This intrinsic ability of α-syn to bind lipids and thus membranes, especially those with negatively charged surfaces, raised the possibility of potential interactions with the mitochondrial

membranes (Shvadchak et al., 2011). The lipid binding properties of α-syn have been extensively investigated (Rhoades et al., 2006) and several studies have demonstrated the ability of α-syn N-terminal domain to adopt an α-helix conformation upon exposure to lipid surfaces (Ulmer et al., 2005). Deletion and/or insertion of charged amino groups in the first 25 residues of α-syn N-terminal domain strongly affected the propensity to adopt an α-helical conformation and also altered the binding to membranes (Perrin et al., 2000; Vamvaca et al., 2009; Bartels et al., 2010). Thus, it is not surprising that the presence of mutations in this domain strongly affected this feature. However, the three most frequent mutations confer different behavior: the A30P perturbs the helical structure leading to a reduction in lipid affinity, the E46K mutation increases the affinity for lipids, while the A53T has no major effect (Perrin et al., 2000; Jo et al., 2002; Bussell and Eliezer, 2004; Perlmutter et al., 2009).

By confocal and immuno-gold electron microscopy techniques a fraction of cellular α-syn has been found to localize at the OMM in dopaminergic neurons (Li et al., 2007) and rat brain neurons (Zhang et al., 2008). High pressure freeze immuno-electron microscopy on SH-SY5Y cells overexpressing α-syn further revealed that the protein directly binds the outer membrane of mitochondria leading to a MFN2 and DRP1-independent mitochondrial fragmentation without affecting the mitochondrial membrane potential or the ATP levels (Kamp et al., 2010). The expression of pathologic A30P and A53T mutants retrieved similar results, despite of in vitro analysis has revealed, at least for the A30P, a reduction in lipid affinity, thus suggesting that the amount of overexpressed protein could also play a major role in membranes binding (Kamp et al., 2010). Accordingly, upon overexpression of wt or A53T and E46K α-syn mutants, but not under conditions of reduced α-syn levels (Nakamura et al., 2011) or upon overexpression of the A30P mutant, a phenotype of mitochondrial fragmentation was observed suggesting that the amount of α-syn bound to mitochondria membranes might play a pivotal role. In line with this possibility, recent findings have shown that the forced delivery of wt and A53T α-syn to the outer membrane of mitochondria caused a reduction in mitochondrial size, while the A30P mutant had no effect (Pozo Devoto et al., 2017).

Compelling evidence for a selective preference of α-syn for mitochondria came from FRET-based and *in vitro* studies demonstrating that it selectively binds to mitochondria independently of the mitochondrial membrane potential, suggesting that the lipid composition rather than the functional state of the organelle is involved in the binding (Nakamura et al., 2008). These studies indicated that cardiolipin, a phospholipid enriched in mitochondrial membranes, is strictly required for α-syn interaction (Ghio et al., 2016).

Additionally, by immune-electron microscopy on HEK293 cells stably expressing α-syn it has been observed that under condition of low intracellular pH α-syn translocates to the OMM, but not within the mitochondrial matrix or the intermembrane space (Cole et al., 2008). Thus, it is tempting to speculate that the mitochondrial targeting of α-syn could be enhanced by cellular stress conditions. Whether the association of α-syn with mitochondria occurs directly with the membranes or is mediated by other proteins is still unclear. Sodium carbonate and proteinase K treatment on isolated mitochondria does not interfere with its association (Cole et al., 2008; Parihar et al., 2008). On the other hand, *in vitro* pull down experiments of mitochondrial extracts with a peptide composed of the last C-terminal 40 amino acids of α-syn retrieved TOM22, TOM40, VDAC 1-2-3 and Samm50 as binding partners (**Figure 2**) and, interestingly, S129 phosphorylation drastically reduced α-syn association with TOM40 and Samm50 (McFarland et al., 2008). Beside a series of α-syn induced mitochondrial alterations (such as increased ROS production and oxidative stress, alteration in complex I and deletions of mtDNA), α-syn accumulation was also accompanied by a decrease in TOM40 expression (Bender et al., 2013). More recently, α-syn binding to TOM20, but not to TOM40, TOM22 or Tim23, has been reported to impair the TOM20/TOM22 assembly, affecting the import of the complex I subunit Ndufs3 and leading to reduced respiration and increased ROS production. Intriguingly, the oligomeric dopamine-modified and the phosphomimic S129E mutant of α-syn, but not the monomeric or the nitrated and fibrillary forms, were reported to impair protein import, suggesting that the trimeric/tetrameric structure may play a role in mediating mitochondrial toxicity (Di Maio et al., 2016). As anticipated above, VDAC is also an important and recurrent α-syn binding partner: co-immunoprecipitation analysis revealed an association in the brainstem, striatum, and cerebral cortex of Thy1-A53T human α-syn transgenic mice (Martin et al., 2014) and *in vitro* studies revealed that recombinant monomeric α-syn at nanomolar concentration was sufficient to reversibly block VDAC1 channel activity in planar lipid bilayer, (Rostovtseva et al., 2015). Interestingly, nigral neurons from brains of PD patients containing α-syn positive inclusions also showed reduced VDAC1 immunoreactivity as compared with those displaying soluble or absent α-syn (Chu et al., 2014), suggesting a strong correlation between α-syn and VDAC in the pathogenesis of PD.

Thus, although α-syn possess the ability to bind cellular membranes directly, with an intrinsic preference for those mirroring the composition of the mitochondrial ones, protein-protein interactions with the components of the mitochondrial import machinery at the OMM have been consistently reported. Albeit a specific molecular mechanism for this selectivity toward the mitochondrial protein import machinery, in particular TOM20 and TOM40, is still missing, it might represent one of the first event in the neurodegenerative process.

Alpha-Synuclein in the Inter-membrane Space (IMS)

Several studies have raised the possibility that a portion of the cellular α-syn can be found at the sub-mitochondrial level [inter membrane space (IMS), the inner membrane or the matrix], implying that a direct translocation from the cytosol across the OMM must also occur. As stated above, a number of OMM partners involved in this process has been found; however, how and why α-syn is imported into the mitochondria is still almost completely unexplored. *In vivo* and *in vitro* import of human

α-syn into the mitochondria depends on the mitochondrial membrane potential and ATP levels and although anti-TOM40 antibodies have been reported to abolish α-syn mitochondrial localization (Devi et al., 2008), direct translocation through VDAC reconstituted into planar lipid bilayer has also been demonstrated (Rostovtseva et al., 2015; Hoogerheide et al., 2017), raising questions on whether there is a selective engagement on one of the two pathways or both components can be equally used based on the cell needs (Neupert and Herrmann, 2007). Understanding if the occurrence of the pathogenic mutations, the post-translational modifications or the aggregation state of the protein drive it to a specific pathway may be useful for a better understanding of α-syn physio/pathological behavior inside mitochondria as well as for the development of pharmacological approach and deserves further investigations.

At the IMS α-syn has been shown to interact with a portion of VDAC1 facing this compartment in the substantia nigra of α-syn overexpressing rats and in dopaminergic MN9D cells (Lu et al., 2013) and with the adenine nucleotide translocase (ANT) (**Figure 3**), one of the most abundant proteins of the inner mitochondrial membrane (IMM; Liu and Chen, 2013) involved in the exchange of ADP/ATP between the matrix and the IMS (Ryan et al., 1999; Halestrap and Brenner, 2003). Interestingly, alterations of mitochondrial membrane potential, increased ROS production and mitochondrial vacuolation, swallowing and loss of cristae observed upon α-syn overexpression are partially reverted by the incubation with the ANT inhibitor bongkrekic acid (BKA), which was shown to reduce the interaction between α-syn and both ANT and VDAC, suggesting that this interaction could be the key for α-syn detrimental activity (Shen et al., 2014). Interestingly, these effects could not be recapitulated by overexpression of a truncated form of α-syn lacking its N-terminus (aminoacids 1–60) or mutated in two key residues important for the targeting (the V63 and N65 residues), indicating that the N-terminus domain is critical for α-syn-induced cytotoxicity, probably affecting its mitochondrial localization (Shen et al., 2014; Zhang et al., 2016). Altogether, these findings suggest that α-syn translocation into the IMS of mitochondria might be a physiologically relevant under basal conditions and that increasing levels of α-syn also at this site could impact on the ability of cell to keep the ATP levels well balanced, contributing to the damage of dopaminergic neurons (Zhu et al., 2011).

Alpha-Synuclein at the Inner Mitochondrial Membrane

Electron paramagnetic resonance (EPR) spectroscopy studies designed to analyze the interactions between α-syn and large unilamellar phospholipid vesicles mimicking either the inner or the OMM, indicated that the protein binds specifically to the IMM through its N-terminus (Robotta et al., 2014). In accordance, fluorescence anisotropy and high resolution nuclear magnetic resonance spectroscopy (NMR) studies confirmed that wt and A30P α-syn mutant could strongly interact with large unilamellar phospholipid vesicles mimicking the IMM but not with those mimicking the OMM. The differential affinity for the two membranes was related to the specific cardiolipin composition, which in the inner membrane is at least 40 times higher than in the outer membrane (de Kroon et al., 1997; Zigoneanu et al., 2012). This evidence was further confirmed in α-syn transgenic mice which showed a predominant α-syn localization at the IMM (Nakamura et al., 2011). Indeed, cardiolipin ablation from inner membrane-like vesicles or its saturation with cardiolipin-binding dye nonyl-acridine orange (NAO) strongly prevented α-syn binding (Cole et al., 2008; Zigoneanu et al., 2012).

Proteomics analysis identified a series of α-syn interactors at the level of the IMM, including mitofilin, a mitochondrial inner membrane protein important for the regulation of cristae morphology (John et al., 2005; McFarland et al., 2008). iTRAQ proteomics on synaptosomes from α-syn$^{+/+}$ and α-syn$^{-/-}$ mice also revealed that α-syn interacts with sideroflexin 3 (SFXN3), a putative iron transporter of the IMM (Fleming et al., 2001; Li et al., 2010) which is important for maintenance of the synaptic morphology and neuromuscular junctions (Amorim et al., 2017) and that has been found down-regulated in substantia nigra of PD-affected patients (Simunovic et al., 2009).

The mitochondrial solute carrier family 25 members (namely the 3, 11, 12 and 13) and the components of the electron transport chain have also been reported as binding partners of α-syn (McFarland et al., 2008). Accordingly, yeast two-hybrid and co-immunoprecipitation assay confirmed a specific interaction of α-syn with complex IV of the mitochondrial transport chain (Elkon et al., 2002). Moreover, association of α-syn with complex I in PD brain has also been reported and found to induce a reduction in complex I activity and increased ROS production in a time and dose-dependent manner (Devi et al., 2008; Liu et al., 2009), suggesting that the progressive accumulation of α-syn at the IMM may impair mitochondrial functions and induce oxidative stress.

Alpha-Synuclein in the Mitochondrial Matrix

To the best of our knowledge, the number of studies indicating a specific localization for α-syn within the mitochondrial matrix is limited and evidence for α-syn translocation through the IMM is missing. Moreover, *ex vivo* proximity ligation assays reveal no interactions between α-syn and Tim23, the major protein translocase of the inner membrane of mitochondria (Di Maio et al., 2016). Nevertheless, some clues suggest the possibility that a minor fraction of α-syn could be localized at the mitochondrial matrix (Devi et al., 2008; Zhang et al., 2008; Liu et al., 2009) and, intriguingly, proteomic analysis revealed α-syn interaction with the B, D and γ chain of the ATP synthase (McFarland et al., 2008).

Evidence obtained at functional level showing that α-syn absence impact essential mitochondrial function further support the possibility that α-syn may interact with ATP synthase. Indeed, primary neuron/glia co-cultures from cerebral cortex of α/β/γ-syn triple knock out (TKO) mice revealed reduced mitochondrial membrane potential, decreased ATP synthase activity and lower ATP levels (Ludtmann et al., 2016), importantly, exogenous addiction of monomeric α-syn, but not

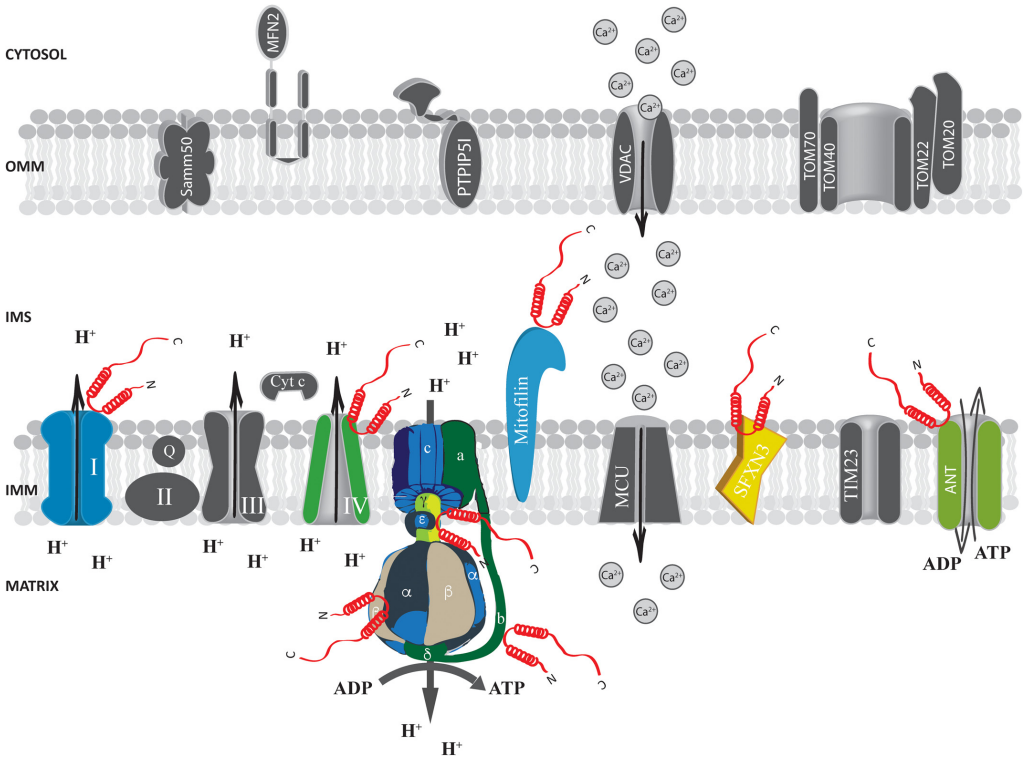

FIGURE 3 | Alpha-syn at the inter membrane space (IMS), inner mitochondrial membrane (IMM) and mitochondrial matrix. Picture shows α-syn binding partners at the IMS, IMM and mitochondrial matrix. The protein's interactors are highlighted in colors. Alpha-syn is shown in red.

of the A30P mutant, to the neuron/glia co-cultures was able to fully revert the phenotypes observed in the TKO neurons by physically interacting with the α subunit of the ATP synthase (**Figure 3**), suggesting that the mitochondrial matrix-resident fraction of α-syn could play a pivotal role in regulating important mitochondrial activities.

This scenario brings to speculate that a pool of α-syn exerts a physiological role inside the mitochondrial matrix where it is able to increase ATP synthase activity through direct binding with its α subunit, thus ensuring mitochondrial health and proper ATP fueling for synaptic functions. Aggregation and/or mutations of the protein could thus result in exaggerated mitochondrial accumulations that may lead to loss of function and initiate the degenerative process in PD.

CONCLUSION AND FUTURE PERSPECTIVES

Mitochondrial dysfunctions and α-syn misfolding/aggregation have both been extensively documented in the pathogenesis of PD (Exner et al., 2012; Bose and Beal, 2016). The possible interplay between the two phenomena is suggested by the evidence that increased levels and/or mutations of α-syn trigger mitochondrial alterations and that mitochondrial impairment causes α-syn accumulation and aggregation (Betarbet et al., 2006; George et al., 2010). Which of the two events comes first in the development of the neurodegenerative process is not yet clear (Zaltieri et al., 2015), but the strong association between α-syn and mitochondria is nowadays believed to play a pivotal role in the pathogenesis of PD. Although substantia nigra pars compacta dopaminergic neurons are among the first neurons to degenerate, additional neuronal populations are also affected in PD (Dauer and Przedborski, 2003; Surmeier et al., 2017; Zhai et al., 2018). Despite the mechanisms governing this selective vulnerability are poorly understood, increasing evidence suggests that α-syn-mediated alteration of mitochondria wellness might be particularly important in dopaminergic neurons from substantia nigra (Calì et al., 2014). Indeed, they show increased cytosolic dopamine, Ca^{2+} entry and mitochondrial oxidative stress that, in turn, induces accumulation of oxidized dopamine and mitochondrial dysfunction, leading to the onset of the PD-related motor symptoms (Burbulla et al., 2017; Lieberman et al., 2017).

Despite the absence of a canonical mitochondrial targeting signal in α-syn sequence, a growing body of literatures indicates that at least a portion of cellular α-syn not only is able to physically interact with mitochondrial membranes by lipids-mediated binding, but also to cross them, possibly through a translocation process mediated by TIM/TOM complex or VDAC protein, reaching the intermembrane space and/or the matrix (Devi and Anandatheerthavarada, 2010; Nakamura, 2013; Abramov et al., 2017; Pozo Devoto and Falzone, 2017). Interestingly, α-syn effects on mitochondria seem to interplay

with other mitochondrial proteins/pathways known to be mutated/altered in PD, further confirming that mitochondrial α-syn can be a major player in the onset of the disease. In fact, it has been proposed that blockage of mitochondrial protein import through α-syn interaction with TOM20 may activate Pink1/Parkin mediated mitophagy, by promoting Pink1 accumulation at the OMM and consequent Parkin recruitment (Di Maio et al., 2016). In accordance, midbrain dopaminergic neurons overexpressing α-syn A53T display an increase in mitochondrial clearance (Chinta et al., 2010), and the overexpression of wt Pink1 or Parkin, but not their PD-associated mutants, is able to rescue the α-syn-induced impairment of mitochondrial morphology (Kamp et al., 2010).

Several aspects of α-syn/mitochondria interplay, however, need to be further elucidated. First, it is worth to investigate whether the different sub-organelle localizations of the protein account for peculiar physiological functions or represent the response to specific stimuli. It is not clear whether α-syn toxic species are delivered to the mitochondria as a pathological or a protective mechanism. In other words, it is still to be proven whether the portion of α-syn that localizes within mitochondria resides there to exert a physiologically relevant function or for other reasons, i.e., to be degraded. An elegant work has shown, in yeast and human cells, that protein aggregates enter the mitochondrial intermembrane space and matrix (through the mitochondrial import machinery) in order to be degraded (Ruan et al., 2017). The possibility that this process also favors the clearance of α-syn deserves further investigations.

In addition, the subtle equilibrium between physiological and pathological roles of α-syn and its propensity to accumulate within the cell in the course of the neurodegenerative process suggest that there might be a threshold of protein amount in the cytosol and mitochondria that discriminates between healthy and detrimental effects. In accordance, it has been shown that the mitochondrial distribution of the wt and A53T α-syn is significantly increased in cells overexpressing the proteins compared to controls (Parihar et al., 2008) and that their induction of mitochondrial fragmentation is directly related to their expression levels (Pozo Devoto et al., 2017). Moreover, only high expression of the A53T mutant in midbrain dopaminergic neurons of transgenic mice significantly increases the number of autophagic mitochondria (Chinta et al., 2010). Finally, we have previously demonstrated that increased endogenous wt α-syn content by valproic acid treatment and the TAT-mediated delivery of high doses of exogenous protein lead to a reduction in mitochondrial Ca^{2+} uptake, while low levels of the protein induce an increase in mitochondrial Ca^{2+} influx. This observation could explain the discrepancies observed on α-syn action at the MAMs, being its effects possibly related to different transfection methods that permit to reach different amount of overexpressed α-syn (Calì et al., 2012; Paillusson et al., 2017).

Differences in α-syn oligomerization states could further complicate the scenario. It is unclear which aggregation state of the protein forces its mitochondrial localization and its detrimental or positive effects on mitochondria. Nevertheless, western blot analysis on rat brain purified mitochondria incubated with aggregated or un-aggregated α-syn revealed that only the oligomeric form of the protein can associate with mitochondria, as confirmed by immuno-gold electron microscopy (Parihar et al., 2008, 2009). Concordantly, only soluble and prefibrillar α-syn oligomers, but not monomeric or fibrillar α-syn, are able to bind TOM20 and block mitochondrial protein import as well as to induce complex I dysfunction and mitochondrial membrane potential dissipation leading to the impairment of mitochondrial Ca^{2+} handling and enhanced cytochrome c release (Luth et al., 2014; Di Maio et al., 2016).

Finally, the impact of known pathogenic mutations on α-syn mitochondrial regulation is not completely understood. Beside their role on protein aggregation, it has been suggested that mutations are able to regulate α-syn-mitochondrial association, with the A30P mutant being particularly defective in mitochondrial membranes binding.

Albeit all this evidence clearly supports a pivotal role for α-syn in the alteration of mitochondrial functions leading to the neurodegenerative process, they also point out the requirement of additional efforts to dissect the intimate relationship between α-syn physio/pathology and mitochondrial dysfunctions, providing new elements for the complete understanding of neuronal degeneration in PD.

AUTHOR CONTRIBUTIONS

MV, DC, MB, and TC contributed to the design and writing of the manuscript.

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PERSPECTIVE
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Quality Over Quantity: Advantages of Using Alpha-Synuclein Preformed Fibril Triggered Synucleinopathy to Model Idiopathic Parkinson's Disease

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Animal models have significantly advanced our understanding of Parkinson's disease (PD). Alpha-synuclein (α-syn) has taken center stage due to its genetic connection to familial PD and localization to Lewy bodies, one pathological hallmark of PD. Animal models developed on the premise of elevated alpha-synuclein via germline manipulation or viral vector-mediated overexpression are used to investigate PD pathophysiology and vet novel therapeutics. While these models represented a step forward compared to their neurotoxicant model predecessors, they rely on overexpression of supraphysiological levels of α-syn to trigger toxicity. However, whereas *SNCA*-linked familial PD is associated with elevated α-syn, elevated α-syn is not associated with idiopathic PD. Therefore, the defining feature of the α-syn overexpression models may fail to appropriately model idiopathic PD. In the last several years a new model has been developed in which α-syn preformed fibrils are injected intrastriatally and trigger normal endogenous levels of α-syn to misfold and accumulate into Lewy body-like inclusions. Following a defined period of inclusion accumulation, distinct phases of neuroinflammation and progressive degeneration can be detected in the nigrostriatal system. In this perspective, we highlight the fact that levels of α-syn achieved in overexpression models generally exceed those observed in idiopathic and even *SNCA* multiplication-linked PD. This raises the possibility that supraphysiological α-syn expression may drive pathophysiological mechanisms not relevant to idiopathic PD. We argue in this perspective that synucleinopathy triggered to form within the context of normal α-syn expression represents a more faithful animal model of idiopathic PD when examining the role of neuroinflammation or the relationship between a-syn aggregation and toxicity.

Keywords: Parkinson's disease, alpha-synuclein, preformed fibrils, synucleinopathy, animal models

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 7–10 million individuals worldwide. Though PD was first described over 200 years ago by James Parkinson, no therapies currently exist to halt or slow nigrostriatal degeneration despite scores of preclinical studies predicting the success of particular disease-modifying treatment strategies. This "translational abyss" may be due in part to the failure of animal models to faithfully recapitulate human disease and, more specifically, failure to use the appropriate PD animal model.

During the past two decades, numerous preclinical studies have used overexpression of human wildtype or mutant alpha-synuclein (α-syn) in *Drosophila*, rodents and non-human primates to model PD. Overexpression is achieved in these models via transgenic engineering or via injection of viral vectors. However, while these α-syn models represented an advance over neurotoxicant models through the incorporation of α-syn, the mechanism of toxicity and the neuropathology generated differ from idiopathic PD in key respects. Specifically, α-syn overexpression paradigms result in α-syn protein expression levels that far exceed levels associated with idiopathic PD, levels which can induce exacerbated neuroinflammation. Further, viral vector-mediated α-syn overexpression results in pathology in limited circuitry and can lack a protracted phase of classical Lewy body-like inclusion pathology. We contend that these shortcomings in the α-syn overexpression-based models have hindered our understanding of the pathogenic contributions of normal endogenous α-syn in idiopathic PD and have handicapped our ability to predict efficacy of novel neuroprotective therapeutics.

Recently, a model of synucleinopathy and nigral degeneration induced by intracerebral administration of sonicated preformed fibrils of α-syn (PFFs) has provided a new platform to study the pathogenic cascade in which normal levels of endogenous α-syn levels are triggered to misfold, template and accumulate. The engagement of physiologic levels of endogenous α-syn in the α-syn PFF model allows for the assessment of differential neural circuit vulnerabilities to α-syn inclusion formation and toxicity. The α-syn PFF model recapitulates many features of human idiopathic PD, namely: a protracted interval of accumulation of insoluble Lewy-like pathology, α-syn inclusion triggered neuroinflammation and degeneration of specific neuronal subpopulations.

Comprehensive comparisons of the viral vector mediated a-syn overexpression model and the a-syn PFF model have been previously conducted (Volpicelli-Daley et al., 2016; Koprich et al., 2017). In this perspective, we highlight pathological features of human idiopathic and *SNCA*-linked familial PD in order to compare these features to those observed in the α-syn PFF model. Ultimately, these sporadic PD and α-syn PFF model features will be placed in the context of the pathology induced by overexpression of α-syn, with a focus on viral vector-mediated α-syn overexpression. We argue the perspective that the synucleinopathy and the inclusion-initiated nigral degeneration resulting from α-syn PFF injection provides a superior platform for comprehending the pathophysiology of idiopathic PD and by extension, a superior platform for evaluating potential neuroprotective therapeutics.

Parkinson's Disease

Alpha-Synuclein (α-Syn) Expression and Localization in Idiopathic and SNCA-Linked Familial PD

While the majority (90–95%) of PD cases do not have an identified genetic component, they share with familial PD a role for the protein α-syn. In the CNS, α-syn accounts for approximately 1% of total protein and is localized primarily in the cytosol of axon terminals, with known roles in membrane bending, synaptic transmission, and plasticity (Bendor et al., 2013; Benskey et al., 2016). In 1997 it was first revealed that an Ala53Thr (A53T) substitution in the α-syn gene (*SNCA*) was associated with young onset, autosomal dominant PD (Polymeropoulos, 1998). Shortly thereafter it was shown in subjects with idiopathic PD that fibrillar α-syn was a major component of Lewy bodies (Spillantini et al., 1998). Subsequent work identified additional point mutations [A30P, E46K, H50Q, and G51D; (Kruger et al., 1998; Somme et al., 2011; Appel-Cresswell et al., 2013; Lesage et al., 2013)] that increase the risk of PD. The effects of these missense mutations on α-syn protein conformational state, solubility, membrane association, and aggregation kinetics have been extensively studied *in vitro* and *in vivo* (Jo et al., 2000; Khalaf et al., 2014; Dettmer et al., 2015; Xu et al., 2016). In addition to point mutations in *SNCA*, duplications or triplications of wildtype *SNCA* are also causative in familial PD. Collectively, this body of work provides compelling evidence that α-syn plays a central role in the pathophysiology of both familial and idiopathic PD.

As expected, studies of α-syn multiplication carriers demonstrate that α-syn mRNA and protein are elevated in these genetic forms of the disease with 1.5–2-fold increases in abundance reported (Singleton et al., 2003; Chartier-Harlin et al., 2004; Farrer et al., 2004; Fuchs et al., 2007; Olgiati et al., 2015) Notably, *SNCA* triplication carriers exhibit earlier onset and more-rapidly progressing PD compared to duplication carriers or sporadic PD patients, further lending support that dose-dependent increases in α-syn are a driving factor in disease onset and severity. However, in idiopathic PD the evidence for either elevated α-syn mRNA or α-syn protein is lacking. Analysis of α-syn mRNA levels within individual nigral neurons from early or late idiopathic PD subjects has revealed conflicting results with no differences reported (Tan et al., 2005; Su et al., 2017), increases reported (Grundemann et al., 2008) or decreases reported (Neystat et al., 1999; Kingsbury et al., 2004). Analysis of total α-syn protein levels in post-mortem idiopathic PD tissue suggests either a modest transient increase or similar levels to age-matched control (Zhou et al., 2011). In contrast to *SNCA*-linked familial PD, the concept that increases in α-syn levels drive pathophysiology in idiopathic PD is less supported.

Whereas total expression levels of α-syn appear to distinguish *SNCA* multiplication carriers from idiopathic PD patients, changes in the solubility, membrane association, and abundance of post-translationally modified forms α-syn are similar in

both patient subgroups. Investigations beyond a focus on α-syn abundance have identified changes in cellular localization and post-translational modifications as potential mechanisms of α-syn-mediated toxicity, as reviewed in Jo et al. (2000), Lee et al. (2002), Auluck et al. (2010), Tong et al. (2010), van Rooijen et al. (2010), Barrett and Timothy Greenamyre (2015), Oueslati (2016), and Burre et al. (2018). In idiopathic PD, studies have consistently demonstrated shifts in the ratio of soluble to insoluble α-syn without concurrent changes in total α-syn levels. Specifically: decreases in soluble monomeric α-syn with concurrent increases in soluble phosphorylated α-syn (pSyn) along with increases in membrane-bound α-syn have been observed in particularly vulnerable regions (SN and cortex) in sporadic PD cases (Gibb and Lees, 1988; Irizarry et al., 1998). Similar observations have also been made in samples derived from *SNCA* triplication carriers, albeit with increased magnitude and less regional specificity (Tong et al., 2010). The fact that both genetic and idiopathic forms of PD are associated with α-syn phosphorylation and increased membrane interactions suggests a role for these phenomena in PD pathophysiology.

Development of Lewy Pathology and Affected Circuitry

Confirmed diagnosis of PD is not made until Lewy bodies (LBs) and Lewy neurites (LNs) are observed upon post-mortem evaluation. LBs are composed of dozens of proteins, which may include α-syn, neurofilament, p62 and ubiquitin. pSyn staining is the most common immunohistochemical method of LB detection in post-mortem tissue, however, it should be noted that pSyn inclusions likely represent end stage LB development. Immature LBs, termed "pale bodies" are more often observed in early disease stages. Pale bodies are strongly immunoreactive for α-syn and manifest as intracellular diffuse, granular eosinophilic material with ill-defined borders. As disease stage advances, mature cytoplasmic LBs predominate over pale bodies and differ slightly in appearance depending on location in the cortex or brainstem (Gibb and Lees, 1988; Irizarry et al., 1998; Stefanis, 2012). With increasing maturity, LB and LN inclusions display a dense core with radiating filaments, and are strongly Thioflavin-S positive for beta-sheet structure and resistant to digestion by proteinase-K (Neumann et al., 2002; Li et al., 2010).

Although Lewy pathology is widespread in PD brain, it occurs in well-defined regions including the substantia nigra pars compacta, amygdala, olfactory bulb, temporal, frontal and parietal cortices (Halliday et al., 2011). Braak and colleagues developed a staging scheme for PD based on the location of pSyn LB and LN inclusions. Braak proposed that inclusions are first found in the olfactory bulb and dorsal motor nucleus of the vagus nerve, and follow an ascending pattern through the brainstem and finally the cortex (Braak et al., 2003; Dickson, 2018), lending to the debate of PD as a prion-like disease (Braak et al., 2003; Halliday et al., 2011; van de Berg et al., 2012). However, it should be noted that ∼50% of cases do not follow this staging scheme (Burke et al., 2008; Jellinger, 2008; Kalaitzakis et al., 2008; Beach et al., 2009). Other theories suggest parallel rather than stepwise accumulation of Lewy pathology based on differential vulnerability profiles of various cell types and regions (Engelender and Isacson, 2017).

Neuroinflammation

In recent years, neuroinflammation has been proposed as a contributor to neurodegeneration in PD and a potential target for disease modification. Early observations of post-mortem tissue describe a local increase in inflammatory markers in the SN associated with microglia, notably human-leukocyte antigen-D related [HLA-DR; (McGeer et al., 1988a,b, 1993; Imamura et al., 2003)], the human analog for major histocompatibility complex-II (MHC-II; antigen presentation). Not only has increased MHC-II expression been observed, it correlates positively with α-syn burden (Croisier et al., 2005). More recent work has implicated mutations in HLA-DR in amplified risk for developing PD, and levels of MHC-II are increased in cases of Incidental Lewy Body Disease [Braak stage I-II; (Dijkstra et al., 2015)] suggesting that inflammation may be at least in part, a contributing factor to ongoing degeneration (Kannarkat et al., 2015). On a broad level, cytokine measurements from patient biofluids (plasma and CSF) have consistently shown deviations from normal proinflammatory and anti-inflammatory cytokine levels compared to controls, although results are conflicting and may stem from variance in subject disease duration and time of sample collection (Mogi et al., 1996; Lindqvist et al., 2013; Eidson et al., 2017). However, while studies of patient tissue and biofluids have suggested that inflammation is involved in PD, these samples represent a single point in time over long disease duration. It is unclear whether neuroinflammatory markers that associate with LBs precede the formation of LBs and if the LB-containing neurons ultimately degenerate. Thus, the time-course of neuroinflammation in relation to α-syn accumulation and aggregation and nigral degeneration in human PD has yet to be determined.

Using the α-Syn PFF Seeded Synucleinopathy to Model Idiopathic PD

PFF-Induced Synucleinopathy in the Context of Normal Levels of Endogenous α-Syn

Given that idiopathic PD is not associated with an increase in total α-syn protein levels, synucleinopathy that arises in the context of normal endogenous α-syn levels would more faithfully recapitulate this key characteristic of the non-genetic form of PD. In contrast, previous models have relied on global overexpression (transgenics) or targeted overexpression (viral vector-mediated) of α-syn. The α-syn preformed fibril (PFF) model represents an approach in which synucleinopathy is induced in an environment of normal α-syn protein levels. The PFF model was first developed *in vitro* by introduction of α-syn PFFs to primary neuronal cultures. Briefly, α-syn fibrils are generated from recombinant α-syn monomers and sonicated to form smaller ∼50 nm fragments which are introduced to cell culture (Volpicelli-Daley et al., 2011). The PFFs are internalized by neurons, template and recruit endogenous α-syn and accumulate as inclusions of insoluble pSyn (Luk et al., 2009; Volpicelli-Daley et al., 2011, 2014). The pSyn inclusions

ultimately lead to neuronal dysfunction and degeneration. This toxicity is not due to introduction of the high quantity of PFFs *per se* but can be directly linked to the recruitment of endogenous α-syn into inclusions as evidenced by the fact that PFFs do not induce toxicity when applied to α-syn$^{-/-}$ primary neurons (Volpicelli-Daley et al., 2011; Luk et al., 2012b).

Development of Abundant Lewy-Like Pathology in Multiple Extra Nigrostriatal Regions

The α-syn PFF model has since been extended to wildtype mice (Luk et al., 2012a) and rats (Paumier et al., 2015; Abdelmotilib et al., 2017; Duffy et al., 2018) and most recently non-human primates (Shimozawa et al., 2017). These studies demonstrate that direct intracerebral injection of α-syn PFFs leads to accumulation of insoluble pSyn inclusions resembling Lewy pathology, all taking place in an environment of normal endogenous α-syn expression levels. Similar to *in vitro* findings, the injection of the high quantity of PFFs in a-syn$^{-/-}$ mice does not result in accumulation formation or degeneration (Luk et al., 2012b), illustrating the contribution and requirement of normal endogenous a-syn levels in the development of the pathological cascade. The similarity between α-syn PFF induced pSyn inclusions and human Lewy pathology in the SNc is particularly striking. At early time points post-injection, nigral pSyn immunoreactive inclusions resemble pale bodies: granular, diffuse and cytoplasmic (**Figure 1B**), whereas cortical inclusions occur abundantly in neurites and appear tendril-like in the soma. Over time, pSyn inclusions condense into more compact aggregates. The observed pSyn inclusions frequently colocalize with markers commonly observed in human LBs including p62 and ubiquitin, consist of α-syn oligomers and fibrils, and are also Thioflavin-S positive and proteinase-K resistant (Paumier et al., 2015; Duffy et al., 2018). Two photon microscopy has confirmed that neurons that form these pSyn inclusions ultimately degenerate (Osterberg Valerie et al., 2015). Similarly, the magnitude of pSyn inclusion formation observed in the substantia nigra 2 months following PFF injection can be used to predict the ultimate extent of nigral degeneration observed at 6 months (Duffy et al., 2018). The protracted course of these aggregation and degeneration events provides investigators the ability to focus on particular phases of the synucleinopathy cascade.

In vivo, the spatial emergence of LB and LN-like pathology is dependent on the location of PFF injection. For example, several groups have demonstrated that intrastriatal injections of PFFs result in pSyn inclusion accumulation in cell bodies of regions innervating the striatum (Wall et al., 2013): namely the substantia nigra, agranular insular and motor cortices, and amygdala (Luk et al., 2012a,b; Paumier et al., 2015; Abdelmotilib et al., 2017; Duffy et al., 2018), suggesting that axon terminals internalize the PFFs. The occurrence of pathology in these defined regions supports the concept of retrograde transport and templating of endogenous α-syn within neurons exposed to the injection site, rather than prion-like spread throughout the extensive brain networks. Importantly, areas in which pathology is observed are implicated in human PD (Halliday et al., 2011).

Neuroinflammation

In addition to generating α-syn pathology in multiple brain regions, intrastriatal injection of α-syn PFFs allows for investigations to delineate the time course of the inflammatory response in the substantia nigra. Due to the fact that the injection site (striatum) is spatially separated from the substantia nigra, inflammation related to surgical injection is minimized. Intrastriatal injection of PFFs, but not vehicle or other protein controls, induces peak reactive **Figure 1D** microglial morphology in the substantia nigra at 2 months, corresponding to the time point in which the greatest number of pSyn inclusions are observed (Duffy et al., 2018). In addition, a pSyn specific increase in MHC-II immunoreactive microglia is observed at this same time point, significantly correlating with pSyn inclusion load. This relationship between MHC-II immunoreactivity and α-syn inclusion burden is reminiscent to what has been reported in idiopathic PD (Croisier et al., 2005) providing face validity to the α-syn PFF model. However, while MHC-II is classically associated with a proinflammatory phenotype, it is unknown whether MHC-IIir microglia play a direct role in neurodegeneration, as a recent study has suggested that decreased expression of MHC-II and its regulator, *Mhc2ta*, augment α-syn pathology and accelerate degeneration (Jimenez-Ferrer et al., 2017). In addition, determining the stimulus of MHC-II expression is warranted (i.e., classical stimuli such as extracellular proteins or alternative induction by IFN-γ secretion). It is possible that the gradual and modest nature of degeneration is insufficient to activate a sufficient immune response, rendering microglia as neutral. In addition, microglia promote tissue healing and repair, as they represent the brain's primary defense system against insult and infection. Therefore, clarifying the beneficial or detrimental role[s] of microglia at different disease stages is warranted. We suggest that the PFF model provides an improved platform for clarifying the role of microglia in synucleinopathy and degeneration, as it avoids the confounds of surgical artifact, compressed time-course, and irrelevant inflammatory responses resulting from continuous supraphysiological expression of human protein.

Is α-Syn Overexpression Analogous to Idiopathic PD?

Reliance on Supraphysiological α-Syn Levels and Lack of Protracted Lewy-Like Pathology

The development of AAV-α-syn overexpression models represents a significant advantage over previous neurotoxicant models which lack synucleinopathy as a component of disease pathology. Notably, AAV-α-syn overexpression models have provided insights into direct mechanisms of α-syn toxicity, including axonal and dopamine transport deficits and impairment of protein degradation pathways (Chung et al., 2009; Gaugler et al., 2012; Volpicelli-Daley et al., 2016; Koprich et al., 2017). Models induced by intranigral injection of adeno-associated (AAV) or lentiviral (LV) overexpressing human wildtype or mutant α-syn often result in dramatic increases in the levels of protein (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002; Yamada et al., 2004; Ulusoy et al., 2010;

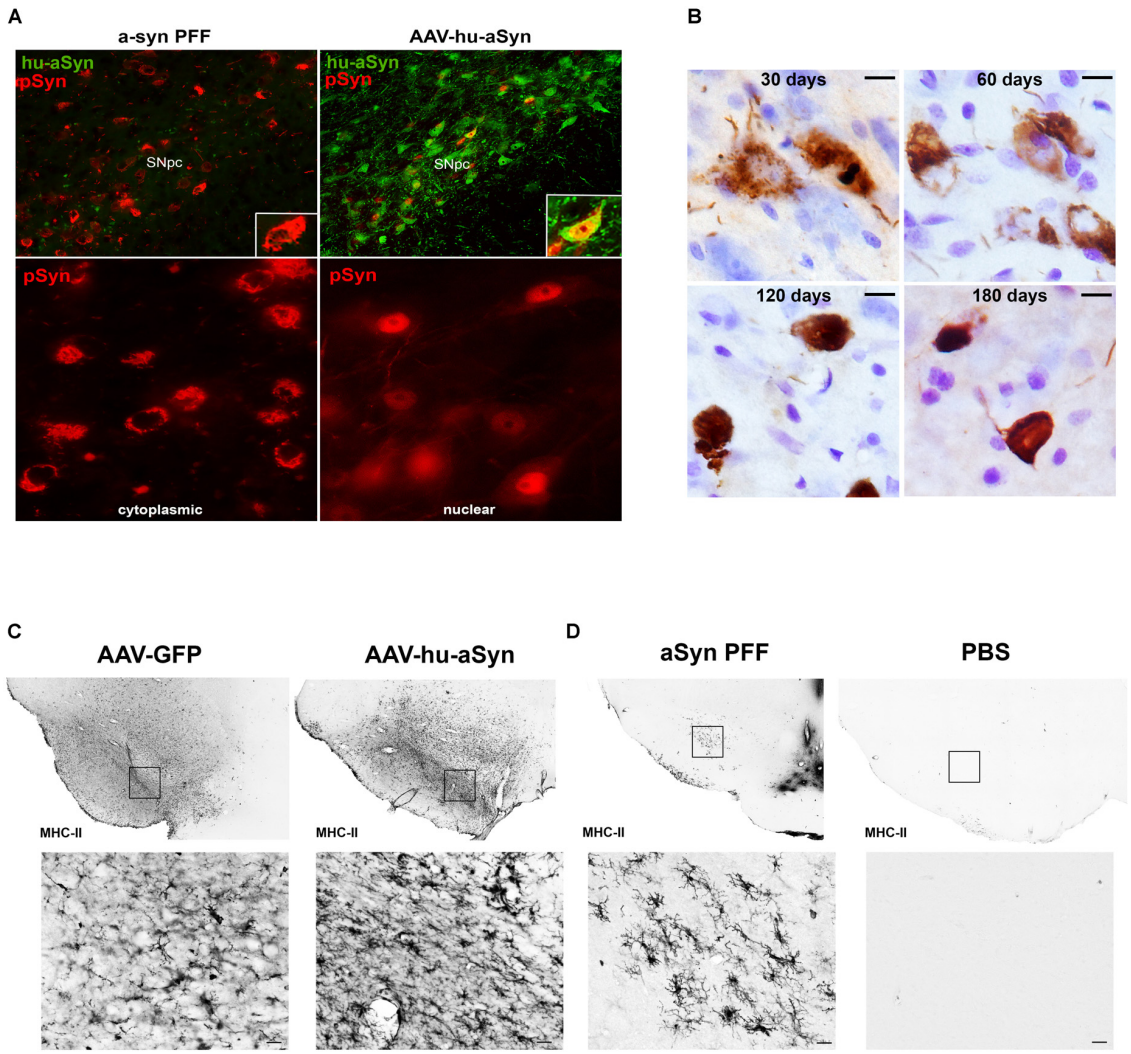

FIGURE 1 | Comparison of alpha-synuclein pathology and inflammation between AAV-overexpression and α-syn preformed fibril models. **(A)** Intrastriatal injection of α-syn PFFs results in accumulation of phosphorylated α-syn (pSyn, red) inclusions in the cytoplasm of SNc neurons, similar to cytoplasmic Lewy bodies in PD. Intranigral delivery of rAAV2/5 expressing human wildtype α-syn results in transduction of many nigral neurons with abundant human α-syn (green), but relatively few pSyn inclusions (red) in the cytoplasm in comparison to PFF-induced pSyn inclusions number. pSyn inclusions induced by AAV-mediated overexpression of α-syn are predominantly nuclear. **(B)** Evolution of Lewy body-like pSyn inclusion formation in the substantia nigra pars compacta induced by intrastriatal PFF injection. Inclusions follow a similar pattern to that observed in human PD, with immature inclusions appearing diffuse and granular and becoming more dense and compact over time. **(C,D)** Comparison of MHC-II expression 8 weeks following intranigral rAAV2/5 GFP or human wildtype α-syn vector injection **(C)** or intrastriatal injection of sonicated preformed murine α-syn fibrils or PBS **(D)**. In all cases no loss of nigral dopamine neurons was observed. Injection of AAV2/5 GFP or human α-syn is associated with robust increases in MHC-II on microglia throughout the mesencephalon, suggesting that the inflammation observed is largely attributable to surgical injection at the transduction site. In contrast, intrastriatal injection of PFFs results in MHC-II expression more localized to the SNc, with few to no MHC-II immunoreactive microglia evident in PBS injected animals. Abbreviations: rAAV2/5, recombinant adeno-associated virus 2/5; α-syn, alpha-synuclein; GFP, green fluorescent protein; MHC-II, major histocompatibility complex-II; PBS, phosphate buffered saline; PFFs, alpha-synuclein preformed fibrils; pSyn, alpha-synuclein phosphorylated at serine 129; SNc, substantia nigra pars compacta.

Lundblad et al., 2012; Mulcahy et al., 2012; Oliveras-Salvá et al., 2013; Van der Perren et al., 2015; Ip et al., 2017). Although final protein levels are titer dependent, many studies report levels of α-syn 2–20× higher than normal endogenous expression levels, as reviewed extensively (Volpicelli-Daley et al., 2016). As these levels far exceed those observed in either idiopathic, duplication and even SNCA triplication carriers, they raise the potential for pathophysiological mechanisms specific to supraphysiological α-syn expression, mechanisms that may not be relevant to idiopathic PD. Indeed, the downregulation of multiple trophic factor responsive genes is observed with fourfold, but not lower α-syn overexpression levels (Decressac et al., 2012; Su et al., 2017). *In vitro* studies show that following transduction, neurons release multiple forms of α-syn (Kim et al., 2013). It is therefore likely that α-syn is similarly released from neurons transduced *in vivo*. It is unclear

TABLE 1 | Considerations when modeling idiopathic PD in rodents.

Feature	AAV-overexpression models	α-syn PFF model
Ability to examine impact of α-synuclein inclusions distinct from degeneration	**Difficult** Simultaneous α-syn overexpression and aggregation progresses rapidly to degeneration over the course of weeks	**Straightforward** Distinct interval of inclusion formation followed by degeneration over a protracted time course
Injection artifact	**Confound** Direct injections into the SN produce marked neuroinflammatory response that can make interpretation difficult	**Less of a factor** Direct injections into the striatum have less of an impact within the SN
α-synuclein levels	**Not analogous to idiopathic PD** Continuous supraphysiological α-syn levels produced by forced overexpression are not analogous to idiopathic PD	**Normal endogenous α-syn levels** Pathophysiology results from templating of normal levels of α-syn
Extranigral α-synuclein pathology	**Not present** Pathology limited to the nigrostriatal system	**α-syn pathology in multiple regions** Allows for the examination of events outside of nigrostriatal system

Abbreviations: AAV, adeno-associated virus; α-syn, alpha-synuclein; PD, Parkinson's disease; PFFs, alpha-synuclein preformed fibrils; SN, substantia nigra.

to what extent inclusion-bearing neurons release α-syn, if at all.

Lewy pathology in PD is widespread and likely develops in multiple regions concurrently. In contrast, AAV and LV-mediated overexpression models drive α-syn expression in discrete circuitries, most often the nigrostriatal system. Another consideration is the form of Lewy pathology generated by α-syn overexpression. Although pSyn immunoreactive and proteinase-K resistant inclusions have been reported they are most often small and punctate (Ip et al., 2017) in contrast to the large, cytoplasmic aggregates seen in human PD and in the α-syn PFF model. Further, frequently the pSyn inclusions generated following α-syn overexpression are localized to the nucleus **Figure 1A** and **Supplementary Material**, unlike the cytoplasmic LBs that are the hallmark of PD and are observed in the PFF model (**Figure 1B**). A dramatic rise in cytosolic pSyn has been documented in PD (Zhou et al., 2011), and thus pSyn localized to the nucleus that is observed with viral vector-mediated α-syn overexpression would prevent pSyn's ability to interact with cytoplasmic proteins and structures.

Transgenic models overexpressing wildtype or mutant α-syn consistently display widespread synuclein pathology including proteinase-K resistant inclusions and behavioral deficits (Tanji et al., 2010; Yamakado et al., 2012). While there are some exceptions (Nuber et al., 2013), the majority of transgenic models do not exhibit robust, protracted nigral degeneration (Matsuoka et al., 2001; Fernagut and Chesselet, 2004). Thus, while transgenic models are adequate for investigating development of Lewy-like pathology as a consequence of germline genetic changes, most fail to recapitulate downstream nigral degeneration and thus have limited translational potential for evaluation of neuroprotective strategies.

Inflammation in AAV and LV-Mediated Overexpression Models: Location, Location, Location

Another feature of viral vector-mediated α-syn overexpression models that is often leveraged is their ability to produce a robust neuroinflammatory response as indicated by microgliosis, MHC-II and CD68 on microglia, in addition to production of proinflammatory cytokines (Chung et al., 2009; Sanchez-Guajardo et al., 2010; Harms et al., 2013). However, there are several considerations when interpreting the disease relevance of the neuroinflammatory response in this paradigm. First, the majority of viral vector models are induced by direct intranigral injection which alone, in the absence of α-syn overexpression, can trigger a pronounced increase in MHC-II immunoreactive microglia (**Figure 1C**). This suggests that a significant component of the inflammatory response resulting from intranigral injections of α-syn vectors is due to the injection itself. Further, as previously stated, α-syn overexpression paradigms can result in the release of supraphysiological levels of α-syn into the immediate environment. This secretion of α-syn likely triggers a neuroinflammatory response in the absence of degeneration (**Figure 1C**) that has little to do with the disease state attempting to be modeled. Lastly, the majority of AAV and LV models overexpress human α-syn, not rodent α-syn, in rats and mice (Fischer et al., 2016; Volpicelli-Daley et al., 2016). As rat and mouse α-syn differs from human α-syn by eight amino acids, it is plausible that overexpression of the foreign human protein may initiate an artificial inflammatory response.

CONCLUSION

The multiple features of the α-syn PFF model and the AAV a-syn model have been extensively reviewed elsewhere (refs). However, we contend that some of these model-specific features deserve specific attention when modeling idiopathic PD (**Table 1**). In particular, we offer that that α-syn PFF model may be better suited for studies of neuroinflammation and the relationship between α-syn aggregation and toxicity in idiopathic PD. The ability to advance our understanding of pathophysiology in idiopathic PD and predict the efficacy of novel therapeutics is dependent on the fidelity of animal models to the disease state. When modeling idiopathic PD, the presence of LB-like α-syn

inclusions within the context of normal endogenous α-syn levels in multiple brain regions, which ultimately results in progressive nigrostriatal degeneration are an essential model feature. While α-syn overexpression models have advanced our understanding of α-syn-mediated toxicity, they depend on focal expression of supraphysiological levels of α-syn in a limited circuitry. In contrast to *SNCA*-linked familial PD, clinicopathologic evidence does not support the concept that increases in α-syn levels drive pathophysiology in idiopathic PD and therefore α-syn overexpression may trigger pathogenic mechanisms that may not be relevant to idiopathic PD. We propose that accumulation of Lewy body-like inclusions in multiple regions induced by injection of PFFs in the context of normal α-syn levels, ultimately resulting in downstream inflammation and progressive nigral degeneration, more faithfully models the sequence of events in idiopathic PD. Additionally, findings from the PFF-model may be applicable to other synucleinopathies. Thus, the synucleinopathy induced by α-syn PFF injections represents an exceptional preclinical PD model to investigate the pathogenic contribution of endogenous α-syn, and assess novel disease-modifying therapeutics.

ETHICS STATEMENT

All procedures performed in studies involving animals were in accordance with the ethical standards of the Institute for Animal Use and Care Committee (IACUC) at Michigan State University.

AUTHOR CONTRIBUTIONS

This manuscript was conceived and organized by MD, TC, and CS and discussed among all authors. Data were generated by MD, CK, CS, DF, and AS. The manuscript was first written and revised by MD and CS, and it was reviewed and critiqued by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00621/full#supplementary-material

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REVIEW
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α-Synuclein and Noradrenergic Modulation of Immune Cells in Parkinson's Disease Pathogenesis

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α-synuclein (α-syn) pathology and loss of noradrenergic neurons in the locus coeruleus (LC) are among the most ubiquitous features of Parkinson's disease (PD). While noradrenergic dysfunction is associated with non-motor symptoms of PD, preclinical research suggests that the loss of LC norepinephrine (NE), and subsequently its immune modulatory and neuroprotective actions, may exacerbate or even accelerate disease progression. In this review, we discuss the mechanisms by which α-syn pathology and loss of central NE may directly impact brain health by interrupting neurotrophic factor signaling, exacerbating neuroinflammation, and altering regulation of innate and adaptive immune cells.

Keywords: α-synuclein, locus coeruleus, Parkinson's disease, neuroinflammation, norepinephrine, immune cell

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INTRODUCTION

Locus coeruleus (LC) degeneration and α-synuclein (α-syn) aggregation are among the most ubiquitous features of Parkinson's disease (PD) (Chui et al., 1986; German et al., 1992; Zarow et al., 2003). Brain regions affected in PD, including the LC, contain large protein-rich intracellular inclusions known as Lewy bodies (LB) or Lewy neurites (LN) accompanied by chronic inflammation and neuron loss (den Hartog and Bethlem, 1960; Spillantini et al., 1997; Tansey and Goldberg, 2010). While LBs and LNs contain numerous proteins, α-syn is the predominant component (Spillantini et al., 1997), and α-syn is the major pathological protein underlying PD pathogenesis. α-syn is a 140-amino acid protein encoded by the *SNCA* gene, which is expressed in many tissue types and which accounts for approximately 1% of cytosolic proteins in neurons (Shibayama-Imazu et al., 1993; Iwai et al., 1995; Stefanis, 2012). It is highly expressed in the presynaptic terminals where it acts as a molecular chaperone in SNARE formation and vesicular trafficking (Burre et al., 2010). Genetic evidence comes from individuals carrying *SNCA* mutations, which confer increased risk of PD, or autosomal dominant forms of PD (Klein and Schlossmacher, 2006). Finally, animal models overexpressing α-syn develop age-dependent α-syn aggregates and PD-like behavioral abnormalities (Masliah et al., 2000; Giasson et al., 2002). The initiating event in α-syn aggregation is unknown, but Lewy pathology (LP) and cell loss are common within discrete neuronal populations in PD.

Extensive dysfunction of catecholaminergic neurons is a well-established feature of PD, and a major hallmark is LP and loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) which induces motor impairments including tremor, muscle rigidity, bradykinesia, and postural instability (Hirsch et al., 1988; Fearnley and Lees, 1991; Parkinson, 2002). A diagnosis of PD is currently dependent on the presence of motor symptoms and striatal dopamine deficiency; however, PD is a multifactorial disease with non-motor symptoms that are associated with alterations in cholinergic, serotonergic, and noradrenergic systems occurring years or even decades prior to the onset of motor dysfunction (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008).

LP and degeneration of several pontine and medullary nuclei (including the dorsal raphe, dorsal motor nucleus of the vagus, pedunculopontine nucleus, and LC) are ubiquitous features of PD (Halliday et al., 1990). The LC is the major source of norepinephrine (NE) to the CNS, and it is among the first brain regions to be affected in PD (Iversen et al., 1983; Mann and Yates, 1983; Braak et al., 2001). NE is the ligand for the adrenergic receptors (ARs) comprised of seven G-protein coupled receptors that are G_q-, $G_{i/o}$-, or G_s-coupled, allowing NE to have diverse functional effects dependent on receptor expression and cell type (Strosberg, 1993). LC neurons are constitutively active and innervate virtually every brain region via extensive and complex axonal arborization that facilitates the release of both synaptic NE and extra-synaptic NE at axonal varicosities (Freedman et al., 1975; Grzanna and Molliver, 1980; Jones and Yang, 1985; Agnati et al., 1995) where LC-NE can be neuroprotective by both direct and indirect mechanisms. Here, we will review evidence that LC dysfunction may exacerbate PD pathophysiology and may represent a tipping point in disease progression.

LC-NE DYSREGULATION COULD PROMOTE THE PROGRESSION OF PD PATHOLOGY

It is unclear why certain neuronal populations like the LC are vulnerable to α-syn pathology, but sensitivity to oxidative stress, pacemaker activity, and extensive contact with blood vessels that may expose LC neurons to circulating toxins have been implicated (Jenner, 2003; Cho, 2014; Pamphlett, 2014). The degree of noradrenergic innervation to a brain region is negatively correlated with DA loss (Tong et al., 2006), indicating that the loss of central NE and its neuroprotective actions may directly influence the rate of PD progression. In PD, loss of LC neurons begins prior to nigral pathology and appears to be of greater magnitude (German et al., 1992; Zarow et al., 2003; Szot et al., 2006; Brunnstrom et al., 2011). Per the Braak staging hypothesis of PD pathology, LP first appears in brainstem nuclei (stage 1), and, as PD progresses, continues along a caudo-rostral axis with LC pathology appearing at stage 2 and SNpc pathology at stage 3, before ultimately extending into cortical regions (Braak et al., 2003). PD brain tissue has marked LC denervation in many brain regions and loss of LC cell bodies that extends throughout its rostral-caudal axis (Javoy-Agid et al., 1984; German et al., 1992; Pavese et al., 2011). Imaging and postmortem histological studies of PD patients reveal a progressive loss of central NE throughout the brain (Pifl et al., 2012) along with accumulation of α-syn and loss of LC neurons (Halliday et al., 1990; Chen et al., 2014; Keren et al., 2015; Isaias et al., 2016). LC neuron vulnerability to α-syn pathology can be replicated experimentally. A recent model targeted viral vector-mediated overexpression of a familial PD mutant α-syn variant to the murine LC region (Henrich et al., 2018). While transgene expression was not restricted to neuronal cells, the resulting progressive α-syn aggregation, gliosis, and LC degeneration are reminiscent of LC pathology found in PD. Enzymes responsible for NE synthesis and NE metabolite levels are reduced in the CSF of PD patients, also supporting these central changes in NE metabolism (Hurst et al., 1985; Goldstein et al., 2012). Evidence of early LC dysfunction can be found in patients who do not meet the diagnostic criteria for PD. In such individuals, decreased neuron density in the LC, but not VTA or dorsal raphe, corresponds to the severity of global parkinsonism (Buchman et al., 2012), suggesting that this state may represent prodromal/preclinical PD. Patients who had LP at autopsy but lacked any of the clinical signs of PD also had reduced LC neuron density as compared to DA neurons in the SNpc, further highlighting the possible early role of LC neuron loss in PD (Dickson et al., 2008).

There is also evidence that α-syn may directly affect NE homeostasis by two separate mechanisms. First, norepinephrine transporter (NET)-expressing cells transfected for α-syn expression reveal that high levels of α-syn negatively regulate NET expression on the cell surface, while relatively lower levels increase NET expression (Wersinger et al., 2006). Second, when α-syn is overexpressed in an NE-producing cell line or transgenic rodent model, it can translocate to the nucleus and directly interfere with transcription of dopamine ß-hydroxylase (DBH), the enzyme involved in the final step of NE synthesis, reducing NE production (Kim et al., 2011, 2014). It is possible that interfering in NE neurotransmission could, in turn, impact α-syn expression as ß-adrenergic receptor (ß-AR) agonists reduce *SNCA* mRNA and α-syn protein expression in induced pluripotent stem cells derived from individuals carrying the *SNCA* triplication mutation (Mittal et al., 2017). Together, these data indicate that α-syn can influence NE metabolism, and that this, in turn, could impact α-syn expression, although additional work is required to determine if this is clinically relevant.

PD NON-MOTOR SYMPTOMS

The LC is the major source of NE to the CNS (Mouton et al., 1994), and dysregulated noradrenergic innervation is associated with many of the non-motor symptoms of PD including anxiety (Casacchia et al., 1975; Stein et al., 1990; Nuti et al., 2004), depression (Shulman et al., 2002; Ravina et al., 2007), rapid eye movement (REM) sleep behavioral disorder (RBD) (Sixel-Doring et al., 2011; Kalaitzakis et al., 2013), and dementia (Chui et al., 1986).

Up to 60% of PD patients report experiencing some form of anxiety (Chaudhuri and Schapira, 2009; Lin et al., 2015; Houser and Tansey, 2017). Dopamine, serotonin, and NE have been implicated in PD anxiety, suggesting that its neurobiological origins are complex (Eskow Jaunarajs et al., 2011; Thobois et al., 2017; Joling et al., 2018). LC neurons are highly active during stress exposure (Bingham et al., 2011; Curtis et al., 2012) and innervate all corticolimbic regions involved in the anxiety response (Aston-Jones et al., 1991, 1999). In PD patients, anxiety severity is inversely correlated with dopamine/NE transporter binding in the LC (Remy et al., 2005), and experimentally, selectively inhibiting LC neurons during stress exposure blocks the subsequent anxiety-like behavior (McCall et al., 2015).

Around 35% of PD patients suffer from depression (Reijnders et al., 2008; Houser and Tansey, 2017). Dysfunction of LC-NE is known to be associated with depression (Moriguchi et al., 2017) and is a common pharmacological target in the treatment of depression (Ressler and Nemeroff, 2001; Remy et al., 2005). Indeed, early investigation of NET expression in the LC reported decreased NET in major depressive disorder (Klimek et al., 1997), although results from subsequent studies have been inconsistent (Moriguchi et al., 2017). While it is unclear if NET is downregulated due to lack of available NE or in order to increase synaptic NE levels, it is clear that NE dysfunction can contribute to depressive symptoms.

LC neuron activity fluctuates diurnally with increased activity immediately prior to waking and during waking hours (Hobson et al., 1975). Sleep disturbances are one of the most common complaints from PD patients (Smith et al., 1997) and can include insomnia (Gjerstad et al., 2007), excessive daytime sleepiness (Rye et al., 2000), and RBD (Comella et al., 1998; Gagnon et al., 2002). A recent study reported that disturbed sleep is positively correlated with anxiety and depression in PD (Rana et al., 2018). In fact, RBD is the most predictive non-motor symptom of synucleinopathies with up to 92% of idiopathic RBD patients receiving a synucleinopathy diagnosis within 14 years (Iranzo et al., 2006; Postuma et al., 2009; Schenck et al., 2013). There is evidence that LC neurons in individuals that have PD with disturbed sleep contain more LP than in those without (Kalaitzakis et al., 2013), and mice lacking DBH (and subsequently, NE) have significantly disturbed sleep behavior (Hunsley and Palmiter, 2003). Together, these data suggest that loss of central NE may directly contribute to the development of sleep disturbances in PD.

An estimated 83% of PD patients will experience some sort of cognitive dysfunction, including dementia (Hely et al., 2008). Dementia is characterized by cognitive impairment, including memory loss, attentional deficits, and loss of executive function (Elizan et al., 1986; Aarsland et al., 2003). While dementia is generally associated with cholinergic deficits and late-stage PD, early executive disturbances may arise from deregulation of LC-NE. PD patients with dementia have more extensive loss of LC-NE in cortical regions than those without (Chan-Palay and Asan, 1989). In fact, degeneration of LC neurons and loss of cortical NE is a central component of dementia of Alzheimer's type (Mann and Yates, 1983; Zarow et al., 2003). In animal models, hippocampal LC-NE is essential for proper memory acquisition and retrieval (Devauges and Sara, 1991; Mello-Carpes et al., 2016), and loss of LC neurons can impact memory and enhance cognitive deficits (Ohno et al., 1997; Chalermpalanupap et al., 2018).

BEYOND THE NON-MOTOR SYMPTOMS

The temporal relationship between LC and SNpc pathology suggests that loss of LC-NE may leave SNpc neurons more vulnerable to α-syn toxicity and potentiate the rate of PD progression. Experimentally, loss of LC-NE exacerbates 6-OHDA- and MPTP-mediated nigral degeneration in rodent and primate models (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Rommelfanger et al., 2007; Yao et al., 2015), while increasing synaptic NE by genetic deletion or pharmacological blockade of the NE transporter (NET) confers resistance (Kilbourn et al., 1998; Rommelfanger et al., 2004). Indeed, individuals with a functional polymorphism in the promoter regions of the *DBH* gene have reduced risk of developing PD (Healy et al., 2004). In sum, these data demonstrate that loss of NE may exacerbate nigral pathology.

NEUROPROTECTIVE EFFECTS

NE can directly act as a neurotrophic factor but can also indirectly stimulate neurotrophic factor expression. Primary mesencephalic cultures treated chronically with NE have a significantly reduced rate of cell death, increased neuritic processes, and reduced production of reactive oxygen species when compared to untreated cultures, and this phenotype resembles cultures treated with traditional antioxidants (Troadec et al., 2001, 2002). Increasing synaptic NE was shown to be protective against neuron loss and inflammation in a model of hypoxic-ischemia (Toshimitsu et al., 2018). While NE ligation of ARs directly facilitates neuroprotection by several mechanisms, the neuroprotective effects are not always blocked by AR antagonists, suggesting NE-mediated protection may also occur indirectly. One candidate mechanism of interest is the neuropeptide brain-derived neurotrophic factor (BDNF), which is synthesized and released by astrocytes and neurons, including those in the LC (Castren et al., 1995). BDNF signaling is primarily mediated by binding to the high affinity tropomyosin-related kinase B receptor (TrkB), which can protect SNpc neurons in experimental models, and BDNF mRNA is reduced in the SNpc in PD (Hyman et al., 1991; Spina et al., 1992; Howells et al., 2000). NE can also enhance BDNF transcription and BDNF/TrkB kinetics (Chen et al., 2007). Activation of the β1-adrenergic receptor stimulates BDNF transcription in astrocytes (Koppel et al., 2018). When BDNF binds to TrkB, signal transduction is mediated by TrkB dimerizing and autophosphorylating (Haniu et al., 1997). NE can induce autophosphorylation of TrkB and is protective against cell death in primary culture (Liu et al., 2015). In addition to loss of NE, α-syn may also directly disrupt the neuroprotective effects of BDNF. A recent study demonstrated that α-syn has the potential to bind the kinase domain on TrkB receptors, preventing the neurotrophic signaling of BDNF/TrkB, and that this exacerbates degeneration of DA neurons (Kang et al., 2017).

CENTRAL INFLAMMATION

Neuroinflammation is a vital mechanism in restoring brain integrity following neuronal insult but is also a core component of PD pathology. In a healthy brain, the inflammatory response resolves relatively quickly, with normal brain function restored (Roth et al., 2014; Laumet et al., 2018). In neurodegenerative diseases, such as PD, sustained neuroinflammation can become

cytotoxic, aggravating neuronal degeneration. It is unclear what triggers the initial inflammation in PD, but extracellular monomeric or aggregated α-syn can be phagocytosed by microglia and induce their activation (Zhang et al., 2005; Hoenen et al., 2016), and neuronal overexpression of α-syn aggravates and prolongs neuroinflammation (Miller et al., 2007; Gao et al., 2011; Sanchez-Guajardo et al., 2013). In PD patients, immune mediators such as IL-1ß, TGFß, IFNγ, and IL-6 are increased in the cerebral spinal fluid (CSF) and nigrostriatal regions (Mogi et al., 1994; Blum-Degen et al., 1995; Mount et al., 2007), and SNpc DA neurons appear particularly sensitive to pro-inflammatory cytokines (McGuire et al., 2001; Mount et al., 2007; Tansey and Goldberg, 2010). In fact, neuroinflammation is detectable prior to signs of neuronal degeneration, suggesting a potential early role for inflammation in PD pathogenesis (Theodore et al., 2008; Watson et al., 2012).

Research indicates that dysregulation of noradrenergic signaling may also play a role in driving inflammation. Like overexpression of neuronal α-syn, lesioning LC neurons using a noradrenergic-specific toxin also induces inflammation (Theodore et al., 2008; Watson et al., 2012; Yao et al., 2015; Song et al., 2018). NE can have activating or inhibitory effects on immune cells depending on adrenergic receptor expression, which varies depending on the cellular environment (Khan et al., 1985; Tanaka et al., 2002). Therefore, LC degeneration and subsequent deficient brain NE may contribute to PD pathology by loss of normal immune cell modulation. Microglia, the brain-resident macrophages, are the sentinels of brain parenchyma, monitoring tissue integrity and responding to infection or injury (Nimmerjahn et al., 2005). When ramified (resting) microglia are activated, they adopt an amoeboid morphology, proliferate, and become phagocytic, releasing pro-inflammatory cytokines which can recruit central and peripheral immune cells to the site of insult (Hayes et al., 1987). There is extensive evidence of sustained microglial over-activation in degenerating brain regions in PD (Kim and Joh, 2006; Tansey and Goldberg, 2010), and inhibiting microglia activation with minocycline prevents DA neuronal loss in mice treated with a DA neuron-specific toxin (Wu et al., 2002).

Microglia express many neurotransmitter receptors, including ARs (Pocock and Kettenmann, 2007). While more studies are required to understand how AR activation affects microglial phenotypes, depletion of NE, as is found in PD, exacerbates microglial inflammatory responses (Heneka et al., 2002; Bharani et al., 2017). AR-mediated modulation of microglia is well documented, although reports of the functional outcome are inconsistent. In murine brain slices, resting microglia appear to preferentially express the excitatory ß2-AR, but shift toward the inhibitory α2-AR receptor expression following activation with the canonical microglial activator lipopolysaccharide (LPS) (Gyoneva and Traynelis, 2013). However, microglial treatment with an ß2-AR agonist is reported to have anti- or pro-inflammatory effects. For example, cultured primary microglia treated with a ß2-AR agonist suppressed microglial proliferation (Fujita et al., 1998), while a subsequent study reported that priming microglia with a ß2-AR agonist prior to LPS treatment significantly increased pro-inflammatory IL-1ß and IL-6 expression (Johnson et al., 2013). The functional outcome of microglial AR activation appears dependent on the physiological context, and further examination is needed to determine how this may influence PD pathology.

PERIPHERAL INFLAMMATION

There is abundant evidence that the inflammatory manifestations of PD are not confined to the CNS. Indicators of inflammation have been found in the colon tissue, stool, and blood as well as in the CSF. Colonic expression of the genes encoding pro-inflammatory cytokines TNF, IFNy, IL-6, and IL-1β is increased in PD, accompanied by evidence of gliosis (Devos et al., 2013). Recently, we reported that IL-1α, IL-1β, CXCL8, and CRP are significantly elevated in stool from PD patients compared to controls (Houser et al., 2018), and that serum levels of TNF, IFNy, and neutrophil gelatinase-associated lipocalin levels are significantly and consistently different in PD over a 24-h period (Eidson et al., 2017). Local α-syn expression has been found to increase under inflammatory conditions in the periphery (Stolzenberg et al., 2017), and α-syn pathology has been observed in the enteric nervous system of PD patients, even from the earliest stages of disease (Stokholm et al., 2016; Barrenschee et al., 2017; Punsoni et al., 2017). These findings demonstrate that similar pathological processes are active in the CNS and the periphery in PD, and there is almost certainly significant crosstalk between them.

Degradation of the blood-brain-barrier (BBB) has been well documented in PD (Kortekaas et al., 2005; Pisani et al., 2012; Gray and Woulfe, 2015), and it has been proposed that this impaired barrier function exposes the CNS to circulating factors that could promote α-syn aggregation (Gray and Woulfe, 2015), immune cell infiltration, neuroinflammation, and, ultimately, neurodegeneration (Rite et al., 2007). Whether through direct effects of reduced signaling through endothelial β-ARs or through increases in vascular permeability-promoting inflammation, LC neurodegeneration compromises the integrity of tight junctions (Kalinin et al., 2006) and increases permeability of the BBB (Nag and Harik, 1987). BBB leakiness enables greater interaction between central and peripheral immune activities, allowing exchange of cytokines, chemokines, and other circulating molecules and potentially even facilitating infiltration of peripheral immune cells into the CNS where loss of central NE modulation could result in aberrant immune cell activity.

As with brain-resident microglia, immune cells originating in the periphery can also be modulated by NE. Peripheral immune cells infiltrate the brain parenchyma in PD (Kannarkat et al., 2013), and these will likely be directly impacted by reduced levels of central NE. Peripheral NE levels may also play important immunomodulatory roles in PD. The NE deficiency found in the CNS in PD is not consistently recapitulated in the periphery, with several studies reporting no difference in NE levels in plasma from PD patients compared to healthy controls (Eldrup et al., 1995; Goldstein et al., 2003). It is likely, however, that at least a subset of PD patients is affected by peripheral NE dysregulation as evinced by the prevalence of neurogenic orthostatic hypotension (NOH) associated with this

disease. NOH is a condition in which insufficient noradrenergic activity results in failure to appropriately increase blood pressure (BP) in response to a postural change such as sitting up or standing. This results in insufficient cerebral blood supply and can produce lightheadedness and dizziness, which increase fall risk (Merola et al., 2016). NOH occurs frequently in conditions involving synucleinopathy, and roughly 30% of PD patients are affected. NOH in PD is attributed to noradrenergic postganglionic sympathetic denervation associated with LP and a subsequent failure to induce sufficient NE production when transitioning to an upright position (reviewed by Loavenbruck and Sandroni, 2015). PD patients with orthostatic hypotension exhibit lower levels of NE in plasma compared to PD patients without NOH that reach levels significantly lower than non-PD controls (Senard et al., 1990; Niimi et al., 1999; Goldstein et al., 2005). This creates the potential for PD-associated NE deficiency to modulate peripheral immune responses as well as central.

Nearly every lymphoid tissue in the body has postganglionic sympathetic innervation, and peripheral innate and adaptive immune cells express ARs, rendering them responsive to NE. Excitatory β2-ARs are the most highly expressed ARs on peripheral immune cells, and their activity likely dominates the immune response to NE. β-AR signaling has potent anti-inflammatory effects on innate immune cells (reviewed by Qiao et al., 2018). In macrophages, which bear close functional resemblance to microglia, it suppresses pro-inflammatory activity and promotes tolerogenic and homeostatic phenotypes (Grailer et al., 2014; Noh et al., 2017). It also limits the number and the effector functions of natural killer (NK) cells (Whalen and Bankhurst, 1990; Takamoto et al., 1991). Adrenergic signaling has been shown to impair the functions of neutrophils and eosinophils as well (Gosain et al., 2009; Brunskole Hummel et al., 2013; Noguchi et al., 2015). Dendritic cells connect the innate and adaptive immune responses by sampling antigens in the local environment and then presenting them with appropriate polarization signals to T cells. β2-AR activation profoundly suppresses dendritic cell functionality, inhibiting their maturation, migration, antigen presentation including cross presentation, and proinflammatory cytokine production while inducing expression of anti-inflammatory factors (Seiffert et al., 2002; Herve et al., 2013; Chen et al., 2016; Qiao et al., 2018). It is important to note that while these anti-inflammatory effects on innate immune cells are well-documented, study designs differ widely, and the effects they observe on these cells vary depending on physiological context, time, AR agonist, and dose. Further research will be necessary to better characterize the relationship between NE and innate immune responses.

CD4+ T helper (Th) cells are indirectly affected by AR agonists due to their suppressive effects on dendritic cells which result in diminished differentiation of effector T cells, particularly Th1s (Wu et al., 2016). Th1 cells also express β2-ARs (McAlees et al., 2011), and their proliferation and activity are inhibited upon ligation of this receptor (Ramer-Quinn et al., 1997; Riether et al., 2011). Since Th2 cells do not express ARs (McAlees et al., 2011), their functionality is not directly modulated by exposure to NE, but NE-mediated suppression of Th1 cells would relieve their negative regulatory pressure on Th2 cells, indirectly promoting Th2-mediated immune activity, which is canonically involved in anti-helminth and allergic immune responses but not classic inflammation (Huang et al., 2015). β2-AR signaling also impairs the activity of CD8+ memory and effector T cells (Chen et al., 2016; Estrada et al., 2016; Bucsek et al., 2017).

The consequences of AR ligation on other T cell subsets are less straightforward. The intricacies of the potential effects of NE on CD4+ Th17 cells are just beginning to be elucidated. These cells are important actors in normal mucosal immunity, but they are also implicated in autoimmune pathology. Several studies have reported that treatment of CD4+ cells with NE promotes differentiation of Th17 cells and increases their activity (IL-17 production) while simultaneously inhibiting Th1 differentiation and activity (IFNγ production) (Carvajal Gonczi et al., 2017; Xu et al., 2018). On the other hand, studies of Th17 cells from both mice and humans with Th17-mediated autoimmune diseases found that treating CD4+ T cells with NE inhibited the differentiation and activity of Th17 cells (IFNγ production was also still reduced) (Boyko et al., 2016; Liu et al., 2018). This indicates that the immunoregulatory effects of NE on Th17 cells are dependent on the physiological context. It is also possible that autoimmune conditions in which pathology is mediated in part by IL-17-producing cells might constitute a unique context in which this alternative regulatory action of NE is observed. For instance, in such conditions, a highly inflammatory cell type that exhibits characteristics of both Th1 and Th17 cells is typically present (Murphy et al., 2010), and it may be that the actions of NE on this particular cell type rather than on canonical Th17s dominate its observed effects in these autoimmune diseases.

Findings on NE modulation of CD4+ T regulatory (Treg) cells, an anti-inflammatory subset which counteracts effector functions of other types of T cells, are even more ambiguous. One study reports that treatment of Tregs with NE prior to transfer in an autoimmune arthritis mouse model rendered them pathological and worsened the disease (Harle et al., 2008). In the same vein, another study found that NE exposure decreased the regulatory activity of Tregs and even induced their apoptosis (Wirth et al., 2014). On the other hand, a study in humans reported that Treg frequencies were elevated under conditions which increased circulating NE levels and that treatment of Tregs with epinephrine, which is chemically similar to NE and binds the same receptors, stimulated Treg proliferation. This effect was blocked by treatment with a β-AR antagonist (Inoue et al., 2017). A final study reported no detectable effects of treatment with NE or epinephrine on human Tregs, though they did determine that they could express three different types of ARs (Cosentino et al., 2007). Obviously, more research is needed to determine the effect of NE on Tregs.

B cells also express β2-ARs, and there is evidence that NE can negatively regulate the magnitude of antibody responses. The effects are highly varied, however, as they are influenced by the effects of NE on T cells, by the stimuli used to activate B cells, and by the immunological and physiological context of the experiment (extensively reviewed by Kin and Sanders, 2006). A couple of more recent studies suggest that, under conditions of autoimmune disease in which B cells contribute to inflammatory activity and pathology, NE exerts a suppressive effect on these

cells which is mediated by decreased IL-7 receptor signaling and enhanced production of anti-inflammatory IL-10 (Pongratz et al., 2012, 2014).

The effects described here do not represent the full extent of peripheral NE-mediated neuroimmune interactions. Most studies to date have focused on the results of β2-AR signaling, but immune cells express other ARs as well which can mediate different effects (Lorton and Bellinger, 2015), and, as in the brain, the relative levels of these receptors change in different immune environments. Activation of the same AR can even produce distinct responses depending on the concentration of the ligand and its temporal relationship to immunogenic stimuli (reviewed by Lorton and Bellinger, 2015). This provides important plasticity for neuroimmune regulatory mechanisms. Nonetheless, many functional studies support the existing literature that indicates a primarily anti-inflammatory impact of peripheral NE. Vagus nerve stimulation is known to have clear immunosuppressive effects (Inoue et al., 2017) and to reduce synuclein expression in the brain (Farrand et al., 2017), and these effects are mediated in large part by NE signaling through β-ARs (Vida et al., 2011). A recent review (Bucsek et al., 2018) summarized numerous studies showing that chemical ablation of sympathetic neurons or β-AR blockade enhanced immune responses to different bacterial, viral, and parasitic infections while AR agonist treatment impaired anti-viral and anti-parasite responses. Several of the studies found that these effects were specific to modulation of peripheral adrenergic activity, but it was also demonstrated that this could induce corresponding immune responses in the CNS. Similarly, another study found that ablation of peripheral and LC noradrenergic neurons prompted an exaggerated acute inflammatory response to peripheral LPS that was observed both in the brain and in the circulation (Bharani et al., 2017).

Taken together, the data on peripheral immune cells and their function when challenged indicate that NE is immunosuppressive, and as such, postganglionic sympathetic denervation and NE deficiency in PD could stimulate pro-inflammatory immune activity. This has implications for PD pathogenesis and the progression of disease pathology. Peripheral and systemic inflammation have been well documented in PD, and it has been proposed that inflammatory mechanisms may contribute to non-motor symptoms and also be responsible for the development and spread of synucleinopathy and the induction of neuroinflammation and neurodegeneration in this disorder (Qin et al., 2016; Houser and Tansey, 2017). PD-associated gastrointestinal abnormalities and dysfunction are consistent with inflammatory conditions in the gut (Houser and Tansey, 2017), and levels of proinflammatory cytokines in the blood correlate positively with the severity of anxiety and depression in PD patients (Wang et al., 2016). α-syn levels increase in the context of immune activation, and some data suggest that peripheral inflammation can induce elevated α-syn expression in the brain (Kelly et al., 2014) and that peripheral α-syn can migrate to the brain through the vagus nerve (Holmqvist et al., 2014). α-syn has also been shown to exert chemoattractant properties on peripheral myeloid cells, including recruiting them into the brain in a rodent PD model (Stolzenberg et al., 2017; Harms et al., 2018). Infiltration of peripheral CD4+ and CD8+ T cells into the brain has also been observed in PD (Brochard et al., 2009), and it has been shown that these T cells (primarily the CD4+ subset) in peripheral blood from PD patients recognize and respond to peptides derived from α-syn (Sulzer et al., 2017). In animal models of parkinsonian neuropathology, invading monocytes and CD4+ T cells have been identified as key mediators of neurodegeneration (Brochard et al., 2009; Harms et al., 2018).

NE deficiency, centrally and/or in the periphery, could potentiate all of these immune-mediated effects in PD. It would impair anti-inflammatory regulatory functions, shifting immune cells toward more pro-inflammatory phenotypes. Innate immune cells affected in this way would be less able to clear α-syn aggregates and neuronal debris effectively and in a toleragenic manner and more likely to recruit additional effector cells, stimulate their pro-inflammatory activities, and perhaps even present α-syn and other neuronal antigens in a context which could induce autoimmune responses (Sulzer et al., 2017). Furthermore, the activity of at least some T cell subsets which may be pathologically involved in PD could be potentiated by a loss of inhibitory NE signaling. Especially in the context of a compromised BBB, these pro-inflammatory immune cells and their products would have greater access to the CNS and could infiltrate and mediate damaging effects on neurons there.

DISCUSSION

Extensive dysfunction of catecholaminergic neurons is a well-established feature of PD, and while a major hallmark is LP and loss of DA neurons in the SNpc, PD is a multifactorial disease with alterations in cholinergic, serotinergic, and noradrenergic systems occurring years earlier and generally associated with PD's non-motor symptoms (Halliday et al., 1990; Braak et al., 2003). α-syn pathology and a progressive decline in LC-NE have been well characterized; still it is unclear why these neurons are among the most vulnerable in PD. Still less is known about how the deficits in LC-NE and the loss of its neuroprotective and neuroimmune modulatory effects could influence the development of synucleinopathy and exacerbate PD pathology (summarized in **Figure 1**). Preclinical research has provided compelling evidence supporting the neuroprotective functions of NE. Experimentally, depletion of NE renders SNpc neurons vulnerable in toxin models of PD (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Rommelfanger et al., 2007), while NE enhancement is protective (Kilbourn et al., 1998; Rommelfanger et al., 2004). Additionally, there is a reciprocal modulatory relationship between α-syn and NE whereby α-syn can modulate NE neurotransmission, both at the level of synthesis (Kim et al., 2014), and by modulating NET expression at the cell surface (Wersinger et al., 2006), and NE can attenuate *SNCA* transcription and α-syn protein expression (Mittal et al., 2017). As PD pathophysiology progresses, LP develops in the SNpc and other brain regions, and LC-NE denervation may exacerbate the rate and/or degree of degeneration during this premotor phase of PD. Experimentally, NE drives BDNF/TrkB

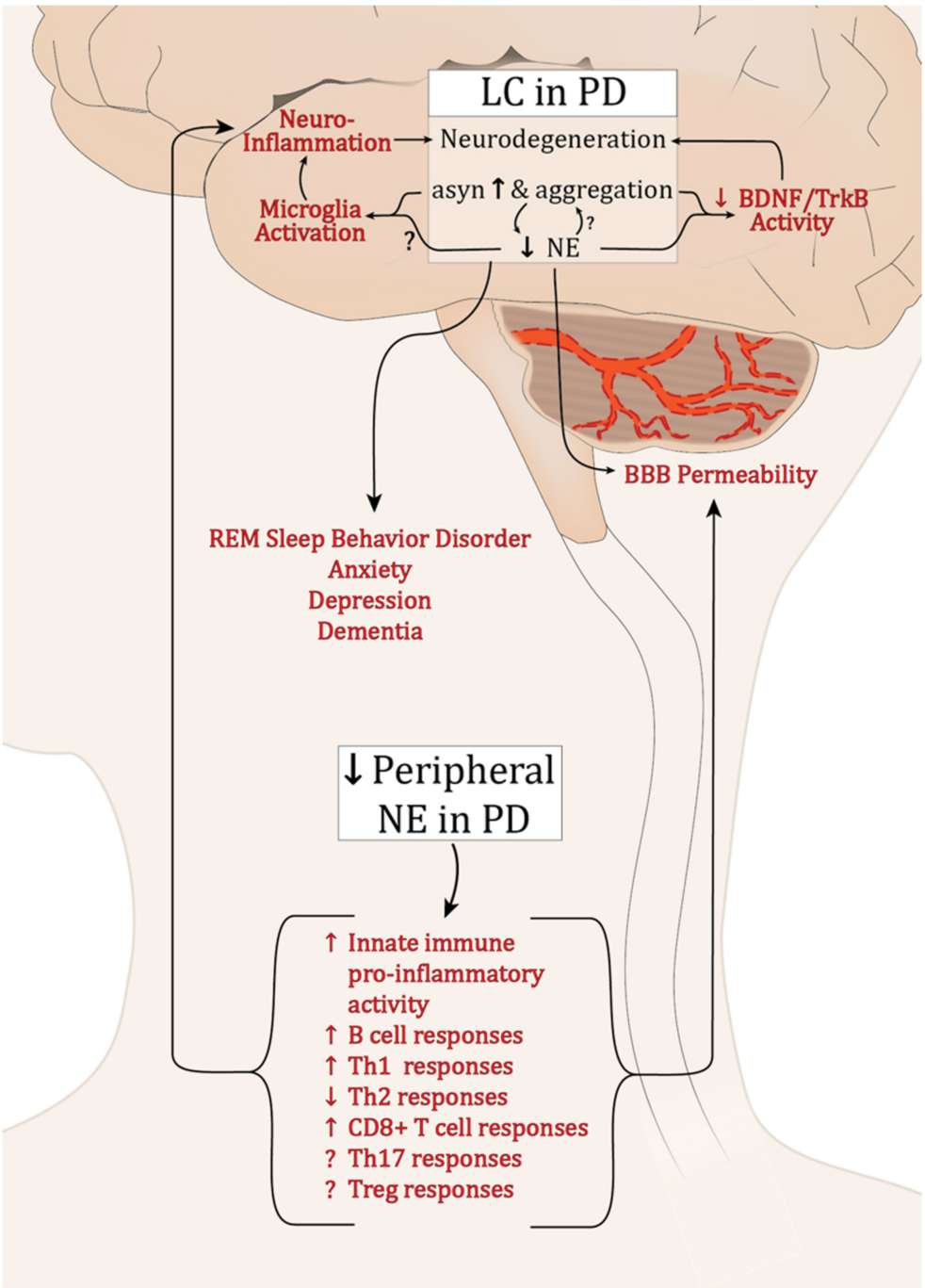

FIGURE 1 | Potential effects of dysregulated NE in PD. Abbreviations: α-syn, α-synuclein; BBB, blood brain barrier; B cell, B lymphocytes; BDNF, brain derived neurotrophic factor; LC, locus coeruleus; PD, Parkinson's disease; NE, norepinephrine; Th1, T helper 1 lymphocytes; Th17, IL-17 producing T helper lymphocytes; Th2, T helper 2 lymphocytes; CD8+, cytotoxic T lymphocytes; Treg, T regulatory lymphocytes; Trkb, tropomyosin-related kinase B receptor.

signal transduction (Liu et al., 2015), while α-syn can interrupt it (Kang et al., 2017). The detrimental effects of declining NE in PD may be compounded by the inhibition of BDNF-mediated neuroprotection by α-syn. This could contribute to the low serum BDNF levels that negatively correlate with motor impairment in later PD (Scalzo et al., 2010).

Neuroinflammation is a cardinal feature of PD and experimentally, both α-syn overexpression and lesion of the LC neurons result in inflammation (Theodore et al., 2008; Watson et al., 2012; Yao et al., 2015). While the experimental outcomes are currently inconsistent, it is clear that NE can modulate microglia activation status (Fujita et al., 1998;

Gyoneva and Traynelis, 2013; Johnson et al., 2013). The decline in brain NE, increase in synucleinopathy, and subsequent modulation of microglia may contribute to the chronic inflammation found in PD brain tissue. Such inflammation is sufficient to induce parkinsonian neurodegeneration (Duffy et al., 2018; Song et al., 2018).

While the brain was once believed to be "immune privileged," the entry of peripheral immune cells through the BBB is now a well-established feature of PD. Numerous immune cell populations are responsive to NE, and its deficiency in the periphery would diminish what seems to be a largely anti-inflammatory regulatory influence. This could promote exaggerated pro-inflammatory immune responses systemically. If the peripheral immune cells were recruited to the brain, reduced local NE levels combined with synuclein pathology would serve to augment and sustain inflammatory activity.

The physiological effects of neuroimmune interactions both centrally and peripherally are myriad, and their subtleties are just beginning to be appreciated and studied in detail. They may have the potential, however, to offer new therapeutic approaches for disorders such as PD for which effective treatments remain elusive. Future research evaluating the incidence of PD among individuals taking β-AR blockers (Mittal et al., 2017), for instance, and the rate of disease progression in PD patients treated with drugs that raise peripheral NE levels, such as droxidopa, could reveal new information about the role of NE in PD pathology.

AUTHOR CONTRIBUTIONS

LB contributed to conception and wrote the first draft of the manuscript. MH wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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REVIEW
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Rationally Designed Variants of α-Synuclein Illuminate Its *in vivo* Structural Properties in Health and Disease

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α-Synuclein (αS) is a conserved and abundant neuronal protein with unusual structural properties. It appears to partition between folded and unstructured states as well as between membrane-bound and aqueously soluble states. In addition, a switch between monomeric and tetrameric/multimeric states has been observed recently. The precise composition, localization and abundance of the multimeric species are under study and remain unsettled. Yet to interfere with disease pathogenesis, we must dissect how small changes in αS homeostasis may give rise to Parkinson's disease (PD), dementia with Lewy bodies (DLB) and other human synucleinopathies. Rationally designed αS point mutations that prevent the protein from populating all states within its normal folding repertoire have continued to be instrumental in bringing new insights into its biochemistry *in vivo*. This review summarizes biochemical and cell biological findings about αS homeostasis from different labs, with a special emphasis on intact-cell approaches that may preserve the complex, metastable native states of αS.

Keywords: α-synuclein, structure, proteotoxicity, mutagenesis, multimerization, Parkinson's disease

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αS IN HEALTH AND DISEASE

"Synucleinopathies" comprise Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy and also Alzheimer's disease. DLB is the most common cause of dementia after AD and vascular dementia (Meeus et al., 2012), while PD is the most common neurodegenerative disease after AD, with an estimated life-time risk of 1.5% globally (Tanner and Goldman, 1996). Typical pathology of synucleinopathies comprises neuronal loss and neuronal/neuritic aggregation of α-synuclein (αS), a protein of 140 amino acids (aa) with an incompletely defined function involving synaptic vesicle trafficking (e.g., Scott and Roy, 2012; Vargas et al., 2014; Wang et al., 2014). Since its discovery as the first causative gene product for PD (Polymeropoulos et al., 1997) and the major constituent of Lewy bodies and Lewy neurites (large aggregate structures) in patients' brains (Spillantini et al., 1997), αS has been increasingly implicated as a key pathogenic protein in sporadic and familial PD (fPD). αS missense mutations, copy number variants, and upregulated expression have each been associated with fPD (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004; Fuchs et al., 2008; Appel-Cresswell et al., 2013; Kiely et al., 2013; Lesage et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014) or an fPD/DLB spectrum [especially E46K (Zarranz et al., 2004)].

Consequently, both native and altered αS folding states are of great interest as regards normal biology and the mechanisms, diagnostics and disease-modifying therapeutics of synucleinopathies. In order to prevent a pathological state of any protein, it is of central importance to understand its physiological state in all details. Nonetheless, αS structure and precisely how it is altered in the synucleinopathies remain unclear. A well-recognized αS species *in vitro* is the soluble unfolded monomer (Weinreb et al., 1996), and a recent publication showed that this state can persist in considerable part when exogenous unfolded recombinant monomers are delivered into cultured mammalian cells by electroporation (Theillet et al., 2016). However, this in-cell liquid phase NMR analysis did not rule out the existence of other αS species, as the method is unable to detect membrane-bound or multimeric αS forms (Alderson and Bax, 2016). At least 10% of cellular αS is found in membrane fractions (Kahle et al., 2000; Fortin et al., 2010; Dettmer et al., 2015a), and transiently membrane-associated αS has been characterized as monomeric and helically folded (George et al., 1995) or multimeric and (presumably) folded (Burré et al., 2014). In addition, previous (Bartels et al., 2011) and recent (Iljina et al., 2016) studies report soluble, multimeric αS forms that have α-helical conformation, resist aggregation, and are distinct from pathological, β-sheet-rich oligomers that are the hallmark of synucleinopathies. If all αS forms that have been repeatedly described are relevant, they likely exist in a dynamic equilibrium with each other. Perturbation of the neuronal αS equilibrium in neurons on the other hand, may be the starting point for pathological αS insolubility and misfolding. Since their discovery, the known fPD/DLB-linked αS missense mutations have been obvious candidates for studying perturbed αS equilibria, but rationally designed variants have been informative as well.

THE REPETITIVE STRUCTURE OF αS

The N-terminal two thirds of αS contain up to nine 11-residue imperfect repeats, with the consensus core motif being KTKEGV (Bendor et al., 2013). **Figure 1A** highlights the KTKEGV motifs within the 140 aa sequence, and **Figure 1B** displays the αS aa sequence after aligning it by the KTKEGV motifs. Repeats 1–5 and 7 are highly conserved, repeat 9 is partially conserved and repeats 6 and 8 are poorly conserved. The repeats are interrupted by 4 aa (ATVA) between repeats 4 and 5. In addition to the core motif (KTKEGV) that encompasses positions 2–7 of the 11-aa repeat, the polar character of positions 1 and 9 as well as the non-polar, hydrophobic character of positions 8, 10, and 11 are relatively well conserved, as visualized by the color-code in **Figure 1C** (see legend). The repeats are highly conserved, both across vertebrate species and among the three homologs α-, β- and γ-synuclein. The KTKEGV motif has not been observed in non-vertebrates, and no similar sequence has been identified outside the synuclein protein family. However, perilipins (Londos et al., 1999) as well as apolipoproteins and certain plant proteins (George et al., 1995) exhibit a similar overall structure, i.e., they contain 11-aa repeats with a similar pattern of charged/polar and hydrophobic aa. Like αS, apolipoproteins interact with lipid membranes via their N-terminal regions and, interestingly, they are the protein class that is over-represented in amyloid diseases: so far four apolipoproteins, SAA, Apo AI, Apo AII, and Apo AIV, have been described in the context of amyloidosis (Sipe et al., 2014). The 11-aa repeats enable such polypeptides to form amphipathic 11/3 helices at membranes (different from true α-helices): after exactly three turns, position 1 of the next 11-aa repeat is reached. **Figure 1D** illustrates in a simplified fashion the 11/3 helix formation of αS for repeats 1–7 in the context of the rest of the protein including the ATVA intervening sequence. The formation of helical αS on membranes was predicted from its sequence and formally demonstrated by binding to artificial membranes *in vitro* (Davidson et al., 1998). In a simplified diagram that focuses on repeats 1–7 and ignores ATVA, **Figure 1E** depicts two forces that attract amphipathic αS helices to cellular membranes: (i) light gray area: hydrophobic interactions between fatty acyl chains (long curved black lines in **Figure 1E**) and the hydrophobic half of the αS amphipathic helix; and (ii) light blue areas: electrostatic interactions between positively charged lysines (K) on opposite sides of the helix and negatively charged membrane lipid headgroups (e.g., phosphatidylserine, phosphatidic acid, phosphatidylinositol headgroups; depicted in red) (Zhu and Fink, 2003). While a "bent" α-helix was detected on small-diameter micelles by nuclear magnetic resonance (Eliezer et al., 2001; Ulmer and Bax, 2005), the extended 11/3 helix was observed for spin-labeled protein on artificial membranes, which have a larger diameter than micelles and are expected to model the *in vivo* conformation better (**Figure 1F**: aa 9–89) (Jao et al., 2004, 2008). Thus, through the membrane interaction of multiple KTKEGV repeats, αS likely forms one long 11/3 helix that lies along the outside surface of cellular vesicle membranes, at least half-buried in the bilayer (Bussell et al., 2005; Jao et al., 2008; Wietek et al., 2013), while the C-terminal ∼30–40 aa remain unfolded. Moreover, the preference of αS for curved membranes (cellular vesicles) instead of relatively flat membranes (ER or plasma membrane) is well established (Middleton and Rhoades, 2010; Jensen et al., 2011). **Figure 1F** illustrates the positions of repeat 4 and ATVA (both color-coded) within the extended 11/3 helix of αS (only aa 9–89 are shown). Moreover, the positions of the lysine "wings" are shown (the hydrophobic half of the helix is below, the hydrophilic half above the plane that is defined by the lysines).

Importantly, purified recombinant αS in solution behaves like a natively unfolded protein *in vitro* (Weinreb et al., 1996; Bertoncini et al., 2005). The *in vivo* occurrence of this αS conformation, presumably in addition to membrane-associated helical monomers, has recently been suggested by intact-cell NMR (Binolfi et al., 2012; Theillet et al., 2016). In addition to those monomeric states, native multimeric αS assemblies have been observed by several groups (Bartels et al., 2011; Wang et al., 2011, 2014; Dettmer et al., 2013; Gurry et al., 2013; Westphal and Chandra, 2013; Burré et al., 2014; Gould et al., 2014; Iljina et al., 2016). The characterization of the multimeric αS species ranges from soluble tetramers (Bartels et al., 2011; Wang et al., 2011) to membrane-associated octamers (Burré et al., 2014). The

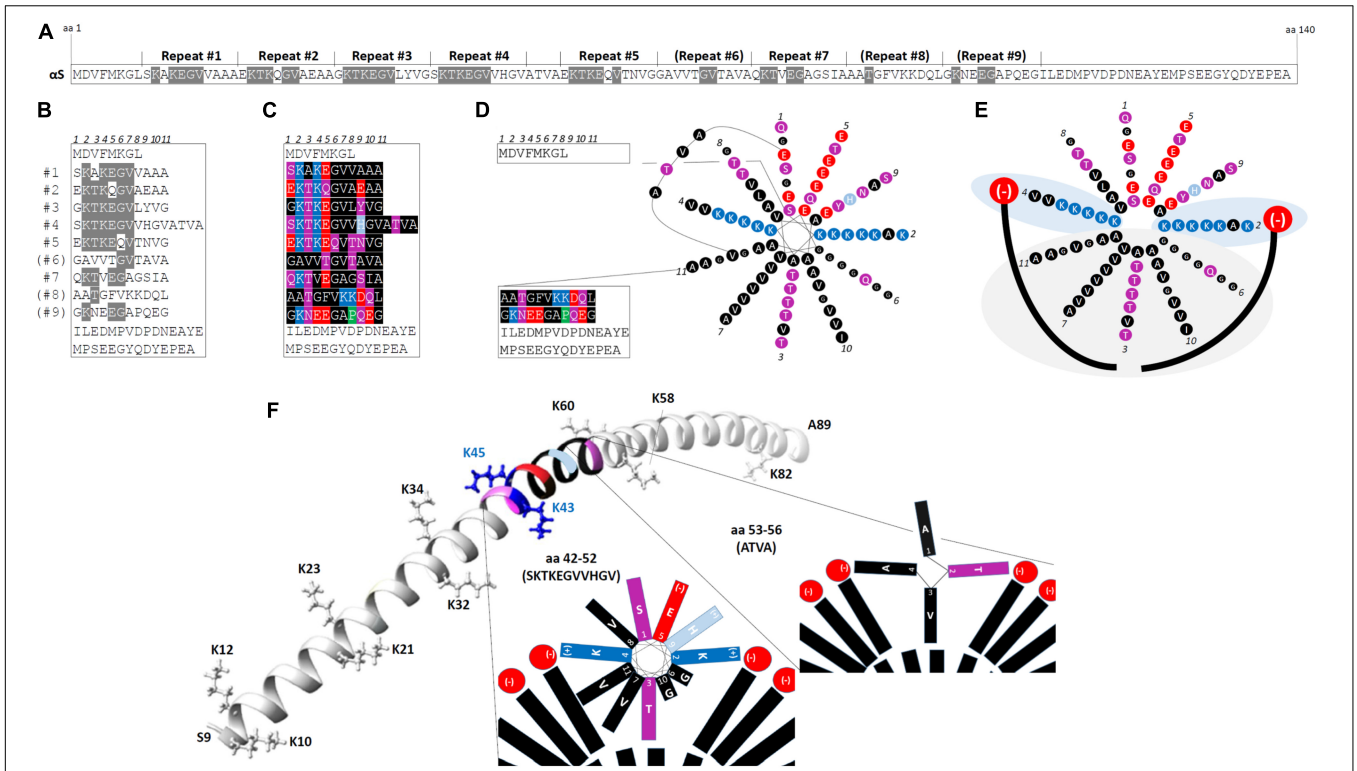

FIGURE 1 | The repetitive structure of αS. **(A)** The linear 140-aa sequence of human wt αS. 11-aa repeats 1–9 with the core consensus motif "KTKEGV" are indicated, poorly conserved repeats are in brackets; aa that fully conform to "KTKEGV" are highlighted in gray. **(B)** Schematic of wt human αS by aligning its aa sequence via the KTKEGV motifs; aa that fully conform to "KTKEGV" are highlighted in gray. Repeats 1–9 are interrupted only once: by "ATVA" between repeat 4 and 5. **(C)** Color-coded schematic of wt human αS by aligning its aa sequence via the KTKEGV motifs. Blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, and black: non-polar residues. In addition to KTKEGV, the polar character of positions 1 and 9 as well as the non-polar, hydrophobic character of positions 8, 10, and 11 are relatively well conserved. **(D)** Color-coded schematic of wt human αS with its 11-aa repeats 1–7 arranged in the helical wheel (11/3 helix: 3 turns over 11 residues) of a membrane-induced amphipathic helix. Blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, and black: non-polar residues. The design of the wheel diagram has been adapted from Bendor et al. (2013). **(E)** Color-coded schematic of αS repeats 1-7 (omitting "ATVA" between repeats 4 and 5) in an 11/3 helical wheel, embedded in the outer leaflet of a curved vesicle membrane (negatively charged lipid head-groups in red, fatty acid 'tails' in black). The helix is stabilized by hydrophobic interactions (gray area) and electrostatic interactions (blue area). **(F)** Position of repeat 4 (aa 42–52) and ATVA (aa 53–56) within the extended 11/3 helix of αS (only aa 9–89 are shown). The positions of the lysine "wings" are indicated (the hydrophobic half of the helix is below, the hydrophilic half above the plane that is defined by the lysines). The structure is based on data by Jao et al. (2008) (courtesy Ralf Langen lab, USC).

relationship and relative abundance of all these species may be highly dependent on biological context and therefore difficult to predict from *in vitro* experiments.

Over the years, several αS missense mutations have each been associated with familial PD (**Figure 2A**: wt αS, **Figure 2B**: mutants). Those are, in chronological order of publication: A53T (Polymeropoulos et al., 1997), A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013; Proukakis et al., 2013), G51D (Kiely et al., 2013; Lesage et al., 2013) and A53E (Pasanen et al., 2014). While all except A30P cluster around aa 50, their relative position in the αS amphipathic helix differs: A30P (11-aa repeat position: 11) and G51D (position 10) are in the hydrophobic half of the helix, E46K (position 5) and H50Q (position 9) are in the hydrophilic half; A53T/E are not found in the 11-aa repeat, but in the ATVA sequence between repeat 4 and 5. Neither is the effect of the substitution on the nature of the respective aa (e.g., charged vs. uncharged) unifying among them. Moreover, A30P binds to membranes less than wt (Jo et al., 2002), E46K binds more (Choi et al., 2004) and the A53T may exhibit similar binding (Bussell and Eliezer, 2004). In contrast to that, the clustering around a "putative protein loop" (Kara et al., 2013) (except A30P) and a negative impact on physiological multimer formation (Dettmer et al., 2015a) have been proposed to be a common feature of fPD-linked mutants. Strategic "exaggerations" and "analogies" of fPD-linked mutants promise to lead to a better understanding of the effect of fPD-linked mutations on αS homeostasis, especially when studied in the cellular context (see below). Many important questions in αS research are ultimately linked to understanding αS conformational homeostasis in intact cells. How are the different αS conformations linked to αS function *in vivo*? How is αS structural homeostasis maintained in health and perturbed in disease? How does αS proteinaceous aggregation start, and is that truly the key pathogenic event in human synucleinopathies? How should we design strategies for therapeutic intervention? This review will emphasize that valuable tools toward answering these

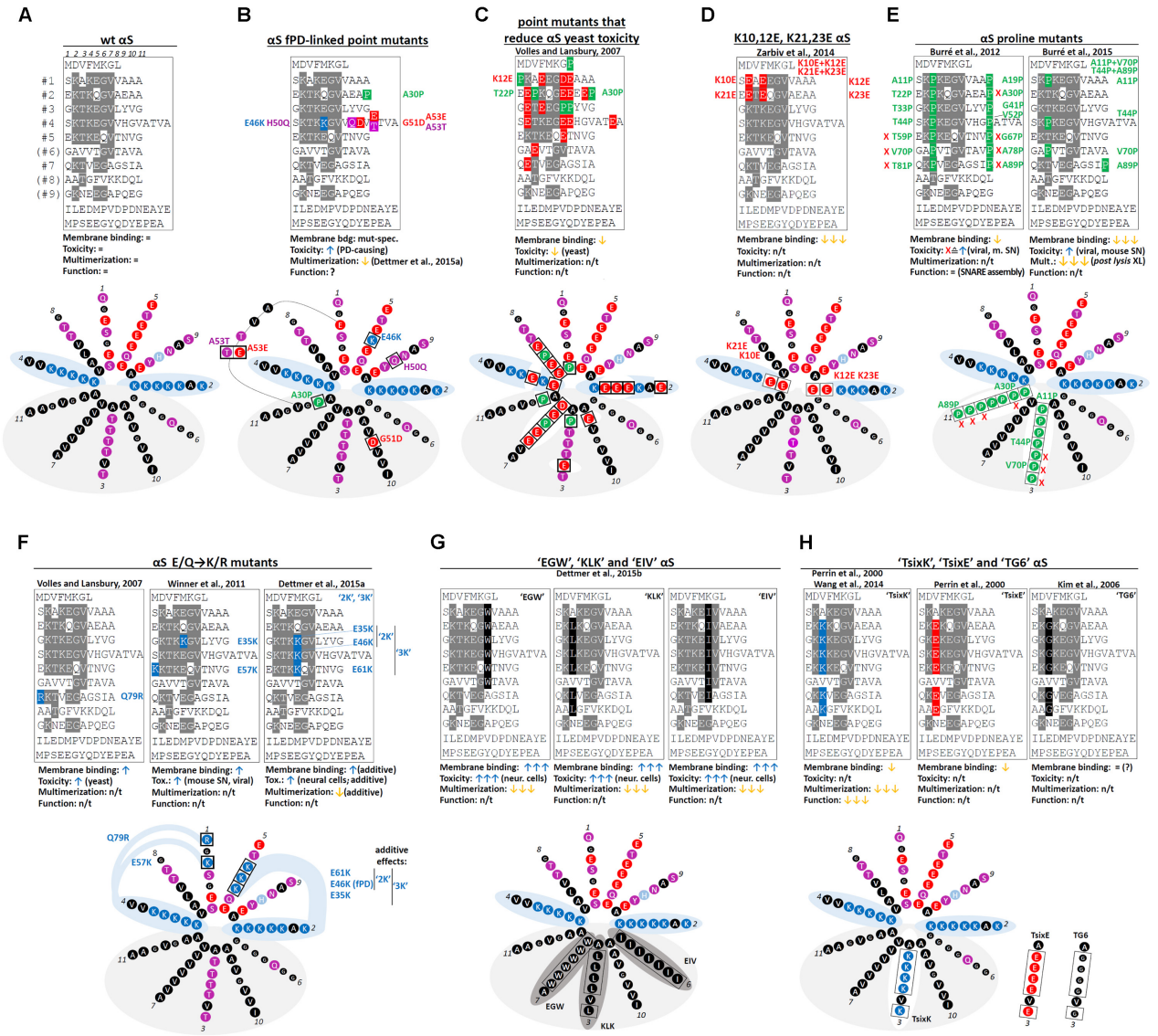

FIGURE 2 | Wt αS, fPD-linked and strategic αS mutations. **(A)** Schematic of wt human αS by aligning its aa sequence via the KTKEGV motifs; aa that fully conform to "KTKEGV" are highlighted in gray (top). Color-coded (see **Figure 1**) schematic of repeats 1–7 (omitting "ATVA" between repeats 4 and 5) in an 11/3 helical wheel (bottom). **(B)** fPD-linked αS missense mutations ("ATVA" was included to illustrate A53T and A53E). **(C)** Non-toxic αS point mutations (in aa 1–89) identified by expression in yeast (most were identified in compound mutants and the focus is on E and P mutations). E at aa positions 3, 7, and 10 of each repeat destabilizes the hydrophobic interaction between αS and lipid tails, E at positions 2 and 4 destabilizes electrostatic interactions with lipid headgroups; the effect of E at position 8 is less clear. P destabilizes the helix independent of its position. **(D)** K10,12E and K21,23E. E substitutions at positions 2 and 4 of each repeat destabilize the electrostatic interaction with lipid headgroups. **(E)** Engineered αS single P mutants (left panel) and compound mutants A11P/V70P and T44P/A89P (right panel). The P substitutions reduce helix formation, thereby increasing the pool of cytosolic unfolded αS. "X" marks toxic single point mutants. **(F)** E/Q-to-K/R mutants. The positive charge of R or K presumably stabilizes the electrostatic interaction with negatively charged headgroups analogous to fPD-linked αS E46K. Left panel: yeast-toxic αS Q79R. Middle panel: E35K and E57K. Right panel: αS "2K" (E35K + E46K) and αS "3K" (E35K + E46K + E61K) amplify/exaggerate E46K. **(G)** Hydrophobic KTKEGV repeat motif mutants. Left: "EGW" (consensus motif: KTKEGW). The bulky, non-polar W instead of V at position 7 adds hydrophobicity to the amphipathic αS helix, leading to strongly increased binding. Middle: Engineered αS mutant "KLK" (KLKEGV). The non-polar L instead of T at position 3 corrects the imperfect hydrophobicity in the amphipathic helix, leading to strongly increased binding. Right: "EIV" (KTKEIV). The non-polar I instead of G at position 6 corrects the imperfect hydrophobicity in the amphipathic αS helix, leading to strongly increased binding. **(H)** KTKEGV repeat motif mutants modifying the "T" position. T at position 3 of each 11-aa repeat causes imperfect hydrophobicity in the hydrophobic face of the amphipathic αS helix, leading to only transient binding. Left: "TsixK" (KKKEGV). K instead of T at position 3 reduces the hydrophobicity in the amphipathic helix. However, the additional positive charge may lead to an aberrant binding to negatively charged lipid head groups, permitting residual, but possibly abnormal binding characterized by less helicity. Middle: "TsixE" (KEKEGV). E instead of T at position 3 reduces the hydrophobicity in the amphipathic helix. Right: "TG6" (KGKEGV). G instead of T at position 3 may have a helix-destabilizing effect, while membrane-binding might be intact. (n/t, not tested; XL, crosslinking; gray areas indicate the hydrophobic face of the amphipathic αS helix; dark gray areas: increased membrane binding/stronger helix formation; white areas: decreased membrane binding/impaired helix formation).

questions can arise from informative point mutations that, e.g., prevent αS from populating all states within its normal folding landscape.

αS TOXICITY AND MEMBRANE BINDING

About one decade after the discovery of αS as the principal Lewy body component and its initial structural characterization, Volles and Lansbury (2007) published a remarkable study on αS fibrillization and yeast toxicity. The authors screened a library of random αS point mutants both *in vitro* and in yeast in order to identify variants that could help elucidate sequence-phenotype relationships. When *in vitro* fibrillization and yeast toxicity of the αS variants were compared, no correlation of toxicity with fibrillization rate was observed, suggesting that fibrillization is not necessary for αS-induced yeast toxicity. A second screen in a library of several thousand yeast clones identified 25 non-toxic αS sequence variants. Most of these contained a mutation to either proline (P) or glutamate (E) that decreased membrane binding (**Figure 2C**) relative to wt αS (**Figure 2A**). The authors hypothesized that αS toxicity in yeast is caused by the protein binding directly to membranes at levels sufficient to non-specifically disrupt membrane homeostasis. Subsequent studies helped characterize the membrane-associated toxicity of αS in more detail: αS expression in yeast (any level of αS expression in the αS-lacking *S. cerevisiae* is "over-expression") was found to lead to vesicle clustering/aggregation (Soper et al., 2008) and vesicle trafficking defects (Cooper et al., 2006). The relevance of these findings beyond the yeast system, and for PD pathogenesis in particular, was highlighted when similar vesicle trafficking defects were recapitulated in patient-derived αS A53T and αS triplication iPS cell cultures (Chung et al., 2013). However, in contrast to the yeast system, no pronounced toxicity was observed in the iPS cells, possibly due to lower expression levels or a better ability of mammalian cells to compensate for αS-induced membrane dyshomeostasis. While this apparent lack of immediate toxicity is consistent with PD being an insidious, relatively late-onset disease, it also suggests that a "mutation amplification" strategy might be necessary to readily detect αS-induced vesicle trafficking defects and their related toxicity in mammalian cells.

A decrease in membrane binding caused by "E mutants" was confirmed upon expression in human cells (HEK and the mesencephalic neuronal cell line MN9D) by Zarbiv et al. (2014). The authors replaced two positive lysine (K) residues with two negative glutamate (E) residues at either the first (K10,12E) or second (K21,23E) KTKEGV repeat motif (**Figure 2D**). Reduced binding of both double-point mutations relative to wt αS was determined by a quantitative phospholipid ELISA assay. In addition, the K-to-E substitutions resulted in strongly reduced levels of soluble αS oligomers, but larger intracellular inclusions. The toxicity of the mutants relative to wt αS was not addressed in the study.

The membrane binding and toxic effects of "P mutants" in neuronal cells were the subject of a comprehensive study by Burré et al. (2012). The authors generated 13 strategic αS proline mutants within seven αS KTKEGV repeats (**Figure 2E**, left panel) and analyzed them relative to wt, A30P, E46K, and A53T αS in seven assays that ranged from biochemical studies on purified αS to examining the toxicity of virally expressing αS in the mouse substantia nigra (SN) by stereotactic injections. Strikingly, proline mutations in the central region of αS, referred to as NAC (non-amyloid β component) domain (residues 61–95), as well as T59P and the fPD-linked mutations A30P, E46K, A53T increased the neurotoxicity of αS. In contrast, *all* P mutants (except G41P) both inside and outside the NAC domain significantly reduced membrane association, as the authors had expected based on the helix-breaking effects of the proline residues. In a follow-up study (Burré et al., 2015), the same group examined proline-rich αS variants A11P/V70P and T44P/A89P (**Figure 2E**, right panel), plus a quadruple mutant A11P/V70P/T44P/A89P. All these compound "P mutants" showed lack of membrane binding in various *in vitro* assays. Moreover, all compound proline mutants were significantly more toxic than wt αS in the cultured cells, and the respective P mutant-injected mice (SN, stereotactic virus injection) showed more motor deficits and more pronounced loss of tyrosine-hydroxylase-positive neurons in the SN compared to wt αS-injected mice (which themselves showed more deficits than control-injected mice). In cultured cells, transfection of A11P/V70P and T44P/A89P as well as the quadruple P mutant caused cytoplasmic inclusion formation that was significantly more pronounced than that of wt αS. While the authors did not address the nature of the inclusions in the study, they assumed that those inclusions were β-sheet-rich and thus different from the vesicle-rich inclusions that can be observed when wt αS is expressed in yeast or when αS variants with enhanced membrane binding are expressed in human cells (see below). At a first glance, these observations by Burré et al. (2015) challenged the assumption of "less membrane-binding = less (immediate) toxicity" as suggested by Volles and Lansbury (2007). However, it should be noted that (i) no proline mutations more C-terminal than V38P had been identified to be protective in yeast, consistent with the idea that NAC-domain mutations are more detrimental; (ii) αS amyloid formation has been reported to be more difficult to achieve in *S. cerevisiae* than in mammalian cells (Soper et al., 2008; Jarosz and Khurana, 2017); (iii) the only real discrepancy between the two studies are the data on A30P. The PD-causing effect of the "P mutant" αS A30P had always been at odds with the study by Volles and Lansbury (2007) and several other αS studies in yeast, where A30P behaved like a negative control for αS toxicity. In that regard, the approaches proposed by Burré et al. might overcome the previous lack of robust αS A30P cellular toxicity models.

Importantly, the study by Volles and Lansbury (2007) (**Figure 2C**) also identified the point mutation Q79R to increase αS toxicity paralleled by an increase in membrane binding (**Figure 2F**, left panel). In this context, a publication by Winner et al. (2011) is of interest, reporting the pronounced toxicity of two engineered αS variants, αS E35K and αS E57K (**Figure 2F**, middle panel), when virally expressed in rat midbrains. The overall effect of these two mutants on αS amphipathic helix formation at membranes might be similar to that of Q79R (**Figure 2F**, left panel), as additional positive charges are added

to the hydrophilic half of the helix in all three cases. More importantly, these charge changes may be analogous to the effect of the fPD-linked αS E46K mutant (Zarranz et al., 2004) (**Figure 2B**). αS E46K is known to bind to membrane phospholipids more tightly than wt αS (Choi et al., 2004). It was suggested that the E46K αS amphipathic helix is more stable at phospholipid membranes due to the possibility of forming an additional salt bridge between the positively charged lysine (K) and a negatively charged phospholipid head group (Perlmutter et al., 2009). This electrostatic attraction is visualized in **Figure 2F**, and the mechanism can likely be extended to E35K and other engineered "K mutants". Similar to E35K, the fPD-linked E46K has been reported to exhibit more pronounced toxicity than wt αS in both yeast (Lázaro et al., 2014) and mammalian cells (Íñigo-Marco et al., 2017). Furthermore, both E35K and E46K occur in the core repeat motif (KTKEGV becomes KTKKGV), and both at position 5 of the 11-aa repeat (E35K: position 5 in repeat 3; E46K: position 5 in repeat 4). This is consistent with the "dose-dependent" effects of the "2K" compound mutant E35K + E46K (**Figure 2F**, right panel) and the further extrapolation to "3K" = E35K + E46K + E61K (**Figure 2F**, right panel) (Dettmer et al., 2015a). Importantly, a "dose-dependent" increase in αS toxicity and membrane binding (PBS insolubility) was observed in mammalian cells with each additional E-to-K mutation. In several assays, transfection of the αS 3K variant into neuroblastoma cells led to strong cytotoxicity, similar to the level caused by the pro-apoptotic protein Bax (Dettmer et al., 2015a). E46K was shown to be more toxic than wt in at least one assay, while the toxicity of αS 2K was in between E46K and 3K. The pronounced cellular toxicity of αS 3K was accompanied by the development of round cytoplasmic inclusions (Dettmer et al., 2015a). Electron microscopy (EM) identified these inclusions as round, dense clusters of vesicles of different sizes that were strongly positive for αS by immunogold (Dettmer et al., 2017). In co-expression experiments, vesicular markers of various origins (endosomal, lysosomal, and Golgi), but not mitochondrial or ER membrane markers, were found to co-localize with the αS 3K inclusions (Dettmer et al., 2017). The lack of 3K overlap with ER membranes is at odds with a report on pronounced ER interactions of E46K (Mbefo et al., 2015), which will need to be consolidated in future studies. Nonetheless, the principal effects of increased toxicity apply to all E/Q-to-K/R mutations in repeat positions 1 and 5, as presented in **Figure 2F**. And this seems to be related to excess interaction with vesicular membranes and/or aberrant binding to non-vesicular membranes. Importantly, the effect of expressing αS 3K in mammalian cells resembles expressing wt human αS in yeast: (immediate) cytotoxicity, inclusion formation and vesicle clusters, linking the 3K effect to wt αS pathobiology.

While no analogy to a known fPD-linked mutant exists, another theoretical strategy of increasing αS membrane binding consists in increasing the hydrophobic interaction between αS and the fatty-acyl chains of phospholipid bilayers. The imperfect hydrophobicity of the hydrophobic half of the αS amphipathic helix (**Figure 1E**) could be enhanced by several strategies: (i) replacing the central threonine (T) of KTEKGV with an uncharged and non-polar aa such as leucine (L), changing the consensus motif to KLKEGV (abbreviated "KLK"; **Figure 2G**, middle panel); (ii) replacing the small glycine (G) with a bulkier non-polar aa such as isoleucine (I), resulting in KTKEIV (abbreviated "EIV"; **Figure 2G**, right panel); or (iii) replacing the non-polar valine (V) with a bulkier non-polar aa such as tryptophan (W), resulting in KTKEGW (abbreviated "EGW"; **Figure 2G**, left panel). Similar to αS 3K, such engineered αS mutants were enriched in membrane fractions, were immediately toxic when transfected into neuroblastoma cells, and led to round cytoplasmic inclusions that were again shown by EM and fluorescence microscopy to be clusters of vesicles of various origins (Dettmer et al., 2015b, 2017). These findings are consistent with earlier observations by Volles and Lansbury (2007), namely, that increased membrane binding and increased toxicity are correlated. A "KLK"-like αS variant termed "T6" (Pranke et al., 2011) (4 T→L and 2 T→F substitutions) has been studied *in vitro*, and the authors concluded that its strongly increased membrane binding was accompanied by a lack of specificity toward target membranes: while wt αS showed a preference for curved membranes, as observed before (e.g., Middleton and Rhoades, 2010; Jensen et al., 2011), αS T6 lost this preference and also bound to flat membranes *in vitro*. However, co-localization analyses with different cellular markers in neuroblastoma cells showed that the highly similar αS "KLK" (**Figure 2G**, middle panel) was associated with vesicular, but not ER or mitochondrial membranes, consistent with a quantitatively, but not qualitatively different binding mode (Dettmer et al., 2017).

Position 2 of the KTKEGV core motif was addressed in two additional studies. Perrin et al. (2000) characterized αS "TsixK" (KTKEGV becomes KKKEGV in six repeats; **Figure 2H**, left panel) and "TsixE" (KTKEGV becomes KEKEGV in six repeats; **Figure 2H**, middle panel). TsixK and TsixE both introduce thermodynamically unfavorable charges into the hydrophobic half of the αS amphipathic helix (TsixK: 6 lysines = 6+; TsixE: 6 glutamates = 6−). Interestingly, the authors observed pronounced membrane repulsion of TsixE, while for certain phospholipid mixtures TsixK still engaged in membrane binding despite diminished lipid-induced helicity (35% helicity instead of 70% in the lipid-bound state). The complete solubility of TsixE in the presence of membranes composed of acidic phospholipids was attributed to both electrostatic repulsion and reduced hydrophobicity in the hydrophobic face of the αS helix. A different study that involved a T substitution strategy, however, questioned to some extent the biological relevance of studying αS membranes interactions *in vitro*: Kim et al. (2006) reported that the interaction of αS with biological membranes may be quite different from that with model membranes. The authors characterized the interaction typically observed with model phospholipid membranes as spontaneous, stable and mainly driven by electrostatic attraction. In contrast, the interaction with cellular membranes was proposed to be highly dynamic and more driven by hydrophobic attraction. Kim et al. (2006) further suggested that the latter mode of αS interaction only occurs in the context of the cytoplasm and that cytoplasmic co-factors assist the interaction, consistent with findings by other researchers (Wislet-Gendebien et al., 2006; Chen et al., 2013). The

authors pursued an "in-lysate" chemical crosslinking strategy and observed that cellular αS-membrane binding was characterized by the trapping of a 17 kDa membrane-bound version of αS (p17), in addition to the expected 14 kDa unmodified monomer. A "TG6" αS mutant (KTKEGV becomes KGKEGV in six repeats; **Figure 2H**, right panel), designed to break the helical structure of the protein, abolished p17 formation, while the mutant protein was apparently still detected in membrane fractions similarly to wt αS [see Figure 4 in Ref. Kim et al. (2006); note that the authors do not explicitly assess the total membrane binding of wt vs. TG6]. Analogous to "TsixK" this could indicate that for TG6 membrane binding was still possible, but helix formation was impaired.

αS MULTIMERIZATION

That cellular αS homeostasis is more complex than just a constant switching between soluble (unfolded) and membrane-associated (helical) monomers was proposed in a study by Bartels et al. (2011). First, using intact-cell crosslinking, the authors observed apparent multimeric species by Western blotting in addition to the 14 kDa monomer. Second, they used native methods to isolate cellular αS under non-denaturing conditions from various natural (non-PD) sources, including neural cells and human erythrocytes. The αS assemblies they isolated had α-helical structure by circular dichroism and were sized by two methods as ∼60 kDa tetramers that were relatively resistant to *in vitro* aggregation, compared to recombinant monomers. Subsequent work from several labs confirmed elements of this new hypothesis of the existence of physiological αS multimers in addition to free monomers and emphasized the dynamic nature of αS tetramers/multimers (Wang et al., 2011; Dettmer et al., 2013; Gurry et al., 2013; Gould et al., 2014; Luth et al., 2015). For example, chemical crosslinkers were found to trap 60 kDa αS (monomer MW: 14 kDa) in intact, living cells, but not in cell lysates (Dettmer et al., 2013). Questions regarding the specificity of crosslinked αS cellular multimers (Fauvet et al., 2012) triggered a search for defined point mutations in the molecule that would abolish αS tetramer/multimer formation, based on the rationale that identifying such mutations would argue for molecular specificity. Focusing on the semi-conserved KTKEGV core motif in the αS 11-aa repeats, Dettmer et al. (2015b) identified missense mutations that, when introduced in several repeats, led to the detection of solely 14 kDa αS monomers, with αS multimers virtually absent. Mapping the identified mutations based on the membrane-induced αS helix model revealed that most αS multimer-abolishing repeated KTKEGV motif variants (6 × KLKEGV = "KLK," 7 × KTKEIV = "EIV," 6 × KTKEGW = "EGW") strongly increased the hydrophobicity of the hydrophobic half of the αS membrane-induced helix (**Figure 2G**). Another multimer-abolishing mutant (6 × KTKKGV = "KGV"; not shown) identified in the study also increased αS membrane helix formation, but likely via additional electrostatic interactions between lysine residues and lipid headgroups. 6 × KTKKGV is a further amplification of αS "3K" (3 × KTKKGV; **Figure 2F**) and, importantly, fPD-linked E46K (1 × KTKKGV; **Figures 2B,F**), which both "dose-dependently" lead to multimer abrogation and increased membrane binding (Dettmer et al., 2015a). In all cases, the increase in membrane binding and loss of αS multimerization was accompanied by inclusion formation and toxicity (Dettmer et al., 2015a,b). This raised the question of what was responsible for the observed toxicity of those particular mutants: the loss of multimerization or the increased membrane binding? If both aspects of αS biology are tightly linked, the question may be hard or impossible to answer.

The study by Dettmer et al. (2015a) also revealed that 5 different fPD-linked αS missense mutations (A30P, E46K, H50Q, G51D, and A53T) all shifted the multimer:monomer equilibrium toward more monomers, albeit not as drastically as the strategic repeated mutants. This is noteworthy because at least A30P (Jensen et al., 1998; Jo et al., 2002) and G51D (Fares et al., 2014) exhibit decreased, not increased, membrane binding, suggesting that the detrimental effect of αS monomer excess goes beyond increased membrane binding. Otherwise, not all five mutants, membrane-enriched such as E46K or cytosol-enriched such as A30P and G51D, would cause PD. The notion that αS A30P reduces both membrane binding and multimerization was supported by Westphal and Chandra (2013), and work by Burré et al. (2015) (**Figure 2E**, right panel) helped generalize the hypothesis that impaired formation of αS membrane-induced amphipathic helices causes αS monomer excess in the cytosol. According to Burré et al. (2015), the lack of membrane interaction of αS A11P/V70P and T44P/A89P monomers causes a failure to form native multimers at the membrane. These findings were consistent with αS TsixK (**Figure 2H**, left panel), which had been described to interfere with αS folding at membranes (Perrin et al., 2000), also abolishing αS multimerization, as assessed via YFP complementation on vesicle surfaces in intact neurons (Wang et al., 2014). Burré et al. (2015) concluded that reduced membrane binding results in the accumulation of monomeric, unfolded αS in the cytosol, as assessed by crosslinking in brain homogenates (but post lysis). It is plausible that an accumulation of unfolded soluble αS is the starting point of αS amyloid formation, since it is well-known that monomeric, unfolded αS can aggregate *in vitro* (Conway et al., 2000). In this context, it is important to note that in pure solutions *in vitro*, where both wt and P mutant αS are natively unfolded, αS A11P/V70P or T44P/A89P were not more aggregation-prone than wt. The P mutants aggregated faster only in the cellular context, in which wt, but not P mutant αS can interact with membranes, once again highlighting the importance of studying consequences of αS structure alterations in intact cells. However, membrane excess of αS – as observed for αS "3K," "KLK" or "EIV" – has also been proposed to be the starting point of αS aggregation (Galvagnion et al., 2015), and the two possibilities are not mutually exclusive. Moreover, the amyloid nature of the observed inclusions in the respective studies reviewed here will require further analysis: EM revealed that "3K," "KLK," or "EIV" αS inclusions are primarily vesicle-rich αS inclusions and may only over time give rise to protein-rich, β-sheet αS inclusions. The exact nature of αS A11P/V70P or T44P/A89P inclusions that Burré et al. (2015) readily observed

upon expressing these in cell culture has not been addressed yet.

In combination, the findings by Dettmer et al. (2015a; 2015b), Burré et al. (2015) and Wang et al. (2014) are consistent with native αS multimerization being the result of an intact αS dynamic equilibrium. In such a dynamic equilibrium, αS is expected to efficiently shuttle between cytosol and membranes, i.e., it constantly binds to and gets released from lipid bilayers. Transient interactions of αS monomers with membranes may drive multimerization (Dettmer et al., 2016, 2017), something that recently was observed in an *in vitro* helical αS (tetramer) reconstitution system (Rovere et al., 2018). A possible scenario is that the induced amphipathic αS helices at lipid bilayers may over time cooperatively interact with each other in such a way that the hydrophobic portions of four monomers face each other, resulting in tetramer/multimer formation and simultaneous release from membranes. Too much or too little membrane association could lead to an accumulation of monomers either in the cytosol (e.g., T44P/A89P) or at membranes (e.g., 3K, KLK), respectively (**Figure 3**).

These considerations could help explain how both membrane-enriched αS E46K (Choi et al., 2004) and cytosol-enriched αS A30P (Jensen et al., 1998; Jo et al., 2002) or G51D (Fares et al., 2014) can cause αS dyshomeostasis culminating in the pathogenesis of PD. Indeed, a loss of αS tetramerization/multimer formation had been proposed as a unifying principle between fPD-linked αS mutants: A30P, E46K, H50Q, G51D, and A53T all shifted the dynamic equilibrium away from tetramers/multimers when studied via intact-cell crosslinking and YFP complementation (Dettmer et al., 2015a). This is consistent with the idea that αS multimers may serve as a "safe" (i.e., not aggregation-prone) storage form in the cytoplasm (Gurry et al., 2013; Westphal and Chandra, 2013). The short-term consequences of αS monomer accumulation at membranes (vesicle-rich αS inclusions; pronounced immediate toxicity) or in the cytosol (β-sheet-rich αS aggregates; more subtle/context-dependent toxicity) may differ. One important unsolved question (see above) remains the aggregation state of αS in the "vesicle-rich" inclusions. By EM [wt αS in yeast (Soper et al., 2008) or 3K/KLK αS in neural cells (Dettmer et al., 2017)], such inclusions are not characterized by fibrillar proteinaceous aggregates. In fact, while immunogold analysis clearly shows strong αS enrichment within the vesicle clusters, it is unclear if there is any direct αS-αS interaction at all, be it native or non-native. Assessed via crosslinking and YFP complementation (Dettmer et al., 2015b), αS 3K, KLK, and EIV appeared largely monomeric (at least the crosslinking assays should be able to detect both β-sheet and helical assemblies of αS). However, the aggregation of αS at membranes has been demonstrated (Galvagnion et al., 2015) and only the kinetics of aggregation and toxicity may differ between αS cytosol and membrane accumulation. Eventually, cytosol and membrane pathways of αS dyshomeostasis may converge in common pathological mechanisms, e.g., after the (relatively slow) formation of β-sheet-rich αS aggregates at membranes (Galvagnion et al., 2015). Alternatively, the principle of selective vulnerability (discussed in Walsh and Selkoe, 2016) may help explain why certain brain regions are susceptible to any αS-related insult, while other regions are not. Engineered αS variants, which have more pronounced effects than the fPD-linked mutations, are expected to continue contributing to a better understanding of these phenomena, especially when tested in animal models.

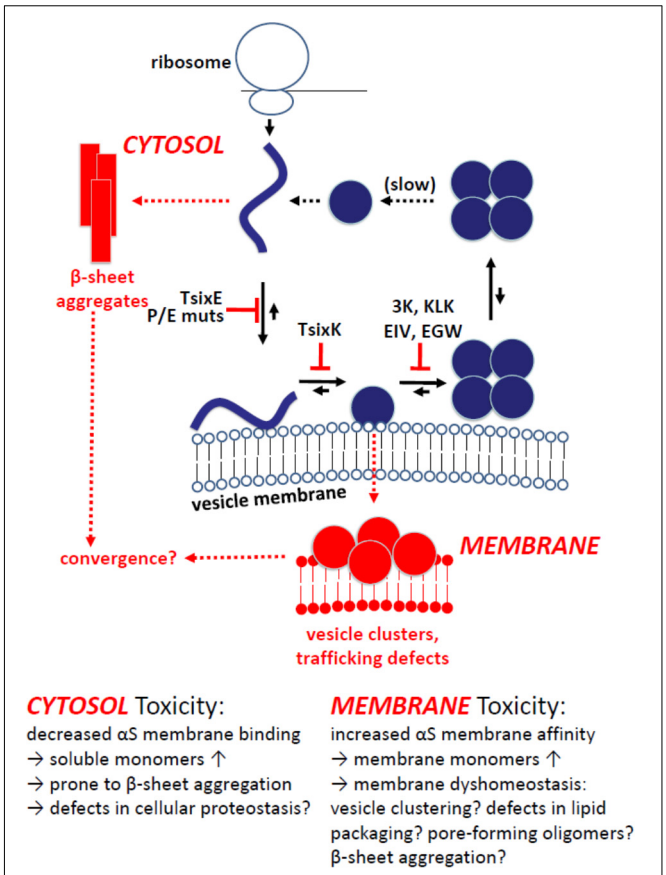

FIGURE 3 | Model of cellular αS homeostasis and the effect of engineered αS mutants. Physiological situation in blue: Coming off the ribosome, αS is soluble, unfolded and monomeric. Upon binding to vesicular membranes, it adopts helical fold. Folded monomers assemble to form multimers/tetramers on membranes. Multimers/tetramers are only weakly membrane-associated and likely in an equilibrium with cytosolic multimers/tetramers. Cytosolic tetramers/multimers may have an intrinsic propensity to disassemble – and eventually unfold, initiating a new cycle. Pathological situation in red: Perturbed cellular αS homeostasis, modeled via engineered αS variants, increases (i) the levels of aggregation-prone unfolded monomers in the cytosol (top left; TsixE, E/P mutants) or (ii) the level of membrane-associated monomeric αS (bottom; 3K, KLK, EIV, EGW mutants). While membrane-associated αS accumulation is generally toxic in *in vivo* models, the toxicity of cytosol-accumulated unfolded αS appears to be context specific, e.g., non-toxic in yeast (Volles and Lansbury, 2007), but more toxic than wt αS when virally expressed in mouse SN (Burré et al., 2015).

The observation that both increased and decreased membrane binding reduces the αS multimer:monomer ratio may indicate that the folding landscape of wt αS is well balanced to minimize both aggregation-prone unfolded, cytoplasmic monomers and membrane-toxicity causing membrane-associated monomers. Consequently, it may not be possible to further "improve" the

native wt αS membrane:cytosol and multimer:monomer ratios by protein engineering. *Disturbed* αS homeostasis, however, may be corrected in the future by interfering with αS membrane-interactions. Interestingly, the drug squalamine has recently been reported to displace αS from lipid vesicles, thereby inhibiting aggregation and reducing toxicity (Perni et al., 2017; Pineda and Burré, 2017). However, an A30P carrier may not benefit from such a treatment because A30P already exhibits decreased membrane binding. Also for sporadic PD cases (wt αS), "precision medicine" might be required to determine if the underlying αS dyshomeostasis can be resolved by an increase or a decrease of αS membrane interaction. Alternatively, compounds that can directly stabilize αS tetramers/multimers, analogous to tafamidis in stabilizing transthyretin tetramers in that amyloidosis (Johnson et al., 2012), could arise as "one-size fit all" drugs. The design of such compounds would be facilitated by resolving the structure of tetrameric αS, which appears highly challenging in light of the reported lysis sensitivity of αS multimers (Dettmer et al., 2013; Luth et al., 2015). It is tempting to speculate that membrane-enriched αS variants such as KLK and EIV might even help stabilize αS multimers *in vitro* if we found methods and conditions to release membrane-enriched αS variants from membranes while the hydrophobic faces of their amphipathic helices engage in ordered synuclein-synuclein interactions at the same time. This approach, of course, would only be valid if the mechanism that drives wt αS multimerization *in vivo* is sufficiently similar to this scenario, for which there is some evidence (Wang et al., 2011; Gurry et al., 2013; Rovere et al., 2018).

αS FUNCTION

Several studies have linked αS function to synaptic vesicle trafficking (Abeliovich et al., 2000; Cooper et al., 2006; Larsen et al., 2006; Nemani et al., 2010; Vargas et al., 2014, 2017; Logan et al., 2017). While the exact details are still under debate, this function seems to be tied to transient αS-membrane interactions, which also seem to play a role in αS multimerization (Rovere et al., 2018). Initial reports focused on effects of αS on directly stabilizing curved membrane structures (Kamp et al., 2010; Varkey et al., 2010), similar to BAR domain proteins (DeWitt and Rhoades, 2013; Westphal and Chandra, 2013). More recent studies described direct effects of αS on membrane clustering Wang et al., 2014) or indirect effects of αS on vesicle fusion via the stabilization of vesicle SNARE complexes (Burré et al., 2010, Burré et al., 2014; Almandoz-Gil et al., 2018). Interestingly, both direct and indirect effects have been proposed to be mediated by αS multimerization. Based on biochemical assays, Burré et al. (2014) proposed a dynamic equilibrium between a natively unfolded form in the cytosol and a physiologically functional, multimeric form at membranes, while the latter but not the former acts as a SNARE complex chaperone at the presynaptic terminal. The study by Wang et al. (2014) coupled YFP complementation and confocal microscopy in cultured primary neurons to propose that αS multimerization occurs on synaptic vesicles and is associated with clustering of the vesicles. Wang et al. (2014) employed the αS TsixK variant (**Figure 2H**, left panel) as a negative control and concluded that an αS variant that does not form multimers also does not have vesicle-clustering activity. YFP signals from wt αS-mediated YFP complementation did not only occur at vesicles, but also remained associated with vesicles, leading the authors to postulate that physiological αS multimers have vesicle-clustering activity. If this vesicle-clustering activity is indeed the function of αS, then the excess vesicle-clustering in αS-expressing yeast (Cooper et al., 2006; Soper et al., 2008) may be only quantitatively, but not qualitatively different from the normal αS activity. However, the membrane-enriched αS 3K, KLK, EIV, EGW were largely monomeric when expressed in neuroblastoma cells, as assessed via crosslinking and YFP complementation (Dettmer et al., 2015b), and still exhibited strong vesicle-clustering activity, indicating that pronounced membrane localization of αS may be sufficient to drive vesicle clustering. Thus, 3K, KLK and similar membrane-favoring αS variants may have to be considered constitutively active gain-of-function mutants with regard to vesicle-clustering activity. Cytosol-enriched αS mutations such as A11P/V70 and T44P/A89P as well as the folding-incompetent TsixK, on the other hand, would be loss-of function mutations (**Figure 2**). In another study, the generation of membrane curvature (another activity of αS that is potentially related to its vesicle-clustering activity), has been assigned to monomeric αS at membranes (Westphal and Chandra, 2013). Conversely, soluble tetrameric αS was described in the same study as a passive storage form of the protein (Westphal and Chandra, 2013). The in part contradictory results summarized here might be resolved eventually by a model in which the protein's ability to rapidly switch between different localizations and folding/assembly states mediates its activity. In light of such a model, the question if monomeric or multimeric αS represents the functional form of the protein becomes obsolete. An analogy for such a scenario would be SNARE proteins that switch between monomeric and (hetero)tetrameric states, thereby driving vesicle fusion events. In the meantime, the αS variants reviewed here represent valuable tools for the field's attempt to better understand αS functions and assign certain aspects of those functions to certain conformations of the protein. As an example, it would be interesting to test if the exocytic fusion-pore dilating effect of wt αS (Logan et al., 2017) is increased or decreased for membrane-enriched αS variants and if cytosol-enriched variants have the opposite effect. In addition to the membrane-associated functions of αS, engineered soluble αS mutants offer opportunities for testing and identifying cytosolic functions of the protein.

AN ATTEMPT TO CATEGORIZE αS MUTATIONS

A simplified wheel diagram of a membrane-induced amphipathic helix of αS (**Figure 1**) helps categorize αS variants into two classes: membrane-enriched and cytosol-enriched variants. Membrane-enriched αS variants stabilize the formation of the αS membrane-induced amphipathic helix, cytosol-enriched variants destabilize the helix.

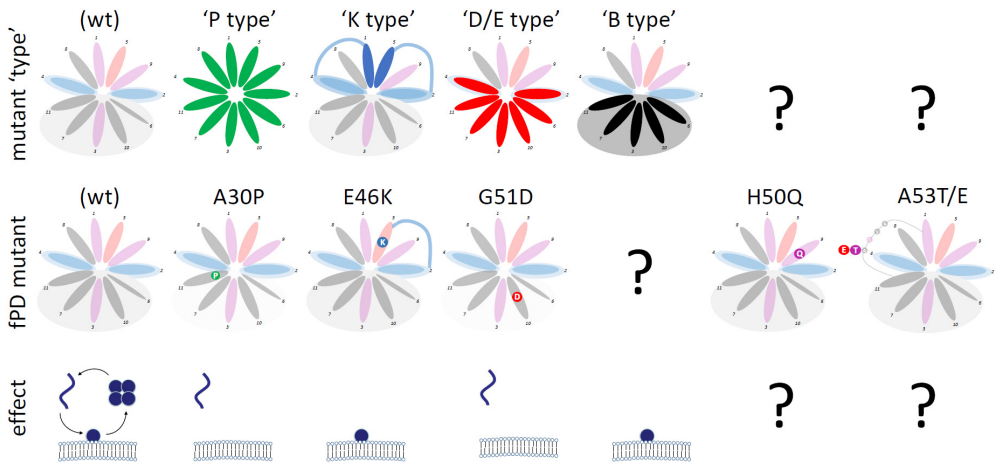

FIGURE 4 | Categories of αS Mutants. Wt αS (shaded; over-simplified by choosing the color-code of the most prevalent aa for each 11-aa repeat position) and αS variants (theoretical: top row; fPD: middle row) are shown. "Mutant type" (upper row) highlights the positions at which the respective mutations are expected to exert the proposed effects. "Effect" (bottom row) shows the (exaggerated) effect of the mutations on αS homeostasis: monomers accumulate at the membrane or in the cytosol.

Cytosol-enriched αS variants can be further classified into (**Figure 4**):

- "P-type" variants: substitutions with proline (P) residues prevent helix formation, leading to the accumulation of cytosolic αS, which is likely unfolded, monomeric and aggregation-prone. P, known as a "helix-breaker", should have this effect at any position in αS. fPD-linked αS A30P (Krüger et al., 1998; Jo et al., 2002) falls into this category; see **Figures 2C,E** for engineered analogs/amplifications.
- "D/E-type" variants: substitutions with aspartate (D) or glutamate (E) residues cause repulsion from lipid acyl chains (D/E in the hydrophobic half of the αS helix). As a result, cytosolic αS accumulates, which is likely unfolded, monomeric and aggregation-prone. fPD-linked αS G51D (Kiely et al., 2013; Lesage et al., 2013; Fares et al., 2014) is one example; see **Figures 2C,H** for engineered analogs/amplifications. D/E substitutions should have similar effects in the "lysine wings" (**Figure 2D**).

The effect of "P-" and "D/E-type" variants on αS toxicity appears to be model- and context-specific and ranges from less toxic than wt in yeast (Volles and Lansbury, 2007) to more toxic than wt in virally injected mouse SN (Burré et al., 2015). This discrepancy, which may have to do with differences in β-sheet-rich aggregation in different cellular environments, will require further elucidation. Of course, human genetics underlines the long-term toxicity of both types: the fPD-linked variants A30P (Krüger et al., 1998; Jo et al., 2002) and G51D (Kiely et al., 2013; Lesage et al., 2013; Fares et al., 2014) cause PD. However, A30P and G51D are likely not readily toxic in yeast or (simple) cellular models, consistent with membrane interactions being important for yeast αS toxicity (Volles and Lansbury, 2007).

Membrane-enriched αS variants can be further classified into (**Figure 4**):

- "K-type variants": substitutions with lysine (K) residues at position 1 or 5 of each 11-aa repeat presumably lead to an energetically favorable interaction with lipid headgroups, in analogy to what has been proposed for fPD-linked αS E46K (Perlmutter et al., 2009) (**Figure 2B**). The result of a stabilized αS membrane-induced amphipathic helix is the accumulation of membrane-associated, helically folded (and monomeric) αS. See **Figure 2F** for analogs and exaggerations of E46K. It should be noted that "K-type" mutations are highly position-specific. All variants discussed here are located at positions 1 and 5 of each 11-aa repeat. A similar effect may occur at positions 8 and 9, but this remains to be tested. The positively charged histidine (position 9 in repeat 4, aa 50 in αS) may stabilize the αS helix in the environment of negatively charged lipid headgroups and therefore the fPD-linked H50Q (Appel-Cresswell et al., 2013) (**Figure 4**, second column from the right) may (slightly) repel αS from membranes. The presence of positively charged K (or R) at the (hydrophobic) positions 3, 6, 7, 10, or 11 will interfere with helix formation and lead to repulsion or a different mode of membrane binding [as discussed by Perrin et al. (2000); **Figure 2H**, left panel].
- "B-type variants": substitutions with "bulky" residues (L, I, W, F) in the hydrophobic half of the αS amphipathic helix enhance hydrophobic interactions, leading to increased membrane dwell-time (**Figure 2G**). "B-type" αS variants do not have a counterpart in an fPD-linked variant. This could be coincidence or indicative of a strong toxicity that has prevented the presence of such variants in humans.

"K-type" αS variants show consistent toxicity in yeast (Volles and Lansbury, 2007), cellular (Dettmer et al., 2015a), and animal (Winner et al., 2011) models, which should most likely also apply to "B-type" variants (Dettmer et al., 2015b). The pronounced toxicity of these variants, however, is possibly caused by disrupting membrane integrity and vesicle trafficking

(Dettmer et al., 2017), and the relationship to β-sheet-rich aggregation will require further elucidation. Thus far, it is not clear if there is any direct αS-αS interaction at all in the vesicle-rich αS inclusions. Recent work (Fusco et al., 2016) suggests that excess αS monomers, that each are in contact with two vesicle membranes, could directly cause aberrant vesicle clustering. Importantly, however, the fPD-linked E46K, a "K-type" mutant, produces typical proteinaceous inclusions, Lewy bodies, in patient brains (Zarranz et al., 2004), arguing against an E46K-specific pathogenic mechanism that is entirely independent of αS proteinaceous aggregation.

LIMITATIONS OF THE PRESENTED STUDIES AND THEIR INTERPRETATION BASED ON A SIMPLIFIED αS MEMBRANE-INDUCED HELIX MODEL

The studies presented and discussed here typically rely on the overexpression of αS, sometimes fused to whole or split fluorescent proteins, in human/rodent cells or even in yeast, an organism that does not possess synuclein. Excess/ectopic expression and the modification with tags that are larger than αS itself may of course affect the localization and structure of the studied variants. Nonetheless, all presented αS variants were compared to wt αS expressed under the same conditions and, therefore, the conclusions relative to wt αS can be expected to be meaningful. Moreover, many key observations such as the toxic effect of adding positive charges to positions 1 or 5 of the 11-aa repeat have been confirmed independent of tagging (untagged, YFP-tagged) or model system (yeast, transfected neural cells, stereotactic viral expression in mouse substantia nigra). However, the effect of expressing folding-impaired P-mutant αS ranges from less toxic (yeast) to more toxic (stereotactic viral expression in mouse substantia nigra) and, while it is obvious to consider the findings in the rodent *in vivo* system more relevant, the study of the "A30P-like" αS toxicity remains challenging.

It also has to be noted again that the synoptic considerations in this review are based on a *simplified* wheel model of αS membrane-induced amphipathic helix formation. The model ignores the "ATVA" interrupting sequence, which leads to a shift (repeats 5–7 relative to repeats 1–4) that is not reflected. The fPD-linked mutations A53T (Polymeropoulos et al., 1997) (ATVA becomes TTVA) and A53E (Pasanen et al., 2014) (ATVA becomes ETVA) cannot be assessed via the simplified wheel model (**Figure 4**, right column). At least for A53E, reduced membrane binding has been reported (Ghosh et al., 2014), while the membrane binding of A53T may be similar to wt (Bussell and Eliezer, 2004). Positions 8 and 9 in the 11-aa repeats seem to be understudied. While located in the hydrophilic half of the αS helix, the nature of the aa found at position 8 are surprisingly hydrophobic, especially in repeats 1–4 (V, A, L, V). This may have to do with membrane-interaction-independent requirements for helix formation and may be addressed in future studies. At position 9, aa with very different characteristics are found: A, E, Y, H, N, A, S. The only positively charged aa, H, is mutated in fPD-linked H50Q and assessing this effect based on the simplified helix model is not straightforward (see above). Moreover, the simplified wheel diagrams in **Figure 2** only depict aa 9–89 while regions up to aa 97 (Fusco et al., 2016) and also the very N-terminus (Bartels et al., 2010) have been proposed to be involved in membrane binding.

It cannot be ruled out that some of the mutants discussed here have very specific effects unrelated to their position in the membrane-induced αS amphipathic helix. For example, E35K and E57K (**Figure 2F**) have been reported to have specific effects on αS aggregation (oligomer formation instead of fast fibrillization) *in vitro*, in the absence of membranes. However, based on the existing data, it appears reasonable to predict that E13K, E83K and, possibly even E105K (all at position 5 of the 11-aa repeat) have similar effects as E46K. The same should apply to E20K (position 1 of 11-aa repeat #2) and mutants generated by replacing the non-E amino acids at positions 1 and 5 of the 11-aa repeats, such as S9K or Q24K, will likely also behave similarly.

The predictive value of the simplified αS wheel diagram will continue to be tested by new strategic αS mutants that researchers will create and possibly even by newly found fPD-linked αS variants. fPD-linked A53T and A53E (see above), which are not in the 11-aa repeat may very well have unique effects and the effect of H50Q, where the only H in αS is mutated, is hard to assess based on the simplified model (see above). H50Q (Khalaf et al., 2014; Rutherford et al., 2014) and A53T (Conway et al., 1998) were reported to form amyloid more readily *in vitro* and – leaving the membrane-induced helix model aside – increased aggregation-propensity of soluble αS in an otherwise "normal" equilibrium of membrane-bound and soluble αS represents a sufficient explanation for long-term toxicity. It should, again, be noted though that the fPD variants A30P, E46K, H50Q, G51D, and A53T were reported to have an αS multimer:monomer ratio that was shifted toward more monomer, suggesting that a reduced multimer:monomer ratio could be the "unifying principle" of αS dyshomeostasis, independent of whether excess monomers accumulate at membranes or in the cytosol (Dettmer et al., 2015a). Lastly, important aspects of αS biology were beyond the scope of this review, which focused mainly on strategic aa mutations in the αS 11-aa repeats studied *in cellulo*. The composition of (vesicular) membranes has been repeatedly shown to influence αS binding (e.g., Nuscher et al., 2004), and the effect of certain αS fPD mutations on, e.g., membrane binding may depend on the exact nature of target membranes. Similarly, certain post-translational modifications such as S129 phosphorylation have been proposed to be an important aspect of αS homeostasis and they have been studied, e.g., via aa substitutions that prevent the respective modifications (e.g., Lázaro et al., 2014). Such aa substitutions were not discussed here. αS function was not the main focus of this review because only two of the key studies presented explicitly addressed it; the proposed function were vesicle clustering (Wang et al., 2014) and SNARE assembly at vesicles (Burré et al., 2012). However, future studies related to other proposed cellular αS interactions such as that with mitochondria (e.g., Kamp et al., 2010) may also benefit from analyzing strategic αS variants.

AUTHOR CONTRIBUTIONS

UD wrote the review.

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Overexpression of Wild-Type Human Alpha-Synuclein Causes Metabolism Abnormalities in Thy1-aSYN Transgenic Mice

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Parkinson's disease is a progressive neurodegenerative disorder characterized by loss of dopaminergic neurons, pathological accumulation of alpha-synuclein and motor symptoms, but also by non-motor symptoms. Metabolic abnormalities including body weight loss have been reported in patients and could precede by several years the emergence of classical motor manifestations. However, our understanding of the pathophysiological mechanisms underlying body weight loss in PD is limited. The present study investigated the links between alpha-synuclein accumulation and energy metabolism in transgenic mice overexpressing Human wild-type (WT) alpha-synuclein under the Thy1 promoter (Thy1-aSYN mice). Results showed that Thy1-aSYN mice gained less body weight throughout life than WT mice, with significant difference observed from 3 months of age. Body composition analysis of 6-month-old transgenic animals showed that body mass loss was due to lower adiposity. Thy1-aSYN mice displayed lower food consumption, increased spontaneous activity, as well as a reduced energy expenditure compared to control mice. While no significant change in glucose or insulin responses were observed, Thy1-aSYN mice had significantly lower plasmatic levels of insulin and leptin than control animals. Moreover, the pathological accumulation of alpha-synuclein in the hypothalamus of 6-month-old Thy1-aSYN mice was associated with a down-regulation of the phosphorylated active form of the signal transducer and activator of transcription 3 (STAT3) and of Rictor (the mTORC2 signaling pathway), known to couple hormonal signals with the maintenance of metabolic and energy homeostasis. Collectively, our results suggest that (i) metabolic alterations are an important phenotype of alpha-synuclein overexpression in mice and that (ii) impaired STAT3 activation and mTORC2 levels in the hypothalamus may underlie the disruption of feeding regulation and energy metabolism in Thy1-aSYN mice.

Keywords: body weight, energy metabolism, insulin, leptin, mTOR, neurodegeneration, parkinsonism, transcription factor STAT3

INTRODUCTION

Parkinson's disease (PD) is the most common movement neurodegenerative disorder in elderly adults. It is characterized by a progressive degeneration of dopaminergic neurons in the *substantia nigra* and by the pathological accumulation of intraneuronal aggregated and hyperphosphorylated alpha-synuclein in Lewy bodies (Bridi and Hirth, 2018). Missense mutations and multiplication of the gene encoding alpha-synuclein *SNCA* (synuclein, alpha [non-A4 component of amyloid precursor]) were identified as genetic abnormalities associated with rare familial forms of PD (Polymeropoulos et al., 1997; Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004). Polymorphisms regulating *SNCA* levels were subsequently associated with sporadic PD (Maraganore et al., 2006; Simon-Sanchez et al., 2009), supporting that alpha-synuclein level is instrumental in most forms of the disease. Dopamine deficit at the striatum -e.g., the striatal area innervated by the *substantia nigra*- is the main factor leading to bradykinesia, resting tremor, rigidity and postural instability. It is generally accepted that these motor symptoms appear only after a substantial proportion of dopaminergic neurons are lost (Bezard et al., 2001).

The motor features of PD can be preceded, sometimes for several years, by non-motor symptoms such as olfactory deficits, sleep disorders, depression and autonomic dysfunction (Schapira et al., 2017). Increasing evidence suggests that unintended body weight change is also a significant feature of PD symptomatology. Weight loss, primarily due to fat rather than muscle loss (Markus et al., 1993), has been frequently documented in PD patients (Chen et al., 2003; Cheshire and Wszolek, 2005; Uc et al., 2006; van der Marck et al., 2012). The majority of patients have lower body mass than control individuals at diagnosis (Sharma and Lewis, 2017). PD patients are moreover four times more likely to lose body mass than healthy elderly individuals (Chen et al., 2003; Cheshire and Wszolek, 2005; Uc et al., 2006; van der Marck et al., 2012; Cumming et al., 2017). Body mass loss during the course of the disease has been associated with poorer clinical outcomes and rapid disease progression (Lorefalt et al., 2004; van der Marck et al., 2012; Sharma and Vassallo, 2014; Pak et al., 2018), suggesting that it could be of prognostic significance for PD severity (Cumming et al., 2017; Sharma and Lewis, 2017; Pak et al., 2018). Thus, to address the links between weight loss and PD is quite necessary (Ma et al., 2018).

Body weight is governed by energy intake and energy expenditure (EE), which are tightly controlled as peripheral hormonal signals integrate in the hypothalamus to regulate food intake and energy outgo (Schwartz et al., 2000). Reduced energy intake, secondary to motor or non-motor symptoms (i.e., hyposmia, gastrointestinal disturbance, depression) has been proposed as a factor contributing to weight loss in PD (Ma et al., 2018). However, other studies showed that mild to moderate PD patients have the same nutritional status compared to controls (Fereshtehnejad et al., 2014) or that weight loss can occur in PD despite an increased energy intake (Chen et al., 2003; Lorefalt et al., 2004). Conflicting results also exist regarding EE, with a study suggesting that increased EE could contribute to weight loss in PD (Markus et al., 1992) while others showed that the total daily EE was not higher in patients with weight loss compared with patients without weight loss (Delikanaki-Skaribas et al., 2009) and healthy controls (Jorgensen et al., 2012). Central regulatory hypothalamic mechanisms in weight disturbance in PD has recently attracted much attention in part due reports of body weight gain after deep brain stimulation of subthalamic nuclei (Bannier et al., 2009; Aiello et al., 2017). Moreover, changes in plasmatic concentrations of hormones regulating energy balance such as leptin occur in PD patients with weight loss (Fiszer et al., 2010). However, our basic understanding of the pathophysiological mechanisms underlying body weight change in PD remains limited. In particular it is not known whether alpha-synuclein accumulation that is central in PD impacts on energy intake/expenditure, levels of peripheral hormones or changes in global function of the hypothalamus.

In the present study, we determined the metabolic phenotype of the well-established Thy1-aSYN mouse model of PD. Thy1-aSYN transgenic mice overexpress full-length Human wild-type (WT) alpha-synuclein under the murine Thy-1 (thymus cell antigen 1, theta) promoter (Thy1-aSYN mice) (Chesselet et al., 2012). We measured the evolution of body mass from weaning to 12 months of age and assessed in 6-month-old (an age preceding any severe motor deficits) animals food intake, spontaneous activity, EE, glucose, and insulin tolerance, as well as alpha-synuclein, insulin, and leptin in the plasma and related signaling pathways in the hypothalamus.

MATERIALS AND METHODS

Animal Procedures

This research was conducted in accordance with the European Union standards for the care and use of laboratory animals and approved by the Nord/Pas-de-Calais Ethical Committee CEEA N°75 (authorization n° O535.02). Thy1-aSYN mice (on a C57Bl6/DBA2 background) were provided by Pr. Marie-Françoise Chesselet (University of California Los Angeles, CA, United states) with the agreement of Prof. Eliezer Masliah (University of California San Diego, CA, United States) and a colony was established in Lille animal facility by breeding transgenic females with WT males (Charles Rivers). Animals were genotyped by PCR from tail DNA samples. Thy1-aSYN and WT littermates male mice were used in the study. Animals were maintained in standard animal cages under specific pathogen-free conditions (12/12 h light/dark cycle; 22°C; grouped housed), with *ad libitum* access to water and standard laboratory chow (RM1A; 14.8 MJ/kg; Special Diets Services). Body weights were measured for different animals housed in our colony, including but not limited to those euthanized for further analyses, at weaning (WT: n = 17, Thy1-aSYN: n = 17), 2 months (WT: n = 17, Thy1-aSYN: n = 17), 3 months (WT: n = 27, Thy1-aSYN: n = 25), 6 months (WT: n = 33, Thy1-aSYN: n = 29), 9 months (WT: n = 10, Thy1-aSYN: n = 9) and 12 months (WT: n = 10, Thy1-aSYN: n = 7) of age. Further behavioral and metabolic

characterizations were performed on 6-month-old animals (WT: $n = 22$, Thy1-aSYN: $n = 19$) or 3-month-old animals (WT: $n = 16$, Thy1-aSYN: $n = 13$). Beam test was performed on 6-month-old animals and lasted 3 days, as described below. The following week, metabolic analyses were carried out using metabolic cages (see below) and glucose tolerance and insulin sensitivity tests were performed (see below). The body compositions were assessed on 12 WT and 10 Thy1-aSYN 6-month-old mice. For blood and plasma analyses, mice were fasted during 6 h before being euthanized. Trunk blood samples were gathered in heparinized tubes and centrifuged at 425 g for 10 min at 4°C. Plasma supernatant was aliquoted and stored at −70°C until assayed. Brains were rapidly removed, hypothalamus dissected out at 4°C and stored at −70°C until use. Inguinal and gluteal adipose tissue were weighted as subcutaneous adipose tissue. The same experimenter dissected all samples for consistency.

Challenging Beam Traversal Test

Motor performance and coordination were measured using a challenging beam traversal procedure (Fleming et al., 2013). Briefly, animals were trained for 2 days in the afternoon to traverse the length of a beam toward their home cage. On the third day, a mesh grid (1 cm squares) was positioned 1 cm above the beam. Mice were then videotaped for a total of five trials. An experimenter blind to genotype watched and rated the videotapes for errors, number of steps made by each animal, and time to traverse for all five trials. An error was counted when, during a forward movement, a limb slipped through the grid and was visible between the grid and the beam surface (Fleming et al., 2013).

Body Composition

Body composition was determined using an "*in vivo* Micro-CT Scanner for Small Lab Animals" (LaTheta LCT-100, Hitachi Aloka Medical Ltd.). Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) mix and about 60 CT slices per mouse were made at 500 μm intervals between shoulders and posterior legs. Slices were analyzed by Aloka software for fat (visceral and subcutaneous) mass, lean mass, and for bone mineral density in the spinal cord. The fat ratio was calculated by the following formula: total fat mass/(total fat mass + lean mass) × 100.

Metabolism, Ambulatory Activity and Energy Expenditure Monitoring

Mice were individually housed in a LabMaster-CaloSys-Calorimetry System (TSE Systems). Animals were placed 3 days in chambers and only the last 24 h were monitored. The locomotor activity was derived from the number of beam breaks (infrared light-beam frame ActiMot2; x- and z- axes sensors). The system measured the volume of O_2 consumed and the volume of CO_2 produced, over a 15 min period, 4 times per hour. These values were averaged to determine the rate of O_2 consumed (VO_2) and CO_2 produced (VCO_2). The EE, the respiratory exchange ratio (RER), and the fatty acid oxidation (FA) were calculated using the following equations RER = VCO_2 / VO_2; EE (kcal/h) = (3.815 + 1.232 × RER) × VO_2) × 1000, FA oxidation (kcal/h) = EE × ((1-RER)/0.3) (Mequinion et al., 2015). Values for RER range between 1.0 to 0.7, with pure carbohydrate oxidation having a value of 1.0 and pure fat oxidation having a value of 0.7.

Food Intake Monitoring/Meal Pattern Analysis

Metabolic cages are equipped with food and drink high precision sensors of 0.01 g and 0.01 ml resolution respectively (TSE Systems, GmbH). Mice had free access to food and water during the whole recording period. Here a meal was defined as a consumption of at least 0.03 g of food separated from the next feeding episode by at least 10 min, as previously described (Hassouna et al., 2013). For each mouse, inter-meal interval, meal duration, meal size and meal rate in dark and light phases were assessed.

Intraperitoneal Glucose Tolerance and Insulin Sensitivity

Intraperitoneal glucose tolerance and insulin sensitivity tests were performed at 2 p.m., after 6 h of fasting. D (+) glucose (1 g/kg in saline; Sigma-Aldrich) or insulin (0.75 units/kg in saline; NovoRapid FlexPen®) were injected intraperitoneally. Blood glucose concentration was measured using a OneTouch Vita® meter (Lifescan) 0, 15, 30, 60, 90 and 120 min following injection.

Blood Glucose and Plasmatic Alpha-Synuclein, Insulin and Leptin Levels

Blood glucose levels were quantified after 6 h of fasting using a OneTouch Vita® meter (Lifescan). Plasma Human alpha-synuclein levels were measured using the Human alpha-Synuclein Kit (Meso Scale Discovery) according to the manufacturer's instructions. Intraassay coefficient of variation was < 14%. Plasma insulin was assayed using the Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia). Intra- and interassay coefficients of variation were <3.4 and <3.0%, respectively. Plasma leptin was measured using the Quantikine® ELISA kit (R&D Systems). Intra- and interassay coefficients of variation were <4.5 and <4.7%, respectively. All the samples were analyzed in duplicate.

Western-Blot Analyses

Tissue was homogenized in 300 μl RIPA buffer (Thermo Scientific) containing 0.5% (w/v) CHAPS (Sigma-Aldrich), protease and phosphatase inhibitors (Complete and PhosSTOP, Roche), using a glass/teflon potter homogenizer (30 strokes), sonicated and let under agitation for 1 h at 4°C. Lysates were centrifuged at 12000 × *g* for 20 min at 4°C. The supernatant was removed and stored at −20°C. Proteins were quantified using the BCA system (Pierce). Protein samples were prepared in reducing conditions (NuPage sample buffer with sample reducing agent, Invitrogen) and heated at 95°C for 10 min. Then, 10 μg of proteins were separated

on a NuPAGE Novex gel (Invitrogen) and transferred to a nitrocellulose or PVDF membrane, for chemiluminescent or fluorescent western-blotting respectively. Membranes were saturated in 5% non-fat dry milk in TNT or 5% Bovine Serum Albumine in Tris–NaCl-Tween buffer and incubated with appropriate primary antibodies allowing detection of actin (Sigma-Aldrich A5441, 1/50000), Akt (Cell Signaling 9272, 1/5000), phospho-Akt (Ser473) (Cell Signaling 12694, 1/2500), Human and murine alpha-synuclein (BD Bioscience 610787; immunogen: Rat synuclein-1 aa. 15-123; 1/10000), phospho-alpha-Synuclein (Ser129) (Cell Signaling 23706; immunogen: synthetic phosphopeptide corresponding to residues surrounding Ser129 of Human alpha-synuclein protein; 1/1000), aggregated alpha-synuclein (Merck MABN389; immunogen: Keyhole limpet hemocyanin-conjugated linear peptide corresponding to Human aggregated alpha-synuclein; 1/2000), IRS-1 (Cell Signaling 2382, 1/1000), phospho-IRS1 (Tyr608) (Merck 09-432, 1/5000), LC3B (Abcam ab48394, 1/2500), mTOR (Cell Signaling 2972, 1/1000), phospho-mTOR (Ser2448) (Cell Signaling 2971, 1/2500), p62 (BD Bioscience 610498, 1/5000), Stat3 (Cell Signaling 9139, 1/2500), phospho-Stat3 (Tyr705) (Cell Signaling 9131, 1/1000), Raptor (Cell Signaling 2280, 1/5000) and Rictor (Cell Signaling 2114, 1/5000). Signals were revealed with horseradish peroxidase conjugated secondary antibodies (Life technologies) and chemiluminescence (ECL Prime Western Blotting Detection Reagent, Amersham Biosciences) using Amersham Imager 600 (GE Healthcare), or with fluorescent secondary antibodies (Life technologies) using Typhoon FLA 9500 GoldSeal (GE Healthcare). Two to eight replicates of each western-blot were performed. Quantification of protein bands densitometry was carried out using ImageJ software version 1.51 w (NIH). Results are normalized to actin levels.

Statistics

The difference in body mass between genotypes was compared across time using a Kruskal–Wallis test followed by Mann–Whitney *U*-test on individual time period. A Mann–Whitney *U*-test was used to compare WT and Thy1-aSYN mice for a given phase or light and dark phases for a given phenotype. Unless otherwise noted, values in the figures and text are mean ± standard error of the mean (SEM). Values of $p < 0.05$ are considered to be statistically significant. Data were analyzed and graphs were plotted by GraphPad Prism® software version 6.05.

RESULTS

Thy1-aSYN Mice Show Impaired Motor Performance and Coordination in Challenging Beam Traversal

Six-month-old WT and Thy1-aSYN mice were tested in the challenging beam traversal test (**Figure 1A**). Time to traverse, number of steps and error per steps were analyzed. Regarding time to traverse, Mann–Whitney *U*-test showed no significant difference between WT and transgenic mice ($p = 0.67$) (**Figure 1B**). Similarly, the number of steps did not differ between WT and transgenic mice ($p = 0.28$; data not shown). Analysis of the number of errors per step indicated that transgenic animals made significantly more errors compared to WT controls (0.77 ± 0.05 vs. 0.38 ± 0.03; $p < 0.0001$) (**Figures 1C,D**). Therefore, our results show that 6-month-old Thy1-aSYN display impairments in the challenging beam traversal test, confirming the deficits of this model in challenging motor tests (Fleming et al., 2004; Chesselet et al., 2012).

Thy1-aSYN Mice Show Reduced Body Weight Gain Over Time

Wild-type and transgenic mice were weighed at 21 days (weaning), 2, 3, 6, 9, and 12 months of age. Kruskal–Wallis analysis showed that both WT and transgenic mice gained weight as they aged ($p < 0.0001$ for both genotype). Mann–Whitney *U*-test on individual time periods revealed that Thy1-aSYN mice weighed significantly less than WT mice from 3 months of age (27.25 ± 0.92 vs. 31.31 ± 0.94 g; $p < 0.001$). Difference increased over time, so that at 12 months of age WT mice reached 42.72 ± 2.13 g, while Thy1-aSYN mice weighted 35.30 ± 2.44 g ($p < 0.05$) (**Figure 2A**). Therefore, our results suggest a slower body weight gain of Thy1-aSYN transgenic mice compared to WT littermates. Next characterizations were conducted on 6-month-old transgenic Thy1-aSYN and WT mice (body mass: Thy1-aSYN: 32.84 ± 1.03 vs. WT: 38.82 ± 1.13 g; $p < 0.001$).

Thy1-aSYN Mice Have Reduced Body Fat Compared to WT Mice

To explore whether lower body mass of Thy1-aSYN compared to WT mice was attributable to change in body composition, X-ray CT Scan imaging was used to evaluate lean and fat masses in anesthetized 6-month-old mice (**Figure 2B**). Data analyses revealed no significant difference in lean mass between transgenic and WT mice (Thy1-aSYN: 17.69 ± 0.63 vs. WT: 19.15 ± 0.54; $p = 0.11$). In contrast, transgenic mice showed a decrease in the total fat mass (2.54 ± 0.26 vs. 8.39 ± 1.35 g; $p < 0.0001$), visceral fat mass (1.22 ± 0.14 vs. 3.90 ± 0.53 g; $p < 0.0001$), and subcutaneous fat mass (1.32 ± 0.125 vs. 4.49 ± 0.82 g; $p < 0.0001$) compared to WT littermates (**Figure 2C**). This was accompanied by a decreased fat ratio (Thy1-aSYN: 12.67 ± 1.36 vs. WT: 28.88 ± 3.29%; $p = 0.0001$). Bone mineral density was also calculated and showed no difference between transgenic and WT animals (data not shown). At euthanasia, the weight of subcutaneous adipose tissue (consisting of inguinal and gluteal adipose tissues) was lower in Thy1-aSYN mice compared to that of WT mice (121.1 ± 10.58 vs. 447.4 ± 77.32 mg; $p < 0.001$) (**Figure 2D**). Therefore, both CT Scan imaging and subcutaneous fat weighting show that Thy1-aSYN mice have a reduced fat accumulation.

Thy1-aSYN Mice Display Reduced Food Intake and Altered Meal Pattern

To better understand the cause of the lower body weight and body fat in transgenic mice, we examined their food

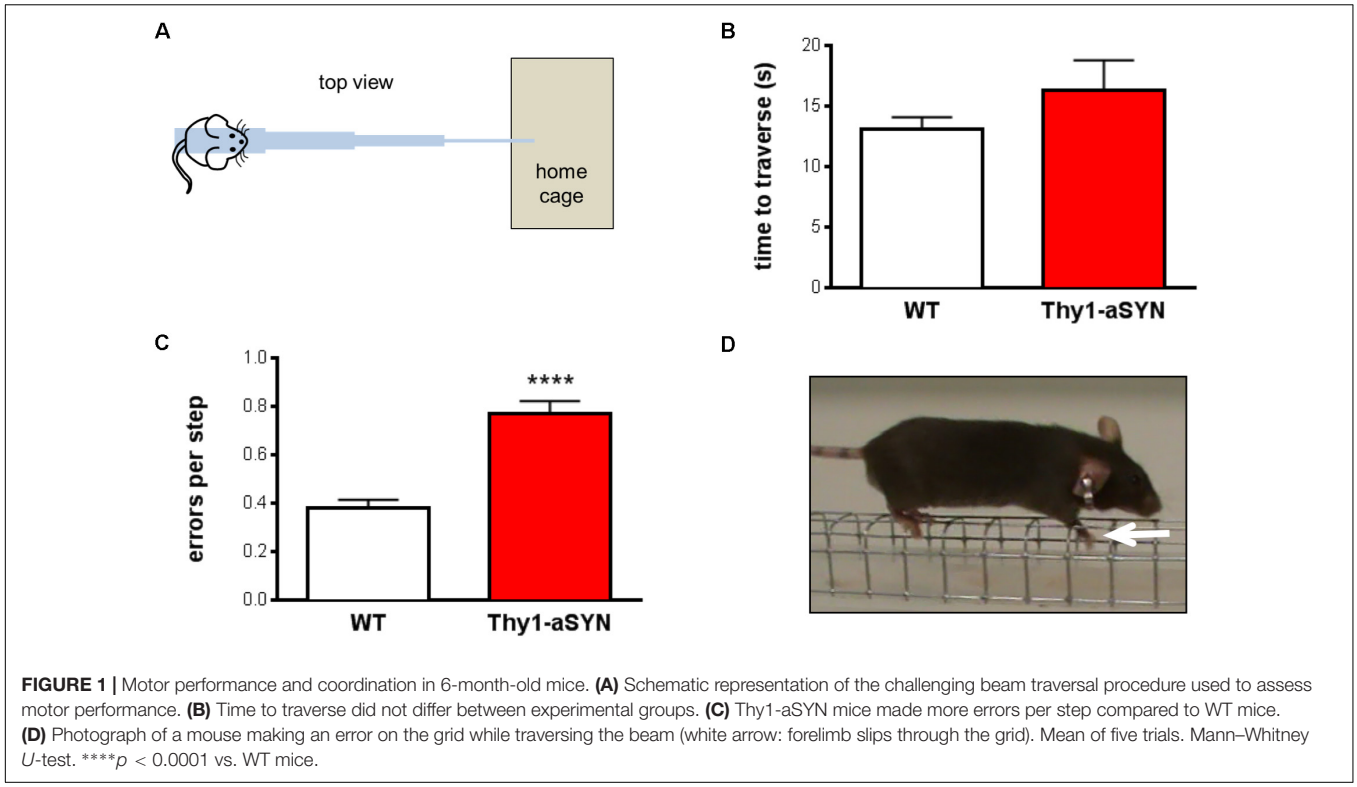

FIGURE 1 | Motor performance and coordination in 6-month-old mice. **(A)** Schematic representation of the challenging beam traversal procedure used to assess motor performance. **(B)** Time to traverse did not differ between experimental groups. **(C)** Thy1-aSYN mice made more errors per step compared to WT mice. **(D)** Photograph of a mouse making an error on the grid while traversing the beam (white arrow: forelimb slips through the grid). Mean of five trials. Mann–Whitney *U*-test. ****$p < 0.0001$ vs. WT mice.

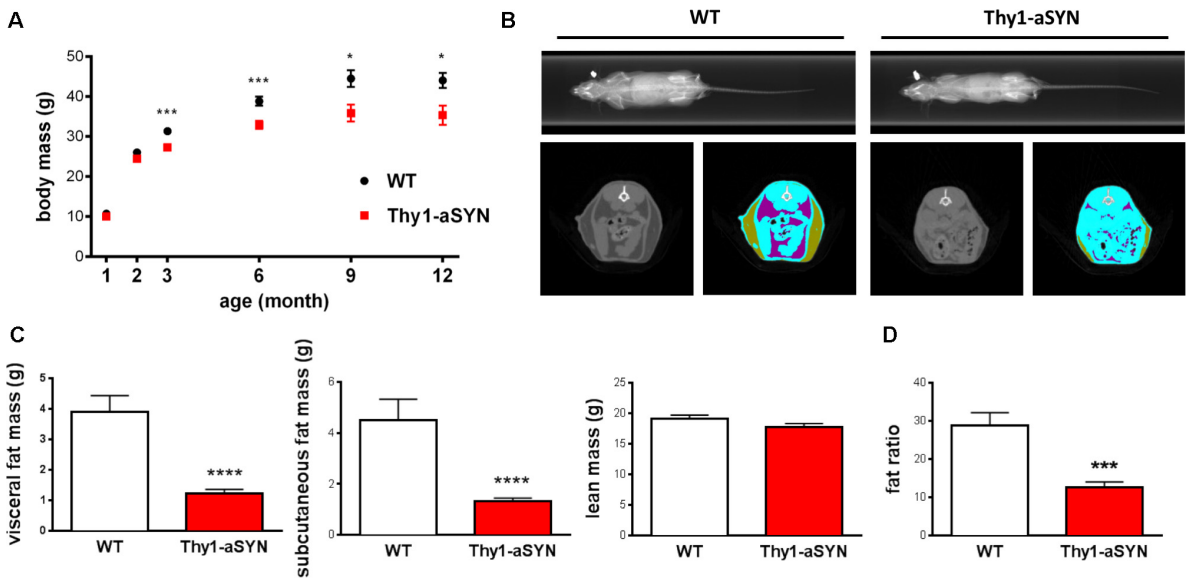

FIGURE 2 | Body weight gain and body composition analysis. **(A)** Thy1-aSYN mice show reduced weight-gain compared to WT littermates from 3 months of age. **(B)** Representative Micro-CT Scans obtained from 6-month-old WT and Thy1-aSYN mice showing selected area of lean (blue), subcutaneous fat (green), visceral fat (purple) tissues. **(C)** Both visceral and subcutaneous fat masses were decreased in 6-month-old Thy1-aSYN mice compared to WT mice, with no change in lean mass. **(D)** Mass of subcutaneous adipose tissue collected at euthanasia was decreased in 6-month-old Thy1-aSYN mice compared to WT mice. Mann–Whitney *U*-test on individual time epochs and for body composition analyses; *$p < 0.05$, ***$p < 0.01$, and ****$p < 0.0001$ vs. WT mice.

intake, ambulatory activity and EE using the LabMaster-CaloSys-Calorimetry system. Transgenic mice consumed 35% less food than their control littermates during the dark cycle (1.85 ± 0.31 vs. 2.86 ± 0.225 g; $p < 0.05$). This was accompanied by a lower consumption of water (1.11 ± 0.26 vs. 2.00 ± 0.23 ml; $p < 0.05$) (**Figure 3A**). Further analyses were performed to compare the inter-meal interval, meal size, meal duration and meal rate. Inter-meal interval analysis revealed no difference

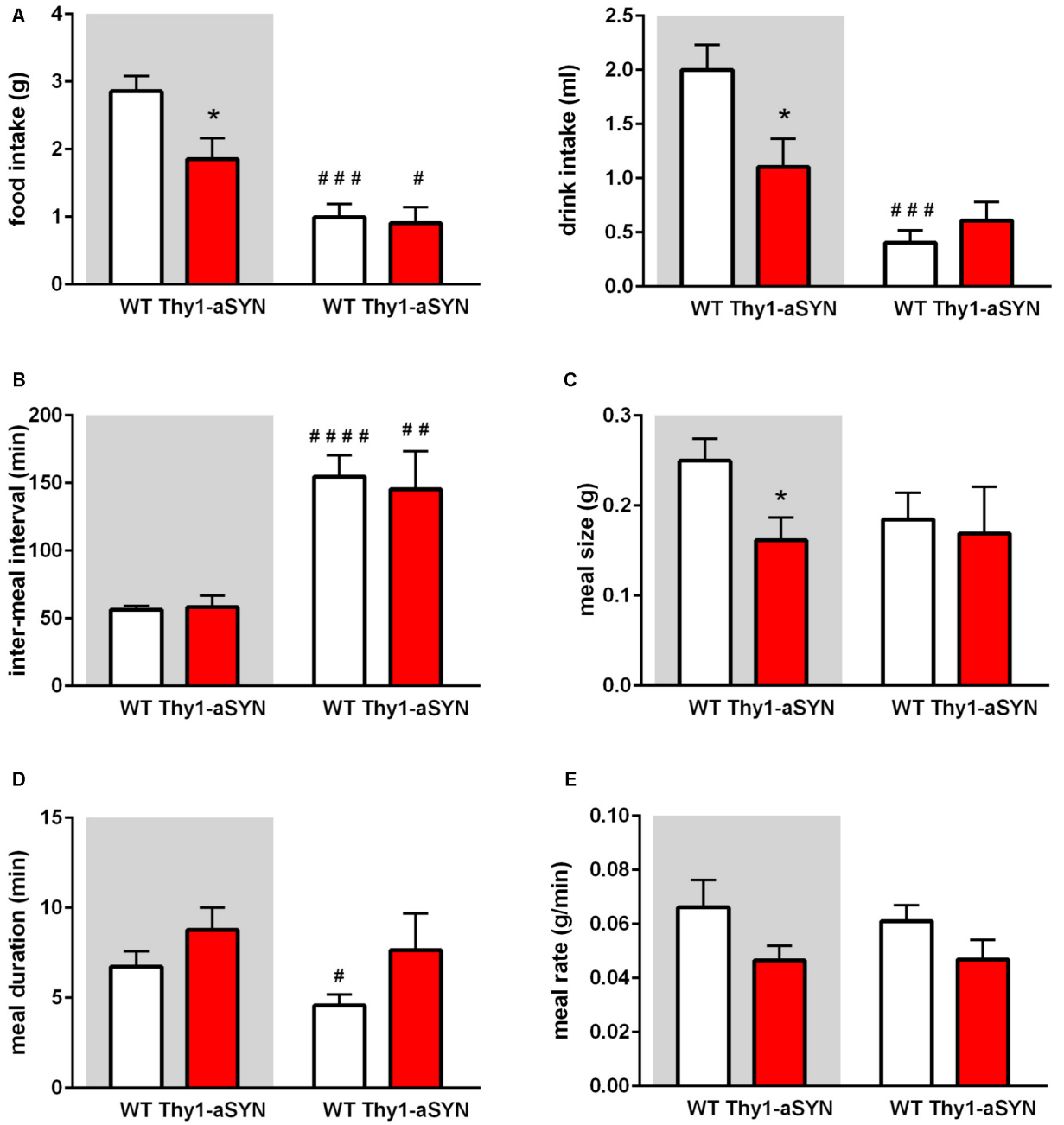

FIGURE 3 | Food intake and meal pattern of 6-month-old mice. **(A)** Thy1-aSYN mice consumed less food and water compared to WT mice during the dark phase. **(B)** Inter-meal interval were more elevated during the light phase than during the dark phase, regardless of the genotype. **(C)** Meal size was decreased in Thy1-aSYN mice compared to WT mice during the dark phase. **(D)** Meal duration and **(E)** Meal rate analyses show no statistically significant difference between genotypes, although Thy1-aSYN mice overall tended to show longer meal duration and decreased meal rate compared to WT mice during the dark phase. Mann–Whitney *U*-test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. dark cycle; $^{*}p < 0.05$ vs. WT mice. White and gray backgrounds represent light and dark phases, respectively.

between transgenic and WT mice and both genotypes showed a more elevated inter-meal interval during the light phase than during the dark phase (**Figure 3B**). Measurement of the meal size showed that it was decreased in Thy1-aSYN mice compared to WT mice during the dark phase (0.162 ± 0.025 vs. 0.250 ± 0.025 g; $p < 0.05$) (**Figure 3C**). Thy1-aSYN mice also tended to show a longer meal duration and a decreased meal rate compared to WT mice, although these differences did not reach statistical significance (**Figures 3D,E**). It however has to be noted that the meal duration was statistically shorter during the light cycle compared to the dark cycle for WT animals (4.58 ± 0.59 vs. 6.73 ± 0.85 min;

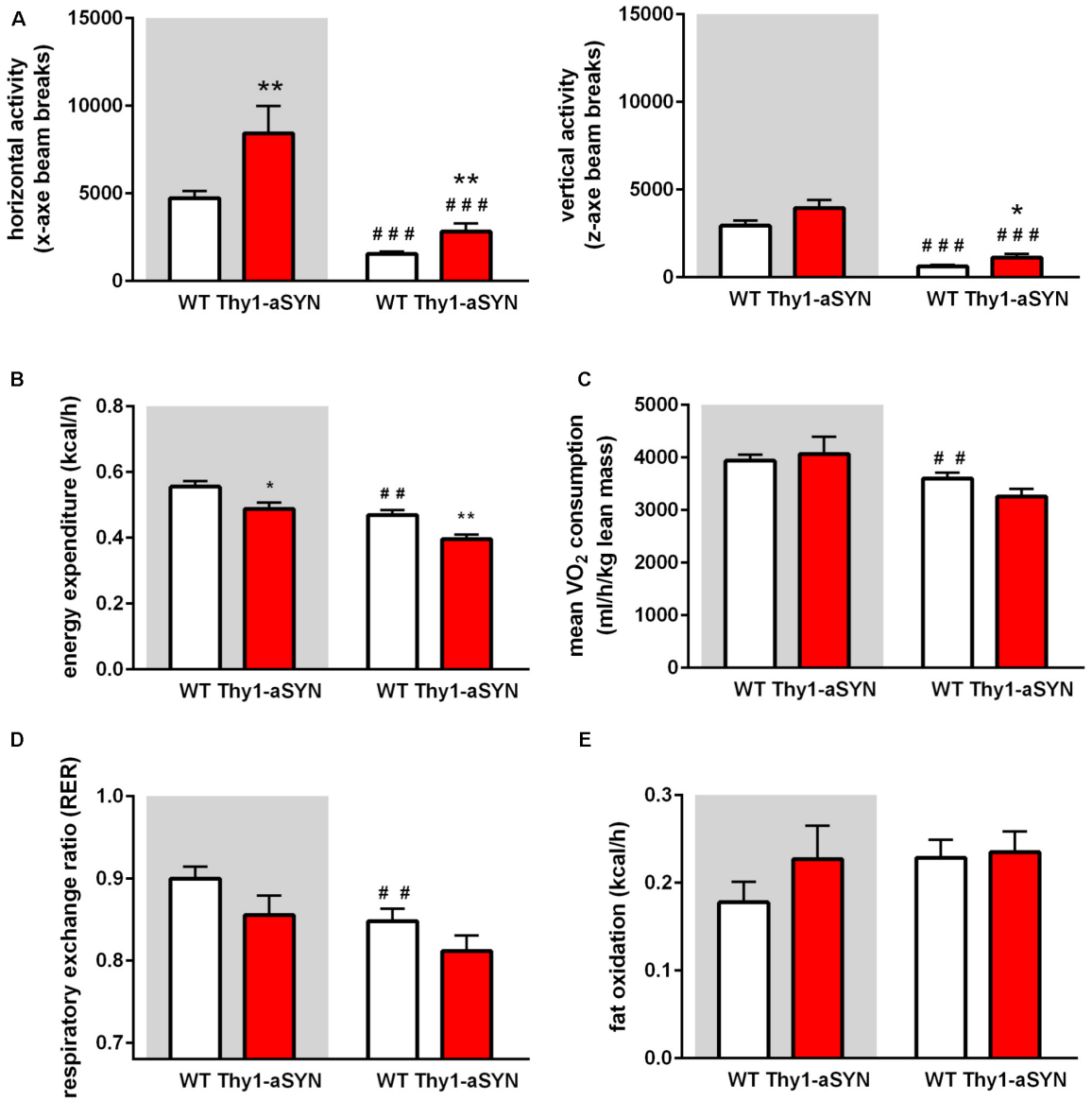

FIGURE 4 | Spontaneous activity and energy metabolism in 6-month-old mice. **(A)** Horizontal (x-axe) and vertical activity (z-axe) of Thy1-aSYN mice and WT mice. **(B)** Energy expenditure was decreased in Thy1-aSYN mice compared to WT mice, both during the dark cycle and light phases. **(C)** Oxygen consumption, **(D)** RER and **(E)** Fat oxidation of Thy1-aSYN and WT mice. Mann–Whitney *U*-test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. dark cycle; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. WT mice. White and gray backgrounds represent light and dark phases, respectively.

$p < 0.05$), but this was not the case for transgenic animals (**Figure 3D**).

Thy1-aSYN Mice Show Increased Activity and Altered Energy Metabolism

The monitoring of locomotor activity showed that the horizontal ambulatory activity (x-axis beam breaks counts; e.g., locomotion) of Thy1-aSYN mice was significantly higher than that of WT mice both during the dark cycle (8425 ± 1552 vs. 4731 ± 401 breaks; $p < 0.05$) and during the light cycle (2828 ± 446 vs. 1558 ± 132 breaks; $p = p < 0.01$). Likewise, the vertical activity (z-axis beam breaks counts; e.g., rearing or jumping) was significantly increased during the light cycle (1128 ± 218 vs. 620 ± 101 breaks; $p < 0.05$) (**Figure 4A**). The measurement of the EE using the LabMaster-CaloSys-Calorimetry system showed that EE was decreased in Thy1-aSYN mice compared to WT mice, both during the dark cycle (0.49 ± 0.02 vs. 0.56 ± 0.02 kcal/h; $p < 0.05$) and during the light cycle (0.40 ± 0.01 vs. 0.47 ± 0.02 kcal/h; $p < 0.01$) (**Figure 4B**). Body composition influences energy metabolism, and there is no consensus on the best normalization tool to use when expressing VO_2 or VCO_2 (Butler and Kozak, 2010; Kaiyala et al., 2010). In our study, we observed no statistically significant change

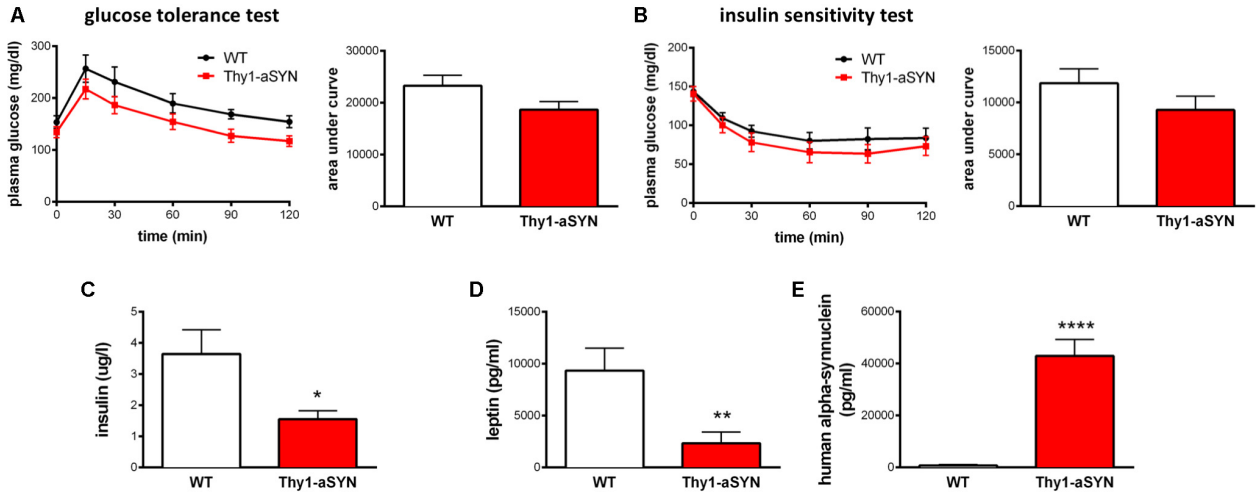

FIGURE 5 | Glucose homeostasis and plasma levels of alpha-synuclein, insulin and leptin in 6-month-old animals. Thy1-aSYN and WT mice showed no significant difference in glycemic response patterns following intraperitoneal injection of **(A)** glucose or **(B)** insulin. Thy1-aSYN mice displayed **(C)** elevated alpha-synuclein levels, **(D)** low insulin and **(E)** low leptin plasma levels compared to WT mice. Mann–Whitney *U*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ vs. WT mice.

in oxygen consumption or carbon dioxide production between transgenic and WT mice, when normalized to either lean mass or total body weight (**Figure 4C** and data not shown). However, our results show that oxygen consumption was decreased during the light cycle compared to the dark cycle for WT animals (3604 ± 110 vs. 3940 ± 115 ml/h/kg lean mass; $p < 0.01$), while this was not the case for the transgenic animals (**Figure 4C**). Similarly, the RER was significantly decreased during the light cycle compared to the dark cycle in WT animals (0.848 ± 0.015 vs 0.900 ± 0.014; $p < 0.01$) but no such difference was observed in Thy1-aSYN mice (**Figure 4D**). Altogether these data suggest that temporal rhythms in energy metabolism may be deregulated in Thy1-aSYN mice. Finally, as the Thy1-aSYN transgenic mice have lower body fat mass than WT mice, we calculated the fat oxidation to better evaluate their use of fat as intrinsic energy source. Our results did not reveal significant difference for this parameter between genotypes (dark cycle: Thy1-aSYN: 0.23 ± 0.04 vs. WT: 0.18 ± 0.02 kcal/h; $p = 0.485$; light cycle: Thy1-aSYN: 0.235 ± 0.02 vs. WT: 0.23 ± 0.02; $p = 0.74$) (**Figure 4E**).

Thy1-aSYN Mice Have Decreased Insulin and Leptin Plasma Levels

Given the change in body weight, fat mass, food consumption, spontaneous activity and EE, we first examined blood glucose levels after 6 h of fasting and observed no significant difference between Thy1-aSYN and WT mice (TR: 160.0 ± 9.75 vs. WT: 172.5 ± 8.32 mg/dl; $p = 0.4732$). To assess whole-body glucose homeostasis, we next performed intraperitoneal glucose tolerance test and insulin sensitivity test on these mice. We detected no differences in the response patterns, with comparable area under the curve for Thy1-aSYN and WT mice in both tests (**Figures 5A,B**). We also measured the plasma levels of insulin and leptin and showed decreased levels of these two hormones in Thy1-aSYN mice compared to WT mice (insulin: 1.56 ± 0.27 vs. 3.64 ± 0.78 μg/l; $p < 0.05$; leptin: 2331 ± 1103 vs. 9348 ± 2163 pg/ml; $p < 0.01$) (**Figures 5C,D**). Finally, the presence of Human alpha-synuclein was detected in the plasma of transgenic mice but not in the plasma of WT mice (42960 ± 6305 pg/ml; $p < 0.0001$ vs. WT mice) (**Figure 5E**).

Thy1-aSYN Mice Display Decreased STAT3 Phosphorylation and Rictor Level in the Hypothalamus

The control of feeding and EE is centrally regulated by the hypothalamus using the information originating from peripheral organs. To determine whether alterations in this brain region could contribute to the metabolic abnormalities in Thy1-aSYN mice, we first evaluated the accumulation of alpha-synuclein in the hypothalamus in our model. We found that alpha-synuclein, detected with an antibody that recognizes both mouse and Human proteins, was expressed in the hypothalamus at a level comparable to that observed in the substantia nigra and the striatum in 6-month-old Thy1-aSYN mice (**Figure 6A**). Further analyses showed that levels of total alpha-synuclein, alpha-synuclein phosphorylation at serine 129 and aggregated alpha-synuclein were higher in the hypothalamus of Thy1-aSYN mice compared to WT mice (928.5 ± 169.7 vs. 100 ± 17.38%; $p < 0.0001$; 1243.1 ± 335.8 vs. 100 ± 21.36%; $p < 0.0001$ and 4133.72 ± 638.6 vs. 100 ± 38.56%; $p < 0.0001$, respectively) (**Figure 6B**). We next compared the expression and activation of receptors and protein complexes known to be critically implicated in the regulation of energy balance and metabolism by the hypothalamus. Western blot of total and active phosphorylated forms of IRS-1 and of STAT3 showed that phosphorylation of STAT3 at tyrosine 705 was decreased in the hypothalamus of 6-month-old Thy1-aSYN mice compared to WT mice (71.84 ± 10.51 vs. 100 ± 9.70%; $p < 0.05$) (**Figure 6B**).

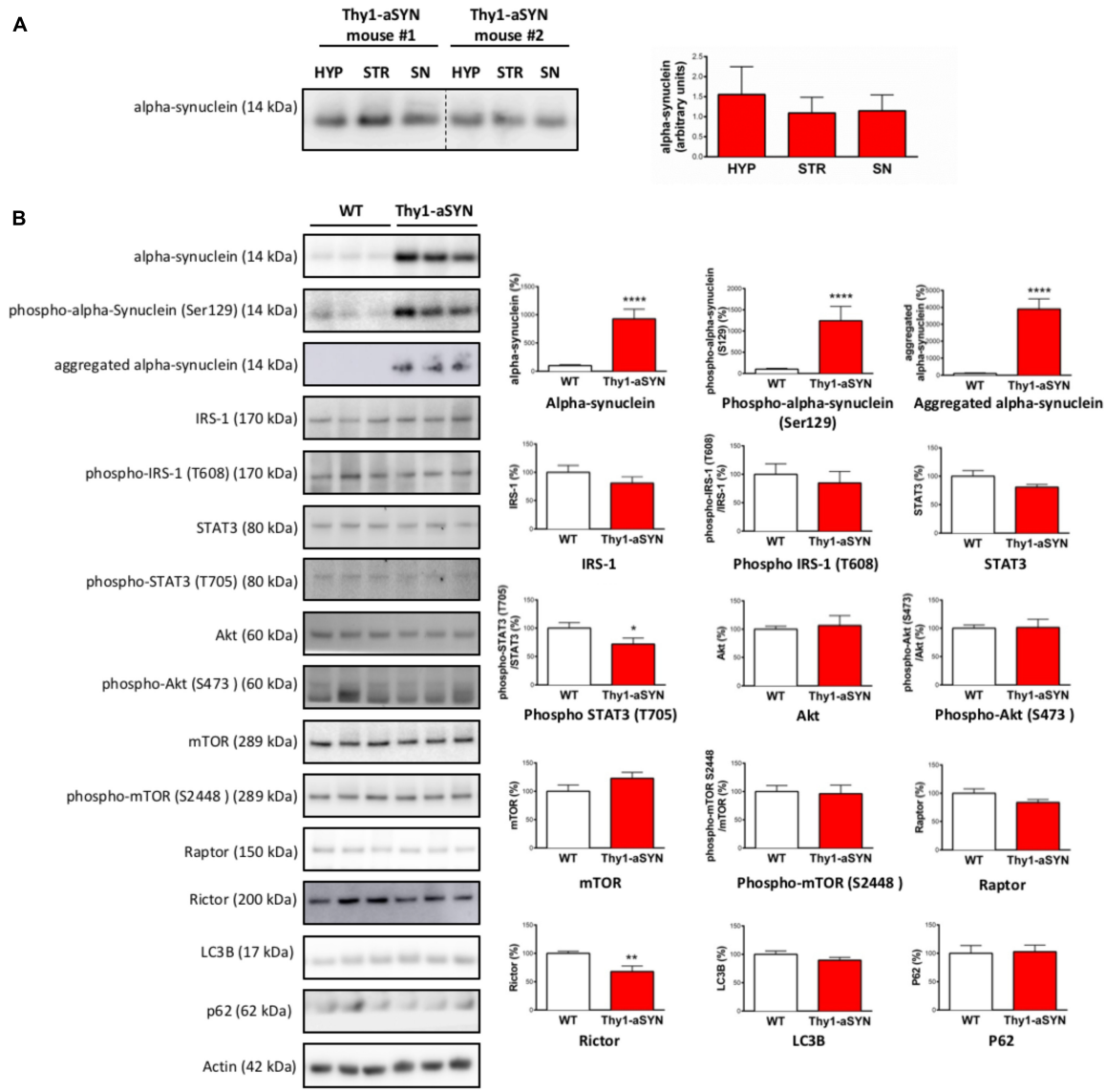

FIGURE 6 | Western blot analysis of hypothalamus protein extracts from 6-month-old Thy1-aSYN and WT mice. **(A)** Alpha-synuclein was expressed at a comparable level in the hypothalamus, the *substantia nigra* and the striatum of 6-month-old Thy1-aSYN mice (Thy1-aSYN: $n = 4$). **(B)** The left images show representative bands for each target protein, and the results are quantified in the right graphs (WT: $n = 12$, Thy1-aSYN: $n = 10$). Thy1-aSYN mice displayed increased levels of total alpha-synuclein, alpha-synuclein phosphorylation at serine 129 and aggregation of alpha-synuclein, as well as decreased phosphorylation at tyrosine 705 of the transcription factor STAT3 and decreased levels of Rictor. Mann–Whitney *U*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ vs. WT mice.

We also compared the major mTOR protein complexes including total and phosphorylated mTOR (that nucleates the two distinct protein complexes mTORC1 and mTORC2), raptor (specific core regulatory protein of the mTORC1 complex) or rictor (specific core regulatory protein of the mTORC2 complex). Obtained data show a significant decrease in the expression of rictor in the hypothalamus of 6-month-old Thy1-aSYN mice (67.97 ± 9.68 vs. $100 \pm 3.66\%$; $p < 0.01$), suggesting that the mTORC2 pathway is deregulated in 6-month-old Thy1-aSYN mice (**Figure 6B**). Given that rictor plays an important role in autophagy induction and that alpha-synuclein is known to alter autophagy signals, the levels of key autophagy-related proteins LC3B and p62 were assessed but showed no significant changes between Thy1-aSYN and WT mice (89.68 ± 4.96 vs. $100 \pm 5.76\%$; $p = 0.27$ and 102.9 ± 11.47 vs. $100 \pm 13.71\%$; $p = 0.64$, respectively).

Finally, we aimed to see whether the deregulation of markers linked to metabolism was observed at an earlier time-point in the Thy1-aSYN mouse model. We therefore compared the phenotype of 3-month-old transgenic and WT animals for both peripheral and central markers previously identified as deregulated. Our results show no change in blood glucose levels and insulinemia and a non-statically decrease of the plasmatic levels of leptin in 3-month-old Thy1-aSYN mice compared to

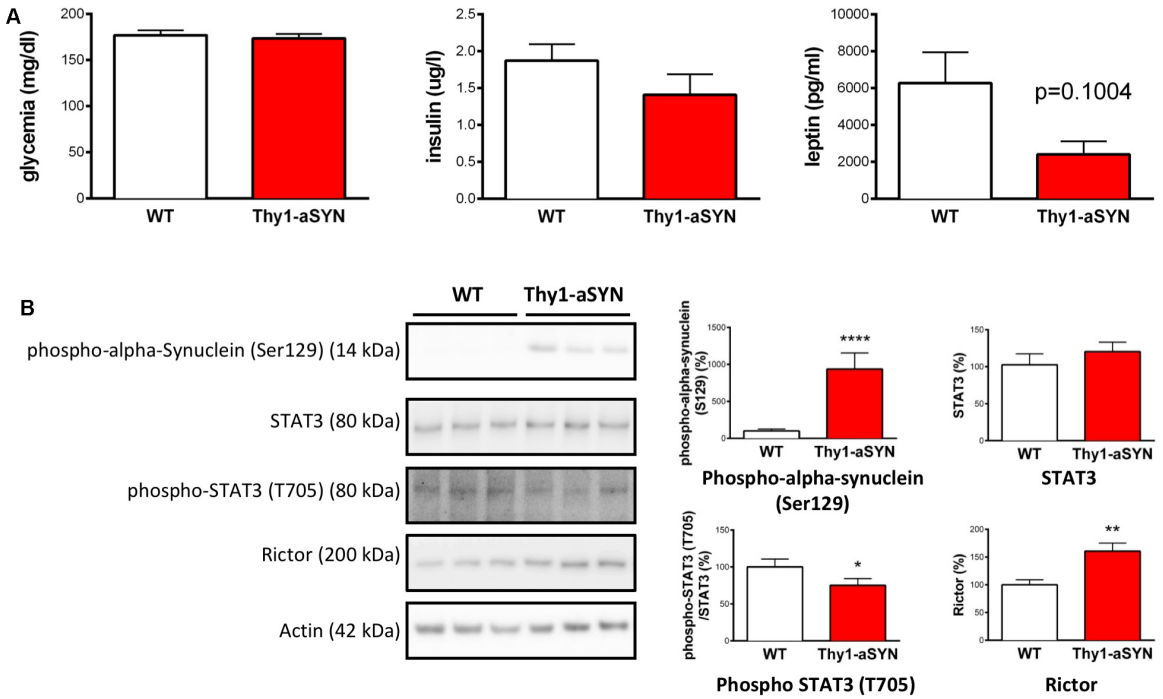

FIGURE 7 | Peripheral hormones and central hypothalamic involvement in 3-month-old animals. **(A)** 3-month-old Thy1-aSYN and WT mice showed no significant difference in blood glucose and plasmatic insulin and leptin levels (WT: $n = 16$, Thy1-aSYN: $n = 11$). **(B)** The left images show representative bands for each target protein, and the results are quantified in the right graphs (WT: $n = 16$, Thy1-aSYN: $n = 13$). Thy1-aSYN mice displayed increased levels of alpha-synuclein phosphorylation at serine 129, as well as decreased STAT3 phosphorylation at tyrosine 705 and increased rictor protein levels. Mann–Whitney *U*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ vs. WT mice.

controls (2416 ± 699 vs. 6273 ± 1670 pg/ml; $p = 0.1004$) (**Figure 7A**). They moreover evidence in 3-month-old Thy1-aSYN mice increased levels of alpha-synuclein phosphorylated at serine 129 (935.5 ± 218.2 vs. 100 ± 26.60%; $p < 0.0001$) together with a decrease in the phosphorylation of STAT3 at tyrosine 705 (75.24 ± 9.06 vs. 100 ± 10.74%; $p < 0.05$) and an increase in rictor protein levels (160.6 ± 14.48 vs. 100 ± 9.08%; $p < 0.01$) (**Figure 7B**).

DISCUSSION

The present study aimed to characterize the metabolic phenotype of the Thy1-aSYN mouse model based on the overexpression of full-length, Human, WT alpha-synuclein. The Thy1-aSYN mouse model reproduces several features evoking sporadic PD, such as alpha-synuclein accumulation in brain regions including the substantia nigra. Later in life - at 14 months of age - these mice lose 40% of striatal dopamine and show sensory-motor deficits that, as in Humans, are partially reversed by L-dopa (Lam et al., 2011). This pathological phenotype may be slower than that of mice that overexpress alpha-synuclein carrying mutations found in patients with rare familial forms of PD (i.e., A53T, A30P) (Chesselet and Richter, 2011). In the present study, we characterized the transgenic mice at an age preceding any dopamine loss and severe sensory-motor deficits (Chesselet et al., 2012) in an attempt to improve our understanding at an earlier phase of the disorder. Consistent with previous publications (Fleming et al., 2004; Lam et al., 2011) we report motor deficits in the challenging beam traversal test, therefore supporting the reproducibility of the Thy1-aSYN phenotype. Our data show for the first time that 6-month-old Thy1-aSYN mice display loss of adiposity, altered feeding behavior, decreased EE, as well as deregulation of peripheral hormones and hypothalamic signaling pathways known to be critically implicated in the regulation of energy homeostasis.

Body Weight and Body Composition of Thy1-aSYN Mice

Thy1-aSYN mice showed a decreased body weight observable from 3 months of age compared to age-matched WT mice, and the difference increased with age. Body composition analysis in 6-month-old animals revealed that Thy1-aSYN mice had lower subcutaneous and visceral fat mass, but no change in lean mass. It has to be noted that Human prospective studies report that PD patients can show decreased body weights several years (2–5 years) before the disease is diagnosed (Durrieu et al., 1992; Chen et al., 2003; Cheshire and Wszolek, 2005; Sharma and Lewis, 2017), after which their average body weights decline. Anthropometric studies show that this weight loss is essentially associated with reduced body fat mass, but not with muscle loss (Markus et al., 1993; Beyer et al., 1995;

Lorefalt et al., 2004, 2009). Therefore, the phenotype of the Thy1-aSYN mouse model recapitulates several features reported in PD patients. Because PD patients are at higher risk of reduced bone mineral density and fractures (Torsney et al., 2014), we aimed to measure bone mineral density in the Thy1-aSYN and WT mice, showing no difference between genotypes.

Altered Feeding Behavior and Energy Metabolism

When individually housed in metabolic cages, 6-month-old transgenic mice showed a decrease in food consumption compared to control mice. This was associated with an altered meal pattern resulting in a smaller meal size. Decreased food consumption and altered meal pattern could arise from a number of factors observed in this transgenic mouse model. First, Thy1-aSYN mice exhibit early and sustained olfactory detection and discrimination deficits without total loss of olfaction, as it is usually reported in patients (Fleming et al., 2008). Olfaction influences food intake (Soria-Gomez et al., 2014) and therefore, such olfactory deficiencies might contribute to a lower food consumption of Thy1-aSYN mice. Second, Thy1-aSYN mice exhibit increased anxiety, that is frequently experienced by PD patients (Mouren et al., 1983). Various studies have associated stress and anxiety to alteration in food intake and body weight (Rivest et al., 1989; Dunn and Berridge, 1990) and this could impact the feeding behavior in our study. Third, Thy1-aSYN mice show reductions in fecal pellet output when exposed to a novelty stress from 2.5–3 months of age, as well as alpha-synuclein accumulation in the colonic myenteric ganglia as reported in 7–8-month-old animals (Wang et al., 2012). Such gastro-intestinal alterations could contribute to weight loss in transgenic animals both by altering their food intake behavior and by causing malabsorption. Thy1-aSYN mice displayed a decreased EE compared to controls, thus arguing against the possibility that an EE dysregulation could contribute to weight loss in our model. Our observations are consistent with the report that weight loss in PD occurs regardless to changes in daily EE (Delikanaki-Skaribas et al., 2009). Analyses of oxygen consumption and RER analyses did not evidence significant changes between transgenic and WT mice. However, they revealed that EE, oxygen consumption and RER were significantly decreased during the light cycle compared to the dark cycle in WT animals but no such differences were observed in Thy1-aSYN mice. This suggests that Thy1-aSYN mice could display alterations in temporal rhythms in energy metabolism and this could be linked to deficits in circadian-regulated behavior (Kudo et al., 2011).

Hyperactivity, Dopamine, and Face-Validity of the Thy1-aSYN Model

Behavioral characterization of 6-month-old transgenic mice showed an increased activity for both horizontal and vertical movements, in line with previous reports of increased open-field activity of Thy1-aSYN mice at 7 months (Lam et al., 2011). Increased activity of Thy1-aSYN mice has to be considered along with the increased extracellular striatal dopamine reported in the Thy1-aSyn mice at 6 months of age and should be distinguished of the later decreased locomotion observed in 14-month-old Thy1-aSYN mice, when striatal dopamine is significantly lost (Lam et al., 2011; Chesselet et al., 2012). Interestingly, an increased activity has also been reported for other mouse models of PD. For instance, knock-in mice bearing the G2019S LRRK2 mutation (a frequent cause of familial PD) have a hyperactive phenotype in the open field test (Longo et al., 2014). Similarly, MitoPark mice (in which the mitochondrial transcription factor Tfam is selectively removed in midbrain dopamine neurons) display at 6 weeks higher activity scores at both horizontal and vertical movements than control mice, while this hyperactivity is reversed to bradykinesia at 12 weeks of age, when dopamine levels get significantly lower in their striatum (Galter et al., 2010). Although it is difficult to extrapolate these data to patients in which levodopa-responsive motor symptoms support the diagnosis, one could hypothesize that early deregulation of dopamine could contribute to presymptomatic motor changes, as suggested in healthy carriers of the LRRK2 G2019S mutation (Mirelman et al., 2011). Noteworthy, the nigrostriatal dopaminergic pathway has also been implicated in feeding (Ungerstedt, 1971; Szczypka et al., 2001) and an excess of dopamine signaling has been reported to inhibit feeding in mice, as demonstrated with non-specific dopamine receptor agonists, DAT inhibitors, or amphetamines (Leibowitz, 1975). As such, changes in dopamine concentration could contribute to hyperactivity, impairments in challenging motor test and feeding abnormalities in 6-month-old Thy1-aSYN mice.

Deregulation in Hormones Controlling Energy Homeostasis

Research on the mechanisms governing body weight, feeding behavior and energy metabolism has provided insight into complex interactions between peripheral signals and the central nervous system (Schwartz et al., 2000). Both plasma insulin and leptin concentrations are decreased in 6-month-old Thy1-aSYN mice. The leptin hormone is produced by white adipose tissue (Considine et al., 1996). Its level declines in Humans and mice after weight loss and accordingly a lower plasma leptin concentration was reported in PD patients with weight loss (Lorefalt et al., 2009; Fiszer et al., 2010). Hypoleptinemia has also been reported in transgenic mice overexpressing A53T alpha-synuclein (Rothman et al., 2014), as well as in mice overexpressing amyloid precursor protein (Ishii et al., 2014) and in two Huntington's disease mouse models (Phan et al., 2009) and might therefore appear as a feature frequently associated with pathological protein accumulation in mice. Insulin is secreted from pancreatic beta cells and its circulating concentrations show a positive correlation with body fat mass (Yu and Kim, 2012). This suggests that the declines in leptin and insulin levels are consequences of the changes in body composition in the Thy1-aSYN mice. In turn, the deregulation of these hormones could alter the phenotype of Thy1-aSYN mice in several ways. For instance, insulin increases adiposity, promotes

lipid synthesis, and inhibits lipolysis (Baskin et al., 1999). Also, both leptin and insulin were shown to raise EE when administered to rodents (Menendez and Atrens, 1989; Morton et al., 2011), therefore directly linking decline in these hormones and reduction in EE in the Thy1-aSYN mice. Insulin and leptin have also neuroprotective properties and can influence synaptic plasticity. Among other studies, leptin administration rescued dopaminergic neurons, decreased the apomorphine-induced rotational behavior and restored striatal catecholamine levels in the unilateral 6-hydroxydopamine mouse model of dopaminergic cell death (Weng et al., 2007). Also, insulin induces the expression of the activity-regulated cytoskeleton-associated gene (Chen et al., 2014), an effector immediate early gene critical to protein-synthesis synaptic plasticity (Rosi, 2011). Leptin receptors and insulin receptors are expressed by dopaminergic neurons in midbrain (Figlewicz et al., 2003) and both hormones act directly on these neurons (Hommel et al., 2006; Mebel et al., 2012). Leptin has been shown to modulate dopamine D2 receptor expression in striatum (Pfaffly et al., 2010). Insulin has been shown to depress dopamine concentration in the ventral tegmental area via increasing its reuptake through dopamine transporter (Mebel et al., 2012). Although these results indicate a potential role of leptin and insulin in regulating the action of dopamine in the brain, the exact molecular mechanisms of such actions are yet to be elucidated.

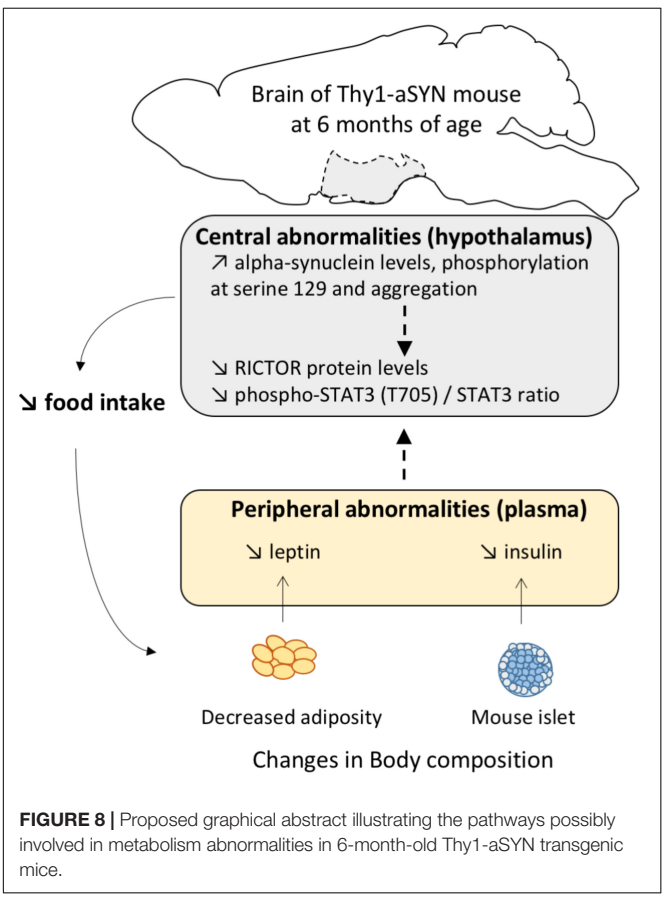

FIGURE 8 | Proposed graphical abstract illustrating the pathways possibly involved in metabolism abnormalities in 6-month-old Thy1-aSYN transgenic mice.

Alpha-Synuclein and Deregulation of Signaling Pathways Regulating Energy Metabolism in the Hypothalamus of Thy1-aSYN Mice

It is now well established that the brain, especially the hypothalamus, maintains body weight homeostasis by effectively adjusting food intake and EE in response to changes in levels of various nutritional status indicators. In PD, alpha-synuclein pathology may spread along neuronal pathways and neuropathological hypothalamic involvement was previously reported (Langston and Forno, 1978; Ansorge et al., 1997), even at preclinical stages (De Pablo-Fernandez et al., 2017). Here, we report for the first time that alpha-synuclein accumulates in the hypothalamus of Thy1-aSYN mice, to levels comparable to those of the *substantia nigra* and the striatum. We also show increased levels of alpha-synuclein phosphorylation at serine 129 and alpha-synuclein aggregation in this brain region. Alpha-synuclein has been suggested to fulfill roles in synaptic function and plasticity and thus it is possible that the alpha-synuclein pathology in the hypothalamus causes functional alterations leading to metabolism abnormalities. Importantly, alpha-synuclein level was also elevated in the plasma of Thy1-aSYN mice compared to WT mice. Although it is not clear how plasma alpha-synuclein levels relate to abnormal aggregated alpha-synuclein in the brain, our results confer to the Thy1-aSYN mouse model a major interest to evaluate the blood as a as a source of neurodegeneration biomarkers. By further analyzing the receptors and protein complexes known to be critically implicated in the regulation of metabolism, we evidenced a decreased phosphorylation at tyrosine 705 of the transcription factor STAT3 in the hypothalamus already present in 3-month-old Thy1-aSYN mice. STAT3 phosphorylation at tyrosine 705 promotes its homodimerization or heterodimerization with other STATs which leads to nucleus translocation and DNA binding. *In vivo* studies have provided evidence indicating that STAT3 activation in the hypothalamus is critical to the regulation of food intake and energy balance, particularly in the functioning of leptin (Bates et al., 2003; Buettner et al., 2006). Thus the downregulation of STAT3 phosphorylation could sign defects in the hypothalamus function in Thy1-aSYN mice. Over the last decade, mTOR complexes 1 and 2 (mTORC1 and mTORC2) have also emerged as critical cellular energy sensors because of their ability to couple hormones (including leptin and insulin) and nutrient signals with the regulation of energy balance and metabolism and activity among others (Haissaguerre et al., 2014). We compared the expression of major mTOR proteins and showed alterations of the protein levels of rictor, a required subunit for mTORC2, with increased protein levels in 3-month-old Thy1-aSYN mice, and decrease protein levels at 6 months of age. We did not observe changes in rictor levels in the *substantia nigra* of 6-month-old Thy1-aSYN (data not shown), suggesting that alpha-synuclein overexpression causes brain region-specific deregulations in our model. Although the function of mTORC2 is not as well-known as the function of mTORC1, it regulates actin polarization and endocytosis (Rispal et al., 2015) and sphingolipid biosynthesis (Roelants et al., 2011). It appears

important to further evaluate the role of mTORC2 in these pathways that we and others reported as notably deregulated in PD (Mutez et al., 2014; Dijkstra et al., 2015). Thus, considering the pivotal roles of the hypothalamus in regulating feeding behavior and energy metabolism, it is reasonable to speculate that the STAT3/mTORC2 alterations could contribute to the metabolic phenotype of Thy1-aSYN mice (**Figure 8**).

CONCLUSION

In conclusion, our data demonstrate a link between alpha-synuclein overexpression and metabolic abnormalities including decreased body weight and adiposity, altered feeding behavior and decreased EE together with hypoleptinemia and hypoinsulinemia in the Thy1-aSYN mouse model. They moreover raise the possibility that alterations of the STAT3/mTORC2 signaling pathways in the hypothalamus of Thy1-aSYN transgenic mice could contribute to the disruption of their feeding behavior and energy metabolism. This emphasizes the need to better characterize the hypothalamus molecular dysregulation in PD to understand the etiology of unintended body weight change in this disease. Future studies are particularly needed to evaluate the interest of the proteins linked to STAT3 and mTORC2 signaling as biomarkers for PD and to test whether strategies aimed to normalize energy metabolism would interfere with alpha-synuclein-linked pathology.

AUTHOR CONTRIBUTIONS

ECu, MM, CL, WS and ASt contributed to the research project execution and to the data and statistical analyses. CV-M, OV, and ECu contributed to the project conception and to the manuscript reviewing. ASt, ASa, KS, TC, PS, M-AB, HC helped with the project execution. ECa supervised the use of the metabolic cages. AL helped with the meal pattern analysis. EM, AD, and BG revised the manuscript providing expertise in PD and cell signaling. M-CC-H contributed to the project conception and organization and edited the manuscript providing expertise in molecular neurodegeneration in PD. KB contributed to the project conception, organization and execution, to the statistical analyses and predominantly contributed to the writing of the article. All authors read and approved the final manuscript.

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REVIEW
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Reduced Cytosolic Calcium as an Early Decisive Cellular State in Parkinson's Disease and Synucleinopathies

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The more than 30-year-old Calcium hypothesis postulates that dysregulation in calcium dependent processes in the aging brain contributes to its increased vulnerability and this concept has been extended to Alzheimer's disease and Parkinson's disease. Central to the hypothesis is that increased levels of intracellular calcium develop and contributes to neuronal demise. We have studied the impact on cells encountering a gradual build-up of aggregated α-synuclein, which is a central process to Parkinson's disease and other synucleinopathies. Surprisingly, we observed a yet unrecognized phase characterized by a reduced cytosolic calcium in cellular and neuronal models of Parkinson's disease, caused by α-synuclein aggregates activating the endoplasmic calcium ATPase, SERCA. Counteracting the initial phase with low calcium rescues the subsequent degenerative phase with increased calcium and cell death – and demonstrates this early phase initiates decisive degenerative signals. In this review, we discuss our findings in relation to literature on calcium dysregulation in Parkinson's disease and dementia.

Keywords: aggregation, α-synuclein, calcium, synucleinopathies, dementia

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INTRODUCTION

In Parkinson's disease (PD), dopaminergic neurons in the substantia nigra pars compacta have been the focus of interest for decades due to their disease-associated loss that gives rise to the striatal dopamine deficiency and motoric symptoms. An intense interest in the cytosolic Ca^{2+} levels of these cells has been based on two independent lines of evidence. First, the autonomous firing of these dopaminergic (DA) neurons along with their large terminal fields causes a continuous and large influx of Ca^{2+} that in order to maintain low normal Ca^{2+} levels has to be balanced by active transport processes at the risk of oxidative stress (Surmeier, 2007; Dryanovski et al., 2013; Surmeier et al., 2017). Second, epidemiological studies have demonstrated that treatment with L-type Ca^{2+} channel antagonists that lowers Ca^{2+} influx reduces the risk of developing PD symptoms (Ritz et al., 2010; Pasternak et al., 2012). We recently reported a novel phenotype with reduced cytosolic Ca^{2+} in neurons that occurs very early in the degenerative processes associated to PD (**Figure 1**). In contrast to previous hypotheses focusing on loss of cells, this observation corroborates the existence of prolonged phase with reduced Ca^{2+} in neurons that encounter progressive build-up of α-synuclein aggregates (Betzer et al., 2018). This phase may contribute to symptomatology by changing the neurons contribution to functional motor and non-motor circuitries.

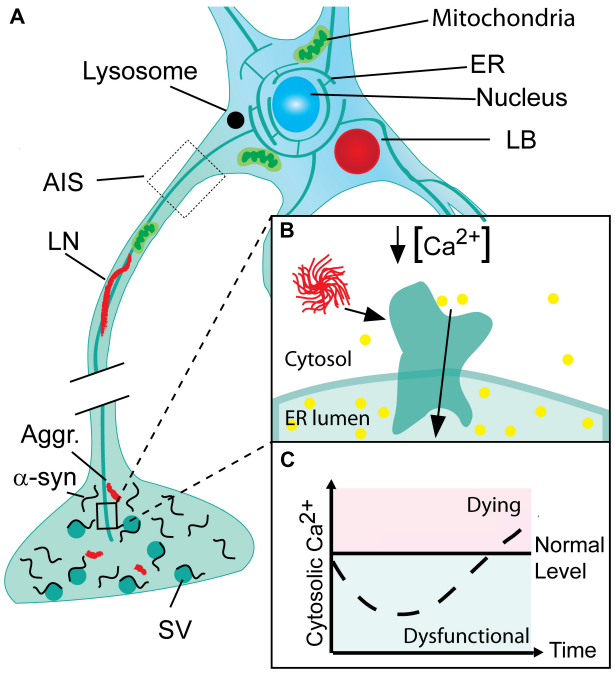

FIGURE 1 | Progressive neuronal accumulation of α-synuclein aggregates in Parkinson's disease cause gradual activation of SERCA and changes in cytosolic Ca^{2+} and Ca^{2+} dependent processes. **(A)** Nerve terminals with their high concentration of α-synuclein are the sites for the initial build-up of α-synuclein (α-syn) aggregates (Aggr.). The aggregates are transported to the cell body by active retrograde axonal transport and during this process accumulate as Lewy neurites (LN). In the cell body, aggregates may accumulate locally as Lewy bodies (LB) if not degraded by lysosomes. **(B)** The endoplasmic reticulum (ER) permeates the entire neuron into its terminals. In Parkinson's disease (PD), α-syn aggregates bind and activate SERCA resulting in reduced cytosolic Ca^{2+} and Ca^{2+} overload in the ER. **(C)** The cytosolic Ca^{2+} level in neurons encountering a progressive build-up of intracellular α-synuclein aggregates displays a biphasic response with a reduced level in the early phase Ca^{2+} ions are translocated into the ER. This phase will be characterized by a range of dysfunctions caused by deranged Ca^{2+} dependent processes. Later the compensatory mechanisms fail and the cell progress into a degenerative state with increased Ca^{2+} that precede cell death.

CYTOSOLIC CALCIUM IONS

Calcium ions are versatile cellular second messengers and proper control of cytosolic Ca^{2+} levels in neurons are crucial for their development and function (Berridge et al., 2000, 2003). The resting cytosolic Ca^{2+} concentration in neurons is approximately 150 nM but this term is a crude measure because most Ca^{2+} signaling is mediated within subcellular microdomains (Rizzuto and Pozzan, 2006). This is particularly true for neurons that are highly polarized with separate microdomains like nerve terminals, axon initial segment and synaptic boutons. Still the experimental use of cytosolic Ca^{2+} sensors like Fura-2 and Fluo3 have given important insights into neuronal Ca^{2+} signaling despite their suboptimal spatial resolution.

The average 150 nM cytosolic Ca^{2+} contrasts the endoplasmic reticulum (ER) and the extracellular compartment where Ca^{2+} concentrations are in the 1 mM range (Berridge et al., 2003; Surmeier and Schumacker, 2013) and these steep gradients are maintained by active transport through efficient pumps and secondary active cotransporters like sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA), plasma membrane ATPases (PMCA), and Na^{+}/ Ca^{2+} exchangers (NCX).

It was early recognized that dysfunctions herein triggered by a broad range of cellular stresses, e.g., decreased ATP and O_2, per default will increase cytosolic Ca^{2+} and ultimately cause cell death (Berridge et al., 1998). However, more subtle changes in leakiness or efficiency of specific pumps could also have fundamental, but slowly progressing effects on the brains ability to compensate with aging and age-related neurodegenerative diseases as put forth in the Ca^{2+}-hypothesis (Khachaturian, 1984, 1994; Berridge, 2010).

PARKINSON'S DISEASE AND α-SYNUCLEIN

Our insight into PD pathophysiology has developed significantly within the last 20 years since (i) mutations within the SNCA gene encoding α-synuclein was identified as causative for autosomal dominant PD in rare families (Zeng et al., 2018), (ii) aggregated α-synuclein was demonstrated as the main component in the neuronal Lewy body inclusions in PD (Spillantini et al., 1997), and (iii) the Braak hypothesis was presented (Braak et al., 2003) on PD being a progressive neurodegenerative disease that continuously involves new but selective areas of the brain thereby bringing increased complexity to the patients symptomatology. Today, it is commonly recognized that many PD patients have suffered from non-motor symptoms, e.g., depression and sleep disturbances, years before they develop motor symptoms leading to PD diagnosis (**Figure 2**). Hence, mechanisms applicable to larger groups of vulnerable neurons rather than the dopaminergic neurons of the substantia nigra pars compacta are attractive targets if the aim is to modify the disease course at different stages from presymptomatic to late-stage disease. The presymptomatic intervention is, as for all late-onset neurodegenerative diseases, especially attractive but requires strong biomarkers to identify at-risk patients. The spreading of pathology is hypothesized to be carried by prion-like aggregates of the normally presynaptic protein α-synuclein that upon uptake in healthy neurons cause progressive conversion of their native α-synuclein into aggregated species. The aggregated species represents a still poorly described group that initially are hypothesized to contain soluble, so-called oligomers or protofibrils, before they convert into amyloid-type filaments. The filaments are deposited in intracellular inclusions localized in axons and cell bodies as Lewy neurites and Lewy bodies that represents pathoanatomical hallmarks of PD (**Figure 1**). The process of α-synuclein aggregation is dose-dependent as evidenced by the autosomal dominant heritability of PD in rare families with elevated levels of normal α-synuclein caused by multiplications of the α-synuclein gene SNCA (Singleton et al., 2003; Chartier-Harlin et al., 2004; Devine et al., 2011). Moreover, GWAS studies demonstrates variations in the SNCA

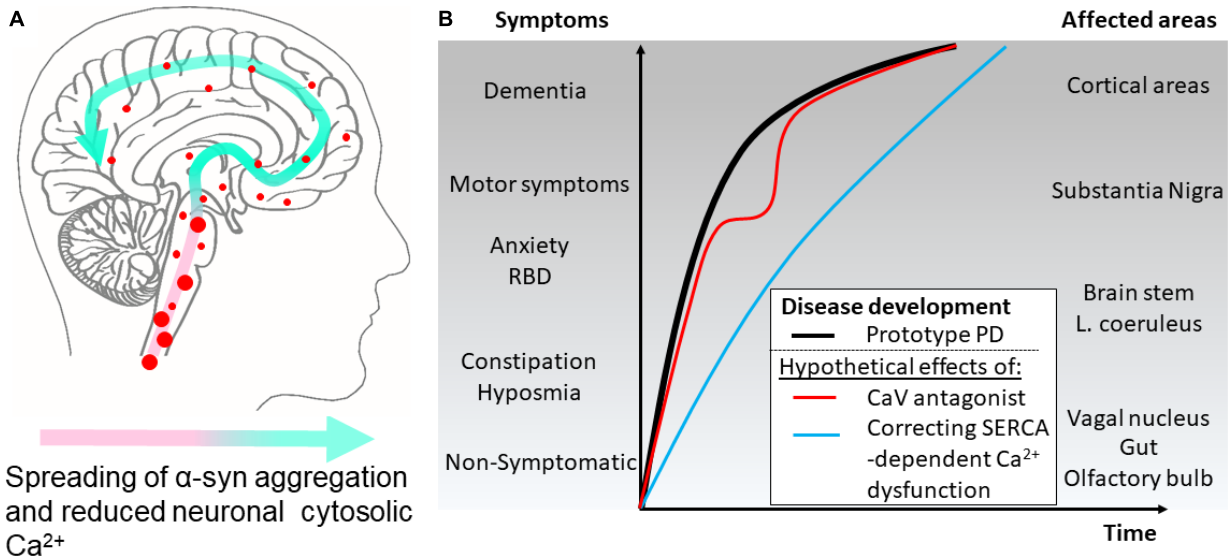

FIGURE 2 | Hypothetical effects on symptomatology in Parkinson's disease by modulating cytosolic calcium in neurons by different mechanisms. **(A)** The disease course for a Parkinson's disease patient can be divided into different phases. According to the Braak hypothesis, the phases represent the sweeping movement of α-synuclein aggregate-dependent neuropathology through the nervous system and is initially detectable in the lower brain stem and the olfactory bulb. **(B)** The presymptomatic phase represent early aggregate build-up in the neurons of the olfactory bulb, the gut and the vagal motor nucleus of the lower brain stem and evolves into the symptomatic phase with hyposmia and constipation as frequent symptoms. Further involvement of the brain stem may add symptoms like sleep disturbances, e.g., REM sleep behavior disorders (RBD), depression, and anxiety. Next, the substantia nigra becomes involved and the patients develop motor symptoms and get diagnosed as PD patients. Finally, higher areas are involved adding cognitive problems to the disease complex. Treatment of patient with L-type Ca^{2+} channel antagonists aims at normalizing Ca^{2+} dysfunctions in the pace-making firing dopaminergic neurons of the substantia nigra pars compacta and other vulnerable neuronal populations. Epidemiological data suggest this treatment lowers the motoric symptoms but not the final progression into dementia as depicted by a red line. A treatment that targets the early Ca^{2+} dysfunctions caused by SERCA activation by α-synuclein aggregates will ideally normalize the early dysfunctions of affected neurons. This could apart from reducing the severity of non-motor symptoms also decrease the rate of disease progression through the brain (blue line).

locus is the largest genetic risk factor for sporadic PD and this effect is hypothesized to be due to increased expression α-synuclein. α-Synuclein is thus considered a key player in the development and progression of PD although it still is unclear which intracellular α-synuclein aggregates harm the neurons and how this is accomplished. Insight into the molecular structure of *in vitro* formed α-synuclein aggregates has only recently reached the level of atomic resolution by CryoEM (Guerrero-Ferreira et al., 2018) and solid state NMR studies (Tuttle et al., 2016). The ability to validate that features of *in vitro* formed aggregates indeed exist *in vivo* has benefitted from the development of aggregate specific antibodies (Lindersson et al., 2004; Vaikath et al., 2015; Lassen et al., 2018; Peng et al., 2018) that allows the validation of specific epitopes on both types of aggregates. Biochemical and structural analyses of α-synuclein aggregates isolated from brain tissue (Gai et al., 2000; Anderson et al., 2006) or cells are sparse but specific targets for α-synuclein aggregates has been identified (Lindersson et al., 2004, 2005; Betzer et al., 2015, 2018; Mao et al., 2016).

We have previously demonstrated that decisive prodegenerative signals, like changes in gene expression and secreted signaling molecules are generated at early time points in human α-synuclein expressing immortalized rat oligodendroglial cells (OLN-93) encountering a progressive build-up of soluble α-synuclein aggregates (Kragh et al., 2009, 2013, 2014). These can be measured in the OLN-93 model at early time points, where no gross dysfunctions are apparent. Recent investigations of this early phase allowed us to demonstrate by use of the ratiometric calcium sensor, Fura-2 that it is characterized by a reduction in cytosolic Ca^{2+} in both mitotic OLN-93 and a non-mitotic human neuroblastoma cell model (SH-SY5Y) of α-synuclein aggregate stress, and in primary cultures of mouse hippocampal neurons expressing human α-synuclein from the Thy1-a-Syn Line 61 (Betzer et al., 2018). The early phase with reduced cytosolic Ca^{2+} preceded the well-known late phase with increased cytosolic Ca^{2+} and increased cell death (**Figure 1**). Mechanistically the decreased cytosolic Ca^{2+} was caused by binding of soluble α-synuclein aggregates to SERCA, leading to increased pumping of Ca^{2+} from cytosol into ER. The increased activity of SERCA was measured by *in vitro* assays using sarcoplasmic microsomes isolated from rabbit muscle as the source of SERCA. Here was measured an increased hydrolysis of ATP, transport of $^{45}Ca^{2+}$, and dephosphorylation rate of the pump. Furthermore, analysis of the rate of Ca^{2+} loading into ER in the OLN-93 cells expressing the calcium sensor aequorin in ER revealed an increased uptake in the presence of α-synuclein aggregates. The Ca^{2+} dysregulation could be treated pharmacologically by the specific SERCA inhibitor cyclopiazonic acid (CPA) leading to reduced cell stress and increased viability in the OLN-93 model, SH-SY5Y model, in the hippocampal neurons, and an *in vivo* C. elegans model, UA44 [baInl1; Pdat-1::AS, Pdat-1::GFP] expressing AS

and GFP in their dopaminergic neurons. The protective CPA dose did not negatively affect the viability of control cells. The abnormal complexes between α-synuclein aggregates and SERCA could be demonstrated by co-immunoprecipitation of SERCA from brain homogenates using *in vitro* formed soluble aggregates as bait and *in situ* in SH-SY5Y cells using anovel α-synuclein/SERCA proximity ligation assay. The proximity ligation assay also revealed positive signals in human brain tissue affected by dementia with Lewy bodies, but not control tissue corroboration this novel mechanism of α-synuclein aggregates engaging with SERCA is relevant to human pathology. Hence, the degenerative phase with reduced cytosolic Ca^{2+} may well be active in human brain. It should be stressed that the mechanisms, whereby α-synuclein aggregates activates SERCA is not restricted to neurons but is generic and will occur in all cells experiencing intracellular α-synuclein aggregates, since SERCA is ubiquitously expressed (Primeau et al., 2018). SERCA is expressed by three genes, ATP2A1, 2 and 3, and their isoform diversity is increased by alternative splicing, giving rise to 13 isoforms (Wuytack et al., 2002; Dally et al., 2010). The expression of SERCA isoforms exhibit both specificity with respect to developmental state and tissue, with SERCA2b and SERCA3 as the predominant isoforms in brain (Uhlen et al., 2015). It will thus apart from PD, Lewy body dementia, and Lewy body variant of Alzheimer's disease (AD), also occur in Multiple systems atrophy, where aggregates accumulate in oligodendrocytes (Gai et al., 1998; Spillantini et al., 1998) as modeled by our OLN-93 models (Betzer et al., 2018). In PD, does astrocytes in proximity of LB containing neurons also contain α-synuclein (Braak et al., 2007) but it does not appear to be aggregated and thus able to affect their SERCA pumps (Takemiya and Yamagata, 2013).

One may ask why this phase with reduced cytosolic Ca^{2+} have not been appreciated before? Firstly, the experimental focus has been on cell models to generate inclusion or pathology that resembles Lewy bodies e.g., phosphorylation of Ser129 on cellular α-synuclein or insoluble α-synuclein species. These phenomena likely require a significant build-up of α-synuclein aggregates and may occur in the late degenerative stage with increased cytosolic Ca^{2+} and enhanced cell death. Secondly, methodologically it is not trivial to demonstrate a 20% decrease in basal cytosolic Ca^{2+} of 150 nM if it is not the aim of the experiment. The genetically encoded Ca^{2+} sensors, like GCAMP6, are designed to demonstrate increases in Ca^{2+} as readouts for neuronal activity. To obtain quantitative measurement of reductions in cytosolic Ca^{2+}, we used the ratiometric Ca^{2+}-sensor Fura-2 that is exited at 340 and 380 nm and has its emission quantified at 510 nm, which upon calibration allows quantitative measurements. In our study, we chose a region of interest to measure the Fura-2 signal in the cell body located outside the nucleus that may reflect neurons average cytosolic Ca^{2+} in the somatodendritic compartment.

Conclusively, when neurons and other cells, like oligodendrocytes in MSA, experience a progressive build-up of α-synuclein aggregation we propose the existence of an early phase characterized by a decreased cytosolic Ca^{2+} and a degree of Ca^{2+} overload in their ER. This phase is not characterized by degenerative death promoting processes, but slowly developing dysfunctions in critical Ca^{2+} dependent processes that compromise the cells ability to contribute to its normal circuitries and thereby brain functions. We propose a neuron-centric mechanisms where Ca^{2+} dysfunctions initiates in neurons that contain the α-synuclein aggregate but one can speculate if it also affect the tripartite synapse coupling neuron and glia (Eroglu and Barres, 2010). The so-called tripartite synapse consists of pre- and postsynaptic neuronal structures along with the astrocyte processes that connects hereto. The astrocytes contribute to the neuron-neuron signaling by releasing glial transmitters that bind to receptors on both pre- and postsynaptic neuronal structures (Eroglu and Barres, 2010). The SERCA activated dysfunctions in neurons may affect both the release of transmitters targeting astrocytes and the receptors that respond to the glial transmitters. Insight into these processes may allow direct targeting the abnormally activated SERCA pump or its downstream signaling pathways. This reorientation of focus from what kills the cells to what make them dysfunctional, may allow us to relieve symptoms caused by dysfunction of neurons and/or glia. Nerve cells often function as parts of closely regulated circuits that by support from glia cells contribute to brain functions and dysfunctions herein cause symptoms. A focus on alleviating cellular dysfunctions may open for more points of therapeutic intervention.

EVIDENCE OF NEURONAL CALCIUM DISTURBANCES IN PD

The Ca^{2+} hypothesis that originally was associated to aging (Khachaturian, 1984) and later AD (Berridge, 2010) has also rationally been adopted in the PD field. First, it was demonstrated that dopaminergic neurons in the substantia nigra pars compacta display autonomous pace-making firing and this combined with their extremely large terminal fields, results in a large influx of Ca^{2+} via L-type voltage-gated Ca^{2+} channels (CaV) (Chan et al., 2007). Maintaining low normal Ca^{2+} levels by active transport processes requires large ATP production by oxidative phosphorylation and carries a risk of oxidative stress (Surmeier, 2007; Dryanovski et al., 2013; Surmeier et al., 2017). The CaV1.3 is largely responsible for the Ca^{2+} influx in pace-making neurons in substantia nigra and has been proposed to be involved in Ca^{2+} dysregulation since it does not fully close during autonomous firing (Wilson and Callaway, 2000; Bean, 2007; Puopolo et al., 2007; Surmeier and Schumacker, 2013).

Second, L-type Ca^{2+} channel blockers of the dihydropyridine type (DiCCBs) used to treat hypertension reduce the risk of developing PD and its associated mortality, but did not reduce the risk of dementia in PD patients (Becker et al., 2008; Ritz et al., 2010; Pasternak et al., 2012). The absent effect on cognition combined with a lack of association between length of previous use of Ca^{2+} channel blockers and its preventive effect, which made the authors suggest "any clinical effects of DiCCBs may be associated with symptomatic relief (preventing the development of clinical symptoms of early disease) rather than having a long-term impact on neurodegeneration" (Pasternak et al., 2012). However, changes to CaV channel expression has been reported

in PD brains suggesting where a dysregulation was demonstrated by immunohistochemistry in early and late stage PD brains (Hurley et al., 2013). The study demonstrated abnormalities in CaV subtype expression with a general increase preceding PD pathology along with a change in the ratio of CaV1.2 vs. CaV1.3 to favor a greater utilization of CaV1.3 channels (Hurley et al., 2013). The mechanism governing these changes, which may allow increased Ca^{2+} influx are unknown, but could in principle be a response to decreased cytosolic Ca^{2+} in neurons. Clinical trials testing the Ca^{2+}-hypothesis in PD are ongoing where the efficacy of CaV1.3 channel antagonist, Israpidine, is being evaluated [1]. However, even if the trials succeed in demonstrating symptomatic relief, they may not modify the progressive and widespread pathology that develops during the course of a PD patients life and which may be caused by prion-like properties of spreading α-synuclein aggregates (Braak et al., 2003).

IS INCREASED CYTOSOLIC CALCIUM ALWAYS BAD OR CAN IT EVEN BE NEUROPROTECTIVE?

We demonstrated that development of α-synuclein aggregates, by activating a Ca^{2+} pump, causes a decrease in cytosolic Ca^{2+}. This demonstrates that not only increased Ca^{2+}, as posited by the Ca^{2+}-hypothesis, but also low Ca^{2+} can have adverse effects (Betzer et al., 2018). Considering the Ca^{2+}-hypothesis, one may ask if increased cytosolic Ca^{2+} always is cytotoxic? Early studies focusing on developmental aspects of primary cultures of peripheral neurons demonstrated increasing intracellular Ca^{2+} levels by chronic depolarization was cytoprotective (Scott and Fisher, 1970; Franklin et al., 1995). This could also be demonstrated *in vivo* where activation of spiral ganglion neurons promoted their survival in the inner ear, which was prevented by blocking Ca^{2+} influx through L-type Ca^{2+} channels by Verapamil (Miller et al., 2003). Treatment with Verapamil was among the CaV channel antagonists that reduced the risk of developing PD symptomatology (Pasternak et al., 2012). A different neuroprotective mechanism has been demonstrated relying on synaptic stimulation of NMDA receptors (NMDAR) (Zhang et al., 2007). The NMDAR stimulation on dendritic spines, but not on extrasynaptic sites, triggers a signaling cascade that causes Ca^{2+} transients in the nucleus. The increase in nuclear Ca^{2+} stimulates transcription of specific genes, among some designated neuronal shield genes that contribute to enhanced neuronal survival and differentiation (Mao et al., 1999; Hardingham et al., 2002; Lee et al., 2005; Papadia et al., 2005; Bok et al., 2007; Zhang et al., 2007; Hardingham, 2009; Bading, 2013). In primary cultures of hippocampal neurons from α-synuclein transgenic mice, we measured the decreased cytosolic Ca^{2+} in the somatodendritic compartment just outside the nucleus (Betzer et al., 2018). Considering the fenestrated nature of the nuclear membrane this will likely result in a decreased nuclear Ca^{2+} that may blunt the protective Ca^{2+} transient elicited by synaptic NMDAR signaling.

[1] https://www.clinicaltrials.gov/

CALCIUM OVERLOAD OF THE ENDOPLASMIC RETICULUM

When α-synuclein aggregates activate SERCA, the transport of Ca^{2+} into the lumen of the ER is increased (Betzer et al., 2018). However, it is unclear how much this increases the Ca^{2+} load and the consequences hereof. Still it is becoming increasingly evident that the level of Ca^{2+} load in the ER plays fundamental roles for a range of important cellular processes. These processes comprise the unfolded protein response, the critical Ca^{2+} filling of mitochondria needed for oxidative phosphorylation, store-operate Ca^{2+} entry (SOCE) of Ca^{2+} across the plasma membrane into the ER (Carreras-Sureda et al., 2018) and the recently described regulation of presynaptic neurotransmitter release probability by axonal and presynaptic ER Ca^{2+} filling (de Juan-Sanz et al., 2017), and specialized overload channels like TMCO1 has been identified that counteracts ER overload (Wang et al., 2016). The significance in these pathways in neurons encountering a gradual build-up of α-synuclein aggregates needs further investigations.

CALCIUM DYSREGULATION AND MITOCHONDRIA

ER represents the major Ca^{2+} storage organelle but other organelles also play important roles in cellular Ca^{2+} homeostasis including mitochondria, Golgi apparatus, secretory vesicles, lysosomes, and peroxisomes (Rodriguez et al., 1997; Pinton et al., 1998; Mitchell et al., 2001; Saris and Carafoli, 2005; Rizzuto and Pozzan, 2006; Drago et al., 2008; Lasorsa et al., 2008). This suggests that α-synuclein aggregate-dependent stimulation of SERCA may affects the functionality of these organelles by the increased ER Ca^{2+}-load and reduced cytosolic Ca^{2+} level.

Mitochondria has for long been considered a key player in PD pathogenesis as evidenced by their decreased complex 1 activity in PD patients (Schapira et al., 1989), the induction of a PD phenotype by the mitochondrial toxin MPTP (Langston et al., 1983), and the identification of mitochondria related PARK2 and PARK4 genes causing autosomal recessive PD (Kitada et al., 1998; Valente et al., 2004). Ca^{2+} influx into mitochondria is carried by diffusion through the voltage-dependent anion channel 1 (VDAC) in the outer mitochondrial membrane and through the mitochondrial Ca^{2+} uniporter (MCU) in the inner mitochondrial membrane (Bathori et al., 2006; Tan and Colombini, 2007). The efficiency of this process rely on the positioning of mitochondria in the proximity of the Ca^{2+}-rich ER by means of mitochondrion-associated membrane (MAM) domains (Rizzuto et al., 2009). An estimate of up to 20% of the mitochondrial surface is in close proximity with the ER (Rizzuto et al., 1998) with a distance of 9–30 nm (Goetz and Nabi, 2006; Csordás et al., 2006). This correspond to the distance of protein complexes tethering the organellar membranes (Perkins et al., 1997). A tethering complex is formed between ER Ca^{2+}-channel, inositol 1, 4, 5-trisphosphate receptor (IP3R) and VDAC1 in the mitochondria by the action of the chaperone GRP75 and this facilitates the flux of Ca^{2+} from ER into mitochondria.

Mitochondria are rich in the lipid cardiolipin that facilitates their α-synuclein binding (Nakamura et al., 2011), and MAM is rich in negatively charged phospholipids (Hayashi and Fujimoto, 2010) that also facilitates binding of α-synuclein (Davidson et al., 1998). The membrane binding ability of α-synuclein and localization to MAM is abolished in α-synuclein carrying the familial PD mutation A30P (Jensen et al., 1998; Guardia-Laguarta et al., 2014) and this is associated with decreased MAM activity and mitochondrial-ER interface (Guardia-Laguarta et al., 2014). This suggests a physiological function of native α-synuclein in facilitating optimal ER-mitochondrial interactions. Aberrant forms of α-synuclein have been demonstrated to affects mitochondrial function by disrupting the Ca^{2+} traffic at ER-mitochondrial interphases (Cali et al., 2012; Guardia-Laguarta et al., 2014; Raffaello et al., 2016) and to increase ER and mitochondrial stress (Smith et al., 2005; Colla et al., 2012; Melachroinou et al., 2013). This may be caused by direct interactions between aberrant α-synuclein forms and VDAC1 (McFarland et al., 2008; Martin et al., 2014; Betzer et al., 2015). However, the significance of these findings and if they only are active in certain species, cellular states and domains has been stressed by recent studies. Mouse neurons lacking α-synuclein alone and all three synuclein forms did not demonstrate significant changes to mitochondrial bioenergetics (Pathak et al., 2017) whereas human iPSC derived neurons expressing mutant α-synuclein displayed mitochondrial dysfunctions (Ryan et al., 2018). Hence, understanding the significance of α-synuclein species interactions with mitochondria and MAM and their relation to the Ca^{2+} homeostasis of the involved organelles needs further investigations.

α-SYNUCLEIN AND AXONAL DYSFUNCTIONS IN PD.

α-synuclein is predominantly a presynaptic protein and its intraneuronal aggregation is likely initiated at this site (Kramer and Schulz-Schaeffer, 2007), which make the axonal transport regulating its anterograde and retrograde transport of key importance. How α-synuclein aggregate dependent SERCA activation affects these processes are currently unknown, but the fundamental role of the Ca^{2+} content in the axonal ER makes α-synuclein aggregation in this compartment especially critical (de Juan-Sanz et al., 2017). Moreover, α-synuclein holds potential of affecting other axonal functions in PD. Axonal pathology is recognized in PD as swellings crowded with organelles shown by electron microscopic studies suggestive of compromised axonal flux (Vital et al., 2014). These finding are corroborated by kinetic analyses of axonally transported biomarkers in cerebrospinal fluids from PD patients (Fanara et al., 2012). The axonal accumulation of aggregated α-synuclein in small, larger granules, and extended inclusions is the defining characteristic of Lewy neurites (Braak et al., 1999) that also can contain other axonally transported cargo like β-synuclein (Galvin et al., 1999) and such abnormalities have also been modeled in α-synuclein transgenic mice (Games et al., 2013). The functional consequences of axonal α-synuclein aggregates are not clear, but transgenic overexpression in neurons demonstrate dysfunctions in vesicle transport, e.g., of Rab7 containing endosomes (Volpicelli-Daley et al., 2014; Koch et al., 2015). α-synuclein containing axonal structures associate to both anterograde kinesin-labeled and retrograde dynein-labeled structures in neurons (Utton et al., 2005) and early reductions in kinesin levels preceded reductions reduction of tyrosine hydroxylase in the dopamine neurons (Chu et al., 2012). Hypothesizing α-synuclein aggregation predominantly initiates in the nerve terminals makes retrograde α-synuclein transport processes of pivotal importance for the clearance (Jensen et al., 1999; Bieri et al., 2018). The retrograde transport is generally considered as an active transport of endosomes and autophagosomes pulled along the microtubules by the dynein motor complex and the process has achieved increased interest because of its involvement in transport of signaling complexes from nerve terminals (Zahavi et al., 2017). α-Synuclein aggregates are able to directly target dynein complex components (Betzer et al., 2015) that may compromise retrograde transport and represent a basis for the accumulation of aggregated α-synuclein in Lewy neurites. However, α-synuclein may also affect other axonal mechanisms by e.g., directly affecting microtubule structures (Alim et al., 2004; Toba et al., 2017), and ER, and mitochondria, which both are organelles present in axons and nerve terminals (Gonzalez and Couve, 2014; Smith and Gallo, 2018). In PD, α-synuclein aggregates accumulate directly on ER (Colla et al., 2012) where they can activate SERCA (Betzer et al., 2018), but they can also bind the ER protein, VAPB (Betzer et al., 2015) thereby disrupting the ER-mitochondria tethering and affecting ATP production (Paillusson et al., 2017). Mechanistic studies that will allow a prioritization of these many interactions and mechanisms are in high demand to allow identification of potential disease modifying targets.

ALZHEIMER DISEASE AND CALCIUM OVERLOAD OF THE ENDOPLASMIC RETICULUM

The Ca^{2+}-hypothesis has been widely embraced in the AD field where the moderate age-related Ca^{2+} remodeling is hypothesized to be pushed into severe Ca^{2+} signal remodeling by components of the amyloidogenic pathway central to AD (Berridge, 2010). Presenilins has turned out as central players in this model. Presenilins 1 and 2 are ER transmembrane proteins that regulate production of amyloidogenic Aβ peptides and causes familial AD in families when mutated (Tolia and De Strooper, 2009). Presenilins are, like aggregated α-synuclein, activators of SERCA pumps (Green et al., 2008). This activity, which tend to increase ER Ca^{2+} levels, is balanced by the presenilins inherent function as low conductance Ca^{2+}-leak channels that allows the Ca^{2+} ions to flow back into the cytosol (Tu et al., 2006). AD causing mutations in presenilin 1 blocks this Ca^{2+}-leak activity thereby causing ER Ca^{2+}-overload with detrimental effects on ER-dependent signaling affecting store operated Ca^{2+} entry (SOCE), expression of ryanodine receptors (RyR), and inositol trisphosphate receptors (IP3R) (Blalock et al., 2003; Emilsson et al., 2006; Bezprozvanny, 2009;

Popugaeva and Bezprozvanny, 2014). This bears resemblance to the situation that we have described for α-synuclein aggregates. Their activation of SERCA will also tend to increase ER Ca^{2+}-load and may therefore share some of these neurobiological effects. It will be motivated in α-synuclein-aggregate dependent models to directly investigate if RyR. IP3R and SOCE functions are affected. SOCE has recently been demomstrated to play a central role in regulating the release probability in nerve terminals that are rich in α-synuclein (de Juan-Sanz et al., 2017).

HYPOTHESIS FOR NEURONAL DYSFUNCTIONS GENERATED BY α-SYNUCLEIN AGGREGATES STIMULATION OF SERCA

Aggregation of α-synuclein in neurons is a progressive process sculpted by the local concentration of α-synuclein, presence of prion-like α-synuclein seeds, and proteostatic balances. When early soluble aggregated α-synuclein species are formed, they will bind to and activate the SERCA pump in the ER. Blocking the α-synuclein aggregate dependent activation by SERCA antagonists protected the affected neurons, thus demonstrating the existence of pivotal downstream signaling pathways. The ER is a dynamic organelle that permeates neurons from nerve terminals through axons, cell bodies into dendritic spines (Toresson and Grant, 2005) and this opens for several independent dysfunctions separated in space and time that contribute to the neurodegenerative process (**Figure 1**). Further insight into the contribution of SERCA-activated Ca^{2+} dysfunctions will open for novel mechanisms to counter the dysfunction associated to PD and other synucleinopathies.

AUTHOR CONTRIBUTIONS

CB and PJ designed and wrote the manuscript.

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Could α-Synuclein Modulation of Insulin and Dopamine Identify a Novel Link Between Parkinson's Disease and Diabetes as Well as Potential Therapies?

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Characterizing the normal function(s) of the protein α-Synuclein (aSyn) has the potential to illuminate links between Parkinson's disease (PD) and diabetes and also point the way toward new therapies for these disorders. Here we provide a perspective for consideration based on our discovery that aSyn normally acts to inhibit insulin secretion from pancreatic β-cells by interacting with the Kir6.2 subunit of the ATP-sensitive potassium channel (K-ATP). It is also known that K-ATP channels act to inhibit brain dopamine secretion, and we have also shown that aSyn is a normal inhibitor of dopamine synthesis. The finding, that aSyn modulates Kir6.2 and other proteins involved in dopamine and insulin secretion, suggests that aSyn interacting proteins may be negatively impacted when aSyn aggregates inside cells, whether in brain or pancreas. Furthermore, identifying therapies for PD that can counteract dysfunction found in diabetes, would be highly beneficial. One such compound may be the multiple sclerosis drug, FTY720, which like aSyn can stimulate the activity of the catalytic subunit of protein phosphatase 2A (PP2Ac) as well as insulin secretion. In aging aSyn transgenic mice given long term oral FTY720, the mice had reduced aSyn pathology and increased levels of the protective molecule, brain derived neurotrophic factor (BDNF) (Vidal-Martinez et al., 2016). In collaboration with medicinal chemists, we made two non-immunosuppressive FTY720s that also enhance PP2Ac activity, and BDNF expression (Vargas-Medrano et al., 2014; Enoru et al., 2016; Segura-Ulate et al., 2017a). FTY720 and our novel FTY720-based-derivatives, may thus have therapeutic potential for both diabetes and PD.

Keywords: alpha-synuclein, dopamine, insulin, Kir6.2, LAG3, Parkinson's disease, type 2 diabetes

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We and others have shown that aSyn protein has important normal functions that are associated with its ability to interact with other molecules in a chaperone-like manner (Jenco et al., 1998; Jensen et al., 1999; Ostrerova et al., 1999; Jo et al., 2000; Murphy et al., 2000; Souza et al., 2000; Hashimoto et al., 2002; Seo et al., 2002; Kim et al., 2004; Acosta-Martinez and Levine, 2007; Martinez et al., 2007; Klegeris et al., 2008; Gorbatyuk et al., 2010b; Aoki and Li, 2011; Jin et al., 2011; Oaks and Sidhu, 2011; Bartels et al., 2014; Lautenschläger et al., 2018). Over the years, our laboratory

has identified several aSyn-interacting proteins and organelles. These include: tyrosine hydroxylase, also called tyrosine 3-monooxygenase (EC 1.14.16.2) (TH), the rate limiting dopamine biosynthetic enzyme that localizes on vesicles and mitochondria with aSyn (Perez et al., 2002; Jin et al., 2007; Alerte et al., 2008); the next enzyme in the dopamine biosynthetic pathway, aromatic amino acid decarboxylase, AADC, also called dopa decarboxylase (Tehranian et al., 2006); the catalytic subunit of protein phosphatase 2A (PP2Ac) (Peng et al., 2005; Lou et al., 2010); and the 14-3-3ζ adapter protein, which also localize to mitochondria to help regulate dopamine synthesis at that organelle (Wang et al., 2009).

With normal aSyn function(s) in mind and knowing that aSyn normally interacts with and regulates many other molecules, we long ago hypothesized that a loss of aSyn function could be especially detrimental to dopaminergic neurons in a manner to contribute to nigral PD pathology (Perez and Hastings, 2004; Porras and Perez, 2014). We have tested this hypothesis in multiple models over the years. These include using aSyn lentivirus in mice, brains from familial PD and Dementia with Lewy Bodies (DLB) subjects, cell free assays, and aSyn transgenic mice where we confirmed that TH and PP2A activities become dysregulated when aSyn aggregates (Alerte et al., 2008; Wu et al., 2012; Farrell et al., 2014). This demonstrates an important normal role for soluble aSyn in the regulation of key aSyn-interacting molecules. Others have also shown that sustaining normal aSyn levels contributes significantly to neuronal viability, further solidifying a major role for soluble aSyn in optimal brain health (Gorbatyuk et al., 2010a; Kanaan and Manfredsson, 2012; Benskey et al., 2016; Collier et al., 2016).

A lesser known function of aSyn is our discovery that the protein is highly expressed in pancreatic beta cells where it interacts with Kir6.2 on insulin secretory granules, acting to downregulate insulin secretion (Geng et al., 2011). In data from co-immunoprecipitation experiments we show that aSyn and Kir6.2 interact with each other in the pancreas and in islet cell cultures, as can be appreciated in **Figure 1**. The methods used for these experiments are detailed in our figure legend. In this same paper, striking immunohistochemical images generated by Drs. Geng and Drain confirm near perfect overlapping localization of aSyn not only with Kir6.2, but also with Sur1, Insulin, and C peptide in beta cells (Geng et al., 2011).

The aSyn/Kir6.2 interaction becomes more intriguing because in brain, neuronal Kir6.2 is found in axons and dendrites (Patel et al., 2011; Trimmer, 2015) where it plays an active role in the downregulation of dopamine secretion (Avshalumov and Rice, 2003; Bao et al., 2005; Shi et al., 2008; Patel et al., 2011; Trimmer, 2015). It remains unknown if Kir6.2 and aSyn interact and colocalize on neurotransmitter secretory vesicles in a manner to downregulate dopamine secretion similar to its effects on Kir6.2 in insulin secretory granules. Still, this possibility and other cumulative findings lead us to propose that a loss of aSyn/Kir6.2 interactions that may occur when aSyn aggregates could produce over-secretion of insulin and dopamine, although this remains largely unexplored. This possibility has further implications because there are multiple emerging lines of evidence supporting links between type 2 diabetes mellitus (T2DM) and PD comorbidity (Hu et al., 2007; Driver et al., 2008; Cereda et al., 2011, 2013; Palacios et al., 2011; Schernhammer et al., 2011; Kotagal et al., 2012; Aviles-Olmos et al., 2013; Santiago and Potashkin, 2013; Marcelo et al., 2014; Zhang and Tian, 2014; Santiago et al., 2017; Foltynie et al., 2018), as has been recently been confirmed (De Pablo-Fernandez et al., 2018).

In this regard, protein misfolding and insulin resistance are common to both T2DM and PD (Athauda and Foltynie, 2016). In diabetes, this protein misfolding implicates the islet amyloid polypeptide protein (IAPP, also known as amylin), which is a short peptide that is packaged and secreted along with insulin from pancreatic beta cells (Moore and Cooper, 1991). IAPP/amylin plays a role in glycemic regulation and is known to adopt abnormal conformations that can permeabilize synthetic vesicles in a pore-like manner akin to findings for aSyn protein (Anguiano et al., 2002). This has led some to propose that IAPP/amylin oligomers may act in a prion-like manner in the pancreatic islet cells of diabetics to spur disease onset and/or progression, as some data tend to support (Mukherjee et al., 2015, 2017). In addition, cross-seeding of aSyn and IAPP/amylin has been shown to accelerate the aggregation of both of these aggregation prone proteins (Horvath and Wittung-Stafshede, 2016), raising the possibility that aSyn may accumulate among the amyloids in pancreatic beta cells. This was recently confirmed in pancreatic tissues from subjects with synucleinopathies (Martinez-Valbuena et al., 2018). In addition, there is evidence that in nigral dopamine neurons of individuals with idiopathic/sporadic PD, there is a dysregulation of miR-126, a microRNA involved in the regulation of insulin/IGF-1/phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) signaling (Kim et al., 2014; Briggs et al., 2015). Further, it is well-appreciated that insulin signaling contributes significantly to normal brain function and becomes dysregulated in neurodegeneration (Bomfim et al., 2012; Bamji-Mirza et al., 2014; Gao et al., 2015). Together these findings provide strong support for an association between T2DM and PD in which aSyn may play a pivotal role.

It is well-established that aSyn misfolding contributes to PD as well as to other synucleinopathies, such as DLB and multiple system atrophy (MSA) (Galvin et al., 2001; Goedert, 2001; Trojanowski and Lee, 2003). It has further been shown that aSyn oligomerization, to form preformed fibrils (PFF), can induce a prion-like spread of aSyn and cell death in PD models (Volpicelli-Daley et al., 2011, 2014; Dryanovski et al., 2013; Polinski et al., 2018). Also, aSyn PFF uptake *in vitro* and *in vivo* is modulated specifically by the LAG3 receptor, which has been shown to contribute to pathological aSyn transmission (Mao et al., 2016). Moreover, LAG3 has also been implicated in autoimmune diabetes (Bettini et al., 2011; Zhang et al., 2017), providing further evidence for potential overlap between diabetes and PD.

It is also becoming accepted that aSyn plays a role in inducing innate and adaptive immunity in PD (Allen Reish and Standaert, 2015), arising, at least in part, by aSyn activating microglial

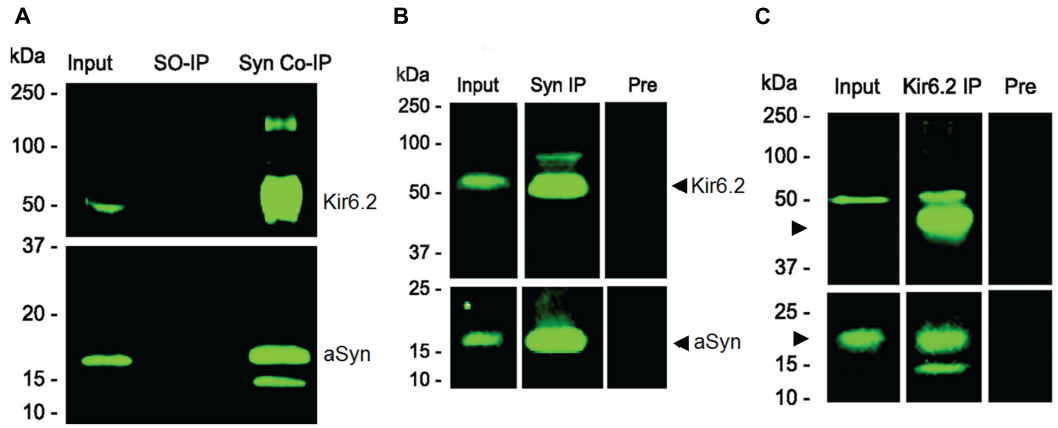

FIGURE 1 | Co-immunoprecipitation (Co-IP) of Kir6.2 with aSyn from pancreas **(A)** and from pancreatic islet cell cultures **(B,C)**. **(A)** Anti-aSyn antibody (BD Biosciences, Cat # BD610787) was used to immunoprecipitate aSyn protein from mouse pancreatic tissue extracts. Immunocomplexes are characterized on immunoblots using anti-Kir6.2 (Santa Cruz Biotechnology, Cat # sc-11228) and anti-aSyn (Santa Cruz Biotechnology, Cat # sc-7011-R) antibodies. Equivalent aliquots of the initial input of each extract (Input) were analyzed. Homogenates in which secondary antibody only was used (SO-IP) served as a negative control. Both aSyn and Kir6.2 were co-immunoprecipitated with the anti-aSyn antibody (Syn Co-IP). **(B)** Binding interactions between Kir6.2 and aSyn are also seen in a representative Co-IP experiment using mouse islet cells grown in culture. Immunoblots were reacted with anti-aSyn antibody (Santa Cruz Biotechnology, Cat # sc-7011-R; in **B** and **C**, bottom panel) or anti-Kir6.2 antibody (Santa Cruz Biotechnology, Cat # sc-20809, H55; in **B** and **C**, top panel). Both Kir6.2 and aSyn are present on immunoblots in initial homogenates (Input), and were enriched after anti-aSyn antibody Co-IP (Syn-1, BD 610787; Syn IP). Specificity was confirmed using pre-adsorbed Syn-1 antibody (Pre), which efficiently reduced levels of protein that were co-immunoprecipitated. (**C**) Binding interactions between Kir6.2 and aSyn in a representative experiment using mouse islet cells also show the presence of Kir6.2 and aSyn in the initial homogenate (Input) as well as in the Co-IP performed using the anti-Kir6.2 antibody (Santa Cruz Biotechnology, Cat # sc-20809, H55; Kir6.2 IP), with specificity demonstrated in a Co-IP using pre-immune serum + beads (Pre). Molecular weights, determined from pre-stained standards, are shown on the left. Data from Geng et al. (2011) reprinted with permission obtained from the Copyright Clearance Center.

cells, which stimulates neuroimmunity (Sanchez-Guajardo et al., 2013). A role for aSyn in metabolism has also been reported in the Thy1 promoter parkinsonian A53T mice, where aSyn pathology was found to drive metabolic abnormalities in that PD model (Rothman et al., 2014). Inflammation and activated innate immunity have been shown to play a role in the pathogenesis of T2DM (Pickup, 2004) and inflammation is known to be common in diabetes and other metabolic disorders (Hotamisligil et al., 1993; Zhong et al., 2017). Based on these findings, it thus would be prudent to evaluate parkinsonian mouse models for potential overlapping pathology related to PD and T2DM.

First described by James Parkinson in the early 1800s, it is remarkable to find that in his initial description of the disorder that was later named after him, he was among the first to suggest that the "shaking palsy" may be caused by "compression of the brain, or dependent on *partial exhaustion of the energy of that organ*" (Parkinson, 2002). This suggests that Parkinson himself had anticipated a potential role for metabolic dysregulation in brain as contributing to the disease pathology. Yet, even 200 years later the scientific community continues to search to identify the cause for PD and for successful therapies that will counteract PD pathology.

In our search to identify protective therapies for PD, we began studying FTY720 (fingolimod, Gilenya), a Food and Drug Administration approved therapy for the demyelinating brain disorder, multiple sclerosis (Brinkmann et al., 2010). We first evaluated FTY720 based on its ability to stimulate PP2A activity (Oaks et al., 2013; Vargas-Medrano et al., 2014). This is because our research had revealed that aSyn is a normal stimulator of PP2A catalytic subunit activity (Peng et al., 2005), and that PP2A activity is significantly diminished *in vivo* if aSyn becomes insoluble and accumulates in Lewy bodies (Wu et al., 2012; Farrell et al., 2014). Later, others showed that FTY720 stimulates the expression of the protective molecule BDNF *in vitro* and *in vivo* (Deogracias et al., 2012). Thus, we began testing FTY720 in aging parkinsonian aSyn A53T transgenic mice and found that the mice not only tolerate long term FTY720 treatment, but also have behavioral improvement, increased BDNF expression, and reduced Lewy body-like aSyn pathology when compared to transgenic littermates treated with a vehicle control solution (Vidal-Martinez et al., 2016). In control experiments Vidal-Martinez et al. (2016) also show that blocking BDNF signaling accelerates aSyn aggregation that is reversed by co-delivering FTY720 with the TrkB blocker, ANA-12. Moreover, in addition to being able to improve both glial and neuronal cell functions (Balatoni et al., 2007; Miron et al., 2008; Kim et al., 2011; Gao et al., 2012; Vargas-Medrano et al., 2014; Cipriani et al., 2015; Segura-Ulate et al., 2017b), FTY720 has been shown to have potent anti-diabetic activity including an ability to stimulate insulin secretion (Fu et al., 2001; Yang et al., 2003; Kendall and Hupfeld, 2008; Zhao et al., 2012; Moon et al., 2013). Remarkably, insulin itself can stimulate dopamine release (Stouffer et al., 2015; Sulzer et al., 2016), confirming

related effects on insulin and dopamine in brain and pancreas that are highly relevant to PD and T2DM. In addition, there is compelling evidence that dopamine itself is produced within beta cells of the human pancreas, where it becomes packaged along with insulin and acts to negatively regulate insulin secretion (Simpson et al., 2012). Future studies will be required to determine if aSyn binding to Kir6.2 occurs in brain to modulate dopamine similarly to its effects on insulin release. Additional studies to assess potential benefits of our novel FTY720-derivative compounds in pancreatic beta cells and neurons are also required. Cumulatively, the findings concerning the comorbidity of diabetes with PD, and the overlapping interactions between aSyn and key regulatory molecules in brain and pancreas open the door to further explore potential novel therapies that may benefit both disorders that affect a large percentage of our rapidly aging population, worldwide.

AUTHOR CONTRIBUTIONS

All authors contributed to literature searches, reading, writing, and/or editing of this manuscript. The review was conceived of by RP who also obtained permission to reprint data via Copyright Clearance Center for **Figure 1**. BY and JV-M provided intellectual content at all stages. After review, recommendations led us to seek the expertise of GV-M to further improve writing and content.

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Conflict of Interest Statement: RP has filed a patent for FTY720 derivative compounds. Compositions and Methods for the Treatment of Parkinson's Disease. Publication# 2015/0290145.

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frontiers in Neuroscience

BRIEF RESEARCH REPORT
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Analysis of α-Synuclein Pathology in PINK1 Knockout Rat Brains

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Mutations in PTEN induced kinase 1 (PINK1) cause autosomal recessive Parkinson's disease (PD). The main pathological hallmarks of PD are loss of dopaminergic neurons in the substantia nigra pars compacta and the formation of protein aggregates containing α-synuclein. Previous studies of PINK1 knockout (PINK1−/−) rats have reported mitochondrial dysfunction, locomotor behavioral deficits, loss of neurons in the substantia nigra and α-synuclein aggregates in various brain regions. We sought to characterize PINK1−/− rats in more detail specifically with respect to α-synuclein pathology because abnormal α-synuclein has been implicated genetically, biophysically and neuropathologically as a mechanism of PD pathogenesis. Moreover, the spontaneous formation of α-synuclein aggregates without α-synuclein overexpression, injection or toxin administration is a rare and important characteristic for an animal model of PD or other synucleinopathies, such as dementia with Lewy bodies and multiple system atrophy. We observed α-synuclein-immunoreactive aggregates in various brain regions of PINK1−/− rats including cortex, thalamus, striatum and ventral midbrain, but nowhere in wild-type (WT) rats. Co-immunofluorescence showed that the α-synuclein-immunoreactive aggregates are both thioflavin S and ubiquitin positive. Many cells in the brains of PINK1−/− rats but not WT rats contained protease-resistant α-synuclein. Total synuclein protein levels were unchanged; however, biochemical fractionation showed a significant shift of α-synuclein from the cytosolic fraction to the synaptic vesicle-enriched fraction of PINK1−/− brain homogenates compared to WT. This data indicates that PINK1 deficiency results in abnormal α-synuclein localization, protease resistance and aggregation *in vivo*. The PINK1−/− rat could be a useful animal model to study the role of abnormal α-synuclein in PD-related neurodegeneration.

Keywords: Parkinson's disease, synuclein, PINK1, Lewy bodies, thioflavin, ubiquitin, inclusions, aggregation

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INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Clinically, PD is defined by slowness of movement, rigidity, postural instability, gait abnormalities and tremor. Neuropathologically, PD is predominantly characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and by the presence of intracellular inclusions, termed Lewy bodies, which are composed mainly of α-synuclein (Spillantini et al., 1998). Point mutations in α-synuclein were the first identified genetic mutations causally linked to PD

(Polymeropoulos et al., 1997; Kruger et al., 1998). The subsequent identification of PD-linked α-synuclein gene duplication and triplication mutations indicates that increased expression of WT α-synuclein (which could promote aggregation by increasing α-synuclein protein concentration) is sufficient to cause PD (Singleton et al., 2003). Despite intensive research, the exact role of α-synuclein aggregation in PD remains unclear (von Bohlen Und Halbach, 2004). In an effort to generate a better animal model of PD and a tool for studying potential mechanisms of disease, the Michael J. Fox Foundation for Parkinson's Research sponsored the generation and initial characterization of PINK1−/− rats as a model of loss-of-function PINK1 mutations causally linked to recessively inherited PD (Valente et al., 2004; Dave et al., 2014). The initial study reported significant motor deficits and age-dependent loss of dopamine neurons in the substantia nigra of PINK1−/− rats (Dave et al., 2014). Subsequent studies of the same line of PINK1−/− rats reported mitochondrial dysfunction, behavioral deficits, loss of neurons in the substantia nigra and locus coeruleus, neurochemical abnormalities and α-synuclein aggregates in various brain regions (Grant et al., 2015; Kelm-Nelson et al., 2015, 2016, 2018; Pultorak et al., 2016; Stauch et al., 2016a,b; Villeneuve et al., 2016a,b). We sought to further characterize PINK1−/− rats specifically with respect to α-synuclein pathology because spontaneous formation of α-synuclein aggregates (without α-synuclein overexpression or injection) is a rare and important feature of PD animal models and because α-synuclein aggregation has been implicated both genetically and biochemically as a mechanism of PD pathogenesis as well as a potential therapeutic target (Goldberg and Lansbury, 2000; Creed and Goldberg, 2018).

MATERIALS AND METHODS

Animals

PINK1−/− rats were obtained from Horizon Discovery and bred to obtain homozygous PINK1−/− and WT Long-Evans controls. Animals were maintained on a 12-h light/dark cycle and were allowed food and water *ad libitum*. This study was carried out in accordance with the recommendations of the NIH Guidelines for the Care and Use of Laboratory Animals. All animal experiments were reviewed and approved in advance by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Immunohistochemistry

Animals were euthanized with CO_2 and immediately perfused with phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Brains were removed and fixed in 10% formalin overnight at 4°C, then transferred to PBS+30% sucrose and maintained at 4°C 2–3 days for cryoprotection. Brains were frozen and sectioned in the coronal plane at 30 μm thickness using a sled microtome. Sections were collected in multi-well plates with each well containing a series of systematically spaced sections (every 10th section). Entire wells of free-floating sections spanning the disease-relevant regions (cortex, striatum, midbrain and thalamus) were blocked in 1% normal goat serum (NGS) in PBS for 1 h, then incubated in primary antibody (BD Biosciences anti-synuclein #610787 diluted 1:1,000) overnight at 4°C. Sections were washed in PBS and incubated with biotinylated goat anti-mouse secondary antibody for 2 h at room temperature, followed by avidin-biotin peroxidase complex solution (Vector Laboratories ABC Elite) for 2 h at room temperature. Sections were washed in PBS, then developed using DAB chromogen (Vector Laboratories). The location and abundance α-synuclein immunoreactive aggregates were scored by an investigator blinded to genotype and age.

Proteinase-K Resistant Immunohistochemistry

Immediately prior to DAB immunohistochemistry, free-floating coronal sections were washed with PBS and treated with 2 mg/ml proteinase K (Fisher Scientific 50-751-7334) for 10 min at room temperature. Sections were then washed thoroughly with PBS and analyzed by immunohistochemistry, as described above. Cells containing proteinase K-resistant α-synuclein immunoreactivity were counted by an investigator blinded to genotype using NIS Elements software. Five sections from each animal were analyzed and averaged.

Thioflavin S Staining

Free-floating coronal sections were mounted on glass slides, allowed to dry overnight, then washed with 70% ethanol followed by 80% ethanol for 1 min each. Slides were then incubated in 1% thioflavin in 80% ethanol for 15 min in the dark, followed by sequential 1-min washes in 80% ethanol and 70% ethanol. For α-synuclein co-immunofluorescence, slides were first treated as above, then incubated with α-synuclein primary antibody (BD Biosciences #610787 diluted 1:250) and analyzed as above.

Microscopy and Image Analysis

Fluorescence images were collected on a Leica TCS-SP5 laser scanning confocal microscope. Images of DAB stained brain sections were acquired on a Nikon Ni-E microscope and analyzed using NIS Elements software.

Western Analysis

Brains were harvested and microdissected immediately following euthanasia, then frozen on dry ice and stored at −80°C. Frozen brain tissue samples were thawed on ice and homogenized with a motorized pestle in RIPA buffer (Boston Bio-products, Bp-115) containing protease and phosphatase inhibitors (Sigma P8340). After incubation on ice for 30 min, homogenates were briefly sonicated, then centrifuged at 1,000 × *g* for 5 min to remove debris. Equal amounts of total protein (measured by Pierce Bradford assay) were mixed with Laemmli buffer, separated by SDS-PAGE and transferred onto 0.2 um PVDF membranes. Membranes were blocked in 1:1 LI-COR Odyssey blocking buffer and TBS with 0.05% Tween 20 (TBS-T) for 1 h at room temperature, then incubated with primary antibody overnight at 4°C, washed, then incubated with LI-COR Odyssey secondary antibodies for 2 h and imaged using a LI-COR Odyssey Scanner.

For biochemical fractionation to obtain cytosolic and synaptic vesicle-enriched fractions according to the methods described by Hallett et al. (2008), cortical tissues from WT and PINK1−/− rats were homogenized in TEVP buffer (10 mM Tris pH 7.5, 5 mM NaF, 1 mM each EDTA, EGTA and Na_3VO_4) containing 320 mM sucrose using a dounce homogenizer and centrifuged for 10 min at 800 × *g* at 4C. The supernatant was centrifuged for 15 min at 9,200 × *g* at 4°C. Following centrifugation, the supernatant was decanted into a clean eppendorf tube and stored on ice (S2). The pellet was then re-suspended in TEVP buffer containing 35.6 mM sucrose and vortexed gently to dislodge and break the pellet, followed by incubation on ice for 30 min. Vortexed samples were centrifuged for 20 min at 25,000 × *g* at 4°C, then the supernatants were transferred to clean centrifuge tubes (LS1). The supernatant, along with the S2 supernatants were centrifuged at 165,00 × *g* for 2 h at 4°C. The resulting supernatant (S3) was used as the cytosolic enriched fraction while the pellet (LP2) was used as the synaptic vesicle enriched fraction.

Statistical Analysis

Statistical analyses were conducted using Graphpad Prism 7 software. Unpaired Student's-*t* tests, two-way ANOVA and non-parametric tests, as indicated, were used to determine significance at the 0.05 level.

RESULTS

α-Synuclein Immunohistochemistry

Because α-synuclein immunoreactive protein aggregates are pathognomonic for PD, and because genetic, biochemical *in vitro* and *in vivo* studies suggest α-synuclein oligomerization or aggregation is linked to the underlying mechanisms of neurodegeneration in PD, we focused our analysis of PINK1−/− rats on characterizing the pattern and age-dependence of α-synuclein immunoreactivity throughout the brain. DAB immunohistochemistry of coronal sections

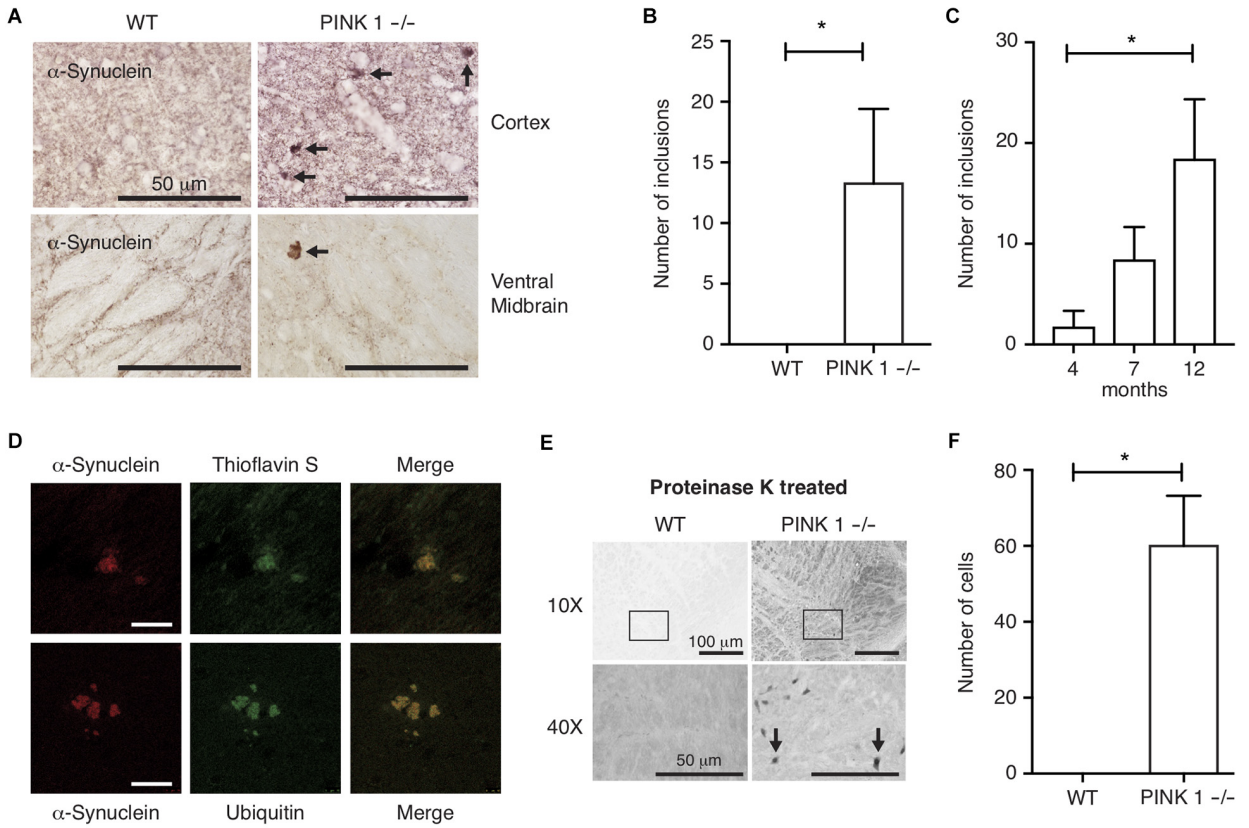

FIGURE 1 | α-synuclein aggregates in PINK1–/– rat brains. **(A)** DAB immunohistochemistry of α-synuclein in the cortex and ventral midbrain of 12-month-old rats. Arrows indicate synuclein-immunoreactive aggregates. **(B)** Mean ± SEM number of α-synuclein inclusions per section, including all brain regions, in (n = 17) WT and (n = 21) PINK1–/– rats. *P < 0.05 by Mann–Whitney U-test. Five systematically spaced sections (every 10th section) were analyzed and averaged for each animal. **(C)** Mean ± SEM number α-synuclein aggregates per section in brains of 4 months (n = 5), 7 months (n = 6), and 12 months (n = 10) PINK1–/– rats. No α-synuclein aggregates were observed in WT rat sections stained and analyzed in parallel (n = 5 at age 4 months, n = 5 at age 7 months, and n = 7 at age 12 months). *P < 0.05 by Tukey's Multiple Comparison test. **(D)** Co-immunofluorescence of α-synuclein and Thioflavin S or α-synuclein and ubiquitin in the midbrain of PINK1–/– rats at 12 months of age. Scale bar = 10 mm. **(E)** DAB α-synuclein immunohistochemistry in the ventral midbrain of 12-month-old WT and PINK1–/– rats following proteinase K treatment. Top panels are 10× magnification bottom panels are 40× magnification. Arrows in bottom panels indicate cells with proteinase K-resistant α-synuclein. **(F)** Mean ± SEM number of cells per section with proteinase K-resistant α-synuclein immunoreactivity in the midbrain of 3 WT and 3 PINK1–/– rats at age 12 months. Five sections were analyzed and averaged for each animal. *P < 0.05 by Mann–Whitney U-test.

using α-synuclein-specific antibodies showed a normal pattern of synaptic α-synuclein throughout the neuropil; however, sparse abnormal α-synuclein immunoreactive aggregates were observed throughout the brains of PINK1−/− but not WT rats (**Figures 1A,B**). Analysis of brain sections from rats at ages 4, 7 and 12 months showed the presence of abnormal α-synuclein immunoreactive aggregates in PINK1−/− rats at all ages, but significantly more aggregates were observed in the older animals (**Figure 1C**) $P < 0.05$ by Tukey's Multiple Comparison test. No α-synuclein aggregates were observed in WT rat brain sections at the same ages stained and analyzed in parallel with the PINK1−/− rat brains. To assess the composition of the α-synuclein immunoreactive aggregates in PINK1−/− rats, we conducted immunofluorescence using α-synuclein and ubiquitin-specific antibodies as well as thioflavin S as a well-established fluorescent stain for amyloid. α-Synuclein immunoreactive aggregates showed thioflavin S and ubiquitin staining consistent with amyloid protein aggregates indicative of neurodegenerative disease (**Figure 1D**).

Proteinase-K Resistant α-Synuclein in PINK1−/− Rats

α-Synuclein is one of the most abundantly expressed proteins in brain and because α-synuclein is prone to aggregation, we sought to assess the extent to which cells could contain

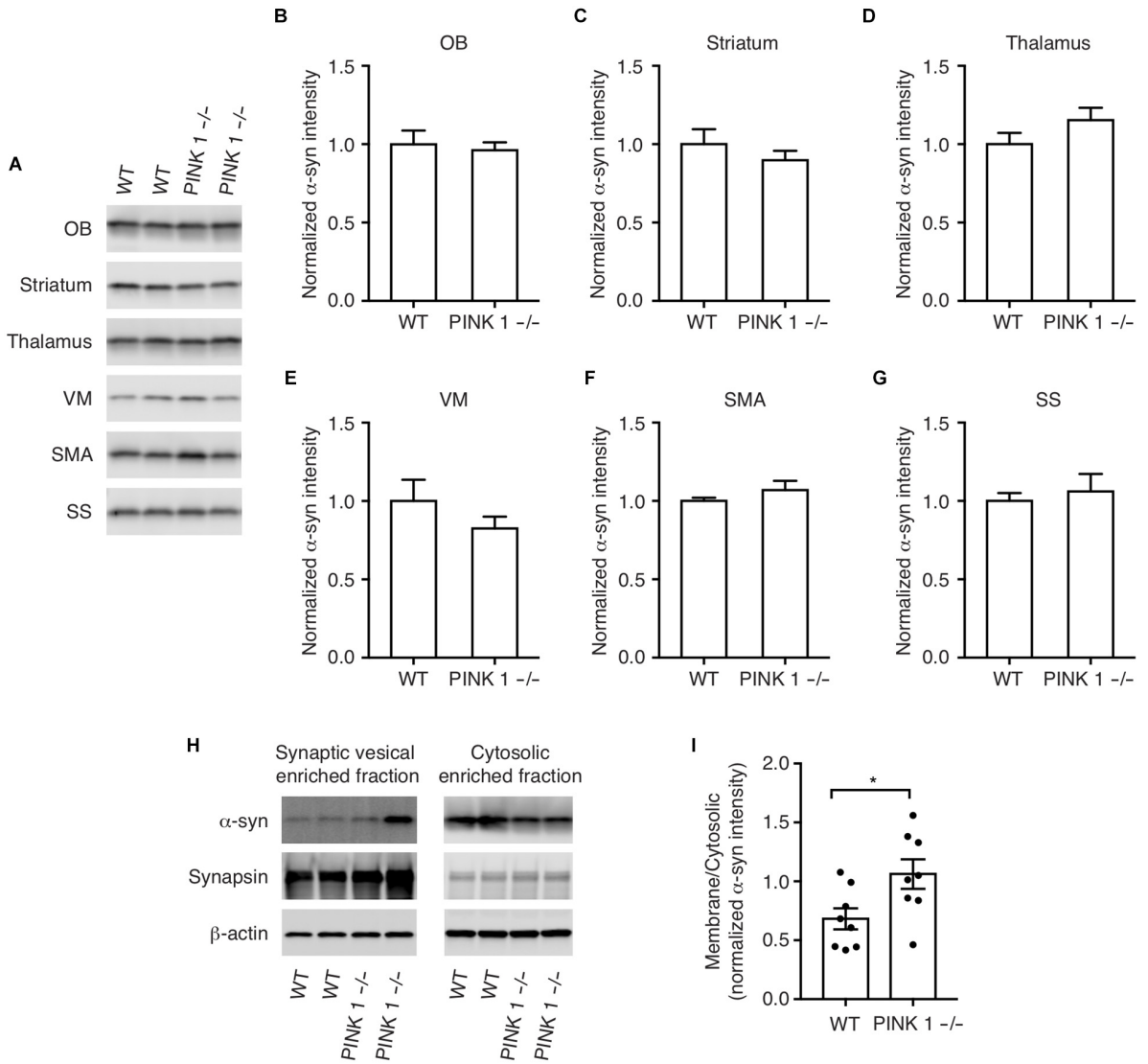

FIGURE 2 | α-synuclein protein levels in WT and PINK1–/– rats. **(A)** Western analysis of α-synuclein in the Olfactory bulb (OB), striatum, thalamus, ventral midbrain (VM), supplementary motor area (SMA), and somatosensory cortex (SS) of WT and PINK1–/– rat brains at age 12 months. **(B–G)** Quantification of western blots from $n = 8$ rats/genotype. Bars represent mean ± SEM. **(H)** Biochemical fractionation and western analysis of cytosolic and synaptic vesicle-enriched fractions of cortex from WT and PINK1–/– rats using antibodies to α-synuclein, synapsin-1, and b-Actin. **(I)** Mean ± SEM ratio of synaptic vesicle bound/cytosolic α-synuclein from $n = 8$ WT and 8 PINK1–/– rats. Asterisk indicates $P < 0.05$ by Student's t-test.

aggregated α-synuclein even if they do not have visible inclusions. To test this, we treated brain sections with proteinase K prior to immunohistochemical analysis, which is a commonly used method to eliminate soluble proteins and to retrieve less accessible epitopes from aggregated proteins, such as aggregated α-synuclein (Beach et al., 2008). Brain sections from 12-month-old WT and PINK1−/− rats were subjected to proteinase K digestion followed by DAB immunohistochemistry with α-synuclein primary antibody. This revealed a large number of cells with proteinase K-resistant α-synuclein in the ventral midbrains of PINK1−/− but not WT rats (**Figures 1E,F**), as previously reported (Grant et al., 2015). Surprisingly, after proteinase K treatment, we did not observe any α-synuclein aggregates similar to those shown in **Figure 1**, indicating that those aggregates are labile to proteinase K treatment even though they are thioflavin S-positive.

α-Synuclein Protein Levels Are Unchanged in PINK1−/− Rats

Overexpression of WT α-synuclein can cause the formation of α-synuclein aggregates (Masliah et al., 2000) and α-synuclein gene duplication and triplication mutations cause PD in humans presumably by increased expression of WT α-synuclein (Singleton et al., 2003). This prompted us to examine more carefully the pattern and cellular distribution of endogenous α-synuclein expression in WT and PINK1−/− rats to determine whether the α-synuclein aggregates are possibly caused by increased localized expression of α-synuclein. Western analysis showed no significant differences between WT and PINK1−/− rats in the pattern of α-synuclein immunoreactivity, even within the ventral midbrain (**Figures 2A–G**).

PINK1−/− Rats Have Increased Synaptic Vesicle Associated α-Synuclein

α-Synuclein loosely interacts with synaptic vesicle membranes and a shift in α-synuclein membrane interaction, possibly by post-translational modifications, oligomerization, interactions with other proteins, altered lipid composition, or by PD-linked point mutations, can affect the propensity of α-synuclein to aggregate (Dikiy and Eliezer, 2012; Galvagnion et al., 2015, 2016; Ysselstein et al., 2015; Samuel et al., 2016; Nuber et al., 2018). To determine whether PINK1 deficiency alters α-synuclein interaction with synaptic vesicles, we analyzed α-synuclein levels in cytosolic and synaptic vesicle enriched fractions of cortex. We found a relative increase in α-synuclein levels in the synaptic vesicle-enriched fraction of PINK1−/− rat brain compared to WT (**Figures 2H,I**). This suggests that the observed α-synuclein inclusions may be due to an increase in the interaction of α-synuclein with synaptic vesicles in PINK1−/− rats.

DISCUSSION

PINK1−/− rats are unusual because α-synuclein immunoreactive aggregates occur spontaneously without synuclein overexpression or administered stresses. In addition to these aggregates, we also detected cells with proteinase K-resistant α-synuclein in the midbrains of the PINK1−/− rats but not WT rats. Our data are consistent with previous reporting of proteinase K-resistant α-synuclein immunoreactivity in PINK1−/− rats (Grant et al., 2015) and α-synuclein aggregates in PINK1−/− rats that could affect neurotransmission underlying motor behaviors (Kelm-Nelson et al., 2016). Although the α-synuclein immunoreactive aggregates we observed in PINK1−/− rats did not resemble Lewy bodies in shape or proteinase K-resistance, we further defined the composition of α-synuclein immunoreactive aggregates by co-localization with thioflavin S and ubiquitin, suggesting that they are composed of amyloid aggregates of α-synuclein, which are characteristics of Lewy bodies (Sakamoto et al., 2002). Even in human brains from PD or Lewy body disease cases, the composition of Lewy bodies and other synuclein aggregates is heterogeneous and this non-uniformity has been proposed to reflect different stages of disease or different phases of synuclein inclusion formation (Sakamoto et al., 2002). Even though total α-synuclein protein levels were unchanged, our biochemical fractionation showed a significant shift of α-synuclein from the cytosolic fraction to the synaptic vesicle-enriched fraction of PINK1−/− brain homogenates compared to WT, which could affect the propensity of α-synuclein to aggregate (Dikiy and Eliezer, 2012; Galvagnion et al., 2015, 2016; Ysselstein et al., 2015; Samuel et al., 2016; Nuber et al., 2018).

CONCLUSION

This study establishes the age-dependent accumulation of α-synuclein aggregates that spontaneously form in the brains of PINK1−/− rats. This supports the use of PINK1−/− rats as a unique model to study the role of spontaneous age-dependent α-synuclein aggregation in PD-related neurodegeneration and cellular mechanisms of familial PD.

AUTHOR CONTRIBUTIONS

RC designed and conducted the study, analyzed the data and drafted the manuscript. MG analyzed the data and wrote the manuscript. Both authors have read and approved the final manuscript.

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