

## Clues to How Alpha-Synuclein Damages Neurons in Parkinson's Disease

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**Abstract:** Alpha-synuclein ( $\alpha$ -syn) appears to normally regulate neurotransmitter release, possibly via calcium-dependent binding and dissociation from lipid domains on secretory vesicles. The pathogenic effects of  $\alpha$ -syn leading to Parkinson's disease (PD) appear to result from alternate toxic effects on lipid membrane. A variety of findings indicate that overexpression of wild-type  $\alpha$ -syn, pathogenic mutations of  $\alpha$ -syn, and dopamine-modified- $\alpha$ -syn promote toxic interaction between  $\alpha$ -syn oligomers and lipids. These may disrupt

transmembrane concentration gradients across secretory vesicles and other organelles and interfere with normal lysosomal or ubiquitin/proteasome mediated protein degradation or mitochondrial function. Additional causes of PD may interfere at other points with normal handling and degradation of  $\alpha$ -syn, providing a variety of entry points to a converging neurodegenerative path underlying the disease.  
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### $\alpha$ -SYN AS A CAUSE OF PARKINSON'S DISEASE

Multiple genetic mutations can cause Parkinson's disease (PD), and in some populations these mutations, particularly in LRRK2, underlie a majority of PD cases.<sup>1</sup> The first gene identified to cause PD was an A53T mutation in alpha-synuclein ( $\alpha$ -syn),<sup>2</sup> which was followed by identification of A30P<sup>3</sup> and E46K  $\alpha$ -syn mutations.<sup>4</sup> A very important subsequent clue to the cause of PD is that overexpression of even wild-type  $\alpha$ -syn protein due to gene multiplication can cause PD<sup>5</sup> with the level of multiplication related to age of onset.<sup>6</sup> A variety of polymorphisms in the  $\alpha$ -syn gene or its promoter may be associated with additional causes of PD, in part by altering the level of the protein.<sup>7</sup>

While the  $\alpha$ -syn pathogenic PD mutations are rare, there is strong evidence that  $\alpha$ -syn is involved at some level in virtually all cases of PD, as it is a major component of the Lewy bodies and Lewy neurites<sup>8</sup> observed in a variety of neurons in PD and "parkinson-plus" disorders such as multiple system atrophy (MSA) and diffuse Lewy body disease.<sup>9</sup>

Animal studies have further confirmed that  $\alpha$ -syn can damage neurons. Viral mediated  $\alpha$ -syn overexpression in rodents can kill SN DA neurons,<sup>10</sup> and expression of pathogenic mutants in transgenic mice can lead to neurodegeneration, although such models to date generally have limited correspondence to PD and hence limited value as PD models.<sup>11</sup> Perhaps most tellingly, deletions of the  $\alpha$ -syn gene are resistant to substantia nigra neuronal death by MPTP toxicity,<sup>12</sup> indicating that the protein can clearly be transduced to play a toxic role.

While evidence is clear that too much wild-type  $\alpha$ -syn protein can be toxic, there is no obvious relationship between  $\alpha$ -syn expression and neuronal damage.  $\alpha$ -Syn expression is far higher during early development than late during maturity<sup>13</sup> when PD occurs, and  $\alpha$ -syn mRNA label is high in neurons both affected and unaffected in PD.<sup>14</sup> It seems that the toxicity of  $\alpha$ -syn must rely on very specific interactions within

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target neurons, and a variety of clues have lately arrived from studies of the protein's normal and presumed morbid functions.

### EXPLORING $\alpha$ -SYN'S NORMAL FUNCTION

The original studies on  $\alpha$ -syn indicated transient interactions with presynaptic terminals,<sup>15,16</sup> while other early findings suggested a role for the protein in neuronal plasticity involved in the seasonal song learning of songbirds.<sup>17</sup> Recent findings are consistent with these roles, with  $\alpha$ -syn appearing to control synaptic vesicle fusion and recycling. Most data indicate that  $\alpha$ -syn inhibits synaptic transmission in an activity-dependent manner, as initially observed in  $\alpha$ -syn deficient mice, where evoked dopamine release recovered more quickly than in the wild-type mouse.<sup>18</sup> This suggested that  $\alpha$ -syn inhibits exocytosis and "refilling" of the population of releasable synaptic vesicles. Subsequent studies on wild-type and pathogenic mutations of  $\alpha$ -syn in chromaffin cells indicate that the inhibition of exocytosis occurs at a late step in exocytosis before vesicle fusion and that this effect of  $\alpha$ -syn was overcome by high levels of activity.<sup>19</sup>

How does  $\alpha$ -syn inhibit exocytosis? The structure of  $\alpha$ -syn shifts between an alpha-helical conformation when bound to membrane lipids to an "unfolded" conformation as a monomer in the cytosol.<sup>20</sup> Acidic phospholipids bind  $\alpha$ -syn, as do polyunsaturated lipids (see below). In secretory vesicles and lysosomes,  $\alpha$ -syn may be particularly prone to binding at "lipid raft" regions,<sup>21-23</sup> although the pathogenic A30P, but not the A53T, mutant protein, binds membrane poorly.

Optical analysis of  $\alpha$ -syn linked to green fluorescent protein (for review see Ref. 24) indicates that  $\alpha$ -syn may dissociate from synaptic vesicles in a calcium- and activity-dependent manner.<sup>22</sup> It may be that  $\alpha$ -syn bound to vesicle membrane interferes with actions of proteins related to vesicle priming and fusion, or to postfusion events involved in vesicle endocytosis recycling (although chromaffin secretory vesicles do not recycle, so that at least some control occurs that is independent of recycling). Together, this evidence suggests a physiological role for  $\alpha$ -syn as a "high pass filter" that enhances neurotransmission via release of its inhibition during periods of high activity.

An alternate function suggested for  $\alpha$ -syn function unrelated to its lipid-binding ability, which is to bind to 14-3-3 protein to negatively regulate tyrosine hydroxylase activity and inhibit DA synthesis, has been presented by Perez et al.<sup>25</sup>

### WHAT MAKES $\alpha$ -SYN TOXIC?

While normal physiological role of  $\alpha$ -syn appears to be dependent on its interactions with membrane lipids, in vitro assays indicate that the protein can form small presumably toxic oligomers known as protofibrils that interact with lipids and disrupt membrane,<sup>26,27</sup> and the pathogenic  $\alpha$ -syn mutants are particularly prone to formation of such oligomers. The pathogenic  $\alpha$ -syn mutants, dopamine  $\alpha$ -syn modification (see below), and the association of  $\alpha$ -syn with polyunsaturated lipids tend to favor the formation of protofibrils.<sup>28-30</sup> As mentioned below, a toxic conformation of the protein with synaptic vesicle, mitochondria, or lysosome membrane could promote degeneration.

Oxidation or nitration of the protein is widely suspected to play a role in oligomerization and aggregation and subsequent cellular damage. The nitration of tyrosine in  $\alpha$ -syn has been reported in a variety of animal PD models<sup>31-34</sup> and may affect oligomerization.<sup>35</sup>

As the cause of parkinsonian motor symptoms in PD is specifically because of the death of SN dopamine neurons, there has been much effort addressing whether an interaction between  $\alpha$ -syn and dopamine may produce toxic forms of  $\alpha$ -syn. Dopamine is prone to oxidation, and its quinone derivative reacts with proteins, generally via a covalent modification of cysteine; dopamine, for example, binds to cysteine residues on parkin, and may promote its aggregation.<sup>36</sup> It is possible that the modification of  $\alpha$ -syn occurs particularly avidly with the monoamine oxidase product of dopamine, 3-4, dihydroxyphenylacetaldehyde (DOPAL).<sup>37</sup>

$\alpha$ -Syn, however, does not possess a cysteine residue. Ischiropoulos and colleagues used  $\alpha$ -syn mutations to identify a series of five amino acids residues (125-129) required for dopamine modification,<sup>38</sup> although protein cleavage may play a role<sup>39</sup>; this association is not covalent, and consequently it has proven impossible to date to detect dopamine-modified  $\alpha$ -syn by mass spectroscopy from cell isolates, or after fixation as an antigen. Nevertheless, in vitro assays indicate that dopamine  $\alpha$ -syn modification, and the pathogenic  $\alpha$ -syn mutants, tend to favor the formation of protofibrils by inhibiting the manufacture of larger presumably less reactive, aggregates,<sup>40</sup> which may eventually produce the Lewy bodies in PD.

### TOXIC ACTIONS AT SECRETORY AND SYNAPTIC VESICLES?

Micromolar concentrations of  $\alpha$ -syn causes proton leakage from isolated chromaffin secretory vesicles,<sup>41</sup>

which would decrease the concentration gradient of catecholamines between the cytosol and vesicle interior.  $\alpha$ -Syn molecules with pathogenic mutations were an order of magnitude more efficacious at disrupting vesicular pH than wild-type protein, and consistent with effects on vesicle pH,  $\alpha$ -syn overexpression in chromaffin cells enhanced cytosolic dopamine levels. The pathogenic mutants may be more effective at disrupting vesicle pH because they are more prone to forming oligomers and “pores” that provide a means for protons and catecholamines to leak through the vesicle membrane.

If dopamine synaptic vesicles in substantia nigra neurons are damaged by pathological interactions with  $\alpha$ -syn, a vicious cycle of dysregulated cytosolic dopamine and further  $\alpha$ -syn damage could ensue, which may explain an important step in the targeting of dopamine neurons in PD.

### **BLOCKADE OF NORMAL PROTEIN DEGRADATION**

$\alpha$ -Syn has also been found to damage lysosomal function.  $\alpha$ -Syn protein appears to be normally degraded by autophagy,<sup>35</sup> i.e., breakdown within lysosomes (for review see Ref. 42). Large aggregates of  $\alpha$ -syn proteins are thought to be degraded intracellularly by a lysosomal pathway known as macroautophagy. Before aggregation,  $\alpha$ -syn can undergo degradation via an alternate lysosomal pathway, chaperone-mediated autophagy (CMA) which entails recognition by the chaperone hsc-70 and accumulation of the substrate into the lysosomal interior.<sup>43</sup>  $\alpha$ -Syn oligomers containing more than two molecules of  $\alpha$ -syn are resistant to CMA.<sup>44</sup>

$\alpha$ -Syn oligomers appear to accumulate on lysosomal membrane, and to inhibit CMA, perhaps by binding to lipid raft-like domains on the lysosome and blocking the activation of the transport required for lysosomal accumulation of proteins substrates. Consistent with  $\alpha$ -syn oligomer blockade of CMA activity, high levels of wild-type  $\alpha$ -syn competitively inhibit uptake of other protein substrates for CMA,<sup>45</sup> while the A30P and A53T  $\alpha$ -syn mutants and dopamine-modified  $\alpha$ -syn are more effective inhibitors than wild-type protein.<sup>44</sup>

### **POTENTIAL INTERACTIONS BETWEEN $\alpha$ -SYN AND THE UPS SYSTEM**

There is evidence that  $\alpha$ -syn can be ubiquitinated<sup>46,47</sup> and thus targeted to lysosomes, aggregates, or the proteasome. The ubiquitinated protein has been difficult to detect in a number of systems. A number of

other proteins that may bind to  $\alpha$ -syn are however, well characterized as ubiquitin substrates, including synphilin.<sup>48</sup> It may be that ubiquitination of such  $\alpha$ -syn interacting proteins provide a signal for lysosomal targeting or other forms of processing.

Several additional mutations that underlie familial PD play roles in the ubiquitination/proteasomal system. In particular, new insights have come from studies on the ubiquitin ligase, parkin, the second gene identified to cause inherited PD.<sup>49</sup> Lewy bodies are not observed in juvenile onset recessive forms of parkin-related PD, suggesting that parkin ubiquitination of an  $\alpha$ -syn interacting protein may be required for the formation of large  $\alpha$ -syn aggregates, although there is also evidence for a toxic gain of function for parkin mutations. Parkin can form a variety of forms of ubiquitin cross protein linkages,<sup>50</sup> including an E48 series that targets synphilin to proteasomal degradation, and an E63 that can target the protein to lysosomes,<sup>48</sup> and monoubiquitination.<sup>51</sup> Parkin may react with cytosolic dopamine, perhaps inhibiting its normal function.<sup>52,53</sup> If disturbance of normal function or a toxic form of parkin also affects the fate of  $\alpha$ -syn, as reported in a mouse model expressing mutant parkin that produces proteinase-K resistant  $\alpha$ -syn aggregates,<sup>34</sup> there will be an important example of how multiple causes of PD may converge at the steps controlling  $\alpha$ -syn degradation.

### **BINDING TO MITOCHONDRIA**

Recent work using fluorescence resonance energy transfer (FRET) imaging indicates that  $\alpha$ -syn binds to mitochondria, where it shifts from a relatively “closed” to an “open” conformation.<sup>54</sup> It is possible that an interaction of the protein with mitochondrial membrane can lead to disruption of membrane normal function or to mitochondrial-driven cell death pathways, including apoptosis.

### **INFLAMMATORY CONSEQUENCES**

$\alpha$ -Syn can trigger inflammation and activation of microglia,<sup>55</sup> and particularly after nitration<sup>32,33</sup> the microglia are able to phagocytose and degrade extracellular  $\alpha$ -syn more avidly than neurons or astrocytes.<sup>7</sup> This inflammatory response would presumably occur after neuronal death, but it is also possible that the protein is released via exocytosis<sup>7</sup> or even that cleaved portions are presented via antigen presentation: in any case, this could lead to vicious cycle of inflammatory response, release of  $\alpha$ -syn or modified  $\alpha$ -syn and further inflammation.

### SUGGESTIONS FOR THERAPEUTIC DIRECTIONS

Although one hopes that this review is rapidly outdated as  $\alpha$ -syn toxicity is characterized, current evidence suggests that multiple steps are required for the development of PD.<sup>56</sup> This may provide multiple points to treat the disease. From the pathways discussed earlier, these include means such as RNA interference to decrease  $\alpha$ -syn expression in disease-prone neurons<sup>57–59</sup> or the use of intracellular antibodies known as intrabodies<sup>60</sup>; to modulate the interactions of  $\alpha$ -syn and lipids that underlie aggregation<sup>31</sup>; to buffer cytosolic dopamine levels by enhanced VMAT2 expression or block its toxic breakdown products, including the use of monoamine oxidase inhibitors<sup>53,61,62</sup>; to provide appropriate ubiquitination of  $\alpha$ -syn or its interacting proteins for targeting to appropriate degradation pathways<sup>47,48,63</sup>; to overcome blockade of lysosomal pathways by enhancing CMA or macroautophagy see Ref. 42; to block the neuroinflammatory cascade.<sup>33</sup>

A range of additional directions now being explored may interact with  $\alpha$ -syn dependent causes of PD; for example, inhibition of neuronal oxyradical stress would block oxidation of cholesterol and associated proteins, buffer cytosolic dopamine, and could correct aberrant proteasomal and autophagic degradation. The recent effort into using L-type calcium channel blockers for therapy (see Ref. 64), has apparent effects on the regulation of cytosolic dopamine and oxyradical production.<sup>62</sup> Attempts at inhibiting neuroinflammation may affect nearly all of these processes, and there are efforts to develop antibody therapies.<sup>65</sup> The recent range of insights into PD provides optimism that clinical treatments may be tailored for individuals depending on the means by which  $\alpha$ -syn causes a particular PD subtype.

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