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Review Article

MISFOLDED α-SYN – FROM RELEASE AND UPTAKE TO CONTAGION OF ADJACENT CELL, ANY LINKS WITH GUT?

Dongya Huang* and Leyla Nazarova

Tongji University affiliated Shanghai East hospital, China, Shanghai.

ABSTRACT

Alpha-synuclein(α-Syn) is an intracellular protein which is constantly synthesized and degraded under physiological conditions. Abnormal aggregation of this protein within neuron is associated with several neurodegenerative disorders, including Parkinson's disease. Although the exact mechanism of initiation of aggregation and misfolding pathway of a-Syn is yet to be discovered; it's mechanism of spreading through interconnected neuronal networks has taken acompelling step. Growing number of studies have investigated step by step mechanisms of a-Syn release from the diseased cell and uptake by normal adjacent cells in molecular basis. Also, some studies have hypothesized probable involvement of gut in this pathogenesis. We will briefly review this prion-like contagion pathway and try to elucidate any connection and relevance to gut to brain theory.

Keywords: alpha-synuclein; α-Syn; Parkinson's disease; PD; vesicle trafficking; protein quality control system; gut-brain axis; microbiome.

INTRODUCTION

Alpha-synuclein. Outlook:

Alpha-synucleins are small proteins containing of 140 amino-acids that are abundantly found in nucleus, mitochondria and presynaptic terminals of brain neurons, as well as other tissues[1]. They are composed of three distinct domains: N-terminal domain, non-amyloid component domain and the C-terminal domain. Non-amyloid component domain consisting of 12 amino-acid sequences is highly hydrophobic, being intrinsic under physiological conditions[2, 3]. When this domain becomes extrinsic, alpha-synuclein loses its normal shape. Hydrophobic sites of different proteins attract each other and destructive α-Syn aggregation and misfolding process begins. However, despite of making up 1 % of all proteins in the cytosol of neurons and intensive research upon its discovery, the exact function of this protein and cascade of initiation of its dysfunction remains unknown.

Protein quality control system. Outlook:

We don't know what mechanism a main driver of abnormal aggregation cascade is. What we know from molecular biology, after translation of proteins by ribosomes, amino-acid sequence has to undergo several steps to gain its three-dimensional functional structure. On this way, proteins can be misfolded and form aggregates. Under physiological conditions, on the way from Endoplasmic reticulum to Golgi body, misfolded protein aggregates undergo Chaperone catalysis. Chaperones, or disaggregates assist them to go back to their normal pathway on gaining their favorable structure[4, 5]. However, if chaperones fail to assist proteins on this state, abnormal aggregates are further recognized by ubiquitin proteosome system or protein degradation network which ultimately breaks down aggregated proteins into peptides and assures cells' well-being[6, 7]. If the capacity of ubiquitin-proteosome route is overwhelmed or activity of this system is declined due to aging, alternative ways of protein quality control system activated. To avoid proteostatic collapse, misfolded proteins are further cleared by aggrephagy pathway, selective form of autophagy[8, 9].

Intracellular neurotransmitter trafficking. Outlook:

Dopamine, or other neurotransmitters deficient in PD are carried out to synaptic cleft by intracellular vesicles destined for extracellular departure named exosomes. The whole complex process of transfer of neurotransmitter to the cell membrane upon its production is classified into following 4 steps: 1) movement of vesicles towards the presynaptic membrane, 2) tethering of vesicles into presynaptic membrane, 3) docking vesicles to the presynaptic membrane and 4) fusion between vesicle and presynaptic membrane. The 1st step is highly mediated by microtubules and associated motor proteins. The 2nd step is believed to be mediated by wide range of tethering proteins. The specificity between vesicle and target membrane maybe conferred by a family of G proteins called Rabs, which act as a master regulator of intracellular tethering processes. With over 60 different Rab genes identified in humans, these proteins constitute the most diverse group of proteins involved in membrane trafficking. They play a key role in vesicle targeting by recruiting specific cytosolic tethering proteins to specific membrane surfaces as well as in recruiting motor proteins, which move

membranous vesicles through the cytoplasm. The main proteins involved in 3rd stage are Ca^{2+} sensor synaptotagmin and the soluble NSF-attachment protein receptors or SNAREs. After receiving action potential stimuli, calcium (Ca^{2+}) channels open, thereby allowing influx of Ca^{2+} into the presynaptic terminal. This Ca^{2+} influx promotes SNARE complex to trigger vesicle trafficking from the closest pool to be fused to the presynaptic plasma membrane at a defined region called the active zone[10]. During transport of neurotransmitters in synaptic vesicles, plasma membrane of the nerve cell contains two t-SNAREs, syntaxin and SNAP-25, whereas the synaptic vesicle membrane contains a single v-SNARE, synaptobrevin. As the synaptic vesicle and presynaptic membrane approach one another, the SNARE motifs of t- and v-SNARE molecules from apposing membranes interact to form four-stranded bundles. Each bundle consists of four α helices, two donated by SNAP-25 and one each donated by syntaxin and synaptobrevin. These parallel α helices zip together to form a tightly interwoven complex that pulls the two apposing lipid bilayers into very close association, which ultimately causes fusion of vesicle membrane with cell membrane and the vesicles are ready to discharge their contents (Figure 1).

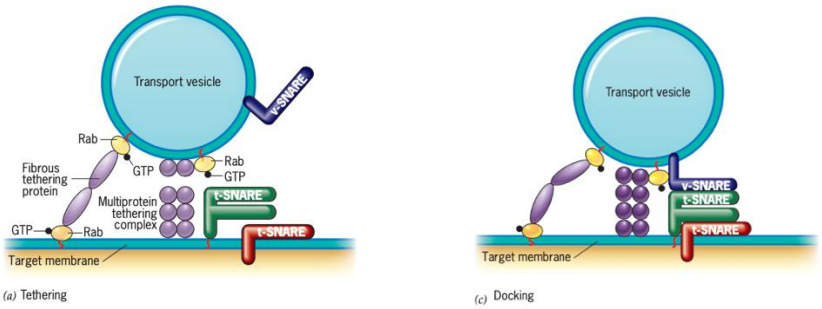

Figure 1: **a)** According to this model, Rab proteins on the vesicle and target membrane are involved in recruiting tethering proteins that mediate initial contact between the two membranes. Two types of tethering proteins are depicted: highly elongated fibrous proteins (e.g., golgins and EEA1) and multiprotein complexes (e.g., the exocyst and TRAPPI). **C)** During the docking stage leading up to membrane fusion, a v-SNARE in the vesicle membrane interacts with the t-SNAREs in the target membrane to form a four-stranded α-helical bundle that brings the two membranes into intimate contact. In the cases described in the text, SNAP-25, one of the t-SNAREs, is a peripheral membrane protein that is bound to the lipid bilayer by a lipid anchor rather than a transmembrane domain. SNAP-25 contributes two helices to the four-helix SNARE bundle.

Source: Karp's Cell and Molecular Biology textbook.

Abnormal aggregation of α-Syn and possible wrong turns:

We find that this portion needs to be reviewed in detail because we think that understanding the whole conception of events related to normal protein degradation and abnormal protein aggregation is key to understand mechanism of neurodegenerative diseases.

Let's have an outlook to normal protein synthesis to figure out in which step the pathway took the wrong turn. Following instructions of SNCA gene, mRNA carries special code by means of nucleotide sequence

to ER, where it undergoes complex process of translation dictated by mRNA and proceeded by ribosomes. Afterwards, under physiological conditions, it's expected to undergo several types of post-translational modifications such as Abelson-Kinase mediated phosphorylation and molecular chaperon packaging and gain its native folded state[11-13]. Only after proper packaging is ensured, the further trafficking could be continued. But what does guide them to their native state? It comes out from the observations of studies in vitro and vivo that sufficient information must be contained in the primary code to guide correct refolding. They also suggested factors like thermal energy from heat, extremes of pH and chemicals such as urea and guanidine at high concentrations can destroy weak covalent interactions that stabilize the native conformation of α-Syn. Unfolding and misfolding of α-Syn in this step is not completely normal but can be expected. Molecular chaperones, a type of proteins found in all organisms from bacteria to human bind and stabilize unfolded protein or counteract misfolding[14]. If succeeded, send that package with monomers to Golgi, if failed, send the package with aggregates to ubiquitination. Burmann et al. proposed that six divergent molecular chaperones recognize a canonical motif in α-synuclein, consisting of the N terminus and a segment around Tyr39, and hinder the aggregation of α-synuclein [13]. Falsone et al. suggested that binding of molecular chaperone HSP90 to α-Syn could prevent α-Syn from vesicle binding and lead to α-Syn oligomer formation[15]. Nevertheless, we assume that main disruptive pathology is not fully due to chaperone dysfunction, since even if it was so, as long as later misfolded protein clearance systems were intact, the cell could be saved. After molecular chaperones fail to mend aggregated α-Syn, latter is further shipped towards ubiquitin-proteosome system. Physiologically, aggregated and misfolded a-Syns are expected to be tagged by ubiquitin, which could be referred as their death sentence. Next, following ubiquitination, where α-Syn aggregates are recognized by their exposed hydrophobic sequences, poly-ubiquitin chain protein cargo delivered to proteosomes, where proteosome, dispersed throughout the cell cytosol cleaves out ubiquitin from α-Syn aggregates in ATP-dependent manner and further degrades the protein into short peptides via its lumen. Ubiquitin mediated cellular garbage disposal system consists of 5 steps: 3 steps of Ubiquitination and 2 steps of proteolysis and involvement of 3 enzymes, namely ubiquitin-activating enzyme(E1), Ubiquitin-conjugating enzyme(E2) and ubiquitin ligase(E3) (Figure 2). Here comes the exciting question, if ubiquitination process was impaired, Lewy bodies - pathological hallmark of PD should be composed of pure protein aggregates, since the product of ubiquitination process is covalently bonded poly-ubiquitin chain protein complex. If Lewy bodies contain a-Syn-ubiquitin complexes, then this step is also intact and has little to do with PD pathogenesis. And indeed, recent studies on the composition of LBs found that they had wide range of other molecules than α-Syn aggregates and were highly enriched with ubiquitin[16, 17]. It means that we should look for guilty around proteosomes and autophagic lysosomes. This could be direct involvement of individual PQC system participant molecules as well as aggregates possessing amyloid like characteristics resistant to proteasomal recycling and autophagy. There is also evidence that protein translation is reduced following negative feedback of increased load on the PQC system[6]. So, if α-Syn aggregates restrict further α-Syn protein translation, the dysfunction

of affected neuron could be proportionally the result of depleted α-Syn monomers and increased α-Syn aggregates, meaning this protein in its different forms is assumably its hosts' enemy and friend.

There also have been studies relating significant alteration in iron composition of Substantia nigra in PD patients to PD pathogenesis[18, 19]. Oligodendrocytes are cells responsible for neuronal myelination in CNS and principal cells in the CNS that stain for iron under physiological conditions. The importance of iron in myelin production has been highlighted by several studies showing that decreased availability of iron in the diet is associated with hypomyelination[20]. Knowing that vulnerable neuronal types in PD are also associated with poor myelination, its rather interesting if these two facts are interrelated or just a coincidence.

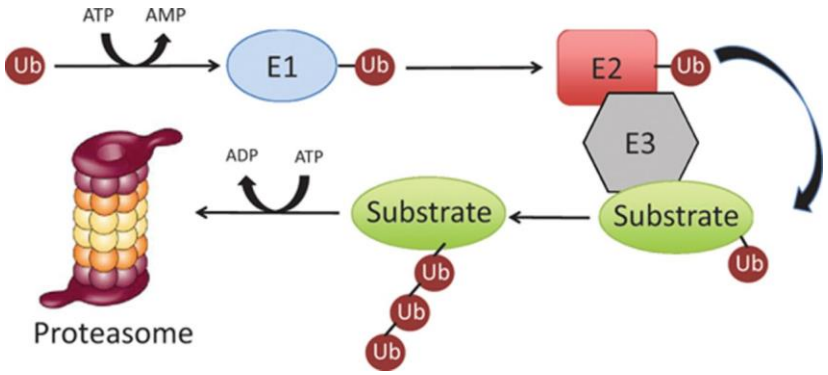

Figure 2: Enzyme 1 (E1) is activated by attachment of ubiquitin (Step1) and then transfers this Ubiquitin molecule to enzyme 2 (E2). Ubiquitin ligase (E3) transfers the bound Ub molecule on E2 to the sidechain NH2 of a lysine residue in a target protein (Step 3). Additional Ub molecules are added to the target protein by repeating steps 1-3, forming a polyubiquitin-protein chain that takes direction toward proteasome (Step4). Ubiquitin is cleaved out; protein is cleaved into small peptides within its lumen (Step5). b) Computer generated structure reveals that proteosome is a cylindrical molecule with a cap at each end of a core region.
Source: Pagan, J., et al., Role of the ubiquitin proteasome system in the heart. Circ Res, 2013.

Updates in evidence on role of α-Syn in neurotransmitter trafficking:

Considerable evidence coming from vitro studies of PD model mice report presence of alteration of a range of proteins involved in synaptic vesicle trafficking and neurotransmitter transmission starting from prodromal phase and maintaining up to cell degeneration. Due to these early-onset synaptic alterations observed prior to dopaminergic neuron degeneration in Substantia nigra, PD has also been classified as a Synaptopathy[21]. Burre and his colleagues reported that maintenance of continuous presynaptic SNARE-complex assembly required a nonclassical chaperone activity mediated by synucleins. Specifically, α-synuclein directly bound to presynaptic membrane phospholipids via its N terminal and to synaptobrevin-2 via its C terminal and promoted SNARE-complex assembly. Interestingly, α-Syn -knock out mice developed age-dependent neurological impairments and displayed decreased SNARE-complex assembly. The role of synucleins was observed to be fully dispensable in young animals, but become essential late in life, which suggests that α-synuclein maintains normal synaptic function during aging. Relying on this evidence they pointed out potential chaperone activity of physiological α-Syn in sustaining normal SNARE-complex assembly

during aging. They also delineated that only the multimeric form of α-Syn upon membrane binding (but not soluble monomeric form) acts as a SNARE complex chaperone at the presynaptic terminal and may protect against neurodegeneration [22, 23]. Facilitation of SNARE-dependent vesicle docking by α-Syn was also supported by other studies[24, 25]. In a recently published study, scientists from Italy isolated neural derived extravesicles from peripheral blood of 32 PD patients and 40 healthy controls and compared the concentrations of oligomeric α-Syn and of the presinaptic SNARE complex proteins: STX-1A, VAMP-2 and SNAP-25. They reported significant alterations in all tested proteins[26]. Although these findings need to be examined further, they have potential to serve as early biomarkers for PD. Furthermore, increase in y-synuclein was reported in many types of cancers, which could be due to enhanced membrane traffic in cancerous cells[27, 28].

In addition to SNARE complex, a line of evidence come from in vitro studies that highlight interplay of a-Syn with several members of Rab GTPase family. The study which used Drosophila models of a-Syn toxicity reported that Rab11 decreases a-Syn aggregation and improve locomotor activity and degeneration of dopaminergic neurons[29]. Same observations were reported by other study, which underlined involvement of other members of Rab family such as Rab8b, Rab13 and Slp5 along with Rab11. They found that these molecules promote a-Syn inclusions and reduce toxicity[30]. Masarachia et al further investigated interplay of other Rab family members and α-Syn by screening their pool. They found that internalized α-Syn partially colocalized with Rab4a-labelled endosomes and later reached autophagy lysosomal pathway. These studies emphasize the involvement of Rab proteins in normal function as well as in internalization and subsequent accumulation of α-Syn. Altogether, these studies indicate that targeting Rab and SNARE families may hold important therapeutic value in PD and other synucleinopathies [31].

The role of α-Syn in regulating presynaptic vesicular dynamics was also emphasized by several studies, who identified that depletion of α-Syn can decrease the availability of reserved synaptic vesicle pool in neurons[32]. Another study reported that sterol depletion enhances α-Syn toxicity and, thus higher membrane sterol concentrations might have synucleinopathy-protective role. However, exactly how sterols affect vesicular trafficking in synucleinopathies is to be investigated[33].

Updates in a-Syn aggregate release, uptake and contagion of adjacent cells:

Supportive evidence suggests that soluble form of α-Syn or monomeric α-Syn can be released within vesicles into extracellular space after being sorted out through sumoylation process, but there is no data that this mechanism also works for α-Syn aggregates[34]. Recent findings from two studies had struck several sparks of light in this area. Lee et al. reported that deubiquitylate USP19 has ability to recruit misfolded proteins within the cytosol to Endoplasmic Reticulum, where they undergo deubiquitylation, encapsulation into endosomes and are excreted from the cell. Their finding suggests that USP19 associated protein quality control pathway's goal is sustaining internal homeostasis, secreting protein aggregates failed proteolysis out of the cell. However, this mechanism also serves as underlying ground of cell-to-cell transmission of pathologic aggregates[35]. Fontaine et al. discovered new DnaJ/Hsc70 chaperone complex which could be involved in

extracellular release of protein aggregates. This function is closely related to intact Hsc70 chaperone activity [36]. However, to this point interconnection of DnaJ/Hsc70 and USP19 pathways remains unclear.

Recent data from several different studies highlighted number of membranous receptors that might take part in uptake of primarily misfolded proteins (but not α-Syn monomers) and interneural propagation of PD pathology. α-Syn fibrils have been observed to bind to cell membrane proteins as heparin sulfate proteoglycans (HSPs), neurexin 1, amyloid b precursor protein 1 (APLP1), Lymphocyte activation gene 3 (LAG3) and Na+/K+ transporting ATPase subunit a3 (a3-NKA)[37-42]. Among those, LAG3 is reported to bind preferably to α-Syn fibrils rather than monomers and cause cell swallow α-Syn-LAG3 complex through endocytosis, which is further transported to cell nucleus. Strikingly, they also reported that LAG3 depletion or LAG3 antibodies significantly delay a α-Syn oligomers transmission, but not completely inhibit it[40]. Although these findings give deeper insight in prion-like transmission of PD pathology, they also indicate that adjacent cell contagion isn't driven by single mechanism, and there still might be several different ones to be discovered. We consider that therapeutic targets designed against these receptors might be limited in their function due to several reasons. First, presentation of PD patients with classical clinical motor symptoms is reported to be after very few dopaminergic neurons are left. And as we know, neuronal death is not subject to remission. Plus, there are number of receptors involved in this process, thus targeting one would give us only partial halt. Antagonists targeting receptors above would come up with excessive accumulation of protein aggregates in extracellular space, which would bring its own consequences.

Any links to gut?

Constipation is the most manifested non-motor symptom of Parkinson Disease patients[43]. PD patients report of long-term constipation suffering when present with first motor symptoms. The Braak model of PD posits that PD related pathology could be initiated in gut years prior to reaching its full extent in CNS. It can be transferred upwards through several pathways; Vagal nerve being primarily involved in retrograde cascade (Figure 3) [44-46]. Braak observed that early lesions of Lewy formations were present in dorsal motor nucleus (DMN) of vagus nerve, anterior olfactory nuclei and VIP neurons of Aurebach plexus. Here we will search for links between gut (primarily intestinal cells but not the enteric neurons) and brain in PD from current clinical data on Parkinsonian pathology.

In a small research from the U.S. investigators examined presence of α-Syn in colonic tissue of 9 PD patients and 24 healthy controls obtained through biopsy. They reported that all PD patients showed staining for α-Syn in the lamina propria in the colonic submucosa and 2 out of 24 controls showed relatively slight staining[47]. Although these findings sound exciting, their study reported using α-Syn antibodies that are not specific for phosphorylated form of alpha-synuclein (which could be used to detect aggregates rather than monomers). Restricted specificity in this study makes its results limited for further interpretation. α-Syn monomers might be expressed in the gut tissue under physiologic conditions, therefore a reliable method must be obtained to differentiate abnormal α-Syn aggregates from normal α-Syn proteins.

Schneider et al. systematically reviewed existing data on assessing monomeric and aggregated α-Syn in Gastro-intestinal (GI) tract respectively. Following α-Syn staining, which can bind to all types of α-Syn proteins, post-mortem samples of PD patients stained positive about 50%–100% compared to controls 0%–52%. Although they reported that α-Syn staining for phosphorylated protein, which could be used to detect α-Syn aggregates, was positive in 23%–93% of PD patients but never in controls, there was high distributional variability of detection of pathology between different gut segments. Therefore, this method, assumably sufficient at estimating true negative rate, cannot be regarded as a perfect predictor.

Transportation of α-Syn from gut to brain was observed after administration of environmental toxin rotenone as well as injection of human α-Syn into Vagal nerve[48, 49]. However, this kind of cell-to-cell transmission is also observed after injecting mice-acquired a-Syn fibrils into dorsal striatum of wild-type mice, which resulted in reduced DA innervations between SN and dorsal striatum and culminated in motor deficits similar to PD[50]. These observations are only suggestive of inter-neuronal transmission across vulnerable zones occurring in CNS and periphery respectively.

Study from Sweden further tested the theory of gut to brain synonucleopathy in mice. They injected α-Syn lysate acquired from post-mortem human PD brain substantia nigra into intestinal wall of wildtype rats. They observed that different forms of α-Syn, including monomeric, oligomeric and fibrillar, all can be transported from intestine to brain DMN region via vagal nerve. Similar observations were recently made by USA scientists, where they injected α-Syn fibrils into the duodenal and pyloric muscularis layer. Spread of aggregated α-Syn in brain was observed first in the DMN following spread of pathology across vulnerable neurons in the exact fashion as described by Braak. Altogether these studies are highly supportive of Braak hypothesis. As mentioned earlier in Braak's staging first involved areas are anterior olfactory nuclei, DMN and Aurebach plexus. Upper mentioned studies only checked for presence retrograde spread cascade from gut to brain. However, Vagal nerve serves as bidirectional pathway. To eliminate biases, we suggest that transportation of α-Syn fibrils downward from DMN to Aurebach plexus should also be tested, and the manner and time of both could be compared.

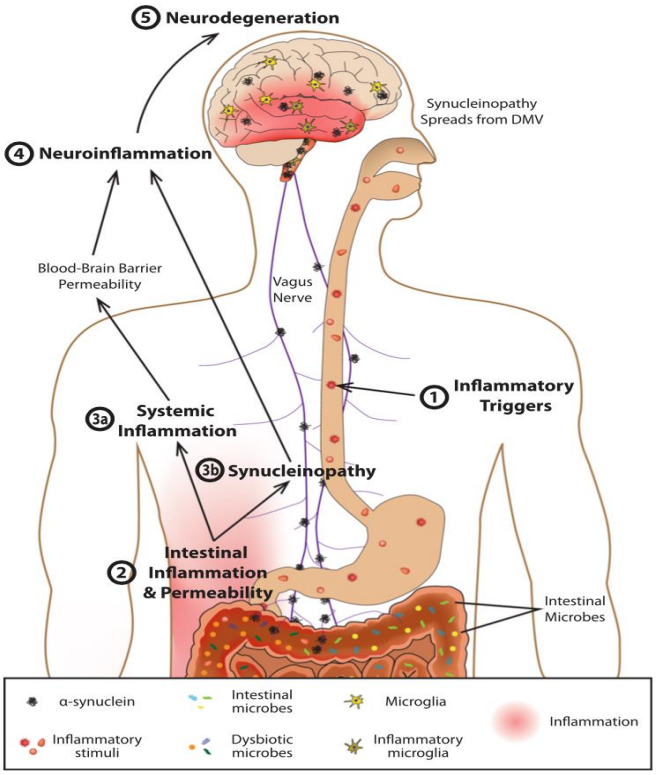

Figure 3: Model of gut originated pathogenesis spreading to brain via Vagal nerve.

Source: Houser, M.C. and M.G. Tansey, The gut-brain axis: is intestinal inflammation a silent driver of Parkinson's disease pathogenesis? NPJ Parkinsons Dis, 2017.

DISCUSSION

As we have advanced with new findings on microbiome and human diseases, we came up with conception that us and our gut microbiota live in a complex union, where both of us are vulnerable to the deeds of the other. Existing data on Parkinsonian pathology from gut to brain can give us some initial insights on presence of α-Syn monomers and oligomers across GI tract. Neither it gives any clues about quantity of the protein of interest, nor the molecular basis of gut synucleinopathy. Methods of intercellular transmission in and from the gut are unclear as well. Any links to gut? Yes! Substantial links? Very hard to say just yet. As we have evidence regarding some molecular receptors playing crucial role in propagation of PD pathogenesis in CNS neuron models, we propose that if hypothesis of retrograde cascade from gut to brain in PD pathology is to be held, research on PD gut models regarding early mentioned receptors and their function in gut brain axis should be further established.

Alpha-Synuclein propagation pattern present in PD brain is not random. It affects certain neuronal types, while others, even located nearby are left unaffected. It certainly follows some rules, and we can try to make some interpretation on the information we have. Exact neurons must have disproportionally long and thin axons which are merely myelinated or unmyelinated to develop α-Syn pathology[51]. This applies to all

neurons involved in center and periphery. Other well myelinated neurons are resistant to pathology. PD pathology is not restricted to Dopaminergic cells either. Widely affected branches of Vagal, existing from very early stage of disease according to Braak's staging category, are Cholinergic. Other involved neurons sharing common morphological features are glutamatergic, noradrenergic, serotonergic as well as histaminergic neurons[46]. Pathology is also observed in nonneuronal cells in other synucleinopathies such us oligodendrocytes, microglia, etc. Although it function is not fully understood, several studies have reported that many cells do not die right after Lewy body formation, but simply lose their function. They also reported that the most toxic form of α-Syn not established, and that Lewy bodies themselves could be harmless inclusions. In that case, as we have evidence on physiological function of α-Syn in neurotransmitter trafficking, it's depletion could be main factor which led to manifestation of Clinical motor and non-motor symptoms of PD, motor: lack of dopamine; non-motor: lack of 5-HT, Ach, and other NTs. Moreover, microglial activation following secretion of α-Syn aggregates into extracellular space is reported across various studies. Neuroinflammation and neuronal loss could be the consequences of secondary process after microglial activation rather than Lewy pathology itself.

Relying on common topographical and morphological features of spreading of PD pathology, we raise questions as what the links between poor myelinated long axons, function of α-Syn in neurotransmitter trafficking and gut are? One hypothesis relates high-energy requirement of poorly myelinated neurons and predicts that any disturbance inducing a negative energy balance could be associated with neuronal malfunctioning, placing them at high risk in case of energy failure, such as mitochondrial dysfunction, which has been shown to play an important role in PD [52]. We also raise suspicions that synaptopathy in vagal nerve terminals and its enteric branches resulted in decreased acetylcholine secretion leading to decreased gastric and intestinal motility, and any reported gut and microbiota involvement could be a consequence rather than cause. The report of research about pathology arising from gut to brain could be just part of bidirectional cargo of synucleopathies, initially coming in gut direction and then going in both directions. PD affects somatic-motor and visceromotor pathways, and only olfactory structures from sensory system. We also wonder what olfactory structures have distinct from other sensory neurons and in common with other affected areas that make them selectively vulnerable.

Although many molecules are found to be and some organisms do seem to be correlated in PD progression, correlation doesn't equal to causation. To learn and expand our horizons further, we need to seek answers to questions lately narrated from existing evidence, as well as from well-designed novel studies through biochemical and translational techniques.

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