Review Article

DOI: http://dx.doi.org/10.53043/2347-3894.acam90034

Tracking the Importance of Enteric a-syn Pathology in Parkinson's Disease

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Asian Journal of Complementary and Alternative Medicine. Volume 10 Issue 02

Published on: 25/04/2022

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Cite this article as: Casini A, Vaccaro R, Vivacqua G, Onori P, Gaudio E et al. *Tracking the Importance of Enteric a-syn Pathology in Parkinson's Disease.* Asian Journal of Complementary and Alternative Medicine, Vol 10(2), 50-57:2022.

ABSTRACT

Alpha-synuclein (α-syn) represents one of the most abundant neuronal proteins and its abnormal aggregation is considered a pathological hallmark of Parkinson's disease (PD). These deposits are frequently found also in the gastrointestinal tract of parkinsonian patients. For that reason, many studies have investigated the distribution of α-syn not only in the central nervous system (CNS) but also in peripheral tissues, including GI track. In the present review, we summarize the last findings regarding the possible correlations between the α-syn pathology and the gut dysfunction in course of PD. According to Braak's hypothesis, in fact, it is supposed that the initial α-syn pathology originates into the gut and transmit anti-dromically to the dorsal motor nucleus (DMNX) by the vagus nerve, from which it can spread up to different rostral and caudal nervous regions. Notwithstanding, it is still poorly known whether α-syn pathology is directly responsible for the enteric disorders of PD patients. The early identification of intestinal symptoms and of their anatomical correlates, might help in identifying PD patients at the early stages of the disease and might contribute to the designing of early disease modifying therapies.

ALPHA-SYNUCLEIN FROM PHYSIOLOGICAL EXPRESSION TO PATHOLOGICAL INVOLVEMENT

α-Syn is a 140 amino acid protein, highly expressed in the mammalian nervous system and belonging to the synuclein family. The first evidence of a synuclein protein came from the electric organ of *Torpedo californica* where the synuclein protein was firstly isolated and named "syn-nuclein" due to its preferential localization in pre-synaptic terminals and at the nuclear envelope [1, 2]. Subsequently, three different synuclein proteins, respectively named as alpha-, beta- and gamma-synuclein were isolated and cloned from the central nervous system of different vertebrates [3].

α-Syn has been detected in the central and peripheral nervous system of different vertebrates from teleosts to humans [4-7], but its precise physiological function is still under debate. Anatomical studies have reported a different sub-cellular localization of α-syn, among different neuronal subtypes [4, 5], thus suggesting a heterogeneous involvement of the protein in neuronal biology [8]. The main part of studies agree on the presence of α-syn in high concentrations at presynaptic level and its preferential localization only in a subset of synaptic vesicles [9]. This suggests that this protein may have a specific function in synaptic vesicle cycling, hence in synaptic transmission [10]. Further studies on the interference of α-syn on the endoplasmic reticulum- Golgi complex support the role of α-syn in vesicle trafficking [11]. In particular, α-syn has been closely linked to the stabilization of the SNARE complex and to the priming and docking of the synaptic vesicles with the pre-synaptic membrane [12]. Furthermore, α-syn presents a biochemical structure characterized by a huge amount of alpha-helical domains, which increase its affinity to the lipid membranes such as mitochondrial membranes and the nuclear envelope [13]. The role of α-syn in these cellular compartments is not widely accepted, although compelling evidence support the possible role of α-syn on mitochondrial function [14] and regulation of gene expression [15]. Few additional reports documented also the physiological expression of α-syn in peripheral mammalian non neuronal cells: it has been detected in some

epithelial cells such as hepatocytes, lung alveolar cells, tubular cells of the kidney and Brunner's glands mucosal cells of the duodenum, as well as in muscle cells such as heart muscle fibers [7, 16].

Alpha-synuclein structural alterations and misfolding as well as its overexpression have been related to the onset and the progression of a group of human neurodegenerative disorders, known as synucleinopathies [17, 18]. Indeed, filamentous inclusions of α-syn are the main component of the Lewy bodies (Lbs) and the Lewy neurites (Lns), which are considered so far, the pathological hallmarks of Parkinson's Disease (PD) and dementia with Lewy bodies (LBD) [19]. Moreover, glial, and neuronal cytoplasmic inclusions characterizing Multiple System Atrophy (MSA), are mainly constituted by insoluble α-syn aggregates [20, 21]. Therefore, the formation of α-syn oligomers and the subsequent aggregation of α-syn in filamentous and insoluble inclusions, plays a central role in the pathology of synucleinopathies.

Recent studies suggest that pathological aggregates of α-syn can spread throughout the nervous system in a process of propagation similar to that characterizing the prion proteins [22]. α-Syn oligomers, in fact, are seeding-competent species leading to the formation of further α-syn aggregates once they enter the target cells. Besides inter-neuronal spreading, intracellular processing of α-syn leads to pathological changes in its binding properties with overexpression and excess of accumulation of α-syn resulting in globally compromised neuronal function, especially targeting synaptic vesicle trafficking and axonal transport [23].

α-Syn and its pathological accumulation are not confined to the central nervous system but have also been reported in the peripheral nervous system [24, 25]. α-Syn pathology can occur in the enteric neurons (ENS) [26], as well in peripheral autonomic ganglia such as submandibular and otic ganglion or paravertebral sympathetic ganglia. α-Synuclein pathology has been reported in salivary glands [27], in the myocardial plexus and in the autonomic fibers innervating salivary glands, sweat glands and pilo-erector muscles of the skin [27-30]. Therefore, synucleinopathies are characterized by a progressive impairment of motor, cognitive and autonomic functions, which might be related to the anatomical distribution of α-syn pathology. Moreover, the presence of α-syn in peripheral nervous system represents an interface with the pathology of the central nervous system which can be also used as a source of molecular biomarkers for disease diagnosis and follow-up.

The lesson coming from the "pure autonomic failure" (PAF), described by Bradbury and Eggleston since 1925 [31], further highlight the importance of the autonomic nervous system in the pre-clinical diagnosis of synucleinopathies. In their original work the authors reported an association of symptoms related to autonomic dysfunctions of the cardiovascular, gastrointestinal, urinary, and reproductive systems. Since then, several studies have described PAF showing degeneration of peripheral autonomic neurons and aggregation of α-syn in Lbs and Lns within sympathetic ganglia and along autonomic fibers of the heart, the bladder, the skin, and the gut [32]. It is well known that some patients affected by PAF undergo to a more severe synucleinopathy, such as PD or MSA, suggesting that, in some cases, PAF might be a pre-motor presentation of them [33]. Whether PAF is a really a pure and independent pathological condition rather than a "neglected presentation of PD" remains under debate, but the importance of the early involvement of the peripheral autonomic nervous system in synucleinopathies has kept increasing attention in the recent years, due to the compelling need of accessible pathological biomarkers.

ALPHA-SYNUCLEIN AND PD: A FOCUS ON THE ENTERIC NERVOUS SYSTEM

PD is the second most common neurodegenerative disorder after Alzheimer's disease. It is a progressive neurodegenerative disorder, which affects several regions within the central and peripheral nervous system, including the ENS. The etiology and pathophysiological events that lead to α-syn misfolding and aggregations are not clear: genetic, and environmental factors are involved, and they probably act either separately as together to induce α-syn pathology. Specific molecular alteration of α-syn have been related to point mutations of α-syn gene and are responsible of autosomal-dominant PD [34]. The formation of small α-syn aggregates, known as α-syn oligomers, represents the first step of α-syn misfolding and aggregation and recently, some cellular and molecular factors have been reported as key drivers of α-syn oligomerization. Among these, the activation of the inflammatory response with the recruitment of immune cells and the production of inflammatory cytokines has been related to the formation of α-syn aggregates and to their propagation from the gut to the brain along the vagus nerve [35].

The ENS is a largely independent neuronal network organized in two major plexuses, which are formed by ganglia and inter-ganglionic fibers and are present within the wall of the entire gastrointestinal tract: the myenteric plexus of Auerbach is mainly involved in the control of smooth muscle activity whereas the submucosal plexus of Meissner regulates glandular secretion and blood flow to the mucosa. Extrinsic sympathetic and parasympathetic nerves contribute to the regulation of motility and secretion. Pre-ganglionic

parasympathetic fibers originate in the dorsal motor nucleus of the vagus (DMV), and reach intra-mural ganglia devoted to the control of the motility of the esophagus, the stomach, the small intestine and the proximal portion of the large intestine until the right half of the transverse colon, whereas pre-ganglionic parasympathetic neurons originating at the level of the intermedio-medial nucleus of the sacral spinal cord contributes to control of motility of the distal colon, from the left half of the transverse colon to the rectum. The sympathetic pre-ganglionic neurons located in the intermedio-lateral nucleus of the thoraco-lumbar spinal cord, modulate the activity of the noradrenergic post-ganglionic neurons placed in the celiac and mesenteric ganglia and involved in the inhibition of gut motility and secretion. The coordinated activity of the ENS and the autonomic nervous system are crucial to maintain the physiological activity of the gastrointestinal tract.

Inflammation and microbiota can interplay a key role in driving the aggregation of α-syn into the gut and in the neurons of the ENS. Remarkably, several reports have shown the presence of Lbs in the ENS, since very early stages of Parkinson's Disease, even before the involvement of the Midbrain Dopaminergic neurons. Therefore, the ENS could be critical in the pathophysiology of PD and the pathological alterations within the ENS could be the anatomical substrate of the gastrointestinal dysfunctions frequently encountered in parkinsonian patients, since the beginning of the disease and often before the exordium of motor symptoms [26].

Friedrich Heinrich Lewy, a German neuropathologist first described in 1912 peculiar cytoplasmic inclusions that bear his name (Lbs) in the *substantia innominata* and in the DMV of patients affected by Paralysis agitans (eponymus of Parkinson's Disease). However, the presence of proteinaceous eosinophilic aggregates outside the central nervous system, in peripheral sympathetic ganglia, was reported since 1960 in patients suffering from the same pathology [36]. Lbs and Lns in the Auerbach's and Meissner's plexuses of patients affected by PD, have been reported in 1988 by Wakabayashi and co-workers [37]. Therefore, even before the discovery of α-syn as the main constituent of LBs, the pathological hallmarks of PD were found in the ENS. These morpho-pathological findings appeared to account for the autonomic symptoms of the alimentary tract frequently seen in PD patients and reported since the late 1950 [38]. More recently, several Authors have dedicated their efforts to detect a-syn aggregates and normal α-syn expression within the intestinal wall, [39]. In normal gut, widespread distribution of α-syn has been reported in the nerve fibers of the lamina propria and of the sub-mucosa, as well as in the ganglia of both submucosal and myenteric plexuses [25] (Figure 1).

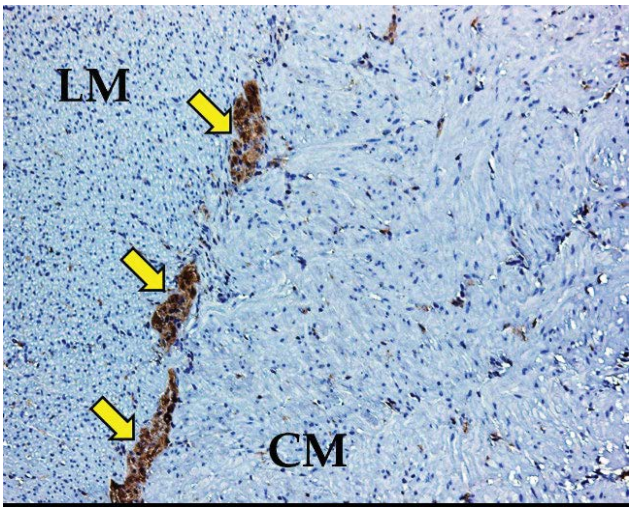

Figure 1. Representative image of several ganglia of the myenteric plexus (yellow arrows) in a normal human ileum. The brown color shows the immunoreaction for α-syn (sc-58480, Santa Cruz Biotechnology). In blue the muscle layers: outer longitudinal layer (LM) and inner circular layer (CM) contrasted with hematoxylin. OM 10x.

Moreover α-syn expression has been found in entero-endocrine chromaffin cells (EECs) where it co-localizes with serotonin [40]. Interestingly, α-syn containing EECs directly connect to α-syn containing nerve fibers, contributing to the formation of a neuro-endocrine circuit between the lumen and the nervous plexa of the gut. The presence of α-syn in this neuroendocrine circuit communicating with the lumen of the gut and exposed to the microbiota, might be involved in the beginning of α-syn misfolding and aggregation into the gut and therefore to the starting of propagation of α-syn pathology from the gut to the brain. In accordance, several studies have reported the presence of pathologic phosphorylated a-syn in different peripheral tissues including the gut [24, 41] and most of them have been made on patients with PD or other neurodegenerative disorders [42].

Following the hypothesis that PD might originate outside of the central nervous system, the large nervous network of the enteric nervous system is placed at a strategic position, being in close relation with a possible front door of pathogens or toxic agents, which are able, through inflammatory mechanism, to trigger the aggregation and the misfolding of α-syn. Moreover, the α-syn dependent degeneration of the enteric nervous system and of the EECs might contribute to the development of gastrointestinal dysregulation commonly found in PD patients and expressing with some key symptoms such as dyspepsia and constipation.

THE BRAAK HYPOTHESIS ON PD PROGRESSION

The idea that the gastrointestinal system is involved in very early stages of PD is widely accepted and supported by clinical and experimental observations [43]. In 2003 Braak and coworkers looking at the brain of patients with confirmed post-mortem diagnosis of PD, outlined a Parkinson's staging system, which describes the regional distribution and progression of protein aggregates in the brain. Interestingly they observed that lesions initially occur in the DMV and in the anterior olfactory nucleus, whereas, only in a subsequent stage, the classic involvement of the Substantia nigra pars compacta occurs [44]. During the same year, Braak theorized that the biological process underlaying PD may begin in the peripheral nervous system before reaching the brain [45].

The Braak hypothesis is based on the connection between the CNS and the ENS, also known as the brain–gut axis, capable of creating a bidirectional connection between the brain and the gastrointestinal tract. It represents a milestone of the current research about PD, because before these studies, PD was considered a disease affecting almost exclusively the CNS. This hypothesis was subsequently integrated with the dual-hit hypothesis, which supposed that an unknown pathogen enters the brain through two doors: the nose, with anterograde progression via the olfactory pathway and the gastrointestinal tract with retrograde progression via the vagus nerve to the brain stem parasympathetic nuclei [46].

The first hypothesis involves the olfactory system, that begins with sensory neurons containing olfactory receptors exposed to the mucus layer. They make synapses with apical dendrites of mitral and tufted cells in neuropil spheres termed glomeruli. Axons of mitral and tufted cells project to several olfactory structures: the anterior olfactory nucleus, olfactory tubercle, piriform cortex, peri-amygdaloid cortex, and the rostral entorhinal cortex [47]. Among olfactory structures, the anterior olfactory nucleus is preferentially involved in synucleinopathies in the early stages of the disease, and it has been hypothesized that this is due to its multiple connections in and out of the olfactory system. The impaired olfactory function has been in fact reported as an early indicator of PD, although the evidence is not homogeneous regarding a simple linear anterograde progression. Instead, progression of α-syn aggregation through the olfactory system can help explain cases of PD that are not related to retrograde vagal spread. Therefore, although the vagal (retrograde) and olfactory (antegrade) pathways appear separate, they can be interconnected at different levels and there is the possibility of multiple simultaneous spread of α-syn [48]. Furthermore, according to Braak's hypothesis, pathogens in the nose could be swallowed with nasal secretions and saliva and reach the gut, which is the second access in the dual-hit hypothesis [46]. The swallowed pathogen may pass through the mucosal barrier of the gastrointestinal tract, infect enteric neurons of myenteric plexus and submucosal plexus, and invade the parasympathetic motor neurons of the vagus nerve in a retrograde fashion. The dual theory is strongly supported by the evidence that Lewy pathology first appears in the olfactory bulb, as well as in parasympathetic neurons of the DMV [49].

The Braak hypothesis, created a revolution in the pathological concept of PD, supporting the hypothesis that the spreading pathogens responsible of the pathological progression were the seeding competent aggregates of α-syn, which originate in the ENS and migrate rostrally to the CNS by the vagal pathways [50]. The presence of α-syn immunoreactive inclusions within the neurons of the submucosal plexus, whose axons project to the mucosa, support Braak's hypothesis of PD [51] and introduce also the concept that luminal factors, such as the molecular products of microbiota might contribute to the development of α-syn aggregates in the mucosal nervous fibers, which are then in turn transmitted to the submucosal plexus and finally to the myenteric plexus, from which they reach the vagal terminations. Additional support for Braak's hypothesis came from functional studies, that underline a significantly greater intestinal permeability in PD subjects compared to the healthy individuals, correlating with increased aggregation of α-syn in the intestinal mucosa [52]. Moreover, another study provides evidence that α-syn is physiologically secreted by enteric neurons and can be up-taken by contiguous neurons [53]. To further corroborate the hypothesis that α-syn could propagate from the ENS to the CNS through the vagus nerve, in 2014 Holmqvist and colleagues isolated different α-syn species (fibrillar, monomeric and oligomeric) from the brain lysate of a patient affected by PD and directly injected them into the submucosa of the gut in rats, demonstrating that α-syn species from the PD brain lysate were able to spread from the intestinal wall to the brain via the vagus nerve [54]. In accordance, different studies in both humans and animal models [55], have reported that truncal vagotomy, decreases the risk of developing PD and the spreading of α-syn pathology from the gut to the brain [56].

Braak's theory must satisfy three criteria. First, there should be an uninterrupted pathway that expresses α-syn throughout its trajectory, that should allow the retrograde transport of the pathological forms of a-syn from the gastrointestinal tract to the CNS. Second, enteric neurons should be able to secrete α-syn to transmit the disease from the ENS trough the vagus nerve. Third, there should be a link between α-syn

and the ENS. The first condition is fulfilled as Holmqvist and colleagues have elegantly shown that α-syn is deposited in a retrograde mode from the ENS to the vagal efferent axons and terminals, which originate from the DMV [54]. Regarding the second condition, Paillusson and coworkers demonstrated that α-syn can be secreted by enteric neurons and thus transmit the pathology from neuron to neuron [53]. The third condition has been satisfied by Chandra and colleagues, who have reported that α-syn is expressed in the EECs. EECs are part of the gut APUD system, and they can directly face the lumen or be placed at the level of the intestinal crypts. They possess neuroendocrine function and highly express α-syn, moreover, they closely connect with the neuronal fibers of the submucosal plexus [40]. For these reasons, they represent an interface between the lumen of the gut and the enteric nervous system and therefore they could be the first affected by α-syn pathology and aggregation [25] (Figure 2). Changes in the microbiota, and development of mucosal inflammatory processes, in fact, might activate EECs and induce the misfolding and the aggregation of α-syn, thus playing a pivotal role in the gut-to-brain transmission of α-syn pathology [57]. These findings support the original Braak's pathogenetic hypothesis that PD could be the result of progressive transmission of α-syn aggregates originating at the level of the mucosal layer of the gastrointestinal tract and reaching the CNS via unmyelinated preganglionic fibers of the vagus nerve [45].

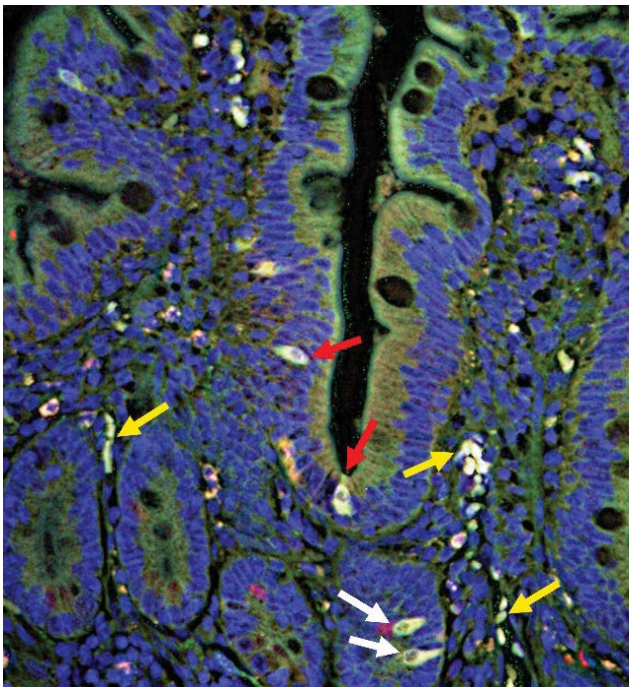

Figure 2. Immunofluorescence for showing the expression of α-syn (sc-58480, Santa Cruz Biotechnology) in normal human jejunum. The presence of α-syn has been studied in EECs of intestinal epithelium and red arrows indicate α-syn-positive cells (with a green fluorochrome) in the villi, whereas white arrows point α-syn positive cells in a gland at the base of the villus. Then, yellow arrows show α-syn positive enteric nerve fibers in contact with epithelial cells. Nuclei (blue) were counterstained with DAPI. OM 20x.

ADVANCES AND CONTROVERSIES OF A-SYN PROPAGATION FROM THE GUT TO THE BRAIN

In support of Braak's hypothesis, recent studies have shown that not only vagotomy but also appendicectomy decreased the risk of developing PD [58]. In fact, has been demonstrated in a large cohort of patients affected by PD, that a subtle inflammation of the vermiform appendix is present and that this leads the aggregation of α-syn within the appendix [59]. From the appendix the aggregates of α-syn could spread to the vagal efferent fibers and reach the nuclei of the brainstem, correlating the subsequent degeneration of the dopaminergic neurons [58]. Over the last years, several experimental evidence have supported the theory of propagation of α-syn aggregates from the gut to the brain, mainly based on the inoculation of α-syn pre-formed fibrils into the intestinal wall [60, 61]. α-Syn fibrils were injected into murine duodenal and pyloric muscularis layer and α-syn aggregates were found in the DMV and in other areas of the brainstem, such as the locus coeruleus and the substantia nigra. In other studies, α-syn fibrils were injected into the duodenum wall of wild-type (WT) and transgenic rats overexpressing human α-syn, showing *trans*-synaptic diffusion of α-syn pathology along the parasympathetic and sympathetic pathways. Moreover, the same authors found α-syn inclusions in the stomach and heart suggesting a secondary anterograde propagation after the initial retrograde transmission. For the first time, they studied the potential secondary anterograde (DMV-to-stomach) transmission of α-syn pathology, after the first retrograde (duodenum-to-DMV) one [62], suggesting that gastrointestinal α-syn pathology could diffuse to the DMV and infect the neuronal nuclei, and then diffuse anterogradely through DMV efferences going back to the gastrointestinal system and the other peripheral organs.

However, clear, and detailed experimental evidence is still lacking regarding the mechanisms by which α-syn pathology of the ENS can diffuse to the brainstem and more specifically to the substantia nigra pars compacta (SNpc), with consequent motor dysfunction. Many controversial experimental data have been reported. For example, the inoculation of PFFs into the gastric wall of WT type mice induced LB-like α-syn aggregates in the DMV, but they decrease in number over

time [63]. Furthermore, a similar inoculation of a-syn PFF into the stomach and colon of rats and non-human primates (NHP) failed to recapitulate a PD-like α-syn pathology into the brains at 12 months post-inoculation. The formation of aggregates was confined to the enteric neurons, with pathological α-syn in the brainstem only present in the vagus nuclei but not at the level of the substantia nigra [60]. For these reasons several Authors consider unlikely that α-syn can reach the brain stem from enteric neurons and diffuse to the substantia nigra inducing classical motor symptoms of PD and suggest that the enteric and the central α-syn pathologies could represent distinct, although concomitant pathological features of PD [42].

The presence of a-syn in the entire gastrointestinal tract and the evidences suggesting the development of enteric α-syn pathology in PD patients, might indicate that α-syn aggregation could be responsible of the functional gastrointestinal disorders reported in PD and maybe due to the synaptic vesicles dysfunction in the enteric nervous system [60]. On the other hand, recent studies are pointing out that PD is a heterogeneous disease and that different PD phenotypes exist, being some of them more subject to inter-neuronal propagation and disease progression [64]. More recently, it has been postulated the possible occurrence of a brain first versus a body first PD [65], being the latter originating in the gut and then propagate to the brainstem with a more severe disease progression comparing to the brain-first subtype.

Therefore, even if Braak's hypothesis remains controversial, the study about the α-syn pathology in the gastrointestinal tract represents a milestone of the current research in PD. Comprehension of the gut-to-brain transmission of α-syn pathology might help in stratifying PD patients according to their clinical and pathological subtype in order to predict disease progression and might shed new light on the mechanisms of α-syn inter-cellular spreading contributing to the development of new therapeutic strategies for PD.

ACKNOWLEDGMENTS

We thank Ludovica Garro, Sapienza University of Rome, for her assistance with the revision of the manuscript and for their comments that improved the present review article.

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