Enteric synucleinopathy: from trendy concept to real entity.

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Submitted: 29 July 2020 · Accepted: 23 August 2020 · Copyedited by: Aivi T. Nguyen · Published: 28 August 2020

Abstract

An accumulating body of literature has emerged in the past 25 years to show that Parkinson’s disease (PD) is not only a disorder of the brain but also of the gastrointestinal tract and more generally of the gut-brain axis. Gastrointestinal symptoms occur in almost every PD patient at some point and in nearly every case examined pathologically autopsy studies find alpha-synuclein deposits, the pathological hallmarks of PD, in the enteric nervous system. This concept of ‘enteric synucleinopathy’ led to the hypothesis that the enteric nervous system might play a pivotal role in the initiation and spreading of PD. Although this hypothesis opens up interesting perspectives on the pathogenesis of neurodegenerative disorders, some important questions are still pending. The present opinion paper describes and compares the physiological and pathophysiological properties of alpha-synuclein in the brain and the enteric nervous system. We conclude that the existing data supports the existence of pathological alpha-synuclein species in the gut in PD. We also discuss if gut-brain interactions are important in other neurodegenerative disorders.

Keywords: Synucleinopathies, Tauopathies, Enteric nervous system, Aggregates, Alpha-synuclein, Parkinson’s disease

Abbreviations

AD - Alzheimer’s disease, ENS - enteric nervous system, GI - Gastrointestinal, LB - Lewy bodies, LN - Lewy neurites, PD - Parkinson’s disease, PMCA - protein misfolding cyclic amplification, PSP - progressive supranuclear palsy

The enteric nervous system (ENS) is an intricate neural network embedded within the gastrointestinal (GI) tract and distributed from the lower oesophagus to the rectum. Compared to other components of the peripheral nervous system, the ENS shows some unique features that closely resemble the CNS and therefore it is sometimes referred to
as ‘the brain-in-the-gut’ or the ‘second brain’ [1] (Figure 1). This close homology between the CNS and ENS suggests that a disease process affecting the CNS could also involve its enteric counterpart. Parkinson’s disease (PD) is the best example of this assumption. PD is the most common synucleinopathy (or synuclein proteinopathy), a group of neurodegenerative disorders characterized by a common pathological lesion composed of aggregates of alpha-synuclein in selectively vulnerable neuron populations in the CNS [2]. Although PD has traditionally been considered a disease of dopaminergic neurons in the substantia nigra, analyses of GI samples from PD patients have consistently found neural pathology, with the presence of alpha-synuclein deposits being detected in the ENS in nearly every PD patient examined [3–11]. This has led to the emerging concept of enteric synucleinopathy. Although these observations open up interesting perspectives on the pathogenesis of neurodegenerative disorders, some important questions are still pending, among which are: what are the biochemical and pathological characteristics of alpha-synuclein deposits in the ENS? Are they similar to those observed in the CNS? Is there any neuronal loss in the ENS in PD? Besides PD, are gut-brain interactions important in other neurodegenerative disorders? Here, we first provide a brief overview of the normal expression profiles of alpha-synuclein in the ENS, before discussing these questions and the arguments for and against the existence of enteric synucleinopathies.

**Alpha-synuclein is physiologically expressed by enteric neurons**

Alpha-synuclein was first isolated in 1988 from the electric organ of the Pacific electric ray _Torpedo californica_. In addition to its strong presynaptic localisation, Maroteaux et al., also identified alpha-synuclein in the nucleus, thus accounting for the name ‘synuclein’ (SYNapse + NUCLEus) [12]. Several studies have documented the physiological function of alpha-synuclein in modulating synaptic vesicle release [13]. Human alpha-synuclein, 140 amino acids in length, is mainly expressed by CNS neurons and erythrocytes and is composed of 3 different functional regions (Figure 2a) [13]. There is still open debate about the physiological structure of brain alpha–synuclein as some research groups concluded that it occurs as a helically folded tetramer [14], while others counterclaim that it exists primarily as a disordered monomer [15]. Alpha-synuclein has a natural propensity to aggregate as amyloid structures in a nucleation-dependent process in which monomers assemble via oligomers into fibrils [16] (Figure 2b).

Alpha-synuclein is also expressed by enteric neurons. In rodents, guinea-pigs and human alpha-synuclein positive neurons are present in the two plexuses of the ENS and along the entire digestive tract [5, 17, 18] (Figure 3). Using amine-reactive cross-linking, we showed that, unlike brain neurons, alpha-synuclein exists primarily as a monomer in enteric neurons [19]. As far as we know, the expression of alpha-synuclein by enteric glial cells, the enteric counterpart of CNS astrocytes, has not been evaluated. Detailed immunohistochemical characterisation showed that alpha-synuclein immunoreactive neurons are mostly cholinergic [17, 18] (Figure 1) and that alpha-synuclein is closely associated with the vesicular apparatus [20]. Although the role of alpha-synuclein in the ENS is still mostly unknown, a recent study suggested that it is involved in the development and electrophysiological properties of enteric cholinergic neurons [21].

**Does the ENS contain pathological aggregated forms of alpha-synuclein?**

Lewy bodies (LB) and Lewy neurites (LN) are the defining neuropathological characteristics of PD. LB typically appear in neuronal somata as eosinophilic, rounded inclusions while LN are strand-like structures observed in axons. In 1997, it was demonstrated that LB and LN isolated from PD brain were highly immunoreactive for alpha-synuclein, thereby suggesting that this protein was one of the main components of Lewy pathology [22]. Subsequent work showed that alpha-synuclein in LB and LN was hyperphosphorylated at serine residue 129 [23] (Figure 2). Because alpha and phospho-alpha-synuclein immunohistochemical staining has a much greater sensitivity than hematoxylin and eosin staining for the detection of LB [24], this method quickly became the method of
Figure 1. Anatomical organisation and local reflexes of the enteric nervous system. The ENS is a neuronal network embedded within the GI tract and distributed from the lower oesophagus to the rectum. It is organized in two major ganglionated plexuses, the myenteric (MP or Auerbach's plexus) mainly involved in the control of smooth muscle activity, and the submucosal (SMP or Meissner's plexus), which regulates secretion (SN, secretory neurons) and microvasculature. Compared to other sections of the peripheral nervous system, the ENS shows unique features that closely resemble some of the CNS: it contains a variety of functionally distinct enteric neurons along with a vast repertoire of neurotransmitters and intercellular messengers which are the basis for enteric neurotransmission. It also harbours a prominent component of glial cells (EGC for enteric glial cells) which, like astrocytes in the CNS, contribute to support, protection and maintenance of the neural networks. Local distention of the intestinal wall and chemical contents in the gut lumen activate intrinsic primary afferent neurons (IPAN) located in both the SMP and MP. The IPAN projects both in oral and anal directions to synapse with interneurons (IN) and motor neurons (MN). Cholinergic (ACh) MN in red are excitatory while nitrergic (NO) MN in green are inhibitory. The peristaltic reflex includes an ascending excitatory reflex mediated by cholinergic MN and elicits contraction of the circular or longitudinal smooth muscles located orally to the site of stimulation. The descending inhibitory reflex involves inhibitory nitrergic MN that elicit relaxation of the circular muscles and longitudinal muscles located anally to the site of stimulation.

choice for the neuropathological diagnosis of PD [25]. Using this approach, several neuropathology laboratories demonstrated that LB and LN-like structures were observed in the ENS (in both the myenteric and submucosal plexus) in the vast majority of PD patients [3–7] (Figure 3). In some of these studies, proteinase K [3] or alkaline protease [7] pre-treatments were used primarily to unmask antigens and enhance immunolabeling, but such treatments might also allow protease-resistant misfolded, aggregated and hyperphosphorylated alpha-synuclein to be distinguished from soluble forms of the protein. On the whole these findings suggest that pathological aggregated alpha-synuclein is present within the GI tract of PD subjects. Two key issues should however be borne in mind. First, the mere detection of alpha-synuclein phosphorylated at serine 129 is not synonymous with aggregation since soluble alpha-synuclein is also physiologically phosphorylated at this residue [26]. Secondly, immunohistochemical approaches are not sufficient to show that alpha-synuclein is misfolded/aggregated; biochemical confirmation of altered solubility is required. Indeed, in the CNS, a comprehensive biochemical characterisation of alpha-synuclein forms in LB has already been car-
ried out. Using one and two-dimensional immunoblot analysis with modification-specific synuclein antibodies and mass spectroscopy, Anderson et al. confirmed that the predominant modification of alpha-synuclein in LB was phosphorylation at serine 129. They also found a set of additional characteristic modifications including ubiquitination at amino-terminal lysine residues and specific carboxy-terminal truncations [27] (Figure 2). An additional property of alpha-synuclein aggregates found in diseased brains is their seeding ability: the amyloid fibrils formed by alpha-synuclein aggregates can act as templates for the conversion of physiological alpha-synuclein, resulting in the growth of the fibrils and spread of alpha-synuclein pathology [28].

How about the biochemical characterisation of alpha-synuclein in diseased ENS? There are some existing data but it is definitely less complete and robust than those available for the CNS. Using one and two-dimensional analysis of colonic biopsies, we were unable to detect any differences in the expression levels, phosphorylation or aggregation status of alpha-synuclein between controls and PD specimens [29]. These negative findings might however be explained by the relative sparsity of neuronal structures and/or alpha-synuclein inclusions in the GI samples that were used; due to a shortage of samples, only 2 routine colonic biopsies per subject were pooled and analysed. However, when we had the opportunity to analyse 4 pooled biopsies per subject, we were able to detect acidic and high molecular weight alpha-synuclein species in the GI tract of PD patients but not controls, which likely represent hyperphosphorylated and aggregated forms of the protein, respectively (Lebouvier-Derkinderen unpublished results, Figure 4). Such a pattern is reminiscent of that observed in PD brain [27] (Figure 4). The limitations of the two-dimensional immunoblotting technique prompted us to use more sensitive approaches, based on the seeding efficiency of alpha-synuclein. Using an assay inspired by the protein misfolding cyclic amplification (PMCA) assay, we showed that GI biopsies from PD patients (2 to 4 biopsies per subject taken from the upper or lower GI tract) seeded alpha-synuclein aggregation in 10 out of 18 cases [30].

More recently, Viviane Labrie and Patrik Brundin’s group focused on the vermiform appendix, a structure which is particularly enriched in alpha-synuclein [31].

Figure 2. Alpha-synuclein structure and molecular mechanism of its oligomerisation and fibrillogenesis. (a) Schematic representation of alpha-synuclein, which is composed of 3 distinct regions: (i) an N-terminal domain (amino acids 1–60) that binds lipids and contains the lysines that are ubiquitinilated (ii) a central domain known as the non-amyloid component (NAC) (amino acids 61–95) which is involved in aggregation, and (iii) a C-terminal acidic tail (amino acids 96–140) accountable for most interactions with other proteins and small molecules and that contains most of the phosphorylation sites including serine 129. Some of the truncated C terminal forms of alpha-synuclein are also shown. (b) Illustration of the molecular steps involved in alpha-synuclein oligomerisation and fibrillogenesis leading to Lewy body formation. Soluble alpha-synuclein is natively unstructured and monomeric. Under pathological conditions, soluble α-synuclein forms β-sheet-like oligomers (protofibrils), which convert into amyloid fibrils and eventually deposit in Lewy bodies.
Figure 3. Physiological expression of alpha-synuclein and phospho-alpha-synuclein histopathology in the ENS. Left: anti-alpha-synuclein antibody Syn-1 was used to detect alpha-synuclein in the myenteric ganglia (colon) in a control subject devoid of neurodegenerative disorders; βIII tubulin antibody was used to label the neuronal network. Scale bar is 50 µm. Right: the colon from a PD patient was microdissected into mucosa, whole mount of submucosa and myenteric plexus. Each part was stained with an antibody specific for the phosphorylated form of alpha-synuclein. Phospho-alpha-synuclein histopathology with a LN-like pattern is observed in the mucosa (arrow, the asterisk is for the crypt) and submucosa, while a LB-like structure is found in the myenteric plexus. Scale bar is 40 µm.

Using mass spectrometry, they identified full-length alpha-synuclein together with a set of 10 truncated forms in the healthy human appendix [32]. Additional experiments performed with an in vitro shaking assay (also inspired by the PMCA approach), showed that appendix lysates from either control or PD subjects seeded aggregation and truncation of alpha-synuclein. These intriguing and provocative findings suggest that aggregated and truncated alpha-synuclein are consistently found in the human healthy appendix, thereby supporting the assumption that the appendix may act as a reservoir for pathogenic forms of alpha-synuclein [31, 32].

It has also been shown that pathological alpha-synuclein obtained from PD brain has the ability to trigger alpha-synuclein pathology in the CNS of rodents and non-human primates [33, 34]. For example, the intranigral inoculation of alpha-synuclein-containing LB extracts obtained from PD brains leads to widespread alpha-synuclein pathology together with dopaminergic neurodegeneration in mice and monkeys [35]. For the sake of comparisons between brain and enteric alpha-synuclein, it is important to know if pathological alpha-synuclein obtained from the GI tract shares the same pathogenic capacity. Although the precise answer to this question remains unknown, a recent study evaluated the effects of alpha-synuclein aggregates from post-mortem PD stellate ganglia (pooled from 3 patients) injected into mouse brain [36]. Stellate ganglion is a paravertebral ganglion, which like the gut, exhibits marked Lewy pathology in almost all PD subjects [4, 37]. In contrast to the findings obtained with substantia nigra-derived alpha-synuclein, no pathological effects were observed when peripheral aggregates from stellate ganglion were injected into the brain of wild-type mice, at least up to 6 months following injection [36]. This intriguing observation, which casts doubt on the pathogenicity of peripheral alpha-synuclein, requires replication using extracts from other peripheral organs affected by Lewy pathology, such as the GI tract or the salivary glands [3, 38].
Is there any enteric neuronal loss in PD?

In the substantia nigra of PD subjects, the presence of alpha-synuclein aggregates is accompanied by severe neuronal loss and clinicopathological findings strongly suggest that the classical PD motor symptoms, including bradykinesia and hypertonia, are driven primarily by neuronal loss rather than only the aggregation of alpha-synuclein [39]. As such, the quantitative evaluation of enteric neuron populations is important to determine the pathological underpinnings of GI symptoms, which are so frequently observed in PD [40]. With that said, and despite the publication of proposed guidelines and consensus techniques, the quantification of submucosal and myenteric neuron number remains challenging [41], mainly because of the fishnet-like architecture of the ENS plexus. In the most comprehensive post-mortem study to date, Annerino et al. used formalin-fixed paraffin embedded sections to compare myenteric neuron density along the length of the GI tract in 6 patients with PD and 12 controls. There were no differences in total myenteric neuron density between controls or patients in any segment examined [42]. Similarly, when whole mounts of submucosa obtained from colonic biopsies were analysed by immunohistochemistry, no major decrease in neuronal density was observed in PD relative to controls in two independent studies that included a total of 58 PD and 30 control cases [8, 43]. On the whole, these data strongly suggest that enteric neuron loss is not a feature of PD.

Figure 4. High molecular weight and post-translationally modified alpha-synuclein species in the GI tract of PD patients. Immunoblots of total mesencephalon lysates (A and B) from a control (A) and a PD patient autopsy sample (B), and immunoblots of colonic biopsy lysates (C and D) from a control (C) and PD patient (D) were resolved by two-dimensional PAGE using pH 3–10 IEF gradients. Immunoblots were probed with the alpha-synuclein antibody Syn-1. White asterisks mark full-length synuclein. Boxes highlight differences between PD patients and controls: acidic modifications of full-length monomer (solid boxes), and high molecular weight species (dotted boxes) are present in PD patients and absent in controls, in both substantia nigra and colonic biopsies (n=2); pI values and positions of molecular weight standards are indicated. An equivalent amount of protein was loaded in A-B and C-D, respectively.
Besides alpha-synuclein, are other aggregated proteins found in the ENS?

The observation that alpha-synuclein deposits are a feature of PD gut logically leads to speculation that such a phenomenon might also occur in other neurodegenerative disorders such as tauopathies. Like alpha-synuclein, tau is physiologically expressed by enteric neurons. However, in contrast to the CNS neurons that express all six tau isoforms, adult human ENS primarily express only two tau isoforms [44]. Tau aggregates found in tauopathies generally contain tau in an elevated state of phosphorylation that is often abnormally cleaved [45]. In a preliminary report, we analysed by Western blot colonic biopsies from 5 patients with probable progressive supranuclear palsy (PSP) using 2 different phospho-tau antibodies and one antibody specific for caspase-cleaved tau [44]. The phosphorylation and truncation patterns of tau in PSP were indistinguishable from those in controls [44]. In a subsequent study, Brittany Dugger et al. performed a comprehensive immunohistochemical analysis of the ENS in tauopathies. Using formalin fixed paraffin embedded sections, they examined the sigmoid colon in 26 PSP, 21 AD and 19 controls using one antibody for total tau and two phospho-tau antibodies [46]. No differences in the staining pattern were observed between colonic specimens from tauopathy patients and controls [46].

So, enteric synucleinopathy: myth or reality?

Even if they are still preliminary, recent findings and in particular the ones obtained with ultra-sensitive amplification techniques such as PMCA, convincingly showed that alpha-synuclein aggregates with seeding capacity are found in the ENS [30, 32]. It therefore seems justified to use the word ‘enteric synucleinopathy’. That said, some outstanding questions remain: (i) existing findings suggest that enteric neuron loss is not a feature of PD but there could be more subtle morphologic changes [47] that could be evaluated in the future using computer-assisted analysis of the enteric neurons [48] (ii) a comprehensive inventory of alpha-synuclein forms present the ENS from patients with PD still need to be carried out, as has already been performed for the CNS [27] (iii) the results obtained with PMCA need to be independently replicated and confirmed by others amplification techniques such as RT-QuIC (real-time quaking-induced conversion) [49]. In addition, it still remains to be determined if pathological alpha-synuclein purified from the ENS is capable of promoting alpha-synuclein pathology when intracerebrally inoculated, as already demonstrated for CNS-derived alpha-synuclein aggregates [50]. This is a critical issue with regard to PD pathogenesis as it has been speculated that enteric alpha-synuclein aggregates may spread to the CNS via the vagal connections. However, performing such experiments, which require purification of LB-containing fractions from the gut, can be challenging due to the low frequency and density of aggregated alpha-synuclein in the ENS [3, 51]. On the whole, current knowledge strongly supports the existence of enteric synucleinopathy and these data encourage future studies aimed at expanding our knowledge of peripheral pathology in neurodegenerative disorders to gain clues that enable further understanding of the differential pathogenesis of these disorders.

References


