The Enteric Nervous System in PD: gateway, bystander victim or source of solutions

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Abstract

Apart from the characteristic and progressive motor and movement related problems, Parkinson's disease (PD) patients also suffer from several non-motor symptoms, including gastrointestinal dysfunction. The fact that the enteric nervous system (ENS) controls motility and that one of the typical PD hallmarks, α -synuclein positive deposits, has also been found in the intestinal wall, have rendered the ENS and the gut a popular subject of study in the context of PD. The possibility that these deposits could serve as an early biomarker, is obviously of tremendous medical benefit, but also the idea that the gut may possibly be a gateway via which the disease is initiated and progressively makes its way via the peripheral nerves to the central nervous system, has increased the interest in the ENS – PD link. Furthermore, the fact that gastrointestinal symptoms are present in PD, suggest that the ENS might be affected as well. However, despite a lot of literature on the topic, the pure role or the actual involvement of the ENS in PD remains elusive. The multitude of experimental approaches and animal models have complicated the interpretation of results and the outcome of different studies does not necessarily align well. In this review, we chose to highlight some elements of interest and some items of confusion, particularly those where research should be focusing. We also list a number of open questions in the field that could serve as a guideline for future, preferably concerted research.

Introduction

Parkinson's disease (PD) is the second most important neurodegenerative disorder after Alzheimer's disease and the most prevalent motor disorder. Apart from typical motor problems (bradykinesia, rigidity, postural instability and resting tremor), PD patients' quality of life is also severely undermined by a variety of non-motor problems such as autonomic insufficiency, sleep and neuropsychiatric symptoms, and cognitive decline. The current weight of evidence supports both genetic and environmental determinants of disease risk.

The pathological hallmarks of PD are loss of dopaminergic neurons in the *substantia nigra* (SN) and other brainstem nuclei, and the presence of cytosolic α -synuclein (AS) rich inclusions called Lewy bodies in the remaining neurons. However, cell death and Lewy pathology in PD are not restricted to the brainstem, but also affect other neuronal populations in the brain and even in the peripheral nervous system. In the last couple of decades the hypothesis that peripheral manifestations of PD might be useful to study or diagnose the central nervous system (CNS) pathology has received increasing attention. Many studies have indeed reported the presence of Lewy body-like pathology in other organs (skin, adrenal medulla, bladder, cardiac and intestinal tissue) (Malek et al. 2014) and in the autonomic nervous system, especially in the Enteric Nervous System (ENS), (Lebouvier et al. 2008, 2010; Pouclet et al. 2012, Shannon et al. 2012) a ganglionated network of nerves embedded in the intestinal wall (**Fig 1**). Recently, there has been particular interest in the gastrointestinal (GI) tract as a potential portal of entry for a causative agent in PD because (1) the GI tract offers the largest surface for interaction with an environmental disease trigger; (2) it has vital functions including its role as a barrier to entry of pathogens and its importance in the innate immune system and (3) the sheer scale of the ENS, often referred to as "the second brain."

The ENS consists of two concentric layers containing neurons and glia, both derived from neural crest cells that migrate into and colonize the gut during embryogenesis (Hao et al. 2015). The submucous plexus (or Meissner plexus) is located closest to the mucosa and is key in controlling blood flow, secretion and absorption, while motility related events are dealt with by the myenteric plexus (or Auerbach's plexus), embedded between the circular and longitudinal muscle layer (**Fig 1**). The coordinated actions of different enteric neuron subtypes and the muscle result in the ability to either propel or stall the luminal contents depending on nutritional or caustic content. Enteric nerves and glial cells also interact with various other cells like the epithelium (Neunlist et al. 2013) with its endocrine cells (Depoortere 2015) and immune cells (Veiga-Fernandez and Pachnis, 2017) to generate a correct balance between tightly sealing the gut wall to defend the host and uptake of nutritional components. Apart from the intrinsic nerves, also extrinsic nerve fibers, (Berthoud et al. 1991, Powley et al. 2011, Brookes et al. 2013) extend into the different layers of the gut to convey mainly sensory information to the central nervous system, either via the vagus nerve or via spinal afferents.

The general and current working hypothesis that brings the gut and PD together is based on a couple of converging observations (GI predictors of PD development, AS aggregation), lines of thought

(environmental toxins) and analogies with progression of other diseases (Prion). Epidemiological studies suggest that constipation predicts the clinical development of PD, and CNS Lewy body pathology at autopsy as well as neuroimaging changes associated with increased risk PD (Petrovich et al. 2009, Ross et al. 2012, Jennings et al. 2014,). Other studies suggest that the risk of developing PD may be lower in people who have had truncal vagotomy, which interrupts parasympathetic innervation of the proximal gut (Svensson et al. 2015, Liu et al. 2017). Braak and others have suggested that the ENS might be a gateway for a pathogenic agent to gain access to the CNS, via the vagus nerve to the dorsal motor nucleus of the vagus in the medulla oblongata, thought by some to be the earliest area affected by the disease (Braak et al. 2002, Lewy 1914). This hypothesis has gained support from human studies suggesting that the abnormal AS conformation might spread from diseased cells to healthy cells (Kordower et al. 2008). Alternatively, a disease causing agent could gain access to the bloodstream and produce CNS disease by a systemic mechanism.

A number of clinical studies have found changes in intestinal function or the intestinal environment that might expose the ENS to toxic or pathogenic substances. These findings include increased intestinal permeability with penetration of bacterial antigens into the intestinal wall, (Forsyth et al. 2011) and a change in the intestinal microbiota favoring pro-inflammatory bacteria across disease severity in PD and even in prodromal PD (Heintz-Buschart et al. 2018; Hill-Burns et al. 2017, Petrov et al. 2017; Scheperjans et al. 2015, Unger et al. 2016). However, the dominant focus in the gut-brain hypothesis in PD has been on the demonstration of AS immunostaining in the mucosa or submucosa of the GI tract. The ease with which tissue samples can be obtained and the common practice of endoscopic colon cancer screenings in the age group at highest risk to develop PD have built interest in this research strategy, and it has face validity given interest in the Braak hypothesis of PD genesis. (Braak et al. 2006). While early studies suggested positive AS immunostaining had high specificity and sensitivity to distinguish PD from healthy subjects (Lebouvier et al. 2010, Shannon et al. 2012). Subsequent studies failed to replicate the findings, a failure due - at least in part - to multiple methodological differences (Ruffmann and Parkinen, 2016). Thus the preponderance of clinical evidence can be described as circumstantial in nature, and the role of the ENS in the genesis or progression of PD remains unknown.

The evidence for ENS, or in more general terms, GI involvement in the pathogenesis of PD has been the subject of a number of comprehensive reviews (Malek et al. 2014, Lionnet et al 2018; Borghammer 2018; Derkinderen et al. 2011; Parashar and Udayambanu, 2017; Houaser and Tansey, 2017; Braak and Del Tredici, 2017; Pellegrini et al 2016; Del Tredici and Braak 2016; Corbille et al. 2016; Mulak and Bonaz, 2015; Klingelhoefer & Reichmann 2015; Comi et al. 2014; Cersosimo and Benaroch 2008; Rao and Gershon 2016; Natale et al 2011; Chung et al. 2016; Cersosimo 2015; Leclair-Visonneau et al 2017, Sanchez-Ferro et al. 2015, Schneider et al. 2016). In this review, we chose to highlight some elements of interest and some items of confusion, particularly those where we think research should be focusing, especially for new people entering the ENS/PD research field. We also try to disentangle some lines of thinking.

Three intermingling lines of thought

Three different, though often-intermingled lines of thought can be identified in the vast body of work concerning the involvement of the GI tract in Parkinson's disease and possibly also other neurodegenerative disorders.

First, early reports of Lewy pathology in the GI tract (esophagus and colon) (Qualman 1984, Kupsky 1987, Wakabashi 1988) gave rise to the concept that GI tissue might prove useful as a disease marker. The frequency of early GI symptoms in PD contributed to the potential importance of this line of research. The discovery that Lewy bodies are largely composed of phosphorylated or misfolded AS provided a useful tool for the identification of specific cellular aggregates in PD. Thus, 20 years after the initial reports of Lewy pathology in the GI tract, Braak (Braak et al. 2006) reported the presence of AS immunoreactive inclusions in the submucous and myenteric plexi of autopsied subjects with incidental cerebral Lewy pathology. He proposed that enteric AS pathology might "spread" via the vagus nerve to the dorsal motor nucleus of the vagus in the medulla oblongata, then spread rostrally in the brain to produce the pattern of CNS AS pathology seen in PD.

Second, is the possibility of an exogenous agent or drug that might cause the disease. Current hypotheses about the genesis of PD suggest an interaction between genetic susceptibility and environmental exposure. The GI tract presents the largest surface area with which environmental agents might interact. The GI epithelium allows the selective entry of beneficial or nutritive agents while excluding toxins and pathogens and is also an essential driver of innate immunity (Veiga-Fernandez and Pachnis, 2017). Alterations in intestinal permeability (Neunlist et al. 2013), mediated by the enteric nervous system, could result in increased exposure to harmful environmental agents. The endogenous protein AS is widely expressed in the ENS, and structural changes in morphology might lead to the spread of aberrant protein conformations in a "prion-like" manner. This links back to the first line of thought.

Third, are possible derangements of GI motility, reflected in symptoms like constipation and dysphagia (Mukherjee et al. 2016, Fasano et al. 2016). Since the ENS controls GI peristalsis, one might conclude that motility abnormalities should be reflected in loss of enteric neurons. Thus neuron counts might be expected to be abnormal in PD. While some studies suggest a loss of neurons in PD patients (Lebouvier et al. 2010, Barrenschée et al. 2017), other studies have not confirmed these results when PD subjects are compared to healthy controls or subjects with GI diseases (Giancola et al. 2016; Desmet et al. 2017).

AS aggregates in the gut: historical facts and current status

In the 1980's, studies (Qualman 1984, Kupsky 1987, Wakabashi 1988) first reported the presence of aggregates in the intestinal wall that were reminiscent of the Lewy body pathology found in postmortem PD brains. This discovery raised the possibility that GI pathology could be a disease

biomarker in readily accessible tissue and that study of peripheral pathology might elucidate disease mechanisms in the CNS. The later discovery that AS was the major component of the Lewy body in most forms of PD, provided a powerful new tool to study PD pathology centrally and in the peripheral nervous system. Several different reports (Braak 2006; Lebouvier et al 2008, 2010; Beach et al. 2016; Böttner et al. 2015) all clearly show that it is indeed possible to detect Lewy body or neurite-like structures in the intestinal wall in living and post-mortem tissue. The fact that AS positive structures can be detected in the periphery has also been shown in several different species and in patients with different backgrounds (sporadic or genetic) (Braak et al 2006, Lebouvier et al. 2010, Derkinderen 2017). Several studies have suggested that there is a rostral-caudal distribution of AS pathology with maximal density in the esophagus, stomach and small intestine. This gradient could reflect the density of vagal innervation of the GI tract (Beach, Adler et al. 2010) which is in line with important Lewy pathology found in the dorsal motor nucleus of the vagus (Braak, Ghebremedin, et al. 2004; Braak, Sastre et al. 2007).

However different reports on AS pathology in the gut are not necessarily fully aligned and consistent. While some initial reports found clear differentiation between PD subjects and controls (Lebouvier et al. 2010, Shannon et al. 2012) others did not (Visanji et al. 2015), furthermore the detection is highly variable, from fully negative to 100% in both controls and PD (Schneider et al. 2016). Though this may in part be due to a semantic problem. AS immunoreactivity is often confused or used interchangeably with Lewy-like AS aggregate detection, it is worthwhile to (re-)consider AS's potential to cause GI symptoms. The fact that extrasynaptic AS is found in age-matched controls (Lebouvier et al 2010, Barrenschee 2017, Desmet et al. 2017), in biopsies from young volunteers (unpublished observations), and conversely that Lewy pathology is not necessarily found in a well-controlled selection of PD patients (Desmet et al. 2017, Bamouratov et al 2016) casts doubt on this popular assumption. Moreover, the unclear relation (Shultz-Schaeffer, 2012) between physiological AS (see below) and the isoforms present pathophysiological Lewy structures further complicates drawing conclusions from AS immunohistochemistry in the gut. As Lewy body and Lewy neurite like lesions predominantly contain AS in its phosphorylated (P-AS) form (90%, Fujiwara 2002), P-AS specific antibodies are more likely to emphasize the detection of pathological P-AS. However, since P-specific antibodies are generally more difficult to work with, the use of regular AS antibodies can still be valuable provided that some morphometric descriptors are included in the analysis. In this way it may be possible to discriminate between pathological occurrence and physiological synaptic expression (**Fig. 1E-F**). Apart from size and shape also, and especially, the density in the gut wall, the numbers of inclusions within the enteric neurons and whether the aggregates appear in a specific location or are rather uniformly distributed, are important factors to consider and report. Probably the best approach in that respect was used in the study by Barrenschee et al. 2017, who not only used P-specific antibodies but also quantified and measured the inclusions per enteric neuron. They show an increased prevalence of P-AS aggregates per neuron in the disease condition. Despite this improved analysis, positive labeling is also found in control patients. Apart from the fact that AS positive deposits are present, it may also be important to differentiate between the different types of AS aggregating species, as they display different toxicity depending on whether they appear as oligomers or fibrils (Peelaerts et al. 2015, and reviewed in Peelaerts & Baekelandt 2016). A worthy attempt in that direction was made by Sharrad et al. (2017)

who used conformation specific antibodies in the ENS of guinea-pigs treated with rotenone, though different AS species were detected it is not clear how this compares to the actual disease and human condition.

A blinded re-analysis of previously reported biopsy specimens confirmed poor reproducibility of the findings, with detection of AS in controls as well as PD subjects (Corbille et al. 2016). In part, the lack of consistency between studies reflected methodological differences, including tissue source (living tissue vs. post-mortem tissue), area sampled within the intestines, depth of the tissue sample, sampling error, primary antibody used, epitope exposure and signal development, as well as detection methods (classic histochemistry or fluorescent detection) and method of image recording (widefield system, confocal microscope). Subsequently, a multicenter methods assessment found that trained raters using an optimal immunohistochemical protocol were able to accurately distinguish PD from control tissue in post-mortem tissue (Beach et al. 2016). Additional study with these defined methods will be necessary to determine the utility of colon biopsies in PD (Corbille et al. 2016).

Physiological expression of AS in the ENS

AS is a soluble 140 kD protein that forms α -helical structures when it is membrane-bound. It is found mainly in presynaptic compartments where it promotes SNARE complex formation (Burré et al. 2010). Since Lewy bodies mainly contain AS species and mutations in AS have been linked to genetic forms of PD, this protein is thought to be critically important in the development of PD. AS can be secreted from synaptic terminals and as this protein is prone to aggregation (Lee 2005), the extracellular aggregates can be taken up by surrounding neurons (Desplats et al PNAS 2009). AS is also expressed by enteric neurons and its expression in different regions of the gut matches the typical synaptic contact patterns (**Fig. 1 D-E**) as present in the submucous and myenteric plexus (Sharrad et al 2013; Phillips et al. 2013a; Böttner 2015; Barrenschee et al. 2017).

In addition to immunohistochemical approaches, genetic approaches (BAC expression technology) Zhong et al. (2017) confirm that the AS promoter is active in enteric neurons, as GFP is expressed in enteric neurons in α -synGFP knock-in animals. At older age an increased expression of α -synGFP was observed, but whether that is due to accumulation or increased promoter activity at older age remains to be determined. Furthermore, it is important to note that mere overexpression of AS, even if that is in its naturally occurring phosphorylated form (~ 5 %) (Fujiwara et al. 2002) does not necessarily reflect pathological or behavioral expression of parkinsonism in the model.

Functional importance of intestinal AS depositions and aggregates

Even if it can be established that AS aggregation in the ENS is a biomarker of PD in man, convincing evidence that this phenomenon is a driver of the disease remains elusive. It will be important to demonstrate that AS expression or aggregation has functional consequences that result in the development of the disease. The prion-like spread of abnormal AS protein, has been proposed as a potential mechanism of disease genesis. The Braak model of spread from the ENS to the medulla followed by spread rostrally supports this hypothesis (Braak et al 2006). However, this pattern of spread has been debated and likely exists in only a subgroup of PD patients (Jellinger et al. 2009;

Kalaitzakis et al. 2008). Spread of AS pathology, including spread from the ENS, has been described in preclinical models of PD.

Despite evidence of AS pathology in the ENS, the functional impact on GI tissues remains unknown, and there is no clear understanding of the relationship, if any, with disease onset or progression. Also it remains elusive as to what that actually means with respect to functionality of that area, how that correlates with the disease or its progression.

The controversy about the extent to which AS pathology in the intestinal wall is involved in the pathogenesis or reflective of the PD disease state remains unresolved.

The gut as a gateway for PD pathology

Though it is not clear what the causal link is nor how and to what extent PD correlates with the occurrence of (occasional) AS containing aggregates in GI tissue, the presence of important GI symptoms in PD can definitely not be ignored. The intriguing Braak hypothesis (Braak, 2006), suggests that the disease progressively extends from the brainstem into the SN and further to the cortical layers. Both the GI symptoms in PD and the occurrence of peripheral aggregates have fueled the idea that PD pathology could even emerge in the periphery and by analogy with Prion diseases progress from the GI tract or the olfactory bulb towards the SN. It is well established that pathological AS (e.g. AS fibrils) can affect and kill neurons when injected in healthy brains (Luk et al. Science 2012). In addition to endogenous, misfolded α -synuclein, exogenous substances, toxins or pathogens have all been suggested as possible triggers for PD. In this case the extrinsic nerve connections between the intestine and the brain would be used as physical tracts for transport of an offending agent to other parts of the nervous system. An increased risk of PD associated with occupational exposure to pesticides (Moretto and Colosio, 2013; Narayan et al 2017; Pouchieu et al. 2018) has been found in a number of studies. The association between parenteral and other routes of exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin and PD is mainly of historical interest, as this led to a commonly-used toxin model of PD (Langston 2017). To what extent gastrointestinal exposure versus blood-borne toxicity is important in the genesis of toxin-induced parkinsonism is unclear.

Several groups have investigated the possibility of peripheral to central transport spread of pathology, either using viral vectors, pathological AS injections or gastrointestinal administration of PD related toxins (e.g. rotenone). Good evidence in support of extrinsic nerve transfer on the cellular level was provided by Pan-Montojo et al. (2012), who co-cultured enteric neurons, transduced to produce AS-mCherry, along with sympathetic neurons. They show that indeed mCherry containing particles were taken up and transported in the sympathetic neurons. The importance of the vagal trajectory was shown in a study by Ulusoy et al. (2013) who injected h-AS coding viral vectors in the vagus nerve of the rat (Ulusoy et al. 2013). This led to expression of h-AS in the ipsi- and not contralateral side of the rat brain. Although this study does not technically confirm the involvement of the peripheral organs, it does provide evidence in support of the vagal spread possibility. Further experimental evidence for the intestino-vagal route comes from a study where human specific AS monomers, oligomers, fibrils or PD lysate (Holmqvist et al. 2014) were injected in the intestinal wall. Using immunohistochemistry for the human isoform the authors were able to show, even shortly after injection, the presence of

spreading AS in the rat vagus nerve, the origins of which are in the DMV. These experiments show indeed that transfer of AS species is technically possible but it remains to be determined whether or not these phenomena truly occur in humans and if so, what are the magnitude and time course of such transfer.

ENS function and PD: lessons from human and animal models

Although PD patients clearly present with GI symptoms including dysphagia, bloating and constipation, the link between GI symptom severity and the classical PD hallmarks like Lewy pathology has not been established. Research addressing this link has been hampered by the fact that no animal models accurately recapitulate all aspects of the disease (Jellinger et al. 2015), and therefore it remains challenging to align all data generated in specific regions of several species by many different labs each using slightly differing technologies.

Loss of (dopaminergic) neurons in PD and related models

The question whether neurons in the enteric nervous system are lost throughout the course of PD has been addressed by several different groups. However it remains elusive whether and which ENS neurons are vulnerable to degeneration in PD and whether there are functional consequences of neuronal loss.

First, it is important to note that there are only very few dopaminergic neurons (tyrosine hydroxylase positive AND dopamine β -hydroxylase negative) in the ENS. Though percentages as high as 15% have been reported, these percentages have not been confirmed in recent literature. Nerve cells that produce dopamine should contain TH, DAT (dopamine transporter) and lack DBH, which differentiates them from other catecholaminergic neurons. Most mouse strains, except for Swiss (CD-1) as reported by Li et al (2004), do not show dopaminergic cell bodies in the ENS and also in human it is doubtful whether dopamine production is intrinsic to the ENS. Obviously a large number of neuronal fibers are TH positive (Fig. 1 C), which can easily be mistaken for enteric dopaminergic nerves. Sharrad et al. (2017) confirmed the extreme paucity ($\sim 0.1\%$) of intrinsic TH neurons in guinea-pig.

Moreover, the relationship between neuronal loss, if any, and changes in GI motility is unclear in PD. While neuronal loss could conceivably give rise to motility changes, other factors may be involved. Even in the absence of overt neuronal loss it is still possible that ENS functionality is altered due to subtle connectivity defects that arise with age or as a consequence of disease. Studies have suggested that aging in rats is associated with about a 25% loss of neurons in the myenteric plexus (Phillips et al 2003). However, inter-species variability and changes related to dietary or other environmental factors make comparison between studies difficult. Although age-related neuronal loss can be as high as $\sim 30\%$ in elderly (Saffrey 2013) considerable variability is also seen in human studies. The rate of neuronal loss in humans has been estimated at about 10% per decade, but there is significant variability among subjects and some studies using gastroduodenal biopsies failed to demonstrate a decline in neuronal populations across a wide age window (Cirillo et al. 2013). In PD subjects, Lebouvier et al. (2010) reported a small reduction in the number of neurofilament M (NF-M) submucous neurons in the colon. This loss, corrected for age, was reported to be a bit more pronounced when severe PD

pathology patients were considered separately. However another study reported no difference in the numbers of duodenal neurons of PD subjects (Desmet et al. 2017).

There remains no evidence that a general or more specific loss of ENS neurons or a change in neuronal function is responsible for GI symptoms in PD. Constipation, a common complaint of PD patients, is common in the general population, and is associated with changes in fermentation, intestinal microbiota and other factors. In one study of VIP expression in PD, changes in expression were related to the presence of constipation rather than to PD (Giancola et al 2016). This underscores the importance of adequate controls in studies of GI function in PD.

GI function in PD animal models

Gut function has been investigated in several different genetic and toxic models for PD. Most of these studies report only mild GI phenotypes, including delayed transit or reduced fecal pellet output.

Using a genetic mouse model, Kuo et al. (2010) showed that the expression of mutant SCNA (A53T or A30P) in a SCNA^{-/-} background, caused delayed colonic motility and made the differences between male and female mice, in a colonic bead propulsion assay, more explicit. Overexpression of WT AS however did not have an effect. Whole gut transit time was increased as well with the mutant AS expression (Kuo et al. 2010). In another AS overexpression study, Sampson et al. (2016) addressed whether AS overexpression (Thy1-AS) is dealt with differently in germ-free, normal or mice given PD microbiota. They found that in the presence of gut microbes the AS overexpression caused not only reduced fecal output but was also necessary for motor deficits to develop.

One popular toxin model of PD is based on the parenteral and intragastric administration of the pesticide and mitochondrial complex 1 blocker rotenone (Pan-Montojo et al. 2010). In rats exposed to rotenone (osmotic administration), Greene et al. reported delayed gastric emptying (Greene et al. 2009) and impaired neural inhibition of the proximal colon but no effect on innervation density or VIP, ChAT and NADPH neuronal numbers. Also Tasselli et al. (2013) report only subtle differences in GI function (reduced fecal pellet output) between control and mice after intragastric rotenone. Anselmi et al. (2017) used a paraquat model, a different PD related toxin, to study the vagal effects on rat stomach function in the context of PD. Using optogenetic inhibition of the dorsal vagal complex they found that this striatal-vagal pathway no longer functions efficiently in rats that had intraperitoneal paraquat injections. This study reveals a monosynaptic nigro-vagal pathway and clearly shows a link between PD related toxins, PD related brain nuclei and vago-GI function. However, the results in this systemic model do not suggest that a local effect on the ENS is involved.

In another recent study (Ulusoy et al. 2017) a viral vector approach was used to overexpress human AS in the DMV and midbrain to test whether h-AS would be transferred from the brain to the stomach. This study clearly shows that h-AS ends up in the lamellar endings of the vagus nerve in the stomach wall. Though it confirms the possibility of spread, it does not provide evidence for ENS dysfunction in relation to the AS expression.

Neuronal measurements in human GI tissues

Most of the studies on human material have been performed using immunohistochemical techniques, which due to regional differences in the GI tract or differing methodological or tissue processing approaches may have generated varying results. Only one study has used a different approach to evaluate functionality and mitochondrial function in live ENS samples obtained from PD patients and controls. Desmet et al. (2017) used live calcium and mitochondrial imaging in non-dispersed neurons in the freshly excised submucous plexus. Their live imaging approach did not reveal any differences in neuronal and mitochondrial functionality between the PD population and their well-matched controls. The outcome of this recent paper (Desmet et al. 2017), casts doubt on the assumption that – given the GI symptoms – the ENS is necessarily affected in the overall PD patient population.

What are the open questions?

Despite the large body of literature focusing on the link between the GI tract and PD, it is clear that more research is needed to clearly establish the nature and importance of GI tract or ENS involvement (active or passive) in PD. It remains important to assess why these studies, have not fully delineated the importance of the ENS-PD connection. One possibility is that the importance of ENS might vary in PD, much as the pattern of spread in the CNS in PD varies. Much remains to be learned about the physiological role of AS in the ENS, the pathophysiology of AS aggregates, the potential that GI AS is a disease biomarker, and most importantly whether there is proof that the GI tract is a portal of entry of a causative agent in PD. For a rational approach to the study of this important issue, it is essential to standardize the methods of study. Below we suggest a couple of open questions that could guide new researchers who enter the exiting ENS-PD field. (**Fig 2**).

- *What does the presence of **(aggregated) AS in the gut wall actually mean?** In what numbers do AS aggregates occur in the gut wall and what is the limit of tolerance above which AS aggregate density can be pathogenic? Is it the aggregates that are harmful, or might aggregation sequester harmful substance and prevent against cellular damage?*

Accurate volumetric quantification of density and aggregate size is important and should replace qualitative illustrations. It will be important to define parameters such as aggregate size and density, and the number and types of neurons that are affected. These metrics will help elucidate the utility of AS as a biomarker as well as to better define the role of AS in ENS integrity.

- *Is it possible that PD effects occur in a **patchy** fashion, either along the length of the gut or in just in specific layers of the gut?*

AS inclusions are not uniformly distributed throughout the gut wall, which raises the spectre of sampling error. Standardized sampling techniques and definitions of positive findings will be helpful to interpret whether AS density is a useful marker of ENS damage or dysfunction as well as the likelihood that central spreading will occur. For those cases, in which inclusion would be extremely sparse, it could be argued that these very confined lesions most probably do not cause a major problem for the organism.

- *Is it possible that PD effects occur specifically in **one of the enteric nerve layers** (submucous or myenteric)?*

As mentioned in the introduction the ENS consists of different layers, the submucous plexus in control of absorption and secretion and the myenteric plexus with neurons that control the muscle. While demonstrated changes in intestinal permeability might indicate changes in the submucous plexus and changes in motility might infer changes in the myenteric plexus, it will be important to define the changes in both layers of the ENS.

- *What if PD related **lesions** in the gut are **transient**?*

Though highly speculative, it is worthwhile to consider the possibility that PD related lesions in the GI tract might be transient, leaving no trace after they have been repaired. If the gut is indeed critically involved or acts as a gateway, the transient nature of a lesion and the capacity to recover from a dramatic one could help explain the variability and the lack of massive effects in patient versus control material (Lebouvier et al. 2010, Baumuratov et al. 2016, Barrenschee et al. 2017, Desmet et al. 2017), either be that when looking at AS positive deposits or at numbers of neurons.

If this were true, it means that the ENS has the capacity to recover from PD related neurodegeneration, which implies that the ENS possibly harbors a solution for other regions affected in PD. It has been shown that glia cells in special conditions, for instance after local damage in the gut, can revert their phenotype to newly generate neurons that replace the dead ones (Laranjeira 2011; Joseph 2011). This raises the interesting hypothesis that the recovery capacity found in these nerve layers, could potentially educate us to stall or reverse pathology elsewhere as well. Obviously this needs thorough testing.

An important unresolved question is whether it is possible that AS positive deposits (and maybe Lewy neurites) are part of a general and dynamic turnover process that occurs also under relatively normal physiological conditions. It could well be that, as long as the tissue is in good condition, macrophage activity is sufficient to keep the ENS environment clean, like microglia do in the brain, and only when they fail AS aggregates are accumulating to a detectable level (Phillips et al. 2013).

- ***Is intestinal inflammation or are recurrent inflammatory insults involved?***

Given that no single genetic or toxic insult accurately models PD, it is likely that multiple converging insults (exotoxins, genetics, energy imbalance, mitochondrial dysfunction) might be required to generate the disease. In addition, it is important to define the role of inflammation in disease genesis. Alterations in inflammatory mediators have been described in human tissue (Pellegrini et al. 2016, Devos et al. 2013) and in toxin-based PD models (Pellegrini et al. 2016). Inflammation has also been proposed as a possible mechanism in GI dysfunction attributed to changes in microbiota profiles (Keshavarzian et al. 2015). Although definitely a worthwhile line of approach, this emerging neuro-inflammation hypothesis has not yet been tested extensively. Villaran et al. (2010) have shown that LPS induced lesions were exacerbated by experimentally induced colitis (Villaran et al. 2010) and in a retrospective study Lin et al. (2016) found an increased risk of PD in a cohort of Taiwanese inflammatory bowel disease (IBD: Crohn's, ulcerative colitis) patients (Lin et al. 2016). However it remains to be determined whether the gut inflammation is cause or consequence of constipation, altered residence time of stools and associated microbiota profiles.

- ***What if GI lesions are reserved for a subset of PD patients?***

Converging evidence suggests that PD is not a uniform disease. There are monogenic forms, and it is likely that different combinations of genetic and environmental influences contribute to development of the disease state in others. As persuasive as was the Braak hypothesis of caudal to rostral disease spread, a growing body of literature suggests that there are different pathological profiles of AS (olfactory bulb only, brainstem predominant, limbic predominant, brainstem and limbic and neocortical) (Jellinger et al. 2009, Kalaitzakis et al 2008, Beach et al., 2009, Kalaitzakis et al 2008, Linazasoro 2007). Increased understanding of heterogeneity in the onset and spread of pathology will help to inform the utility of AS as a biomarker as well as a tool to understand the biology of the disease.

- ***What if specific microbiota are critically involved or protective?***

The preponderance of evidence supporting a change in microbiota in PD suggests that increased pro-inflammatory and decreased anti-inflammatory microbes can be demonstrated in PD (Keshavarzian et al. 2015, Petrov et al 2017, Scheperjans et al. 2015, Hopfner et al. 2017, Li et al. 2017, Pereira et al. 2017). There is preclinical evidence that microbial alterations in PD might potentiate pathology and behavioral abnormalities in a transgenic mouse model (Sampson et al. 2016). In the human studies, it is possible that the changes in microbe population are secondary to the disease process itself or to disease complications such as constipation. Studies in well-defined prodromal populations (subjects with rapid eye movement sleep disorder, or with anosmia and subclinical changes in dopamine neuroimaging) may help clarify this issue.

Conclusions and future prospects

The link between the ENS and PD has been a topic of study for a couple of decades. Originally and mainly because the earlier reports on the presence of AS positive aggregates in the gut wall of some PD patients suggested that this could possibly be used as an early detection biomarker. However, due to the variability in detection, combined with the fact that the approach has limited predictive value gut α -synuclein inclusions may not survive as a PD biomarker candidate.

The other line of thought is that the gut or the ENS might act as a gateway via which a possible toxin or a misfolded protein might be transported to gradually inflict damage to the (most vulnerable) neurons on its path. Though shown to be possible in in-vitro settings and overexpression mouse models, it remains to be determined if, and to what extent this happens in real life.

The fact that constipation and dysphagia are very common symptoms in PD patients is a third reason why researchers have focused on the ENS in its relation to PD. However at present there is no hard evidence that the ENS is necessarily dysfunctional. Live imaging or other technology will be necessary perform in-depth analyses of ENS functionality in different gut regions and stages of disease. One important confounding factor is of course constipation itself, which severely complicates drawing conclusions about cause or consequence, as exposure to noxious substances or a malicious microbiome is naturally higher simply just because of increased transit time.

Further, preferably concerted research actions will be needed to fully clarify the link between ENS function and PD, and to determine whether it is truly a part of entry or an whether the ENS being trained by evolution to resist noxious agents, has potential to lead to a solution. Though there is still a lot of work ahead, the investigation the GUT-PD link remains an extremely interesting research topic.

Figures

Figure 1: Immunostainings of the enteric nervous system. **A.** Radial section through the mouse small intestine showing the gross architecture in green (autofluorescence), with myenteric (MP) and submucous plexus (SMP) layers in magenta (Tuj1) and neuronal fibers extending into the villus tips. **B.** Example of a human duodenal submucous plexus ganglion as present in routine biopsies (widefield fluorescence). Neurons are labeled with HuCD (green), Tuj1 (blue) and Neurofilament-M (red) antibodies. **C.** Typical mouse myenteric ganglion in the mouse colon (widefield fluorescence). Neurons are labeled with HuCD (green), tyrosine hydroxylase (TH, red) and Tuj1 (blue) antibodies. Note that the TH staining labels extrinsic fibers and varicosities in this C57/Bl6 mouse. **D.** 3D confocal view of a human submucous ganglion labeled with HuC/D (green) to identify neurons and to reveal the physiological synaptic expression of α -synuclein (red, antibody: SC-7011-R). **E.** Confocal slice of the mouse submucous plexus with neurons in green (HuC/D) and α -synuclein (antibody: SC-7011-R) in red. **Bars A-E:** 50 μ m.

Figure 2: Schematic overview of the open questions and key features of the Gut-PD link. **1|** Is the entire gut wall involved? Are some regions of the gut more prone to be involved? **2|** Does the pathology affect, if any, one or both nerve layers **3|** What's the role of the mucosa, the epithelial barrier and the microbiota, toxins or noxious agents in the lumen? **4|** What role does the immune system play? **5|** When and how becomes physiological α -synuclein problematic? **6|** In what density, how sparsely do aggregates occur in normal and PD conditions? **7|** What about transport of noxious agents or aggregates via the extrinsic nerves? **8|** Can constipation, because of the prolonged transit time, be the origin rather than the consequence of PD? **9|** Assuming that PD-related lesion in the ENS get repaired, is there potential to learn from the ENS? **10|** Might different subpopulations of PD patients exist, some of which have a more pronounced gut link, while for others this is limited or even absent.

Box 1

Experimental and methodological aspects to consider in GUT-ENS-PD research

The wealth of a models, preparations, tissues (live or post-mortem), approaches, the older sometimes anecdotal evidence and nowadays the highly complex experimental designs, have made the available information on the topic hard to digest. In this bullet-point list, we suggest a number of aspects that can be used as a guideline for designing new experiments:

- Is the research focus on:
 - ENS or entire GUT wall
 - Or specific layers ?
 - The immune system?
 - The microbiome?
 - α -synuclein
 - exogenous factors: nutrients / toxins ?
- What region of the gut is studied ?
- Experimental animals or human tissue ?
- Postmortem or (live) biopsies material ?
- Patients: PD patients or controls, the latter with and without constipation ?
 - prodromal – disease state ?
- Methodology:
 - Immunohistochemistry
 - What antibodies will be considered? α -synuclein or phospho- α -synuclein specific ones? Quantification of expression or of aggregates
 - Sparsity / density (number per mm^2 , mm^3 , neuron, ...)
 - Morphometry (size shape)
 - size of the aggregates, uniformity
 - What read-out will be used?
 - Classic histochemical labeling or immunofluorescence? In case of the latter will widefield, confocal or enhanced/superresolution techniques be used to record and analyze?
 - Neuron number countings (per mm^2 , per ganglion)?
 - Molecular techniques
 - Sequencing, bioinformatics
 - Live techniques?
 - Imaging
 - Gut function
 - Transit times
 - Organ bath experiments
- Confounding factor: constipation !

- may prolonged exposure to
 - Microbiome
 - Fermentation products
 - Toxins

lead to observed alterations (α -synuclein, neuronal numbers, neuronal functionality)

- Role of the immune system ?

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