

Animal models of brain-first and body-first Parkinson's disease

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ABSTRACT

Alpha-synuclein aggregates are the hallmark pathology of Parkinson's disease, which can propagate in a stereotypical pattern along the brain-body axis. Parkinson's disease patients not only display heterogeneous symptoms but also show variable patterns of alpha-synuclein pathology and affected neuronal systems during the disease course, complicating early and accurate diagnosis. Emerging data from post-mortem and imaging studies strongly suggest that disease heterogeneity could, at least in part, be explained by variable disease onset site, i.e. brain or body. This has led to the recently hypothesized formulation of two Parkinson's disease-subtypes, a body-first subtype where pathogenic alpha-synuclein arises in the body and spreads to the brain, and a brain-first subtype where pathogenic alpha-synuclein arises in the brain and spreads to the body. From a preclinical perspective, several animal models have been adapted or developed to reproduce Parkinson's disease-like pathology in the brain or periphery aiming to address the site of disease onset. Here, we review the current rodent and primate models that aim to reproduce Parkinson's disease pathology development and spreading in the brain and/or body and discuss the value and shortcomings of these models for the development of potential future applications in clinical trials and personalized medicine.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder that affects motor functions of patients while causing several non-motor symptoms ranging from cognitive impairment to gastrointestinal issues. Its pathological hallmarks include neurodegeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta and the presence of pathogenic neuronal alpha-synuclein (asyn) aggregates, also known as Lewy bodies and Lewy neurites. The brain pathology however, is not limited to the SN and asyn accumulates in many other areas throughout the brain. Remarkably, asyn accumulation does not occur simultaneously in these brain regions. The groundbreaking findings of Braak and colleagues have described that asyn pathology is dynamic and spreads during the course of the disease from one brain region to another in a stereotypical pattern (Braak et al., 2003a). Based on these extensive post-mortem studies investigating brains from PD patients and control subjects, asyn pathology first appears in the olfactory bulb (OB) and the dorsal motor nucleus of the vagus nerve (DMV) then spreads towards the pontine tegmentum

specifically affecting neurons in the locus coeruleus (LC) and lower raphe nuclei and thereafter reaches the SN and amygdala. Later on, during the final stages of the disease the pathology reaches to cortical areas (Braak et al., 2003a). Importantly, these predilection sites that are affected by Lewy pathology throughout the disease progression are synaptically connected to each other (Braak et al., 2003b). The spread of these asyn aggregates through synaptically coupled networks is likely to be a crucial pathogenic factor in PD and has led to the hypothesis that the disease may be initiated in the nerve terminals of the enteric nervous system (ENS), after which pathology spreads via parasympathetic and sympathetic connections to the DMV and intermediolateral cell columns of the spinal cord (IML), respectively (Braak et al., 2003a). This is often referred to as Braak's gut-first hypothesis and in contrast to what has been believed for over a century, i.e. PD is not just a brain disease but a systemic disease. Indeed, several evidence has supported this view and illustrated that Lewy pathology and neurodegeneration not only occurs in the central nervous system (CNS), but in multiple peripheral organs as well (Beach et al., 2010; Gelpi et al., 2014).

Several clinical and histopathological evidence supports Braak's gut-

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first hypothesis. Asyn aggregates have been found in gut tissue from PD patients collected at early stages, which are associated with Lewy pathology in the DMV (Stokholm et al., 2016 reviewed in Chiang and Lin, 2019). Moreover, symptoms of gut dysfunction including constipation appear up to 20 years before motor symptoms (reviewed in Skjærbæk et al., 2021). Further epidemiological evidence for the gut-first hypothesis comes from patients that went through vagotomy (Svensson et al., 2015). The study compared over 5000 patients that underwent truncal vagotomy and showed that truncal vagotomy reduced the risk of PD by 40–50% at 10–20 years of follow-up (Svensson et al., 2015; Borghammer, 2018). Histopathological evidence suggests that the presence of peripheral asyn pathology in PD patients is not limited to the gut and parasympathetic connections. Additional asyn pathology in PD patients has been detected in the sympathetic cardiac nerve and heart tissue (Orimo et al., 2008) as well as in the skin (Donadio et al., 2016), and one study reported that 5% of Lewy pathology-positive cases showed inclusions solely in the autonomic nervous system (Tanei et al., 2021), supporting the involvement of sympathetic connections in PD pathogenesis. Accordingly, non-motor autonomic symptoms that could be linked to peripheral asyn pathologies such as constipation, orthostatic hypotension and dry skin are common during prodromal PD (Cheon et al., 2008; Borghammer, 2018; Palma and Kaufmann, 2020).

Nevertheless, evidence from neuropathological studies suggests that not all PD patients conform to the gut-first hypothesis as several studies reported that ~20–50% of PD patients do not follow the Braak staging scheme, and 7–17% do not show pathology in the DMV (Attems and Jellinger, 2008; Parkkinen et al., 2008). For example, researchers describe a high neuropathological clinical heterogeneity among PD patients in a longitudinal neuropathological evaluation of the Sydney multicenter study (Halliday et al., 2008). The study reports 50% of cases to be consistent with the Braak staging, showing a brainstem-predominant Lewy pathology with slow disease progression that is in line with Braak's gut-first PD subtype. The remaining 50% of the PD brains that they have analyzed showed either severe neocortical pathology consistent of dementia with Lewy Bodies, or with late onset, shorter survival PD characterized by widespread limbic pathology with additional proteinopathies in the brain. In a more recent population-based neuropathological study in 124 elderly patients with Lewy pathology, Raunio et al. (2019) described that two third of samples represent a clear caudo-rostral pattern, i.e. brainstem-predominant pathology similar to what Braak et al. (2003a) have described, and the rest were characterized with strong asyn accumulation in the amygdala or limbic areas, and with milder caudal (brainstem, medulla and spinal cord) and rostral neocortical pathologies (Borghammer et al., 2021).

2. Brain-first vs. body-first PD: a new hypothesis

Overall, post-mortem studies have reported two principal types of pathology patterns: a brainstem-predominant type with stronger asyn accumulation in the brainstem than more rostral structures and a limbic/amygdala-predominant type, with stronger midbrain pathology compared to more caudal structures. Note that the 'limbic/amygdala-predominant' type does not imply that the amygdala is the initiation site of pathogenic asyn accumulation in all cases, but rather suggests that these structures represent the site of dominant pathology.

This variable histopathological profile of PD patients is also associated with diverse symptoms and clinical phenotypes (De Pablo-Fernandez et al., 2019). In the late 1980's researchers already considered that PD may be a syndrome caused by different pathogenic mechanisms, rather than a single disease and attempted describing PD subtypes based on clinical manifestations (Calne, 1989). The initial studies focused on variable motor phenotypes in early PD stages (Janjovic et al., 1990). More recently however, attempts have also been made in stratifying PD subtypes based on non-motor symptoms. Rationale for using non-motor symptoms as a classifier for PD subtypes are based on a number of considerations. First, non-motor symptoms such as

cognitive impairment, constipation, olfactory dysfunction, rapid eye movement sleep behavior disorder (RBD) and autonomic dysfunction accompany motor symptoms exclusively, although to a varying degree. This variability in non-motor symptoms however, may represent classifiers of clinical subtypes of PD and may correlate with the initiation and development of asyn pathology (Fereshtehnejad et al., 2017; Horsager et al., 2020). Second, non-motor symptoms often manifest prior to the motor symptoms of PD and therefore are seen as the ideal time window for applying personalized disease-modifying therapy (Oertel and Schulz, 2016).

In line with these considerations, and based on imaging data of diagnosed and prodromal PD patients, researchers recently postulated that disease heterogeneity can be explained in part by defining two PD subtypes: "brain-first" or "body-first" PD subtype. The concept is based on the strong correlation of phenotypic and imaging data of early PD patients (Borghammer and Van Den Berge, 2019; Horsager et al., 2020). Body-first cases are associated with RBD during the prodromal phase. RBD is a sleep disorder characterized by atypical motor, behavioral or cognitive episodes during sleep. Over 90% of patients with isolated RBD eventually develop a synucleinopathy, and at least 50% phenoconvert to PD (Schenck et al., 2013; Postuma et al., 2017, 2019). In a recent study using a wide range of imaging techniques such as neuromelanin MRI, ¹²³I-MIBG SPECT, ¹⁸F-FDG-PET, and dopamine scans, researchers showed distinct damage to the peripheral autonomic nervous system and lower brainstem in isolated RBD cases, in contrast to newly diagnosed RBD-negative PD cases. These patients manifested reduced parasympathetic function in the gut, alleviated sympathetic function in the heart and loss of neuromelanin in the lower brainstem, without pathological changes in the nigrostriatal dopaminergic system, implicating that pathological changes at the periphery and the lower brainstem occur prior to the involvement of higher brain regions in these patients (Horsager et al., 2020). In line with these imaging studies, histopathological analyses revealed that patients with RBD-positive PD displayed much higher frequencies of Lewy pathology in peripheral tissues than patients with RBD-negative PD, further supporting that the presence of RBD is indeed a strong determinant of body-first asyn accumulation (Leclair-Visonneau et al., 2017). In contrast, early PD patients that were negative for RBD, exhibited relatively healthy tissue and innervation at the peripheral sites but were characterized with increased pathological alterations in the upper brainstem and telencephalon, and typically displayed dopaminergic deficits prior to peripheral damage. Notably, the dopaminergic dysfunction was predominantly unilateral in these patients (Knudsen et al., 2021). Most importantly, both disease subtypes will evolve to a similar advanced disease stage over time where the entire brain and several peripheral organs are affected (Horsager et al., 2020). These studies however, were performed on early brain-first PD cases rather than prodromal brain-first PD, as detection of prodromal phases of brain-first PD is not possible with our current knowledge and imaging techniques. It will be most valuable to directly test this hypothesis once further detection techniques, such as an asyn PET tracer, becomes available.

An additional scenario of disease onset, which does not exclude the prior hypothesis is that the pathology enters the brain via two routes: (i) nasal and (ii) gastric (Hawkes et al., 2007, 2009). Although there are plenty of data and models exploring the latter, the olfactory bulb (OB) initiation route is relatively less explored as early studies did not detect spreading beyond the olfactory cortex (Braak et al., 2003b). Nonetheless, OB pathology and olfactory deficits in PD are common and should not be overlooked. Indeed, emerging data have reported isolated OB pathology cases, shedding light into the involvement of asyn pathology in PD development. The OB is the most common single-site location of pathology in cases with incidental Lewy body disease (Adler and Beach, 2016) and is likely to be associated with limbic-predominant Lewy pathology in Dementia with Lewy bodies (DLB) (Beach et al., 2009; Rey et al., 2019). It is however important to note that these cases are not common. According to a recent post-mortem brain study of Lewy body

disease cases, three out of 178 cases exhibited isolated OB pathology, whereas 29 cases had isolated pathology elsewhere but not in the OB (Tanej et al., 2021). Nevertheless, these clinical findings, together with data from in vivo studies showing spreading from the OB to other brain areas (Rey et al., 2018), suggest a possible spreading route of asyn pathology from the OB to the limbic system. Another possibility was proposed by Braak et al. (2003b) suggesting that ‘OB-first’ pathology is essentially part of body-first PD. In fact, the observed isolated OB pathology in certain studies (Beach et al., 2009; Adler and Beach, 2016) does not rule out the co-existence of pathogenic asyn in the gut, prior to its spread to the DMV. It should be stressed that the likelihood of detecting asyn pathology in the OB compared to an 8 m-long gut is higher. In support, the majority of iRBD patients (i.e. prodromal body-first PD patients) exhibiting cardiac denervation without reduced striatal dopamine, are hyposmic (Knudsen et al., 2018; Postuma et al., 2019). Furthermore, it is conceivable that an inhaled toxic or viral pathogen affects the olfactory epithelium bilaterally, initiating accumulation of pathogenic asyn in the OB bilaterally, ultimately leading to a bilateral spreading pattern of pathogenic asyn in the brain. A bilaterally affected OB would accompany hyposmia, and bilateral spreading would accompany little or no motor asymmetry, as observed in body-first PD. On the other hand, if a random accumulation of pathogenic asyn were to start in the unilateral OB, this would lead to an asymmetric propagation pattern within the brain, most likely accompanied by less hyposmia and more motor asymmetry, similar to brain-first PD. The importance of isolated OB pathology and whether or not OB-first pathology is associated with brain-first or body-first PD, is still under

debate. The latter might be associated with the level of asymmetry of the initiating pathology (Borghammer et al., 2021; Borghammer, 2021).

Fundamental questions remain to be addressed for the validation and translation of the body-first and brain-first hypothesis: how do brain- and body-first subtypes differ in their initiation mechanisms and progression patterns (especially in the prodromal phase), and how such knowledge could be exploited for tailored subtype-specific interventions. Since clinical investigations alone are not sufficient to address these questions, other models that can recapitulate the pathology of these two PD subtypes are required. Experimental animal models provide an excellent platform to explore the body-brain link in PD pathogenesis from a causal and mechanistic point of view.

Several animal models and tools to reproduce PD-like pathology already exist and new advances in the field have introduced novel models to address molecular and cellular mechanisms leading to the initiation, accumulation and spreading of asyn in specific brain regions and organs. Here, we summarize common and recently developed animal models recapitulating PD pathology (incl. Asyn accumulation, aggregation and propagation, as well as neurodegeneration) from a brain-first and body-first PD perspective and discuss their merits and shortcomings. It is important to note that stratification between body-first and brain-first PD is not absolute and overlapping phenotypes across PD subtypes occur throughout the progression. One of the most challenging issues regarding classification of a model as brain-first or body-first is due to the fact that most early models of PD pathology (e.g. neurotoxin models), belonged to an era in which the importance of non-nigral pathology was not fully recognized. Therefore data regarding

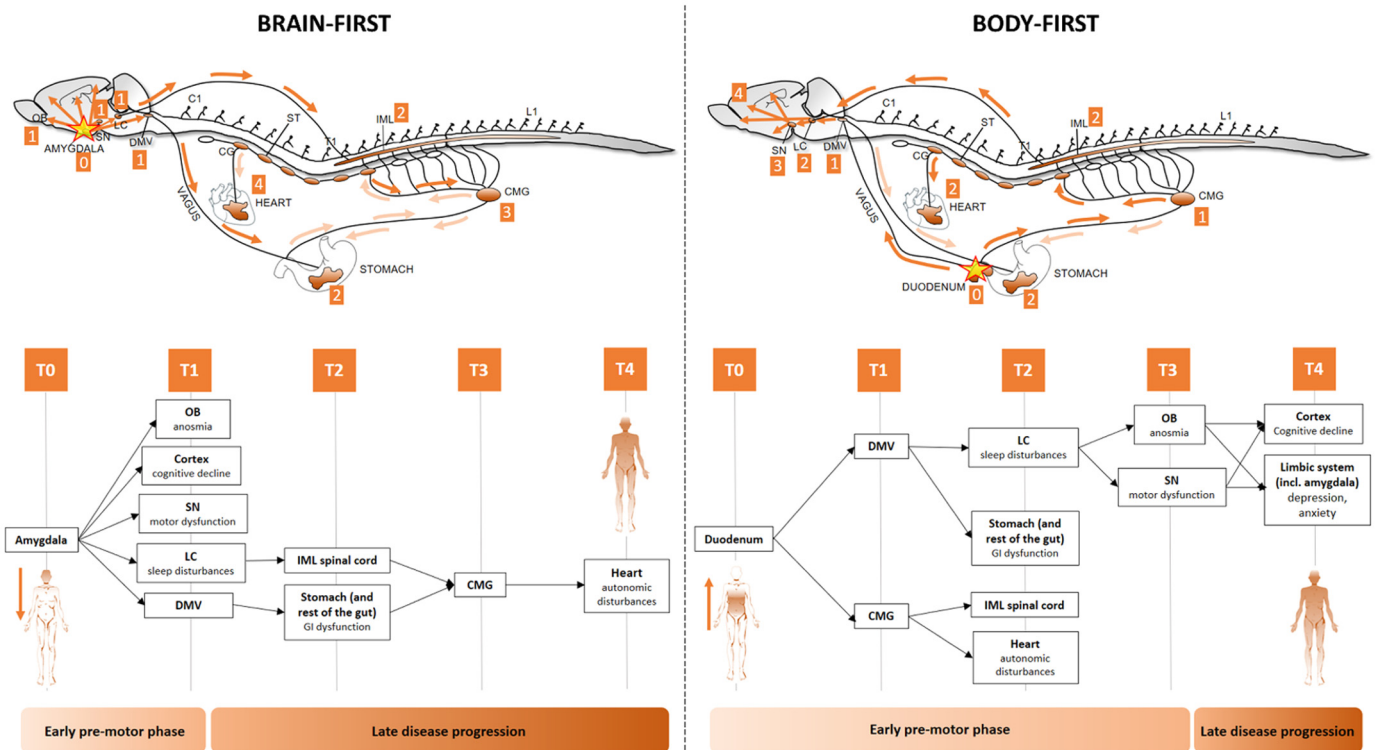


Fig. 1. Modeling the postulated spread of pathology through the connectome in brain-first (left) and body-first (right) PD in rodents. Brain-first PD can be modelled by artificially initiating pathology in the brain of rodents. Body-first PD can be modelled by artificially initiating pathology in the gut of rodents, which is the most likely peripheral initiation site in PD. The schematic below lists the structures affected by asyn pathology at a given time point (T₀-T₄) and the corresponding PD symptoms associated with the pathology. Note that a lag-time from appearance of the first detectable pathogenic asyn in a given structure, to the appearance of symptoms derived from that structure, is to be expected. The time points T₀-T₄ are arbitrary and their length depend on factors that affect disease progression, such as age or genetic background (e.g. asyn overexpressing rodents) of the animal model used in the study. The body-first model is characterized by a longer pre-motor phase compared to the brain-first model. Note, the frequency and importance of initiating OB of OB-first pathology is still unclear and is therefore not included in this figure. Depending on the level of asymmetry of the OB-first pathology, an OB-first model could be associated with either brain-first (unilateral initiation) or body-first (bilateral initiation) PD. Abbreviations: CG: cervical ganglia; CMG: celiac ganglia; DMV: dorsal motor nucleus of the vagus nerve; LC: locus coeruleus; GI: gastro-intestinal; IML: intermediolateral nucleus; OB: olfactory bulb; SN: substantia nigra.

non-nigral pathologies, in particular peripheral asyn accumulation, as well as non-motor symptoms are often unavailable. Moreover, systemic insults or models using elevated basal asyn levels to induce PD-like pathology and phenotype affect both body and brain simultaneously in varying degrees, challenging stratification. Here, we classified the models based on available data. In this review, we argue for the importance of new models that mimic multiple aspects of human PD, such as variable disease onset sites. Future research that takes multiple PD initiation sites into account in their experimental set-up could prevent inconsistent outcomes and contribute to successful translation of basic biomedical research into clinical applications.

Fig. 1 illustrates the brain-first and body-first hypothesis, applied to rodent models of PD pathology. This scientific model posits that pathogenic asyn can start its aggregation in different places of the nervous system, including peripheral organs or CNS, leading to a body-first (i.e. not-brain-first) and brain-first subtype; and that the stereotypical spreading of pathogenic asyn is fundamental for shaping disease course in each subtype (Borghammer and Van Den Berge, 2019). The model acknowledges the rare possibility of multifocal disease initiation, where multiple foci of Lewy pathology arise in different parts of the nervous system simultaneously. In such rare cases, disease course will be driven from multiple sites in parallel, accelerating disease progression (Borghammer et al., 2021). Future applications of this hypothesis in animal models should substantially increase our fundamental knowledge of PD.

3. Brain-first animal models

From a historical point of view, until the early 2000's, PD research was focused primarily on nigrostriatal system dysfunction. In line with this research focus, the use and development of animal models aimed to reproduce nigral dopaminergic neurodegeneration and to develop treatments based on dopamine replacement therapies. Today our knowledge and interest in PD has expanded to extra-nigral pathologies, non-motor symptoms and peripheral asyn accumulation, and novel animal models are being developed to reproduce these pathologies. Nevertheless, two major reasons argue for the indispensability of animal models focused on PD pathology in the brain. First, as discussed above, there is a significant population that are consistent with a brain-first subtype of PD. Second, despite temporal differences in the initiation of SN pathology, all PD patients eventually develop motor symptoms that occur as a consequence of nigrostriatal degeneration. In this section, we will give an overview of these models and discuss the use of these models to recapitulate PD pathology in the brain.

3.1. Neurotoxin models with focus on CNS

Neurotoxin models are the most well-established and commonly used animal models for translational studies in the PD field. Discoveries of all current treatment used in PD therapy including medications such as levodopa, dopamine agonists and monoamine oxidase-B inhibitors, surgical interventions such as deep brain stimulation and experimental fetal cell transplantation therapies, are led by experiments using animal models based on depletion of dopamine in the nigrostriatal system using drugs or toxins. Although they were initially developed to mimic nigral neurodegeneration, most of these neurotoxins also induce non-nigral pathologies and some level of asyn accumulation in neurons.

6-OHDA: The first neurotoxin model that was developed as a PD model involved injection of 6-hydroxydopamine (6-OHDA) into the brain (Ungerstedt, 1968). The specificity of 6-OHDA stems from its ability to bind to dopamine uptake sites (Decker et al., 1993). Once taken up by the neurons, it easily forms free radicals and inhibits mitochondrial respiratory chain complexes I and IV (Glinka and Youdim, 1995). 6-OHDA does not pass the blood brain barrier and therefore needs to be administered directly into the brain for its neurotoxic effects. Typically, 6-OHDA injections target striatal terminals, SN or medial forebrain bundle (Sauer and Oertel, 1994; Kirik et al., 1998). Surgeries

are often performed unilaterally in rats and non-human primates, which allows internal comparison of pathology between two hemispheres and use of specialized behavioral tests to detect asymmetric basal ganglia output as a result of unilateral nigrostriatal lesions (Kirik et al., 1998; Grealish et al., 2010). These animals are characterized by motor symptoms such as bradykinesia and reduced ability to perform motor tasks with the affected side of the body. Of note, older animals seem to have higher susceptibility to 6-OHDA as compared to younger animals (Barata-Antunes et al., 2020). Importantly, 6-OHDA-mediated motor behavioral dysfunction can be reversed by levodopa treatment. Chronic levodopa treatment in 6-OHDA lesioned animals causes involuntary movements similar to levodopa induced-dyskinesia, one of the major side effect of levodopa therapy in PD patients making the model useful for translational studies aiming at dopamine replacement and levodopa induced dyskinesia therapies (Lundblad et al., 2002, 2004).

A major benefit of the 6-OHDA model is its selective properties that allow dissecting the specific impact of nigrostriatal damage. Although 6-OHDA injections specifically target dopaminergic neurons, dopaminergic depletion in the striatum has consequences on other neuronal systems. Several labs have reported that selective dopaminergic depletion in the nigrostriatal system induces dysfunctions in cognition, gut motility and ENS, olfaction and sleep-wake cycle, suggesting that dopamine depletion may be responsible, at least in part, for non-motor symptoms of PD (Bonito-Oliva et al., 2014a, 2014b; Requejo et al., 2020; Colucci et al., 2012). One important limitation of the 6-OHDA model is the lack of asyn pathology that is seen in PD brains, which is to some degree present in other neurotoxin models of PD pathology.

MPTP: MPTP is another commonly used toxin for inducing PD-like pathology in both mice and non-human primates. Animals systemically treated with MPTP are characterized with a selective destruction of dopaminergic neurons of the nigrostriatal tract and related motor deficits (Burns et al., 1983; Jenner et al., 1984; Langston et al., 1984). In non-human primates, the motor phenotype is very similar to the one seen in PD patients, i.e. akinesia, rigidity, flexed posture and difficulty swallowing (Burns et al., 1983). Non-motor symptoms such as olfactory deficits, cognitive decline and gastrointestinal dysfunction have also been reported in mice treated with MPTP (Han et al., 2021). In marmosets, change in sleep patterns and circadian rhythm was observed following a subchronic MPTP treatment regime (Choudhury and Daadi, 2018).

One major difference between 6-OHDA and MPTP model is the extent of affected brain areas. As 6-OHDA is targeted to the nigrostriatal pathway, the direct effect is limited to the nigral dopaminergic system and related neuronal networks (Kirik et al., 1998; Bonito-Oliva et al., 2014a, 2014b; Requejo et al., 2020; Colucci et al., 2012). Whereas, following systemic administration, MPTP passes the blood brain barrier, affecting brain regions also beyond the SN, especially brain areas that are known to be vulnerable to PD pathology, such as the LC (Forno et al., 1986, 1993). Therefore, it is reasonable to suggest that this model more closely recapitulates the whole brain PD pathology and is more likely to provide insights about selective vulnerability of non-dopaminergic neurons associated with PD pathology, as compared to the 6-OHDA model.

Notably, MPTP treatment induces asyn mRNA upregulation and aggregation in the brain (Vila et al., 2000; Kowall et al., 2000; Purisai et al., 2005; Hu et al., 2020). Asyn response to MPTP treatment occurs shortly after the administration. Already 1 week after a single MPTP injection Purisai et al. (2005) observed a marked increase in asyn mRNA and protein levels in squirrel monkeys. At this early time point, neurodegeneration in the nigra was estimated to be around 10%. At later time points, MPTP treatment led to a more robust neurodegeneration (40%), yet asyn levels remained elevated suggesting a persistent accumulation of asyn in nigral neurons rather than a transient upregulation. It is also important to note that proteinopathy observed in the MPTP model is not limited to synucleinopathy. Accumulation of tau in mice and macaques, following MPTP treatment was also reported (Deffains et al., 2021;

Porras et al., 2012; Hu et al., 2020).

Rotenone: Systemic administration of pesticides are also capable of generating dopamine specific neurodegeneration in the nigrostriatal system accompanied with asyn accumulation. Rotenone is an insecticide that has been used extensively as a mitochondrial toxin (oxidative phosphorylation inhibitor) in *in vitro* studies. Exposure to it has been linked to a higher risk of PD (Tanner et al., 2011). Several different routes of rotenone administration, such as intravenous (i.v.), intraperitoneal (i.p.) or intracerebral, has shown to induce dopaminergic dysfunction. The treatment has its challenges in terms of establishing working concentrations: at higher doses rotenone results in high mortality due to cardiovascular toxicity and a careful dosing is required to be able to reach an effective window capable of inducing neurodegeneration (Betarbet et al., 2000; Cannon et al., 2009). Moreover, some animals are resistant to treatment. Betarbet et al. (2000) reported nigral dopaminergic neurodegeneration in 12 out of 25 rats that received i.v. rotenone treatment and Sherer et al. (2003a) reported 18 out of 35 rats that received subcutaneous administration of rotenone. Importantly, pathology observed in rotenone treated animals share features that are similar to PD pathology. Oxidative damage and mitochondrial complex 1 inhibition is the major cellular dysfunction in this model and is detected in the midbrain, olfactory bulb, striatum and cortex of rats treated with rotenone (Sherer et al., 2003b). Apart from dopaminergic neuron loss that may affect 50% of the SN (Cannon et al., 2009), loss of noradrenergic neurons in the LC is also reported (Betarbet et al., 2000). Asyn accumulation in dopaminergic neurons in the form of insoluble and phosphorylated asyn is also one of the important features of rotenone treatment (Di Maio et al., 2016; Betarbet et al., 2000). Indeed, *in vitro* experiments show that rotenone not only induces oxidative damage but also act directly on asyn and induce beta-sheet rich oligomer and fibril formation (Srivastava et al., 2020). Apart from its effect on the CNS, rotenone treatment also induces peripheral pathology, which we will touch upon in the body-first models section (Pan-Montojo et al., 2010, 2012; Morais et al., 2018; Dodiya et al., 2020).

Paraquat: Paraquat is a herbicide that was used worldwide (Tanner et al., 2011). It causes oxidative damage through redox cycling. This reduction is catalyzed by enzymes such as NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and NOS (nitric oxide synthase), and forms superoxide radicals (Di Monte et al., 2002). Systemic treatment of paraquat in mice causes a dose and age-dependent loss of tyrosine hydroxylase (TH) neurons (McCormack et al., 2002; Manning-Bog et al., 2002). McCormack et al. (2002) showed that three times weekly i.p. injections of 10 mg/ml paraquat caused 28% selective loss of nigral dopaminergic neurons and gliosis without any systemic effect. Moreover, paraquat induced upregulation of asyn *in vitro* and accumulation of asyn in the SN (Manning-Bog et al., 2002, 2003). Accumulation of pathological asyn is not limited to the SN after a single paraquat administration in wild-type mice and is observed in other brain regions such as the frontal cortex (Manning-Bog et al., 2002). Interestingly, in mice overexpressing mutant asyn, 6–8 weeks chronic oral paraquat treatment accelerated asyn pathology formation in the ENS (Naudet et al., 2017). Data suggested that accumulation of asyn pathology in the ENS occurs prior to the pathology formation in the brain in these mice. Unfortunately, peripheral pathology was not analyzed in earlier studies investigating asyn accumulation following paraquat treatment. However, given the fact that asyn upregulation in the brain following i.p. injections started already 2 days after injection, it is plausible to speculate that the administration route of toxins may define the onset site of PD pathology, i.e. i.p. treatment induces earlier changes in the brain and oral treatment affects the gastrointestinal system prior to the CNS. In conclusion, mechanisms of toxicity i.e. oxidative stress and mitochondrial dysfunction by which paraquat generates its effects as well as the consequences of the treatment leading to asyn accumulation make paraquat another valuable tool for studying PD related mechanisms, such as selective vulnerability of neurons against PD-related pathology and insults.

Other compounds: Above, we have detailed the most commonly used toxin models to recapitulate PD pathology in the brain. It is important to note that although not as commonly used as the ones listed above, other compounds such as systemic or local stereotaxic administration of lipopolysaccharides (LPS) (Choi et al., 2009; Liu and Bing, 2011; Bodea et al., 2014; Deng et al., 2021) and stereotaxic injections of proteasome inhibitors (reviewed in Bentea et al., 2017) induces PD-like features in animal models, including dopaminergic neurodegeneration and pathogenic asyn aggregation. Finally, peripheral administration of a neurotoxin to simulate PD pathology and neurodegeneration in the brain, could in essence be considered a body-first model as the pathogen is exposed to the PNS. The above-mentioned studies investigating the effect of systemic exposure of a certain neurotoxin investigated the brain pathology but data from PNS were not available. Importantly, the lack of non-motor symptom investigation does not rule out the presence of non-motor symptoms and PNS involvement in the reported studies categorized under brain-first PD. We will further discuss the possibility of body-first pathology initiation by neurotoxins below (see Table 3).

3.2. Viral vector-mediated asyn overexpression

Viral vector-mediated transgene delivery to express genes of interests *in vivo* has become an important tool for experimental modeling over the last decades. Here we will focus on AAV-mediated asyn overexpression and review the pathology that is observed using these models. It is however, important to note that the use of viral vectors in PD research is not limited to asyn overexpression using adeno-associated viral vectors (AAV) (Fischer et al., 2016).

The first vector-mediated asyn overexpression animal models were generated in rats using either recombinant adeno-associated viral (AAV) or lentiviral (LV) vectors (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002). Both vector types led to specific neurodegeneration of nigral dopaminergic neurons but the AAV vectors were more effective in inducing specific neuropathology as compared to the first generation LV vectors used in these studies as they provided better neuronal transduction reaching up to 95% efficiency in nigral dopaminergic neurons with relatively low inflammation (Ulusoy et al., 2008, 2010a). Therefore, AAV vectors have become more popular for gene delivery approaches for CNS diseases. Initial studies typically used AAV capsid serotype 2 (AAV2). These experiments successfully induced 50–80% dopaminergic cell death in the SN gradually developing up to 8 weeks post surgery (Kirik et al., 2002). AAV2 however, has a limited transduction volume in the brain and yields lower titers (Burger et al., 2004). The next generation AAV-mediated asyn delivery studies therefore employed different capsid serotypes of AAV, and have also reported consistent nigral neurodegeneration ranging between 30 and 80% in mice, rats and non-human primates (St Martin et al., 2007; Ulusoy et al., 2010, 2012; Ulusoy et al., 2012; Ulusoy et al., 2017; Decressac et al., 2012; Eslamboli, 2005; Oliveras-Salva et al., 2013; Bourdenx et al., 2015; Kirik et al., 2002; Klein et al., 2002; Kirik et al., 2003).

The pathology in AAV-mediated asyn overexpression models are very well characterized. One of the most important facilitators for unambiguous detection of the exogenous asyn protein is the presence of specific and sensitive antibodies against human asyn. Unlike other synucleinopathy models that depend on the detection of endogenous pathology, in human asyn overexpression models, presence of exogenous asyn protein can be easily and sensitively detected. Neuropathology in the AAV model closely features asyn pathology in human PD. Upon AAV-mediated overexpression of asyn, independent studies have reported accumulation of pathogenic asyn in nigral dopaminergic neurons and striatal projections, consequent decrease in dopamine levels as well as synaptic dysfunction (Decressac et al., 2012; Ulusoy et al., 2010; Kirik et al., 2002; Oliveras-Salva et al., 2013; Bourdenx et al., 2015; Lundblad et al., 2012; Tozzi et al., 2016; Ostergaard et al., 2020). Post-translationally modified forms of asyn, associated with PD pathology, were also detected in asyn transduced neurons following AAV injections,

i.e. asyn phosphorylated at Serine 129 and nitrosylated asyn (Yamada et al., 2004; Koprich et al., 2010; Ulusoy et al., 2010; Decressac et al., 2012; Oliveras-Salva et al., 2013; Bourdenx et al., 2015; Daher et al., 2014; Daher et al., 2015). Furthermore, asyn overexpression also led to accumulation of higher molecular weight asyn species, detected biochemically by proteinase K resistance and Tris-buffered-saline/urea fractionation, or detected immunohistochemically using conformation-specific antibodies targeting oligomeric and fibrillar forms of asyn (Koprich et al., 2010; Oliveras-Salva et al., 2013; Azeredo da Silveira et al., 2009; Taschenberger et al., 2012; Ulusoy et al., 2017). Common post-translationally modified forms of asyn found in PD brains were also subject to investigations using AAV-mediated transgene delivery approaches. Phosphorylation of asyn at Ser129 (p-asyn) is the most abundant form in LBs in PD and is commonly used to detect aberrant accumulation of asyn in patient brains as well as in animal models of PD (Tenreiro et al., 2014). The approach employed to study the role of p-asyn involved introducing a negatively charged amino acid residue into the S129 residue to mimic phosphorylation (Chen and Feany, 2005). However, results obtained in *Drosophila* and rodents were not conclusive in defining whether accumulation of p-asyn is more deleterious or not, as compared to non-phosphorylated asyn (Chen and Feany, 2005; Gorbatyuk et al., 2008; McFarland et al., 2009; Febbraro et al., 2013). Perhaps phosphorylation of asyn at the Ser129 residue itself is not toxic but is a mere marker of a pathogenic process in the brain (Buck et al., 2015). Another abundant post-translational modification of asyn in LBs is the C-terminal truncated forms. C-terminally truncated asyn is not only enriched in pathological asyn aggregates in PD brains but also has been shown to be more prone to aggregate and seed wild-type asyn in vitro (Serpell et al., 2000; Murray et al., 2003; Li et al., 2005; Ma et al., 2018). Indeed, co-expression of wild-type full length and C-terminally truncated asyn in the SN of rats using AAV vectors induced more enhanced asyn pathology in the nigra and striatal terminals as compared to single expression of the full-length protein (Ulusoy et al., 2010b).

AAV-mediated asyn overexpression in the nigrostriatal system models most features of human PD pathology in the midbrain dopaminergic system. Similar to the 6-OHDA model, the pathology is mostly confined within the nigrostriatal system. In order to model asyn pathology in other neuronal populations, alternative approaches were used by targeting non-nigral brain regions such as the ventral tegmental area, medial septum, raphe nucleus and hippocampus (Hall et al., 2014; Wan et al., 2016; Maingay et al., 2005; Cinar et al., 2020). An interesting example of these studies involved bilateral injections of AAV vectors into the nigra and hippocampus of rats (Cinar et al., 2020). Bilateral asyn overexpression in the SN and dentate gyrus not only successfully generated a combination of motor impairment and cognitive deficits (i.e. dysfunctional short-term memory and spatial learning) but also highlighted a role of the CA2 hippocampal region in the development of asyn-mediated behavioral changes (Cinar et al., 2020).

Almost exclusively, studies using AAV vectors aimed to study local pathology at the injection site. However, considering clinical and neuropathological findings from PD patients, it is plausible to expect asyn to spread from the initial injection site to another brain area in a stereotypical pattern. As peripheral asyn pathology was not well recognized at the time when the first viral-mediated asyn overexpression models were developed, asyn spreading in these earlier studies has not been reported. Recently however, researchers investigated whether pathogenic asyn can spread from the brain to the gastrointestinal system upon unilateral AAV injections into the midbrain of rats (Ulusoy et al., 2017). Two months after unilateral injections into the SN with AAV vectors encoding for human asyn, exogenous pathogenic asyn was detected in cholinergic neurons of the DMV. Importantly, DMV neurons project exclusively to the periphery; therefore the presence of human asyn in these neurons implicates neuron-to-neuron protein transfer. At 6 months, human asyn reached vagal nerve endings, more specifically motor terminals, in the stomach wall. Importantly, no sign of GFP was found in the DMV, vagus nerve or the gastrointestinal system in rats

injected with AAV-GFP, suggesting that the AAV vector used in this study is not capable of being transported from one neuron to another, and that the transfer of asyn from the midbrain to other regions is a specific property of the asyn protein. These findings reveal that asyn protein is capable of being transported from the brain to the periphery through efferent vagal projections (Ulusoy et al., 2017). Consequences of nigral asyn overexpression in the ENS was later investigated by O'Donovan et al. (2020). Although they failed to detect human asyn in the myenteric plexus at the analyzed time points, nigral asyn overexpression in the SN caused significant neuronal loss in the submucosal plexus and alterations in the gut microbiome (O'Donovan et al., 2020).

It is important to add a final note about viral vector-mediated asyn overexpression models, prior to the next section reviewing asyn seeding models: despite the presence of fibrillar asyn species in transduced neurons, transfer of asyn from one neuron to another in the AAV models involves non-fibrillar forms of asyn such as monomeric and oligomeric species (Helwig et al., 2016; Ulusoy et al., 2017). Together with other mechanistic studies, these data suggest that asyn is typically released in oligomeric forms and can induce spreading of asyn pathology through neuron-to-neuron transfer (Emmanouilidou et al., 2010; Danzer et al., 2007; Kim et al., 2013). Other studies that we will discuss below however, suggest that at least experimentally, other mechanisms are involved in pathological spreading of PD pathology.

3.3. Injection of fibrillar forms of asyn into the brain – ‘seeding’

The mechanisms by which protein pathology propagate from the gut to the brain or from the brain to the gut in PD has been one of the major gaps in our knowledge regarding disease progression. Although the notion that asyn spreading can occur along the body-brain axis is relatively new, it is long known from the field of prion diseases that protein pathology is not limited to one region and spreads trans-synaptically (Jan et al., 2021). Initially, a presence of a pathogen such as a virus or prion was proposed to explain the progressive nature of protein pathology in neurodegenerative diseases (Gajdusek et al., 1977; Prusiner, 1984; Braak et al., 2003b). The hypothesis became even more accepted following post-mortem findings of PD patient brains transplanted with fetal cells. 11 and 16 years post-transplantation of fetal nigral tissue into the striatum of PD patients, pathological asyn was detected in the surviving graft neurons (Kordower et al., 2008; Li et al., 2008). The presence of Lewy body pathology in 10–16 year old fetal neurons was indeed unexpected and raised questions on whether the host pathology could be transmitted in a prion-like manner, i.e. templated seeding, into the grafted neurons (Kordower and Brundin, 2009).

These initial findings and following debates accelerated a new line of research leading to the development of asyn seeding models of PD, where pathogenic ‘seeds’ in the form of artificial asyn pre-formed fibrils (PFFs) or human PD brain lysate/ homogenate are injected. PFFs are produced in vitro from recombinant monomeric human or mouse asyn. Fibrils are sonicated for fragmentation prior to use, to facilitate uptake. These pathogenic seeds elicit aggregation, hyperphosphorylation and ubiquitination of endogenous asyn (Aulic et al., 2014; Luk et al., 2009; Volpicelli-Daley et al., 2011). Injection of PFFs into the brain of rodents or primates trigger aggregation of endogenous asyn, typically detected by immunostaining against p-asyn (Luk et al., 2012a, 2012b; Masuda-Suzukake et al., 2013; Paumier et al., 2015; Patterson et al., 2019; Shimozaawa et al., 2017). Although sites other than the nigrostriatal system have been targeted (Luk et al., 2012a; Rey et al., 2018), so far the most common site of injection for intracerebral seeding is the striatum. Following PFF injection into the striatum, asyn accumulation is observed typically in brain regions that are connected to the injection site. Aggregated and p-asyn can be detected at 1 month post seeding in brain regions including but not limited to the cortex, amygdala, SN, hypothalamus and olfactory bulb (Luk et al., 2012b; Paumier et al., 2015; Masuda-Suzukake et al., 2013; Duffy et al., 2018). Rey and colleagues have shown that unilateral seeding of the OB with pathogenic

asyn leads to asymmetric spreading of pathology in mice (Rey et al., 2013, 2016, 2018). Unilateral asymmetric OB-first pathology in these studies represent a brain-first PD model.

3.4. Seeding effectivity

One important aspect defining the intensity and magnitude of pathology in intracerebral seeding models is the species compatibility of the seeding fibrils and endogenous asyn. Several studies observed that mouse PFFs, when injected into the rodent brain, are capable of inducing more asyn aggregation and trigger a more widespread pathology (i.e. also in regions that are not directly connected to the injections site) as compared to human PFFs (Luk et al., 2012b; Masuda-Suzukake et al., 2013; Luk et al., 2016). Small sequence differences between human and mouse asyn may reduce the interaction between the pathogenic seed and its substrate, limiting the toxicity of human asyn seeds in rodent models (Luk et al., 2016). Alternatively, mouse asyn may have higher pathological properties. In a recent study, PFFs generated from mouse asyn produced more asyn accumulation in rats than fibrils generated from rat asyn suggesting that additional factors beyond sequence homology between host asyn and PFF may play a role in this phenomenon (Howe et al., 2021).

The pathology that is characterized by p-asyn, nigral neurodegeneration and motor deficits is progressive and dose-dependent. Patterson et al. (2019) have characterized rats that were unilaterally injected with 8 and 16 μ g mouse PFFs into the striatum. Two months post seeding, nigral neuron numbers did not decline but a significant reduction in striatal fiber density was detected. At this early time point, 20–40% of the nigral neurons contained p-asyn pathology. Four months after seeding, around 25% loss of nigral neurons was detected in midbrain sections from rats that received 16 μ g PFFs, and no cell loss was observed in the lower dose group. At 6 months post seeding however, authors reported 25 and 50% nigral neurodegeneration in midbrain sections from animals that received lower and higher PFF doses, respectively. These detailed quantitative assessments clearly demonstrate that asyn fibril seeding of the striatum is capable of generating a dose- and time-dependent pathology in the brain. One important observation in this study that is worth further discussing is the decreased number of p-asyn containing neurons at later time points as compared to earlier points (Patterson et al., 2019). This is in fact not the first report of time-dependent reduction of asyn aggregates in a fibril seeding model. In a study where asyn PFFs were injected into the OB, researchers reported reduced density of asyn aggregation 18 months post seeding in some brain regions (Rey et al., 2018). The study by Patterson and colleagues was able to show this stark reduction by stereological quantification of nigral DAergic neurons and p-asyn containing nigral neurons, and reported a significant decline of p-asyn containing nigral neurons from around 5000 neurons (ca. 40% of total nigral neurons) at 2 months to around 600 neurons (ca. 5% of total nigral neurons) at 6 months post seeding. Taken together, these data suggest that asyn pathology is transient, partly due to neurodegeneration but also due to the ability of the surviving neurons to eliminate the pathology. Although transient pathology following asyn seeding requires further attention and investigations from a disease mechanism point of view, this is beyond the topic of this review. From an in vivo disease modeling point of view, these findings should be taken into consideration while designing experiments aiming to assess asyn pathology and preferably earlier time points should be used for such studies (Patterson et al., 2019).

3.5. Transgenic models: the role of elevated asyn levels

Endogenous levels of asyn are without doubt a critical determinant for the induction of PD pathology. Apart from the missense mutations (e.g. A30P, A53T, and E46K), multiplications in the asyn gene caused by duplication or triplication are found in families with PD (Vekrellis et al.,

2011; Schapira et al., 2014). These multiplications suggested that not only changes in asyn amino acid sequence but also elevated levels of asyn are sufficient to induce PD (Singleton et al., 2003; Kara et al., 2014). Such patients demonstrate increased asyn gene expression in the brain compared to healthy controls suggesting a direct correlation between asyn expression and PD development (Farrer et al., 2004). Even modest changes in asyn levels caused by changes in gene translation and transcription can increase the risk for developing sporadic PD (Farrer et al., 2001; Chiba-Falek and Nussbaum, 2001; Kingsbury et al., 2004). Indeed, analysis of surviving nigral dopaminergic neurons from sporadic PD patient brains using laser capture dissection show increased levels of asyn mRNA, further strengthening the role of upregulated asyn gene in PD pathogenesis (Grundemann et al., 2008). Based on these observations, researchers used ubiquitous upregulation of asyn as means of triggering PD pathology and developed several lines of transgenic mice overexpressing wild-type or mutated forms. These models typically reproduce progressive age-dependent asyn pathology in the brain and are often characterized by predominant cortical and limbic pathology with early involvement of the OB. Unfortunately, a major drawback of these models is the lack of nigrostriatal degeneration with few exceptions (reviewed in Hatami and Chesselet, 2015; Koprich et al., 2017).

Overexpression of asyn in transgenic models is not limited to wild-type or mutated forms. Truncated forms of asyn were also a subject of investigation (Tofaris et al., 2006; Wakamatsu et al., 2008; Daher et al., 2009). The rationale for these studies are based on findings that C-terminally truncated asyn is an abundant post-translational modification in the PD brain and is associated with enhanced aggregation (Serpell et al., 2000; Murray et al., 2003; Li et al., 2005; Ma et al., 2018). Initially, truncated asyn was expressed under the rat TH promoter on a mouse asyn null background. Although mice developed a progressive dopaminergic dysfunction and loss of motor function at old age, researchers were not able to detect frank neurodegeneration in the SN (Tofaris et al., 2006; Michell et al., 2007). Interestingly however, when truncated asyn was expressed on asyn wild-type background, i.e. with normal endogenous asyn present, mice manifested a more severe dopaminergic neurodegeneration, either in the form of age-dependent reduction in DA release (Garcia-Reitboeck et al., 2010) or frank neurodegeneration in the SN (Wakamatsu et al., 2008). Taken together, these findings suggest that basal levels of asyn play a role in asyn pathology development and are also an important determinant in animal models of asyn pathology. Therefore, a number of investigators combined models of induced asyn pathology with animal models with elevated basal levels of asyn to induce more severe pathology. We will discuss these below.

3.6. Combination brain-first models

Transgenic mice overexpressing asyn have often been used to induce more severe pathology in PFF-seeding models. Luk et al. demonstrated that injecting human asyn PFFs into the striatum of transgenic mice overexpressing human mutated (A53T) asyn induced rapid p-asyn pathology with more widespread distribution to other brain regions as compared to wild-type mice (Luk et al., 2012b). Similar to transgenic mice, AAV-mediated overexpression of asyn was also used in studies to increase basal levels of asyn prior to PFF-seeding. Combination of nigral AAV-asyn injections with striatal fibril injections led to a stark increase in nigral cell loss compared to AAV-asyn injection alone (Peelaerts et al., 2015). Using a similar approach in terms of combining AAV-mediated asyn overexpression and a fibril seeding model, Thakur et al. (2017) induced asyn pathology by injecting both AAV vectors and PFFs into the SN. Rats received a mild dose of AAV vectors 1 month before fibril injections. Asyn pathology was already present at 10 days post PFF-seeding. The group that received a combination of AAV and PFF demonstrated a significantly higher loss of nigral neurons, around 50% at 3 weeks post PFF-seeding, compared to 10 and 25% loss of nigral neurons in rats injected solely with AAV or PFFs (Thakur et al., 2017). The successful aggravation of PFF- (Luk et al., 2012a; Peelaerts et al.,

2015; Thakur et al., 2017; Schaser et al., 2020) or toxin-induced asyn pathology (Naudet et al., 2017; Musgrove et al., 2019) in animals overexpressing asyn using AAV vectors or transgenic models not only support the importance of basal asyn levels in PD pathogenesis but also provide basis for developing valuable animal models of brain-first and, as will be describe below, body-first PD pathology models.

4. Body-first animal models

In 2003, Braak postulated that pathological conversion of endogenous into pathogenic asyn may be initiated in the vagal nerve endings in the gut plexi, upon exposure to foreign pathogens. Thereafter pathology propagates through the vagus nerve into the CNS. Although several neuropathological evidence supported this hypothesis, the experimental validation of Braak's gut-first hypothesis required generating new animal models. Studies typically used two separate approaches to induce asyn accumulation: seeding endogenous asyn pathology in the peripheral organs, and overexpressing asyn using viral vectors. We will discuss seeding models that use gut as the initiation site and other organs (e.g. muscle) as initiation sites separately. However, it is important to note that, all body-first seeding models appear to induce similar CNS pathology patterns regardless of their peripheral initiation site. This is not surprising since body-to-brain propagation of pathology is trafficked along parasympathetic and sympathetic nerves that always enter the CNS in the same bilateral lower brainstem structures, after which pathology further progresses bilaterally in the brain in a predictable fashion. In contrast, in brain-first models, CNS pathology distribution heavily depends on the initiation site. Therefore, phenotypic variability is usually higher in brain-first models compared to body-first models. Here we first review gut-first and other body-first seeding models, as well as other models involving the use of viral vector, transgenic and neurotoxin models that explore the body-brain axis in PD.

4.1. Gut-first seeding models

These studies aimed to reproduce gut-to-brain spread of pathology along vagal and sympathetic nerves. To seed endogenous asyn pathology in the gut, researchers inoculated either PFFs or Lewy-body enriched

PD brain homogenates into the gastro-intestinal tract (GIT). The choice of injection site was based on earlier tracing studies showing enriched efferent vagal innervation in the pyloric stomach and proximal duodenum (Berthoud et al., 1991). Indeed, injection of asyn seeds into the upper GIT led to the transport of the protein into the vagus nerve and thereafter to the DMV (Holmqvist et al., 2014). Table 1 provides an overview of animal studies that have tested Braak's gut-first hypothesis using gut seeding. The table includes the inoculation site, studied timepoints, affected organs or fluids, symptoms, neurodegeneration or other markers. Abbreviations: p.i.: post injection, N.A.: not applicable.

Following this initial proof-of-concept work, researchers showed that seeding asyn into the GIT not only induces vagal transport of the injected material (i.e. pathogenic asyn) into the brain, but also seeds endogenous asyn. The OB and/or lower brainstem structures are affected earliest in all studies. Studies where pathology ultimately seems to affect the entire brain are characterized by a progressive brainstem-predominant pathology pattern, indicating trans-synaptic spread from lower brainstem structures to SN to limbic and cortical areas (Van Den Berge et al., 2019, 2021; Kim et al., 2019). Overall, literature seems to be consistent with time-dependent trans-synaptic caudal to rostral spread of pathology, including key regions heavily affected in PD such as the LC, SN, amygdala, and OB. Somewhat controversial, asyn pathology observed in the lower brainstem structures appears to be relatively transient upon single initiation of pathology in the gut, in all studies with longer follow-up (but not the rest of the brain), including in studies reporting nigral degeneration (Manfredsson et al., 2018; Uemura et al., 2018, 2020; Kim et al., 2019; Van Den Berge et al., 2021). The lack of asyn accumulation in longer follow up studies have raised questions about the mechanisms by which peripheral asyn is transported to the brain. In a study using adult non-human primates, researchers were not able to detect asyn pathology in the DMV nor in the vagus nerve 2 years post gut-seeding. Consequently, the authors argued against Braak's hypothesis and suggested an alternative route by a systemic gut-to-brain spread via blood rather than the vagus nerve (Arotcarena et al., 2020). Data at earlier time points in this study investigating DMV pathology is not available. Based on other studies however, asyn accumulation in the DMV occurs at early time points but then seems to disappear over time following gut seeding (Uemura et al., 2018, 2020; Manfredsson et al., 2018; Van Den

Table 1
Seeding studies investigating the gut-first hypothesis.

Author (year)	Inoculum, inoculation site/route	Animal strain	Time-point	Affected organs	Neurodegeneration and symptoms	Body-first hypothesis
Holmqvist et al. (2014)	Monomeric/oligomeric/PFF/lysate, duodenum	Young WT rats	1–6 days p.i.	DMV, vagus nerve	N.A.	Gut-to-brain transport
Uemura et al. (2018)	PFF, gastric wall	Young WT mice	1, 4, 8, 12 months p.i.	DMV (transient), ENS	N.A.	Transient gut-to-brain spread
Manfredsson et al. (2018)	PFF, descending colon	Young WT rats	1, 6, 12 months p.i.	DMV/LC (transient), ENS	Transient gut dysmotility	Transient gut-to-brain spread
Van Den Berge et al. (2019)	PFF, duodenum	BAC rats	2, 4 months p.i.	CNS (brainwide), heart, ENS	Synaptic dysfunction ENS	Persistent bidirectional gut-to-brain and brain-to-gut spread
Kim et al. (2019)	PFF, duodenum	Young WT mice	1, 3, 7, 10 months p.i.	CNS (brainwide but transient n.s. trend in DMV), ENS	Nigral neurodegeneration, incl. Motor, cognitive, psychiatric, olfactory and gastric dysfunction	Persistent gut-to-brain spread, prevented by vagotomy
Arotcarena et al. (2020)	Lysate, ventral stomach/duodenum	Young, adult, aged baboons	2 years p.i.	CNS (excl. DMV), blood, ENS	Nigrostriatal neurodegeneration	Against hypothesis, systemic periphery-to-brain spread
Challis et al. (2020)	PFF, duodenum	Adult and aged WT mice	7, 30, 60, 120 days p.i.	DMV (aged only), ENS	Reduced striatal dopamine, disruptions of ENS network connectivity and the endoplasmic reticulum-Golgi-lysosome pathway	Early gut-to-brain spread in aged mice only
Uemura et al. (2020)	PFF, gastric wall	Young BAC mice	1, 2, 4, 6, 8 months p.i.	DMV (transient), ENS	N.A.	Transient gut-to-brain spread
Van Den Berge et al. (2021)	PFF, duodenum	Aged WT rats	10 and 20 weeks p.i.	CNS (brainwide but DMV/LC transient), heart, skin, muscle, ENS	Synaptic dysfunction ENS and heart, incl. Gastric dysfunction	Bidirectional gut-to-brain and brain-to-gut spread, facilitated by ageing

Berge et al., 2021). It is therefore likely that gut-to-brain propagation of pathogenic asyn in the study conducted by Arotcarena et al. (2020) did occur via the vagus nerve, but that this pathology has disappeared at the investigated 2-year time-point.

Nevertheless, although time-dependent reduction of asyn aggregates in a fibril seeding model has been reported earlier (Patterson et al., 2019; Rey et al., 2018), it is true that this observation in animal models is at odds with human data, since 90% of body-first PD patients have pathology in the DMV in all stages of disease progression (Braak et al., 2003a). It is however likely that in gut-first patients, the pathology is triggered in the gut by recurring inflammation (or exposure to toxins) (Devos et al., 2013) at different sites across the ENS, creating recurring formation of pathology and transport to the DMV. This results in the typical pathology profile of body-first patients with pathology in the DMV > LC > SN. One can therefore speculate that in animal models, DMV pathology as result of a single asyn PFF injection into the gut is reversible due to lack of a constant trigger that induces asyn accumulation. Indeed, similar to observation from animal studies, although rare, some PD cases exhibit brainstem-predominant pathology without pathology in the DMV (Raunio et al., 2019). It is likely that these cases represent gut origin with transient or dynamic pathology in the DMV, due to a single or few insults in the gut that create a single or few formations and transport of pathogenic asyn to the DMV. It could be postulated that as soon as the influx of pathogenic asyn stops, the pathology gets degraded in the DMV, whereas other brainstem areas like the LC and SN continue to host and template pathology. This would result in a brainstem-predominant pathology profile without DMV pathology. Future PD animal studies employing single and chronic seeding are necessary to elucidate the potential dynamic nature of DMV pathology in PD.

Interestingly, pathology induced by gut-seeding is not only transmitted to the brain, but also to other organs known to be affected in human PD, via autonomic nerves. Van Den Berge et al. (2019) was among the first to show trans-synaptic gut-to-brain and brain-to-gut propagation of induced pathology along the vagus nerve, and also sympathetic structures including the autonomic ganglia and heart. The presence of asyn pathology in the stomach and heart suggested a secondary anterograde propagation after initial retrograde spreading. Simultaneously, Kim et al. (2019) demonstrated that vagotomy prevents gut-to-brain propagation, providing evidence that the vagus nerve serves as a gateway for gut-to-brain propagation of pathology. In a follow-up study, Van Den Berge et al. (2021) observed p-asyn accumulation not only in the gut and brain, but also in the heart, autonomic ganglia and skin of old wild-type rodents at 20 weeks post seeding, further supporting earlier in vivo studies suggesting a secondary anterograde propagation. Interestingly, the pathology involved both parasympathetic and sympathetic denervation of the gut and heart, respectively, as well as delayed gastric transit, in the prodromal disease phase of gut-seeded rats (Van Den Berge et al., 2021), similar to human body-first PD. Importantly, this study highlights that ageing in otherwise healthy animals substantially promotes p-asyn accumulation, and that old age is crucial for complete propagation to heart and skin. This is not surprising, since old age is considered the strongest risk factor in PD. It is well-known that pathogenic asyn mimics the behavior of prions, and the process of ageing has also been noted to increase vulnerability in the context of true prion disorders (Gasperini and Legname, 2014). It is conceivable that also in PD, the combination of both age-related cellular changes of the native protein and failing compensatory clearance mechanisms contribute to the enhanced risk of disease progression in older subjects (Reeve et al., 2014). Therefore, the use of old wild-type rodents seems to better recapitulate human body-first PD, and could be relevant clinically to optimize diagnostics and therapeutics in aged patients.

4.2. Other body-first propagation models

Besides the gut, also other peripheral routes (intramuscular (i.m.), i. v., intranasal (i.n.), and intraglossal (i.g.)), have been studied to investigate peripheral-to-brain propagation of pathogenic seeds. These studies are listed in Table 2 and also include special body-first animal models with seeding in lower brainstem structures associated with pre-motor symptoms in body-first PD. The table includes the inoculation site, studied timepoints, organ or fluids involved, symptoms, neurodegeneration or other markers.

Most studies investigating peripheral spreading routes employed the M83 transgenic mouse model that expresses human asyn with the A53T mutation (Sacino et al., 2014; Breid et al., 2016; Ayers et al., 2017, 2018; Lohmann et al., 2019; Wang et al., 2020; Ferreira et al., 2021; Macdonald et al., 2021). Homozygous M83 mice develop motor deficit from 8 months of age. Some studies also use M20 transgenic mice expressing wild-type human asyn driven by the mouse prion protein promoter, which also develop motor impairment from 6 months of age (Sacino et al., 2014; Ayers et al., 2018; Giraldo et al., 2018). In these studies fibrils, or asyn seeds, are administered i.m., i.p., i.v., i.n. or i.g. Across studies, i.p. and i.m. inoculation of fibrils seem to induce the most robust asyn pathology in the spinal cords and brains of transgenic mice, as well as gliosis and hindleg paralysis (Sacino et al., 2014; Breid et al., 2016; Ayers et al., 2017, 2018; Lohmann et al., 2019; Wang et al., 2020; Ferreira et al., 2021). In case of unilateral i.m. injection, motor deficit started unilateral. Motor phenotypes in both M83 and M20 mouse models are accelerated upon i.m., i.p. or i.v. injection of mouse fibrils, although less efficient in M20 models, compared to old naive mice. Upon seeding, pathology appears first in the lumbar spinal cord and progresses rostrally to several brain nuclei whose axons synapse directly on ventral motor neurons in the spinal cord. These studies therefore indicate axonal transport of pathogenic asyn along direct neuroanatomical connections. Moreover, transection of the sciatic nerve delayed disease progression indicating retrograde transport of pathogenic asyn to the spinal cord as a primary mechanism of CNS neuroinvasion upon i.m. seeding. It has to be noted that the distribution of pathogenic asyn in the CNS was similar to that of old naive M83 or M20 mice. Authors therefore speculated that the distribution is likely due to retrograde spread along neuroanatomical connections, as well as selective vulnerability, perhaps due to the mouse prion promoter used, to drive the expression of asyn in these transgenic mice models (Ayers et al., 2018).

Route of administration seems to play a role in asyn pathology development and propagation. For example, administration of PFFs through the blood does not consistently generate CNS pathology and motor deficits in mice, across studies, suggesting that i.v. route may be less efficient in inducing PD pathology as compared to i.m. and i.p. administration (Ayers et al., 2018; Lohmann et al., 2019). Similarly, in a comparative study, treatment of PFFs through oral gavage led to a slow transmission of asyn pathology as compared i.m., i.p. and i.v. delivery, probably because the orally administered seeds are partially eliminated through digestion and defecation (Lohmann et al., 2019). Though, i.v., i. n. and i.g. have also been proven effective seeding routes in heterozygous M83 mice, resulting in widespread CNS immunoreactivity against asyn pathology (similar to homozygous M83), a severe motor phenotype and shortened lifespan from 20 to 10 months (Macdonald et al., 2021). Discrepancy between studies could be due to administration of different doses as i.v. induction of pathology in heterozygous M83 mice is dose-dependent (Macdonald et al., 2021). Another study performing i.n. seeding in young wild-type rodents did not yield CNS disease (Masuda-Suzukake et al., 2013). This indicates that the seeding and propagation of pathogenic asyn depends not only on the administration route and seeds, but also on the basal levels of endogenous asyn present in the animal model used. It has to be noted that increased basal levels of endogenous asyn prior to peripheral seeding contribute to a phenotype that is not strictly body-first compared to peripheral seeding of wild-type animals, or other transgenic strains that are characterized by a more

Table 2

Other seeding studies investigating the body-first hypothesis.

Author (year)	Inoculum, inoculation site/route	Animal strain	Time point	Organs involved	Neurodegeneration/Symptoms/Other markers	Body-first hypothesis
Masuda-Suzukake et al. (2013)	PFF, intranasal	Young wild-type	21 months p.i.	No	N.A.	No peripheral-to-CNS transmission
Sacino et al. (2014)	PFF, bilateral or left gastrocnemius muscle	Young +/+ M20, M83 mice	4, 8, 12 months p.i. (M20) end-stage (M83)	CNS (spinal cord, medulla, pons, motor cortex)	Micro- and astrogliosis, microgliosis, rapid motor phenotype in M83	Peripheral-to-CNS spread, delayed by transection of sciatic nerve
Breid et al. (2016)	PFF, i.g., i.p.	Young +/-M83 mice	End-stage (229 days post i.g., 285 days post i.p.)	CNS (brain-wide)	Gliosis and motor phenotype (4/5 i.p., 1/5 i.g.)	Peripheral-to-CNS spread, most effective after i.p. seeding
Ayers et al. (2017)	PFF, tail vein, i.p., bilateral gastrocnemius muscle	Young +/+ M83 (i.v.), +/-M83 (i.p.) mice	4, 6 months p.i., end-stage	CNS (spinal cord, medulla, pons, thalamus)	Motor phenotype, most rapid after i.m., but also after i.p.. CNS involvement w.o. motor phenotype after i.v.	Peripheral-to-CNS spread after i.m., i.p. and i.v. seeding
Ayers et al. (2018)	PFF, unilateral sciatic nerve	Young +/-M20, M83 mice	1, 2 months p.i., end-stage	CNS (dorsal root ganglia, spinal cord, medulla, pons)	Micro- and astrogliosis, microgliosis, motor phenotype in M20 & M83	Peripheral-to-CNS spread
Lohmann et al. (2019)	PFF, i.g., i.p., i.v.	Young +/-M83 mice	End-stage (384 days post i.g., 208 days post i.v., 202 days post i.p.)	CNS (brain-wide)	Gliosis and motor phenotype (4/8 i.g. high dose, 10/10 i.v., 10/10 i.p.)	Against hypothesis, systemic periphery-to-brain spread
Schaser et al. (2020)	PFF, right gastrocnemius muscle	Young A53T SynGFP mice	4, 8 months p.i.	CNS (cortex, midbrain, pons)	Neurodegeneration, delayed formation of astrocytic inclusions	Progressive peripheral-to-brain spread
Macdonald et al. (2021)	A53T PFF, i.g., i.n., i.v., i.p.	Young +/-M83 mice	End-stage (i.g., i.n., i.v., i.p. 1, 3, 5 months)	CNS (brain-wide, spinal cord), similar to M83 +/-	Neurodegeneration, motor phenotype & shortened lifespan (i.n. more severe than i.g., i.v. dose-dependent), reduced spinal cord motor neurons when i.p., shift in microglial cell morphology	Progressive peripheral-to-brain spread, irrespective of inoculation route and seed used
Ferreira et al. (2021)	MSA homogenate (type II), i.m., i.v., i.p. PFF (PD, MSA), bilateral gastrocnemius muscle	Young +/-M83 mice	End-stage (i.m., i.v., i.p. 5 months) End-stage	CNS (brainstem, midbrain) strain-specific	Motor phenotype most severe with p25 (MSA) PFF strain	Peripheral-to-brain spread, most effective with MSA-strain
Wang et al. (2020)	PFF, celiac and stellate ganglia	Young +/-M83 mice	1, 2, 3, 4, 5 months p.i.	CNS (medulla, pons, midbrain), heart, gut. Skin	Autonomic dysfunction (orthostatic hypotension, constipation, hypohidrosis and hyposmia)	Progressive peripheral-to-brain spread
Seeding lower brainstem structures to model RBD, depression, anxiety (prodromal body-first PD)						
Shen et al. (2020)	PFF, tegmental nucleus	Young wild-type mice	1, 3, 5, 8 months p.i.	CNS (brain-wide)	REM-sleep disorder, locomotor dysfunction, depression, hyposmia, gastrointestinal dysmotility	Progressive brainstem-to-brain-wide spread, and brain-to-gut spread
Henrich et al. (2020)	PFF, PPN	Young wild-type	1, 6, 12 weeks p.i.	CNS (brain-wide)	Gliosis (unilateral), spread depending on connectome and cellular vulnerability	Retrograde trans-synaptic spread

slowly progressing phenotype, including the occurrence of enteric pathology prior to CNS pathology.

The underlying mechanism of how peripheral seeding can induce pathogenic asyn in the CNS remains to be elucidated. In case of i.p. or i.g. inoculation (Breid et al., 2016; Lohmann et al., 2019; Macdonald et al., 2021), the injected seeds may cross the mucosal barrier of the GIT, after which they are taken up by the vagal terminals of the myenteric plexus and propagate along parasympathetic and sympathetic pathways through the autonomic connectome. Alternatively, upon oral or i.v. inoculation, pathogenic asyn might be transported to the CNS via blood, penetrate the blood-brain barrier and cause CNS disease. Despite differences in experimental design such as observation timepoints, inoculum properties and animal model used, almost all peripheral seeding studies listed here demonstrate similarities to the pathogenesis with prions, with some exceptions (e.g. transient pathology in some neurons such as the ones in the DMV). Similarities include the apparent ability to spread from the periphery to the CNS via autonomic pathways, as well as neuroinvasive properties that lead to CNS pathology and disease.

The fact that disease onset in M83 and M20 mice can be shortened and predicted through a simple i.m. (> 50% faster) or i.p. (slightly faster) provides a valuable model to accelerate studies designed to investigate mechanisms underlying induction of inclusion pathology,

neurodegeneration and motor deficit. These models enable a more rapid and cost-effective preclinical testing of novel PD therapies in manifest disease stages, rather than prodromal disease stages.

Especially in body-first PD, the longer pre-motor phase allows time to attempt disease-modifying therapy. Therefore, studies have recently been focusing to model the pre-motor body-first PD, which is characterized by the presence of non-motor symptoms like RBD, depression, anxiety and/or autonomic disturbances, in the lack of motor symptoms. For example, a recent study aiming to model autonomic dysfunction in synucleinopathies injected PFFs into the autonomic stellate and celiac ganglia in M83 mice (Wang et al., 2020). Targeted seeding of the autonomic ganglia led to a phenotype that could be classified as a pre-motor body-first PD pathology model, characterized with progressive brainstem-predominant CNS involvement, pathology in gut, skin and heart without the presence of motor symptoms. Importantly, these mice developed autonomic dysfunctions such as orthostatic hypotension, hyposmia and constipation (Wang et al., 2020).

4.3. Seeding effectivity

Similar to intracerebral seeding, seeding effectivity appears to depend on the strain properties of the seeds in relation to the animal

model employed. Mouse fibrils seems to be more potent than human fibrils to induce peripheral-to-brain propagation upon i.m. seeding of M83/M20 mice and upon gut seeding of wild-type rats (Sacino et al., 2014; Ayers et al., 2018; Van Den Berge et al., 2021), which may reflect a species incompatibility or 'species barrier'.

The strain-hypothesis in synucleinopathies (PD, DLB, MSA) suggests that clinical heterogeneity seen in patients could be explained by the presence of distinct asyn strains that are characterized by different morphological and biochemical traits influencing e.g. cell tropism and toxicity of the asyn strain. Interestingly, some strains appear to be more seeding efficient or toxic within a certain animal model, supporting the strain-hypothesis. The P25a PFFs (MSA-strain) results in more rapid disease onset, shorter life span, and strain-specific distribution of pathogenic inclusions in the CNS of M83 mice, compared to mouse PFFs (Ferreira et al., 2021).

4.4. Body-first models by seeding the lower brainstem

Finally, we would like to review a number of studies that targeted the brain with the aim of reproducing the pathways affected in body-first PD. Pathology in the sublateralodorsal tegmental nucleus, pedunculopontine nucleus (PPN) and LC is associated with anxiety, depression, and (isolated) RBD, which is a robust biomarker of body-first PD (if RBD occurs prior to motor symptoms) (Andersen et al., 2020; Horsager et al., 2020). It is hypothesized that pathology propagates from the body to the lower brainstem via the vagus nerve in Braak stage 1 and 2 (gut > VN > DMV/IML (Braak stage 1) > LC/PPN (Braak stage 2)), leading to a body-first phenotype and pathology profile in the brain. By (bilateral) seeding of these lower brainstem-nuclei, a brainstem-predominant pathology profile is obtained in the early disease stages, accompanied by sleep and other autonomic disturbances, as observed in pre-motor body-first PD cases. Therefore, seeding studies targeting lower brainstem structures are considered body-first PD models.

The sublateralodorsal tegmental nucleus is involved in the maintenance of muscle atonia during REM sleep and lesions in the nucleus lead to RBD-like behavior (Lu et al., 2006; Fuller et al., 2007; Fort et al., 2009; Luppi et al., 2011). With an aim to recapitulate RBD-like behavior and investigate whether asyn induced RBD-like behavior would lead to PD-like pathology and behavior, researchers co-injected asyn PFFs with AAV vectors encoding for human asyn into the sublateralodorsal tegmental nucleus of wild-type mice. Indeed, asyn seeded mice developed RBD-like behavior characterized by electromyographic features and motor activity during REM sleep at 1 month post-injection. Mice also developed a time-dependent PD-like phenotype characterized by nigral neurodegeneration starting at 3 months post-injection and behavioral changes starting at 5 months post-injection that involved motor dysfunction, depression, hyposmia and gut dysmotility (Shen et al., 2020). Another set of experiments aiming the brain to model early body-first PD, targeted LC or PPN. In these studies, retrograde trans-synaptic spread of pathogenic asyn seems to be correlated to synaptic connections as well as other cellular or regional factors (Henrich et al., 2020). P-asyn pathology was localized in cholinergic (but not in neighbouring non-cholinergic neurons) of the PPN upon seeding, and all neurons projecting to PPN cholinergics displayed p-asyn, albeit pathology levels were not correlated to connection strength (Henrich et al., 2020).

4.5. Viral vector-mediated body-first models

Besides seeding with PFFs, viral-mediated overexpression of asyn has also been used to investigate body-first asyn propagation patterns in the brain. Targeted viral vector-mediated overexpression of human mutant A53T-asyn in LC neurons caused progressive noradrenergic neurodegeneration over 9 weeks, including formation of p-asyn pathology, micro- and astrogliosis. Apart from local pathology, extensive expression of asyn within the axons were observed in LC output regions,

indicating anterograde axonal transport of A53T-asyn (Henrich et al., 2018). Despite the broad asyn immunoreactivity in LC output regions, the authors were not able to detect asyn in neuronal cell bodies outside the LC within the experimental observation time (i.e. 9 weeks). As the authors suggest, this may indeed be due to relatively short observation time. Alternatively, the lack of neuron-to-neuron spreading following AAV-mediated asyn overexpression could also be attributed to the abundance of exogenous asyn expression that may mask the detection of asyn in other neurons in the brain. Although the model developed by Henrich et al. (2018) did not recapitulate asyn pathology spreading, it is merited in being an animal model of asyn mediated noradrenergic degeneration and may provide further insights in the involvement of the noradrenergic system in PD pathogenesis.

In an attempt to recapitulate the stereotypic caudo-rostral asyn spreading in PD, researchers used the vagus nerve as an entry route for human asyn protein (Ulusoy et al., 2013). AAV vectors encoding for human asyn were injected in the vagus nerve at the level of the neck. The rationale behind the intravagal injection was two folds: the vagus nerve projects to the medulla oblongata and terminates at these nuclei. Therefore, detection of any exogenous asyn in the more rostral areas such as pons, midbrain and forebrain indicates neuron-to-neuron spreading. Second, the vagus nerve connects the brain to the gastrointestinal system, where PD pathology is also present and the vagus nerve is presumed to be a conduit for transporting pathology from the peripheral nervous system to the DMV (Braak et al., 2003b).

Unilateral injections of AAV vectors encoding either for human asyn or GFP into the rat or mouse vagus nerve specifically transduced the neurons receiving efferent vagus projections located in the DMV and nucleus ambiguus, and the afferent vagal terminals in the nucleus of the tractus solitarius (Ulusoy et al., 2013; Ulusoy et al., 2015; Helwig et al., 2016; Rusconi et al., 2018; Musgrove et al., 2019; Chiu et al., 2021). At early time points, human asyn expression was limited to these medulla oblongata areas. At later time points however, human asyn carrying axons, but not GFP, were observed in the pontine reticular formation areas, parabrachialis nucleus and LC /subcoeruleus areas located in the pons as well as the midbrain reticular formation areas, dorsal raphe and periaqueductal gray at the caudal midbrain regions, and hypothalamic and central amygdala regions in the basal forebrain (Ulusoy et al., 2013; Ulusoy et al., 2015; Helwig et al., 2016). A common feature of these brain regions is that they all project directly into the medulla oblongata (Ter Horst et al., 1991; van der Kooy et al., 1984), supporting a mechanism of neuron-to-neuron human asyn transmission via anatomically interconnected pathways. Vagal AAV injections in both rats and mice illustrated that increased transduction of the neurons in the DMV results in faster and more pronounced asyn spreading towards more rostral brain regions (Helwig et al., 2016; Ulusoy et al., 2015). Subsequent studies showed that asyn spreading is contingent upon viable DMV neurons and neurodegeneration in the DMV would interrupt neuron-to-neuron asyn transport (Ulusoy et al., 2015; Rusconi et al., 2017). In fact, prolonged expression human asyn in the DMV neurons results in neurodegeneration and cessation of asyn spreading to more rostral brain regions (Rusconi et al., 2017). Interestingly, asyn induced neurodegeneration in the DMV neurons have long lasting consequences in brain regions indicated in body-first PD, including neurodegeneration and glial activation in the SN, LC and gliosis in the central amygdala (Rusconi et al., 2017). Consequences in gastrointestinal function following asyn overexpression in the DMV were recently investigated by another independent group. Chiu et al. (2021) showed that AAV-mediated mutant asyn expression through the vagal connections induces selective dysfunction of DMV neurons, specifically by modulating Kv4 channel current density. Asyn-mediated changes in Kv4 channel activity in DMV neurons in turn caused reduced autonomous firing rate, hereby reducing the gut motility (Chiu et al., 2021).

One important aspect of asyn spreading is the role of fibrillar forms. Following injection of asyn PFFs or Lewy body extracts from patients, extracellular fibrils can be internalized into the neurons, possibly by

interacting with cell surface proteins (Mao et al., 2016; Shrivastava et al., 2015), after which they spread from the injection site through seeding endogenous forms of the protein (Luk et al., 2012b; Masuda-Suzukake et al., 2013; Peelaerts et al., 2015; Recasens et al., 2014); Rey et al., 2016). In AAV-mediated asyn overexpression models however, exogenous protein is transcribed as the monomeric asyn and post-translational modifications including oligomerization and fibril formation occurs within the cell through natural events. Even though asyn within the neuron forms fibrillar structures, neuron-to-neuron transport likely involves monomeric and oligomeric asyn species. Indeed, Helwig et al. illustrated that asyn propagation following intravagal AAV injections in mice involved interneuronal transfer of non-fibrillar asyn, suggesting that smaller species, i.e. monomeric and oligomeric asyn may mediate early pathological process during disease development. Interestingly, this type of asyn transfer did not require seeding of endogenous protein. Indeed, studies performed using asyn null mice demonstrated that the endogenous protein is not required for the transport of these soluble forms (Helwig et al., 2016). Together with other studies performed by fibril injections, as briefly discussed in the brain-first PD model section, these data suggest that asyn spreading occurs through at least two mechanisms; transfer of fibrillar asyn to the recipient neuron and its subsequent self-propagation through seeding endogenous asyn, and inter-neuronal transfer of soluble protein species (i.e. monomeric and oligomeric) that diffuses via axonal projections and damages the recipient neurons.

4.6. BAC transgenic body-first models

Recent studies using bacterial artificial chromosome (BAC) transgenic rodents expressing human asyn show age-related prodromal symptoms as well as dopaminergic degeneration (Taguchi et al., 2020; Nuber et al., 2013). Although asyn is ubiquitously expressed in the body, pathology in the gut precedes basal ganglia pathology making these models more suitable for investigating body-first PD pathologies, compared to other transgenic models like M83 mice. BAC transgenic mice develop age-related aggregation of asyn in enteric neurons, which contributes to spread of pathogenic asyn along the vagus nerve and gastrointestinal dysfunction (Zhong et al., 2017).

BAC transgenic mice overexpressing A53T mutated asyn exhibit age-related pathology in the OB, cerebral cortex, REM sleep regulating structures, striatum and SN, including a mild dopaminergic degeneration without motor deficits at 18 months of age. In addition, these mice develop body-first prodromal PD symptoms such as a RBD-like dysfunction without atonia from 5 months of age and hyposmia from 9 months of age (Taguchi et al., 2020). Thus, this transgenic strain seems to be particularly valuable to study prodromal events and mechanisms that takes place in body-first PD (Taguchi et al., 2020). Similarly, BAC transgenic mice overexpressing another familial form of asyn, i.e. A30P mutated asyn, exhibit early aggregation of asyn in the gut, related to gut dysmotility and molecular dysregulations in the gut, prior to motor dysfunction (Gries et al., 2021).

4.7. Environmental models with focus on PNS and non-motor dysfunction

Since Braak's hypothesis, and subsequent detection of asyn pathology in gut biopsies (Braak et al., 2006), it has been suggested that the GIT may be prone to asyn misfolding and may provide entry to environmental toxins that promote asyn misfolding, ultimately leading to PD (Killinger et al., 2018; Jan et al., 2021). In human PD studies, EGCs, cholinergic cells and enteric endocrine cells (EECs) have been described as vulnerable cell populations for expression of asyn pathology (Neunlist et al., 2014; Clairembault et al., 2015; Chandra et al., 2017). Furthermore, an altered gut microbiome and intestinal permeability, as well as enteric inflammation have been associated to accumulation of pathogenic asyn (Forsyth et al., 2011; Scheperjans et al., 2015; Rolli-Derkin-Deren et al., 2020; Benvenuti et al., 2020). Gut inflammation and EGC

upregulation appear to coincide with gut permeability, as inflammation and glial activation modulate the intestinal epithelial barrier causing a hyperpermeable gut in human PD (Soret et al., 2013; Devos et al., 2013; Neunlist et al., 2014). Accordingly, leakage of gut bacteria or environmental toxins may trigger oxidative stress, inflammation and consequently asyn aggregation (Glass et al., 2010; Scheperjans et al., 2015). Since EECs directly connect to the gut lumen and enteric nerves, forming a neural loop between the gut epithelium and the ENS, it has also been postulated that uptake of gut bacteria or environmental toxins by EECs may trigger asyn misfolding (Chandra et al., 2017). Data from PD patients are typically limited and characterized by a large variability in diet, age, body mass, antibiotics/medicine, exercise, etc. As rodent models allow controlled experimental conditions, they enable investigations on the causality and interactions of pathology trigger factors such as gut microbiome, intestinal permeability and inflammation in both body-first and brain-first PD. Fig. 2 provides a schematic representation of these disease initiation factors in body-first PD. It remains to be elucidated how these gut factors are affected in brain-first PD.

In animal studies, a wide range of environmental toxins has been used to induce enteric inflammation (including dextran sulfate sodium salt (DSS) treatment, LPS, paraquat, rotenone and amyloid-producing bacteria) to recapitulate clinical neuroinflammation and other neuropathological markers of PD. In this paragraph, we review environmental toxin models that focus on the investigation of peripheral pathology, non-motor symptom development and PNS dysfunction. Table 3 presents an overview of most recent studies per toxin, including the animal strain used, administration method and key findings. We refer to recent in-depth review studies for a complete list.

DSS has been used extensively to model colitis as it induces enteric inflammation and disrupts the integrity of the intestinal barrier. DSS-induced enteric inflammation has been associated with CNS inflammation (Chassaing et al., 2014; Do and Woo, 2018), an altered gut microbiome (Dwyer et al., 2021), enteric asyn pathology (Resnikoff et al., 2019; Grathwohl et al., 2021), CNS asyn pathology and dopaminergic cell death (Kishimoto et al., 2019; Gil-Martínez et al., 2019). Importantly, several studies investigating the gut-brain axis in PD rodent models have shown that lesioning of SN results in altered gut pathology, physiology and dysmotility; and that enteric inflammation has a negative effect on nigrostriatal homeostasis (Anselmi et al., 2017; Garrido-Gil et al., 2018; O'Donovan et al., 2020; Pellegrini et al., 2016; Ulusoy et al., 2017). These studies provide evidence for a bidirectional link between the gut and PD associated neurocircuitry in the brain. And, a disturbed gut-brain interaction in PD patients might explain prodromal gut dysfunction, as well as increased vulnerability of PD neurocircuitry upon gut inflammation. However, it remains to be elucidated whether gut inflammation is directly related or a secondary side-effect of PD-associated neurodegeneration.

Additionally, systemic and intranasal LPS-induced inflammation has shown to promote neuroinflammation and dopaminergic cell death, including motor symptoms and non-motor symptoms such as gastrointestinal-, olfactory-, psychiatric-, and cognitive dysfunction (reviewed in Deng et al., 2020; Deng et al., 2021; Bhattacharyya and Bhunia, 2021). Note, the studies that failed to induce pathology in the CNS and/or neurodegeneration and a motor phenotype upon exposure of WT rodents to DSS or LPS all used young WT mice.

Also exposure to the pesticide rotenone has shown to induce an altered gut microbiome (Dodiya et al., 2020; Bhattarai et al., 2021), enteric and CNS inflammation (Dodiya et al., 2020), enteric asyn pathology (Pan-Montojo et al., 2010, 2012), as well as gut, olfactory and cognitive dysfunction, including immunological and neurotransmitter alterations in the pre-motor phase (Morais et al., 2018; Zhang et al., 2021), and even nigral cells loss with motor dysfunction (Bhattarai et al., 2021). In two separate studies by Pan-Montojo et al. (2010, 2012), oral rotenone treatment resulted in the formation of pathogenic asyn in the DMV and medullary preganglionic neurons of the IML. Importantly, this progressive pathology was prevented by hemivagotomy and partial

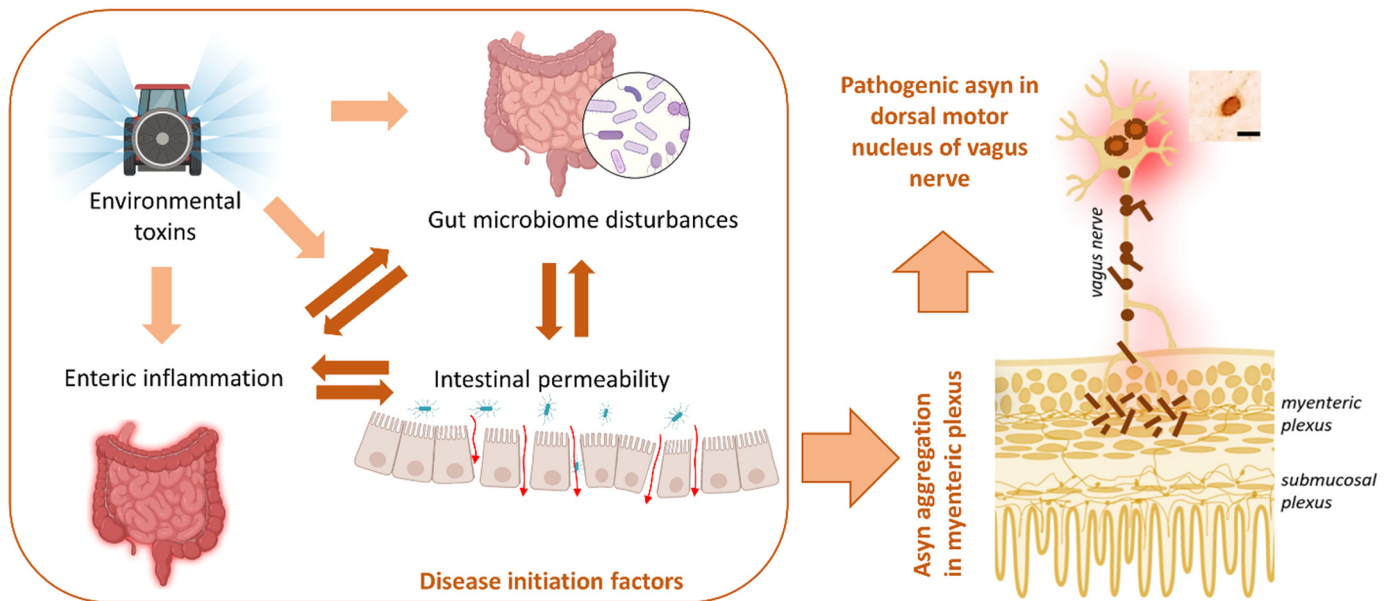


Fig. 2. Schematic representation of complex interplay of disease initiation factors in body-first PD.

sympatectomy implicating the direct role of the vagus nerve in the development of CNS pathology following rotenone treatment (Pan-Montojo et al., 2012). Despite using a higher dose, in contrary to the studies conducted by Pan-Montojo et al. (2010, 2012), Morais et al. (2018) reported a lack of asyn pathology in OB and did not report asyn pathology development in other brain regions following oral rotenone treatment. However, note that the former studies employed 1-year old wild-type animals, which may have facilitated the formation of asyn pathology in the CNS. It is likely that a combination of exposure to rotenone and ageing contributes to CNS pathology in these models.

The gut microbiome is not limited to induction of inflammation, as it also produces functional amyloids, which have self-aggregating properties. *E. coli* bacteria produce the functional bacterial amyloid curli, which is a protein that can efficiently cross-seed asyn and promote its aggregation. It is shown that exposure to *E. coli* bacteria induced pathogenic asyn in both the ENS and CNS of aged Fischer 344 rats (Chen et al., 2016; Kochman et al., 2021). Interestingly, the Fischer 344 wild-type rat strain develops spontaneous asyn pathology in the ENS at old age (Phillips et al., 2009; Van Den Berge et al., 2021). Taken together, these studies suggest that ageing is a contributing factor to the development of CNS pathology upon *E. coli* exposure as well. Also in over-expressing human asyn mice, colonization with curli-producing *E. coli* bacteria, but not with the *E. coli* strain that does not produce curli protein, promotes asyn pathology in the gut and brain, including motor deficit and gastrointestinal dysfunction. Importantly, curli-colonized mice exhibited increased p-asyn deposition in the SN, suggesting a spreading pattern in line with Braak's hypothesis and body-first PD. Oral treatment with a gut-restricted amyloid inhibitor interfered with curli-mediated acceleration of asyn pathology and behavioral deficits in these mice, supporting that exposure to microbial amyloids in the gut alone is capable of triggering PD-related pathology in the gut, as well as in the brain (Sampson et al., 2020).

Finally, it has been reported that probiotics and fecal microbiota transplantation (FMT) treatment may preserve gut homeostasis and play a protective role in prodromal PD (Metta et al., 2021). Preclinical probiotic and FMT studies in a controlled experimental set-up are needed to investigate whether this treatment strategy is able to affect brain functioning through gut-brain signaling.

5. The SOC model

In extension of the brain-first vs. body-first hypothesis, a hypothesized stratification of PD subtypes based on disease onset site, a novel model of PD etiopathogenesis has been proposed: the asyn origin site and connectome (SOC) model. According to this model the clinical and histopathological profile is determined by (Adler and Beach, 2016) the anatomical location of the initial pathogenic asyn (the disease onset site), and (Alafuzoff et al., 2009) the neural connectome, which plays a crucial role in determining how pathogenic asyn propagates along the brain-body axis (Borghammer, 2021). Since human brain connectivity is dominated by ipsilateral connections, this model implies that, in brain-first cases, any initiating pathogen in a single hemisphere propagates predominantly within the ipsilateral hemisphere. On the other hand, the myenteric plexus is innervated by both the posterior and anterior vagus nerve and thus in gut-first cases, retrograde propagation of pathology from the gut to the DMV occurs bilaterally, after which pathology further propagates within the brain bilaterally. Importantly, this onset-site-dependent unilaterality of pathology explains the degree of asymmetry in motor and non-motor symptoms that PD patients exhibit. In support of this model, it has been shown recently that nigrostriatal degeneration, measured with FDOPA PET and DaT SPECT, was significantly more symmetric in body-first PD patients (i.e. patients with isolated RBD or de novo PD patients with RBD) versus brain-first PD patients (i.e. PD patients without RBD). These data confirm that brain-first PD is characterized by more asymmetric distribution most likely due to predominant unilateral propagation of pathogenic asyn within the brain, compared to body-first PD (Knudsen et al., 2021).

The predictive value of this model remains to be tested. Brain banks rarely own brains from which both hemispheres are processed and stored in equal conditions. Also most animal studies analyze and portray propagation in a single hemisphere, and therefore evidence to support this model is limited. However, animal studies that do include bilateral investigation of asyn pathology distribution do support the validity of these concepts, with symmetric initial involvement of the DMV, followed by symmetric involvement of the LC, SN, limbic, and cortical regions (Van Den Berge et al., 2019, 2021; Kim et al., 2019). Similar symmetric involvement is seen with i.v., oral and i.p. administration of seeds (Breid et al., 2016; Lohmann et al., 2019). This is not surprising since all peripheral pathology is trafficked along the same vagal and sympathetic nerves, after which it enters the brain through the same

Table 3
Environmental toxin studies investigating the gut-first hypothesis.

Author (year)	Animal strain	Injection site, dose	Key pathological findings
Paraquat			
Naudet et al. (2017)	M83 mice (A53T mutant asyn-overexpressing mice under prion promoter)	oral daily for 6–8 weeks, 10 mg/kg	p-asyn accumulation in ENS and increased GFAP, no p-asyn accumulation in the brain
DSS			
Do and Woo (2018)	WT mice	oral daily for 3 or 7 days, 3% (w/v)	alterations in GFAP and brain-derived neurotrophic factor depending on brain region (hippocampus and hypothalamus upregulated, amygdala downregulated)
Resnikoff et al. (2019)	monkeys	N.A.	increased expression of p-asyn, inflammatory markers and oxidative stress in ENS
Kishimoto et al. (2019)	M83 mice	oral daily for 12 weeks, 0.5% (w/v)	increased accumulation of p-asyn in ENS and CNS, accompanied with accelerated nigral cell loss and motor dysfunction
Gil-Martínez et al. (2019)	MPTP mice		accelerated nigral cell loss and significant exacerbation of microglial and astrocytes activation
Grathwohl et al. (2021)	WT or (Thy1)-h [A30P]asyn	oral daily for 5 days, 1 cycle (acute) or 3–4 cycles (chronic), 1%, 2.5% or 5% (w/v)	age-dependent accumulation of p-asyn in ENS and SN of 21-but not 9-month-old transgenic, accompanied with loss of TH-positive nigral neurons
Dwyer et al. (2021)	WT mice pretreated with i.c. LPS injection and i.p. paraquat injections (every other day for 11 days 10 mg/kg)	oral daily for 5 days, 250 mg/ml	altered gut microbiome, ENS inflammation, no effect on LPS-induced neurodegeneration
LPS (see Table 1 from Deng et al., 2020; and Bhattacharyya and Bhunia, 2021), in addition:			
Grathwohl et al. (2021)	WT or (Thy1)-h [A30P]asyn	i.p. at day 0 and day 3, 0.5 mg/kg	no accumulation of p-asyn in ENS
Deng et al. (2021)	WT mice	i.p. daily for 4 days, 0.3 mg/kg	olfactory dysfunction and anxiety-like behavior, but no nigral cell loss or motor dysfunction; brain-region specific changes in inflammation and oxidative stress
Rotenone (see Table 1 from Miyazaki and Masato, 2020), in addition:			
Dodiya et al. (2020)	WT mice with chronic stress daily for 12 weeks	oral daily for 6 weeks, 10 mg/kg/day	increased intestinal permeability, microbiota dysbiosis, neuroinflammation, and neurodegeneration in SN, worsened by chronic stress
Bhattarai et al. (2021)	WT mice (germ-free (GF) or conventionally raised (CR))	oral daily for 6 weeks, 10 mg/kg	altered gut microbiome, increased intestinal permeability in CR mice only; loss of TH-positive neurons, motor

Table 3 (continued)

Author (year)	Animal strain	Injection site, dose	Key pathological findings
Zhang et al. (2021)	WT mice	i.p. daily for 3 weeks, 0.75 or 1.5 mg/kg	phenotype in CR mice only cognitive decline, associated with neurodegeneration, synaptic loss, accumulation of p-asyn and microglial activation in the hippocampus and cortical regions

lower brainstem structures, i.e. bilateral DMV and LC. Further bilateral spreading through the brain occurs hereafter in a predictable fashion, including evolving symptoms. In contrast, unilateral injection of pathogenic asyn in the OB or striatum causes progressive predominant ipsilateral CNS involvement that is 3 to 10 times higher than in the contralateral hemisphere (Rey et al., 2016; Okuzumi et al., 2018; Masuda-Suzukake et al., 2013; Henderson et al., 2019, 2020; Meziás et al., 2020; Thomsen et al., 2021). Note, pathology levels are often represented using colormaps with logarithmic scale due to large asymmetry in pathology levels. Interestingly, the pathology distribution in the contralateral hemisphere generally resembles the pattern in the ipsilateral hemisphere at the previous time point, indicating further propagation of CNS pathology occurs via commissural, homolog-to-homolog connections from the affected brain structure to its contralateral homolog (Borghammer, 2021). Thus, intracerebral spreading of pathology is highly dependent on the disease onset site in brain-first PD, and disease progression and symptoms are therefore more heterogeneous in brain-first PD, compared to body-first PD. Fig. 3 portrays pathology distribution in the brain of a gut-first and a brain-first seeding model.

6. Shortcomings

Non-human primates represent the best approximation of the human PNS and CNS, and their use is indispensable for validating therapeutics prior to clinical trials. Although not as similar to human PNS and CNS as compared to non-human primates, the higher complexity of the rodent nervous system makes them suited as model species to investigate PD pathogenesis and pathophysiology, in particular as it allows the simultaneous assessment of behavioral features, in vivo imaging and pathological assessments. One major advantage of rodent models is the availability of tools to modulate gene expression through transgenesis or viral vectors (i.e. overexpression, knockout or knockdown of disease-related genes) that enable mechanistic investigation of novel mediators of pathology and neurodegeneration. Yet, there are four major shortcomings of current PD animal models that hamper accurate recapitulation of human PD: (Adler and Beach, 2016) shortage of PD models recapitulating the prodromal disease stages, (Alafuzoff et al., 2009) use of young animals, (Andersen et al., 2020) shortage of multi-variate approaches (i.e. linking gut microbiome and inflammation to PD progression), (Angers et al., 2010) lack of whole-brain studies. We will discuss each of these points below:

To date, there is no therapy available to cure or slow down PD and the early or pre-motor disease phases are likely to be most suitable for applying personalized disease-modifying therapy. The shortage of animal models aiming to recapitulate prodromal PD pathology, such as the early involvement of the autonomic nervous system in body-first PD and non-nigral pathologies in brain-first PD, has significantly slowed down our understanding of PD pathogenesis. Considering that the onset of non-motor symptoms precedes the onset of motor symptoms up to 20 years, it is crucial to study prodromal non-motor aspects of PD, which could assist significantly early diagnosis and interventions. Another

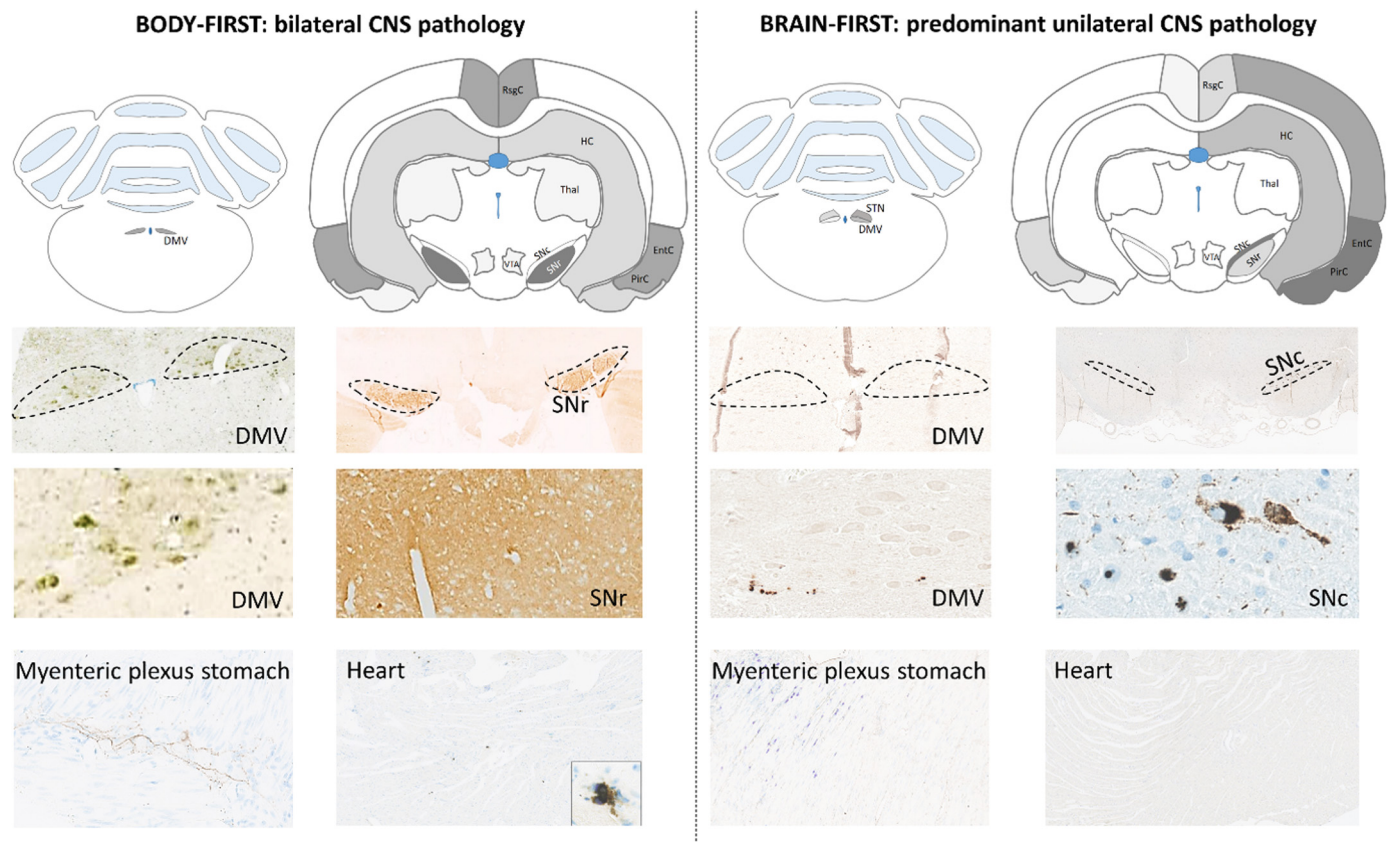


Fig. 3. IHC-detection of p- α syn in multiple organs of body-first (gut-seeded) and brain-first (amygdala-seeded) rodents indicating bilateral spread in body-first and predominant unilateral spread in the CNS of brain-first rodents at 10–12 weeks post seeding. Our data indicate early involvement of heart and stomach, including gut dysfunction, in the body-first animal model at 10 weeks post seeding (Van Den Berge et al., 2019). The entire DMV, LC and neuropil of the SN pars reticulata (SNr) are heavily affected in the gut-seeded model, indicative of propagation of α syn from the LC and other structures. In contrast, α syn deposits are detected primarily in ipsilateral limbic and cortical structures upon unilateral seeding of the amygdala, with clear cytosolic inclusions in the ipsilateral SNc, but not SNr, indicative of propagation of α syn from the amygdala. Only minor contralateral pathology and no PNS pathology is observed at early time points (data not published). Later time points need to be studied to investigate brain-to-body propagation of pathogenic α syn in the brain-first model.

issue regarding PD disease modeling is the lack of models capturing the entire course of the disease, i.e. models that can reproduce both peripheral and central pathologies, and display nigrostriatal degeneration and motor symptoms. Ideally, a PD animal model should recapitulate the entire disease progression manifesting a clear prodromal phase that eventually progress to nigral dopaminergic neurodegeneration, displaying both motor- and non-motor symptoms. Such complete models would be very valuable to test personalized disease-modifying treatment strategies. Nevertheless, one should also keep in mind that the relatively short lifespan of animals used in *in vivo* modeling may not allow an ideal case scenario, and it is highly possible that one can never generate such a perfect model, instead, different models may be needed to mimic partial disease phenotypes and progression. In this light, focus on modeling a clear prodromal phase is crucial as it allows differentiation between subtypes at early disease stages. Body-first and brain-first PD phenotypes are highly overlapping at middle and late disease-stages as PD eventually affects the entire autonomic nervous system.

Although the importance of age in PD pathology development is well known, the use of aged animals for developing models of PD pathology so far has been relatively neglected. This is probably due to time and cost related concerns. Recent *in vivo* experimental studies however, further substantiate the role of ageing in endogenous and induced α syn pathology development and implicate the role age as a facilitator for PD pathology (Phillips et al., 2009; Pan-Montojo et al., 2010; Challis et al., 2020; Van Den Berge et al., 2021). The mechanisms by which ageing promotes α syn pathology requires further investigations and such studies could help define novel targets against α syn aggregation. The use

of aged animals should therefore be taken into account while designing future experiments as an aggravating factor in gut-first and brain-first PD animal models, in order to elucidate the nature of age-dependent interactions with α syn pathology.

Despite a growing body of evidence highlighting the involvement of the gut health in PD pathogenesis the exact mechanism linking enteric inflammation and neurodegeneration in PD pathogenesis remains to be elucidated. Presence of α syn in the gut likely induces enteric pathology and dysfunction, which can in turn trigger enteric inflammation, EGC dysfunction, gut dysbiosis and gut hyperpermeability, suggesting that all these variables may be important factors in α syn pathology development and spreading. Although an increasing number of studies recently focused on some of these variables (Kelly et al., 2014; Chen et al., 2016; Chen et al., 2018; Gorecki et al., 2019; O'Donovan et al., 2020; Sampson et al., 2020), further studies characterizing multiple pathological gut variables in a single experimental set-up and correlating these to disease progression are warranted. Future body-first and brain-first animal models require a multi-variate approach to elucidate the subtype-specific pathological contributions of alterations in gut permeability, inflammation and microbiome.

Finally, an important shortcoming of *in vivo* studies, in regards to modeling and understanding asymmetric progression in (brain-first) PD cases, is the lack of reports detailing pathology development in each hemisphere. We currently have scarce data on how unilateral neurodegeneration and α syn pathology affects the contralateral hemisphere. A number of studies using unilateral pathology induction paradigms, reporting contralateral α syn pathology, clearly suggest that initiation of

asyn pathology from a single site is capable of inducing asyn pathology in the contralateral hemisphere (Ulusoy et al., 2013; Paumier et al., 2015; Duffy et al., 2018; Patterson et al., 2019). Moreover, reports suggest that unilateral injections of PFFs into the striatum lead to nigral neurodegeneration in the contralateral side without the presence of asyn pathology, yet the mechanisms of neurodegeneration is not known (Paumier et al., 2015; Patterson et al., 2019). The SOC model provides a whole body disease case and also emphasizes the importance of studying asyn propagation in both hemispheres. Future animal studies will need to verify the predictability and applicability of the SOC model. Nevertheless, taken together, these studies emphasize the importance of a whole-brain analysis to study the involvement of predominant symmetric or asymmetric CNS pathologies in gut-initiated or brain-initiated PD pathologies respectively.

7. Translational value and future applications

Although the involvement of genetic/transcriptional changes in the asyn gene caused by mutations, ageing, and exposure to environmental toxins are well accepted risk factors of PD, it is unlikely that PD stems solely from a single factor, but instead from the interactions between these factors. Animal models do not typically incorporate all possible factors that may trigger PD pathology nor is there a single animal model that can mimic all clinical features of PD. Given that PD may have variable causes as well as different sites of onset (i.e. body-first and brain-first subtypes), presence of a single model would not be meaningful either. Perhaps one of the major failures of PD animal models in terms of their predictive value for translational studies is the use of a single animal model recapitulating an isolated pathology (e.g. nigrostriatal pathology) based on a single disease risk factor (e.g. mitochondrial dysfunction or asyn overexpression) (Decressac et al., 2011; Barker and Bjorklund, 2020). We have discussed advantages and shortcomings of animal models reproducing PD pathology above, without doubt they need to be improved in terms of recapitulating the actual disease mechanisms and stages in order to improve translational outcome. From a clinical diagnosis and study design point of view however, one should also not underestimate the potential of patient stratification in improving the success from the bench to the bedside. Early stratification of PD subtypes are not only valuable in terms of improving translational studies for developing disease-modifying therapies, but also are necessary for early intervention.

Although the stratification of body-first and brain-first PD in the prodromal phase is yet to be proven, emerging novel techniques may be instrumental for identification and phenotyping prodromal stages. For example, recent developments in asyn PET tracers and CNS biomarkers provide invaluable possibilities for early stratification and detailed investigation of PD subtypes (Fayyad et al., 2019; Kuebler et al., 2021). Research towards developing such biomarkers is highly important and animal models will undoubtedly play an important role in validating these markers. Recently developed, more sensitive, investigative tools such as PMCA (Protein-Misfolding Cyclic Amplification) (Shahnawaz et al., 2020), RT-QuIC (Real-Time Quaking-Induced Conversion) (Groverman et al., 2018), PLA (Proximity Ligation Assay) (Roberts et al., 2019) and thiophene-based assays (Shahnawaz et al., 2020) are other examples of valuable tools for detecting prodromal markers of PD to investigate the relation between disease onset site, but also possible variations in asyn strain characteristics leading to different synucleinopathies and PD subtypes. Identification of subtype-specific asyn aggregates from easily accessible peripheral fluids or tissues using these emerging techniques may further contribute stratification of PD patients.

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