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Experimental models of gut-first Parkinson's Disease: a systematic review

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Abstract and keywords

Background: There is strong support from studies in humans and in animal models that Parkinson's Disease (PD) may begin in the gut. This brings about a unique opportunity for researchers in the field of Neurogastroenterology to contribute to advancing the field and making contributions that could lead to the ability to diagnose and treat PD in the premotor stages. Lack of familiarity with some of the aspects of the experimental approaches used in these studies may present a barrier for neurogastroenterology researchers to enter the field. Much remains to be understood about intestinal-specific components of gut-first PD pathogenesis and the field would benefit from contributions of enteric and central nervous system (CNS) neuroscientists.

Purpose: To address these issues, we have conducted a systematic review of the two most frequently used experimental models of gut-first PD: transneuronal propagation of α-synuclein preformed fibrils (PFFs) and oral exposure to environmental toxins. We have reviewed the details of these studies and present methodological considerations for the use of these models. Our aim is that this review will serve as a framework and useful reference for neuroscientists, gastroenterologists, and neurologists interested in applying their expertise to advancing our understanding of gut-first PD.

Keywords: alpha-synuclein, animal models, gut-brain axis, Parkinson's Disease, pesticides, vagus nerve, gut-first PD

Introduction to gut-first PD and review objectives

Parkinson's Disease (PD) is a prevalent neurodegenerative disease characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which results in the cardinal motor symptoms of tremor, rigidity and bradykinesia. PD is a significant source of disability and morbidity among the aging population and its prevalence is increasing[1]. The understanding that the gut is affected in PD dates to the earliest observations of the disease phenotype and pathology. Constipation, which is seen in up to 80% of PD patients[2], was described by James Parkinson in his initial case reports describing the disease.[3] Lewy Pathology, the pathological hallmark of PD, was first observed in the colon in 1928[4,5].

More recently, Braak and colleagues theorized that PD may *begin* in the gut[6-8]. This theory was based on observations that Lewy Pathology, now termed Lewy-type synucleinopathy (LTS) spread regionally in the brain from areas more closely connected to the periphery in association with disease duration[6-8]. Further pathological studies identified LTS in autonomic fibers innervating the gut[9,10], in Auerbach's and Meissner's plexi of the enteric nervous system (ENS)[9,11,12], and in the submucosal plexus of biopsies obtained during colonoscopy[9,13,14]. Epidemiologic studies provided further support for this theory demonstrating a decreased risk of PD with truncal vs. super-selective vagotomy[15-17]. Plausibility for the initiation of PD in the ENS is supported in preclinical disease models[18]. Additionally multi-modal imaging studies and an analysis of two autopsy case series have identified patterns of pathology that are highly supportive of both brain-first and body-first disease paradigms in human PD[5,19].

At the onset of the parkinsonian motor symptoms, 50%-60% of dopaminergic neurons have already been lost[20]; thus, slowing or stopping disease progression in the gut, could be a mean to preventing debilitating motor symptoms. PD, however, is a complex disease and the factors that promote gut-brain spread of α-synuclein (α-Syn), and those that determine whether the spread results in neurodegeneration are yet to be completely understood.

Our objective is to provide a systematic review of two of the most frequently used models of gut-first PD: transneuronal propagation of α-Syn preformed fibrils (PFFs) and oral exposure to environmental toxins. Through critical comparison of studies using these models, our aim is to provide a roadmap for enteric and CNS neuroscientists to successfully implement these models. In addition, evaluation of variability in outcomes may uncover areas where further study is most warranted.

Search strategy and results of search

We limited our search to full manuscripts in English which were indexed in PubMed through August 1st, 2022. We used the following initial search with terms limited to title/abstract: (vagus AND (synuclein OR Parkinson*)) AND (gut OR intestin* OR enteric OR gastro* OR duoden* OR colon). This yielded 145 records which were independently reviewed by two authors (EJV, XT). Additional studies were identified by searching reference lists of reviews and studies from the initial search. We included preclinical studies that used intestinal-specific delivery of PFF or toxins and evaluated CNS disease pathology. For each study, data extraction was performed independently by two authors (XT or AY and EJV). Where there were discrepancies, we discussed the study and were able to arrive at consensus among the three primary authors or following consultation with the senior author (RAT). When there

were questions regarding the interpretation of the data presented in the studies, the original study authors were contacted for clarification.

PD models of intestinal exposure to environmental toxins

Background

Toxin models are well-established and commonly used animal models for translational studies in the PD field. Due to reliable selective toxicity to dopaminergic neurons, these models are frequently used to investigate therapeutics, such as medications, surgical interventions and experimental fetal cell transplantation therapies[18]. Toxin-induced degeneration of dopaminergic neurons can replicate some aspects of PD in animal models through exposure to and administration of various environmental variables. The GI tract is a major route of human exposure to environmental toxins, thus overall interest in gut-first PD has increased the interest in animal models using oral toxin exposure. While GI dysfunction and ENS pathology in PD patients are now widely acknowledged, there is debate on the onset, severity, and contribution of the ENS to GI dysfunction and PD development. Here we have discussed the most common and recently developed oral environmental toxin animal models that replicate parkinsonian pathology.

Rotenone

Rotenone is an odorless naturally occurring toxin derived from the roots and stems of *Derris*, *Tephrosia*, *Lonchocarpus*, and *Mundulea* plant species[21]. It is a broad-spectrum insecticide, piscicide, and pesticide effectively used in the agricultural industry as well as in fish management[22]. Rotenone has a short half-life in water and its degradation is accelerated by light and elevated temperatures; thus, the risk to

terrestrial animal health is considered low in comparison to synthetic pesticides[23]. Because rotenone is highly lipophilic, it easily crosses the blood brain barrier and biological membranes. Once inside the cell, it builds up in subcellular organelles, such as the mitochondria. Here, it binds specifically to complex I of the mitochondrial electron transport chain, causing disruption of mitochondrial respiration, overproduction of reactive oxygen species (ROS), and oxidative stress[24,25]. Interest in rotenone in the PD field expanded following the demonstration by Betarbet et al. that rotenone infusion replicated the main symptoms of PD in rats in 2000[26]. Systemic application of rotenone causes the PD-like pathology of nigrostriatal neurodegeneration with formation of α-Syn inclusions that are similar to Lewy bodies, but also leads to neurodegeneration of dopaminergic neurons in the ventral tegmental area, which is an adjacent area typically spared in PD[26,27].

Oral administration of rotenone was first used in 2007, by Inden and colleagues[28]. Mice (2 months old) given different doses (0.25-30 mg/kg) of rotenone orally every day for 28 days showed dose-dependent motor impairments, and PD-like CNS pathology (see Box 1 for an explanation of these findings), including nigrostriatal DA neurodegeneration, and up-regulation of α-synuclein in the surviving DA neurons[28]. Interestingly, oral rotenone at a higher dose (100 mg/kg) and longer treatment duration (56 days) would significantly increase mortality to 90% (40% mortality was observed with the 30 mg/kg dose)[29]. DA neuron loss with the 100 mg/kg dose was also more variable in comparison to 30 mg/kg, which produced a consistent loss of tyrosine hydroxylase (TH)-positive neurons and behavioral impairment[29]. Therefore, 30 mg/kg was frequently employed as a high-dose regimen for oral treatment. In addition, the effects of 100 mg/kg were more likely to be the result of acute toxicity.

Most but not all studies using rotenone have shown an intestinal phenotype. Tasselli et al. replicated the neurodegeneration in the SNpc, but a decrease in stool frequency was the only GI phenotype alteration and there were no neuropathological changes in the ENS[30]. However, when Xiaodong et al. administered rotenone, behavioral and neuropathological features of PD were accompanied by colon inflammation and GI motor dysfunction[31]. More importantly, in these mice, constipation was prodromal to motor deficits[31]. The primary difference between these studies was that evaluation of GI function by the former group was performed three weeks after the final rotenone dose (Table 1)[30]. Thus, it is possible that GI dysfunctions were somehow recovered following rotenone treatment.

In 2010, in the first use of rotenone to specifically model gut-first PD, Pan-Montojo et al. used a low dose (5 mg/kg) of rotenone but a longer oral administration (1.5 and 3 months) to induce parkinsonian neuropathology in mice[32]. The ENS, dorsal motor nucleus of the vagus (DMV), and spinal cord intermediolateral nucleus all developed α-synuclein aggregates after 1.5 months of treatment, with the exception of SNpc, which displayed a comparable pathology after 3 months of treatment. Surprisingly, the amount rotenone in the brain and plasma, measured by high performance liquid chromatography, was too low to elicit parkinsonian signs[32]. Mitigation of rotenone-induced CNS PD pathology by vagotomy provided additional evidence that PD may begin in the ENS and spread via the vagus nerve to the lower brainstem, a process that precedes degeneration of the dopaminergic nigrostriatal system[32,33]. Notably, rotenone increased α-synuclein accumulation and exocytosis in primary cultures of enteric neurons, and α-synuclein was taken up by sympathetic neurons in co-culture and transported in a retrograde fashion[32].

Low dose oral rotenone (3-10 mg/kg) has been widely used by various teams to replicate gut first PD[32-39]. The primary phenotypes of this model include SNpc neurodegeneration, motor deficits, and decreased gut motility. This phenotype is observed in young and old mice using both high (30 mg/kg) or low (3-10 mg/kg)-dose regimens, suggesting that age is not a crucial factor (Table 1). In terms of the dosing regimen, many studies implement this model by administering rotenone every day (high dose) or five or six times per week (low dose) (Table 1). Less frequent dosing may not be effective in producing SNpc neurodegeneration, as was seen in one study where rotenone (5 mg/kg) was given twice weekly even though the treatment duration was extended to 50 weeks[40]. However, review of this study highlights an additional important methodological factor as, in this study, rotenone was not dissolved in a lipophilic medium, such as DMSO or chloroform, which would have facilitated rotenone absorption and avoided liver metabolism[24]. Optimization of the dose and the drug vehicle to achieve effective absorption and target a narrow toxicity index is considered to be the primary challenge in the use of this model.

Paraquat

Paraquat (PQ^{2+}, 1,1′-dimethyl-4,4′-bipyridinium dichloride) is an herbicide widely used in many developing countries because it is affordable, non-selective, and fast-acting. Using paraquat in agriculture is comparatively safe because dermal or air exposure often only results in minor, localized harm[41,42]. However, oral exposure can result in acute toxicity, which is associated both with damage to the lungs, liver and kidneys, as well as a high case-fatality rate[43]. The structural similarity of paraquat to MPP+ (1-methyl-4-phenylpyridinium), the active metabolite of the well-known Parkinsonism-inducing neurotoxin MPTP (1-methyl-4-phenyl-1, 2, 3, 6-

tetrahydropyridin), prompted evaluation of its link to PD[44,45]. Paraquat is metabolized to PQ^+ by NADPH oxidase. PQ^+, like MPTP, is taken up by neurons via the dopamine transporter (DAT), neutral amino acid transporter and organic cation transporter[44,46]. It inhibits the activity of mitochondrial complex I and induces neuronal oxidative stress through mitochondrial ROS generation, mechanisms strongly implicated in PD pathogenesis[45,47,48].

Preclinical studies in rodents have shown that intraperitoneal injection of paraquat produces dose-dependent PD pathology in the CNS, including synucleinopathy, deficits in nigrostiatal dopaminergic signaling, and neurodegeneration (see Box 1)[47,49-51]. While the most frequent route of exposure to paraquat in human and animals is oral ingestion, surprisingly, neither intragastric gavage of paraquat at 5 mg/kg/day in Wistar-derived rats for two weeks nor chronic drinking water exposure of paraquat (50 mg/mL) daily for six weeks in C57BL/6 mice produced PD pathology[52,53]. However, these negative findings do not preclude that toxicity may be induced with protocols using different parameters of delivery, dose, duration of exposure, and oral bioavailability. Studies using radiolabeled paraquat found that it was poorly metabolized in rat brain, as showed by the fact that it significantly accumulated in the brain following repeated oral administrations[42,53]. Additionally, orally delivered of paraquat significantly accelerated the formation of α-synuclein aggregates in the ENS of TgM83 human α-synuclein transgenic mice and induced ENS reactive gliosis in C57BL/6 mice, however, this study did not evaluate as to whether these findings were associated with acceleration of central PD pathology[52].

In 2018, Anselmi et al. introduced a novel PD model in rats by oral coadministration of 1 mg/kg (a dose that is subthreshold for pathology with IP administration) of paraquat combined with lectin, a glycoprotein capable of specific recognition of and

reversible binding to carbohydrate residues of the complex glycoconjugates on the cell membrane[32]. Lectins are commonly utilized to enhance drug absorption at a variety of sites because cell surface carbohydrate expression is a widespread phenomenon[54]. Lectin can enter the cell through receptor mediated endocytosis or under conditions in which there is increased transcellular permeability[55,56]. There is evidence in *Caenorhabditis elegans,* that dietary lectins can propagate trans-synaptically and have toxicity to dopaminergic neurons[57,58]. Anselmi et al. found that when they administered paraquat in combination with lectin (P+L) to rats by oral gavage daily for one week, the rats developed a parkinsonian phenotype, which included both central (motor deficits, degeneration of SNpc TH-IR neurons, presence of pathologic α-synuclein (p-α-Syn) in the brainstem and midbrain) and intestinal (decreased gastric motility, enteric p-α-Syn) features[57]. Notably, motor deficits were reversible by L-dopa administration. In addition, prior to the onset of central PD pathology, there was alteration of nigrovagal modulation of gut motility, indicated by attenuation of changes in gastric motility that result from chemical stimulation of nigrovagal pathways[59]. Furthermore, vagotomy prevented the development of central parkinsonian symptoms and constrained p-α-Syn to myenteric neurons, supporting a vagally mediated progression of the synucleinopathy[57,60]. These data demonstrate that the oral administration of subthreshold doses of paraquat and lectin is a promising model of prodromal environmental toxin-associated PD. Additional studies are needed to further characterize this model and to determine whether it can be modified for use in mouse models.

Trichloroethylene

Trichloroethylene (TCE) is a major nonflammable industrial solvent currently used in the manufacturing of adhesives, lubricants, paint strippers, pesticides, metal degreasing and waterless drying[61-63]. Prior to its industrial use, TCE was used as a light anesthetic agent and an extractant in food processing[62]. Its use in medicine and food processing was outlawed in the United States in 1977 after it was found to be a neurotoxin and multisystemic carcinogen[61].

In vivo studies have shown that TCE is metabolized to chloral, a precursor of TaClo, which is structurally similar to MPTP[64,65]. TaClo is strongly cytotoxic to dopaminergic neurons through inhibition of the mitochondrial respiratory chain complex I and subsequent induction of oxidative stress and neuroinflammation, a common etiopathologic theme in toxin-mediated PD[65-67]. Converging evidence from epidemiological and *in vivo* studies support that exposure to TCE increased the risk of developing PD[63,67,68]. In 2008, Gash et al. showed that oral administration of TCE (1000 mg/kg/day) to Fisher 344 rats five days per week for six weeks resulted in selective complex 1 mitochondrial impairment in the midbrain with concomitant neurodegeneration of DA neurons[67]. These findings were replicated by Liu et al. with increasing doses of TCE (200, 500, 1000 mg/kg)[66]. Rats orally treated with TCE showed motor deficits, a significant dose-dependent loss of SNpc dopaminergic neurons, and accumulation of intracellular α-synuclein in the dorsal vagal complex (DVC) and SNpc, but not in the cerebral cortex[66]. Interestingly, striatal levels of the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid were significantly reduced in TCE-treated rats, whereas DA levels were not altered, which could potentially represent an early stage of axon terminal dysfunction[66]. The same group extended the studies for 8 months at the same dosing frequency on C57BL/6

mice[63]. Chronic oral exposure of TCE (400 mg/kg/day) resulted in progressive and selective loss of 50% DA neurons in the SNpc and a 50% loss of DA, as well as 72% loss of DOPAC[63]. The prolonged studies suggest that the unaltered DA level in the rats might be due to short exposure of TCE.

These novel TCE rodent models present characteristic features of parkinsonism, which may aid in better understanding of how environmental risk factors contribute to gut-first PD. The aforementioned studies examining the impact of oral TCE, investigated brain pathology but data from the peripheral nervous system were not available yet. It is necessary to conduct more research to determine how TCE impacts the ENS and whether the accumulation of intracellular α-synuclein in the DVC and SNpc is the result of vagal transmissions from the GI system or local toxicity from TaClo.

Strengths and limitations of environmental toxin models

Mitochondrial dysfunction and oxidative stress have long been implicated as pathophysiologic mechanisms underlying PD. An ideal experimental model of PD should reproduce the progressive, selective nigrostriatal dopaminergic degeneration and Lewy body formation seen in PD, test the relevance of systemic defects in complex I activity, and explain the potential involvement of toxin exposure in development of parkinsonism. Unfortunately, there is no animal model that can recapitulate all aspects faithfully. There has been criticism that the levels of toxins in the experimental animals do not occur in the natural environment. However, the goal of using animals to model PD is to replicate in days or weeks what may require years of exposure in people. The advantages of these models include a specific PD phenotype and a shorter experimental period. PD

animal models using toxins have made significant contributions to the creation of symptom-targeted medications, particularly for motor symptoms. In addition, studies have shown that environmental toxins trigger release of α-synuclein from enteric and sympathetic neurons into the extracellular space, where it can be endocytosed by neighboring neurons and transferred to non-neuronal cells, making toxin models potentially useful for the study of enteric synucleinopathy. It is important to note that additional toxins are also used, although less commonly. These include organophosphate Chlorpyrifos, maneb, and dieldrin.

PD models using intestinal delivery of exogenous α-Syn preformed fibrils (PFFs)

Background

A pivotal advance in the PD field was the identification of α-synuclein as the primary component of Lewy Pathology following the discovery of familial mutation in the α-synuclein gene (*SNCA*) in 1997[69]. Several critical breakthroughs were made leading to the current widely accepted paradigm that spread of p-α-Syn is a key component of PD pathogenesis[70-73]. α-synuclein is a 14-kilodalton soluble protein in monomer form, however, under “favorable” conditions, monomers will form oligomers and oligomers will form fibrils[74]. Mechanisms leading to initial formation of pathologic strains are not fully understood, but conditions that are associated include increased monomer concentration, mutations in the *SNCA* gene[74], and exogenous compounds such as lipolysaccharide[75] and environmental toxins[49]. These pathologic seeds induce misfolding of additional α-synuclein in a prion-like manner and spread transneuronally. It should be noted that while fibrillar forms prompt seeding of

endogenous α-synuclein, evidence supports that that the oligomeric forms may be responsible for toxicity[76].

The findings that α-synuclein was present in vagal preganglionic fibers in the gut[77], and that intragastric rotenone resulted in the presence of p-α-Syn in the brainstem, which was prevented by vagotomy[32], were followed by the first study demonstrating that p-α-Syn was directly taken up by vagal efferent fibers in the gut and transported in a retrograde fashion to the DMV[78]. This and subsequent studies involving injection of p-α-Syn into the intestinal wall[78-87], are the primary focus of this section of the review. For comparison, we have also carefully reviewed models in which p-α-Syn is administered intraabdominally by other routes, including injection into autonomic ganglia[88], intraperitoneal injection[89-92], and oral administration[89,93].

Intestinal wall injection of PFFs

We identified ten studies that met our inclusion criteria[78-87]. These studies were published by eight separate groups, and used rat[78,79,82,84,87], mouse[80,81,84-86] and non-human primate (NHP)[82,83] models. Characteristics and findings from these studies are shown in Tables 2 and 3. Figure 1 summarizes studies in rodents with stomach and/or duodenal injection as a *Parallel Sets* diagram to aid in visualizing which combinations of experimental factors resulted in pathology above the brainstem.

Studies in rats

The first report of α-synuclein propagation from the gut to the brain via the vagus nerve was made by Holmqvuist et al. in 2014[78]. Their study built on findings that intragastric administration of rotenone produced CNS pathology via the vagus nerve (reviewed above)[32]. Five sites in the wall of the stomach and duodenum of adult WT Sprague Dawley rats were injected with PD brain lysate, recombinant human α-

synuclein monomers, PFFs or bovine serum albumin (BSA). Sections of intestine, vagus nerve and DMV were evaluated for α-synuclein by immunohistochemistry (IHC). Co-injection of labeled Atto-550-α-synuclein with fluorogold was used to confirm that the injected α-synuclein was taken up by vagal efferent fibers. This study was focused primarily on demonstrating initial propagation via the vagus nerve and evaluated pathology shortly after injection (12 hours to 6 days). α-synuclein in both PFF- and PD lysate-injected animals was detectable in the DMV in three to six days. Higher level brain regions, including the locus ceruleus (LC) were not affected nor was there evidence of neuronal cell death at 6 days.

Manfredsson, et al.[82] also used WT Sprague Dawley rats, but their study differed from Holmqvist et al.[78] in several ways. These included the use of murine PFFs (mPFFs) instead of human PFFs (hPFFs), injection into the colon instead of the stomach and duodenum, use of a larger total PFF dose (60 µg vs. 15 µg), and evaluation for pathology at later time points (1, 6, and 12 months). They confirmed propagation of p-α-Syn to the DMV but found that it was transient and was not present at six or twelve months.

The third and fourth studies in rats were conducted by Van Den Berge et al.[79,87]. Both studies injected PFFs into six sites in the pylorus and duodenum. In the first study, adult rats expressing human α-synuclein (BAC-*SNCA*) and WT littermates were injected with a total of 18 µg hPFFs and pathology was evaluated at two and four months post-injection[87]. For both studies, hPFFs contained a mutation preventing phosphorylation at serine-129, which ensured the typical antibodies for p-α-Syn, which are specific for phosphorylation at this site, would only detect endogenous p-α-Syn. While both PFF- and PBS-injected BAC-*SNCA* rats showed p-α-Syn in multiple cortical brain regions, only PFF-injected animals showed pathology in

the DMV and LC. Additionally, the pathology in the SNr was stronger in PFF- than PBS-injected BAC-*SNCA* rats. WT rats showed no p-α-Syn in the CNS or ENS. PFF-injected BAC-*SNCA* rats also showed p-α-Syn in the heart and stomach, which is suspected to be due to anterograde spread as shown in other models[88,94]. In the second study, they injected young (3-month), adult (10-12-month) or aged (18-month) WT Fischer rats with a higher dose of hPFFs (60 µg) and evaluated pathology at 10 and 20 weeks (young and adult only)[79]. Young rats were also injected with mPFFs and evaluated at 10 weeks. Similar to their prior study, young rats did not develop pathology when injected with hPFFs; however, injection with mPFFs resulted in CNS pathology in the DMV, LC and SNpc after 10 weeks. In contrast to young mice, hPFFs did result in seeding of endogenous α-synuclein in both adult and aged mice[79]. p-α-Syn was detected in the DMV, LC and SNpc of adult rats at 10 and 20 weeks post injection and in aged rats it was detected at 10 weeks (20 weeks was not evaluated for this group). Quantitative measures of p-α-Syn showed an accelerated rate of progression in aged rats with a significant increase in SNpc immunoreactivity (IR) at 10 weeks (vs. adult PBS injected) in comparison to 20 weeks for adults. ENS α-synuclein was proteinase-K resistant only in old rats at 10 weeks and adult rats at 20 weeks. The use of mutated PFFs is a strength of this study as it ensured that the detected pathology resulted from the seeding of endogenous α-synuclein. The absence of even transient pathology in the DMV using mutated fibrils suggests that some p-α-Syn seen in other studies may be exogenous.

Studies in mice

In the first published study in mice, Uemura et al. injected the stomach of WT C57BL/6J mice at two months of age with 48 µg mPFFs and evaluated pathology at

23 and 45 days, and at 4, 8, and 12 months[81]. p-α-Syn was seen in the DMV at 23 days and persisted through the last time point (12 months), but it declined in intensity in both the DMV and the ENS at time points after 45 days. Providing support for a vagal route of propagation, DMV pathology was not seen in mice that underwent cervical vagotomy and no pathology was seen in the spinal cord. α-synuclein pathology was not observed in brain regions other than the DMV, except for limited pathology in the nucleus ambiguous.

In contrast to Uemura et al., progressive PD pathology including loss of nigrostriatal dopaminergic neurons was observed in young (2-month) WT C57B/6J mice following injection of 25 µg mPFFs in the pylorus and duodenum by Kim et al.[85]. Pathology was detectable in the DMV at one month post-injection and had progressed to the olfactory bulb (OB) at 10 months post-injection. hPFFs did not result in an increase in CNS p-α-Syn, nor was pathology evident in animals following truncal vagotomy or in animals lacking endogenous expression of α-synuclein (*Snca*-KO). Phenotypically, PFF-injected animals showed GI and sensorimotor deficits similar to those seen in other models of parkinsonism. Loss of DA neurons, measured by unbiased stereology, was present at seven months. The authors posited that the difference in findings between this study and the study by Uemura et al.[81] may have been related to the length of the injected fibrils. This explanation initially seemed plausible as fibril length has been linked to potency[74,95]; however, Uemura et al., later published a correction stating that the image in their publication was mislabeled and showed PFFs prior to (rather than following) sonication[96]. The authors have confirmed that the PFFs were the same used in their later study (66.8 nm, N. Uemura, personal communication, November 19, 2022). In this second study, Uemura et al. used the same protocol in A53T-SNCA-BAC mice and while the pathology was increased over

WT, it still did not progress beyond the brainstem[80]. There is in vitro evidence that mPFFs are less efficient at seeding human α-synuclein[76], but mPFFs and hPFFs have both frequently been used in intracerebral injection models[95]. Could the difference in outcome be related to PFF dose? Uemura et al., used a higher total dose of PFFs than Kim et al., but this is unlikely to decrease seeding potential based on a study indicating the absence of a "ceiling effect" in cell culture[95]. The studies also differed in the injection site (pylorus and duodenum vs. stomach). It is possible that this accounts for the differences in findings. There is heterogeneity in vagal neurons innervating the upper gut[59], but Uemura et al. achieved efficient vagal propagation to the DMV from the stomach, which suggests a potential role for factors unrelated to gut-to-brain transmission but perhaps representing a "second-hit" necessary for disease. In human biopsies, immune cell distribution and cytokine expression was vastly different between the stomach and the duodenal bulb[97], and based on the known importance of inflammation in PD[98], the role of the inflammatory response in gut-first PD deserves additional investigation. Differences in the microbiome, other environmental factors, and variability in PFF pathogenicity, which may arise from any differences in buffers or storage conditions[99], could also account for different outcomes.

The final two studies in mice provide further examples of the importance both of PFF-related factors and host physiology in disease pathogenesis. Ahn et al.[84] found that the fragments of α-synuclein and Tau following cleavage by asparagine endopeptidase, form highly neurotoxic fibrils that result in neurodegeneration three months following injection into the colon wall of mice overexpressing WT *SNCA,* a model of age-dependent neurodegeneration which does not display neuronal loss at this age[100]. Challis et al.[86] used a very small dose of mPFFs (6 µg total injected into

two sites in the duodenum) to initiate seeding in the gut. They observed only transient p-α-Syn present in the brainstem in young (2-month) mice. In aged mice, however, motor and GI deficits and p-α-Syn in the CNS persisted at 120 days and this was accompanied by reduced striatal dopamine content. Interestingly, increasing autophagy via GBA-1 gene transfer reduced synucleinopathy and improved GI function, suggesting that age-related changes in physiology may be just as important as PFF pathogenicity.

Non-human primate models

Two studies have evaluated gut seeding in NHP models. In addition to injection in rats, Manfredsson et al.[82] injected NHPs (Macaca fascicularis) with hPFFs or α-Syn monomers (200 µg over nine sites in the colon and one stomach site) or an adenoviral vector expressing A53T mutated α-synuclein to the same sites. In contrast to their findings in rats, they were not able to detect any p-α-Syn in the CNS at 12 months post-injection. All α-synuclein forms increased insoluble α-synuclein in the ENS, but the increase was highest for the viral vector and lowest for monomers. The degree of pathology was similar in the colon and stomach. Arotcarena et al.[83] injected a very small dose (2.4 ng) of PD brain lysate into the stomach and duodenum or the striatum of baboons. At 24 months post injection, regardless of the injection site, p-α-Syn was seen in the SNpc and other brain regions and there was a loss of nigrostriatal dopaminergic neurons. Increased burden of enteric p-α-Syn was associated with reduced IR for TH in the SNpc; however, no p-α-Syn was seen in the DMV or vagus nerve. In addition, blood levels of α-synuclein were increased for both injection sites.

Other intraabdominal administration of PFFs

Our search strategy also identified six studies that administered PFFs via intraabdominal routes[88-93]. These studies are summarized in Table 4. Interestingly, all studies were conducted in M83 mice which express A53T mutant human α-synuclein under the prion promoter. Most of these used intraperitoneal (IP) injection[89-93]. While IP is a systemic route of delivery and it is not specific to the intestine, it may be relevant for disease models that rely on transneuronal propagation. Thus, we have included these studies in the table. Three studies deserve special mention. First, Wang et al.[88] have demonstrated that injecting PFFs into the stellate and celiac ganglia produces a model that recapitulates multiple prodromal autonomic parkinsonian symptoms, including delayed intestinal transit. Second, in two studies, oral administration of PFFs resulted in acceleration of neurodegeneration[89,93], with oral delivery daily for 28 days equivalent to a single IP injection[89]. It will be important to determine whether oral administration is capable of producing pathology in additional models, both for insight into pathogenesis and for simplification of animal models.

Strengths and limitations of PFF injection models

PFF gut-seeding models are useful gut-first models primarily because they recapitulate a model of disease progression that increasingly appears to be relevant in human disease. Importantly, they model the phase of disease that occurs after there is pathologic misfolded α-synuclein and may be less relevant to address questions regarding this initial event. There are still many aspects of this model that are not understood, including the variability in disease progression ranging from brainstem-restricted pathology to neurodegeneration. As a model of a complex human phenotype, this variability can also be seen as a strength since determining

predictors of progressive pathology may uncover disease-modifying therapeutic targets.

There are key methodical considerations when adopting this model. First, consideration must be taken in choice of host strain and α-synuclein origin species. WT mouse strains show variability in the response to PFFs[95] and strains that may be more susceptible, such as C3H/Hej, have not yet been used[95]. Second, PFF characteristics are likely to be highly important, and our understanding of what is important specifically for gut-first pathogenesis is, at the moment, limited. Results in gut-seeding studies appear consistent with intracerebral PFF studies with regard to increased pathogenicity of shorter fibrils[101]; however, it should be noted that in cell culture, fibril length did not have an effect on pathogenicity, while concentration did, and the molecular concentration may be underestimated for shorter fibrils when using mass-based measures of concentration[95]. Additionally, the pathogenicity of fibril solutions with shorter fibrils may also relate to the presence of other conformations following longer sonication times[74]. Finally, there is variability in which outcomes are used to measure gut-to-brain transmission. The resistance of synucleinopathy to treatment with proteinase K is a marker of mature Lewy pathology and increases the specificity of immunodetection. The use of this proteinase K for detection of ENS synucleinopathy in animal models is inconsistent in general and also among gut-seeding studies. Further studies are needed to determine how best to quantify pathology in these models. It was noted by Van Den Berge et al.[79] that while ENS pathology was proteinase K resistant in adult and aged rats, CNS pathology was not. Kim et al. measured total p-α-Syn, as well as Triton x-100 soluble and insoluble α-synuclein[85]. Group differences were almost identical for total p-α-Syn and insoluble α-synuclein both in the gut and CNS[85].

Additional and emerging rodent models of gut-first PD

While the majority of gut-first PD models have used environmental toxins and/or PFFs, there are other experimental paradigms. Two types of models that are being used with more frequency are “multi-hit” models using a gut-directed second hit, and microbial toxin models. “Multi-hit” models use a combination of pathogenic factors. In addition to combining genetic overexpression of α-synuclein with gut delivery of environmental toxins[39,52] or PFFs[80,84,87], gut inflammation can be used to accelerate parkinsonian pathology[102-105]. Pathogenesis in these models may not always involve ascending pathology and may require systemic inflammation[103]; however, peripheral mechanisms are likely involved[106]. Microbial toxin models have shed light on potential microbiome-linked pathogenic mechanisms through studying isolated proteins or metabolites produced by microbes. For example, the bacterial amyloid curli accelerated pathology in mice overexpressing α-synuclein[107]. While the gut microbiome contains abundant curli-producing microbes, exposure to some microbial toxins is via the environment. β-*N*-methylamino-L-alanine (BMAA), is a neurotoxin produced by cyanobacteria which has been linked to amyotrophic lateral sclerosis and PD. Oral administration of BMAA to wild-type C57B/6 mice resulted in gut inflammation accompanied by increased α-synuclein in an ascending pattern from the gut through the SN with pathologic α-synuclein through the DMV[108]. Despite the availability of viral vectors that target the peripheral nervous system following systemic administration[109], viral vector-mediated overexpression of α-synuclein has seldom been used to model gut-first PD[82]. PFFs are likely to be more pathogenic than overexpression of monomers, but viral-vector based overexpression may be useful in multi-hit models and may be increase feasibility in a laboratory without available PFF expertise. For extensive and comprehensive

discussion of additional animal models of both brain-first and body-first PD, we direct the reader to a recent review by Van Den Berge and Ulusoy [18].

Conclusion

While progress on the role of the gut in PD has been rapid, this field is young and we are far from the level of mechanistic understanding required to develop targeted therapies. Oral administration of environmental toxins and intestinal injection of α-Syn PFF are two models of PD that may be useful in identifying gut-specific disease mechanisms and targets. These models remain challenging to implement. An ideal model would be non-invasive to reduce variability arising from operator experience. Future studies might combine features of successful models, such as testing oral delivery of PFFs with lectin to increase absorption. The most advanced results in gut-first PD still originate from analysis of PD patients, including the initial observations of Braak and his team, and subsequent supportive evidence from patient cohorts and retrospective studies[5,6,8,16,17,19]. This evidence should prompt new research on the early premotor phase of PD and the role of the gastrointestinal tract in this highly disabling neurodegenerative disease.

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Box 1: Central Neuropathology in PD models

Loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc)

The process of neurodegeneration will eventually result in the loss of neurons; thus, the most definitive finding in PD models is a decreased number of dopaminergic neurons in the SNpc. The gold standard for detecting this is through unbiased stereological counting of neuronal cell bodies and calculation of the percentage cell bodies identified with a nuclear stain (Nissl) that express a specific marker for dopaminergic neurons, typically tyrosine hydroxylase (TH)[110,111].

Axonal degeneration

Before the loss of neuronal cell bodies, there is axonal degeneration. Dopaminergic neurons extend from the SNpc to the striatum. TH is present in the axon terminals; thus, a decrease striatal TH immunoreactivity indicates loss of dopaminergic axon projections. This is typically quantified by densitometric analysis of immunolabeled tissue sections, but western blot is also used[112].

Nigrostriatal dysfunction

Impaired function of dopaminergic neurons is presumed to be an early stage in the process of neurodegeneration. A pathological correlate of this dysfunction is a reduction in levels of dopamine in the striatum, which can measured by high performance liquid chromatography (HPLC) with electrochemical detection[113].

Synucleinopathy

The presence of pathological forms of α-synuclein is closely associated with disease progression in PD models. In earliest stages, this may be detected by antibodies specific for α-synuclein phosphorylated at serine 129. Conformation specific antibodies are available that can detect oligomers, fibrils and mature Lewy

Pathology (LP), also referred to as Lewy Type Synucleinopathy (LTS). Progression of pathologic α-synuclein is not sufficient for the development of neurodegeneration.

Table 1: Summary of oral environmental toxin PD animal models

Environmental toxin	Dose	Animal strain, sex and age	Treatment duration†, ¶	Neuropathology				GI pathology		Notes	References
				Striatal DA level	SNpc TH-IR neuron	SNpc α-syn/ p-α-syn	Motor function	GI motility	ENS α-syn/ p-α-syn		
Rotenone	**High dose**										
	0.25, 1.0, 2.5, 5.0, 10 or 30 mg/kg	C57BL/6N mice, male, 8w	28d	↓	↓	↑ a-syn	↓	n.r	n.r	Neuropathological symptom was shown from the dose of 10mg/kg and in a dosing term-dependent manner.	Inden et al. 2007[28], Takeuchi et al. 2009[114], Inden et al. 2009[115]
	30 or 100 mg/kg	C57BL/6N mice, male, 8w	56d	n.r	↓	↑ a-syn	↓	n.r	n.r	High dose increased the lethal rate.	Inden et al. 2011[29],Sun et al. 2017[116]
	30 mg /kg	C57BL/6N mice, n.r, 1y	28d¶	n.r	↓	↑	↓	-	↓ a-syn	Assessment time (post-treatment): 1-3w for motor test, 4w for tissue collection. No gross GI dysfunction.	Tasselli et al. 2013[30]
	30 mg/kg	C57BL/6 mice, male, 8-9w	1w to 4w	n.r	↓	↑	↓	↓	↑	GI dysfunction preceding motor deficits.	Yang et al. 2017[31]
	Intermediate dose										
	10 mg/kg	C57BL/6 mice, male, 7w	28d	n.r	↓	↑	↓	↓	↑	Increased GI inflammation and barrier dysfunction.	Perez-Pardo et al. 2018[36], Perez-Pardo et al. 2019[37]
	6.25 mg/kg	C57BL/6J mice, n.r, 1y	6d/w for 4m	n.r	n.r	n.r	↓	↓	n.r	n/a	Wagner et al. 2013[117]
	6.25 mg/kg	C57BL/6 mice, n.r, 1y	5d/w for 1m and 3m	n.r	↓	1.5m: - 3m: ↑	1.5m: - 3m: ↓	n.r	n.r	Spatial learning and memory abilities were improved in 3m group.	Jia et al. 2014[118]
	6.25 mg/kg	C57BL/6 mice, male, 8w	5d/w for 1m-4m¶	n.r	↓	n.r	↓	n.r	n.r	Assessment time (post-treatment): no gap vs 2m treatment+4m washout period; diffusion kurtosis imaging (DKI) by MRI showed involvement of thalamus.	Khairnar et al. 2021[38]
	Low dose										
	5 mg/kg	C57BL/6J mice, n.r, 1y	1.5 and 3m	↓	↓	1.5m: - 3m: ↑ a-syn	1.5m: - 3m: ↓	n.r	↑ p-a-syn	Rotenone was not detectable in the blood or brain when daily dose was lower than 5mg/kg; a-syn aggregated within DMV, IML after 1.5m	Pan-Montojo et al. 2010[32], Kuang, Cao, and Lu 2017[34], Hu et al. 2016[119]
	5 mg/kg	C57Bl/6J mice, n.r, 1y	6d/w for 2m and 4m	n.r	↓	↑ a-syn	↓	↓	↑ a-syn	Vagotomy prevented the PD-like pathology progression; rotenone stimulated a-syn secretion from enteric neurons.	Pan-Montojo et al. 2012[33]

	5 mg/kg	C57BL/6J mice, n.r, 8w	5d/w for 4m	n.r	n.r	n.r	n.r	↓	↑ a-syn	Decreases density of sympathetic neurites innervating the gut.	Arnhold et al. 2016[35]
	5 mg/kg	C57BL/6 N and LRRK2^{R1441G} knockin mice, male, 30w	Twice weekly for 50w	n.r	-	n.r	↓	n.r	n.r	Rotenone dissolving vehicle contained no DMSO or chloroform; increased apoptotic cells in cortex, striatum and SNpc.	Liu et al. 2017[40]
	3 mg/kg	M83$^{+/-}$ mice, n.r, 12-36w	5d/w for 2m	n.r	↓	n.r	n.r	↓	n.r	Olfactory dysfunction; PD-like pathology was induced in an age-dependent manner.	Chen et al. 2022[39]
Paraquat	10 mg/kg	C57BL/6 mice and TgM83, mix, 8w	In drinking water for 8w[¶]	n.r	-	-	-	n.r	↑ in M83 mice	Assessment time (post-treatment): 1w; no PD-like pathology in C57BL/6 mice; do not affect DVC or SNpc.	Naudet et al. 2017[52]
Paraquat + lectins	1 mg/kg paraquat +0.05% lectin	Sprague–Dawley rats, male, n.r	7d[¶]	n.r	↓	↑	↓	↓	↑	Assessment time(post-treatment): 2-4w; aggregation of p-α-syn in ENS, DVC, SNpc.	Anselmi et al. 2018[57]
Trichloroethylene (TCE)	1000 mg/kg	Fischer 344 rats, male, 5m	5d/w for 6w	↓	↓	↑	n.r	n.r	n.r	TCE induced complex 1 mitochondrial impairment in midbrain and SN.	Gash et al. 2008[67]
	200, 500, or 1000 mg/kg	Fischer 344 rats, male	5d/w for 6w	↓	↓	↑	↓	n.r	n.r	TCE-induced dopaminergic neuron degeneration in a dose-dependent manner.	Liu et al. 2010[66]
	400 mg/kg	C57BL/6 mice	5d/w for 8m	↓	↓	↑	↓	n.r	n.r	TaClo—the metabolite of TCE induced PD-like pathology.	Liu et al. 2018[63]

[†]: Once daily unless otherwise noted; [¶]:Pathology was assessed at the end of treatment except where noted; Underline: major dose/treatment duration; n.r: not reported; -: no change; DA: dopamine; TH-IR: tyrosine tyrosinase immunoreactive; a-syn: a-synuclein; p-a-syn: phosphorylated a-synuclein (); GI: gastroenterology; ENS: enteric nervous system; d: day; w: week; y: year; DMV: dorsal motor nucleus of the vagus; IML: spinal cord intermediolateral column; PD: Parkinson disease; SNpc: substantia nigra pars compacta.

Table 2: Characteristics of studies using α-Syn PFFs injected into the intestinal wall

Citation	Species, strain, age	Fibril type (characteristics); total dose	Control	Injection site and method (n injections)	Time points (post-injection)
Holmqvist, et al, 2014[78]	R, Sprague Dawley, Adult (250g)	PD lysate, 2 µg/µl (15 µg aSyn)	BSA in PBS	stomach, duodenum (5)	12h, 1, 2 ,3 & 6d
		hPFFs (>100nm, 1µg/µl), 15µg			
Manfredsson, et al., 2018[82]	R, Sprague-Dawley, young adult (220g)	mPFF (55.9nm, 2µg/µl) 60µg	Monomers, Saline, AAV2/5-GFP	desc. colon (6)	1, 6 &12m
	NHP-Macaca fascicularis, n.r.	hPFF (75.8nm, 2µg/µl), 200µg AAV2/5-alpha syn A53T		desc. colon (9), stomach (1)	
Uemura, et al., 2018[81]	M, C57BL/6J, 2m	mPFF (66.8nm[†], 2µg/µl), 48µg	PBS	stomach body and pylorus (8)	23 & 45d, 4, 8 & 12m
Kim, et al., 2019[85]	M, C57BL/6J, 3m	mPFF (64.7nm, 2.5µg/µl), 25µg	PBS	pylorus (2), duodenum (2)	1, 3, 7 &10m
Van Den Berge, et al., 2019[87]	R, BAC-*SNCA*[§], Sprague-Dawley, 4m (300-400 g)	hPFF (44.2nm, 1µg/µl), 18µg	PBS	pylorus, duodenum (6)	2 & 4m
Ahn, et al., 2020[84]	M, *SNCA*-OVX, 3m[¶]	hPFFs (aSyn N103/Tau N368, ~150nm, 1.5µg/µl); 3µg	PBS	descending colon (colonoscopy)	9-12d, 3m
Uemura, et al., 2020[80]	M, A53T-*SNCA*-BAC$^{+/-}$, WT-C57BL/6J, 2-3m	mPFF (66.8nm, 2µg/µl), 48µg	PBS	stomach body and pylorus (8)	1, 2, 4, 6 & 8m
Challis, et al., 2020[86]	M, C57BL/6N, 2m & 16m	mPFF (50nm[‡], 1µg/µl), 6µg	Monomer, BSA	duodenum (2)	7, 21, 60, 90 & 120d
Arotcarena, et al., 2020[83]	NHP, WT Baboon, 1-14y	PD brain lysate, 24pg/µl, 2.4ng	Intrastriatal, no injection	stomach, duodenum	12 & 24m
Van Den Berge, et al., 2021[79]	R, Fischer 3m, 10-12	hPFF (44.2nm, 2µg/µl), 60µg	PBS	pylorus, duodenum (6)	10 & 20w
	R, Fischer 18m	hPFF (44.2nm, 2µg/µl), 60µg	PBS	pylorus, duodenum (6)	10w
	R, Fischer 3m	mPFF (2µg/µl), 60µg	PBS	pylorus, duodenum (6)	10w

[†]N. Uemura, personal communication, November 19, 2022

[‡]V. Gradinaru, personal communication, November 15, 2022
[§]Expression of WT human α-Syn under normal regulatory elements
[¶]We include only N103/Tau N368 PFFs which were most efficiently propogated
PD: Parkinson's disease; WT, wild type; R: Rats; M: Mice; NHP: non-human primate; h: Hour; d: Day; w: Week; m : Month ; y: year; PFF: preformed fibril; hPFF, human PFF ; mPFF, mouse PFFs ; BSA: Bovine serum albumin; PBS: Phosphate buffered saline; n.r: not reported; SNCA-OVX, deletion of murine *Snca* and overexpression of human WT *SNCA* (Jackson Labs #023837)

Table 3: Findings of studies using α-Syn PFFs injected into the intestinal wall

Citation		Time (Post-injection)	Motor deficits	CNS p-asyn IR	CNS p-α-Syn quant[†]	↓STR DA	↓TH IR	DA neuron loss	ENS p-α-Syn	Delayed transit
Holmqvist, et al, 2014[78]		12h	n.r.	n.d.	n.r.	n.r.	n.r.	n.r.	yes	n.r.
		2d		vagus					n.r.	
		6d		DMV						
Manfredsson, et al., 2018[82]	R	1m	n.r.	DMV, LC (small)	n.r.	n.r.	n.r.	no	yes	yes
		6m		n.d.						n.s
		12m								no
	NHP	6m, 12m		n.d.	n.r.	n.r.	n.r.	n.r.	yes	n.r.
Uemura, et al., 2018[81]		23d, 45d	n.r.	DMV	n.s.	n.r.	n.r.	n.r.	high	n.r.
		4m		DMV (<45d)	n.r.				moderate	
		8m		DMV (<4m)					few	
		12m		DMV (≈8m)					rare	
Kim, et al., 2019[85]		1m	n.r.	DMV, LC (small)	Pons	no	no	no	n.s.	yes
		3m	n.r.	+AMG, SNc, HYP, PFC	+MO, AMG				yes	n.r.
		7m	yes	+HIP, STR, OB	+VMB, HIP, STR, PFC	yes	STR, VMB	yes		
		10m	n.r.	↑HIP, PFC, OB, STR, CTX; ↓AMG, VMB, MO	+OB	n.r.				
Van Den Berge, et al., 2019[87]	R,BAC-*SNCA*	2m	n.r.	DMV, LC, SNr	DMV, LC	n.r.	no	n.r.	yes	n.r.
		4m			+SNr					
	R, WT	2m, 4m		n.d.	n.s.				n.d.	
Ahn, et al., 2020[84]		9-12d	n.r.	Bst	n.r.	n.r	no	n.r.	n.r.	n.r.
		3m	yes	DMV, SN, striatum, LC	STR, SN, AMG, CTX		yes	yes	yes	
Uemura, et al., 2020[80]		1m	n.r.	DMV	DMV	n.r.	n.r.	n.r.	yes	n.r.
		2m		DMV (<1m)						
		4m		DMV (≈2m), nuc. amb.					n.r.	

Citation			Time (Post-injection)	Motor deficits	CNS p-asyn IR	CNS p-α-Syn quant†	↓STR DA	↓TH IR	DA neuron loss	ENS p-α-Syn	Delayed transit
			6m, 8m		DMV (<4m)					rare	
Challis, et al., 2020[86]	M, 2m		7d, 21d	no	nodose ganglion	n.s.	no	n.r.	n.r.	n.s.	no
			60d	yes						yes	yes
			90d							n.r.	n.r.
			120d							yes	yes
	M, 16m		60d	no	n.r.	n.r.	no	n.r.	no	n.r.	yes
			120d		DMV, SN	n.s.	yes		no		
Arotcarena, et al., 2020[83]			24m	n.r.	SNpc and PHIPC	n.s.	yes	SNpc‡	yes	yes	n.r.
Van Den Berge, et al., 2021[79]	mPFFs	young (3m)	10w	n.r.	DMV, LC, SN	DMV, LC, SN	n.r.	no	n.r.	yes	n.r.
	hPFFs	young (3m)	10w		n.d.	n.d.		no		yes	n.s.
			20w							n.s.	n.s.
		adult (10-12m)	10w		DMV, LC, SN	DMV, LC		n.s.		yes	n.s.
			20w			SN		yes			yes
		aged§ (18m)	10w			DMV, LC, SN		yes			n.r.

†Quantitative measures used for the table included: number of p129-α-Syn positive neurons (IHC)[80,81,114], densitometric analysis of Triton-X insoluble p129-α-Syn (western blot)[85], densitometric analysis of p-α-Syn immunohistochemistry[79,83,85,87], p129-α-Syn+/TH+ neurons[84]

‡subthreshold for motor deficits

§vs. Adult PBS-injected

“+” indicates regions are in addition to what was observed at earlier timepoints.

CNS, central nervous system; IR, immunoreactivity; STR: Striatum; DA, dopamine; TH-IR: tyrosine hydroxylase; ENS, enteric nervous system; h: Hour; d: Day; w: Week; WT, wild type; R: Rats; M: Mice; NHP: non-human primate ; p-αSyn: Phosphorylated αsyn; DMV: dorsal motor nucleus of the vagus nerve; AMG, Amygdala; LC, locus coeruleus; HIP, Hippocampus; OB = olfactory bulb; MO: Medulla oblongata; SNr: substantia nigra pars reticulata; HYP: hypothalamus; Bst: brainstem tissues; VMB:Ventral midbrain; PFC: Prefrontal cortex; ; n.r: not reported; n.s: not significant ; n.d., not detected ; PFF: preformed fibril; hPFF, human PFF ; mPFF, mouse PFFs ; BSA: Bovine serum albumin; PBS: Phosphate buffered saline; PHIPC, parahippocampal corttex; nuc. amb., nucleus ambiguous

Table 4: Findings of studies using α-Syn PFFs with additional intrabdominal delivery routes

Citation	Route†	Methods	Findings
Breid, et. al., 2016[92]	IP	M83$^{+/-}$ mice (6-8w) were injected with hPFFs (~100nm, 50 µg total dose)	4/5 affected (median incubation time of 229±17 days.)
Ayers, et al., 2017[91]	IP	M83$^{+/-}$ mice (2m) were injected with mPFFs (~100nm, 50 µg)	At 180 d (predetermined), 3/6 affected, 6/6 had p129-α-Syn in thalamus, hypothalamus, periaqueductal gray, brain stem, and spinal cord.
Sargent, et al., 2017[90]	IP	M83$^{+/-}$ and M83$^{+/+}$ neonates were injected with M83 brain lysates IP and IC	M83$^{+/+}$ IP injected had longer survival than IC (<8m vs 4–14 m). For M83$^{+/-}$, 10/19 IP affected. P129-α-Syn distribution was similar in IP and IC.
Lohmann, et all., 2019[93]	Oral, IP	M83$^{+/-}$ mice (6-8w)were injected with 50 µg hPFFs (50 nm), or gavaged with 50 µg or 500 µg	IP: 10/10 affected, mean survival (SD) 200 (35) d Oral, 50 µg: 2/8 affected (220 and 350 d) Oral, 500 µg:4/8 affected , mean survival (SD) 384 (32)d All routes showed p-α-Syn to cortex with high density in more caudal regions .
Wang, et al., 2020[88]	Stellate and celiac ganglia	M83$^{+/-}$ (2m) were injected with 11 µg hPFFs (47.8 nm) 11 µg into celiac and stellate ganglia	p129-α-Syn throughout brainstem and midbrain. Autonomic dysfunction and prolonged tranist preceded motor symptoms.
Macdonald, et al., 2021[89]	Oral, IP	M83$^{+/-}$ mice (6-8w) received A53T hPFFs (57 m) orally (200 µL of 400 µM) for 28 d or one injection IP	Oral administration resulted in motor deficits at 6m. At 5m, p129-α-Syn most dense in thalamus, hypothalamus, amygdala, basal forebrain, stria terminalis, periaqueductal grey, reticular formation and spinal cord (present but less dense in SN). Simiilar to IP.

†Only intraabdominal routes are described

IP: Intraperitoneal; IC, intracerebral; h: Hour; d: Day; w: Week; m : Month ; y: year; PFF: preformed fibril; hPFF, human PFF ; M83, expression of human A53T mutated *SNCA* under prion promoter (Jackson Labs #004479); SD, standard deviation; SN, substantia nigra

Figures

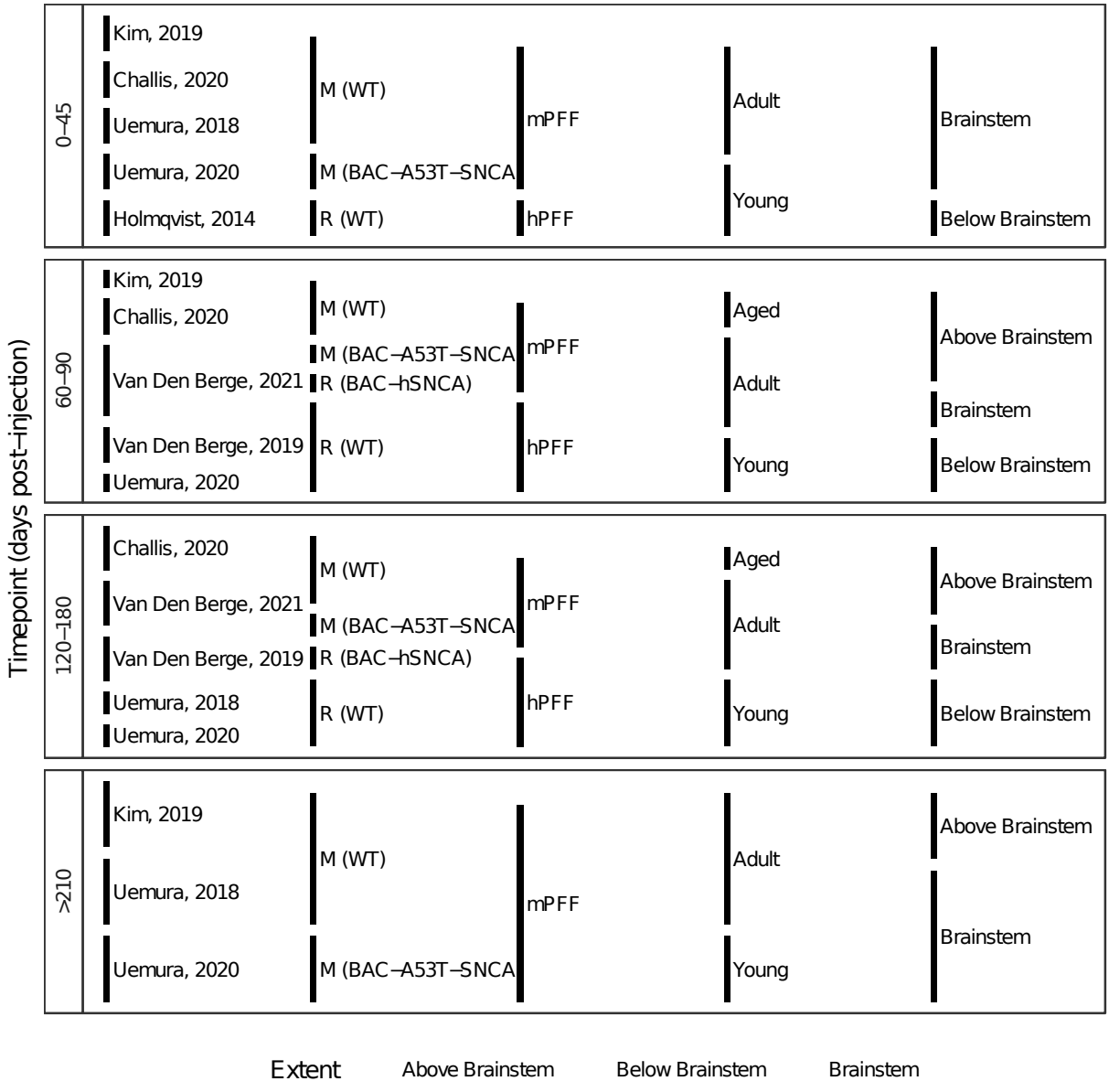

Figure 1: Characteristics of studies with proximal gastrointestinal injection of α-ynuclein preformed fibrils (PFFs). A parallel sets diagram is used to diagram the combinations of selected study characteristics that were associated with progression of p-α-Syn. Each ribbon corresponds to an experimental group. The experimental factors are indicated by the passage of the ribbon through the nodes (vertical black lines). The color of the ribbon corresponds to the furthest location of p-α-Syn at that time point.
M, mouse; R, rat; PFF: preformed fibril; hPFF, human PFF; WT, wild type